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RESEARCH ARTICLE

The relative roles of vision and chemosensation in mate recognition of *Drosophila melanogaster*

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ABSTRACT

Animals rely on sensory cues to classify objects in their environment and respond appropriately. However, the spatial structure of those sensory cues can greatly impact when, where and how they are perceived. In this study, we examined the relative roles of visual and chemosensory cues in the mate recognition behavior of fruit flies (*Drosophila melanogaster*) using a robotic fly dummy that was programmed to interact with individual males. By pairing male flies with dummies of various shapes, sizes and speeds, or coated with different pheromones, we 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 with 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 did influence how long they would continue chasing. Male pheromone both inhibits chase initiation and shortens chase duration. Collectively, these results suggest that male *D. melanogaster* 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.

KEY WORDS: Courtship, Pheromones, Object recognition

INTRODUCTION

For many insects, visual object recognition is essential for a variety of behaviors, including foraging, prey capture (Nordström, 2013; Olberg et al., 2000) and courtship (Boeddeker et al., 2003; Collett and Land, 1975; Trischler et al., 2010). However, little is known about visual object classification in the fruit fly *Drosophila melanogaster* Meigen. Whereas the overall circuitry of the motion vision pathway has been thoroughly studied (Borst, 2009; Paulk et al., 2013), previous research on the role of vision in *Drosophila* has largely focused on vertical landmarks [i.e. 'stripe fixation' (Götz, 1987), looming shapes (Card and Dickinson, 2008) and large-field optomotor responses (Götz, 1968)]. Flying *Drosophila* exhibit innate turning reactions that depend on object size (Maimon et al., 2008) and can be trained to steer towards specific visual scenes (Dill et al., 1993; Tang et al., 2004; Wolf and Heisenberg, 1991), suggesting that they possess some crude ability to recognize particular shapes or patterns.

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One behavior during which visual object recognition might be particularly important is courtship. Courtship in *Drosophila* involves a sequence of highly stereotyped maneuvers made by the male in order to assess, attract and copulate with a female (Greenspan and Ferveur, 2000; Sturtevant, 1915). Flies can reproduce in the dark (Spieth and Hsu, 1950), demonstrating that vision is not required for successful courtship. However, males in the dark or with impaired vision take longer to initiate and complete courtship (Markow, 1975) and blind males are out-competed by individuals with intact vision (Connolly et al., 1969). Furthermore, vision is required for certain components of courtship such as orientation and chasing (Cook, 1979). In laboratory studies, the role of vision is likely further diminished because courtship experiments are often performed using small chambers (radius <1 cm) in which the female and male flies are the only objects present (e.g. de la Paz Fernández et al., 2010). Males in the wild face a much more difficult task because 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.

Vision is certainly not the only cue available to males when assessing an object's identity. Flies also produce species- and sex-specific pheromones that are important in courtship (Billeter and Levine, 2013; Cobb and Jallon, 1990; Ferveur, 2005; Wicker-Thomas, 2007). However, most of these compounds are heavy, long-chain hydrocarbons with limited volatility. Large groups of *Drosophila* in small spaces may collectively emit detectable levels of pheromones into the surrounding air or deposit them on the substratum (Farine et al., 2012), but the sources of such chemosensory cues would be difficult for any individual to localize. Males would undoubtedly benefit from the ability to find females at a distance using vision or other cues.

In this study, we examined the relative roles of visual and chemosensory cues in mate recognition using a remotely actuated fly dummy. By testing males with different dummies, we assessed the influence of size, shape, speed and pheromone content on courtship. Males prefer chasing objects that are similar in size to a female. However, whereas males were less likely to begin chasing larger dummies, once they started, they continued chasing for a similar length of time regardless of the target's shape or speed. Adding female pheromones to the dummy did not alter the probability that a male would start chasing it, but did increase the duration of the chase. Collectively, these results suggest 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.

RESULTS

For our experiments, we used a behavioral apparatus called 'Flyatar' (Zabala et al., 2012), consisting of a remotely actuated fly dummy

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List of abbreviations

CH	cuticular hydrocarbon
cVA	cis-vaccenyl acetate
GCMS	gas chromatography–mass spectrometry
oe$^-$	with genetically ablated oenocytes

(Fig. 1A). We first paired males with moving dummies of various shapes and sizes and measured the total time spent chasing. Overall, chases with the dummy appeared qualitatively similar to those with actual females (Fig. 1B; supplementary material Movie 1). We never saw males performing courtship maneuvers not directed at the dummy nor in the dark (data not shown). Chase sequences were identified in post-processing using an automated behavioral classifier (Fig. 1C). As seen in Fig. 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, we systematically studied the influence of shape by pairing males with cylindrical dummies of constant height (0.8 mm) but increasing diameter (Fig. 2B), or cuboidal dummies of constant width (1.6×1.6 mm) but increasing height (Fig. 2C). Males paired with dummies of smaller radius or decreased height spent the most time chasing (Fig. 2B and 2C, respectively). For a subset of behavioral trials, we scored each sequence for the presence of unilateral wing extension, a courtship-specific male behavior. We found that males not only chased less when paired with taller dummies, they also demonstrated fewer wing extensions during those chases (Fig. 2D). We were not able to record song production, and do not know the extent to which wing extension correlates with singing.

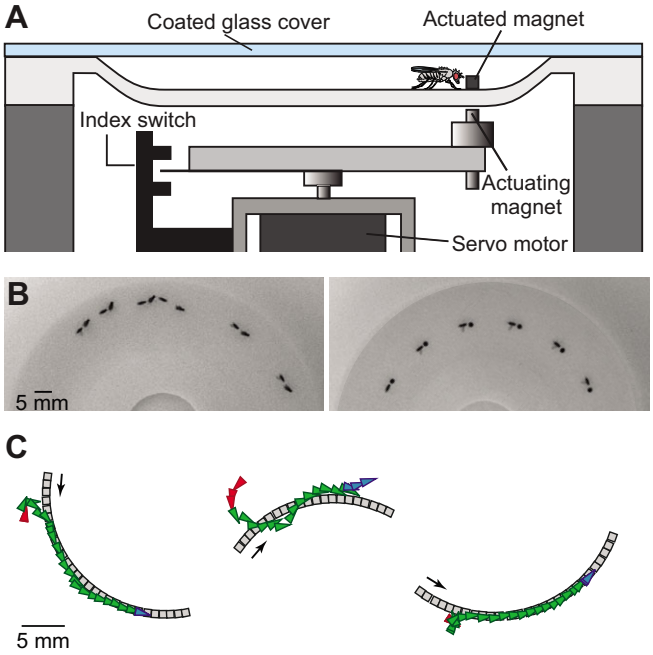

Fig. 1. Flyatar is a viable system for understanding social interactions in *Drosophila melanogaster*. (A) Schematic representation of Flyatar, shown in vertical cross-section, not drawn to scale. (B) Series of overlaid stills progressing left to right. Left panel depicts an example chase between a male and a female *D. melanogaster*. Right panel depicts an example chase between a male *D. melanogaster* and the dummy. (C) Three example chases (at 3 frames s^{-1}) identified by our 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, green during the chase, and blue after the end of the chase. Black arrows indicate the direction the dummy is initially moving.

In order to determine whether size or shape of the dummy affects the distance at which males first notice the dummy, we calculated the distance between the fly and dummy when the fly first oriented towards and visually fixated the dummy (Fig. 3). This fixating maneuver is the earliest courtship behavior we can measure. Because males do not always first fixate the dummy before approaching and chasing it, we had to first identify those chases that began with a fixating maneuver (Fig. 3A), which we defined as a turn made by the male $\geq\pi/8$ rad that brought the dummy into the frontal third of the male's field of view. The distance between the male and 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 we manipulated dummy height, males paired with the tallest dummies initiated fewer chases (Fig. 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. 4A, top). Even as we varied the dummy's speed over a large range from 1.0 to 13.4 mm s^{-1}, the chase duration remained roughly constant at 10 s (Fig. 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 mm s^{-1} tended to stay directly behind the dummy and made few lateral, circling motions (Fig. 5B,C; supplementary material Movie 2). In contrast, males chasing a dummy moving at 1 mm s^{-1} spent more time moving laterally and circling to the sides of the dummy.

Males initiated fewer chases when paired with wider dummies (Fig. 4B, bottom). Chase duration also generally decreased as dummy width increased (Fig. 4B, top). Taken at face value, this result suggests that the width of an object influences male behavior more than the 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 Fig. 4A and Fig. 5A suggest that object shape is 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.

We next examined whether chemical cues, such as pheromones, could influence chase initiation or duration. We coated dummies with pheromone mixtures extracted from male or virgin female flies. In each experiment, we used gas chromatography–mass spectrometry (GCMS) to quantify the amount of pheromone applied to the dummy. Although we tried to apply a consistent and physiologically relevant amount of pheromone in each trial, the amount of pheromone deposited on the dummy varied (Fig. 6). Additionally, because of differences in the surface chemistry and morphology of the dummies compared with real flies, we do not know how much of the hexane-

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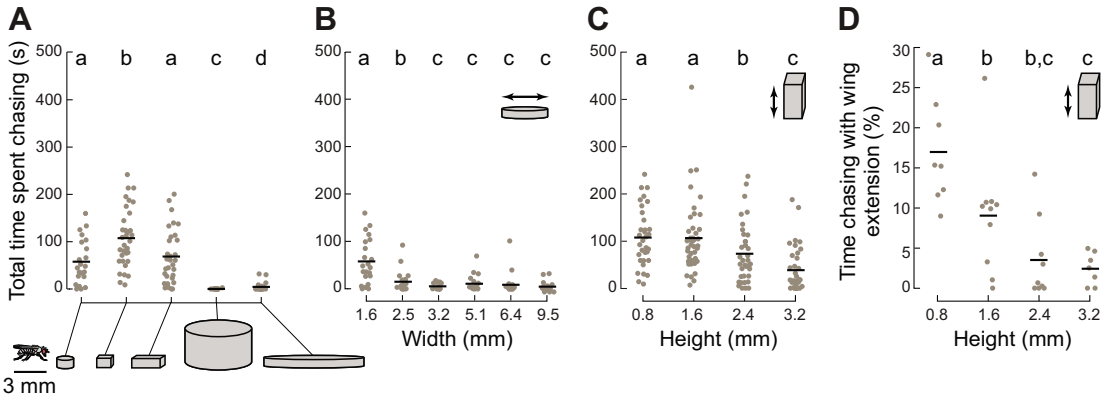

Fig. 2. The shape and size of a dummy influence males' likelihood of courtship. Gray circles indicate the response of a single male during a 10 min behavioral trial. Black dashes plot the population mean. Groups with the same letter are not significantly different (P<0.05, plus Bonferroni correction). (A–C) Total time males spent chasing when paired with a particular dummy. Dummy shape is indicated along the horizontal axis. In A, dummies have the following dimensions, from left to right (in mm): 1.6×0.8 (n=22), 1.6×1.6×0.8 (n=33), 1.6×3.2×0.8 (n=31), 6.4×3.2 (n=20), 9.6×0.8 (n=24). In B and C, dummy shape is modified along a single axis, as represented by the shape in the upper right corner of each panel. In B, sample sizes from left to right are as follows: n=22, n=20, n=20, n=20, n=23, n=27. In C, sample sizes from left to right are as follows: n=33, n=42, n=36, n=36. Data from the first columns of B and C are replicated in A. (D) Percent of time spent chasing that males also had a wing extended. Ten flies were randomly selected and examined for each dummy shape, but data for only those that demonstrated chasing are shown. From left to right: n=8, n=10, n=9, n=7.

extracted pheromone is actually detectable by males. Therefore, we also conducted complementary experiments with immobilized, wild-type flies mounted on top of the dummy.

Males paired with dummies perfumed with female cuticular hydrocarbons (CHs) spent more time chasing compared with males paired with blank dummies (Fig. 7A). Males were not any more likely to initiate chases towards a perfumed dummy (Fig. 7C); instead, they chased the perfumed dummies approximately twice as long as blank ones (Fig. 7B). We 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. 7A). This result was due to both shorter chase durations (Fig. 7B) and decreased numbers of chases initiated (Fig. 7C). To examine whether this repression of chase initiation was due to the immediate influence of male-specific pheromones or a change in behavior over time, we plotted a cumulative distribution of when, over the duration of a behavioral experiment, males initiated chases (Fig. 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. We also tracked wing extension for a subset of these trials (Fig. 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

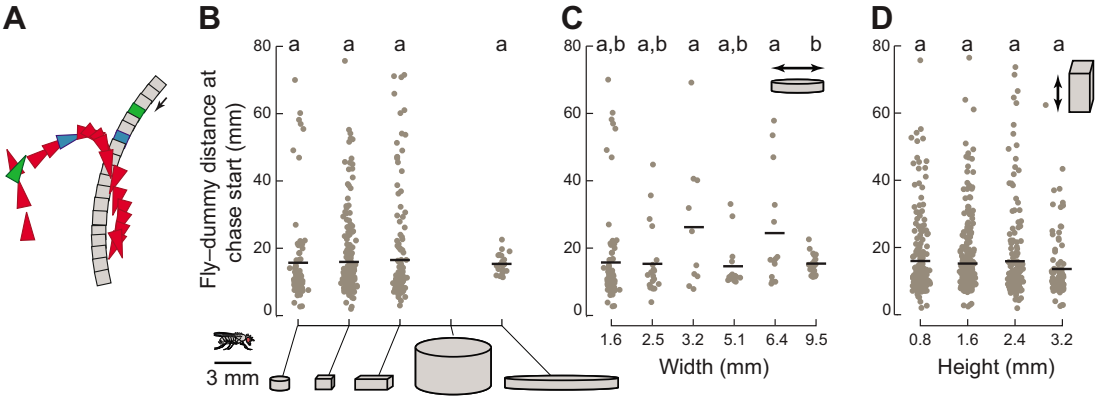

Fig. 3. Males' distance from the dummy when visually fixating and initiating chasing does not vary with dummy shape. (A) Example chase. Triangles indicate the positions and orientations of male flies, and the squares indicate the positions and orientations of the dummy. The black arrow shows the initial direction of dummy travel. Blue shapes indicate the first frame that is classified as a chase by our classifier (see Materials and methods). We 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 ≥π/8 (green shapes), the fly was classified as having made an orienting maneuver, which is the earliest courtship behavior we 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 10 min behavioral trial. Black dashes represent the mean of each trial type. In B, dummies have the following dimensions, from left to right (in mm): 1.6×0.8 (n=76), 1.6×1.6×0.8 (n=204), 1.6×3.2×0.8 (n=155), 6.4×3.2 (n=0), 9.6×0.8 (n=21). Dummy shape is indicated along the horizontal axis. In C and D, dummy shape is modified along a single axis, as represented by the shape in the upper right corner of each panel. In C, sample sizes from left to right are as follows: n=76, n=20, n=10, n=15, n=14, n=21. In D, sample sizes from left to right are as follows: n=204, n=317, n=186, n=99.

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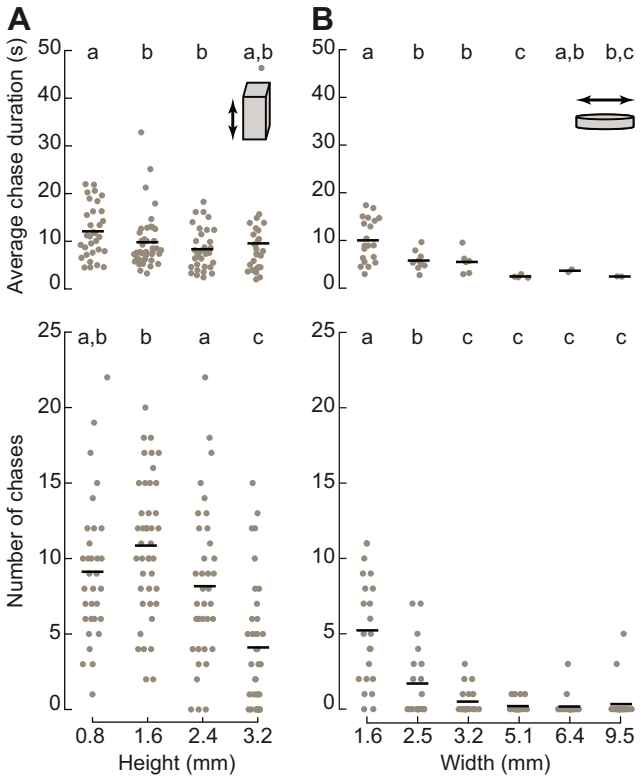

Fig. 4. Dummy shape affects males' likelihood of initiating a chase but not chase duration. Gray dots indicate the response of a single male during a 10 min behavioral trial. Black dashes plot the population mean. Groups with the same letter are not significantly different (P<0.05, plus Bonferroni correction). Top panels plot the average chase duration during the 10 min 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 x1.6 mm base. In the top and bottom panels, sample sizes from left to right are as follows: n=32, n=41, n=32, n=26. (B) Responses to changes in width of cylindrical dummy with a 0.8 mm height. In the top and bottom panels, sample sizes from left to right are as follows: n=20, n=9, n=6, n=4, n=2, n=2.

male-perfumed dummies (P<0.05), suggesting that female pheromones promote wing extension but male pheromones do not inhibit it.

In addition to wild-type flies, we 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 we 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. 7A), suggesting that the presence of the fly body does increase their responsiveness. However, our 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 was 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. 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. We also examined whether the presence of pheromone or a fly body altered the distance at which the male first fixated the dummy (Fig. 7F). Again, no conditions tested appeared to have an effect, with the exception of the dummy mounted with an oe$^-$ male.

So far, all our experiments were performed using moving stimuli. We next tested whether males might be attracted to a stationary dummy or a stationary patch of pheromones that was not associated with a visual object. To present a pheromone stimulus in the absence of a strong visual cue, we placed a small circle of filter paper coated with 400 µl of either hexane or hexane with dissolved female CHs in the arena. As shown by the spatial distributions plotted in Fig. 8, males spent little time in the vicinity of either the pheromone patch or the hexane solvent (filter paper with hexane: 0.92±0.63 s, filter paper with hexane and female CHs: 0.58±0.40 s). However, when we placed a stationary dummy in the same location as the filter paper, males spent significantly more time near the dummy compared to the filter paper (P<0.05), suggesting that a stationary visual object is an attractive cue. Coating the stationary dummy with female pheromones increased its attractiveness, as males spent significantly more time near a coated dummy than a blank dummy (219.34±173.30 s versus 71.63±53.39 s, P<0.05). By 'attractiveness', we refer simply to a male's tendency to spend time near an object and do not distinguish between long-distance attraction and local preference.

Pheromones are important for distinguishing not only the gender of a potential mate but also its species identity (Wyatt, 2003). We hypothesized that males should chase dummies perfumed with pheromones from other species less than those perfumed with conspecific pheromones. We 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. 9, males spend less time chasing dummies perfumed with female *D. simulans* pheromones compared with 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* pheromones promote 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 pheromones may be shared between the two species.

DISCUSSION

By pairing male flies with dummies of various shapes, sizes, speeds and pheromone coatings, we determined that visual and chemical cues are important at different points in the courtship sequence of *D. melanogaster* (Fig. 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 4, 5). With more information, such as the presence of cuticular pheromones, males will choose to either stop or continue chasing (Fig. 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. 7). Both the shape of the dummy and its pheromone coating influence the amount of wing extension exhibited during a courtship bout (Fig. 2D, Fig. 7E). Finally, *D. simulans* pheromone does not increase

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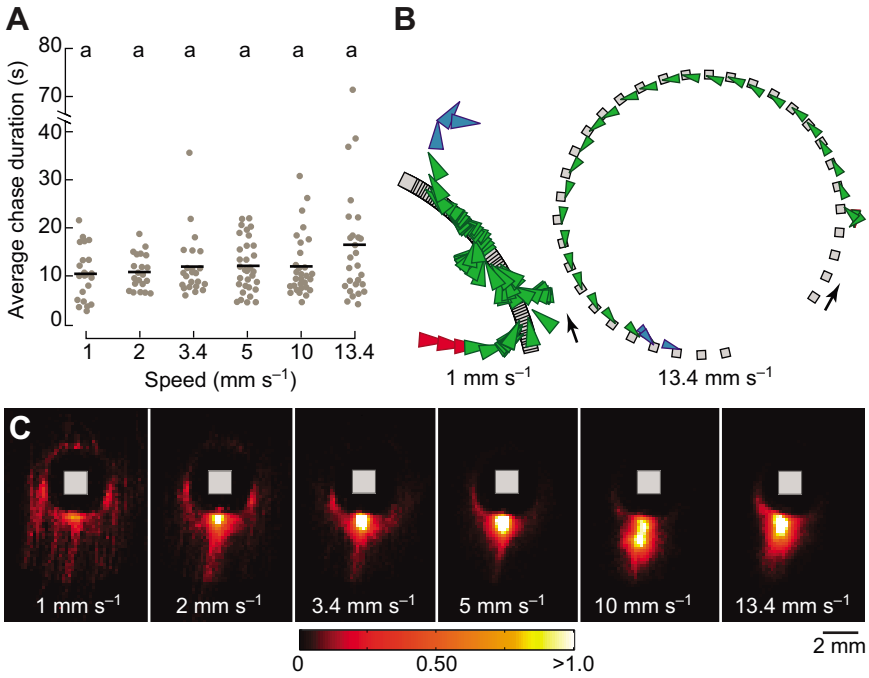

Fig. 5. Dummy speed does not affect chase duration, but does affect males' circling tendency. (A) Average chase duration. Males were paired with cuboid dummies (0.8×1.6×1.6 mm^3) moving at the indicated speed. Gray circles indicate the single male responses during a 10 min behavioral trial. Black dashes plot the population mean. Groups with the same letter are not significantly different (P<0.05, plus Bonferroni correction). Sample sizes from left to right are: n=22, n=21, n=22, n=31, n=29, n=27. (B) Example traces of a male chasing a cuboid dummy moving at 1 mm s^{-1} (left) or 13.4 mm s^{-1} (right), 2.5 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 the end of the chase. Black arrows indicate the direction the dummy is initially moving. Drawn to different scales. (C) 2D 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 are pooled for each panel and the speed of the dummy is indicated at the bottom of each panel. Sample sizes are the same as in A. Color scale represents percent occupancy.

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 *D. 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., 2003) and horseshoe crabs (Barlow et al., 1982). Our 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.

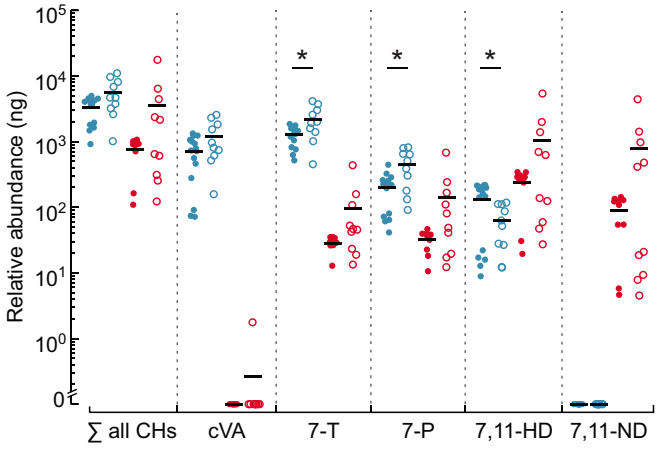

Fig. 6. Dummies were perfumed with a physiologically relevant amount of pheromone. Blue and red filled and unfilled circles indicate the amount of pheromone component found on an individual fly (filled) or dummy (unfilled). Color indicates gender, with blue indicating pheromone component extracted from male flies or male-perfumed dummies and red indicating the pheromone component extracted from females or female-perfumed dummies. Black dashes represent the population mean, and asterisks indicate statistically significant differences (*P<0.05 plus Bonferroni correction). Abundance was determined by comparison to an internal standard, octadecane. CH, cuticular hydrocarbon; cVA, cis-vaccenyl acetate; 7-T, 7-tricosene; 7-P, 7-pentacosene; 7,11-HD, 7,11-heptacosadiene; 7,11-ND, 7,11-nonacosadiene. Sample sizes, from left to right: n=15, n=10, n=10, n=10.

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 our experiments, males occasionally chased dummies that were very dissimilar in size and shape to a female (Fig. 2). Furthermore, 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 our 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, our 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 (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

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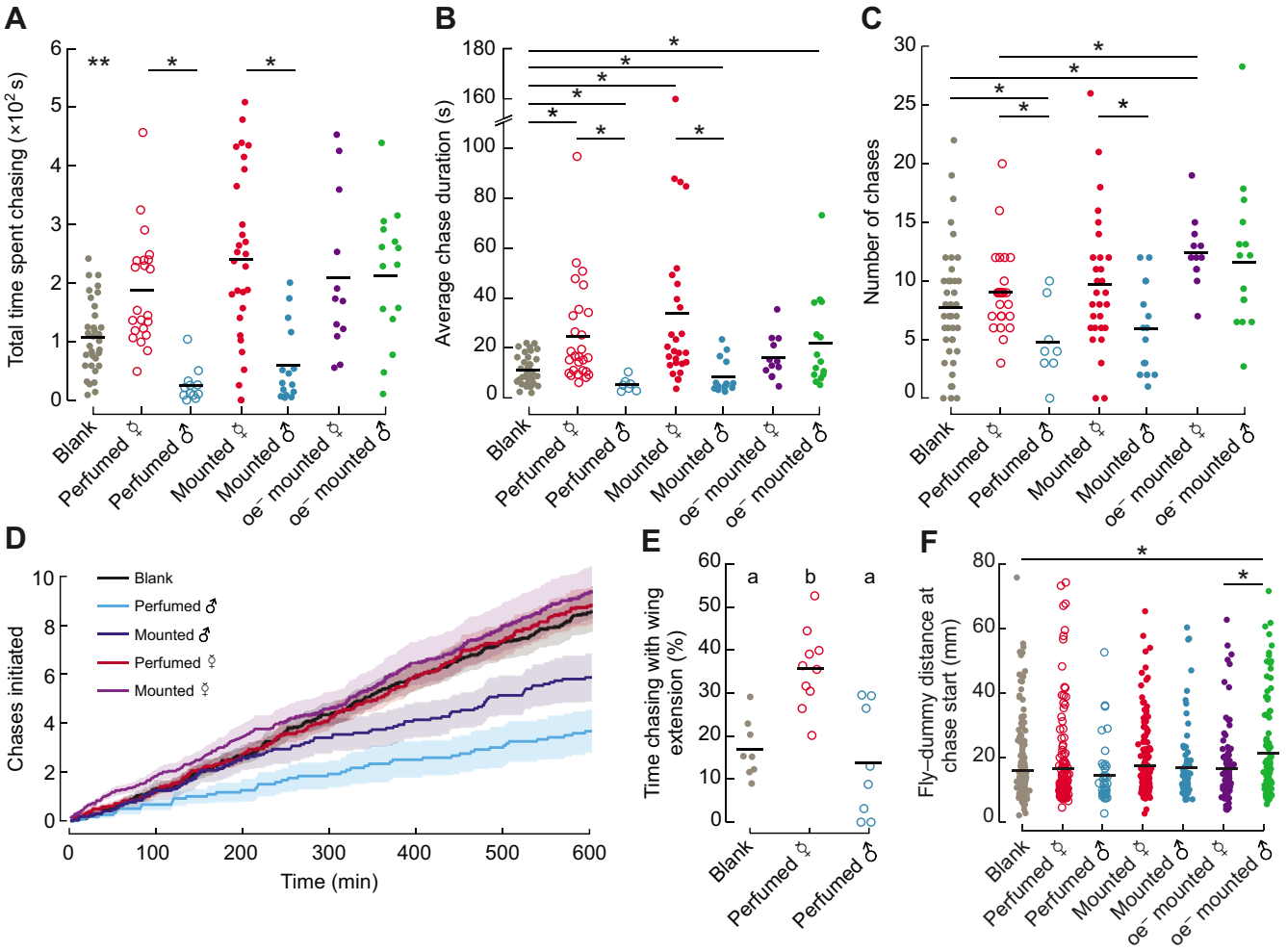

Fig. 7. The presence of pheromones affects chase duration but not the number of chasing bouts. (A–C,E,F) Colored and gray filled and unfilled circles represent the responses of single males during a 10 min behavioral trial. In all cases, the dummy used was cuboid (0.8×1.6×1.6 mm^3), which was then further 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. Sample sizes from left to right: n=33, n=22, n=12, n=29, n=15, n=12, n=15. (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 10 min behavioral trial, males initiated a chase. Data for all males were 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). Ten flies were randomly selected and examined for each condition, but only those that demonstrated chasing are shown. From left to right: n=8, n=10, n=8. (F) As described in Fig. 3, we 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. From left to right: n=204, n=150, n=41, n=181, n=72, n=101, n=104.

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 our experiments with other recent studies examining courtship initiation (Kohatsu et al., 2011; Pan et al., 2012). In both of these prior studies, vision alone was insufficient to evoke male courtship. For example, Kohatsu and co-workers (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 our results is that tethering may raise the arousal threshold necessary to elicit chasing. In the other study, Pan and co-workers (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 two-dimensional, because the rubber band dummies were located underneath a clear arena floor on which the flies walked. Our experiments demonstrate that height is an important factor in courtship initiation and the stimulus used by Pan and co-workers 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, our 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.

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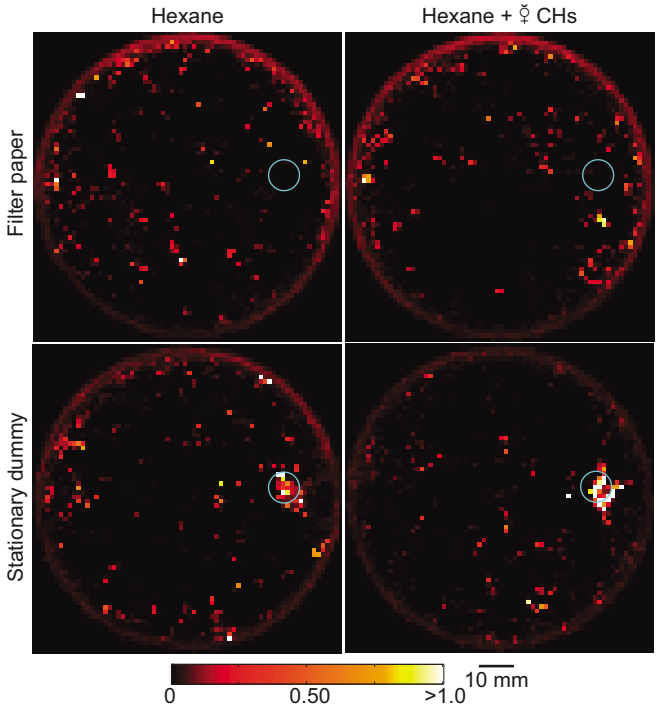

Fig. 8. Males are not attracted to a stationary source of pheromone without a visual cue. 2D histogram of the positions of male flies in an arena with either a circle of filter paper (top panels) or a stationary dummy measuring 0.8×1.6×1.6 mm^3 (bottom panels). The filter paper and dummy were coated either in hexane (left panels) or hexane with dissolved female CHs (right panels). Color scale represents percent occupancy. The blue circle surrounds an area 10 mm in diameter around the filter paper or dummy and total occupancy (s) was measured inside the circle. Average occupancy values with 95% confidence intervals are as follows, from left to right: top panels, 0.92±0.63 s and 0.58±0.40 s; bottom panels, 71.63±53.59 s, 219±173.30 s. From left to right, sample sizes for top panels: *n*=11, *n*=10 and for bottom panels: *n*=17, *n*=17.

Farine and co-workers (Farine et al., 2012) found that approximately 0.2% of a fly's CHs are volatile enough to be recovered using solid phase microextraction fiber under natural conditions, but collecting detectable amounts required placing 10–20 flies in small 8 ml vials for 2 h. 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 and Hall (Tompkins and Hall, 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 our experiments, we found that males could fixate and approach dummies at distances greater than 8 mm (Fig. 3). This result, combined with our 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 our 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 cis-vaccenyl acetate (cVA), are light compared with other CHs and volatile to some degree (Farine et al., 2012; Jallon et al., 1981), and could function as a long-distance cue that inhibits courtship initiation. Whether the same male pheromones are responsible for both inhibiting male chase initiation and truncating chases is currently unknown. Another male-specific pheromone, 7-tricosene, which is heavier than cVA, has also been shown to decrease male–male courtship (Billeter et al., 2009; Wang et al., 2011) and might be involved in decreasing chase duration.

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, our 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 we measured during a typical chase (Fig. 5C), males were certainly close enough to do so. The pheromones responsible for increasing the duration of chasing bouts are not known, but several female-specific pheromones have been shown to stimulate male wing extension and inhibit

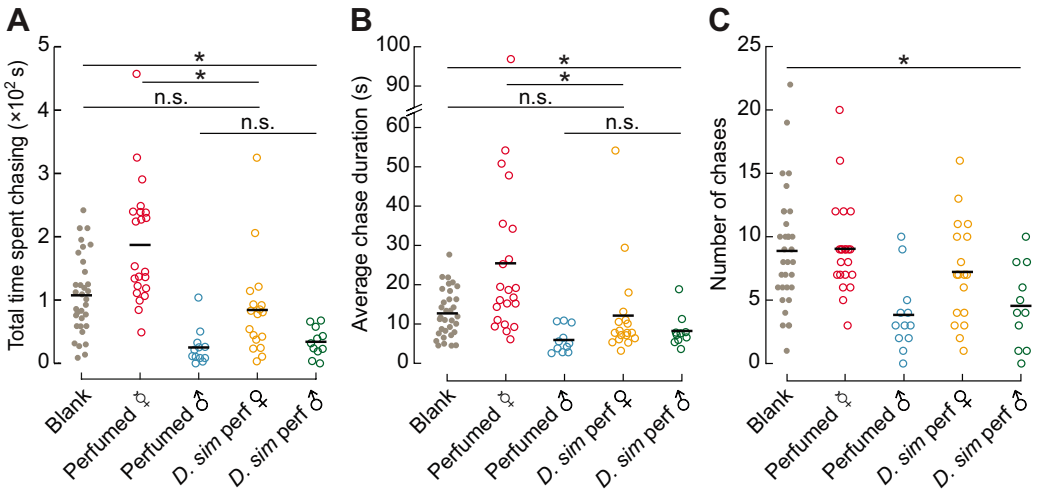

Fig. 9. Allospecific pheromone does not elicit an increase in chasing bout length. Colored and gray filled and unfilled circles represent the responses of individual males during a 10 min behavioral trial. In all cases, the dummy used was cuboid (0.8×1.6×1.6 mm^3), which was then further modified as indicated on the horizontal axis. *D. sim* perf, *Drosophila simulans* perfumed. Black dashes represent the population mean, and asterisks indicate statistically significant differences (*P<0.05 plus Bonferroni correction; n.s., not significant). (A) Total time males spent chasing. Sample sizes, from left to right: *n*=33, *n*=22, *n*=12, *n*=18, *n*=11. (B) Average chase duration, as initiated by males towards the dummy. (C) Total number of chases initiated by the male towards the dummy.

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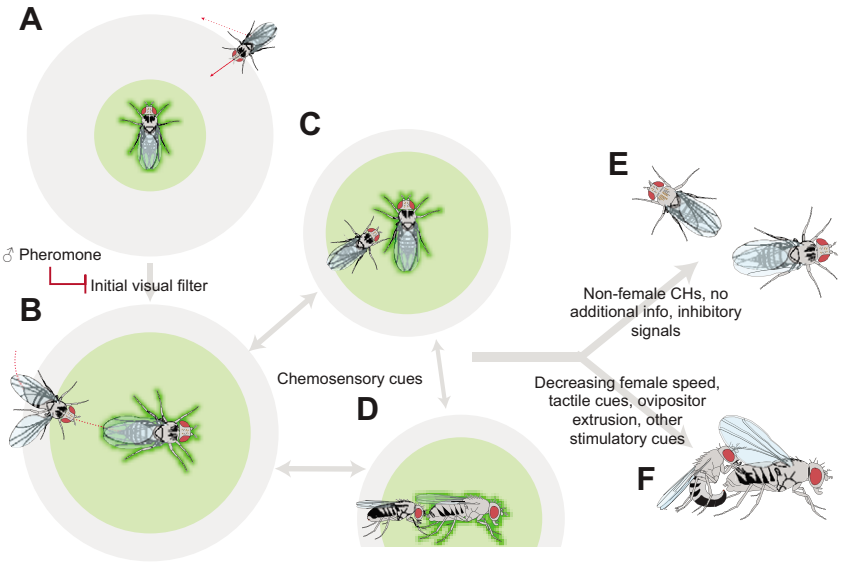

Fig. 10. Schematic representation of courtship and the range of sensory cues encountered. Dark green outline represents the likely spatial scale of gustatory cues, light green the range of olfactory cues and light gray the range of visual cues, not drawn to scale. Courtship begins (A) when the male fly first orients towards the likely female. Using a coarse initial visual filter, the male decides whether 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).

interspecies courtship, including the 7,11-dienes 7-11-nonacosadiene and 7-11-heptacosadiene (Antony and Jallon, 1982; Marcillac et al., 2005).

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 crucially 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, we 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 we are to better understand the neural mechanisms underlying the early stages of male courtship behavior.

MATERIALS AND METHODS

Animals

Unless otherwise noted, all flies were reared on standard medium in a 16 h:8 h light:dark cycle at 25°C. Behavioral experiments were performed on 2- to 4-day-old male fruit flies, *D. melanogaster* of the Canton-S strain. Males were collected under light anesthesia (CO_2) 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 ablated adult oenocytes by crossing '+: *PromE(800)-Gal4, tubP-Gal80^{ts}*;+' with '+: *UAS-StingerII, UAS-hid/CyO*;+'. Progeny were kept at 18°C until eclosion. Adult progeny were collected under CO_2 anesthesia and kept at 25°C for at least 24 h. 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. We verified ablations by examining flies for green fluorescent protein fluorescence on day 5.

For experiments in which a fly was mounted on the dummy, we 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 (CHs) were extracted from 2- to 7-day-old male and virgin female *D. melanogaster* and male and female *D. simulans*. Flies were collected under CO_2 anesthesia and housed in food vials in same-sex groups. To extract CHs for behavioral experiments, groups of several hundred same-sex flies were anesthetized (CO_2) and placed into 20 ml scintillation vials with 20 µl hexane per fly. The fly–hexane mixture was agitated for 2 min. The hexane with dissolved CHs was pipetted into glass microvials (Microliter Analytical Supplies, Suwanee, GA, USA) and the flies were 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^{-1} of octadecane (Sigma-Aldrich, St Louis, MO, USA) as an internal standard, agitated for 2 min, and then the fly or dummy was removed. The extract was then analyzed using a 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 min), ramped at 42.5°C min^{-1} to 135°C, followed by a ramp of 25°C min^{-1} to 235°C and a ramp of 3°C min^{-1} to 285°C, where it was held for 10 min. 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).

Hydrocarbon application

Prior to application of CH extracts, dummies were washed with hexane. We then applied 400 µl 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. To apply pheromone to filter paper, we first used nitrogen to evaporate 400 µl of CH extract or hexane to a reduced volume between 5 and 30 µl. This volume was then pipetted onto the filter paper in 8 µl increments. The hexane was then allowed to evaporate until the filter paper was visibly dry.

Behavioral assays

The dummies used in all experiments were nickel-coated neodynium 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 our 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×3.5 mm deep (styled after Simon et al., 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 min. Room lights were turned off to ensure that 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 (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 mm s^{-1} unless otherwise stated.

Data and statistical analysis

Data were analyzed using custom code written in MATLAB and Python. We 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 ([–π /3, π /3]), the fly to dummy distance had to be ≤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. We further confirmed the accuracy of our classifier using a technique similar to that used by Schneider et al. (Schneider et al., 2012) by developing a test set of data (taken from trials involving a 0.8×1.6×1.6 mm^3 dummy moving at 5 mm s^{-1}) with the dummy trajectories shifted ahead by 20 s (0.5 times the length of one circling of the arena). Over this test set, our classifier falsely identified only one chase, compared with the 293 chases identified from the original data. Except for Fig. 3, we 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, most of our data had non-homogeneous variances, and, as determined by the Kolmogorov–Smirnov test, were not normally distributed ($P<0.05$). As such, we developed a nonparametric resampling method (Fisher's exact test) whereby we could make pair-wise comparisons using the difference of means as our test statistic. In all comparisons we 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. Significance for Fig. 8 was determined using a two-sample *t*-test.

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Competing interests

The authors declare no competing financial interests.

Author contributions

S.A. and M.H.D. designed the experiments. S.A. conducted all experiments and analyzed the resulting data. S.A. extracted and analyzed pheromones. S.A. and S.S. built the experimental hardware. S.S. wrote the software controlling the experimental hardware and tracking the flies. S.A. and M.H.D. wrote the manuscript, and all authors contributed comments and edits.

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Supplementary material

Supplementary material available online at
http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.105817/-/DC1

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Movie 1. Male *D. melanogaster* chasing dummy. Videos of three example chases are provided. All chases are with a 1.6×1.6×0.8 mm^3 dummy moving at 5 mm s^{-1}.

Movie 2. 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 1.6×1.6×0.8 mm^3 dummy. The first three chases are with the dummy moving at its slowest speed, 1 mm s^{-1}. The second three chases are with the dummy moving at its fastest speed, 13.4 mm s^{-1}.