Intense light unleashes male-male courtship behavior in wild-type *Drosophila*

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***Drosophila* courtship studies have elucidated several principles of the neurogenetic organization of complex behavior. Through an integration across sensory modalities, males perform stereotypic patterns of chasing, courtship song production, and copulation attempts. Here we report a serendipitous finding that intense light not only enhances courtship toward female targets but also triggers unexpected courtship behaviors among male flies. Strikingly, in wild-type male-only chambers, we observed extreme behavioral manifestations, such as "chaining" and "wheeling", resembling previously reported male-male courtship behaviors in *fruitless* mutants and in transformants with ectopic *mini-white*$^{+}$ overexpression. This male-male courtship was greatly diminished in a variety of visual system mutants, including disrupted phototransduction (*norpA*), eliminated eye-color screening pigments (*white*), or deletion of the R7 photoreceptor cells (*sevenless*). However, light-induced courtship was unhampered in wing-cut flies, despite their inability to produce courtship song, a major acoustic signal during courtship. Unexpectedly the olfactory mutants *orco* and *sbl* displayed unrestrained male-male courtship. Particularly, *orco* males attained maximum courtship scores under either dim or intense light conditions. Together, our observations support the notion that the innate male courtship behavior is restrained by olfactory cues under normal conditions but can be unleashed by strong visual stimulation in *Drosophila*.**

Introduction

The male courtship behavior repertoire in *Drosophila melanogaster* emerges from a complex integration of external sensory information (1–3), prior experiences(4–6), and internal drive(7–9). A large collection of discrete visual(10–12), olfactory(13–15), gustatory(16–18) and mechanosensory(19) cues serve to direct courtship behavior towards receptive females. These signals in-turn trigger stereotypic sequences of chasing, licking, wing extension, courtship 'song' production and eventual mounting (20, 21). Genetic perturbations in this sensory-motor integration process can reveal contributions of the genetic-organizations and neural circuit-mechanisms underlying this complex behavioral program(22–24).

A striking phenotype arising from certain genetic manipulations is male-male courtship behaviors. For example, certain *fruitless* mutants display remarkable 'chains' of courting males(25, 26). The *fruitless* gene product undergoes alternative splicing to produce female- and male-specific transcripts, fru^F and fru^M , respectively(27, 28). In fru^M-disrupted males, male-male courtship is often observed(27). Dysfunction of fru^M-positive sensory neurons disrupt reception of the male-specific repulsive cues, 7-tricosine and cis-vaccenyl acetate, leading to male-male courtship(29–33). Furthermore, activation of fru^M-expressing neurons, through optogenetic means, induces male-male courtship(34, 35). Male-male courtship has also been reported in studies using *painless* TrpA channel mutants(36), dopaminergic signaling mutants(37, 38), or transformants for forced expression of mini-*white* constructs(39, 40).

Here, we describe a serendipitous finding of male-male courtship behaviors in wild-type (WT) *Drosophila melanogaster* evoked by intense light. We observed the light intensity-dependent increase in chasing, wing extension courtship song and chaining in different strains of WT males, using LED-, incandescent- and sun-light illumination. We examined the phenomena in flies with genetic and surgical manipulations to elucidate the key roles of the visual and olfactory systems in gating this male-male courtship behavior.

Results

Intense light riggers courtship behavior in male flies. A modified semi-automated *Drosophila* tracking system, IowaFLI Tracker(41) was employed to analyze activity in each circular arena containing eight flies (Figure 1). The tracking system consisted of a clear polyacrylic sheet containing four arenas which were housed in a light-shielded cylindrical chamber equipped with an inner circular strip of LED lighting (Figure 1A). A webcam mounted on top of the ceiling captured fly behaviors. In male-only arenas, we unexpectedly discovered a high frequency of courtship behaviors at intense light setting. Seconds after light on, we regularly observed chasing and attempting to court among male flies (Figure 1B, Movie 1). Strikingly, we often saw chains of courting males (Figure 1B, Movie 1), reminiscent to phenotypes previously found in mini-*white* transgenic lines(39, 40) and *fruitless* mutants(25, 42). In a modified arena equipped with a microphone, we recorded light-induced pulse and sine courtship songs (Figure 1C) which correlated with courtship-related wing extensions in the arena.

To quantify light-induced courtship, we developed a protocol where the eight flies in the arena were first subjected to 2-

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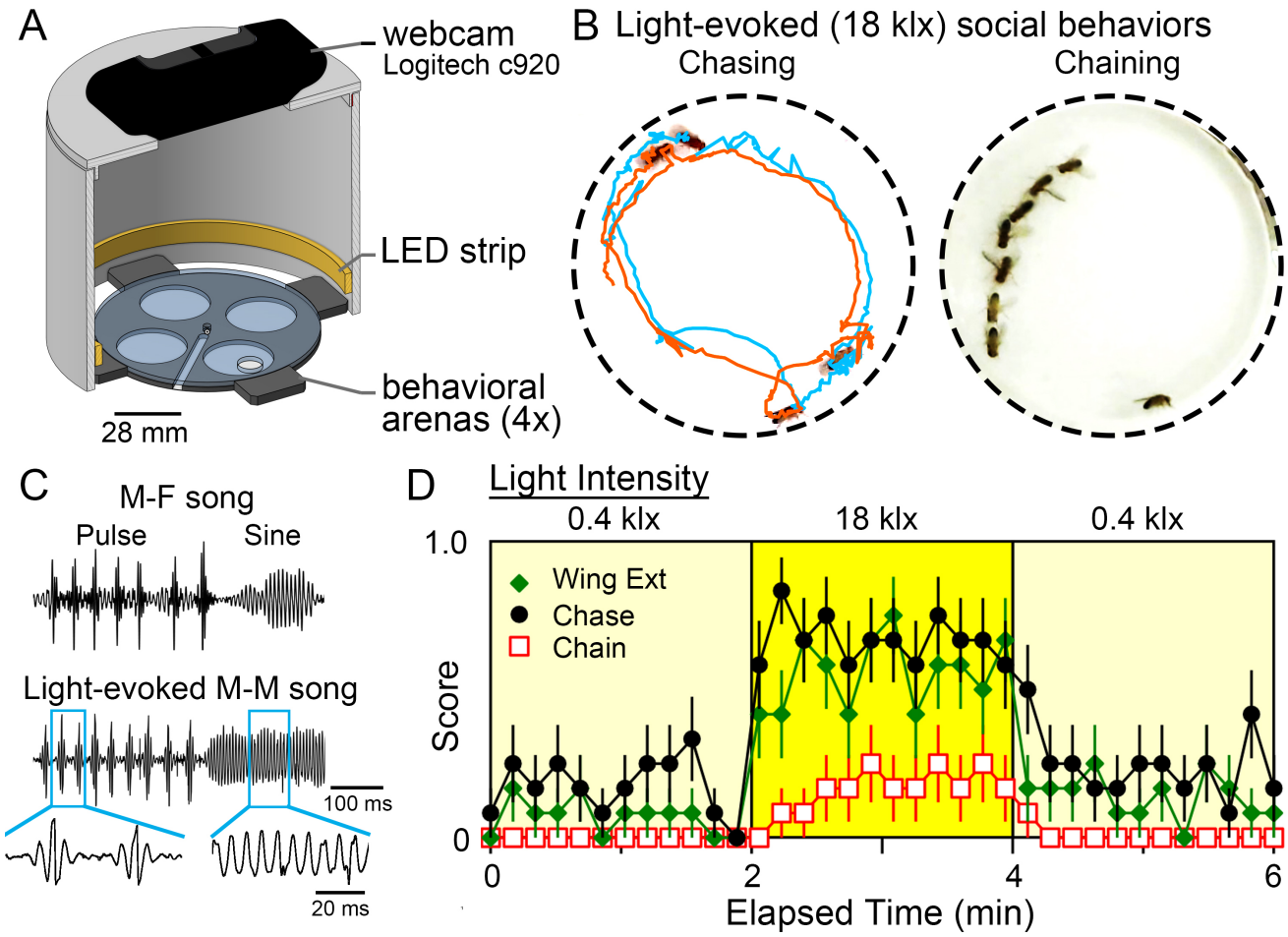

Fig. 1. Light-evoked male-male courtship behavior in *Drosophila*. (A) Schematic drawing of the behavioral arena, LED strip lighting and webcam configuration. Eight males were loaded into each of the four circular behavioral arenas (regions of interest, ROIs). An LED strip surrounding the arenas illuminated fly behavior with minimal shadowing, and a webcam above recorded activity. (B) Intense light triggers male-male courtship behaviors including chasing (left, orange track chasing cyan track), as well as chaining and wing extensions (right). (C) In modified arenas equipped with a microphone (see Methods), intense light triggered pulse and sine songs in male-only arenas (lower waveform), resembling qualitatively songs recorded in mixed (4 male & 4 female) arenas (upper waveform) (D) Quantification of light-induced courtship behaviors. Eight males were subjected to 2-min low-light intensity period, followed by 2-min of high-intensity light, and a 2-min low-light recovery period. The presence or absence of respective behaviors was scored during successive 10-s intervals. Data points represent the average score for each time bin (10 s) depicting fraction of positive detection among the observations (n = 12 ROIs) for the various behaviors (see Methods). Error bars indicate SEM for the binominal distribution.

min illumination of relatively low intensity (0.4 klx, within the normal range of room lighting) followed by 2-min of high-intensity light (18 klx) and subsequently, another low-intensity light period (Figure 1C). For each successive 10-s interval, we manually scored the occurrence of chasing, wing extensions and chaining in the arena. The total number of intervals during which the respective behaviors were observed in the arena was reported as the "total score" (ranging from 0 to 12, Figure S1; see Methods). During the high-light period, the increase in total chasing score was more than 700% ($p = 0.0001$), ∼400%. ($p = 0.0002$) for wing extension, and greater than 800% ($p = 0.045$) for chasing (Figure S1).

Generality and specificity of light-induced courtship behaviors. To determine whether light-induced male-male courtship is a specific phenomenon restricted to our WT *Canton-S* (CS) strain used in most of the experiments, we tested and found the same male-male courtship behaviors in other *melanogaster* strains, as shown in Figure 2A for a second WT strain, *Berlin*. We observed similar high-light-induced increases in wing extension and chasing behavior as CS flies, with occasional chaining observed as well (Figure 2A). Although a comprehensive analysis of light-induced courtship across a large collection of WT strains is beyond our scope, our observations thus far suggest light-triggered male-male courtship may be a pervasive behavior among strains of WT *D. melanogaster*. We also compared mixed-sex arenas (4 male and 4 female flies) to examine how high-intensity illumination affects male-female interactions, in order to determine whether the intense light-triggered courtship behavior was restricted towards other males only. As shown in Figure 2B, in these mixed-sex arenas, high-intensity lighting not only evoked male-male, but also similarly increased male-female courtship behaviors (∼300%. $p < 0.001$). Together, these findings suggest intense light intensifies courtship drive of male flies toward both male and female flies, rather than switches their sex preference from females to males (Figure S1).

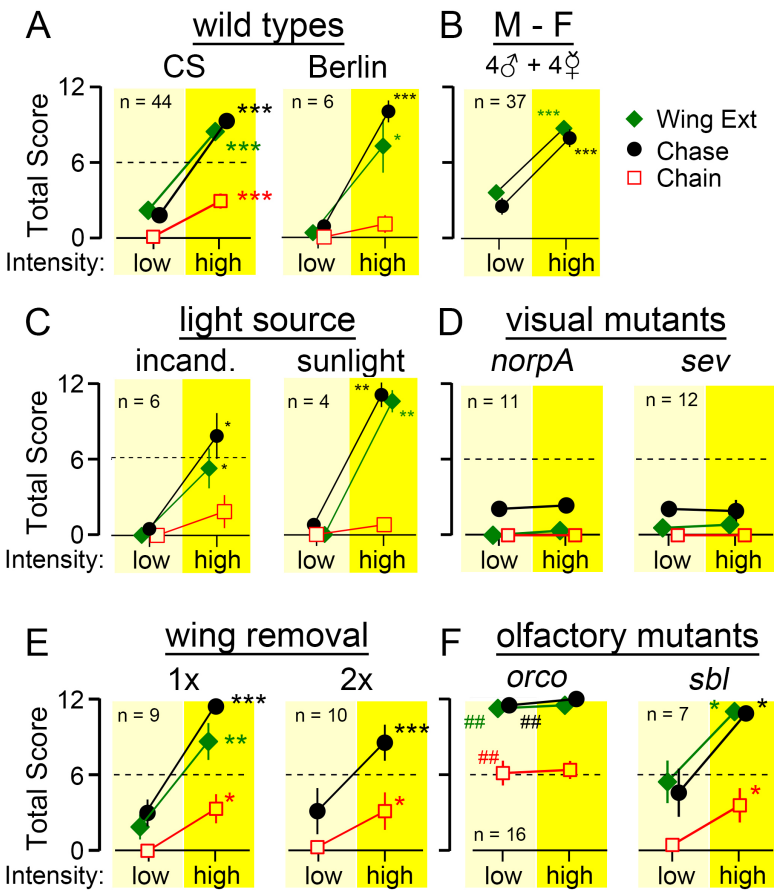

Fig. 2. Genetic and environmental factors affecting light-evoked male-male courtship. Flies were subjected to a 2-min period of low light intensity followed by 2-min of high light intensity (0.4 klx and 18 klx respectively, except for panel C with different light sources). (A) Light-evoked male-male courtship in the CS and Berlin WT Drosophila strains. (B) Courtship behavior in mixed sex arenas with 4 males and 4 females. (C) Male-male courtship in arenas under incandescent light (left) or in direct sunlight (right). For incandescent illumination, light was delivered by two light pipes from an USHIO EKZ 10.8 V, 30 W bulb ('low' intensity: 0.4 klx, 'high' intensity 25 klx). For sunlight experiments, the behavioral arena was placed in a shaded area for the 'low' period (0.4 klx) and moved to direct sunlight (90 klx) for 'high' period. Experiments were done in the Biology Building courtyard on the University of Iowa campus on 19/05/2022 at 15:00 CDT (temp 29 ℃). (D) Lack of light-triggered courtship behaviors in visual mutants (norpA and sev). (E) Wing-cut flies and (F) Olfactory mutants (orco and sbl). Note the light-induced increase in courtship behavior is absent in visual mutants, but unimpaired in flies with one (1x) or both (2x) wings removed. Olfactory mutants show greatly enhanced courtship activity, with orco attaining nearly maximum scores even at low-light intensity. Data are shown as mean +/- SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, paired t-test (high vs. low). For each panel, the sample size (ROIs) is indicated.

Roles of sensory cues in light-triggered male-male courtship. We first characterized the efficacy of different lighting conditions for triggering the male-male phenomenon. By adjusting the arena LED light intensity, we found that the relationship between light intensity and incidence of wing extensions, chasing and chaining behaviors was monotonic and that the proportionally steepest increases occurred between 2.0 and 6.0 klx (Figure S2). We then asked whether the LED light source we used was specifically required, or some other sources could also evoke the effect. As shown in Figure 2C, intense incandescent light (25 klx) also elicited courtship behavior. We further asked if natural light (e.g., intense sunlight on a spring day) could induce male-male courtship activity. Remarkably, after moving the arena from a shaded location ($\sim$ 0.4 klx) to a location under direct bright sunlight ($\sim$90 klx), we observed robust courtship activity (Figure 2C). Taken together, these results indicate a wide variety of sufficiently intense, broad-spectrum visible light sources can effectively induce male-male courtship behavior.

We introduced experimental perturbations to the various sensory systems for indications of their roles in the light-induced male-male courtship. First, we examined males with a disrupted visual system. The gene *norpA* encodes the enzyme phospholipase C required for phototransduction, and several mutations eliminate photoreceptor potential, rendering mutant flies completely blind(43). In blind $norpA^{P12}$ mutants, we found intense light did not induce courtship activity (Figure 2D, Movie 1). To monitor the activity patterns among the male flies, an automated analysis of kinematic properties of individual flies as well as social interactions among them. We employed IowaFLI Tracker(41) with an interaction proximity criterion of 3.5 mm, which was found optimal for capturing chasing events in WT flies. As Figure 3 shows, unlike WT flies, upon high-intensity illumination, *norpA* flies displayed neither a drastic increase in activity levels (left panels, continuous locomotion tracks with individual flies color-coded) nor a greatly enhanced level of social interactions (right panels, colored time segments registered individuals active within the 3.5 mm vicinity).

We further took advantage of anatomical mutations affecting a particular category of photoreceptors. The *Drosophila*

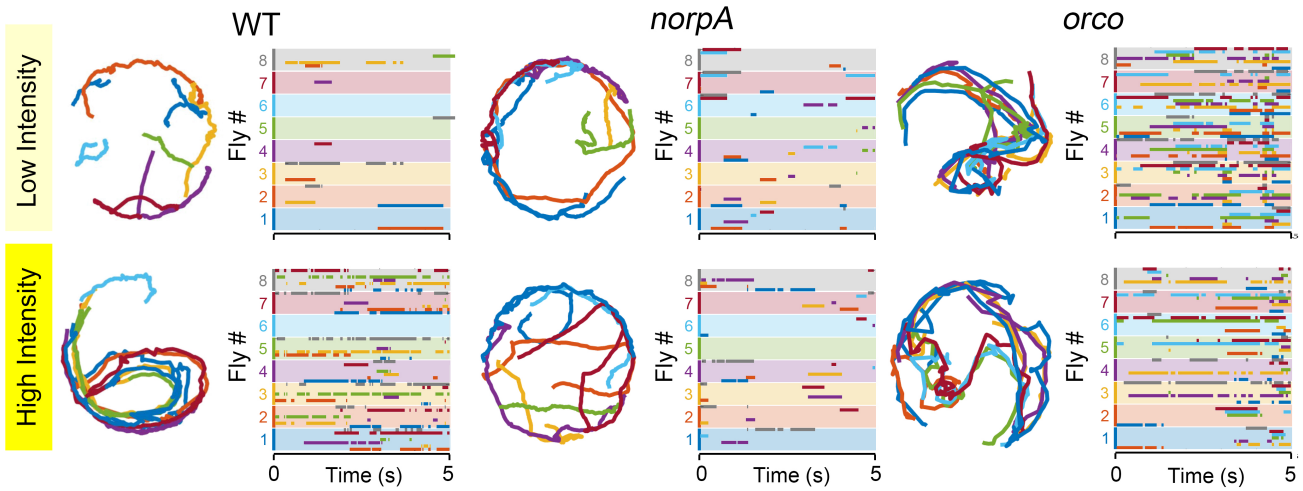

Fig. 3. Fly tracks and interactogram of light-induced male-male courtship. Representative behavioral trajectories (left panels for each genotype; 5-s sample) and interactograms (right panels; corresponding to the left trajectories) of WT, *norpA*, and *orco* males under low and high light. Note that flies are chasing others under high light in WT, and under both low and high light in *orco* mutants. However, *norpA* flies displayed no increase in levels of locomotion or social interactions. To monitor fly activity patterns, the IowaFLI Tracker program (41) was used for automated analysis of kinematic properties of individual flies as well as social interactions among them. An interaction proximity criterion of 3.5 mm, optimal for capturing chasing events, was adopted. For each genotype, individual flies (#1-8) were color-coded to depict their continuous locomotion tracks (Left), and to register events of individual fly's encountering others (each color-coded, falling within the 3.5-mm mutual distance) along the 5-s duration (Right). In the horizontal shaded bands of 8 different colors, interaction of the fly of the designated band color with all other individuals were registered with darker color-coded line segments to indicate the time intervals when their mutual distance < 3.5 mm.

compound eye is composed of about 800 facets, each cylindrical rhabdomere below consisting of 6 outer photoreceptors (R1 - R6, green light sensitive) and two central photoreceptors (R7 and R8, UV and blue light, respectively). In *sevenless* (*sev*) mutants, the high-acuity channels composed of central R7 and R8 is functionally impaired because R7 is absent, which also disrupts the light guide for R8 underneath, while the high sensitivity channel, consisting of R1-R6 are intact(44). The results showed that light-evoked male-male courtship was absent in *sev* mutants (Figure 2D), implying a critical role for the R7/R8 system in light-triggered male-male courtship. Visual processing depends on integration of photoreceptor responses from individual rhabdomeres. Because of the screening pigments, each acts as an independent light guide for input from a narrow angular visual field, in isolation of its neighbors. Mutations of the *white* (*w*) gene are devoid of the screening pigments and thus degrade visual image processing. In w^{1118} flies, despite their greater sensitivity to light and active locomotion in the arena, the male-male courtship behavior was missing (Figure S3). These observations indicate the central role of visual function in the light-evoked male-male courtship.

In contrast to the visual system, manipulations of mechanosensory or auditory cues revealed drastically different outcomes. In groups of males, courtship behaviors are known to be triggered by playback of courtship song(19). However, we found that in wing-cut male flies, which had disrupted wing mechanosensory inputs and were unable to produce courtship song, intense light nevertheless reliably triggered chasing and chaining (Figure 2E). Notably, flies with one wing or both wings cut displayed similar chasing and chaining scores and flies with only a single wing could attain a frequency of wing-extension comparable to WT. This result demonstrates the auditory cue generated by wing beats and the mechanosensory receptors along the wing blades are not required in the light-evoked male-male courtship.

To determine the role of olfactory systems, we examined two mutants *smellblind* (*sbl*), a *para* Na^+ channel allele with disrupted odor-evoked behaviors(45) and *odorant receptor co-receptor* (*orco*), encoding a requisite olfactory receptor subunit(46). Surprisingly, at low light intensities, both mutants displayed strikingly elevated courtship behaviors compared to WT males (Figure 2F, Figure 3). Indeed, recurrent male-male courtship in *orco* flies was so prevalent (Movie 1), frequently approaching the maximum score of 12, such that drastic increase in courtship was not apparent during the intense light periods. However, in *sbl* males, which showed milder increases in low-light scores, intense-light could further promote courtship activities to attain the maximum score (Figure 2F), prompting a further investigation into interactions between intense light and olfactory processes in gating male courtship behaviors.

Discussion

The role of visual system function in male-male courtship. Our findings add a striking case of male-male courtship behavior in WT flies, in contrast to previous reports from a number of genetic variants, including *fru*(25, 42), *painless*(36), an unidentified mutation (on Chr. 3L) reported by Sharma(47), as well as several mini-*white*$^+$ transformants(39, 40). A strong visual contribution to such behaviors was suggested by Sharma's observation that blue light inhibits male-male courtship(47). Similarly, blue and UV lights have been reported to suppress male-female courtship(6). In a preliminary follow-on experiment, we subjected orco1 and $orco^2$ males to intense blue light and found that male-male courtship was severely suppressed (Table S1). It is important to note that the two *orco* alleles employed in

this study carry the mini-w^+ construct (in place of the *orco* gene) in a w^- background (See Methods for the construct). Their eye color appeared normal indicating strong mini-w^+ expression. Therefore, the extreme phenotypes of *w;;orco*^{1} and *w;;orco*^{1} may have had contributions from mini-w^+ expression. Our preliminary analysis of *orco*^{1} and *orco*^{2} heterozygotes (*orco*/+) generated from crossing *orco/orco* to w^+ crosses displayed a relationship between eye-color density and male-male courtship activity. We observed darker eye color in *w;;orco*^{1}/+ than *w;;orco*^{2}/+, indicating differences in mini-w^+ expression, and accordingly a stronger male-male courtship behaviors in *w;;orco*^{1}/+ (Table S2). In terms of male-male courtship scores, we observed in the following sequence as shown in Table S2:
$w;orco^1/orco^1 = w;orco^2/orco^2 > w;orco^1/+ > w;orco^2/+$.
Furthermore, *norpA*^{P12}*/Y;;orco*^{1}/+ and *sev*^{LY3}*/Y;;orco*^{1}/+ males have been obtained and the behavior test suggests total suppression of *orco*^{1}/+ phenotype, behaving like *norpA*^{P12} and *sev*^{LY3} males (Table S2). Taken together, our results confirm a critical role of visual system function in male-male courtship.

The effective light intensity range and underlying candidate neural circuits. The above findings demonstrate that light plays a major role in courtship behavior in *Drosophila melanogaster*. Besides facilitating visually guided motor behaviors, intense light serves as a potent modulator promoting male sex drive as indicated by increased courtship behavior towards females as well as males. Notably, the light intensity range required to effectively trigger male-male courtship (between 2 and 6 klx, Figure S2) is several folds greater than common light sources encountered in the lab, including room lights or incubator lighting (∼0.5 klx, measurements in our lab). However, this light intensity is within the range that flies encounter in the wild, as direct sunlight can be upwards of 100 klx(48). Importantly, our findings with both incandescent light and sunlight (Figure 2C) indicate a variety of sufficiently intense broad-band light sources can induce male-male courtship. Although resolving the circuit-level processes gating light-evoked male-male courtship is beyond the scope of this report, it is tempting to speculate based on recent advances in identification and manipulation of neuronal components of various circuits involved in male courtship behavior. Our analysis suggests an interplay of visual and olfactory processing. Indeed, in a model previously proposed by Wang et al.(36), male flies have an intrinsic drive to court that is normally restrained by olfactory processing of certain signals from other males (e.g., cis-vaccenyl acetate(22)). Flies deficient in olfactory processing such as *orco* and *sbl* would lack these restraints, consistent with the increased male-male courtship shown in Figures 2 and 3. Conceivably, intense light could serve to unleash the intrinsic courtship drive either by supercharging the courtship motor program to overcome olfactory inhibition or by directly suppressing the inhibitory olfactory processing in WT flies. Within the broad-band illumination used in this study, the shorter wavelength UV and blue light preferentially activate R7 and R8, respectively. UV and blue light are known to suppress male-female courtship(6) and blue light was found to inhibit male-male courtship in the mutant flies reported by Sharma(47). Our analysis of *norpA* and *sev* mutants implies that R7 and/or R8 processing plays an important role in light-induced courtship (Figure 2B). Photoreceptors R7 and R8 directly target several classes of medulla neurons, including Dm8 amacrine-like cells, and Tm5 and Tm9 cells which project to the lobula plate(49). It is possible that these medulla neurons directly or indirectly influence excitability of *fru*-expressing P1 neurons in the lobula plate, which exert strong modulation on the activity of LC10a circuit that has been shown to be essential for controlling courtship chasing performance(3, 50). Future analysis will elucidate the action and modulation of the neural circuit underlying light-evoked male-male courtship. Our findings provide a new context of courtship behavior and can add constraints for data interpretation in the studies of neural circuits responsible for the repertoires in male courtship behaviors.

Methods

***Drosophila* Stocks and Husbandry.** All flies were reared at room temperature (23 °C) under a 12:12 light-dark cycle in vials containing cornmeal media(51, 52). All flies studied were age-controlled as specified. Throughout, WT strain used was *Canton-S* (except for Figure 2A, where WT-*Berlin* was used). Other mutant lines studied included: *smellblind* (*sbl*^{1}, *sbl*^{2}), EMS-induced mutant alleles of the voltage-gated sodium channel gene *paralytic* (gift from J. Carlson(45)); *orco*^{1} and *orco*^{1} (*w*;; TIw+mW.hs=TIorco*^{1} and *w*;; TIw+mW.hs=TIorco*^{2}; Bloomington 23129 and 23130), two insertion alleles of the gene *odorant receptor co-receptor* (*orco*); *norpA*^{P12}, an EMS-induced allele of *no receptor potential A*, encoding a phospholipase C required for visual transduction (gift from W.L. Pak(43)); and *sevenless*^{LY3} (*sev*^{LY3}) lacking R7 photoreceptors (gift from W.L. Pak(54)).

Courtship Behavioral Recording. Fly courtship behavior was observed in a custom-built chamber which consisted of an arena piece, and arena ceiling cover, and a light-shielded cylindrical chamber (Figure 1A). The arena piece consisted of a plexiglass sheet (2 mm thick) with four milled circular chambers referred to as "Regions of Interest" or ROIs, (26 mm diameter) seated on filter paper (Whatman #1) forming 2 mm-high behavioral arenas (4 ROIs). The arena ceiling cover was a plexiglass sheet of the same specification. The light-shielded chamber was constructed from a 4" PVC pipe coupler (NIBCO #4801) with a custom milled PVC lid. A 21 LED strip (STN-A30K80-A3A-10B5M-12V, SuperBrightLEDs.com; Figure S4) was glued along the inner rim next to the base of the light chamber. LEDs were powered by a custom-built current-controlled power supply (Figure S5). For Figure 2C experiments, incandescent light was delivered via fiber optic light pipes from an USHIO EKZ 10.8 V, 30 W bulb. Fly behaviors were captured by a Logitech c920 webcam connected to a PC. For microphone recordings (Figure 1B), a modified arena of a larger dimension was constructed

with a hole drilled in the side of the arena. A high-gain microphone (FC-23629-P16, Knowles Electronics, Itasca, IL, USA; see also(55)) was placed in the opening to pick up fly sounds. Light intensity measurements (Figure S2) were performed using a digital light meter (DLM1, Universal Enterprise Inc. Portland, OR, USA) following manufacturer's instructions.

Behavioral recordings were performed by aspirating 8 flies into each arena (without anesthesia). Following a brief acclimatization period (< 5 min), flies were recorded for 2 min under 'low-light' conditions, immediately followed by 2-min of 'high-light' conditions. In some experiments (Figure 1C-D), we included a 2-min 'low-light' recovery period.

Quantification and Statistical Analysis. Behavioral scoring was carried out in groups of 8 flies confined in the arena. (For male-female courtship, 4 males and 4 females were used). Criteria for chasing and wing extension behaviors were based on Hall(20), and we define chaining behavior as the formation of a mobile chain of 4 or more flies (cf. Zhang & Odenwald(39) and Hing & Carlson(40)). Scoring was performed by a pair of operators (an observer and a recorder), either on-site during live video recording or by viewing playback of videos. Recording and scoring were performed on pairs of ROIs, one for experimental and one for control (or comparison) flies. Each successive 10-second interval in the 2-min observation periods was divided into two 5-second scoring windows and experimental and control ROIs were scored alternately (i.e., 5 s for one ROI followed by 5 s for the other). the presence or absence of each of the courtship behaviors in an ROI was recorded in the 5-second window. The total number of 5-s windows (out of 12 possible) in which the respective behaviors were observed, reported as the "total score," ranging from 0-12. In addition, automated analysis of walking kinematics was performed offline with IowaFLI Tracker as described in Iyengar et al.(41).

All statistical analyses were performed in MATLAB r2021b. Unless otherwise indicated, all statistically significance values are reported from paired t-tests comparing the 'low' vs 'high' light conditions. Some experiments in Figure S1 employed one-way ANOVA as specified.

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Ethics: N/A

Data, code and materials: An updated version of IowaFLI Tracker (version 3.1) and the original data sets for figure and table construction can be found on github ($https://github.com/IyengarAtulya/IowaFLI_tracker$).

Competing Interests: The Authors have no competing interests to declare.

Contributions: A.U.: conceptualization, data curation, formal analysis, investigation, methodology, supervision, visualization, writing-review & editing; A.B.: formal analysis, investigation, supervision; T.K.: conceptualization, investigation, visualization; S.L.: investigation; M.R.: formal analysis, investigation, visualization; E.C.: investigation; C.F.W.: conceptualization, funding acquisition, investigation, methodology, project administration, visualization, writing - review & editing; A.I. conceptualization, data curation, formal analysis, investigation, methodology, resources, software, supervision, validation, visualization, writing – original draft.

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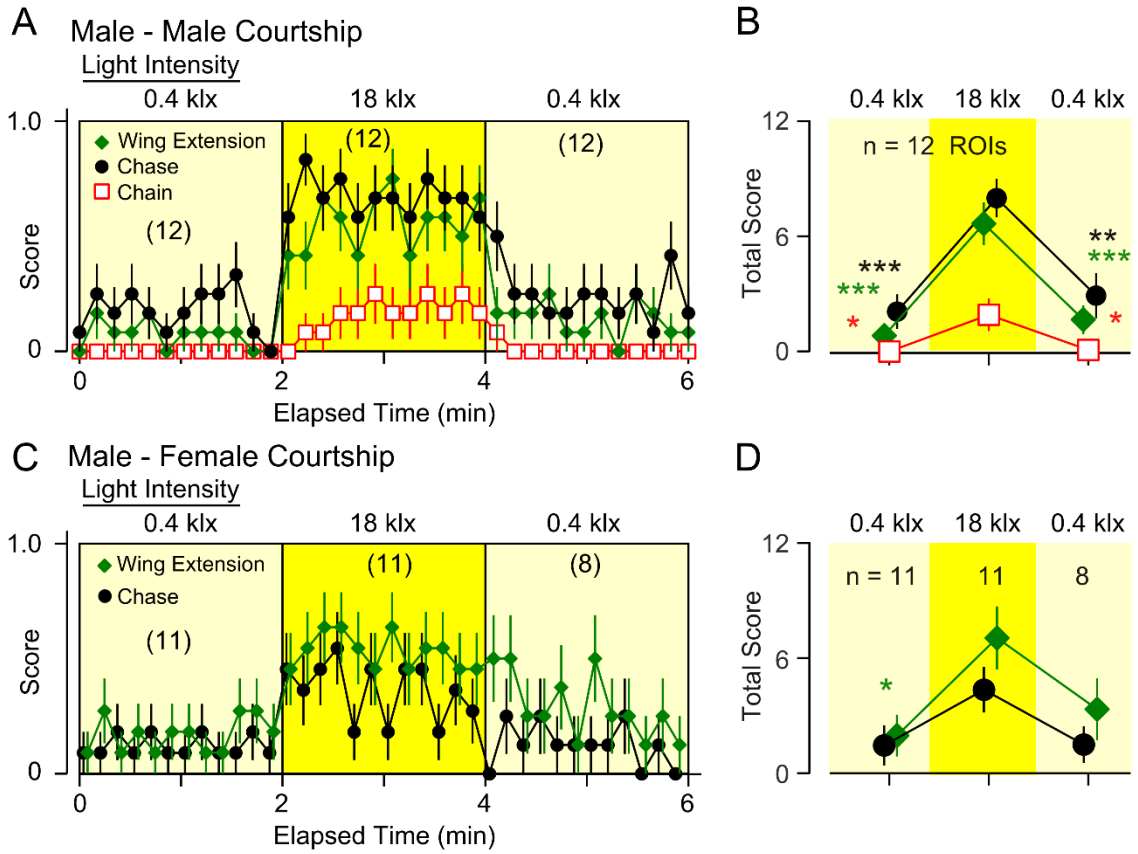

Figure S1. Light-induced male-male and male-female courtship behavior. (A) Kinetics of light-induced courtship behaviors between male flies. 8 male flies were in the arena (ROI). High-intensity (18 klx) illumination immediately induced courtship behavior, which was terminated rapidly after switching back to 0.4 klx. Error bars indicate SEM. # ROIs are indicated in parenthesis. (B) Total scores in A are summed over 2-min periods and shown with SEM and # ROIs. (C) Kinetics of light-enhanced courtship behaviors between males and females. 4 males and 4 females were in the arena. Courtship behaviors between males and females was also increased by 18 klx illumination, but unlike male-male courtship, switching back to 0.4 klx only gradually reduced the male-female courtship scores. Error bars indicate SEM. # ROIs are indicated in parenthesis. (D) Scores in C are summed over 2-min periods. # ROIs and SEM are indicated. One-way ANOVA, Bonferroni-corrected t-test *post hoc* was applied for comparisons between 1st low-light and high-light, as well as between high-light and the 2nd low-light. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. For comparison, Figure 1D is duplicated in panel A.

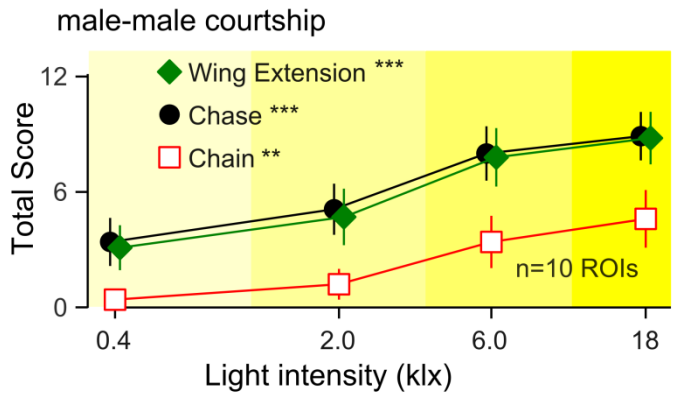

Figure S2. Light-intensity dependence of male-male courtship behavior among 8 male flies in an observation arena. Wing extension, chase, and chain were scored for 2 min under different illumination intensities of 0.4, 2.0, 6.0, and 18 klx. *** and ** indicates $p < 0.001$ and 0.01, respectively (Page's L Trend Test).

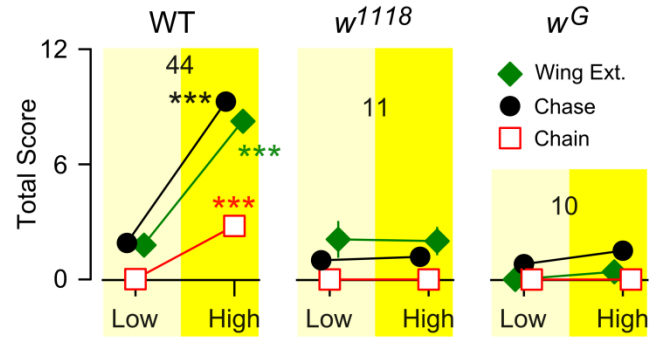

Figure S3. Lack of light-induced male-male courtship behavior in *w* mutants. 8 white-eyed male flies were introduced into the observation arena and their courtship behavior was recorded under low illumination (0.5 klx) for 2 min and then under high-intensity illumination (18 klx) for 2 min. *** $p < 0.001$, paired t-test (high vs. low). WT data replotted from Figure 2A for comparison.

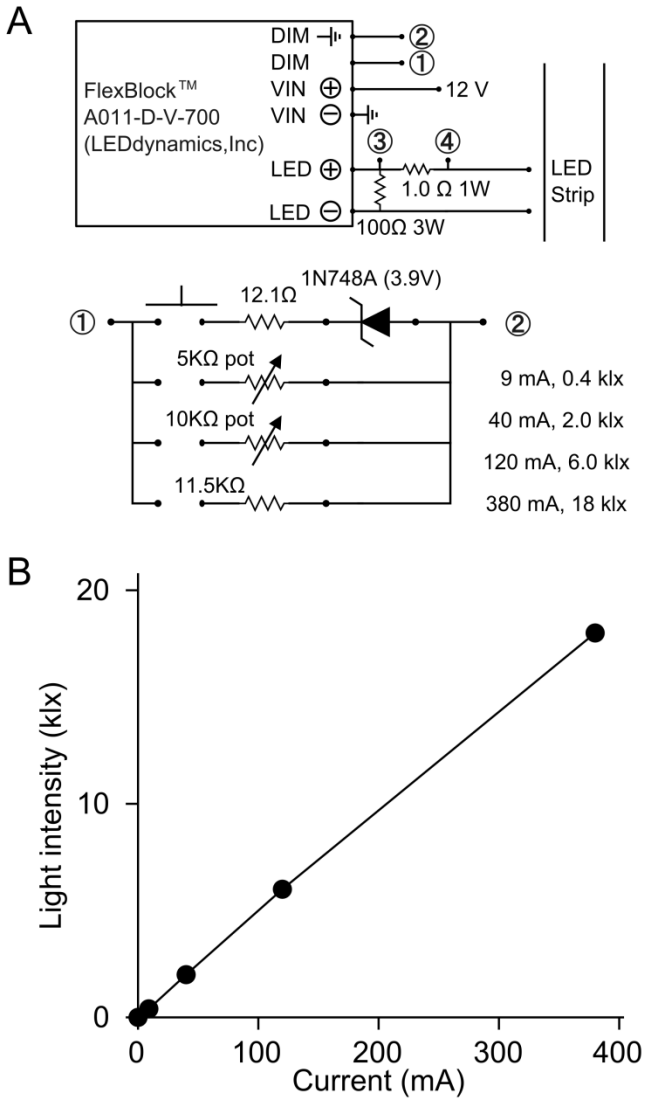

Figure S4. (A) Circuit diagram and components of the current regulator used for a LED strip (STN-A30K80-A3A-10B5M-12V, SuperBrightLEDs.com) in the behavior chamber. Two potentiometers were adjusted to achieve the designated brightness (2.0 and 6.0 klx). (B) Current-brightness correlation in the behavior chamber. Current was measured as a voltage drop between the terminals 3 and 4 shown in panel A.

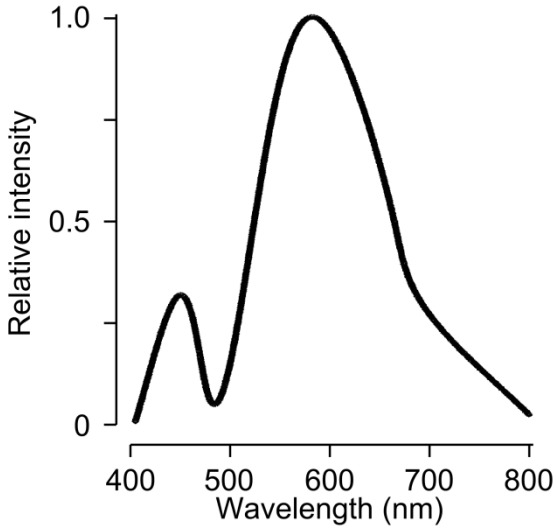

Figure. S5. Spectral characteristics of the LED strip (STN-A30K80-A3A-10B5M-12V, SuperBrightLEDs.com) used in the behavioral observation chamber. I_F = 20 mA. Ta = 25℃. Modified from manufacturer's specifications.

		Low white (0.4 klx)	High white (18 klx)	Blue (13 klx)
WT	Wing Ext	0.17 ± 0.18	8.2 ± 1.6	0.50 ± 0.37
	Chase	2.2 ± 0.87	10.7 ± 0.54	1.3 ± 0.78
	Chain	0.33 ± 0.37	4.2 ± 1.7	0.17 ± 0.18
orco	Wing Ext	7.7 ± 2.4	7.2 ± 1.6	0.00 ± 0.00
	Chase	8.2 ± 1.8	10.7 ± 0.67	0.50 ± 0.37
	Chain	2.2 ± 1.2	2.7 ± 0.78	0.00 ± 0.00

Table S1. Male-male courtship-like behaviors of WT and *orco* mutant flies under different color illuminations. Note that courtship behaviors were suppressed under blue light even though the blue light was blight (13 klx), suggesting that behaviors of *orco* can still be influenced by illumination. $orco^1$ (n = 3) and $orco^2$ flies (n = 3) were combined. # ROI for WT = 6. Error indicates SEM. To see the effect of illumination more clearly, the 'chain' criterion was relaxed from four animals in a row to three animals in a row for this data set.

Genotype	Eye color		Low white (0.4 klx)	High white (18 klx)	# ROI
$w^{1118}; \frac{orco^{1}}{+}$	Dark orange	Wing Ext	4.6 ± 2.6	4.2 ± 2.3	5
		Chase	5.8 ± 2.4	5.8 ± 1.9	
		Chain	1.8 ± 1.1	0.60 ± 0.40	
$w^{1118}; \frac{orco^{2}}{+}$	Pale orange	Wing Ext	0.43 ± 0.30	0.14 ± 0.14	7
		Chase	0.29 ± 0.18	2.3 ± 0.84	
		Chain	0.0 ± 0.0	0.0 ± 0.0	
$sev^{LY3}; \frac{orco^{1}}{+}$	Red (WT)	Wing Ext	0.0 ± 0.0	0.88 ± 0.74	8
		Chase	0.0 ± 0.0	1.4 ± 0.82	
		Chain	0.0 ± 0.0	0.0 ± 0.0	
$norpA^{P12}; \frac{orco^{1}}{+}$	Red (WT)	Wing Ext	0.0 ± 0.0	0.0 ± 0.0	8
		Chase	0.13 ± 0.13	0.0 ± 0.0	
		Chain	0.0 ± 0.0	0.0 ± 0.0	

Table S2. Interactions between *orco* heterozygous and visual mutations in light-induced male-male courtship behavior. *w*; *orco*/+ showed higher behavioral scores for darker eye color. With visual mutations, behavior scores were as low as those of visual mutants even though the eye color was indistinguishable from WT.