

Fly, Robot: The use of a controllable fly robot to explore object recognition and visual tracking  
during courtship in *Drosophila*

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**Abstract**

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Though the application of genetic approaches has greatly increased our understanding of motion vision in the fruit fly, *Drosophila melanogaster*, little is known about higher-order functions of the visual system such as object recognition. Visual object recognition likely plays an important role during courtship: for successful courtship, males must be able to distinguish an object from the background and further categorize it as conspecific, female, and receptive. Because courtship between two flies happens at a close distance, typically within a few millimeters, the fly's eye could possibly distinguish fine-scale patterns for use in object recognition. Even at a greater distance, the male would have access to large-scale cues like shape, size, and color, as well as motion cues like speed and patterns of motion, to aid in its visual identification of objects. In the following sets of experiments, I used a modifiable, programmable fly dummy coupled with automated behavioral tracking to explore how males use visual cues to dynamically modulate their courtship and the neurons that may be involved in these behaviors.

**I. The relative roles of vision and chemosensation in mate recognition:** Animals rely on sensory cues to appropriately classify and respond to objects in their environment. However, the spatial structure of those sensory cues can greatly impact when and how they are perceived. In this study, I examined the relative roles of visual and chemosensory cues in mate recognition by pairing male flies with dummies of various shapes, sizes, and speeds, or coated with different pheromones. I determined that visual and chemical cues play specific roles at different points in the courtship sequence. Vision is essential for determining whether to approach a moving object and initiate courtship, and males were more likely to begin chasing objects that were the same approximate dimensions as another fly. However, whereas males were less likely to begin chasing larger dummies, once started, they would continue chasing for a similar length of time regardless of the dummy's shape. The presence of female pheromones on the moving dummy did not affect whether males would initiate a chase, but it did influence how long they would continue chasing. Collectively, these results demonstrate that males can visually distinguish potential mates and that different sensory cues play a dominant role at different stages of courtship.

**II. Pigmentation and shape affect male's positioning during courtship chases:** Beyond helping a male identify a mate from afar, vision continues to be an important cue after courtship

has begun. Vision allows a male to track and pursue a female, and it also influences how the male positions himself around the female during these chases. I found that as dummies increase in width or height, males demonstrate less wing extension and position themselves further from the dummy. I further found a role for dummy color in modulating mate attractiveness: males are not strongly attracted towards very dark or very light objects, but do show a robust courtship response towards gray-painted dummies. These experiments are the first to suggest that males use color to distinguish potential mates. I also found that male flies preferentially bias their chasing and courtship maneuvers towards the abdomen—and not the head—of female flies, irrespective of her direction of movement. This preference is maintained to some degree towards females that do not produce pheromones, suggesting that males can use vision to distinguish different parts of the female body. Color could be one of the visual cues males use to position themselves around the female, as males paired with dummies painted multiple colors bias their position towards the preferred color.

**III. Despite concurrent activation of P1 neurons, males still use visual cues to dynamically modulate courtship:** The P1 neurons are a population of ~20 *Drosophila* male-specific, fru<sup>M</sup>-expressing neurons in the posterior brain that have recently been identified as the putative site where sensory cues are integrated and translated into persistent courtship behavior. Males with activated P1 neurons direct their courtship maneuvers towards nearby moving objects, and functional imaging studies demonstrate activation of these cells by both chemosensory and visual stimuli. I activated P1 neurons in male *Drosophila* using the heat-sensitive cation channel, TRPA1, and examined the effect on their courtship responses towards dummies of varying shapes or chemosensory content. While overall courtship levels were elevated, males still showed a preference for smaller, more fly-sized objects. Furthermore, males still used dummy shape or color to modulate their levels of wing extension and chasing position relative to the dummy. However, males with activated P1 neurons no longer modulated their chasing position in response to the dummy's pheromone coating. Thus, visual information is able to modulate male courtship independent of P1 activation, but P1 neurons are important for modulating a males' response to pheromones.

*For my Umma, one of the strongest women I know.*

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SA  
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## Chapter 1. Introduction

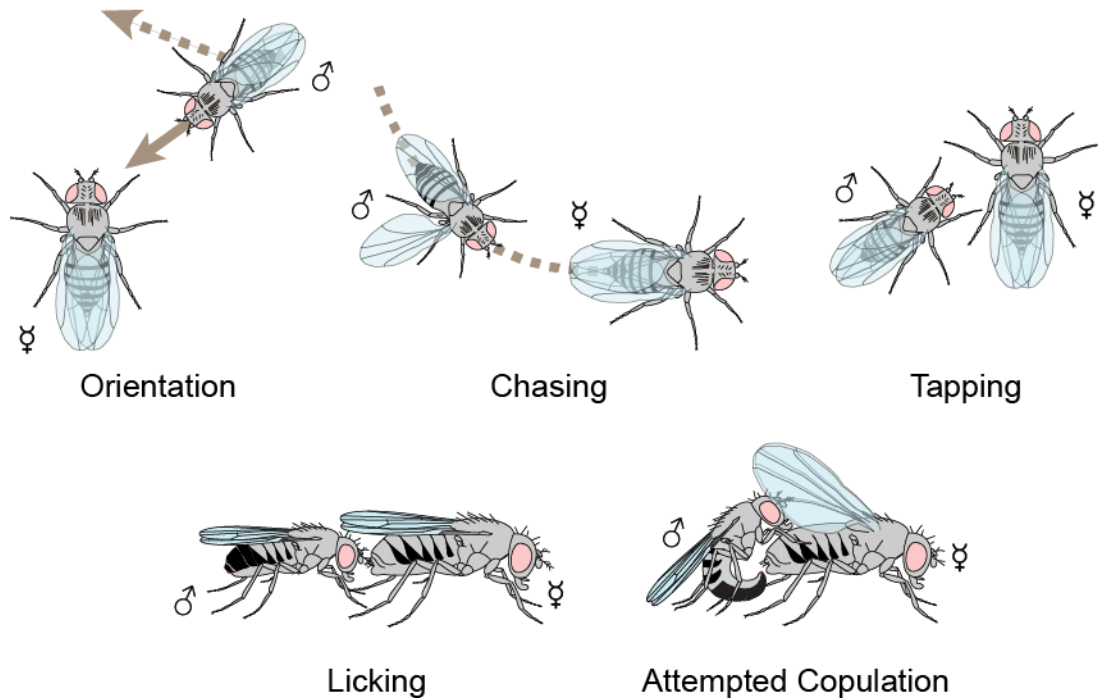
For many insects, visual object recognition is essential for a variety of behaviors, including foraging, prey capture (Nordström, 2013; Olberg and Worthington, 2000), and courtship (Boeddeker *et al.*, 2003b; Collett and Land, 1975; Trischler *et al.*, 2010). However, little is known about visual object classification in the fruit fly, *Drosophila melanogaster*. Whereas the overall circuitry of the motion vision pathway has been thoroughly studied (Borst, 2009; Paulk *et al.*, 2013), previous research has largely focused on motion or landmark cues and their role in navigation (i.e. ‘stripe fixation’ (Götz, 1987), looming shapes (Card and Dickinson, 2008), and large field optomotor responses (Götz, 1968)). This lack may in part be due to the fact that *Drosophila* does not display behaviors typically associated with exemplary feats of object recognition such as predation or central place foraging, nor does this species have an eye that is optimized for the detection of fine detail (Land, 1997).

Nevertheless, one behavior during which visual object recognition likely plays an important role is courtship. Courtship in *Drosophila* involves a sequence of highly stereotyped and elaborate maneuvers made by the male in order to assess, attract, and mate with a female (Greenspan and Ferveur, 2000; Sturtevant, 1915) (Fig. 1.1). For successful courtship, the male must be able to distinguish an object from the background and further categorize it as conspecific, female, and receptive. Because courtship between two flies happens at a close distance, typically within a few millimeters (Branson *et al.*, 2009; Sturtevant, 1915), the fly’s eye could possibly distinguish fine-scale patterns for use in object recognition. Even at a greater distance, the male would have access to large-scale cues like shape, size, and color, as well as motion cues like speed and patterns of motion, to aid in its visual identification of objects. Below, I will review some of the literature related to *Drosophila* visual processing, the potential role of vision during courtship, and how vision may impact the use of other courtship cues like the detection of pheromones.

## 1.1 The fly visual system

In order to understand how male flies use vision during courtship, I must first understand the limitations and capabilities of their visual system. The majority of visual input to the fly brain comes from the compound eyes. Each eye consists of about 700 ommatidia, and each ommatidium is composed of a lens and 8 photoreceptor neurons, R1-8 (Heisenberg and Wolf, 1984). R1-6 are thought of as the “achromatic” photoreceptors, as they all express the same opsin that responds to a broad spectrum of wavelengths (Kirschfeld and Franceschini, 1977). R7 and R8, in contrast, respond specifically to UV and blue-green wavelengths (Kirschfeld and Franceschini, 1977; Salcedo *et al.*, 1999). Visual information from the ommatidia is then retinotopically projected to a series of neuropil where visual information is subsequently processed, first to the lamina, then medulla, followed by the lobula complex. R7-8 axons skip the lamina and project directly to the medulla. From the lobula complex, visual information is further processed in the optic glomeruli of the lateral protocerebrum (Borst, 2009; Cajal and Sánchez, 1915; Heisenberg and Wolf, 1984). Roughly half the fly’s brain is dedicated to processing visual information (Rein *et al.*, 2002).

Flies possess one of the fastest visual systems in the animal kingdom, but suffer greatly when it comes to resolution. Due to their structure, compound eyes are severely limited in the resolutions they can achieve. With only 700 ommatidia with an interommatidial angle of  $5^\circ$  (Land, 1997), each eye would be roughly equivalent to a 25x25 pixel camera, which spread across the fly’s wide field of view, translates into very little resolution per visual arc. However, despite their poor visual resolution, recent studies do suggest that *D. melanogaster* possess at least some crude ability to recognize particular shapes or patterns. Flying flies exhibit innate turning reactions that depend on object size – they are attracted towards long vertical objects and strongly repulsed by smaller visual stimuli (Maimon *et al.*, 2008). Flies can also be trained to steer towards specific visual scenes (Dill *et al.*, 1993; Tang *et al.*, 2004; Wolf and Heisenberg, 1991). Less is known about visual navigation in walking flies, but studies do show that they will pursue salient objects (Strauss and Pichler, 1998) and can be trained to use visual landmark cues (Ofstad *et al.*, 2011).



**Figure 1.1. The major male courtship behaviors.** Depicted are the main steps of *D. melanogaster* courtship as performed by the male. Courtship generally proceeds such that earlier behaviors are in the upper left progressing to later behaviors in the lower right.

## 1.2 Vision is not required for *Drosophila* courtship

Courtship begins when the male orients towards the female and fixates her in the center of his field of view (Fig. 1.1). He then approaches the female, who typically runs away, leading to a pursuit, or chasing, phase. As the male pursues the female, he will periodically extend one of his wings and vibrate it to produce a species specific song. He will also approach the female and tap her with his front tarsi in an attempt to taste the pheromones present on her cuticle, or else circle behind her and lick her genitals with his proboscis. Eventually, after exhibiting the above behaviors multiple times, the male will attempt to copulate by bending his abdomen and thrusting his genitals at the female (Bastock and Manning, 1955; Greenspan and Ferveur, 2000).

Vision is not required for successful courtship – flies can reproduce in the dark (Spieth and Hsu, 1950). However, males in the dark or with impaired vision take longer to initiate and complete courtship (Markow, 1975), and blind males are outcompeted by individuals with intact vision (Connolly *et al.*, 1969). Additionally, vision is required for certain components of courtship, such as orientation and chasing, and flies chasing in the dark do not exhibit either behavior (Cook, 1979).

Furthermore, laboratory experiments are not typically designed to assess the role of vision during courtship and likely underemphasize the importance of this sense. Courtship experiments are often performed in small chambers in which the female and male flies are the only objects present (for example, see Fernández *et al.*, 2010). In such a setting, the male would gain little additional information from the visual channel regarding the identity of the other “object” (the female), and he would very quickly encounter other, potentially more informative sensory cues, such as the cuticular pheromones. Males in the wild face a much more difficult task – natural rots attract many individuals of different species (Sturtevant, 1915). To find an appropriate mate, males must not only visually distinguish an object from the background, they must also determine its gender, species identity, and receptivity. They are also not immediately placed within close proximity of the putative female, but must typically approach from a distance.

What types of visual cues could the male be using? Vision is likely important to assess both an object’s location and its identity. Males could use cues like shape, color, patterns of motion and speed to determine if an object is a potential mate and whether to begin pursuit. Then, during the chase, males likely use vision to track and chase females and when deciding which wing to extend for song. Vision could be further important to detect inhibitory cues from unreceptive females, like wing flipping. Finally, the male must determine a female’s polarity and the location of her genitals in order to successfully copulate – could vision be important then too?

### **1.3 Visual object recognition in other insects**

Many species of insects, including some flies, demonstrate an amazing ability to pursue and track small targets, often at high speeds and against a cluttered background. Dragonflies are well-known for their skill in pursuing and catching prey (Mischianti *et al.*, 2014; Olberg and Worthington, 2000), and males of many Dipteran species (e.g., smaller hoverflies, blowflies, and houseflies) track conspecifics while flying as part of their courtship ritual (Boeddeker *et al.*, 2003b; Collett and Land, 1975; Collett and Land, 1978; Land, 1993; Tricca and Trujillo-cenoz, 1980). Many specializations have been found that aid in these difficult tasks. Foremost, when pursuing their target, flying insects tend to do so from behind and below, thereby fixating the target on the dorsal region of their eyes (Boeddeker *et al.*, 2003b; Collett and Land, 1975; Land

and Collett, 1974; Mischianti *et al.*, 2014; Olberg *et al.*, 2007). As a result, the target is viewed against the bright sky, increasing the relative object/background contrast. Furthermore, hoverflies, blowflies, and houseflies all have a region of eye with smaller interommatidial angles and large facet diameters (often referred to as a “love-spot”), resulting in a zone of acute, increased visual resolution (Hardie, 1985; Land, 1997; Land and Eckert, 1985). The underlying retina of the love-spot also demonstrates adaptations providing higher sensitivity with stronger and faster responses compared to females (Burton, 2003; Hornstein *et al.*, 2000), thus enabling males to detect conspecifics at greater distances and higher velocities.

Male *D. melanogaster* also chase potential mates, but without any of the advantages mentioned above. Because their courtship does not occur during flight, males do not have the option of chasing a potential female from below and thereby increasing the contrast of the female against background. Furthermore, male *D. melanogaster* do not have any sort of a specialized region of their eyes like the love-spot found in other flies (Land, 1997). Perhaps because *Drosophila* males pursue mates at a lower speed (Branson *et al.*, 2009), these adaptations are unnecessary. Additionally, male and female *D. melanogaster* are typically much closer to one another during courtship pursuit compared to the species discussed above (Branson *et al.*, 2009), thereby increasing the resolution of the image the female casts on the male retina and perhaps rendering a love-spot unnecessary.

#### **1.4 Males also use chemosensory cues to recognize mates**

Vision is, of course, not the only cue available to males when assessing an object’s identity. Past research has shown that males also likely use chemosensory, tactile, and auditory cues during courtship (Billeter and Levine, 2013; Greenspan and Ferveur, 2000). Again, although no single one of these modalities appears to be necessary for courtship, their lack does impair courtship significantly (Greenspan and Ferveur, 2000).

The best studied of these additional modalities is chemosensation. Flies produce both species- and sex-specific pheromones (Table 1.1), most of which are hydrocarbons found on the cuticle and synthesized in specialized cells, called oenocytes, found on the inner surface of the abdominal cuticle (Billeter *et al.*, 2009). Compared to females, males produce high levels of the monoalkene (Z)-7-tricosene (7-T) and (Z)-7-pentacosene (7-P) (Antony and Jallon, 1982; Farine *et al.*, 2012), which are repulsive to other males (Antony and Jallon, 1982; Ferveur, 2005;

Compound name	Abbrev.	M	F
<i>n</i> -Heneicosane	<i>n</i> -C21	+	+
(Z)-11-Vaccenyl Acetate	cVA	+	-
(Z)-T-Docosene	7-D	+	+
<i>n</i> -Docosane	<i>n</i> -C22	+	+
(Z,Z)-7,11-Tricosadiene	7,11,TD	-	+
2-Methyldocosane	23-Br	+	+
(Z)-9-Tricosene	9-T	+	+
(Z)-7-Tricosene	7-T	++	+
(Z)-5-Tricosene	5-T	+	+
<i>n</i> -Tricosane	<i>n</i> -C23	+	+
<i>n</i> -Tetracosane	<i>n</i> -C24	+	+
(Z,Z)-9,13-Pentacosadiene	9,13-PD	-	+
(Z,Z)-7,11-Pentacosadiene	7,11-PD	-	+
(x)-12-Pentacosene	12-P	+	+
2-Methyltetracosane	25-Br	+	+
(Z)-9-Pentacosene	9-P	+	+
(Z)-7-Pentacosene	7-P	++	+
(Z)-5-Pentacosene	5-P	+	+
<i>n</i> -Pentacosane	<i>n</i> -C25	+	+
(Z,Z)-9,13-Heptacosadiene	9,13-HD	-	+
(Z,Z)-7,11-Heptacosadiene	7,11-HD	-	+
2-Methylhexacosane	27-Br	+	+
(Z)-7-Heptacosene	7-H	+	+
<i>n</i> -Heptacosane	<i>n</i> -C27	+	+
<i>n</i> -Octacosane	<i>n</i> -C28	+	+
(Z,Z)-9,13-Nonacosadiene	9,13-ND	-	+
(Z,Z)-7,11-Nonacosadiene	7,11-ND	-	+
2-Methyloctacosane	29-Br	+	+
<i>n</i> -Nonacosane	<i>n</i> -C29	+	+
2-Methyltriacontane	31-Br	+	+
3- <i>O</i> -acetyl-1,3-dihydroxy-octacos-11,19-diene	CH503	+	-
Methyl laurate	ML	+	+
Methyl myristate	MM	+	+
Methyl palmitate	MP	+	+

**Table 1.1 List of major compounds found on *D. melanogaster*.** The last two columns indicate presence on males (M) or females (F). (-) indicates a lack of that compound, (+) indicates compound is found, and (++) indicates that the compound is especially enriched. Modified from Farine et al. (2012).

Lacaille *et al.*, 2007) and increase females' receptivity to mating attempts (Grillet *et al.*, 2006). Females, in contrast, produce sex- and species-specific dienes such as (7Z,11Z)-heptacosadiene (7,11-HD) and (7Z,11Z)-nonacosadiene (7,11-ND), which have been shown to have an aphrodisiac effect on males (Tompkins *et al.*, 1980; Venard and Jallon, 1980). Males will court males that have been genetically modified to express female hydrocarbons, demonstrating the importance of these pheromones for conveying sexual identity (Ferveur *et al.*, 1997). However, males will also court and attempt to copulate with males and females with ablated oenocytes (*oe<sup>-</sup>*), often even demonstrating a preference for *oe<sup>-</sup>* females compared to wildtype females (Billeter *et al.*, 2009). These results indicate that oenocyte-synthesized pheromones are not the only means of stimulating courtship and that these pheromones may even act to somehow slow courtship. Males from sibling species will also court females lacking cuticular hydrocarbons, and re-application of dienes on *D. melanogaster* females or conspecific females blocks courtship from these males, demonstrating that pheromones are also an important cue for species-identity (Billeter *et al.*, 2009).

The oenocytes are not the only site of pheromone production—many other pheromones important for courtship, some only recently discovered, are likely synthesized elsewhere. The best studied of these additional pheromones is 11-*cis*-vaccenyl acetate (cVA), which is produced in the male ejaculatory bulb and transferred to females upon copulation (Butterworth, 1969; Datta *et al.*, 2008; Jallon *et al.*, 1981; Kurtovic *et al.*, 2007; Wang *et al.*, 2011; Watanabe *et al.*, 2011). The presence of cVA reduces female attractiveness and male ardor, thereby acting as a cue identifying other males and mated females. cVA has also been demonstrated to have many other effects on fly behavior, depending on context: it can act as an aggregation cue and stimulates male-male aggression and female oviposition (Bartelt *et al.*, 1985; Ejima, 2015; Wang and Anderson, 2010; Wang *et al.*, 2011). CH503 is another male-specific pheromone hypothesized to be synthesized in the male reproductive tract. Like cVA, CH503 is transferred to females upon mating and inhibits male courtship. However, CH503 appears to be much longer-lived on the female cuticle (Fernández *et al.*, 2010; Yew *et al.*, 2009). Finally, a third pheromone, methyl laurate (ML) was only recently discovered by Dweck *et al.* (2015). Its site of synthesis is unknown, but is independent of the oenocytes. ML is produced by both males and females in small quantities and has been shown to have a significant effect stimulating male

courtship and mating. ML is thought to be one of the cues mediating male attraction to oe- males and females. ML, like cVA, also mediates short-range aggregation (Dweck *et al.*, 2015).

Flies sense pheromones via both olfaction and gustation. The majority of hydrocarbons produced by this species are heavy, long-chain molecules with limited volatility, and are thus thought to be sensed via gustation. Gustatory receptor neurons (GRNs) express either gustatory receptors (GRs) or ionotropic receptors (IRs), and are found on the distal end of the proboscis, the pharynx, the tarsi, and anterior wing margins (Kohl *et al.*, 2015). Of these, both the proboscis and foretarsi have been directly implicated in male courtship behavior (Greenspan and Ferveur, 2000; Spieth, 1974). Loss of the gustatory receptors Gr68a (Bray and Amrein, 2003) and Gr39a (Watanabe *et al.*, 2011) reduces male to female courtship, implying they may be important in detecting female dienes (Billeter and Levine, 2013). Loss of Gr32a (Miyamoto and Amrein, 2008; Wang *et al.*, 2011) and Gr33a (Jiao *et al.*, 2007) results in enhanced male-male courtship, suggesting they detect male-specific inhibitory signals. GRNs expressing receptors of the pickpocket (Ppk) family have also been implicated in mate discrimination – ppk23 neurons demonstrate heterogeneous responses, with one population responding to male CHs and the other responding to female CHs (Thistle *et al.*, 2012). Activation of ppk23-expressing neurons triggers male-female courtship and inhibits male-male courtship (Kohl *et al.*, 2015; Lin *et al.*, 2005; Liu *et al.*, 2012; Starostina *et al.*, 2012; Toda *et al.*, 2012; Vijayan *et al.*, 2014).

Some pheromones, including cVA and ML, are thought to have some limited volatility and are likely sensed via olfaction (Dweck *et al.*, 2015; Yew *et al.*, 2009). Olfactory receptor neurons (ORNs) include neurons expressing either odorant receptors (ORs) or IRs, and are found on both the antennae and maxillary palps (Kaupp, 2010; Kohl *et al.*, 2015; van der Goes van Naters and Carlson, 2007). Of the three main morphological ORN types, only trichoid ORNs seem to be sensitive to fly odors (van der Goes van Naters and Carlson, 2007). Four odorant receptors respond to fly extracts: Or67d, Or47b, Or88a, and Or65a. Or67d and Or65a respond to cVA (Kohl *et al.*, 2015), whereas ML activates both Or47b and Or88a (Dweck *et al.*, 2015). Or88a also responds to methyl myristate and methyl palmitate (Dweck *et al.*, 2015). ORN information travels to the antennal lobe, and from there goes to either the lateral horn or the mushroom body (Kohl *et al.*, 2015; van der Goes van Naters and Carlson, 2007).

Thus far, it has been difficult to estimate just how far fly pheromones can diffuse away from their source and still be detectable by other flies. A study by Farine *et al.* (2012) did find

that about 0.2% of flies' lightest HCs volatilized natural conditions. However, to collect compounds, Farine used small vials (about 8 mL of available space) with a high density of flies (10-20) for two hours, thus representing the best case scenario for concentrating and capturing volatilized CHs. Several studies that placed males in a behavioral chamber and wafted in air that had been piped over virgin females failed to detect an increase in male courtship (Antony and Jallon, 1982; Tompkins *et al.*, 1980). A single study by Tompkins *et al.* (1980) did show that female extract could increase male courtship behavior at a distance, but at 8 mm the extract's effect was no longer significant. More recently, Clowney *et al.* (2015) demonstrated that the DA1 glomerulus, which receives input from cVA-sensing ORNs, was activated when a male abdomen was brought within 3-6 mm of a tethered male's antennae.

Recent evidence also suggests that pheromones may, beyond enabling flies to determine the source's gender, also help males locate a female's abdomen versus her head. Kimura *et al.* (2015) found that wildtype males are especially attracted to the abdomen of immobile females, and that they were able to maintain this attraction and ability to locate the abdomen as long as they had either intact vision or olfaction. However, olfaction-deficient males (Or83b<sup>2</sup> mutants) were no longer able to position themselves near the abdomen in the dark.

Finally, some evidence suggests that the chemosensory pathway could somehow be mediating the saliency of the visual pathway. Ejima and Griffith (2008) showed that males with silenced Gr68a neurons displayed normal courtship towards immobilized (headless) females but had impaired courtship towards intact females, suggesting a potential role for Gr68a-positive mechanosensory neurons in motion detection and courtship initiation.

### **1.5 Putative neurons involved in visual target tracking**

Insect neurons that respond specifically to small moving objects (small-target movement detectors, STMDs) were first described in the optic lobes of hawkmoths and hoverflies (Collett, 1971; Nordström and O'Carroll, 2009), and have since been identified in a variety of other insects, including hoverflies and dragonflies (Barnett *et al.*, 2007; Nordström and O'Carroll, 2009; Nordström *et al.*, 2006; O'Carroll, 1993). STMDs are characterized by highly selective responses to small moving targets (<3° of the visual field), while being inhibited by or indifferent to large bars and wide-field stimuli (Nordström and O'Carroll, 2009). Many STMDs demonstrate robust responses towards a target moving against a cluttered background, even when there is no velocity difference between the target and background, and even if the target was smaller than

the receptive field of a single ommatidium, suggesting these cells have complex physiological properties (Barnett *et al.*, 2007; Nordström *et al.*, 2006).

The number of STMDs found vary from species to species. Based on receptive field location, size, directional selectivity, and other physiological responses, hoverflies may have as many as 20 different STMD types (Nordström *et al.*, 2006). Their receptive fields tend to cluster in the dorso-frontal visual field, corresponding closely to the location of the love spot in males, strongly suggesting a role in male target pursuit (Barnett *et al.*, 2007; Nordström *et al.*, 2006). Some dragonfly STMDs have been shown to terminate in pre-motor areas of the lateral midbrain, where they likely provide direct and/or indirect input to direction-selective, target-selective descending neurons (TSDNs) (Nordström, 2012; Olberg, 2012). TSDNs have been implicated in steering and wing control during prey pursuit (Frye and Olberg, 1995; Olberg, 1981; Olberg, 1986).

STMD-like cells have only very recently been described in *D. melanogaster*. Aptekar *et al.* (2015) used calcium imaging to examine activity in the dendrites of cells projecting from the lobula to optic glomeruli in the central brain, and found cells that respond to “figure-like” stimuli, such as the leading edge of Fourier bar. Kim *et al.* (2015) recorded from interneurons of the optic glomeruli (OGINs) of the lateral protocerebrum, and found that the majority of OGINs responded to small moving targets, but not at all to wide field motion, and without directional selectivity. Whether they are involved in courtship remains to be determined.

Sex-specific splicing of two genes, *fruitless* (*fru*) and *doublesex* (*dsx*) primarily determines a fly’s sex and is responsible for most of the sexual dimorphism seen between male and female brains (Arthur *et al.*, 1998; Dickson, 2008; Kimura *et al.*, 2008; Pan *et al.*, 2011; Yamamoto and Koganezawa, 2013). Because chasing is a male-specific behavior, males may demonstrate special neural specializations for visual target tracking. A number of *Drosophila* visual lobe neurons are positive for male-specific *fru<sup>M</sup>* isoforms and will likely be good candidates for visual neurons involved in courtship (Yu *et al.*, 2010). Another population of ~20 *Drosophila* male-specific, *fru<sup>M</sup>*-expressing neurons in the posterior brain—the P1 neurons—has recently been identified as the putative site where sensory cues are integrated and translated into persistent courtship behavior. Activation (Inagaki *et al.*, 2013; Kohatsu *et al.*, 2011; Pan *et al.*, 2012) or masculinization (Kimura *et al.*, 2008) of these cells stimulates courtship. If an object, especially a moving object, is present, males with activated P1 neurons will direct their courtship

maneuvers towards that object (Bath *et al.*, 2014; Kohatsu *et al.*, 2011; Pan *et al.*, 2012). If P1 cell-signaling is disrupted using *shibire<sup>ts</sup>*, courtship is not entirely abolished, but courtship latency significantly increases and the courtship index decreases (Pan *et al.*, 2012). Calcium imaging demonstrates that P1 neurons are activated when the male fly senses virgin female contact pheromones via his foretarsi, and this response is attenuated if a male-specific pheromone such as cVA is present or if the male touches the abdomen of a mated conspecific female or female of another species (Clowney *et al.*, 2015; Kohatsu *et al.*, 2011). Furthermore, after contacting female pheromones, P1 cells show a response to moving visual stimuli as well (Kohatsu and Yamamoto, 2015). This response, however, is not present if the male does not first contact female pheromone, suggesting that chemosensory cues somehow gate the visual sensitivity. As such, it is clear that these cells are involved in the earliest stages of courtship initiation, but how exactly visual information is being used in these cells is currently unclear.

## **1.6 Courtship is a stigmergic behavior**

The concept of stigmergy was first introduced in 1959 by the French zoologist Pierre-Paul Grasse to help understand the behavior of groups of social animals, and how coordination is able to arise from their collective actions (Grasse, 1959). Essentially, stigmergy theorizes that some trace left in the environment by an action stimulates the performance of the next action. This action then likely leaves some new or transformed trace, and stimulates some other action, either by the same or differing agent (Theraulaz and Bonabeau, 1999).

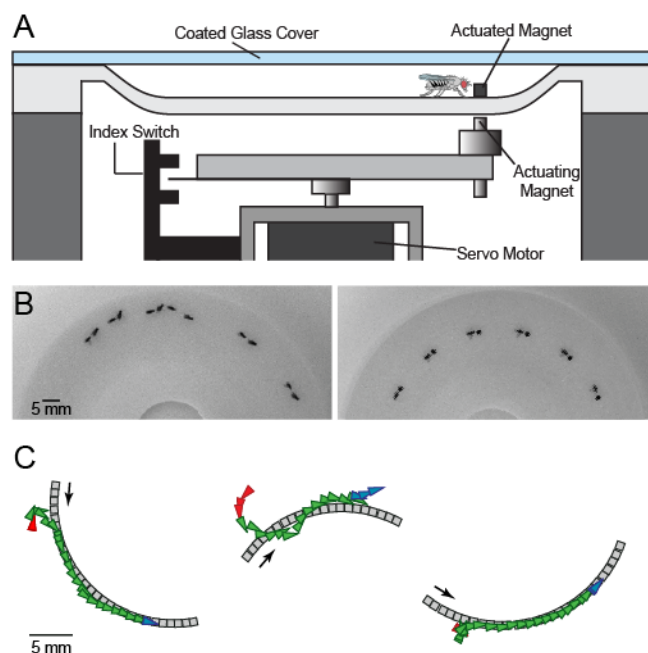
Courtship is very much a stigmergic behavior – the actions of the male and female are coordinated by each of their actions and the signals they send and receive. Thus, a seemingly complex courtship “dance” can arise from a series of simple, externally ordered behaviors. An important consequence, then, is that the context in which a cue is presented becomes important: certain cues may only be perceived or may only be salient at particular stages of behavior or when the receiver is in a particular state. Furthermore, the spatial and temporal nature of these cues can greatly influence the dynamics of sequential behaviors and how they unfold. As a result, any analysis of the role of vision during courtship must take into account the different stages of courtship and the other cues that are available to the male. I seek to understand not just whether vision is used during courtship, but more importantly *how*.

### 1.7 Development of the behavioral apparatus “Flyatar” to study vision in courtship

In order to understand how male flies may use vision during courtship, I developed a behavioral set-up, dubbed “Flyatar” (Zabala *et al.*, 2012), involving an artificial fly dummy (a magnet) that can be programmed to interact with individual or groups of flies (Fig. 1.1A). I can freely modify the dummy’s appearance and patterns of motions, and I can further couple the dummy’s motions to those of the other flies via a sophisticated machine-vision system. The dummy is actuated by a servo motor that sits beneath the arena, hidden from the fly’s view. Chase sequences were identified in post-processing using an automated behavioral classifier. Overall, chases with the dummy appeared qualitatively similar to those with actual females (Fig. 1.1B, video S1).

Flyatar offers several advantages: foremost, it enables us to uncouple chemosensory and visual cues and manipulate just one modality at a time. As a result, I can begin to understand how each of these cues is used during courtship, and how the two modalities may interact. Furthermore, the behavioral arena used is much larger than those traditionally used for courtship studies. By using a larger arena in which males are free to approach or disengage with dummy flies, I can dissect at which stages of courtship different sensory cues become relevant, and thus how they might be used by males to progress through the courtship sequence.

However, Flyatar does present a few key limitations: because the behavioral chamber is so large, I am unable to capture a high resolution image of the male to score behaviors like tapping and licking. I am also limited in the size and types of dummies I can present, in large



**Figure 1.2. Flyatar is a viable system for understanding social interactions in *Drosophila melanogaster*.** (A) Schematic representation of Flyatar, shown in vertical cross-section, not drawn to scale. (B) Series of overlaid stills progressing left to right. Left panel depicts an example chase between a male and female *D. melanogaster*. Right panel depicts an example chase between a male *D. melanogaster* and dummy. (C) Three example chases (at 3 frames  $s^{-1}$ ) identified by my automated behavior classifier. Triangles indicate the positions and orientations of male flies, and the gray squares indicate the positions and orientations of the dummy. Red triangles represent fly position before the chase, black during the chase, and blue after chase end. Black arrows indicate the direction the dummy is initially moving.

part because as dummy magnets get shorter or narrower, their field strength diminishes such that they are no longer compatible with the Flyatar actuating system. And finally, males demonstrate a great deal of variability in their behavior towards the dummies, requiring many trials per manipulation to achieve statistical power. Nevertheless, Flyatar is a powerful system for understanding how males use different sensory cues to progress through the courtship sequence.

### **1.8 Thesis overview**

In the first part of the following thesis, I explored the relative contributions of dummy shape and chemosensation in shaping courtship dynamics, especially courtship initiation. I found that different sensory cues play a dominant role at different stages of courtship: visual cues determine whether males will approach a prospective target, whereas chemosensory cues determine how long the male continues to chase. Next, I examined how vision may be influencing the male's decision on where to position himself relative to a female when chasing and singing. I glued females to the dummy in various orientations, and looked at how well the male can determine the female's polarity, and the cues he uses to do so. I then further looked at how pigmentation affects male chasing, and whether it could be a putative cue affecting male positioning. Finally, I attempted to understand the role of a particular cluster of neurons, the P1 neurons, in courtship, and the types of visual information this cluster may be receiving and acting upon. I activated P1 using TrpA1 and then examined how courtship dynamics were affected.

## Chapter 2. The relative roles of vision and chemosensation in mate recognition of *Drosophila*

### 2.1 Introduction

Animals rely on sensory cues to appropriately classify and respond to objects in their environment. However, the spatial structure of those sensory cues can greatly impact when and how they are perceived. In this first chapter, I examined the relative roles of two sensory modalities, vision and chemosensation, in mate recognition. By pairing male flies with dummies of various shapes, sizes, and speeds, or coated with different pheromones, I determined that visual and chemical cues play specific roles at different points in the courtship sequence. Vision is essential for determining whether to approach a moving object and initiate courtship, and males were more likely to begin chasing objects that were the same approximate dimensions as another fly. However, whereas males were less likely to begin chasing larger dummies, once started, they would continue chasing for a similar length of time regardless of the dummy's shape. The presence of female pheromones on the moving dummy did not affect the probability that males would initiate a chase, but it did influence how long they would continue chasing. Male pheromone inhibited chase initiation and also shortened chase duration. Collectively, these results suggest that male *Drosophila* use different sensory cues to progress through the courtship sequence: visual cues are dominant when deciding whether to approach an object whereas chemosensory cues determine how long the male pursues its target.

### 2.2 Methods

#### *Animals*

Unless otherwise noted, all flies were reared on standard medium in a 16:8 h light/dark cycle at 25°C. The majority of behavioral experiments were performed on 2-4 day old male fruit flies, *D. melanogaster* Meigen of the *Canton-S* strain. Some behavioral experiments (when noted) were performed on males of the *Oregon-R* strain. Males were collected under light anesthesia (CO<sub>2</sub>)

within a few hours of eclosion and housed individually in food vials. A single male was aspirated into the behavioral chamber at the start of each behavioral trial.

I ablated adult oenocytes by crossing ‘+: *PromE(800)-Gal4, tubP-Gal80<sup>ts</sup>*;+’ to ‘+: *UAS-StingerII, UAS-hid/CyO*;+.’ Progeny were kept at 18°C until eclosion. Adult progeny were collected under CO<sub>2</sub> anesthesia and kept at 25°C for at least 24h. Adults were then subjected to three overnight heat treatments at 30°C (on days 2, 3, and 4) and returned to 25°C between treatments. I verified ablations by examining flies for GFP fluorescence on day 5.

For experiments in which a fly was mounted on the dummy, I first anesthetized flies with cold, removed their legs and wings, and then glued them on top of the dummy using UV-cured glue (Newall XUVG-1, Loctite 3104). The dummy was placed into the behavioral chamber and rotated to move head first.

#### *Hydrocarbon extraction and analysis*

Cuticular hydrocarbons were extracted from 2-7 day old male and virgin female *D. melanogaster* and male and female *D. simulans*. Flies were collected under CO<sub>2</sub> anesthesia and housed in food vials in same-sex groups. To extract cuticular hydrocarbons (CHs) for behavioral experiments, groups of several hundred same-sex flies were anesthetized (CO<sub>2</sub>) and placed into 20 mL scintillation vials with 20 µl hexane per fly. The fly-hexane mixture was agitated for two minutes. The hexane with dissolved CHs was pipetted into glass microvials (Microliter Analytical Supplies (Suwanee, GA USA)) and the flies discarded. To extract CHs for quantification purposes, individual flies or dummies perfumed with pheromone were placed in glass microvials containing 50 µl hexane spiked with 10 ng/ml of octadecane (Sigma-Aldrich (St. Louis, MO USA)) as an internal standard, agitated for two minutes, and then the fly or dummy was removed. The extract was then analyzed using a gas chromatography–mass spectrometer (GCMS) consisting of an HP 7890A GC, a 5975C Network Mass Detector (Agilent Technologies (Palo Alto, CA USA)), and a DB5 GC column (J&W Scientific (Folsom, CA USA); 30 m, 0.25 mm, 0.25 µm) with helium as carrier gas. The column temperature profile began at 50° C (held for 4 minutes), ramped at 42.5° C min<sup>-1</sup> to 135° C, followed by a ramp of 25° C min<sup>-1</sup> to 235° C and a ramp of 3° C min<sup>-1</sup> to 285° C, where it was held for 10 minutes. Chromatogram peaks were tentatively identified using the NIST mass spectral library (*ca.* 120,000 spectra) and verified by chromatography with authentic standards and published Kovats

indices. Peak areas for each compound were integrated using ChemStation software (Agilent Technologies).

### *Perfuming of dummy*

Prior to application of CH extracts, dummies were washed with hexane. I then applied 400  $\mu$ l of fly pheromone dissolved in hexane onto a dummy in a glass microvial. The hexane was evaporated under nitrogen, leaving the CHs as a residue lightly coating the dummy.

### *Behavioral assays*

The dummies used in all experiments were nickel-coated neodymium magnets (Armstrong Magnetics, Inc. (Bellingham, WA USA) and K&J Magnetics, Inc. (Pipersville, PA USA)). Dummy shapes were limited as magnet manufacturers could not make smaller dummies. Also, as the magnets get shorter or narrower, their field strength diminishes such that they are no longer compatible with the actuating system.

All dummies were washed in hexane prior to the start of the experiment unless perfumed with pheromone. The behavioral chamber (100 mm wide x 3.5 mm deep, styled after Simon and Dickinson (2010)) was cleaned with acetone and 70% ethanol every three trials when performing experiments with non-perfumed or fly-mounted dummies, and between every trial when using perfumed dummies. After cleaning, a dummy was placed in the behavioral chamber. A fresh dummy was used every three trials when unperfumed or mounted, and every trial when perfumed. Males were then aspirated into the behavioral chamber. The experiment initiated once the male began walking. If the male did not walk for several minutes, or if its wings were damaged, the male was replaced. Experiments ran for 10 minutes. Room lights were turned off to ensure light levels stayed constant between experiments. A camera (acA640-100gm; Basler) above the behavioral chamber automatically tracked and recorded the movements of the fly and the dummy using custom software (downloadable at <https://github.com/ssafarik/Flylab>) based on the Robot Operating System (ROS; Willow Garage (Menlo Park, CA USA)). The dummy was programmed to move in a circle around the arena with a radius of 31 mm with a constant speed of  $5\text{mm s}^{-1}$  unless otherwise stated.

### *Data and statistical analysis*

Data were analyzed using custom code written in Matlab and Python. I developed a behavioral classifier to automatically identify chases. This classifier was based on three criteria: the dummy must be within the front third of the fly's field of view ( $[-\pi/3, \pi/3]$ ), the fly to dummy distance had to be  $\leq 7$  mm, and both conditions must be satisfied for at least 2 s. All identified chases were examined and verified, and obvious false positives were manually removed. I further confirmed the accuracy of my classifier using a technique similar to that used by Schneider, *et al* (2012) by developing a test set of data (taken from trials involving a  $0.8 \times 1.6 \times 1.6$  mm<sup>3</sup> dummy moving at  $5$  mm s<sup>-1</sup>) with the dummy trajectories shifted ahead by 20 seconds (0.5 times the length of one circling of the arena). Over this test set, the classifier falsely identified only 1 chase, compared to the 293 chases identified from the original data. Except for Fig. 3, I defined the start of the chase as not when the male first fixated the dummy, but rather when he approached within 7 mm. Wing extension was scored manually.

According to a two-sample f-test, my data consisted of non-homogeneous variances, and, as determined by the Kolmogorov-Smirnov test, was also not normally distributed ( $p < 0.05$ ). As such, I developed a nonparametric resampling method (Fisher's exact test) whereby I could make pair-wise comparisons using the difference of means as my test statistic. In all comparisons I set a significance level of 5%, with Bonferroni corrections for number of comparisons made. In each figure, significance is denoted in two ways: either letter code when every pair is compared or brackets when only specific comparisons were made.

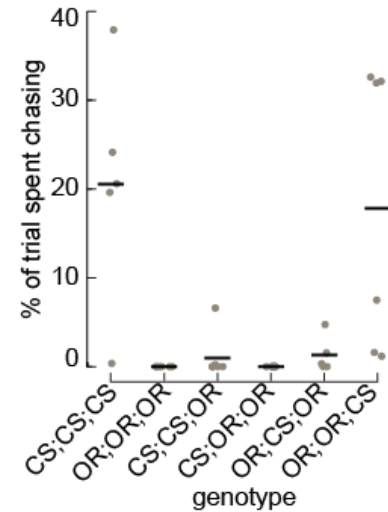
## **2.3 Results**

When first developing my courtship assay, I found that levels of chasing varies in different wild type strains, and not all strains will chase the dummy equally. Specifically, I found that males from two classic *D. melanogaster* strains – Oregon-R and Canton-S—differ in their willingness to chase the Flyatar dummy (Fig. 2.1). By testing hybrids that differ in which chromosome comes from which strain, I find that the third chromosome appears to underlie the difference in chasing levels (Fig 2.1). The difference in chasing levels could be because Oregon-R has lost the ability to chase, or because Canton-S has become less selective. To determine which might be the case, I collected wild *D. melanogaster*, established a new laboratory strain, and found that

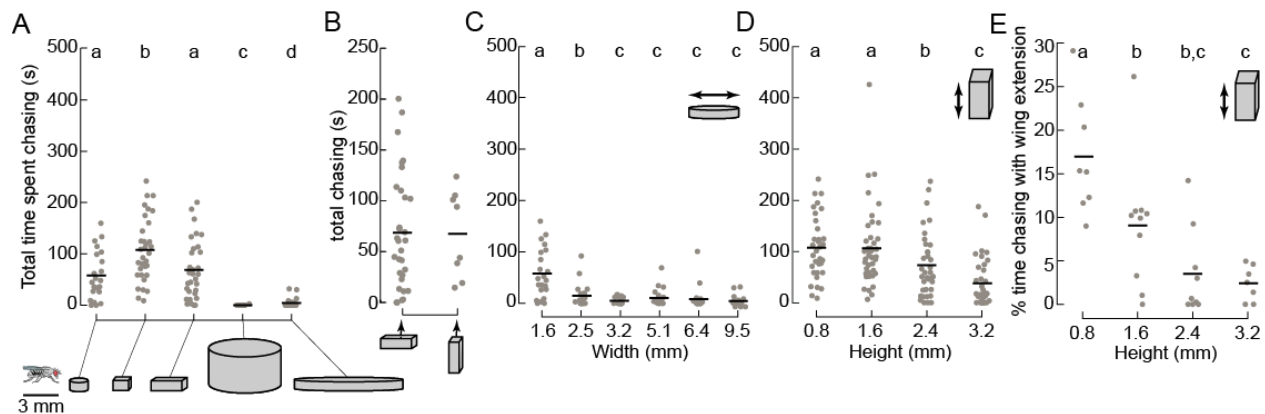
males of this new strain still chased the Flyatar dummy (data not shown). I decided to use the Canton-S strain for the remainder of my experiments.

I paired males with moving dummies of various shapes and sizes and measured the total time spent chasing. I never saw males performing courtship maneuvers not directed at the dummy nor in the dark (data not shown). As seen in Fig. 2.2A, the size and shape of the dummy clearly influence males' propensity for chasing. Males spent little time chasing when paired with dummies that were larger or very differently shaped than another fly. Males also spent little time chasing a larger, isometrically-scaled version of a small attractive dummy. These results suggest that males can indeed distinguish and behave differently towards different objects.

Next, I systematically studied the influence of shape by pairing males with cylindrical dummies of constant height (0.8 mm) but increasing diameter (Fig. 2.2C), or cuboidal dummies of



**Figure 2.1. Chasing responses of different wildtype strains.** Gray dots indicate the response of a single male during a behavioral trial. Black dashes plot the population mean. Males were of the genotype indicated: each chromosome came from one of two strains, either Canton-S (CS) or Oregon-R (OR). Dummy was 1.6 x 0.8 mm large.

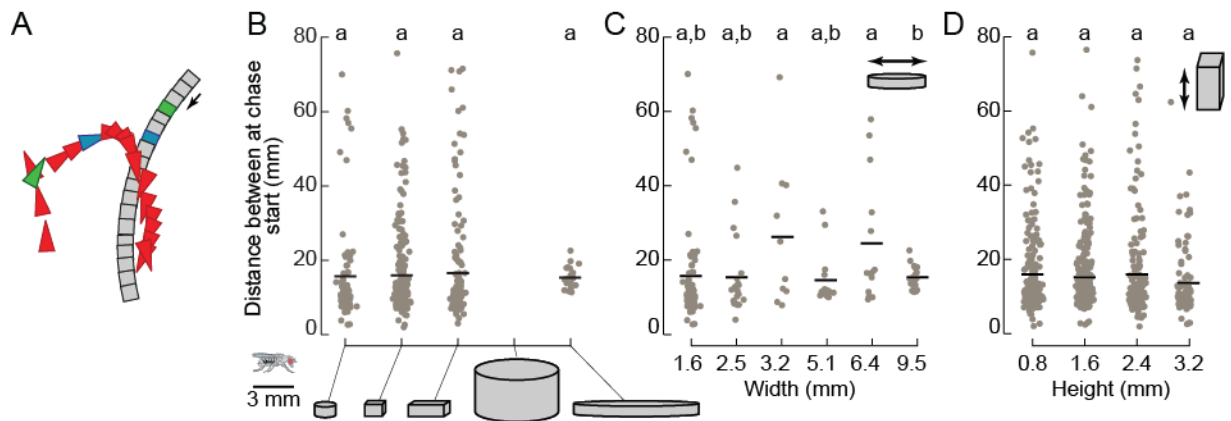


**Figure 2.2. The shape and size of a dummy influence males' likelihood of initiating courtship.** Gray dots indicate the response of a single male during a ten-minute behavioral trial. Black dashes plot the population mean. Groups with the same letter are not significantly different ( $p < 0.05$ , plus Bonferroni correction). (A-D) Total time males spent chasing when paired with a particular dummy. Dummy shape is indicated along the horizontal axis. In panel (A), dummies have following dimensions, from left to right (in mm): 1.6 x 0.8, 1.6 x 1.6 x 0.8, 1.6 x 3.2 x 0.8, 6.4 x 3.2, 9.6 x 0.8. In panel (B), dummy shape with dimensions 1.6 x 3.2 x 0.8 mm is moved so that its long axis is either perpendicular (first column) or parallel (second column) with the direction of motion. Data from the third column of (A) is replicated in the first column of (B). In panels (C) and (D), dummy shape is modified along a single axis, as represented by the shape in the upper right corner of each panel. Data from the first columns of panels (C) and (D) are replicated in panel (A). (E) Percent of time spent chasing that males also had a wing extended.

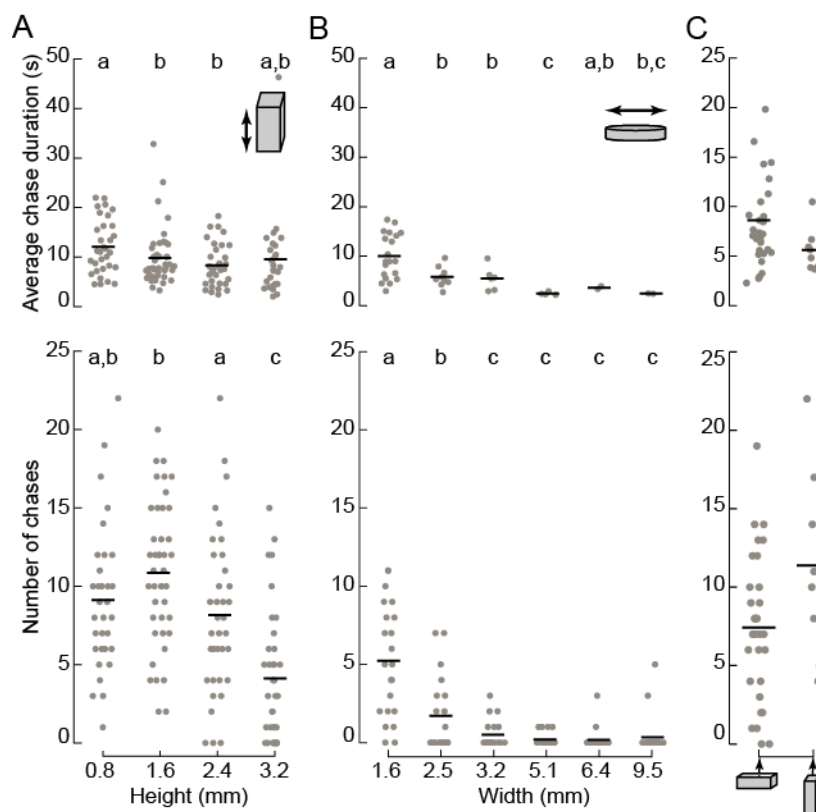
constant width (1.6 mm x 1.6 mm) but increasing height (Fig. 2.2D). Males paired with dummies of smaller radius or decreased height spent the most time chasing (Fig. 2.2C, D, respectively). Males equally chased a non-radially symmetric dummy even when it was rotated by 90° such that its long axis was now perpendicular with the direction of motion (Fig. 2.2B)

For a subset of behavioral trials, I scored each sequence for the presence of unilateral wing extension, a courtship-specific male behavior. I found that males not only chased less when paired with taller dummies, they also demonstrated fewer wing extensions during those chases (Fig. 2.2E). I were not able to record song production, and do not know the extent to which wing extension correlates with singing.

In order to determine if size or shape of the dummy affects the distance at which males first notice the dummy, I calculated the distance between the fly and dummy when the fly first oriented towards and visually fixated the dummy (Fig. 2.3). This fixating maneuver is the earliest courtship behavior I can measure. Because males do not always first fixate the dummy before approaching and chasing it, I had to first identify those chases that began with a fixating maneuver (Fig. 2.3A), which I defined as a turn made by the male  $\geq \pi/8$  radians that brought the dummy into the frontal third of the male's field of view. The distance between the male and



**Figure 2.3. Males' distance from the dummy when visually fixating and initiating chasing does not vary with dummy shape.** (A) Example chase. Triangles indicate the positions and orientations of male flies, and the squares indicate the positions and orientations of the dummy. The black arrow shows the initial direction of dummy travel. Blue shapes indicate the first frame that is classified as a chase by my classifier (see Materials and Methods). I then evaluated the prior frames to find the frame in which the dummy first entered the frontal third of the fly's visual field assuming no head rotation. If, in order to bring the dummy into the frontal third of its visual field, the fly made a turning maneuver  $\geq \pi/8$  (green shapes), the fly was classified as having made an orienting maneuver, which is the earliest courtship behavior I can measure. The distance between the fly and the dummy when this orienting maneuver was made is plotted in B-D. Gray circles represent the responses of males per chase during a ten minute behavioral trial. Black dashes represent the mean of each trial type. (B-C) Dummy shape is modified along a single axis, as represented by the shape in the upper right corner of each panel. (D) The dummy used was cuboid (0.8 x 1.6 x 1.6 mm<sup>3</sup>) and its speed of movement was modified as indicated on the horizontal axis.



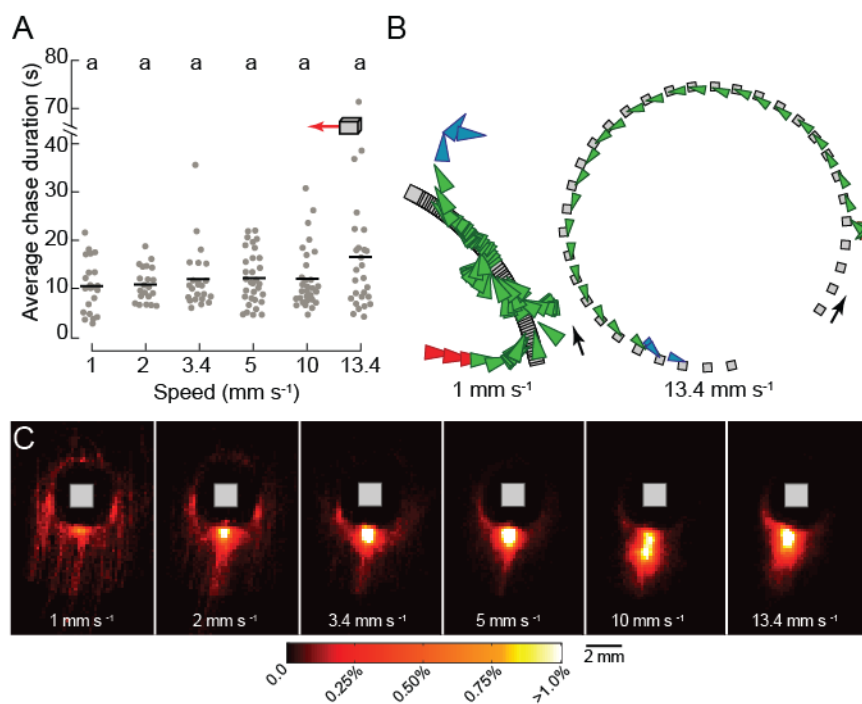
**Figure 2.4. Dummy shape affects males' likelihood of initiating a chase but not chase duration.** Gray dots indicate the response of a single male during a ten minute behavioral trial. Black dashes plot the population mean. Groups with the same letter are not significantly different ( $p < 0.05$ , plus Bonferroni correction). Top panels plot the average chase duration during the ten minute behavioral trials; bottom panels plot the total number of chases males initiated towards the dummy. (A) Responses to changes in height of cuboid dummy with a 1.6 x 1.6 mm base. (B) Responses to changes in width of cylindrical dummy with a 0.8 mm height. (C) Responses to dummy with dimensions 1.6 x 3.2 x 0.8 mm moved so that its long axis is either perpendicular (first column) or parallel (second column) with the direction of motion.

dummy when this turn occurs is plotted in Fig. 3. Overall, dummy size and shape do not appear to influence the distribution of distances at which the male will fixate the dummy.

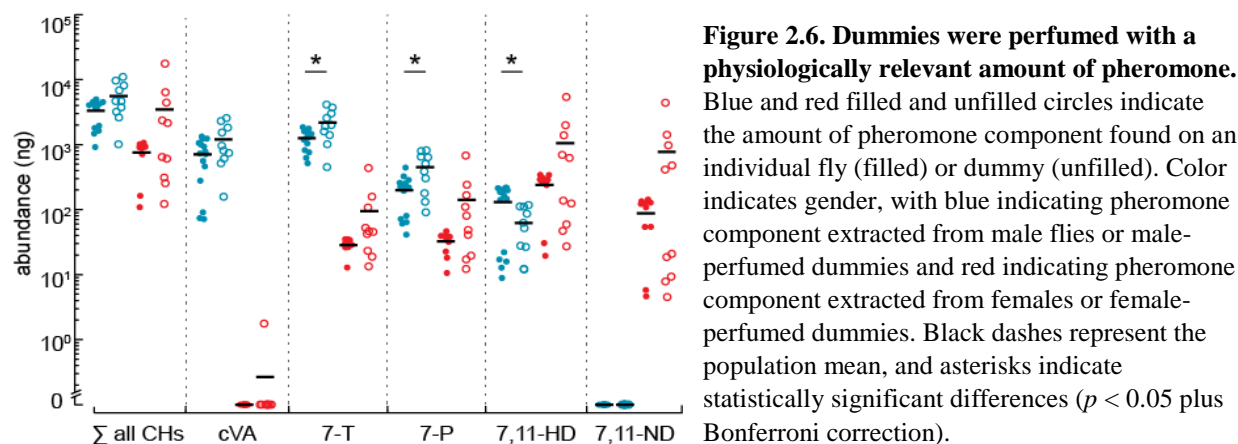
The total chasing time is a function of the number of chases initiated and the durations of those chases. In experiments in which I manipulated dummy height, males paired with the tallest dummies initiated fewer chases (Fig. 2.4A, bottom). Once they had initiated a chase, however, males continued chasing for a similar amount of time regardless of the dummy's height (Fig. 2.4A, top). Even as I varied the dummy's speed over a large range from 1.0 to 13.4  $\text{mm s}^{-1}$ , the chase duration remained roughly constant at 10 s (Fig. 2.5A). This result suggests that the temporal duration of the chase might be controlled by an internal clock that is not strongly influenced by the speed of the target or proprioceptive feedback during walking. There were, however, consistent differences between fast and slow chases. For example, males chasing a

dummy moving at  $13.4 \text{ mm s}^{-1}$  tended to stay directly behind the dummy and made few lateral, circling motions. In contrast, males chasing a dummy moving at  $1 \text{ mm s}^{-1}$  spent more time moving laterally and circling to the sides of the dummy (Fig. 2.5B, C, video S2).

Males initiated fewer chases when paired with wider dummies (Fig. 2.4B, bottom). Chase duration also generally decreased as dummy width increased (Fig. 2.4B, top). Taken at face value, this result suggests that the width of an object influences male behavior more than height. However, the measurements of chase duration are statistically problematic because males rarely chased the larger dummies, resulting in few observations. In addition, unlike increasing height, increasing the dummy's width increases its contact area with the arena floor, which may have increased the vibrations caused by the dummy's motion, inadvertently affecting the male's courtship behavior. Nevertheless, the results of Figs 2.4A and 2.5A suggest that object shape is



**Figure 2.5. Dummy speed does not affect chase duration, but does affect males' circling tendency.** (A) Plot indicates the average chase duration. Males were paired with cuboid dummies ( $0.8 \times 1.6 \times 1.6 \text{ mm}^3$ ) moving at the indicated speed. Gray dots indicate the single male responses during a ten minute behavioral trial. Black dashes plot the population mean. Groups with the same letter are not significantly different ( $p < 0.05$ , plus Bonferroni correction). (B) Example traces of a male chasing a cuboid dummy moving at  $1 \text{ mm s}^{-1}$  (left) or  $13.4 \text{ mm s}^{-1}$  (right),  $2.5 \text{ frames s}^{-1}$ . Triangles indicate the positions and orientations of male flies, and the gray squares indicate the positions and orientations of the dummy. Red triangles represent fly position before the chase, green during the chase, and blue after chase end. Black arrows indicate the direction the dummy is initially moving. Drawn to different scales. (C) Histogram of the positions of male flies during chases in dummy-centered coordinates. The gray square indicates the dummy's position and size. Fly position is measured from its center. Data from all chases from all males is pooled for each panel. Speed of dummy is indicated at bottom of each panel.

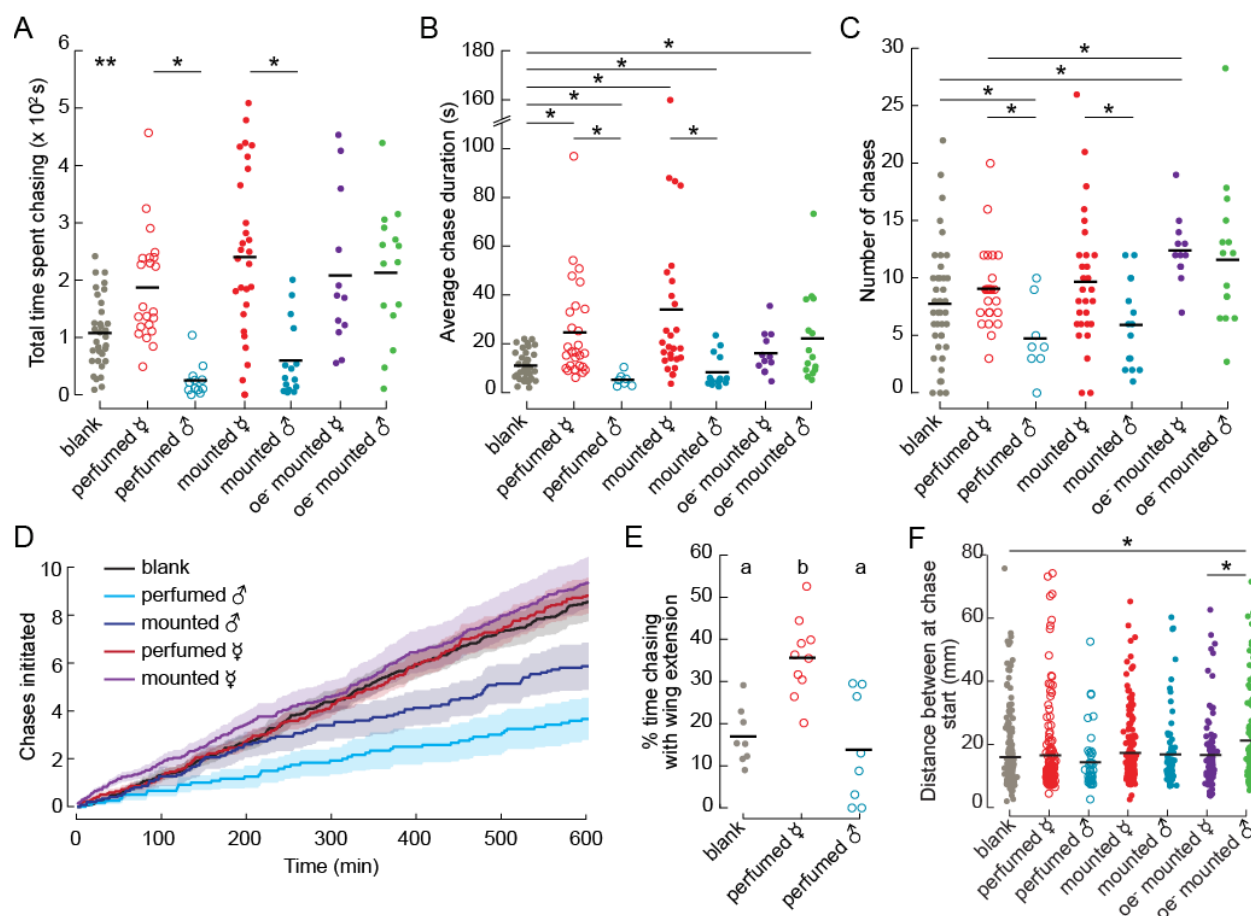


most important for determining initial attractiveness, but once males begin chasing, they will continue chasing for a fixed amount of time if no other information is available. Again, the dummy's orientation relative to its direction of motion did not appear to influence either chasing initiation or duration (Fig. 2.4C).

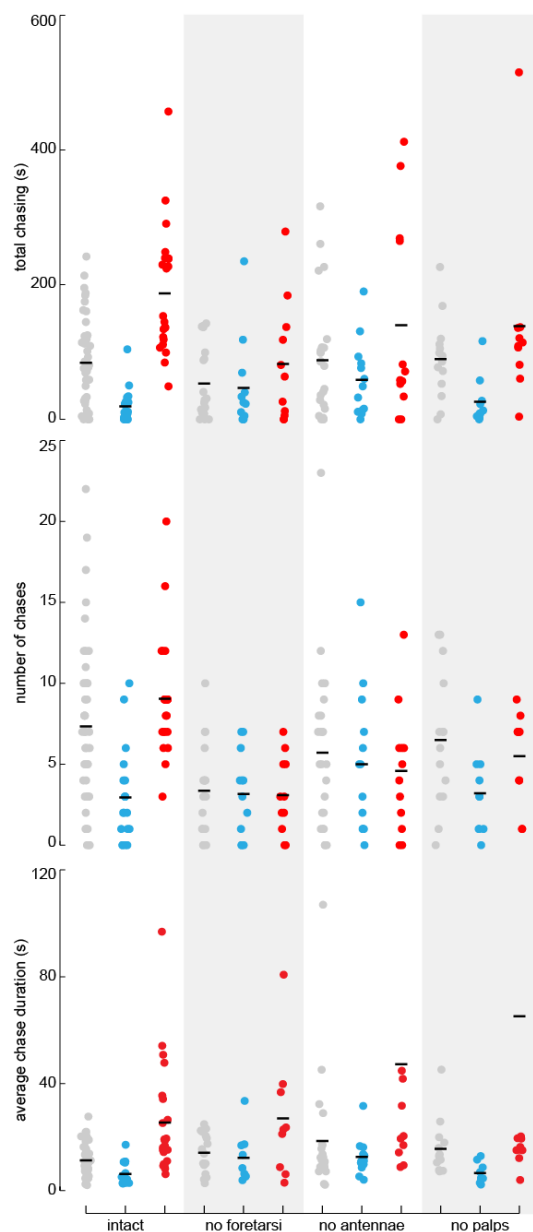
We next examined whether chemical cues, such as pheromones, could influence chase initiation or duration. I coated dummies with pheromone mixtures extracted from male or virgin female flies. In each experiment, I used gas chromatography-mass spectrometry (GCMS) to quantify the amount of pheromone applied to the dummy. Although I tried to apply a consistent and physiologically relevant amount of pheromone in each trial, the amount of pheromone deposited on the dummy varied (Fig. 2.6). Additionally, because of differences in the surface chemistry and morphology of the dummies compared to real flies, I do not know how much of the hexane-extracted pheromone is actually detectable by males. Therefore, I also conducted complimentary experiments with immobilized, wildtype flies mounted on top of the dummy.

Males paired with dummies perfumed with female cuticular hydrocarbons (CHs) spent more time chasing compared to males paired with blank dummies (Fig. 2.7A). Males were not any more likely to initiate chases towards a perfumed dummy (Fig. 2.7C); instead, they chased the perfumed dummies about twice as long as blank ones (Fig. 2.7B). I observed similar results when males were presented with dummies on which a female fly was mounted. The presence of the female roughly doubled the chase duration but did not influence the number of chases initiated.

Males paired with dummies perfumed with male pheromone or dummies with males mounted on them spent less time chasing (Fig. 2.7A). This result was due to both shorter chase durations (Fig. 2.7B) and decreased numbers of chases initiated (Fig. 2.7C). To examine whether



**Figure 2.7. The presence of pheromones affects chase duration but not the number of chasing bouts.** (A-C, E-F) Colored and gray filled and unfilled circles represent the responses of single males during a ten minute behavioral trial. In all cases, the dummy used was cuboid (0.8 x 1.6 x 1.6 mm<sup>3</sup>), which was then further modified as indicated on the horizontal axis. “oe” mounted” refers to dummies mounted with a fly with genetically ablated oenocytes (see materials and methods). Black dashes represent the population mean, and asterisks indicate statistically significant differences ( $p < 0.05$  plus Bonferroni correction). The double asterisk indicates that the population was significantly different from all other conditions in that panel ( $p < 0.05$  plus Bonferroni correction). (A) Total time males spent chasing when paired with the indicated dummy. (B) Average chase duration as performed by the male towards the dummy. (C) Number of chases initiated by the male towards the dummy. (D) Cumulative distribution of when, over the ten minute behavioral trial, males initiated a chase. Data for all males was averaged (dark line) with surrounding fill indicating S.E.M. (E) Percent of time spent chasing that males also had a wing extended. Groups with the same letter are not significantly different ( $p < 0.05$  plus Bonferroni correction). (F) As described in Fig. 3, I identified chases that began with the male making an orienting maneuver to fixate the dummy prior to approaching and chasing the dummy. The distance between the fly and the dummy when this orienting maneuver was made is plotted in F.



**Figure 2.8. Ablation of specific body parts affects male perception of pheromone.**

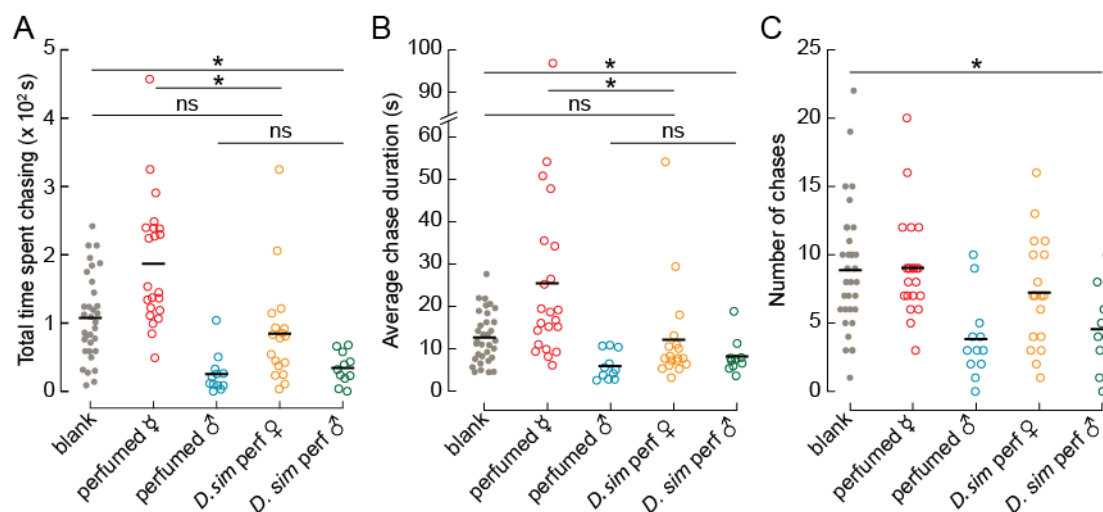
Colored and gray filled circles represent the responses of single males during 10 min behavioral trials. Black dashes represent the population mean. In all cases, the dummy used was cuboid ( $0.8 \times 1.6 \times 1.6 \text{ mm}^3$ ), which was then further modified with no pheromone (gray), male pheromone (blue) or female pheromone (red). Males were manipulated as indicated on the horizontal axis—either intact, or with a bilateral ablation in the foretarsi, antennae, or palps. Note: 2 outliers omitted from data in bottom figure, last column.

this repression of chase initiation was due to the immediate influence of male-specific pheromones or a change in behavior over time, I plotted a cumulative distribution of when, during a behavioral experiment, males initiated chases (Fig. 2.7D). The slopes of all traces are remarkably constant over the 10 min trial, showing that males were not gradually learning to avoid the male-perfumed and male-mounted dummies. This result implies that male flies can sense male pheromones over a great enough distance to prevent chase initiation. I also tracked wing extension for a subset of these trials (Fig. 2.7E).

Males extended their wings significantly more to female-perfumed dummies than to blank dummies, but they did not extend their wings significantly less to male-perfumed dummies ( $p < 0.05$ ), suggesting that female pheromones promote wing extension but male pheromones do not inhibit it. In addition to wildtype flies, I also mounted onto dummies flies that had been manipulated to no longer produce CHs via genetic ablation of their oenocytes ( $oe^-$  flies).  $Oe^-$  flies constitute the most fly-like visual stimuli I can provide that also lack the majority of chemosensory cues normally available. As seen in Fig. 7A, males' responses to dummies mounted with either  $oe^-$  males or  $oe^-$  females were identical, suggesting that males cannot distinguish between male and female flies when oenocytes are absent. Males did chase dummies mounted with  $oe^-$  flies more than they chased unperfumed dummies (Fig. 2.7A), suggesting that the presence of the fly body does increase their

responsiveness. However, my data do not resolve whether this effect was due to a change in chase initiation or chase duration. The mean chase duration of males paired with  $oe^-$  fly-mounted dummies is slightly greater than that of males paired with unperfumed dummies and slightly less than that of males paired with female-perfumed or female-mounted dummies (Fig. 2.7B). However, none of these effects were statistically significant at the  $p < 0.05$  level. The number of chases did increase for males paired with dummies mounted with  $oe^-$  flies, but these results were only significant at the  $p < 0.05$  level when compared with blank dummies or the female-perfumed dummies. My results are not consistent with previous findings that  $oe^-$  flies can act as a super stimulus for male courtship behavior (Billeter *et al.*, 2009): in my experiments, dummies mounted with  $oe^-$  flies did not evoke more chasing from males compared to dummies mounted with wildtype females. I also examined whether the presence of pheromone or fly body affected the distance at which the male first fixated the dummy (Fig. 2.7F). Again, no conditions tested appeared to have an effect.

To assess which groups of chemoreceptors could be mediating the responses to male and female pheromone, I next tested males with ablations in one of the following: foretarsi, antennae, and palps, and paired them with plain dummies, or dummies coated in either male or female pheromone (Fig. 2.8). Males lacking palps or foretarsi do not demonstrate the increased chasing towards dummies coated with female pheromone, with the effect being much greater with



**Figure 2.9. Allospecific pheromone does not elicit an increase in chasing bout length.** Colored and gray filled and unfilled circles represent the responses of individual males during a ten-minute behavioral trial. In all cases, the dummy used was cuboid (0.8 x 1.6 x 1.6 mm<sup>3</sup>), which was then further modified as indicated on the horizontal axis. Black dashes represent the population mean, and asterisks indicate statistically significant differences ( $p < 0.05$  plus Bonferroni correction). Not significant is indicated as ‘ns’. (A) Total time males spent chasing. (B) Average chase duration, as initiated by males towards the dummy. (C) Total number of chases initiated by the male towards the dummy.

removal of the palps. Males lacking foretarsi also do not show decreased chasing towards male pheromone. Males lacking antennae also appear to have a reduced response to male pheromone.

Pheromones are important not only for distinguishing the gender of a potential mate but also its species identity (Wyatt, 2003). I hypothesized that males should chase dummies perfumed with pheromones from other species less than those perfumed with conspecific pheromones. I extracted and perfumed the dummies with pheromone from a closely related species, *Drosophila simulans*. This species co-occurs with *D. melanogaster* in the wild and is visually identical (Sturtevant, 1920). As demonstrated in Fig. 2.9, males spend less time chasing dummies perfumed with female *D. simulans* pheromones compared to dummies perfumed with conspecific female pheromones. Indeed, the response to dummies perfumed with female *D. simulans* pheromones was not statistically different from the response to non-perfumed dummies ( $p < 0.05$ ). This result suggests that *D. simulans* female pheromones do not have an inhibitory effect on *D. melanogaster* males, but rather that female *D. melanogaster* pheromone promotes courtship. The response to dummies perfumed with male *D. simulans* pheromones was identical to the response of males presented with dummies perfumed with male *D. melanogaster* pheromones, suggesting that the inhibitory component of male pheromone may be shared between the two species.

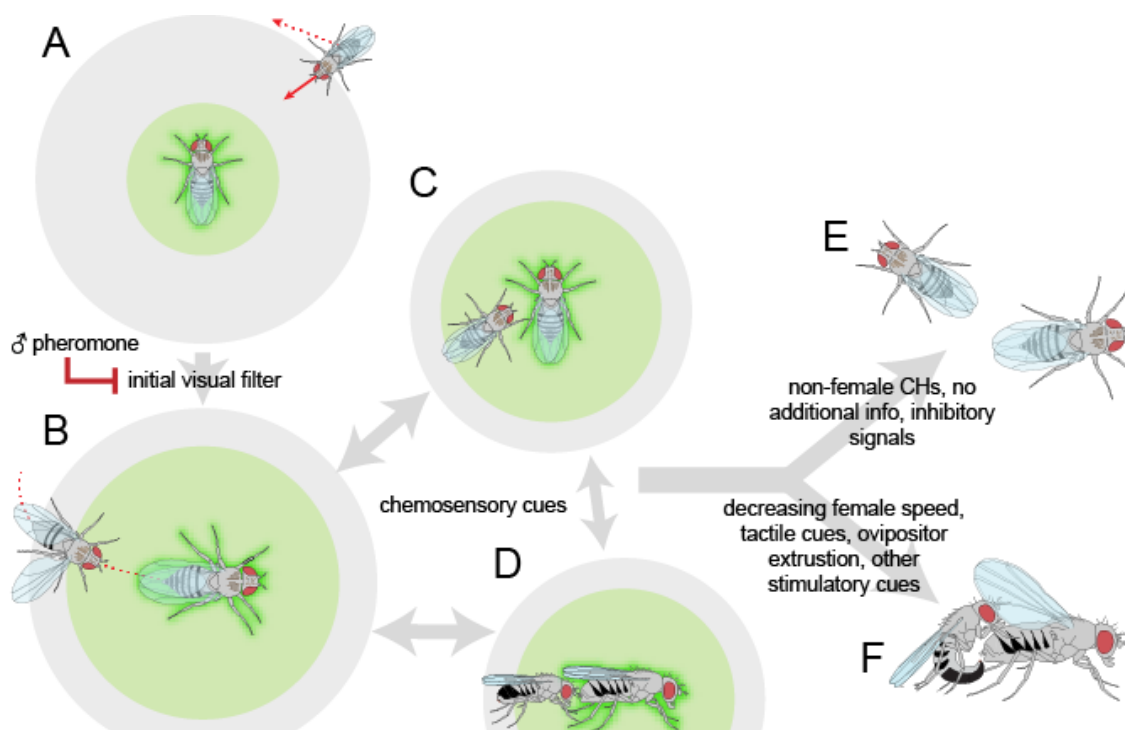
## 2.4 Discussion

By pairing male flies with dummies of various shapes, sizes, speeds, and pheromone coatings, I determined that visual and chemical cues are important at different points in the courtship sequence of *Drosophila* (Fig. 2.10). Males appear to use a simple visual filter to decide whether to approach a moving object, and then continue chasing for a fixed amount of time in the absence of additional cues (Figs 2.4, 2.5). With more information, such as the presence of cuticular pheromones, males will choose to either stop or continue chasing (Fig. 2.7). Female CHs do not affect whether the male will initiate a chase; they only influence how long the male continues chasing once he has already begun. Male pheromones, however, are likely detected at a greater distance and can inhibit chase initiation (Fig. 2.7). Both the shape of the dummy and its pheromone coating influence the amount of wing extension exhibited during a courtship bout (Figs 2.2D, 2.7E). Finally, *D. simulans* pheromone does not increase male chase duration (Fig. 9), implying that female *D. simulans* lack the CH components that prolong chases. Males

responded similarly to dummies whether they were perfumed with male *D. melanogaster* or *simulans* pheromone, perhaps because males of both species produce similar inhibitory compounds.

The ability to visually distinguish among dummies and selectively pursue only those that match specific criteria has been extensively studied in other arthropods (Wehner, 1981) including dragonflies (Olberg *et al.*, 2005), blowflies (Boeddeker *et al.*, 2003a), and horseshoe crabs (Barlow *et al.*, 1982). My study suggests that *D. melanogaster* males can also distinguish amongst objects using a simple visual filter in the initial stages of courtship. This hypothetical filter assesses the shape of the target and its size and influences both whether the male will initiate a chase and how much time it spends extending a wing.

Given the poor spatial resolution of *Drosophila* eyes (Buchner, 1984), it is not surprising that the visual filter that governs courtship is quite coarse. In my experiments, males occasionally chased dummies that were very dissimilar in size and shape to a female (Fig. 2). Furthermore,



**Figure 2.10. Schematic representation of courtship and the ranges of sensory cues encountered.** Dark green outline represents the likely spatial scale of gustatory cues (< 2 mm), light green the range of olfactory cues (0-8 mm), and light gray the range of visual cues (> 0 mm), not drawn to scale. Courtship begins in (A) when the male fly first orients towards the likely female. Using a coarse initial visual filter, the male decides whether or not to approach and chase the female (B). This approach brings the male into range of the female's volatilized pheromones. Based on the gathering of olfactory and gustatory information by tapping (C) or licking (D) the female, males will continue chasing for a given amount of time. The lack of further sensory information or the presence of inhibitory signals will cause the male to abort courtship (E). Alternatively, the presence of other encouraging or excitatory signals will lead to eventual copulation (F).

males were unable to distinguish between male- and female-mounted dummies in the absence of pheromones (Fig. 7), suggesting that they cannot distinguish between sexes using visual cues. Nevertheless, given the importance of courtship for reproductive success, one might have expected the males to exhibit a bit more selectivity. One explanation is that my experimental procedure of isolating males soon after eclosion yielded less selective animals. Alternatively, males may simply not need to discriminate fine details in the early stages of courtship, and perhaps being less selective even offers an advantage when competing with other males. Also, in situations in which the male is presented with a choice of many objects with different shapes, the small bias in the male's shape preference may result in his chasing the most female-like shape. Nonetheless, my study shows that males behave differently towards different visual objects and thus exhibit some level of discrimination during the earliest stage of courtship.

By raising flies in complete darkness, Spieth and Hsu (1950) proved that vision is not necessary for mating in *D. melanogaster*. However, later studies revealed that male courtship behavior differs markedly in the light and dark. Whereas males in both situations exhibit many of the same courtship maneuvers, males in the dark never orient towards and fixate a female, nor do they extensively chase (Cook, 1980). Rather, males use a very different strategy in the dark to locate females and initialize courtship: they extend both wings and walk in a zig-zag pattern of motion until they collide with another fly (Cook, 1980; Crossley and Zuill, 1970; Krstic *et al.*, 2009). Should the male thus encounter a fly in the dark, he would already be close enough to sample its pheromones and choose whether to court it. In contrast, males in the light typically begin courtship by visually fixating the potential mate at a distance and then approaching and chasing her (Greenspan and Ferveur, 2000). Thus, males appear to have two different modes for locating females and initiating courtship. In the light, visual cues dominate the male's search, whereas in the dark, tactile and chemosensory cues take precedence

The fact that both visual and the combination of chemosensory and tactile cues can initiate courtship helps reconcile the results of my experiments with other recent studies examining courtship initiation (Kohatsu *et al.*, 2011; Pan *et al.*, 2012). In both these prior studies, vision alone was insufficient to evoke male courtship. For example, Kohatsu and coworkers (Kohatsu *et al.*, 2011) presented tethered males with a female abdomen but observed no chasing unless males first touched the abdomen. One possibility for the discrepancy with my results is that tethering may raise the arousal threshold necessary to elicit chasing. In the other

study, Pan and coworkers (Pan *et al.*, 2012) reported that males would not chase simple rubber band dummies until they concurrently activated P1 neurons. However, the visual stimulus used in this prior study was essentially 2-dimensional, because the rubber band dummies were located underneath a clear arena floor on which the flies walked. My experiments demonstrate that height is an important factor in courtship initiation and the stimulus used by Pan and coworkers may not have been the right shape (or could not be viewed on the correct region of the retina) to initiate courtship without concurrent neuronal activation.

Once a male has initiated courtship, my experiments demonstrate that pheromones then influence chase duration. How and at what distance are the males detecting the pheromones? The majority of *Drosophila* pheromones are heavy hydrocarbons of low volatility. A study by Farine *et al.* (2012) found that about 0.2% of a fly's CHs are light enough to be volatile under natural conditions, but collecting detectable amounts required placing 10-20 flies in small 8 ml vials for two hours. Furthermore, several studies that have placed males in a behavioral chamber in the presence of air that had been piped over virgin females failed to detect an increase in male courtship (Antony and Jallon, 1982; Tompkins *et al.*, 1980). A study by Tompkins *et al.* (1981) did show that female extract could increase male courtship behavior at a distance, but at 8 mm the effect was no longer significant. In my experiments, I found that males could fixate and approach dummies at distances greater than 8 mm (Fig. 3). This result, combined with my observation that female pheromones did not increase the number of chases initiated, suggests that vision, when available, is the predominant cue used by males to initiate chases. However, in my experiments, male pheromone did lower males' propensity to initiate courtship, suggesting that males can sense male pheromones at a far enough distance so as to inhibit courtship initiation. Several male-specific pheromones, such as cVA, are light compared to other CHs and volatile to some degree (Farine *et al.*, 2012; Jallon *et al.*, 1981).

Although fly pheromones may not act as the primary cue to initiate courtship, once a male approaches the perfumed dummy, he might detect the CHs by either olfaction or gustation. Both olfactory (van der Goes van Naters and Carlson, 2007) and gustatory (Bray and Amrein, 2003; Miyamoto and Amrein, 2008; Watanabe *et al.*, 2011) neurons respond to fly CHs. Unfortunately, my experiments do not provide the visual resolution necessary to determine whether males actually contacted the dummies with their legs, wings, or mouthparts during

chases. However, given the distribution of fly-dummy distances I measured during a typical chase (Fig. 5C), males were certainly close enough to do so.

Sensory cues have a spatial structure that will greatly impact how and when animals perceive them. As a result, when determining which sensory cues are relevant for a given behavior, it is critically important to study such behaviors on a spatial scale that is ethologically plausible. By using a larger arena in which males were free to approach or disengage with their targets, I were able to dissect at which stages of courtship different sensory cues become relevant, and thus how they are used by males to progress through the courtship sequence. This approach is essential if I are to better understand the neural mechanisms underlying the early stages of male courtship behavior.

## Chapter 3. Pigmentation and shape affect male's positioning during courtship chases

### 3.1 Introduction

Thus far, I have mainly examined the role of visual cues when approaching a potential mate from afar. However, vision is doubtless important during a chase, if not least to enable a male to track and pursue a female. In the dark, males rarely chase (Agrawal *et al.*, 2014; Cook, 1980), and the chases they do make are severely abbreviated, presumably lasting only as long as the male can maintain contact (Connolly *et al.*, 1969; Cook, 1980). Furthermore, vision may also influence how a male positions himself around a female and when he chooses to sing. Successful copulation requires appropriate positioning of the male around the female – ultimately, the male must target copulation attempts towards the female's abdomen. Males may additionally aim their song towards the female by using the wing closest to her when singing (Cook, 1980; Kohatsu *et al.*, 2011). Evidence from a recent study from the Yamamoto lab (Kimura *et al.*, 2015) suggests that in the absence of olfactory cues, males use vision to locate a stationary female's abdomen: courting *Or83b*<sup>2</sup> mutant males still bias their position towards a female's abdomen unless placed in dark conditions. Coen *et al.* (2014) found that blind males, compared to intact males, produce song over a wider range of inter-fly distances and orientations.

In this chapter, I examine whether males actively modulate their position during chases in response to visual cues. I find that males maintain a specific distance between themselves and the dummy during the chase that depends on dummy shape and pigmentation. Pigmentation also affects chasing duration, and males demonstrate a marked preference for a dummies of an intermediate, gray pigmentation. I also find that males will bias their position when chasing towards a female's abdomen, regardless of her direction of motion, and that this bias still occurs towards females lacking oenocytes. Males chasing dummies painted multiple colors are able to recapitulate these results.

## 3.2 Methods

### *Preparation of dummy*

For experiments in which a female fly was attached to the dummy, I first anesthetized flies with cold, removed their legs and wings, and then glued them either on top or to the side of the dummy using UV-cured glue (Newall XUVG-1, Loctite 3104). The dummy was placed into the behavioral chamber and rotated to move so that the abdomen was pointed in the directions indicated. For painted dummies, I used Flat Ultra Behr paint (ultra pure white or supernova), and for black-painted dummies, a top coating of black India ink (Winsor and Newton). Dummies were painted in just clear nail polish (Wet n' Wild) or with paint and nail polish as the final coating. Dummies were then left out for at least 24 hours before use.

### *Statistical analysis*

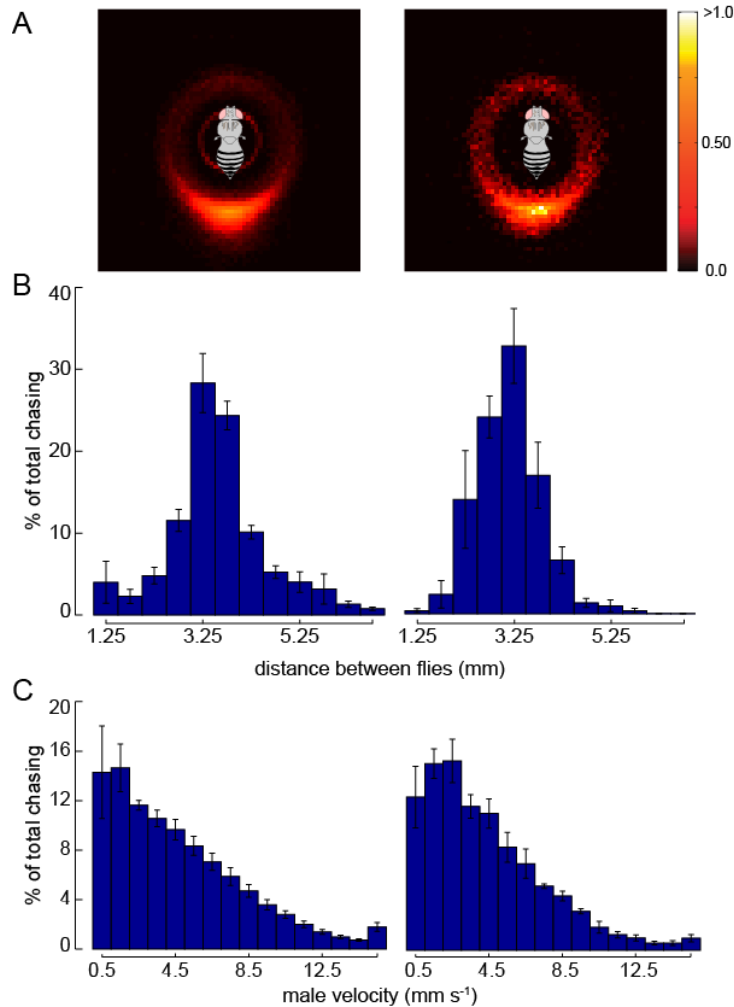
A two-sample t-test was used to compare the means of all histograms. All other statistical analysis is the same as noted in 2.2.

*All other methods remain as noted in 2.2.*

## 3.3 Results

I began by first analyzing chase sequences between a male and virgin female fly to understand how the male positions himself during a typical chase (Fig. 3.1). Males would vary the distance between themselves and the female throughout a chase, but were generally about 1-2 body lengths away (about 3.25 mm) (Fig 3.1B). Males would additionally extend a wing to sing for up to 20% of the total time spent chasing (data not shown). When extending a wing, males were much more likely to be located around the sides or in front of the female (Fig. 3.1A). They were also likely to be located closer to the female compared to their location when not singing (Fig. 3.1B).

In order to assess whether males use visual cues to position themselves behind a female, I more closely examined males' position when chasing shapes of increasing height or width. As dummy height increased, the distance between the male and dummy also increased (Fig. 3.2A). Thus, even though chase duration stayed relatively constant regardless of the dummy height (see Ch. 2), I found that male chasing was affected by dummy shape after all: males chase taller

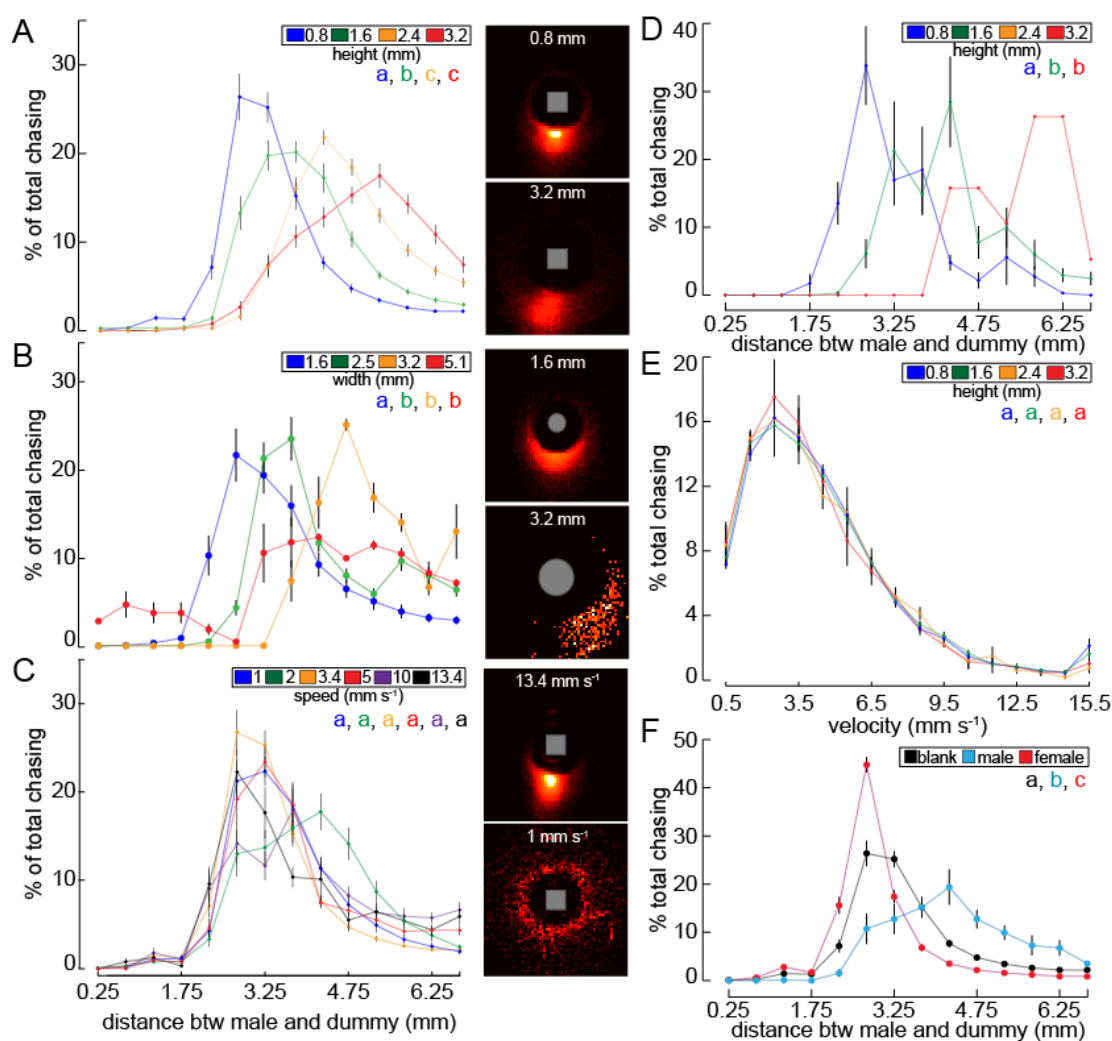


**Figure 3.1. Males' position relative to a female during chases subtly changes when he extends a wing to sing.** Left panels include all data when male is identified as chasing. Right panels include data only from when male is additionally extending a wing. (A) 2-D histogram of the positions of male flies during chases in female-centered coordinates. Fly position is measured from the male's center. Data from all chases from all males is pooled. Scale bar indicates percent of data. (B) Histogram of relative distance between male and female during chases (as measured from their centers). (C) Histogram of male's velocity during chases. Error bars depict standard deviation, and bars plot averages over flies. Data in this panel comes from 8 different males.

Surprisingly, males showed a strong preference for the gray magnet and rarely chased the white or black magnet. To further verify that the preference was not due to a chemical property of the paint, I combined the black and white paints to make a gray similar to the other gray tested, and found that males continued to demonstrate a strong chasing response to this new, "mixed" gray dummy (Fig. 3.3A). I also assessed male attraction to the differently painted

dummies from further away. Similarly, males' position shifted further from the dummy as the dummy's width increased (Fig. 3.2B), though this shift was harder to assess since males so rarely chased the wider dummies. The distance between the male and dummy was not influenced by the dummy's speed (Fig. 3.2C) but was influenced by the dummy's pheromone coating (Fig. 3.2F). Males did not change their velocity when chasing dummies of different heights (3.2E), but do demonstrate a shift in the relative distance when the male also extends his wing (3.2D).

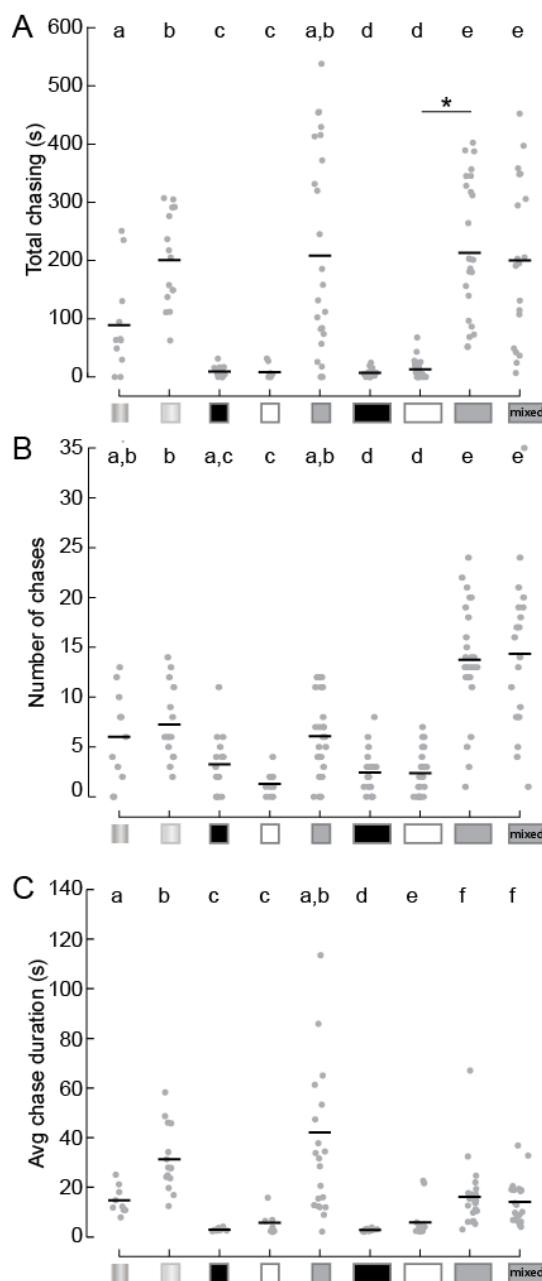
I wondered whether dummy color, and thus its relative contrast to the background, could also influence male chasing and positioning. I painted dummies one of three shades: white, gray, or black, and then examined levels of total chasing (Fig. 3.3A). All dummies tested were coated in the same clear coating to control for chemosensory content of



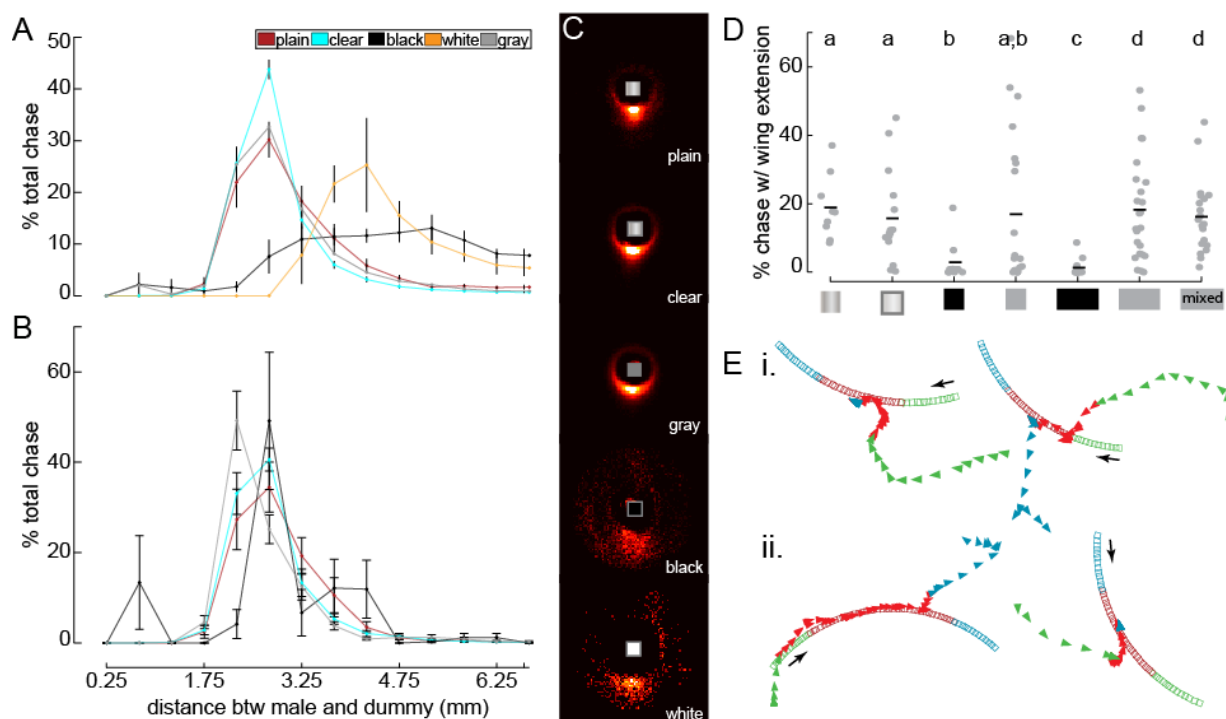
**Figure 3.2. Males shift to chasing from further away as dummy height or width increases.** (A-C, F) Histogram of relative distance between male and dummy during chases (as measured from their centers). Right panels are 2-D histogram of the positions of male flies during chases in dummy-centered coordinates when chasing the indicated dummy. Color-scale is the same as in Fig. 3.1. Data is oriented such that the female-dummy would be moving towards the upper left corner and the left side is towards the center of the arena. (A) Chasing response towards dummies of increasing height. From left to right,  $n = 32, 42, 36, 36$ . (B) Chasing response towards dummies of increasing width. Traces have been adjusted to counter the increasing width of the dummy by subtracting the difference in radius from the narrowest dummy. From left to right,  $n = 22, 20, 20, 20$ . (C) Chasing responses towards dummies of decreasing velocity. From left to right,  $n = 24, 22, 23, 33, 33, 30$ . (D) Histogram of relative distance between male and dummy during chases when the male is also extending a wing. Color-scale is the same as in Fig. 3.1. (E) Histogram of male's velocity during chases. (F) Chasing responses towards dummies of differing chemosensory coatings. From left to right,  $n = 32, 22, 19$ . Error bars depict standard deviation, and data points plot averages over flies. Groups with the same letter do not have significantly different means (two sample t-test,  $p < 0.05$ , plus Bonferroni correction).

dummies on a black background. However, males placed in a black behavioral chamber never demonstrated any chases towards any of the dummies regardless of their coating (data not shown).

Unlike the responses to differently shaped dummies (Fig. 2.4), males chasing the white or black dummy demonstrated decreased levels of not only chase initiation, but also chase duration (Fig. 3.3B, C). Thus, males both approached these dummies less and also pursued them for a shorter period of time. I then examined chase positioning and noticed that although the male's maintained distance between himself and the dummy was similar whether chasing the plain, clear-coated, or gray painted dummies, when chasing the black and white dummies, the male never approached as closely (Fig. 3.4A). This pattern is especially apparent in distributions of the male's position around the dummy (Fig. 3.4C). This difference could be because the male terminates his chase before getting much closer, or because during the chase, he overall maintains a greater distance. An examination of chase trajectories reveals that during almost all chases towards the black dummy, the male terminates the chase soon after beginning the approach (Fig. 3.4Ei, video S3). Lengthier chases are much more common when males court the white dummy (Fig. 3.4Eii, video S3). When chasing the black dummy, males also spend less time with an extended wing (Fig. 3.4D), but dummy color does not appear to



**Figure 3.3. Males demonstrate a preference for gray-painted dummies.** Gray dots indicate the response of a single male during a 10 min behavioral trial. Black dashes plot the population mean. Dummy coating is indicated along the horizontal axis: plain, clear-coated, black, white, gray, etc. In last column, black and white paint was mixed to create a new, “mixed” gray. In the first 5 columns, dummy was 1.6 x 1.6 x 0.8mm. In the last 4 columns, dummy was 1.6 x 3.2 x 0.8 mm. From left to right, n = 11, 15, 15, 10, 24, 18, 22, 25, 20. Groups with the same letter do not have significantly different means (Fischer’s exact,  $p < 0.05$ , plus Bonferroni correction). (A) Total time males spent chasing. (B) The total number of chases males initiated towards the dummy (C) Average chase duration

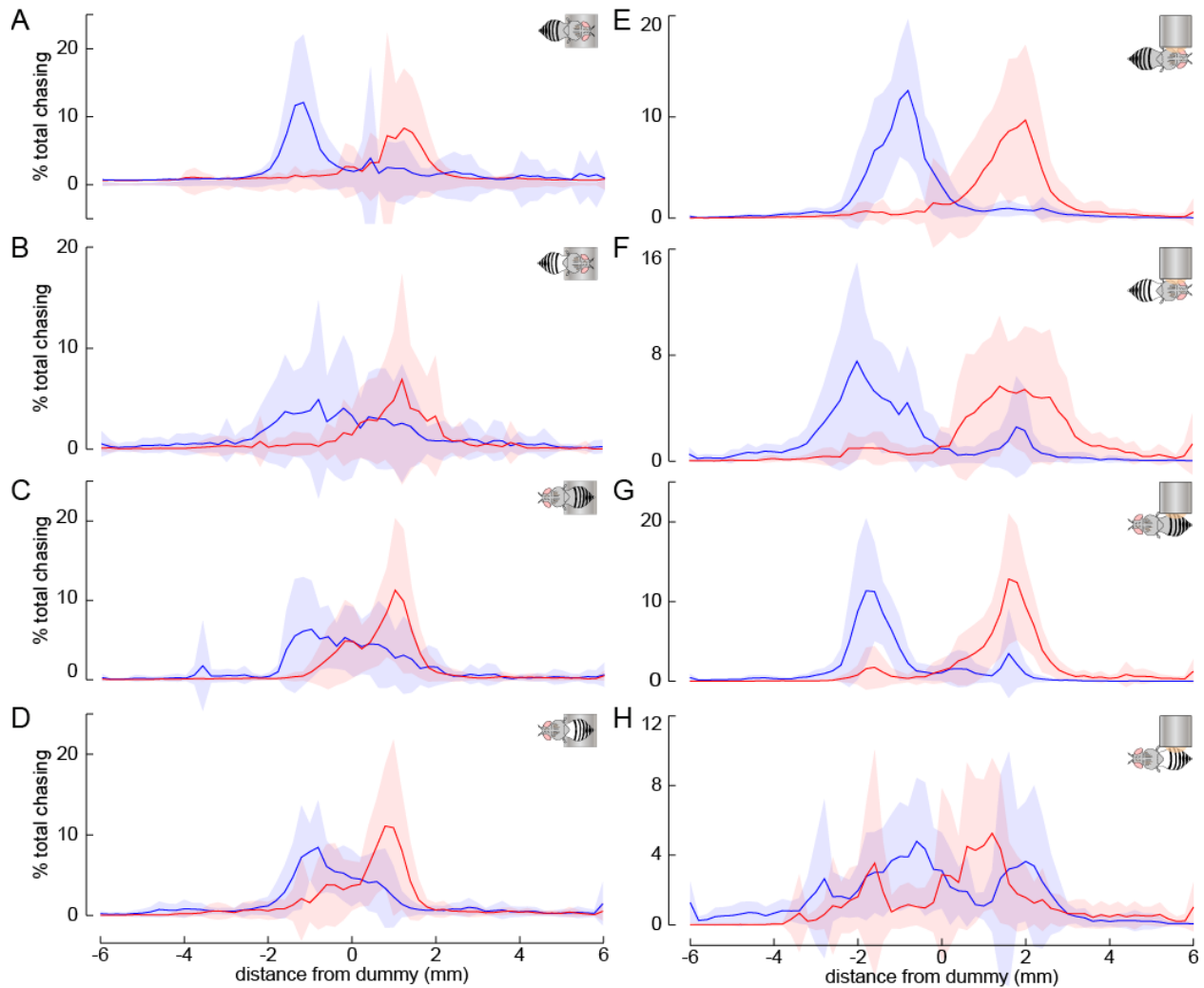


**Figure 3.4. Males rarely closely approach black or white dummies and demonstrate very little wing extension during these chases.** (A-B) Histogram of relative distance between male and dummy during chases (as measured from their centers). Error bars depict standard deviation, and data points plot averages over flies. From left to right,  $n = 11, 15, 15, 10, 24$ . (A) includes data from all chases. Traces for black and white dummy are significantly different from the rest (two-sample t-test,  $p < 0.05$ , plus Bonferroni correction). (B) includes data from only when male is also extending a wing. No traces are significantly different (two-sample t-test,  $p < 0.05$ , plus Bonferroni correction) (C) 2-D histogram of the positions of male flies during chases in dummy-centered coordinates when chasing the indicated dummy. Color-scale is the same as in Fig. 3.1. Data is oriented such that the female-dummy would be moving towards the upper left corner and the left side is towards the center of the arena. (D) Percent of time spent chasing that males also had a wing extended. Gray dots indicate the response of a single male during a 10 min behavioral trial. Black dashes plot the population mean. Dummy coating is indicated along the horizontal axis: plain, clear-coated, black, gray, etc. In last column, black and white paint was mixed to create a new, “mixed” gray. In the first 4 columns, dummy was  $1.6 \times 1.6 \times 0.8$  mm. In the last 3 columns, dummy was  $1.6 \times 3.2 \times 0.8$  mm. Groups with the same letter do not have significantly different means (Fischer’s exact,  $p < 0.05$ , plus Bonferroni correction). (E) Example traces of a male chasing a black (i) or white (ii) dummy,  $2$  frames  $s^{-1}$ . Triangles indicate the positions and orientations of male flies, and the squares indicate the positions and orientations of the dummy. Red represents position before the chase, green during the chase, and blue after chase end. Black arrows indicate the direction the dummy is initially moving.

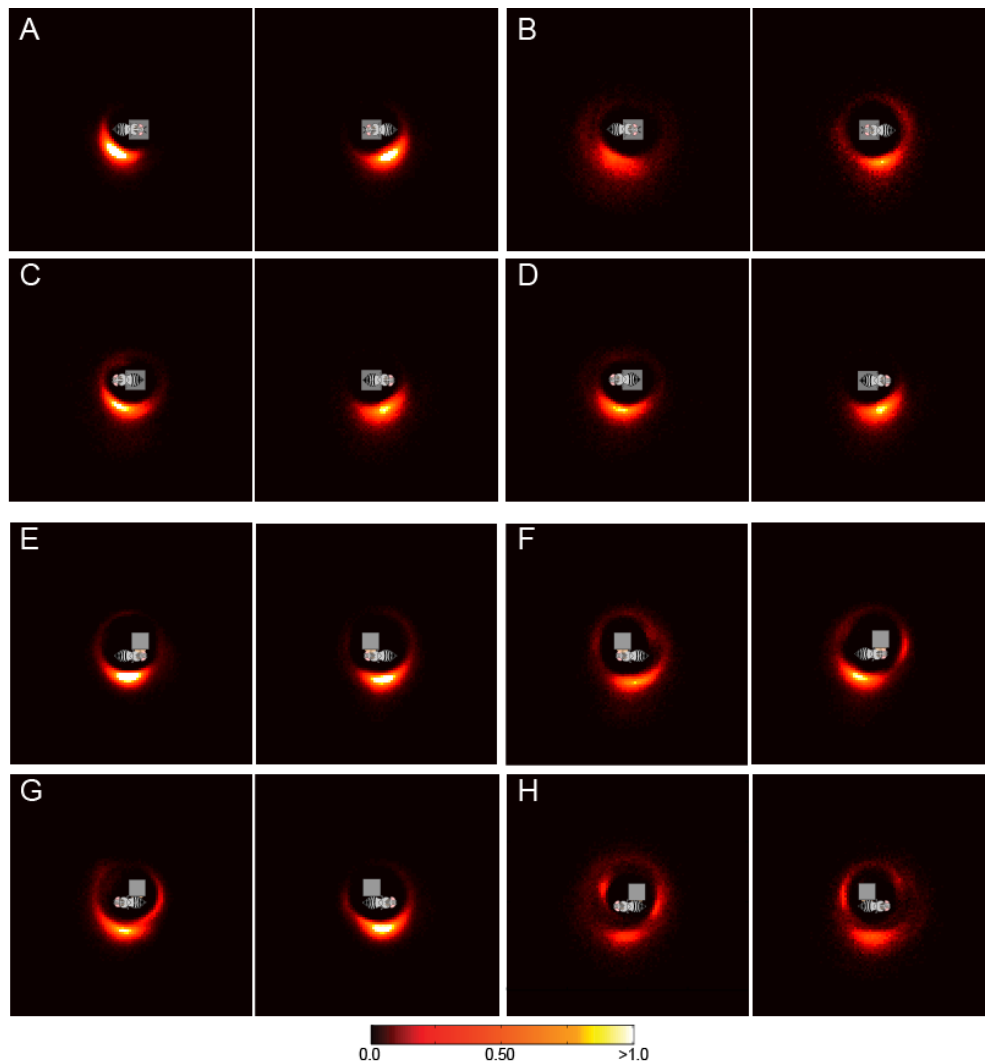
influence the distance between the male and dummy when the male extends his wing (Fig. 3.4B).

Previous research has shown that males will bias their courtship attempts towards the female's abdomen and may be able to use vision to do so. To test this hypothesis, I glued female *D. melanogaster* to either the top of a plain dummy with their abdomen (Fig. 3.5A, B) or head (Fig. 3.5C, D) hanging off, or to the side, with either their head (Fig. 3.5E, F) or abdomen attached (Fig. 3.5G, H) and the other side free. This dummy-female is then moved around the arena such that she appears to be moving sideways, with either the head or abdomen pointed towards the center of the arena. Fig. 3.6 shows the two-dimensional histogram of male positioning around the dummy-females described above. When the female is glued to the top of the dummy, I found that while the female's direction of motion does have some influence on male positioning, in response to a wildtype female whose head is attached to the dummy with her abdomen free, males will nevertheless robustly bias their chasing towards the female's abdomen regardless of her direction of motion (Figs. 3.5A, 3.6A). This preference is maintained even when an oenocyteless ( $oe^-$ ) female is used, though the preference does degrade and the male's position is not quite as tightly coupled to the abdomen (Figs. 3.5B, 3.6B). When the female is rotated so that her abdomen is attached to the dummy and her head free, I do still see a slight preference for the female end not glued to the dummy, suggesting that the dummy or glue is having some effect on the male's preference (Figs. 3.5C, D, 3.6C, D). Nevertheless, the preference for the free head is definitely decreased compared to the free abdomen, and the biasing towards the abdomen is similar whether males were chasing wildtype or  $oe^-$  dummy-females.

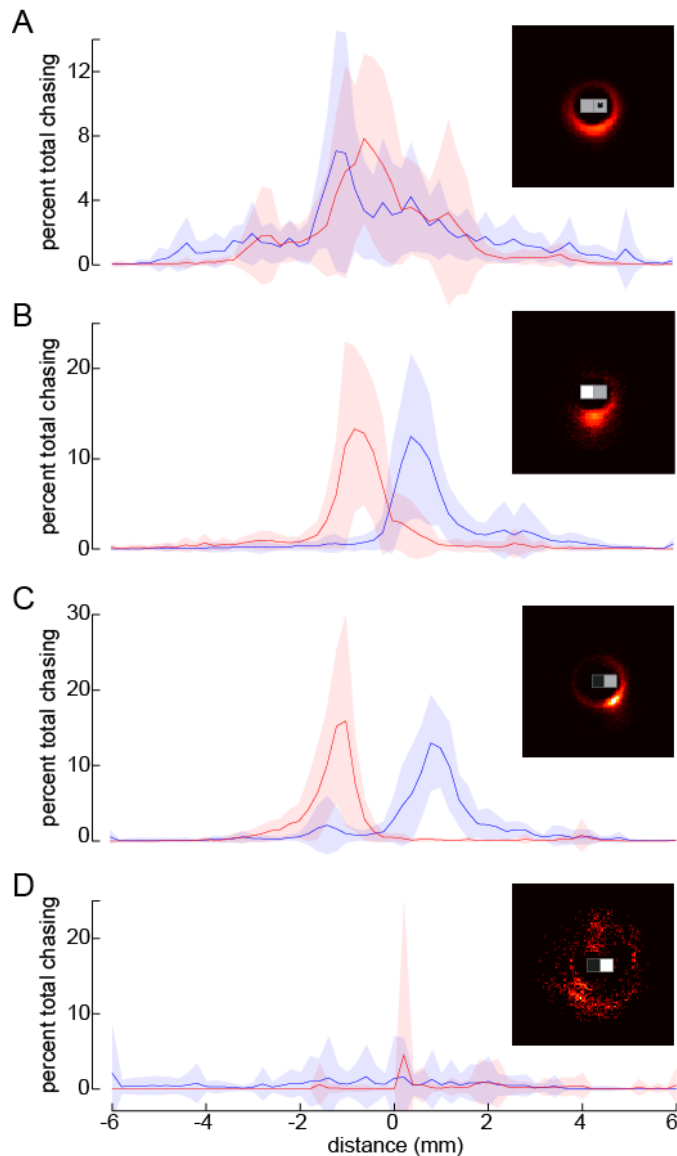
The data from males chasing a female glued to the side of the dummy is harder to interpret (Figs. 3.5E-H, 3.6E-H). An advantage of gluing the female to the side of the dummy is that the entirety of the female is at the same horizontal position on the male's retina. However, the shape is instead disrupted if the male views the dummy from the sides where the female is not attached. Males do demonstrate a strong preference for the abdomen whether chasing a wildtype or  $oe^-$  female (Figs. 3.5E-F, 3.6E-F), but when the wildtype female is rotated so that her abdomen is attached to the dummy, the male continues to strongly pursue the unattached female end (Figs. 3.5G, 3.6G). However, there is a slight increase in chasing of the other side closer to the abdomen. When males chase a rotated  $oe^-$  female whose abdomen is attached to the dummy, this preference disappears, and the male is no longer strongly biased to either dummy end (Figs.



**Figure 3.5. Males bias their chasing towards the female abdomen.** Histograms plotting male position while chasing along radial axis extending from arena center (left) to arena edge (right), centered so that 0 reflects the dummy's center. Icon in upper right corner indicates the dummy used and its orientation in the blue trace. For red traces, dummy-female is horizontally flipped such that the non-attached end is now oriented towards the outside of the arena. Dark line plots population mean, shaded portion standard deviation. White abdomen indicates oenocyteless female. (A-D) Female is glued to the top of the dummy. (E-H) Female is glued to the side of the dummy. (B, D, F, H) Female's oenocytes have been genetically ablated.



**Figure 3.6. Males bias their chasing towards the female abdomen.** 2-D histograms of male positions during chases in dummy-centered coordinates when chasing the indicated dummy-female. Data is oriented such that the female-dummy would be moving towards the upper left corner, and the left side is towards the center of the arena. White abdomen indicates oenocyteless female. Scale bar indicates percent of data. (A-D) Female is glued to the top of the dummy. (E-H) Female is glued to the side of the dummy. (B, D, F, H) Female's oenocytes have been genetically ablated.

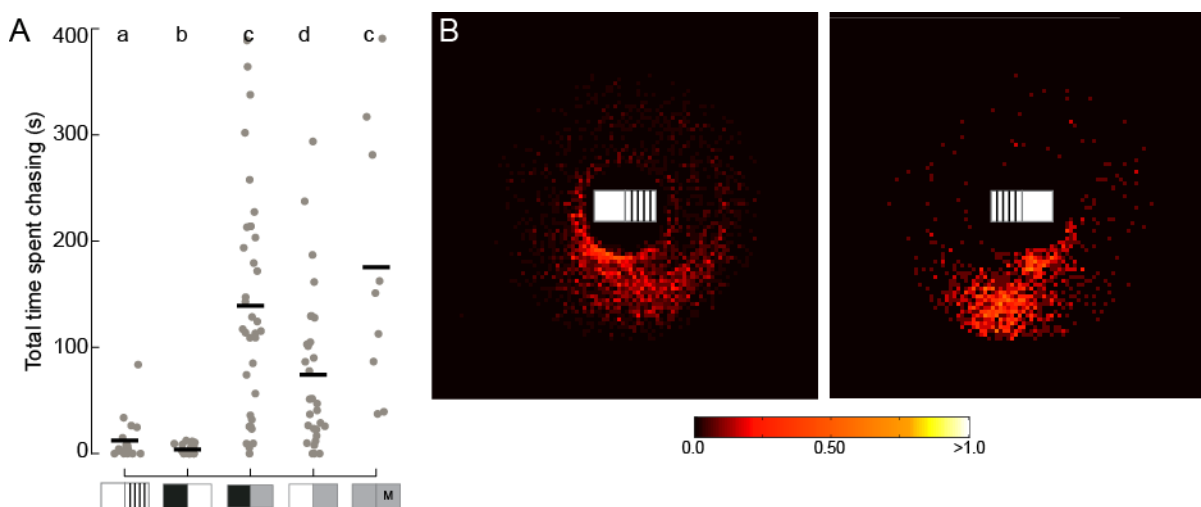


**Figure 3.7. Males paired with dummies painted multiple colors will bias their position towards the preferred color.** Histograms plotting male position while chasing along radial axis extending from arena center (left) to arena edge (right), centered so that 0 reflects the dummy's center. Icon in inset panel indicates the dummy used and its orientation in the blue trace. For red traces, dummy is rotated by 180°. Dark line plots population mean, shaded portion standard deviation. Inset: 2-D histograms of male positions during chases in dummy-centered coordinates when chasing the indicated dummy. Data is oriented such that the dummy would be moving towards the upper left corner. Color-scale is the same as in Fig. 3.6.

mechanism underlying the abdomen preference.

3.5H, 3.6H). If anything, the male appears to be slightly biased towards the abdomen and the aversive effect of the dummy or glue seems to have disappeared. Together, these results suggest that the male does have a preference for the wildtype female abdomen, but whether he can locate the abdomen in the absence of chemosensory cues synthesized by the oenocytes remains inconclusive.

I next assessed whether pigmentation could be a potential cue enabling males to bias their positioning. To do so, I painted a dummy half one color and half another color (Fig. 3.7). When the ends were painted with the two grays, males demonstrated no preference for either end of the dummy (Fig. 3.7A). Similarly, when the dummy was painted half black, half white, the males did not demonstrate a strong preference towards either end, though their pattern in positioning was similar compared to either color alone (Fig. 3.7D). However, when painted half gray and half black or white, males demonstrated a strong preference for positioning themselves near the gray end (Fig. 3.7B-C). Thus, pigmentation could indeed be a potential



**Figure 3.8. Males are not strongly attracted to a black and white striped dummy.** (A) Total time males spent chasing. Gray dots indicate the response of a single male during a 10 min behavioral trial. Black dashes plot the population mean. Dummy coating is indicated along the horizontal axis: half white, half striped; half black, half white; half black, half gray; half white, half gray; half gray, half mixed gray. Dummy measured 1.6 x 3.2 x 0.8 mm. From left to right,  $n = 18, 21, 35, 30, 9$ . Groups with the same letter do not have significantly different means (Fischer's exact,  $p < 0.05$ , plus Bonferroni correction). (B) 2-D histograms of male position during chases in dummy-centered coordinates when chasing the indicated dummy. Data is oriented such that the female-dummy would be moving towards the upper left corner and the left side is towards the center of the arena.  $n = 9$  for each.

Given the low resolution of the male's vision, I reasoned that a series of thin black and white stripes may appear gray, and thus attractive, to the male. Thus, I painted a dummy with half black and white stripes, and the other half white. Males still rarely chased the striped dummy (Fig. 3.8A). However, in the little chasing data collected, males demonstrated an interesting patterning to their positioning around the dummy: at a greater distance, males demonstrated at most a small bias towards the striped end of the dummy. As they neared the dummy, they demonstrated an increasingly stronger bias towards the white end of the dummy.

### 3.4 Discussion

Vision, specifically dummy shape and size, was in the previous chapter shown to be an important long range cue used by the male when deciding whether or not to initiate courtship. In this chapter, I have shown that color could be another such cue: dummy color definitely influences male's likelihood of chasing. However, I have also found that vision continues to be an important cue during the chase itself after the male has already approached the dummy and begun chasing. Males use visual cues such as shape and color to decide how to position themselves around the female, especially when determining the distance at which they should

chase. These visual cues also influence how much time a male spends extending a wing and therefore the amount of song he will produce. Shape may even influence the distance between the male and female at which the male will extend his wing. And finally, pigmentation is sufficient to bias male positioning around a dummy.

How a male positions himself around females during courtship can significantly impact his attractiveness as assessed by the female. For example, the further a male is from a female, the more the amplitude of his song will be attenuated. Behavior experiments have demonstrated that females use song amplitude as a measure of male quality (Shirangi *et al.*, 2013). Evidence from the Murthy lab suggests that males can dynamically modulate song patterning based on inter-fly distance, and vision may be crucial to making such judgements (Coen *et al.*, 2014). Additionally, the male's ability to position himself around the female may also aid in pursuit and preventing competitors from stealing his courtship target. Eventually, in order to achieve successful copulation, the male must locate the female's genitals.

We are still unable to conclusively determine whether or not males can use visual cues to locate a female's abdomen. My experiments demonstrate that males do appear to have some ability to do so. My results may not be as clean as those obtained by Kimura *et al.* (2015) because I used a moving, versus stationary, dummy-female. Cook (1980) found that males were much more precise when tracking the abdomen of a stationary female. Additionally, because it is very difficult to make the dummy invisible to the male, and because I had to use glue to attach the female to the dummy, the dummy likely influenced the male and his chasing. Ideally, I would want a behavioral experiment in which I could modulate a female's direction of motion but still have all means of doing so completely hidden from the male. Finally, because females do produce pheromones independent of the oenocytes, I were unable to completely eliminate all chemosensory cues coming from the female simply by ablating the oenocytes. Eventually, I will need to additionally disrupt the male's ability to detect chemosensory cues to definitely prove that they do not underlie the male's ability to target the female's abdomen.

That males were so much more attracted to the gray dummy compared to the black dummy was very surprising. I expected that, because of its high contrast relative to the white background, males would actually demonstrate a heightened attraction towards the black dummy. This result suggests that males use pigmentation as a cue when deciding if an object is potentially a female. I hypothesize that different mechanisms mediate the shortened bout

duration and difference in positioning seen in males courting the black or white dummy: in the case of the white dummy, males may simply not be able to easily distinguish the dummy from the white background. Because of this difficulty, they frequently lose track of the white dummy after initiating a chase, hence the shortened bout duration. However, occasionally the male is able to track the white dummy for extended bout durations, perhaps because he is able to maintain physical contact with it, thus leading to some of the longer chases that I occasionally observed. In the case of the black dummy, males are certainly able to see it, as demonstrated by another series of experiments using Flyatar (Zabala *et al.*, 2012). Rather, males must be using some sort of visual filter that inhibits initiating chases towards darkly pigmented objects. In the few cases where chases are initiated, they are quickly ended. One possible mechanism underlying rapid chase termination is a strong expansion cue from a high-contrast object.

These experiments are among the first to suggest that males use pigmentation information to make courtship-related decisions. I have certainly not explored the full space of colors to determine how tightly tuned male preference is, and how it may relate to female cuticle pigmentation. Interesting follow-up experiments include testing males of other, more darkly pigmented species to see if they demonstrate a different preference compared to *D. melanogaster* males. These experiments also offer a potentially powerful means of behaviorally assessing the neural circuitry underlying male color vision.

## **Chapter 4. Despite concurrent activation of P1 neurons, males still use visual cues to dynamically modulate courtship.**

### **4.1 Introduction**

Recent studies have implicated one particular cluster of neurons, the P1 cells, as the site of integration of sensory cues during courtship. These cells are activated by female pheromones and visual motion and inhibited by male pheromones (Clowney *et al.*, 2015; Kohatsu and Yamamoto, 2015; Kohatsu *et al.*, 2011). Concurrent activation of P1 neurons and presentation of moving or stationary dummies elicits male courtship towards the dummy, even when the male otherwise demonstrates no interest in the dummy (Inagaki *et al.*, 2013; Pan *et al.*, 2012).

Flyatar offers a unique advantage in examining the role of the P1 neurons during courtship and chasing. Because of the large arena in which males can engage with the dummy over multiple chasing bouts, I can assess whether the P1 neurons are involved in the early decisions of whether to court an object, the decision to continue courting, or some combination. Second, studies have shown that P1 neurons respond to visual motion, but only after first contacting chemosensory cues (Kohatsu and Yamamoto, 2015). According to my model of how males use visual cues to guide courtship (Fig. 2.10), this result suggests that the P1 neurons are not the source of the initial visual filter when males decide whether an object looks female-like enough to initiate courtship. Because I can present differently shaped dummies, I can test whether activating P1 neurons disrupts this initial visual filter or if males maintain their shape selectivity. Finally, I can also assess whether male positioning during P1-activated courtship is “normal” – does the male maintain the ability to change his position in response to visual cues?

In the following chapter, I activated P1 neurons using the heat-sensitive cation channel TRPA1, and then examined the resulting changes in amounts of chasing, chase structure, and chase positioning.

## 4.2 Methods

### *Animals*

All flies were reared on standard medium in a 16:8 h light/dark cycle at 25°C. Behavioral experiments were performed on 2-5 day old male fruit flies, *D. melanogaster* Meigen of either the *Canton-S* strain or males expressing dTRPA1 in genetically specified neurons. Males were collected under light anesthesia (CO<sub>2</sub>) within a few hours of eclosion and housed individually in food vials. A single male was aspirated into the behavioral chamber at the start of each behavioral trial.

We activated P1 by crossing *UAS-dTrpA1* to either one of two lines: *R71G01-GAL4* (provided by B. Baker) or *P1-GAL4* (a “split” GAL4 line) (provided by D. Anderson). All strains were backcrossed into the Canton-S genetic background. Expression patterns of both GAL4 lines was verified via confocal imaging.

### *Behavioral assays*

Chamber remained at room temperature (aprox. 20-22°C) for control condition, or was heated to either 27°C or 30°C using a ceramic infrared heat emitter (Zoo Med 150W). Otherwise, behavioral assays were conducted as described in 2.2.

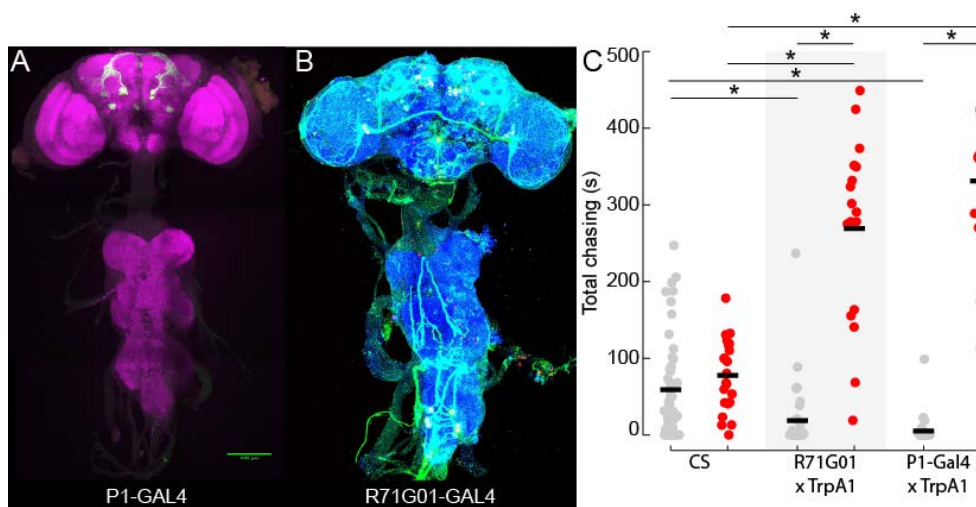
### *Behavioral tracking*

Videos of behavioral trials were analyzed using DuoTrax, software designed by the Branson lab at Janelia Research Campus (<https://github.com/kristinbranson/cbtrack>).

*All other methods remain as noted in 2.2.*

## 4.3 Results

We had two different Gal4 lines to label and activate P1 cells. *P1-GAL4* is highly selective, but only labels about 9-10 cells, or about half, of the total cluster (Fig. 4.1A). *R71G01-GAL4* is much less selective (Fig. 4.1B). I expressed dTRPA1 in males of both genotypes and looked for differences in their chasing behavior when activated by heat. The baseline chasing of *P1-GAL4* and *R71G01-GAL4* males without heat activation was fairly low (Fig. 4.1C). When the behavioral chamber was heated to 30°C, males spent the majority of the ten-minute behavioral



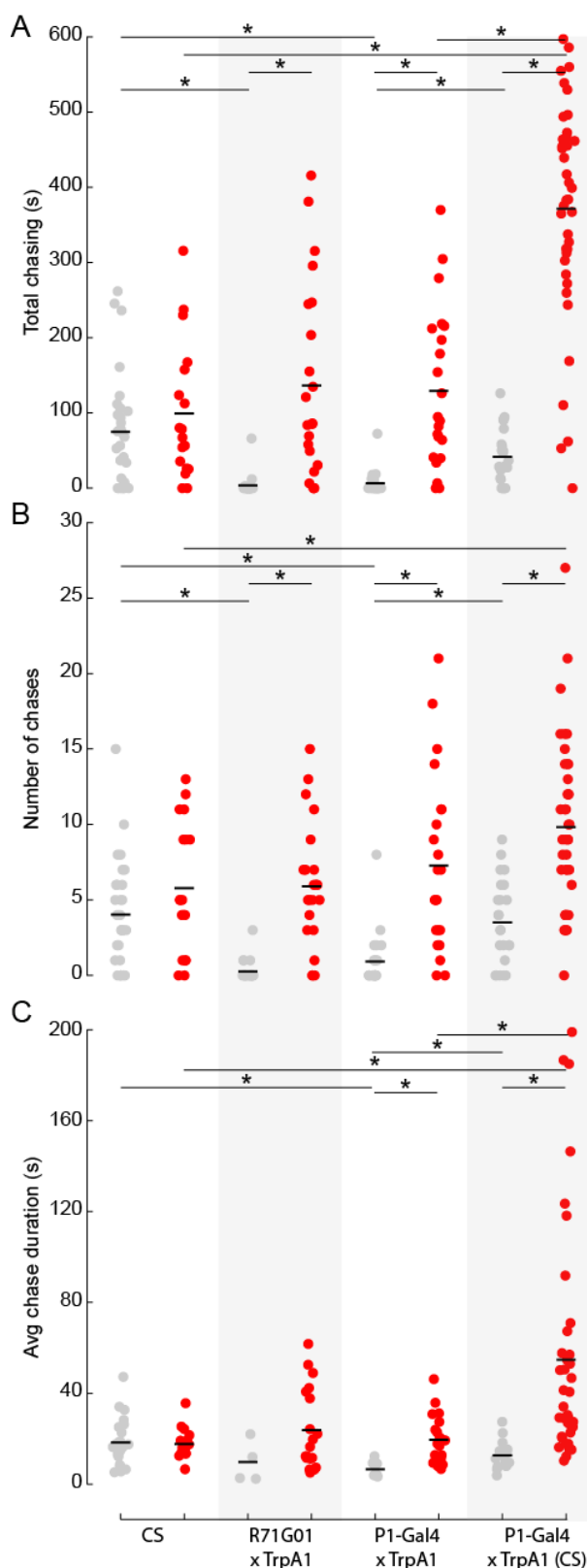
**Figure 4.1. Males with activated P1 neurons demonstrate increased levels of chasing.** (A-B) Confocal images of the two GAL4 lines labelled with green fluorescent protein (GFP). (C) Total time males spent chasing. Red and gray dots indicate the response of a single male during a 10 min behavioral trial. Black dashes plot the population mean. Male genotype is indicated along the horizontal axis. Red dots indicate trials in which the behavioral chamber was heated to 30°C. Dummy was 1.6 x 1.6 x 0.8mm and moved at 5 mm s<sup>-1</sup>. From left to right, n = 49, 22, 31, 17, 32, 16. Asterisks indicate statistically significant differences (Fischer's exact,  $p < 0.05$  plus Bonferroni correction).

trial chasing and frequently demonstrated what appeared to be instances of abdomen curling and jumping onto the dummy. Additionally, it became difficult to separate chasing bouts: during chases, males would frequently look away from the dummy and then immediately back (movie S4). I was unsure if these movements away indicated chasing bout ends as males so quickly reengaged with the dummy.

As such, I backcrossed one of the lines, P1-Gal4, into the Canton-S background and further decided to lower the activation temperature to 27°C. I additionally increased the dummy's speed from 5 mm s<sup>-1</sup> to 13.4 mm s<sup>-1</sup>. Lowering the temperature and increasing the dummy's speed abolished the instances of abdomen curling, jumping, and rapid maneuvers away and back towards the dummy (movie S4). Backcrossing lines into the Canton-S background did increase the baseline level of chasing significantly, though it also interestingly increased levels of chasing in the heated fly as well (Fig. 4.2A). The increase in total chasing seen in males with activated P1 neurons is due to an increase in both chase duration and chase initiation (Fig. 4.2B, C).

Next, I paired males with their shape, but still demonstrated a decrease in chasing response as the dummy increased in height (Fig. 4.3A). This increase in total chasing of shorter dummies is mostly due to an increase in the number of chasing bouts initiated (Fig. 4.3B, C). Male's ability to retain shape selectivity argues against the P1 neurons I activated being the site

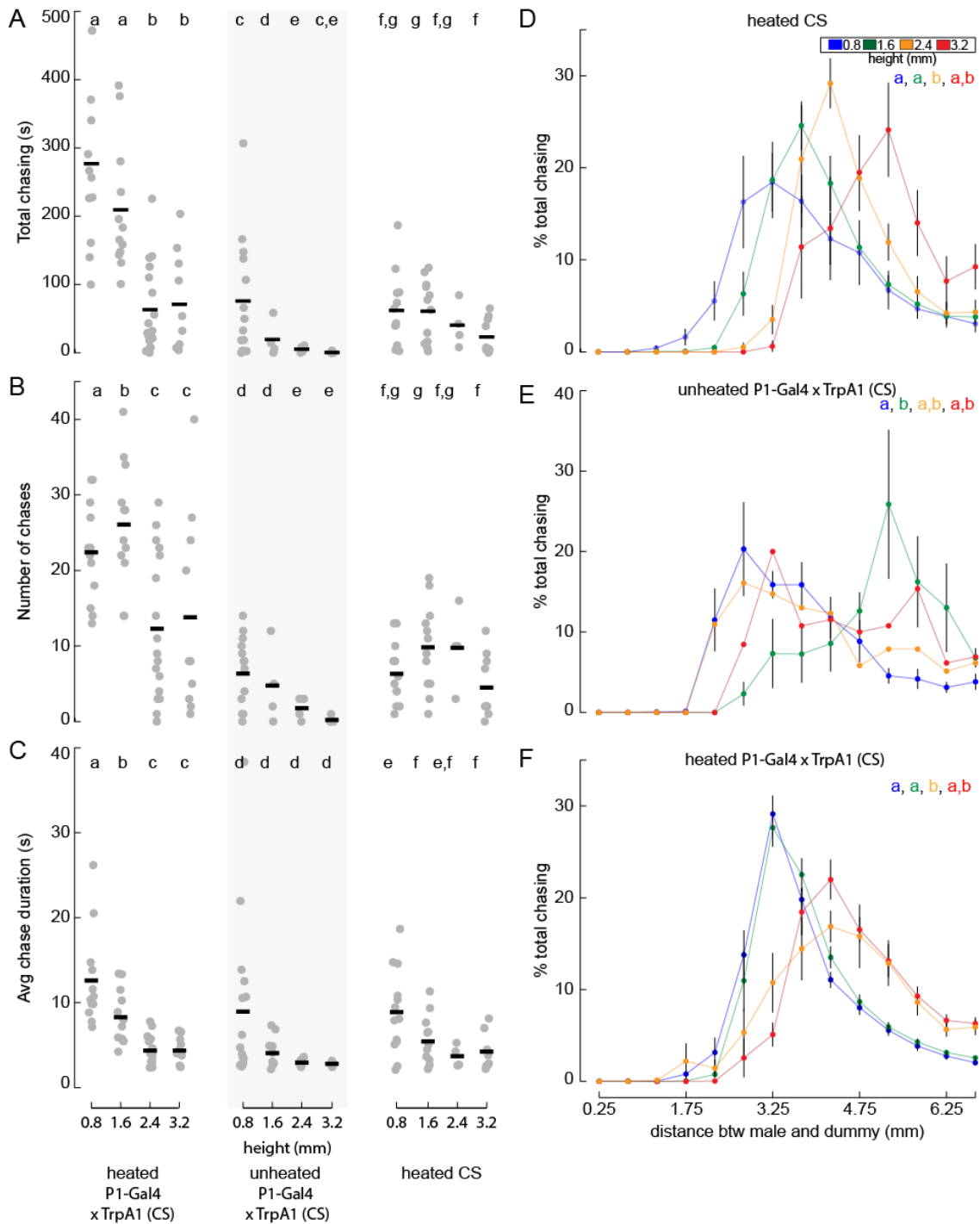
of the initial visual filter. I next examined how males positioned themselves while chasing. Unfortunately, I was not able to resolve how well control males without P1-activation were able



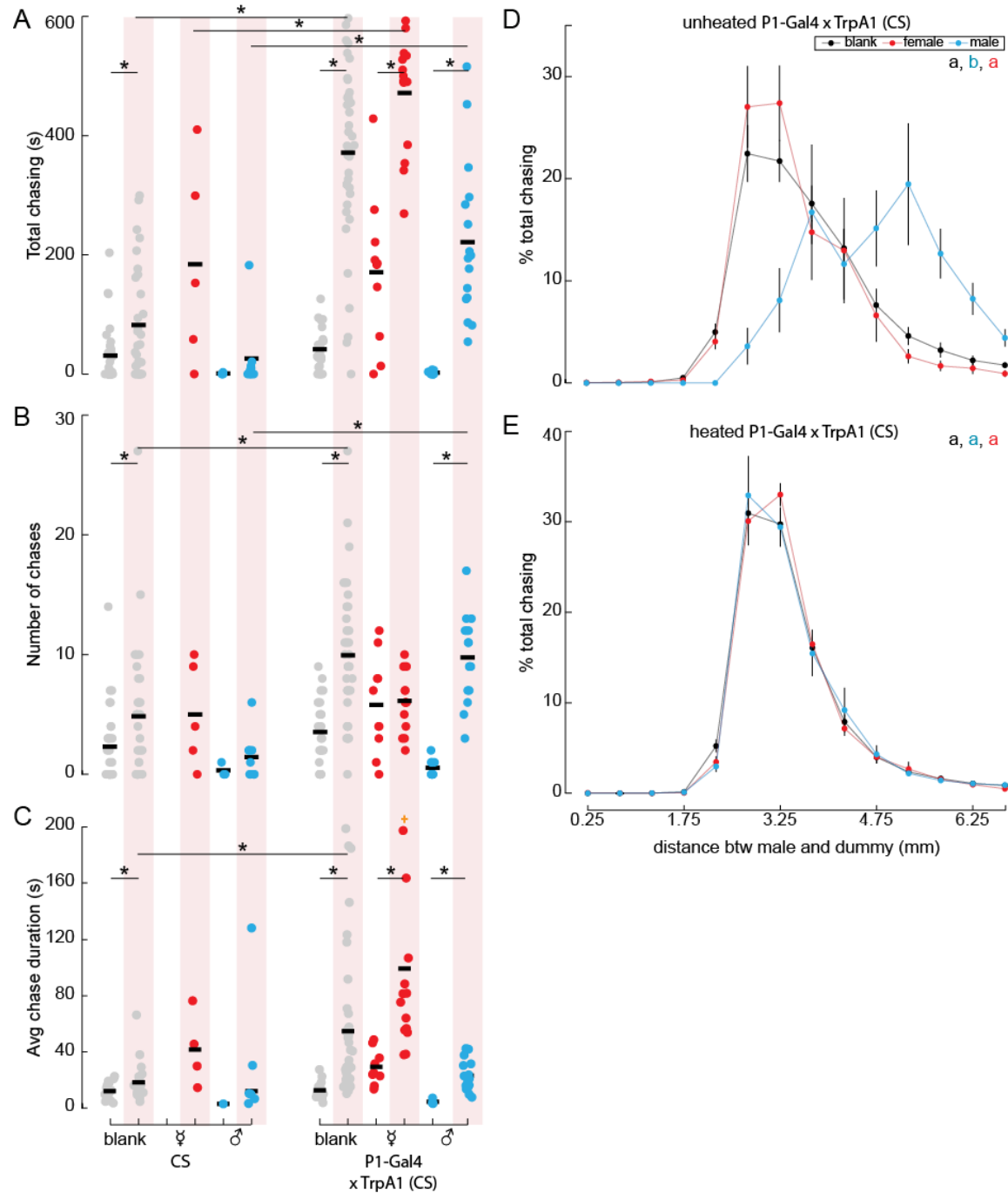
to modulate their position in response to dummy height (Fig. 4.3E), probably because these males had a very low baseline level of chasing towards the taller dummies. Instead, I made comparisons to heated wildtype, *Canton-S* males (Fig. 4.3D). Overall, activation of P1 neurons does appear to slightly skew males towards chasing the shortest dummy from further away (Fig. 4.3F). Males with activated P1 neurons still modulate their position in response to dummy height to a limited degree – when paired with the two taller dummies, males chase at a greater distance compared to males paired with the shortest two dummies. However, males with activated P1 neurons definitely do not display the same range of modulation seen in *Canton-S* males.

I next paired males with dummies perfumed with female or male pheromone. Similar to wildtype males, P1-activated males chased female pheromone perfumed dummies more and male pheromone perfumed dummies

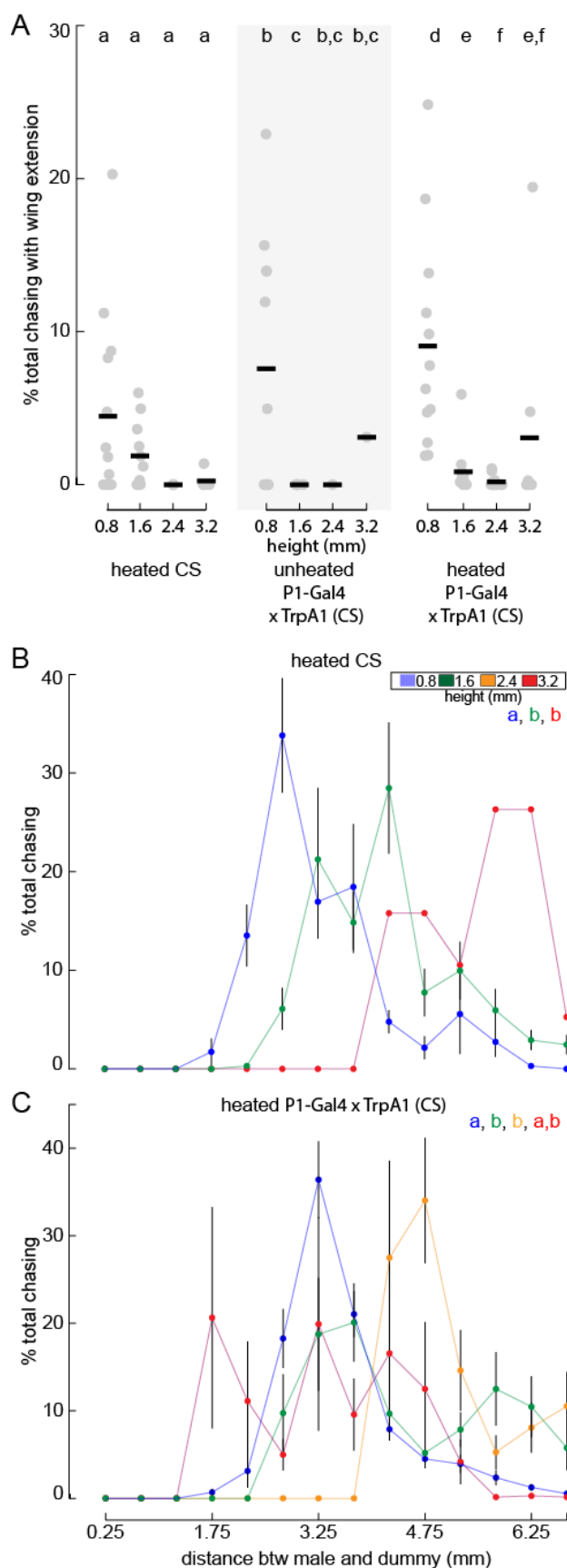
**Figure 4.2. Genetic background affects both the baseline and activated levels of chasing.** Red and gray dots indicate the response of a single male during a 10 min. behavioral trial. Black dashes plot the population mean. Male genotype is indicated along the horizontal axis. Red dots indicate trials in which the behavioral chamber was heated to 27°C. Dummy was 1.6 x 1.6 x 0.8mm and moved at 13.4 mm s<sup>-1</sup>. (A) Total time males spent chasing. (B) The total number of chases males initiated towards the dummy (C) Average chase duration. Asterisks indicate statistically significant differences (Fisher's exact,  $p < 0.05$  plus Bonferroni correction).



**Figure 4.3. Males with activated P1 cells maintain their shape preference.** (A-C) Gray dots indicate the response of a single male during a 10 min behavioral trial. Black dashes plot the population mean. Male genotype is indicated along the horizontal axis. In all trials, dummy was  $1.6 \times 1.6 \times 0.8$  mm and moved  $5 \text{ mm s}^{-1}$ . In heated trials, behavioral chamber was heated to  $27^\circ\text{C}$ . From left to right,  $n = 12, 12, 17, 10, 18, 12, 9, 10, 13, 12, 4, 10$ . Groups with the same letter do not have significantly different means (Fisher's exact,  $p < 0.05$ , plus Bonferroni correction). (A) Total time males spent chasing. (B) The total number of chases males initiated towards the dummy (C) Average chase duration. (D-F) Histogram of relative distance between male and dummy during chases (as measured from their centers). Male genotype is indicated beneath each histogram. Error bars depict standard deviation, and data points plot averages over flies. Groups with the same letter do not have significantly different means (two sample t-test,  $p < 0.05$ , plus Bonferroni correction).



**Figure 4.4. P1 activation can alter males' responses to dummies' pheromone coating.** (A-C) Dots indicate the response of a single male during a 10 min behavioral trial. Black dashes plot the population mean. Male genotype is indicated along the horizontal axis. Red dots indicate trials in which the dummy was perfumed with female virgin pheromone extract and blue dots indicate trials when the dummy was coated with male pheromone extract. In all trials, dummy was 1.6 x 1.6 x 0.8 mm and moved 13.4 mm s<sup>-1</sup>. Pink background denotes a trial in which the behavioral chamber was heated to 27°C. From left to right, n = 30, 31, 0, 5, 3, 9, 23, 39, 10, 14, 9, 16. Asterisks indicate statistically significant differences (Fisher's exact,  $p < 0.05$  plus Bonferroni correction). (A) Total time males spent chasing. (B) The total number of chases males initiated towards the dummy (C) Average chase duration. (D-E) Histogram of relative distance between male and dummy during chases (as measured from their centers). Male genotype is indicated beneath each histogram, and color denotes pheromone coating of dummy. Error bars depict standard deviation, and data points plot averages over flies. Groups with the same letter do not have significantly different means (two sample t-test,  $p < 0.05$ , plus Bonferroni correction).



less (Fig. 4.4A). P1-activation was able to counter the inhibitory effect of the male pheromone to some degree, but those males still chased a blank dummy more. Interestingly, P1 activation did remove the inhibitory effect of male pheromone on chasing bout initiation (Fig. 4.4B). Also, P1-activated males chased all dummies, regardless of pheromone content, from about the same distance (Fig. 4.4 E). Thus, P1-activation does appear to disrupt males' ability to sense male pheromone and respond accordingly.

Finally, I examined P1-activated males' patterns of wing extension. Males with activated P1 neurons still demonstrated decreased amounts of wing extension towards dummies of increasing height (Fig. 4.5A). However, because males demonstrated so little wing extension towards taller dummies, I was

**Figure 4.4. Males still demonstrate decreased levels of wing extension towards dummies of increasing height despite concurrent P1 activation.** (A) Total proportion of time spent chasing with wing extended. Gray dots indicate the response of a single male during a 10 min behavioral trial. Black dashes plot the population mean. Male genotype is indicated along the horizontal axis. In all trials, dummy was 1.6 x 1.6 x 0.8 mm and moved 5 mm s<sup>-1</sup>. From left to right, n = 13, 12, 4, 10, 18, 12, 9, 10, 12, 12, 17, 10. Groups with the same letter do not have significantly different means (Fisher's exact,  $p < 0.05$ , plus Bonferroni correction). (B-C) Histogram of relative distance between male and dummy when male had a wing extended (as measured from their centers). Male genotype is indicated beneath each histogram, and color denotes dummy height. Error bars depict standard deviation, and data points plot averages over flies. Groups with the same letter do not have significantly different means (two sample t-test,  $p < 0.05$ , plus Bonferroni correction).

unable to determine if these males, similar to wildtype males, sang from greater distances as dummy height increased (Fig. 4.5B, C).

#### 4.4 Discussion

Previous studies of P1 neurons have focused on how this cluster integrates multiple sensory cues to activate courtship. However, males with activated P1 are unique in that they don't just demonstrate the motor patterns of courtship, but do so in an object-oriented manner. Clearly these males can still utilize visual information to track and court moving objects. In this chapter, I sought to understand how P1 activation increases male courtship behavior and whether it disrupts males' ability to modulate chasing in response to visual cues. P1 activation increased both the number of chases initiated by males as well as the lengths of their chasing bouts. Males also demonstrated increased chasing of all objects, but still demonstrated decreases in chasing response as dummy height increased, similar to wildtype males. Furthermore, males with activated P1 neurons still demonstrated some limited ability to modulate their position relative to the dummy as its shape changed, but males were not able to modulate their position in response to the dummy's pheromone content. I was not able to determine whether P1 activation affected the patterning of males' wing extension.

Overall, it was difficult to determine if P1 neurons are involved in only courtship initiation or maintenance – my results suggest that these neurons could mediate both. However, an increase in courtship initiation does not mean that these neurons are responsible for the courtship initiations seen towards blank dummies detected from afar (see Ch. 2 for examples). Males will initiate courtship with females even in the dark (Krstic *et al.*, 2009; Spieth and Hsu, 1950). These courtship bouts are thought to be the result of an initial chemosensory or tactile contact, rather than visual stimulation. Because P1 neurons clearly respond to chemosensory cues, they may be responsible for initiating courtship in such situations.

However, though P1 neurons are active, male flies do not chase all moving objects. Males are still able to selectively chase specific dummy shapes. Where information about dummy shape feeds into the courtship pathway is currently unknown. Perhaps information about shape modulates P1 neuron activity, and is thus able to modify a male's ardor. Or, shape information may be feeding into the courtship pathway downstream of P1 neurons.

P1 activation selectively interfered with males' ability to modulate their position in response to the pheromone content of the dummy, and all chases shared similarity with chases made by wildtype males towards female-perfumed dummies. Again, a lot of evidence points to chemosensory information modulating P1 activity (Clowney *et al.*, 2015; Kohatsu and Yamamoto, 2015; Kohatsu *et al.*, 2011). P1 activation may mimic activation caused by detection of female pheromones, and thus males react to the dummy as if it were coated in such chemosensory cues.

Throughout these experiments, I was tonically stimulating P1 neurons. Such a pattern of activity is likely very artificial. Interestingly, if I used a higher temperature to activate cells, I saw an assortment of courtship behaviors, including substantial increases in chasing, jumping on the dummy, and potential abdomen bending. This result suggests that increased activation of this group of cells somehow also increases incident of all courtship behaviors, and perhaps also increases the proportion of later courtship behaviors.

## Chapter 5. Conclusion

### Summary of findings

In this thesis, I explored how male *D. melanogaster* use visual cues during courtship. By pairing male flies with dummies of various shapes, sizes, speeds, and pheromone coatings, I determined that visual and chemical cues are important at different points in the courtship sequence. Males use a simple visual filter to decide whether to approach a moving object, and the presence of cuticular pheromones influences how long males continue chasing. Dummy color can also influence males' likelihood of initiating courtship, and males were far more likely to chase a gray dummy compared to a black or white dummy. Furthermore, vision continues to be an important cue used by the male during the chase as well. Males use visual cues such as shape and color to decide how to position themselves around the female, especially when determining the distance at which they should chase. These visual cues also influence how much time a male spends extending a wing and therefore the amount of song he will produce. The P1 cluster of neurons appear to be involved in both chase initiation and maintenance, but activation of these cells does not abolish males' shape selectivity. P1 activation does interfere with males' ability to modulate their position while chasing in response to the dummy's chemosensory coating.

### A new technique for studying courtship

Flyatar is a powerful new tool for studying male courtship behavior and especially the role of vision during courtship. Because I can easily modify the dummy's appearance or coating, I can uncouple chemosensory and visual cues and manipulate just one modality at a time. As a result, I can begin to understand how each of these cues is used during courtship, and how the two modalities may interact. Consequently, my experiments are the first to show that males use information about shape and pigmentation when determining whether to court an object. Furthermore, because the behavioral arena I used is much larger than those traditionally used for courtship studies, I can observe and analyze multiple chasing bouts in their entirety to develop hypotheses regarding at which stages of courtship different sensory cues become relevant, and

thus how they might be used by males to progress through the courtship sequence. Such a spatial scale is much more ethologically relevant, and we can thus begin to understand the challenges faced by flies outside the laboratory.

### **Future experiments and impact on related work**

The experiments described in this thesis are among the first to demonstrate how males use vision to find and court females and set the ground work for an examination of the neural circuitry underlying visual object detection. Recent studies have found object-responsive neurons in the fly visual system that respond to object motion in flying flies (Aptekar *et al.*, 2015; Kim *et al.*, 2015). However, no such neurons have yet been found for walking flies, and it is unknown to what degree neurons responsible for object detection in walking or flying flies may overlap. Flyatar experiments, coupled with activation or inactivation of these cells, may yield insights into their importance for courtship and mate tracking. We could further develop a tethered walking prep in which males are stimulated to chase an object, and use functional imaging to examine how activity of these object-tracking neurons changes over time.

The results involving painted dummies are especially interesting and suggest a powerful assay for examinations of color vision in *Drosophila*. I have only just begun an exploration of the pigmentation cues males could be using to determine an object's identity. Further experiments with other shades of gray, and other colors, are required to measure how fine-tuned males' pigmentation preferences are. Furthermore, because the pigmentation preference in the half-painted dummies is so robust, one could further assay the photoreceptors or downstream visual neurons involved in such discriminations by testing how well males show a preference for either dummy end when mutant for specific neurons.

Additionally, I have only just begun to explore the types of discriminations made by courting males. Evidence from the literature suggests male can not only distinguish males from females and conspecifics from allospecifics, but also the female's age and general fitness (Billeter and Levine, 2013; Krupp *et al.*, 2008). By using pheromone extracts from females of different ages and fitness levels, we can use Flyatar to assess whether males use chemosensory cues for such discriminations.

Finally, because during Flyatar experiments the fly and dummy are tracked in real time, we can actually "close the loop" between the male and the dummy by adjusting the dummy's

motions in response to the male's activity. Such experiments enables us to ask questions related to the stigmergic nature of courtship. For example – a popular hypothesis in the literature suggests that males use female locomotor speed as a measure of receptivity. Females that slow in response to courtship attempts are thought to be more receptive than females that do not slow. Therefore, do changes in the female's locomotor speed affect male ardor? We could run experiments in which once a male starts chasing, we either slow or speed the dummy's motions, and then examine the effect on male courtship behaviors. We could further look instead at female receptivity, by switching Flyatar from the pursued to the pursuer. Does the female alter her locomotor patterns in response to chases made by differently shaped or coated dummies?

*Drosophila* courtship is a robust, stereotyped, and yet complex behavior. Males and females use multiple sensory cues to attract and assess the fitness of a potential mate, and the use of these cues evolves over the course of an interaction. By using Flyatar to isolate these sensory cues and understand how and when they become important, we can begin to better understand how such a complicated behavior progresses. Such characterizations will also doubtless facilitate future investigations into the underlying neural mechanisms.

## Supplemental movies

**Video S1. Male *D. melanogaster* chasing dummy.** Videos of three example chases are provided. All chases are with a  $1.6 \times 1.6 \times 0.8 \text{ mm}^3$  dummy moving at  $5 \text{ mm s}^{-1}$ .

**Video S2. Male *D. melanogaster* chasing dummies moving at various speeds.** Videos of three example chases are provided for the fastest and slowest dummy speed tested. All chases are with a  $1.6 \times 1.6 \times 0.8 \text{ mm}^3$  dummy. The first three chases are with the dummy moving at its slowest speed,  $1 \text{ mm s}^{-1}$ . The second three chases are with the dummy moving at its fastest speed,  $13.4 \text{ mm s}^{-1}$ .

**Video S3. Male *D. melanogaster* chasing black or white dummies.** Videos of example chases are provided for the white and black dummies. All chases are with a  $1.6 \times 1.6 \times 0.8 \text{ mm}^3$  dummy moving at  $5 \text{ mm s}^{-1}$ .

**Video S4. Male *D. melanogaster* with activated P1 neurons chasing dummy.** Videos of example chases are provided. All chases are with a  $1.6 \times 1.6 \times 0.8 \text{ mm}^3$  dummy moving at  $5 \text{ mm s}^{-1}$ . The behavioral arena has been heated to  $30^\circ\text{C}$ . Males are of the genotype *P1-Gal4 x UAS-dTrpA1*.

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