RESEARCH ARTICLE

The effects of target contrast on *Drosophila* courtship

Sweta Agrawal[1],* and Michael H. Dickinson[2]

ABSTRACT

Many animals use visual cues such as object shape, color and motion to detect and pursue conspecific mates. Contrast is another possibly informative visual cue, but has not been studied in great detail. In this study, we presented male *Drosophila melanogaster* with small, fly-sized, moving objects painted either black, white or gray to test whether they use contrast cues to identify mates. We found that males frequently chased gray objects and rarely chased white or black objects. Although males started chasing black objects as often as gray objects, the resulting chases were much shorter. To test whether the attraction to gray objects was mediated via contrast, we fabricated black and gray behavioral chambers. However, wild-type males almost never chased any objects in these darkly colored chambers. To circumvent this limitation, we increased baseline levels of chasing by thermogenetically activating P1 neurons to promote courtship. Males with thermogenetically activated P1 neurons maintained a similar preference for gray objects despite elevated levels of courtship behavior. When placed in a black chamber, males with activated P1 neurons switched their preference and chased black objects more than gray objects. We also tested whether males use contrast cues to orient to particular parts of the female's body during courtship. When presented with moving objects painted two colors, males positioned themselves next to the gray half regardless of whether the other half was painted black or white. These results suggest that males can use contrast to recognize potential mates and to position themselves during courtship.

KEY WORDS: Mate recognition, P1 neurons, Object recognition

INTRODUCTION

Many animals use vision to recognize and approach conspecific mates, including horseshoe crabs (Barlow et al., 1982; Herzog et al., 1996), crustaceans (Díaz and Thiel, 2004; Murai and Backwell, 2006; Perez et al., 2012; Tsai et al., 2017), stickleback fish (Nordeide, 2002; Pelkwijk and Tinbergen, 1937; Tinbergen, 1948), birds (Cooper and Forshaw, 1977), flies (Boeddeker et al., 2003; Land and Collett, 1974; Trischler, 2010) and spiders (Clark and Uetz, 1990; Foelix, 2011; Girard et al., 2015). The relevant visual cues used in mating behavior include shape, motion and color. For example, male blowflies paired with dummy mates typically pursued and captured only those dummies most similar in size to another blowfly, and relied on contrast and motion to detect and track the target (Boeddeker et al., 2003). In his classic experiments on three-spined stickleback fish, Nikolas Tinbergen found that the

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red coloring of male bellies is an important, sexually dimorphic cue: when presented with a dummy fish with a red underside, males reacted with aggressive displays as if it were another male. However, when the red coloring was absent, males were more likely to court the dummy as if it were female (Pelkwijk and Tinbergen, 1937; Tinbergen, 1948).

In addition to color, contrast (i.e. the relative intensity of an object compared with its background) can be an important visual cue for mate recognition. For example, when Barlow and co-workers placed black, white or gray cement castings of female horseshoe crabs on a Massachusetts beach, they found that male horseshoe crabs were most attracted to the black and gray castings and least attracted to the white castings (Barlow et al., 1982). In addition, although related to prey capture rather than mating behavior, anurans provide compelling examples of how animals use contrast to recognize objects. Many frog species prefer pursuing black prey dummies, whereas certain toad species prefer pursuing white prey dummies (Ewert and Seifert, 1974). Experience can alter this preference; frogs presented repeatedly with a threatening black object began to prefer white prey targets (Mikulka et al., 1980), and these preferences have been directly linked to the activity of visual neurons in the retina and pretectum (reviewed in Ingle, 1983).

Although the fruit fly *Drosophila melanogaster* uses object shape to recognize, approach and court potential mates (Agrawal et al., 2014; Cook, 1979), it is not known whether contrast also influences this behavior. Cuticle pigmentation varies in intensity across the *Drosophila* genus (Wittkopp et al., 2003), and thus contrast could provide a meaningful cue to distinguish different species of drosophilids. Furthermore, some species, such as *D. melanogaster*, also exhibit sexually dimorphic pigmentation: the dorsal abdomen of males are darkly pigmented whereas in females, pigmentation is restricted to a posterior stripe on each abdominal segment (Williams et al., 2008). Past efforts to assess whether differences in pigmentation could affect sexual selection are difficult to interpret because they relied on mutations in pigmentation genes that have many pleiotropic effects on behavior (Anaka et al., 2008; Connolly et al., 1969; Kopp et al., 2000; Takahashi, 2013; True, 2003; Wittkopp and Beldade, 2009). For example, *ebony* mutants demonstrate impaired phototactic and optomotor responses, abnormal circadian rhythms, and deficient dopamine signaling (reviewed in Takahashi, 2013). Furthermore, mutations in pigmentation genes such as *ebony* and *tan* may alter the composition of cuticular hydrocarbons, which are also important cues during courtship (Massey et al., 2019 preprint).

To circumvent confounds associated with pigment mutations, we examined the importance of object contrast in *D. melanogaster* courtship by presenting male flies with moving, fly-sized objects painted either black, white or gray and then quantified the resulting courtship behavior. We found that males strongly preferred chasing low-contrast gray targets. Males initiated chases of high-contrast black targets as often as gray targets, but terminated those chases more quickly, resulting in less total chasing of black targets. Unfortunately, wild-type males rarely chased any targets when

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moving against the background of a darkly colored chamber. However, males with thermogenetically activated P1 neurons, which demonstrated a similar preference for gray targets in the white chambers, switched their preference and robustly chased black targets when placed in a darkly colored chamber. This result suggests that males prefer chasing low-contrast targets. Furthermore, when presented with objects painted half gray and either half black or half white, males positioned themselves next to the gray half of the object and avoided the other half. Together, these results demonstrate that males can use contrast to recognize and court potential mates.

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. The majority of behavioral experiments were performed on 2- to 4-day-old male fruit flies, *Drosophila melanogaster* Meigen 1830 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. Each male only experienced a single experimental condition. For experiments in which a fly was mounted on or behind a magnet, we first anesthetized flies with cold, removed their legs and wings, and then glued them onto the magnet using UV-cured glue (Newall XUVG-1, Loctite 3104). The magnet was placed into the behavioral chamber and rotated such that the abdomen of the attached female was pointed in the directions indicated. These dummies were used no later than 3 h after the fly was glued to the magnet.

The following stock was obtained from the Bloomington *Drosophila* Stock Center (no. 26263): w[*]; P{y[+t7.7] w[+mC]=UAS-TrpA1(B).K}attP16. The P1 split-GAL4 line [*w^{1118};pJRFC12-10XUAS-IVS-myr::GFP (su(Hw)attP5)/R15A01-AD (attP40);R71G01-DBD (attP2)/+*] was kindly provided by David Anderson, California Institute of Technology (Hoopfer et al., 2015; Inagaki et al., 2014). Both lines were backcrossed into a Canton-S background.

Preparation of dummies
The fly-sized targets, which we hereafter call dummies, were 1.6×1.6×0.8 mm (square) or 1.6×3.2×0.8 mm (rectangular) nickel-coated neodynium magnets (Armstrong Magnetics, Inc., Bellingham, WA, USA), washed in hexane and then painted with Behr Ultra paint (Santa Anna, CA, USA). We used the colors Ultra Pure White for white dummies and Supernova for gray. For black dummies, we painted a coating of black India ink (Winsor and Newton, London, UK) onto white-painted dummies. After painting, all dummies were coated with two layers of clear nail polish (Wet n' Wild, City of Industry, CA, USA) and allowed to dry for at least 24 h before use. The nail polish coating protected the paint from chipping and controlled for differences in the chemosensory content of the paint by providing a uniform outer shell. Clear-coated dummies lacked any paint but were still coated with two layers of clear nail polish.

Behavioral assays
We used the Flyatar apparatus (Agrawal et al., 2014) for all behavioral assays (Fig. 1A). A 100 mm wide×3.5 mm deep chamber was based on a previous design with gently sloping walls (Simon and Dickinson, 2010). Unless otherwise noted, the behavioral chamber was made of white PVC plastic. To reduce build-up of chemical cues, we cleaned the chamber with acetone and 70% ethanol every three to five trials. After cleaning, a fresh dummy was placed in the behavioral chamber. At the start of each experiment, one male was aspirated into the chamber and the trial began once the male started to walk. If the male did not walk for several minutes, or if its wings were damaged, it was replaced with a new individual. A camera (Basler, acA640-100gm) above the chamber was used to record the movements of the fly and the dummy, which were tracked using custom software (https://github.com/ssafarik/Flylab) implemented within 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 at a constant speed of 5 mm s^{-1}. Each trial ran for 10 min. Unless otherwise noted, the average ambient temperature in the chamber was approximately 22°C.

Data and statistical analysis
Data were analyzed using custom code written in MATLAB and Python (available from https://github.com/sagrawal/Flylab_analysis). Data can be downloaded from the Dryad Digital Repository (https://doi.org/10.5061/dryad.k74670v). We designed a behavioral classifier to automatically identify chases. This classifier required that the dummy reside within the 120 deg, 7 mm sector in front of the fly for at least 2 s. All identified chases were examined and verified, and obvious false positives were manually removed. Wing extension was scored using DuoTrax, software written and provided by Kristin Branson (Janelia Research Campus).

According to a two-sample *F*-test and the Kolmogorov–Smirnov test ($P<0.05$), our data consisted of non-homogeneous variances with non-normal distributions. We thus used a nonparametric resampling method (Fisher's exact test) in which we made pair-wise comparisons using the difference of means as a test statistic. In all comparisons, we set a significance level of 5%, with Bonferroni–Holm corrections for number of comparisons made. In each figure, significance is denoted using a letter code, in which data labeled with a different letter were significantly different from one another.

RESULTS
We first tested whether the color of the dummy could influence males' chasing behavior. We painted square or rectangular dummies one of three shades: white, gray or black, each coated with clear nail polish to control for the chemosensory content of the paint. We measured the chromaticity of each paint, and found that they all reflected a similarly broad spectrum of light (Fig. 1B). Therefore, we ignored any influence of chromaticity and considered the painted dummies to be identical with respect to spectral content. We also tested plain dummies lacking any paint but still coated with the clear nail polish (clear-coated). These unpainted dummies were dully metallic in appearance owing to their nickel surface.

We show some example trajectories of chases towards gray, black and white square dummies in Fig. 1C. Surprisingly, males showed a strong preference for courting and chasing the gray dummy and rarely chased the white or black dummy, regardless of its shape (Fig. 1D). Males even chased the white and black dummies less than an unpainted (but still clear-coated) dummy. Males initiated a similar number of chases towards the square black and gray dummies, but initiated fewer chases towards the rectangular black dummy compared with the rectangular gray dummy. Males initiated fewer chases towards the white dummies (Fig. 1E), and pursued both white and black dummies for notably shorter periods of time than gray dummies (Fig. 1F). An examination of chase trajectories revealed that during many chases towards the black or white dummy, males terminated the chase soon after beginning their approach (Fig. 1C,F, Movie 1). Males chased the white dummy a bit
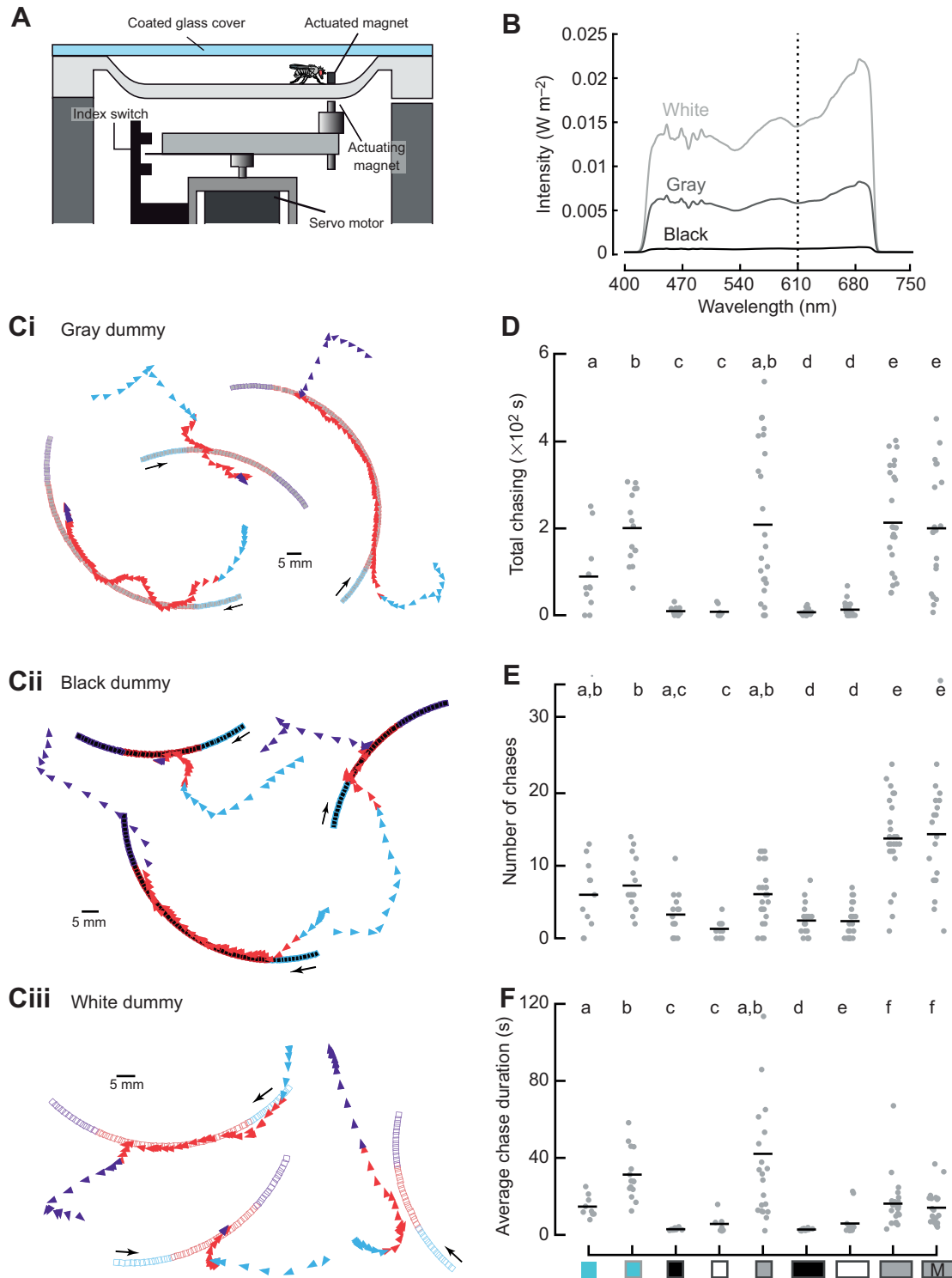
Fig. 1. Male *Drosophila melanogaster* flies prefer chasing gray dummies. (A) Schematic representation of Flyatar, shown in vertical cross-section, not drawn to scale. (B) Spectral intensity of the different paints under the same light source as used during the behavioral assays, measured using a Konica Minolta CS-200. Dotted line represents the estimated limit of spectral sensitivity of the retina of *D. melanogaster* (Stavenga and Arikawa, 2008). (C) Example traces (three each) of a male chasing a gray, black or white dummy, displayed at 2 frames s^{-1}. Triangles indicate the positions and orientations of male flies, and the squares indicate the positions and orientations of the dummy. Blue represents position before the chase, red during the chase and purple after chase end. Black arrows indicate the direction the dummy is initially moving. (D) Total time males spent chasing. (E) The total number of chases males initiated towards the dummy. (F) Average chase duration. Gray dots indicate the response of single males during a 10 min behavioral trial. Black dashes plot the population mean. Groups with the same letter do not have significantly different means (Fisher's exact test, $P<0.05$, plus Bonferroni correction). Dummy coating is indicated along the horizontal axis in F (from left to right): plain square, clear-coated square, black square, white square, gray square, black rectangular, white rectangular and gray rectangular. In the last column, white paint and black ink were mixed to create a new, mixed gray (denoted by an 'M'). All dummies, except for the first column, had a final clear-coat. In the first five columns, the dummy measured 1.6×1.6×0.8 mm. In the last four columns, the dummy measured 1.6×3.2×0.8 mm. From left to right, n=11, 15, 15, 10, 24, 18, 22, 25, 20.

longer than they did a black dummy, but these events were quite infrequent (Fig. 1C,E,F, Movie 1). To further verify that the preference for the gray dummy was not due to a chemical property of the paint, we combined the white paint and black ink to make a gray similar in tone to the Supernova gray paint. We found that males also exhibited a strong chasing response to dummies painted with this mixed gray paint, and this response was indistinguishable from the response to the original gray paint (Fig. 1D–F).

We further examined males' position relative to the dummy when chasing. Although wild-type males maintained a distance between themselves and the dummy that was similar whether chasing the plain, clear-coated or gray-painted dummies, males never approached as closely when chasing the black or white dummies (Fig. 2A,B). Because chases towards the black and white dummies were so much shorter, this difference is likely because males terminated chases before getting very close to the dummy. We did not observe any examples of wing extension towards the white dummy, and males also spent less time extending a wing and singing when chasing the black dummy (Fig. 2D). Dummy color did not influence the distance between the male and dummy when males extended their wings (Fig. 2C), suggesting that wing extension tends to occur at a fairly stereotyped inter-fly distance independent of target contrast.

To test whether attraction to the gray dummy was mediated via contrast, and therefore intensity relative to the background, we fabricated two new behavioral chambers from black and gray PVC plastic. These chambers were otherwise identical to the original white chamber. However, when wild-type Canton-S flies were placed in either the black or gray chambers, they spent almost no time courting any dummies regardless of their color (Fig. 3A). Consequently, we chose to raise the baseline level of chasing by thermogenetically activating P1 neurons, which have been previously shown to mediate courtship activity (Kimura et al., 2008; Kohatsu et al., 2011; Pan et al., 2012; von Philipsborn et al., 2011). Males expressing TrpA1 under the control of a split-GAL4 driver targeting P1 cells (P1-GAL4, from Hoopfer et al., 2015) were placed into either the white, gray or black PVC chambers at an average ambient temperature of 27°C. We initially tested P1-GAL4×TrpA1 males at a higher temperature (29°C) more similar to past thermogenetic experiments. However, we found that at this increased temperature, males exhibited atypical behaviors rarely exhibited by wild-type Canton-S males, such as frequently jumping on the dummies. Because P1 neurons have also been implicated in gating aggressive behaviors, we opted to use a lower temperature for all experiments to avoid any possible confounds of different behavioral states.

Overall, males with activated P1 cells did chase the dummies more compared with wild-type CS flies, although they, too, spent much less time chasing in the black or gray chambers than in the white chamber (Fig. 3B). Both wild-type flies and males with activated P1 neurons demonstrated a similar, strong preference for the gray dummy when in the white chamber; as a result, we believe that data using the thermogenetically manipulated males can be interpreted to explain the behavior of wild-type males.

In the gray chamber, males chased the gray dummy more than the black or white dummies. In contrast, in the black chamber, males paired with black dummies spent slightly more time chasing than males paired with the gray or white dummies (Fig. 3B). In the black chamber, the black dummy would generate the least contrast against the background, suggesting that males indeed prefer chasing low-contrast targets. However, although males paired with the black dummy spent the most time chasing compared with all other males in the black chamber, there were no significant differences between
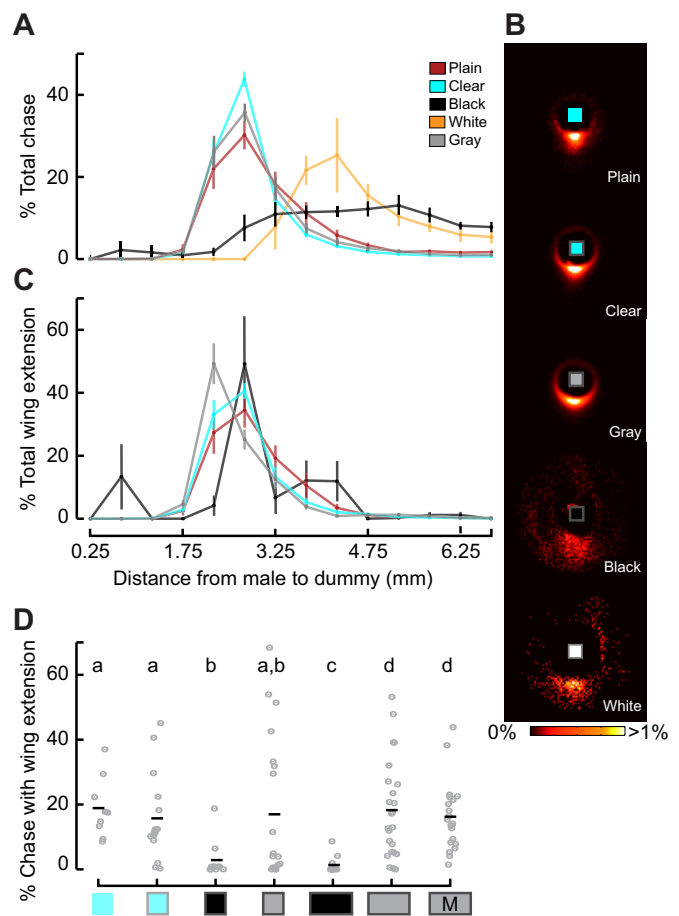
Fig. 2. Males rarely approach black or white dummies and demonstrate very little wing extension. (A,B) Histogram of relative distance between male and dummy during chases (as measured from their centers). Error bars depict standard deviation, and data points plot averages over flies; n=11 (plain), 15 (clear-coated), 15 (black), 10 (white), 24 (gray). (A) Includes data from all chases. Distributions for black and white dummy are significantly different from the rest (two-sample t-test, $P<0.05$, plus Bonferroni correction). (B) 2-D histogram of the positions of male flies during chases in dummy-centered coordinates when chasing the indicated dummy. Data are oriented such that the dummy would be moving towards the upper left corner. (C) Data from sequences when the male is also extending a wing. No distributions are significantly different from one another (two-sample t-test, $P<0.05$, plus Bonferroni correction). (D) Percent of time spent chasing in which males also extended a wing. Gray dots indicate the response of single males 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 (denoted by an 'M'). In the first four columns, the dummy was 1.6×1.6×0.8 mm. In the last three columns, dummy was 1.6×3.2×0.8 mm. Groups with the same letter do not have significantly different means (Fisher's exact test, $P<0.05$, plus Bonferroni correction).

the amount of chasing exhibited by males paired with a black dummy in the black chamber and males paired with a black dummy in the white chamber. We believe this lack of difference is because overall, chasing behavior was suppressed in the black chamber. Males placed in the white chamber traveled farther (Fig. 3C) and walked faster (Fig. 3D) than males in the dark chamber, regardless of dummy type. Thus, males in the dark chamber may simply encounter the dummy less often because they are not as active. To control for this possible confound, we instead examined only instances when the dummy and fly were less than 7 mm apart, and calculated the percentage of these close encounters that also resulted
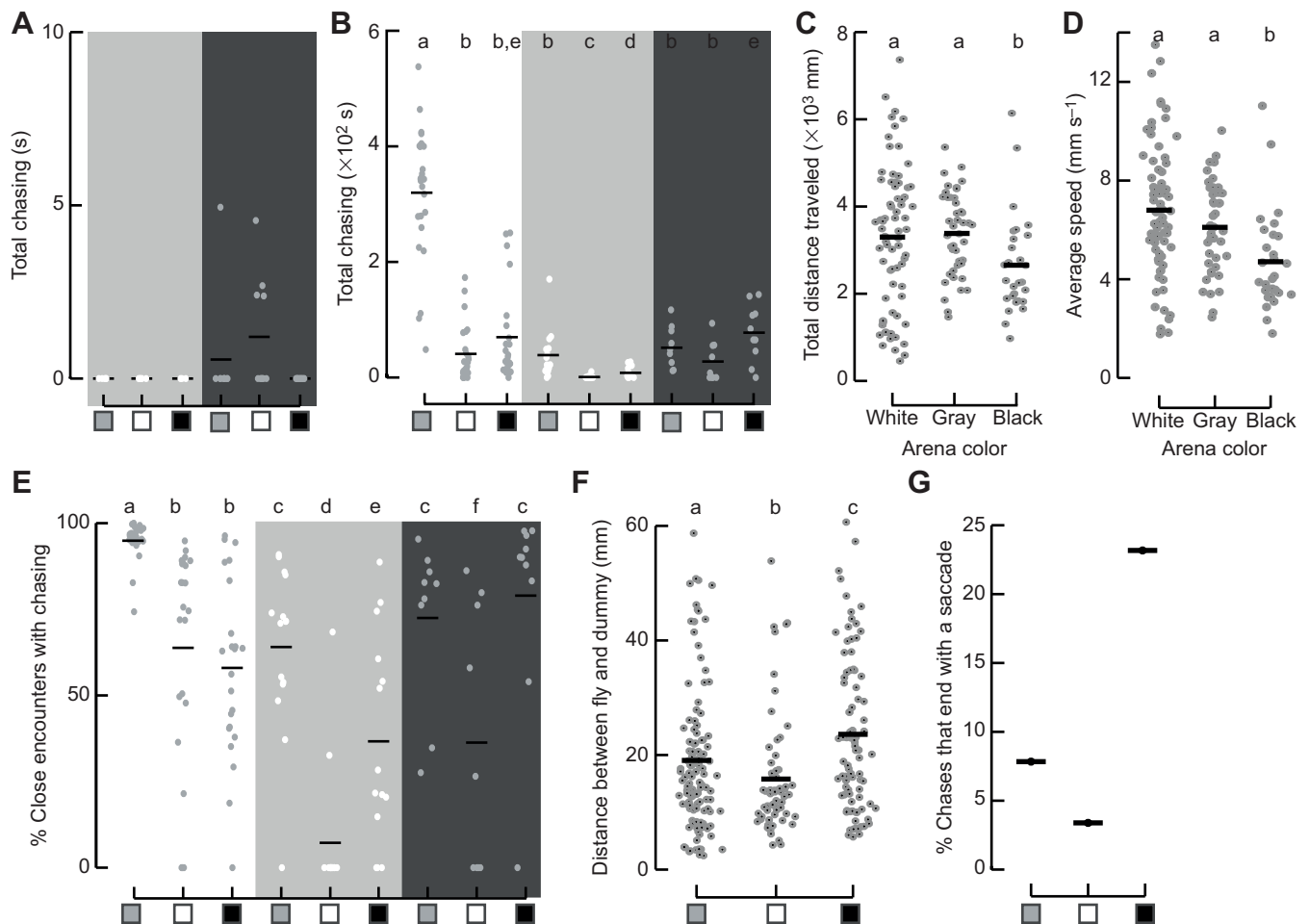
Fig. 3. Relative contrast influences male chasing behavior. (A–F) Gray dots indicate the response of single males during a 10 min behavioral trial. Black dashes plot the population mean. (A) Canton-S wild-type males at room temperature (~22°C) paired with the indicated dummy (gray square, white square or black square) in either a gray chamber (left three columns) or a black chamber (right three columns). From left to right, n=5, 5, 3, 9, 10, 10. Total time spent chasing is plotted. (B) Experiments using P1-GAL4×UAS-TrpA1 male flies in a 27°C chamber. Males were paired with dummies in either a white chamber (left three columns), gray chamber (middle three columns) or black chamber (right three columns) with the indicated dummy (gray square, white square or black square). From left to right, n=25, 24, 23, 15, 14, 14, 9, 9, 10. Total time spent chasing is plotted. (C) Total distance traveled by individual P1-GAL4×UAS-TrpA1 male flies in a 27°C chamber that was either white, gray or black. From left to right, n=72, 43, 28. (D) Average velocity of P1-GAL4×UAS-TrpA1 male flies in a 27°C chamber that was either white, gray or black. From left to right, n=72, 43, 28. (E) Percent of time when the P1-GAL4×UAS-TrpA1 male fly was less than 7 mm from the dummy and also chasing. Males were paired with dummies in a chamber that was either white (left three columns), gray (middle three columns) or black (right three columns) with the indicated dummy (gray square, white square or black square). From left to right, n=25, 24, 23, 15, 14, 14, 9, 9, 10. (F) Distance between P1-GAL4×UAS-TrpA1 male fly and dummy when male first makes a fixating saccade, centering the dummy in the center of his field of view. Dummies were painted either gray, white or black. From left to right, n=117, 63, 92. (G) Percent of chases that ended with P1-GAL4×UAS-TrpA1 males turning away from the dummy. From left to right, total number of chases=804, 177, 246.

in chasing (Fig. 3E). Using this metric, we found that males paired with a black dummy in the black chamber indeed spent more time chasing than males paired with a black dummy in the white chamber, suggesting that when the contrast of the black dummy decreases, it is more attractive to male flies.

Wild-type Canton-S males rarely chased the black or white dummies. However, males with thermogenetically activated P1 neurons spent more time overall chasing all three dummies. As a result, by examining the many resulting chase bouts made by P1-Gal4xTrpA1 males, we could better understand the influence of contrast on both chase initiation and chase termination. First, we examined whether the increased contrast of the black dummy enabled males to perceive the dummy from farther distances. We identified chase bouts that were preceded by a fixating saccade in which the male rotated more than 22.5 deg to center the dummy in his field of view, as this saccade is the earliest courtship-related

maneuver we are able to resolve in our videos. We found that, on average, males paired with black dummies made fixating saccades at greater distances from the dummy whereas males paired with white dummies made fixating saccades at shorter distances from the dummy (Fig. 3F). Thus, increased contrast may enable males to notice potential mates at greater distances. We next looked at how contrast influences chasing bouts termination. We found that males paired with the black dummy were more likely to end a chasing bout by turning away from the dummy (versus, for example, freezing) than when paired with the gray or white dummy (Fig. 3G).

Kimura et al. (2015) demonstrated that males can distinguish a female's anterior end from her posterior, and that vision is sufficient for this orientating behavior. Indeed, we also found that when males chased a female glued to a magnet, they oriented themselves so as to be closer to the female's abdomen (Fig. 4A). In these experiments, we rotated the female's abdomen so that it pointed either towards the
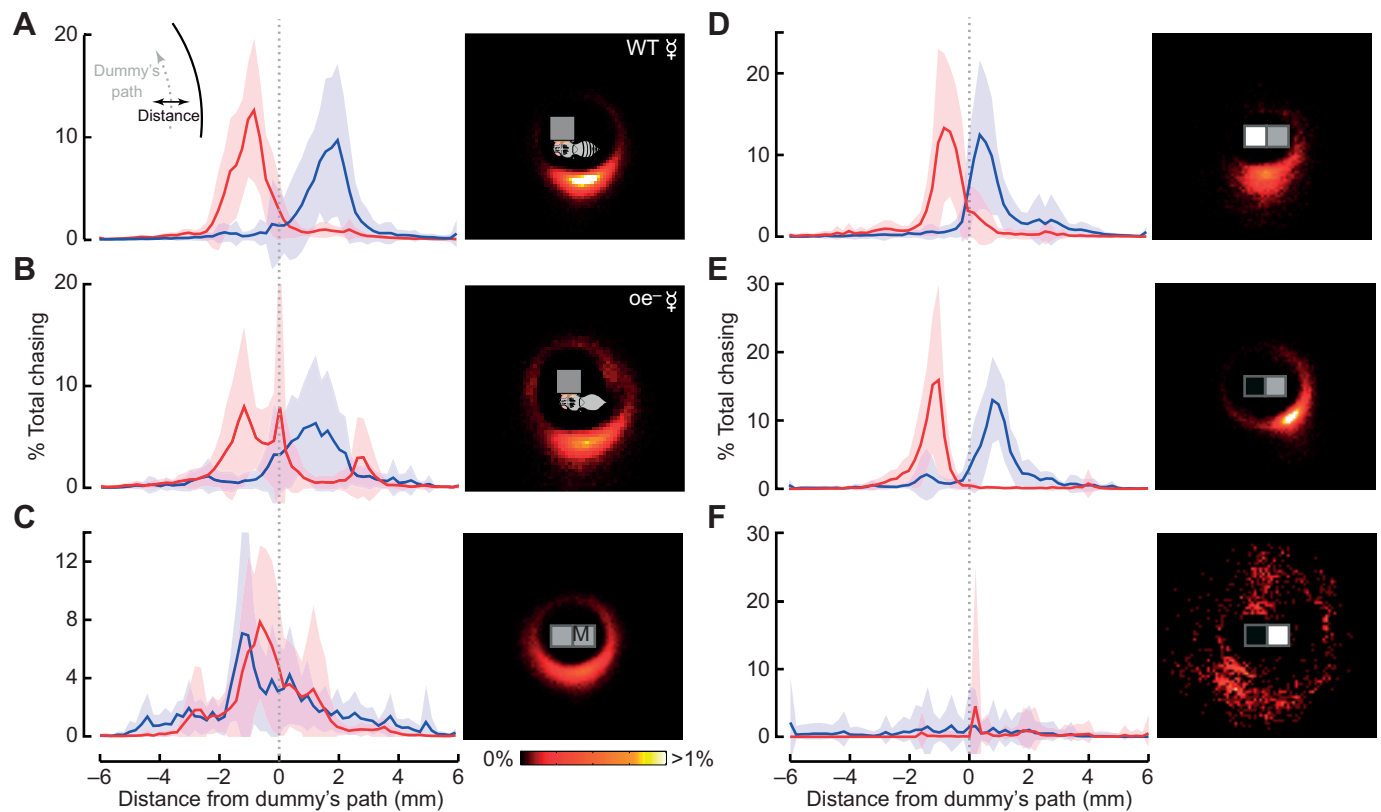
Fig. 4. Males paired with dummies painted multiple colors bias their position towards the preferred color. Histograms plot male position while chasing along radial axis extending from arena center (left, negative values) to arena edge (right, positive values), centered so that 0 reflects the dummy's center (as depicted in the left inset in A). Dark line plots population mean, shaded portion plots standard deviation. Right panels: 2-D histograms of male positions during chases in dummy-centered coordinates when chasing the indicated dummy, and correspond to data in the blue trace. Data are oriented such that the dummy would be moving towards the upper left corner. For red traces, dummy is rotated by 180 deg. In A and B, the dummy measured 1.6×1.6×0.8 mm and had a wingless, legless wild-type CS female (A; n=15, 24 for red and blue traces, respectively) or a female with pheromones genetically ablated (oe⁻) (B; n=17, 16 for red and blue traces, respectively) glued to the dummy as indicated. The mercury symbol in the right panels denotes virgin female. (C–F) The dummy measured 1.6×3.2×0.8 mm and was painted as indicated in the right panel. (C) n=10, 9 for red and blue traces, respectively; (D) n=16, 20 for red and blue traces, respectively; (E) n=14, 20 for red and blue traces, respectively; (F) n=10, 9 for red and blue traces, respectively.

center of the arena or towards the outer edge of the arena as the dummy moved. Males chasing a female with her abdomen pointed towards the center of the arena tended to be closer to the center of the arena, whereas males chasing a female with her abdomen pointed towards the edge of the arena tended to be further from the center of the arena. When we instead glued females to the magnet that had been manipulated to no longer produce cuticular pheromones via genetic ablation of their oenocytes (oe⁻ flies), males still biased their chasing to be closer to the oe⁻ female abdomens (Fig. 4B).

We further assessed whether dummy color could bias males' position during a chase. To do so, we painted dummies using two different paints, each covering one half of the rectangular target (Fig. 4C–F). When the dummies were painted uniformly gray (either with the original gray paint or the gray that was a mix of the white paint and black ink), males demonstrated no preference for either end of the dummy (Fig. 4C). Similarly, when the dummy was painted half black and half white, males did not demonstrate a strong preference towards either end (Fig. 4F). However, when paired with a dummy painted half gray and either half black or half white, males demonstrated a strong tendency to position themselves near the gray half, as if they interpreted that half to be a female abdomen (Fig. 4D,E). We do not know whether this behavior is due to attraction to the gray color or aversion to the black and white. Nevertheless, these results suggest that males could potentially use

pigmentation pattern as a cue to position themselves closer to a female's abdomen during courtship.

Given the low resolution of fly vision, we reasoned that a series of thin black and white stripes might appear gray, and thus attractive, to the male. We painted a half of a dummy with thin black and white stripes, and the other half white. Unfortunately, males rarely chased the striped dummy (Fig. 5A), responding to it similarly as to a black, white, or half-black and half-white painted dummy. However, in the little chasing data that we observed, males demonstrated a curious pattern: at a greater distance, they demonstrated a slight bias towards the striped end of the dummy, whereas as they neared the dummy, they demonstrated an increasingly stronger bias towards the white end (Fig. 5B).

DISCUSSION

All animals face the challenge of exhibiting appropriate behaviors in appropriate contexts. Key to this challenge of proper action selection is correctly identifying objects in the environment as predators, prey or potential mates. Male *D. melanogaster* use a combination of chemosensory and visual cues to identify and pursue conspecific females (Dickson, 2008). However, owing to the heavy molecular weight and poor diffusivity of most species-specific pheromones, males may rely on visual cues at greater distances. We have previously shown that object shape and size are important long-range
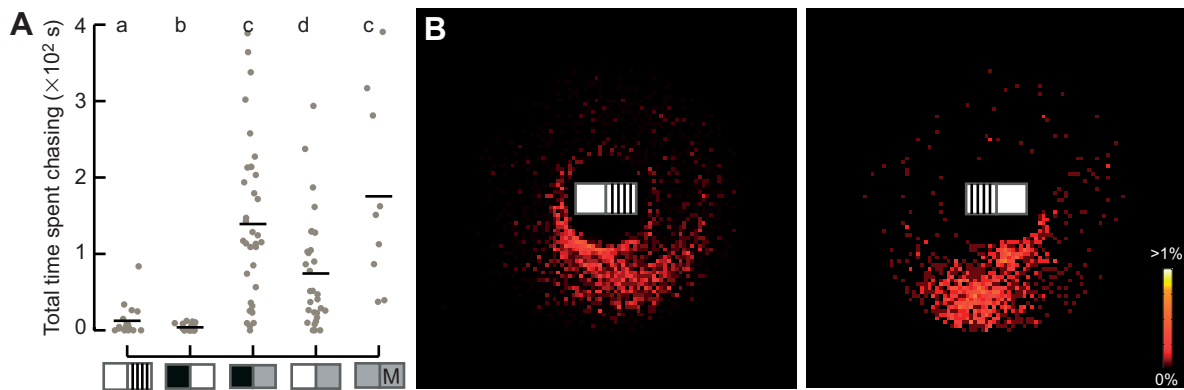
Fig. 5. Males are not strongly attracted to a black-and-white striped dummy. (A) Total time males spent chasing. Gray dots indicate the response of single males during a 10 min behavioral trial. Black dashes plot the population mean. Dummy measured 1.6×3.2×0.8 mm. Dummy coating is indicated along the horizontal axis: half white, half striped; half black, half white; half black, half gray; half white, half gray; half gray, half mixed gray. From left to right, n=18, 21, 35, 30, 9. Groups with the same letter do not have significantly different means (Fisher'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 are oriented such that the dummy would be moving towards the upper left corner and the left side is towards the center of the arena. n=9 for each.

visual cues (Agrawal et al., 2014). In this study, we further demonstrate that an object's contrast is another important visual cue, and males avoid chasing high-contrast targets (Fig. 6).

As might be expected, in the present study, males did not spend much time chasing white dummies. They both initiated fewer chases

towards white dummies (Fig. 1E), never exhibited any wing extension (Fig. 2) and were also closer to the dummy before making a fixating saccade at the start of a chase (Fig. 3F). We hypothesize that the lack of strong contrast of the white dummy in the white chamber decreased the male's ability to follow and chase the
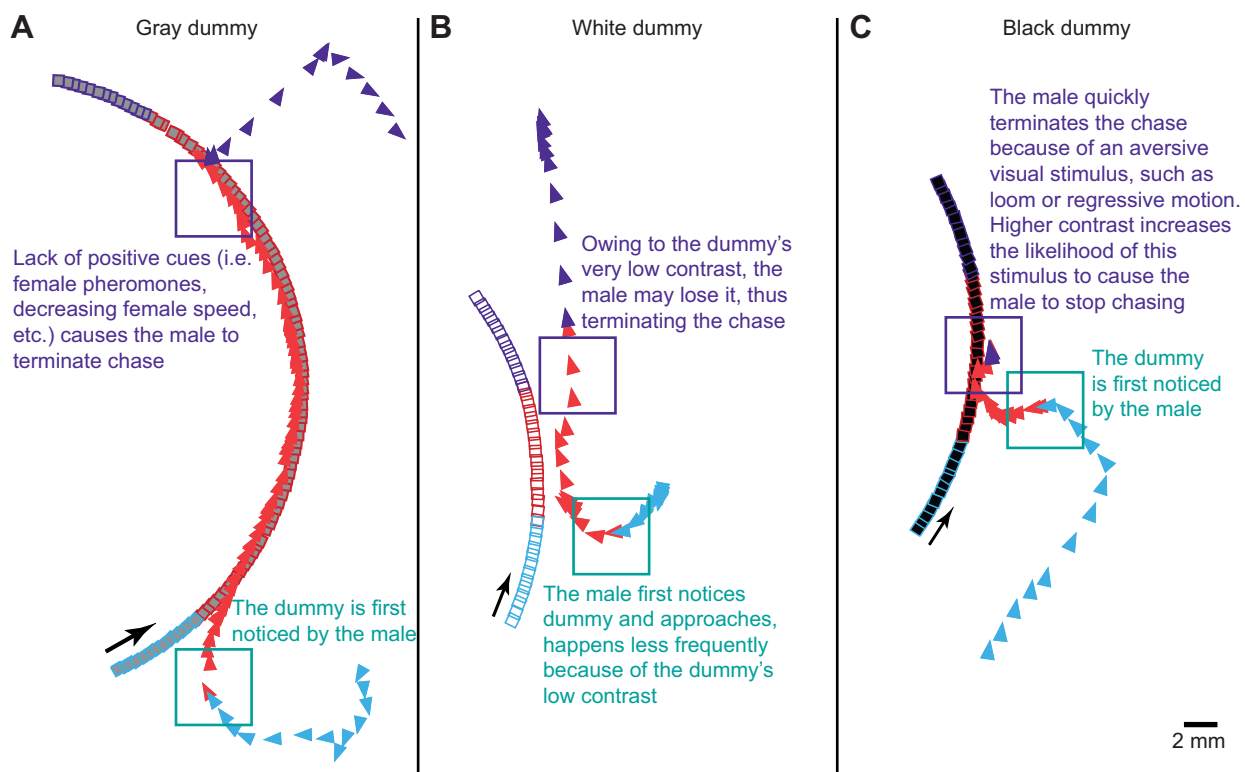
Fig. 6. Male preference for low-contrast targets is due to an interaction of multiple, visually guided reflexes. Schematic representation of the role of visual contrast during *Drosophila* courtship behavior. Each panel depicts an actual chase made by a male of the indicated dummy (from left to right: gray, white, black). Green boxes highlight the start of the chase, when the male first notices and orients towards the dummy. This orientation, and the male's subsequent decision to approach and chase, relies on sufficient contrast for the male to be able to discriminate the target. If an object has too low a contrast, such as the white dummy, fewer chases are initialized because the male is less able to distinguish the dummy from the background. Purple boxes highlight the end of the chase. Regardless of the dummy's appearance, the absence of other requisite signals will lead the male to eventually terminate the chase. In the case of very low contrast objects such as the white dummy, the male may terminate the chase simply because he has lost the object and can no longer fixate and pursue it. In the case of high-contrast objects such as the black dummy, as the male chases the target, aversive visual stimuli such as a high-contrast loom or regressive motion would cause the male to freeze or turn away, thus terminating courtship. The higher the contrast of the target, the more likely that a looming or regressively moving stimulus would cause the male to stop chasing.

dummy. However, even when we increased the contrast of the white dummy by switching the background chamber, males still did not spend much time chasing the white dummy (Fig. 3). This result suggests that the sign of the contrast may be important as well.

That males would so strongly prefer an object of intermediate contrast is striking. For animals with a low-resolution compound eye, contrast is often thought to mediate object detection, with higher contrast indicating greater behavioral salience (Dvorak et al., 1980; Götz, 1965; Land, 1997). Some insects, such as hoverflies, have adaptations such as larger ommatidial facets to increase contrast sensitivity so as to better detect mates (Straw et al., 2006). As such, we would have expected that males would more often pursue black dummies, or at least pursue black and gray dummies equally, because the higher contrast objects would be more easily detected. Although we did find that increased contrast increased the distance at which males first make a fixating saccade towards the dummies (Fig. 3F), we found that males strongly prefer chasing the lower contrast gray dummies. Males were most likely to court a black dummy when it was on a black background, thus reducing the relative contrast of the target (Fig. 6).

Sensitivity to contrast may be a species-specific cue. Many drosophilids vary in their pigmentation, and several are particularly darkly colored (Wittkopp et al., 2003). These species may prefer chasing higher-contrast targets, as compared with *D. melanogaster*, which has a lightly pigmented cuticle. Males could also use pigmentation to distinguish specific female anatomy, information that would then be used to pattern courtship. *Drosophila* possess a characteristic pattern of pigmentation that includes a large red eye at the head end, and an abdomen that is a light yellow on the ventral side and striped with dark pigments on the dorsal side. Past research suggests that males are indeed able to discriminate the abdomen from the head of the female in order to position themselves during courtship (Cook, 1980; Kimura et al., 2015). Whereas chemosensory cues are definitely important for this orienting behavior, olfaction-defective $Or83b^2$ homozygous males still position themselves appropriately. When we glued a female to a moving magnet, we also found that males preferentially positioned themselves near the female's abdomen when chasing. We were able to recapitulate the positioning phenotype by painting a target dummy two different colors: males always preferred positioning themselves near the gray end of the dummy and away from the white or black end. We do not know, however, whether this behavior is the result of the males actually perceiving the entire painted dummy as a female with varying pigmentation. Males may simply be attracted to the gray color, find white or black aversive, or both. To our knowledge, we do not know of any behaviors that require the male to be specifically oriented towards the head or abdomen, with the exception of genital licking (which our assay lacks the resolution to measure), and, of course, copulation (which does not occur with dummies). Some past observations suggest that males may direct song towards the female head by consistently extending the wing closest to the head (Bastock and Manning, 1955; Cook, 1979, 1980); we were never able to recapitulate this observation. Thus, although our experiments do not conclusively prove that males are using pigmentation to visually locate a female's abdomen, they do suggest that pigmentation and relative contrast could be two such visual cues.

The preference for gray targets that we observed could arise at many different points in the courtship circuitry. Object-detecting, visual projection neurons that relay visual information to courtship-controlling circuits might simply be more sensitive to gray or intermediate contrast objects, resulting in a matched filter for low-contrast targets. For example, work by Keleş and colleagues (2017)

describes a lobular columnar neuron LC11 that is sensitive to small moving targets and demonstrates a preference for low-contrast targets. However, during our behavioral experiments, we found that males paired with black square dummies initiated a similar number of chases compared with males paired with gray square dummies, suggesting that their initial attraction towards the black or gray square targets does not differ significantly. Additionally, the lobular columnar neuron, LC10 – which has been directly shown to be necessary for courtship (Ribeiro et al., 2018) – does not demonstrate any differences in response when presented with dark or bright square bars. Accordingly, in contrast to what we previously found when varying dummy shape (Agrawal et al., 2014), we do not think that males' preference for gray dummies is due to contrast sensitivity in a mate-detecting visual filter. Rather, we hypothesize that males' preference for gray dummies could arise via an aversive reflex that is triggered most often when pursuing black dummies, thus truncating any such chases (Fig. 6). In other words, we suggest that our results are explained by an interaction of multiple visual reflexes as males approach a target, the rapid movement will result in a looming stimulus as the distance to the target decreases. Flies are sensitive to and navigate away from looming stimuli, and past experiments suggest that a dark, high-contrast looming stimulus is particularly effective at triggering an escape response (Gabbiani et al., 1999). Looming is not the only visual motion stimulus that could interfere with males' courtship: walking females will also freeze in response to a regressively moving black target (Zabala et al., 2012). Our results are consistent with the hypothesis that rather than preferring low-contrast objects, males find high-contrast objects aversive. Chases of black dummies were shorter (Fig. 1F), males never approached the black dummies as closely (Fig. 2A), perhaps because they truncated chases so soon after approaching (Movie 1), and males were also more likely to end chases of black dummies by turning away. Future experiments using a tethered walking fly surrounded by a visual virtual reality set-up could address the extent to which looming or sudden regressive motion could cause termination of courtship behaviors.

Data availability
Data are available from the Dryad Digital Repository (Agrawal and Dickinson, 2019): https://doi.org/10.5061/dryad.k74670v

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.203414.supplemental

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