UNIVERSITY OF CALIFORNIA SAN DIEGO

The Effects of External Cues on Or47b-Mediated Promotion of Male Courtship in Drosophila

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in

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by

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The Thesis of Bharat Sekar is approved, and it is acceptable in quality and for publication on microfilm and electronically:

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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ABSTRACT OF THE THESIS

The Effects of External Cues on Or47b-Mediated Promotion of Male Courtship in *Drosophila*

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Professor Jing W. Wang, Chair

Mating drive is not only determined by the internal state of the organism but also its external sensory stimuli. In *Drosophila melanogaster*, the Or47b olfactory receptor neurons respond to aphrodisiac pheromones. The pheromone sensitivity of these receptor neurons in male flies is regulated by a reproductive hormone that signals their fertility state. Thus, the Or47b olfactory receptor neurons can be considered as a critical integration node for internal reproductive state and external sensory stimuli. However, it remains unclear whether the impact of Or47b input is also affected by contextual external cues. Here we perform a series of behavioral assays to investigate the influence of food odor, female age, size of courtship chamber and light on the outcome of courtship

competition between wild type and Or47b mutant males. We find that the copulation advantage of wild type over Or47b mutant males persists in all tested conditions, despite the concomitant differences in total copulation rates. Together, these results indicate that Or47b input remains a key determinant of courtship decision in varying environments, thereby highlighting its importance in the neural circuitry controlling male mating behavior.

INTRODUCTION

Olfaction is a key regulator of mating behavior in a multitude of species. An extensive array of genetic tools along with an anatomically simpler, yet representative, olfactory system (Ache & Young, 2005; Bargmann, 2006; Hildebrand & Shepherd, 1997; Kaupp, 2010; Laurent, 2002) makes *Drosophila melanogaster* an attractive model organism to study how sensory detection of pheromone cues affects mating behavior.

In the last decade, a lot of progress has been made in understanding the role of pheromonal cues in the promotion of male courtship in *Drosophila*, beginning with van der Goes van Naters and Carlson's (2007) discovery that the Or47b receptor detected a cuticular hydrocarbon. At around the same time, Root and colleagues (2007) demonstrated that disrupting GABAergic signaling to Or47b ORNs interfered with male courtship. Then, in 2011, Wang and colleagues showed that the Or47b receptor promotes male courtship of oenocyte-eliminated males and that this effect is subordinate to the suppression of male courtship by male pheromones. In 2015, Dweck and colleagues identified the cuticular hydrocarbons methyl laurate, methyl myristate and methyl palmitate as ligands of the Or47b receptor and showed that Or47b signaling is required for pheromone-based promotion of male courtship. Lin, Cao and colleagues (2016) furthered this research with their findings that the Or47b receptor responds most strongly to palmitoleic acid, and that the sensitivity of Or47b olfactory receptor neurons (ORNs) in male flies changes with age, peaking at 7 days. In this study, they developed a courtship competition assay similar to the comparative mating assay used by Dweck and colleagues (2015). In this assay two male flies, one from the control group and another

from the experimental group, are loaded into a chamber along with one female fly. The flies are observed for a period of two hours and the male that copulated with the female first and the latency to copulation are recorded. We use their competitive courtship assay and control conditions as a starting point for our experiments.

Another olfactory receptor shown to promote male courtship is the Ir84a receptor, which responds to phenyl acetic acid, a food odorant found in natural and laboratory foods (Grosjean et al, 2011). Here, we investigate the effect of presence of food odor on the promotion of male courtship through Or47b signaling using the courtship competition assay. As both receptors increase male arousal, they may have redundant effects, where reduced signaling through one receptor is compensated by activity in the other. Alternatively, promotion of male courtship by one of the two receptors may be dependent on the activation of the other receptor. In this case, pheromonal promotion of courtship may be dependent on Ir84a activation, signaling the presence of food, or the promotion of courtship by odors from food sources may rely on Or47b activation, signaling the presence of other flies. A third possibility is that signaling through these two receptors is independent of the activation of the other receptor and have an additive effect on promotion of male courtship. This possibility is the one we hypothesize to be true, as no current research suggests otherwise. We verified that Ir84a activity promotes courtship in the courtship competition assay, and then tested the interaction between Ir84a and Or47b receptor mediated promotion of courtship and found the signals to act independently of each other in promoting courtship.

The cuticular hydrocarbon profile of female flies changes as they age. Specifically, the proportion of palmitoleic acid out of total fatty acid increases steadily by around 5% in the first ten days of adulthood on a fat-free diet at about 0.5% per day (Green & Geer, 1978; Pennanec'h, Bricard, Kunesch & Jallon, 1997). Or47b ORNs in seven-day-old males shows responses to nanogram quantities of palmitoleic acid in single sensillum recordings (SSRs) (Lin et al, 2016). Because of this high sensitivity, we investigated whether the single-day increase in the proportion of palmitoleic acid between 2-day-old and 3-day-old females is enough to produce a detectable increase in Or47b-mediated promotion of male courtship. We also investigated whether the increase over a longer period from 2-day-old to 7-day-old females could produce a significant increase in male arousal caused by the Or47b receptor. We did not investigate females older than seven days as the receptivity of female *Drosophila melanogaster* to male courtship begins to decrease after this age (Manning, 1967). As the broad dose-response curves from SSRs of Or47b ORNs suggest that there will be a very small change in ORN response to slight increases in ligand, we do not expect the increased proportion of palmitoleic acid in older females to have a significant effect on male courtship. We found no change in the wildtype advantage from using older females. Using older females produced an insignificant increase in total copulation.

Drosophila courtship in the wild commonly ends with either ovipositor extension or decampment by the female (Gromko & Markow, 1993). The size of a courtship chamber acts as a physical barrier to the female's ability to reject courtship by decamping. This could affect the study of the Or47b receptor's promotion of male

courtship using the courtship competition assay in 2 cm diameter chambers by artificially inflating the courtship success of one group of males. This could benefit wild type males by making it easier for them to locate females after they decamp as palmitoleic acid is a low volatility compound and the females are limited in the distance they can walk, jump or fly away. Conversely, this could benefit the Or47b males as they may be more successful due to the higher probability of chance encounters that could lead to courtship driven by non-Or47b neural circuits. Here, we compared the results from 2 cm and 4 cm diameter chambers. If the 2 cm chamber's restriction of female decamping benefitted one group of males over the other, we expect that this advantage would be reduced in the 4 cm diameter chambers. As no evidence suggests that the limitation of decampment would benefit one group of males over the other, we hypothesize that the restriction of female decampment by the chamber has benefitted both groups of males equally and because of this the wildtype male's advantage over the Or47b mutant male will not change though total copulation should reduce. We found no change in the wildtype male's advantage over the *Or47b*$^{2/2}$ males, but the total copulation was reduced in the 4 cm diameter chambers.

Vision is an important sensory input promoting male courtship in *Drosophila melanogaster* (Krstic, Boll & Noll, 2009; Agrawal, Safarik & Dickinson, 2014). Previous studies of pheromone-based promotion of male courtship through the Or47b receptor were performed under 660 nm red light illumination (Dweck et al, 2015; Lin et al, 2016), which is not visible to flies (Salcedo et al, 1999). As such, it is unknown how these two courtship-promoting signals interact. It is possible that vision-based promotion of

courtship is limited by Or47b signaling or vice versa. Alternatively, Or47b activation may have a diminished role in promoting courtship in the presence of light, primarily acting when visual input is not available. The Or47b mutant was generated by inserting a *mini-white* allele into the Or47b gene, which increases male-male courtship in the light, and had a mutant form of the *white* gene, which inhibits courtship in the light due to optomotor blindness and dazzling from excess light due to the lack of retinal pigmentation (Anaka et al, 2008; Krstic, Boll & Noll, 2013; Xiao, Qiu & Robertson, 2017; Yin Hing & Carlson, 1996; Zhang & Odenwald, 1995). Therefore, we generated a red-eyed Or47b mutant by replacing the mutant *white* allele with the wildtype *white* allele to prevent these factors from interfering with results in the light condition. We found that the wildtype males have a reduction in their competitive advantage over the w^{+}*; Or47b*$^{2/2}$ mutant flies in the light.

The white gene is a sex-linked gene that encodes part of an ATP-binding cassette transporter involved in the uptake of precursors of retinal pigments (Ewart & Howells, 1998; Sullivan, Grillo & Kitos, 1974; Summers, Howells & Pyliotis, 1982) and precursors of the neurotransmitters, dopamine and serotonin (J. Borycz, J. A. Borycz, Kubow, Lloyd & Meinertzhagen, 2008). Any influence of the *white* gene in the darkness would be a result of its extra-retinal expression. Different studies have differing views on whether the *white* gene affects courtship in the darkness. Krstic, Boll and Noll (2013) found no difference in the ability of wildtype and *white* mutant flies to court females in the darkness using a ten-minute-long assay. Reed and Reed (1950) found that wildtype males were more successful in their 24-hour-long assay, accounting for ~57% of total

copulation. Xiao, Qui and Robertson did not observe any copulation in white mutants under red-light illumination, suggesting a severe courtship defect. As the Or47b mutant line used for most previous experiments had a mutant form of the *white* gene, we investigated whether *white* mutation affected courtship in our assay using Berlin wildtype flies and w^-(Berlin) flies in both the light and darkness. A truncated form of the wildtype gene, the *mini-white* gene, is used as a marker in the Or47b mutant line. Expression levels of *mini-white* are affected by the chromosomal insertion site and orientation (Klemenz, Weber & Gehring, 1987; Levis, Hazelrigg & Rubin, 1985a, 1985b; Pirrotta, Steller & Bozzetti, 1985; Silicheva et al, 2010). As we found that *white* mutation creates a courtship defect in the dark, we tested if this courtship defect affects the Or47b mutant flies by comparing the red-eyed w^+*; Or47b*$^{2/2}$ flies we created for our study of the influence of light with the original w^-*; Or47b*$^{2/2}$ line and found no courtship defect in these flies.

METHODS

Fly Lines and Rearing Conditions

Flies were raised in groups of 10 in vials containing standard fly food containing molasses (UCSD Drosophila Recharge Facility) at 25°C ±1°C and 50-60% humidity in a 12:12 light-dark cycle. If eye color could not be used to differentiate the two males, fluorescent dye (UVXPBR, LDP LLC) was used to identify males (Figure 1A-C, Figure 5). One male line was dusted with the dye two or three days prior to the assay, with the genotype that was dusted alternated between trials to minimize any dye-induced behavioral bias.

Table 1. Fly Lines Used for Male Flies in Experiments, along with BDSC stock references and papers describing fly line generation where available. *Ir84a$^{Gal4/Gal4}$ is referred to as Ir84a$^{-/-}$ or Ir84a^{Gal4} in Grosjean et al (2011).

Genotype	BDSC Stock	Original Paper	Experiments Used For
Berlin wild type (WT)	N/A	N/A	Fig 1, 2, 3, 4, 5, 6A-C
w^{-}(Berlin)	N/A	N/A	Fig 6A-C
Or47b$^{2/2}$	RRID:BDSC_51306	Wang et al, 2011	Fig 1D-F, 2, 3, 4, 6D-F
*Ir84a$^{Gal4/Gal4}$**	RRID:BDSC_41750	Grosjean et al, 2011	Fig 1A-C
w^{+}; Or47b$^{2/2}$	N/A	N/A	Fig 5, 6D-F

Or47b$^{2/2}$ flies were backcrossed to w^{-}(Berlin) background for six generations and had mini-*white* markers. *Ir84a$^{Gal4/Gal4}$* flies were backcrossed to Berlin wild type flies for

six generations. Additionally, the backcrossed *Or47b*$^{2/2}$ flies were crossed to Berlin wild type flies to create the w^{+}*; Or47b*$^{2/2}$ line. PCR and gel electrophoresis were used to ensure *Ir84a*$^{Gal4/Gal4}$ and w^{+}*; Or47b*$^{2/2}$ were homozygous for their respective mutant alleles using the primers in Table 2. All male flies used in experiments were seven days old and naive.

Table 2. Primers Used for Allele Identification. P2 and P3 are the same sequences used in Grosjean et al (2011). Or47b.2-for and –rev are the same sequences used in Wang et al (2011).

Gene	*Primer*	*Allele(s)*	*Sequence* (*5'-3'*)
Ir84a	P3 (O129)	Both	CGC ACG ATG AAT CTG TAG GTT A
	P2 (O665)	Wild type	TTA CTC ACT TCT GGG TGT GGC AG
	Gal4 R	Mutant (*Ir84a*^{Gal4})	TGA AGC CAA TCT ATC TGT GAC GGC ATC
Or47b	*Or47b*.2-for	Both	CAT GTG CAA TGT GAT GAC CA
	Or47b.2-rev	Wild type	CGA TGC AAA GCA ACT TGA GA
	5'*miniwhite*Reverse	Mutant (*Or47b*^{2})	TGA GGT TCT CGG CTA GTT GG

The Canton S line was used for the female flies. Female flies used in experiments were two days old, except for those used for the experiments investigating the effects of changing the age of the female, where three-day-old (Figure 2) and seven-day-old females (Figure 3) were used, as well as the effects of chamber size, where three-day-old females were used (Figure 4). Two-day-old females were all collected within the first six hours of the photoperiod, before Circadian Time 6 (CT6). Older females were collected throughout the photoperiod.

Loading Tube

The loading tube used to load the flies into the mating chambers was constructed from Tygon tubing and two micropipette tips. Specifically, the wider end of a 1000-µL micropipette tip, cut in half, was inserted in one end as the mouthpiece. At the other end of the tubing, another 1000-µL micropipette tip, with a piece of cotton inserted in the wide end as a stopper and about 1 cm of the narrow end removed to allow flies to pass through, served as the vessel to transport flies from the vial to the mating chambers. Flies were collected from vials by inhaling them into the tip of the loading tube. To load the flies into the chambers, the tip of the tube was inserted into the chamber and one fly was allowed to walk into the chamber or was gently aspirated into the chamber.

Chamber Preparation

The 2 cm (3.14 cm^3) and 4 cm (12.57 cm^3) chambers are composed of four parts: the Teflon chamber with an internal diameter of approximately 2 or 4 cm and a height of 2 cm, a Teflon loading ring placed around the top half of the chamber, a metal screen to separate the top and bottom halves of the chamber and a glass cover placed on top of the chamber, allowing observation of the flies as the Teflon is opaque white. The screen allows the chamber to be perfumed without allowing the flies to contact the odorant source, by restricting the flies to the upper 1 cm of the chambers. Rotating the loading ring allows the chamber to be opened and closed for loading by aligning a 0.5 cm hole in the loading ring to a 0.3 cm hole in the wall of the upper part of the chamber. The 2 cm

chambers were used for all experiments except those investigating the effects of chamber size (Figure 4) and are the same as those used in Lin and Cao et al (2016).

Food Dish Preparation

Open Petri dishes (Corning Life Sciences #351008, 35*10 mm for 2 cm chambers; Fisher Scientific #FB0875712, 100*15 mm for 4 cm chambers) containing a 1:1 mixture by weight of standard fly food containing molasses and water were placed under chambers for all experiments except the following. For the no odor condition (Figure 1), the food-water mixture was replaced by a 10 g/L agar solution. This concentration of agar was chosen as it is half the concentration used for the holidic medium described in Piper et al (2013).

Courtship Competition Assay

The assay was performed at 25°C ±1°C and 50-60% humidity under 660 nm red light, which flies cannot see (Salcedo et al, 1999). The flies were allowed to acclimate under experimental condition for half an hour before being loaded into the mating chamber. One male each from the control and experimental group are gently aspirated into the chamber. The genotype loaded first is alternated between trials. Finally, one female was loaded into the chamber and the chamber was placed atop a dish containing fly food, with the start time noted. Around 25-30 chambers were loaded in each trial. The flies are observed for 2 hours from the start time or until copulation occurs. The time at which copulation occurs and the male which copulates with the female were recorded.

The males were identified either by eye color using a white flashlight, or by fluorescent dye using a UV flashlight.

For the light condition (Figures 5 & 6), flies were placed under experimental conditions with illumination from both the 660 nm red light and white tube lights half an hour prior to loading. The assay was shortened to 1 hour in the light as copulation occurred in 95% of the chambers within this time. After either 1 hour passed or copulation occurred in all the chambers loaded, the white tube lights were turned off, and after half an hour, flies were loaded in the dark condition.

The copulation rate is defined as the proportion of chambers in which copulation occurred out of the total chambers loaded. The copulation percentage of the indicated male genotype is defined as the percentage of chambers in which a male of that genotype copulated out of the chambers in which copulation occurred. As trials were performed in parallel with flies from the same stocks, paired t-test is used to compare results between two conditions. As random chance predicts a copulation percentage of 50%, chi-square test for goodness of fit was used to determine if the observed copulation percentage is significantly different from chance (indicated on figures by a dashed line).

RESULTS

Food Odor and Pheromones Promote Male Courtship Independently in *Drosophila*

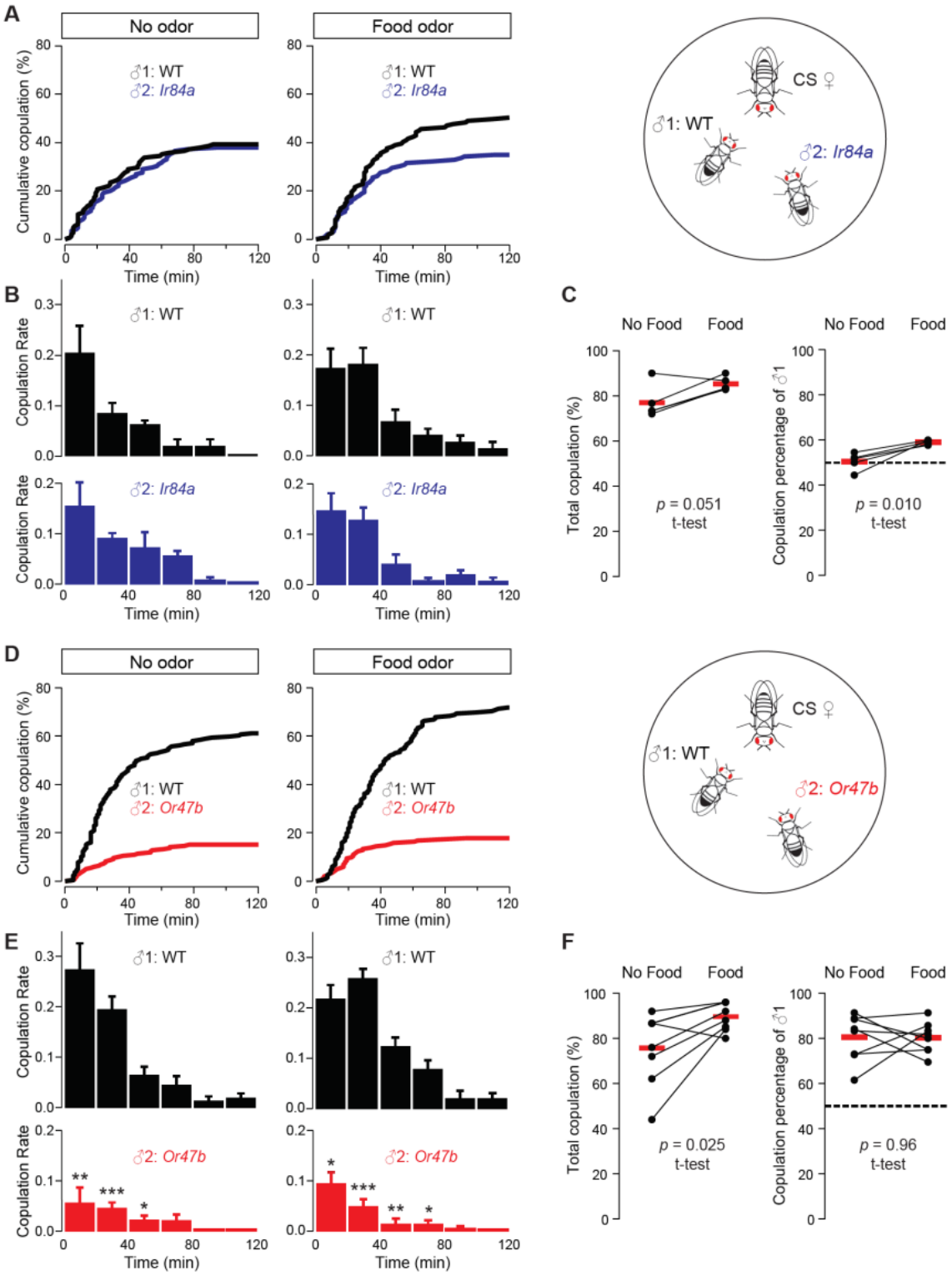
Previous studies (Dweck et al, 2015; Grosjean et al, 2011; Lin et al, 2016; Yew & Chung, 2017) have shown that mate availability and food abundance promote male courtship through the Or47b and Ir84a olfactory receptor neurons (ORNs), respectively. Here, we use receptor mutants to address the question of whether Or47b receptor mediated promotion of male courtship is independent of Ir84a receptor signaling using the courtship competition assay. First, we confirmed that the Ir84a receptor is required for food-odor dependent promotion of male courtship and that the assay is sensitive enough to measure this effect (Figure 1A-C). The wild type male only had an advantage over the Ir84a mutant in the presence of food odor. We then found that the advantage of wild type males over Or47b mutant males is independent of food odor (Figure 1D-F). Furthermore, the total copulation was increased by food odor, suggesting that food odor promotes courtship behavior independent of the Or47b circuit. These results suggest that food abundance, detected by Ir84a ORNs, and mate availability, detected by Or47b ORNs, regulate male courtship behavior through independent neural circuits.

Female Age Does Not Significantly Affect Male Courtship

As females age, their cuticular hydrocarbon profiles change, with the proportion of palmitoleic acid, a ligand of the Or47b receptor (Lin et al, 2016), in the cuticle steadily increasing (Green & Geer, 1978; Pennanec'h et al, 1997). To investigate whether the single-day increases in proportion of palmitoleic acid could affect the wildtype male's

Figure 1. Food Odor Promotes Male Courtship Behavior Through the Ir84a Receptor.

(A) Courtship competition assay – one virgin Canton S female and one male each from the Berlin wild type (WT) and $Ir84a^{Gal4/Gal4}$ genotypes (right column) – was used to measure male courtship behavior. Cumulative copulation rates are plotted against time for each male genotype in the absence (left column) and presence (middle column) of food odor. (B) The copulation distributions in the absence (left) and presence (right) binned into twenty-minute segments is plotted for each male genotype. Significances are indicated on the figures (paired t-test). (C) The overall copulation rates observed in the two conditions are shown on the left, with lines connecting parallel experiments. There is no significant difference between the overall copulation rates between the two conditions ($p > 0.05$, $n = 5$, paired t-test). Copulation percentages of the wild type males are shown on the right, in which parallel experiments are connected by lines. The wild type male had a significant advantage over the mutant male only in the presence of food odor and differed significantly from the no odor condition(no odor: $p = 8.50 * 10^{-1}$, $n = 145$, chi-squared test; food odor: $p = 4.13 * 10^{-2}$, $n = 149$, chi-squared test; $p < 0.05$, $n = 5$, paired t-test). Red bars denote average copulation percentage for each condition. The dashed lines indicate chance level of 50%. (D) Courtship competition assay – one virgin Canton S female and one male each from the Berlin wild type (WT) and $Or47b^{2/2}$ genotypes (right column) – was used to measure male courtship behavior. Cumulative copulation rates are plotted against time for each male genotype in the absence (left column) and presence (middle column) of food odor. (E) The copulation distributions in the absence (left) and presence (right) binned into twenty-minute segments is plotted for each male genotype. Significances are indicated on the figure (paired t-test). (F) The overall copulation rates observed in the two conditions are shown on the left, with lines connecting parallel experiments. There is a significant increase in the overall copulation rate from the absence to the presence of food odor ($p < 0.05$, $n = 8$, paired t-test). Copulation percentages of the wild type males are shown on the right, in which parallel experiments are connected by lines. The wild type male had a significant advantage over the mutant male in both the absence and presence of food odor and there was no significant difference between the two conditions (no odor: $p = 5.47 * 10^{-15}$, $n = 219$, chi-squared test; food odor: $p = 5.41 * 10^{-16}$, $n = 202$, chi-squared test; $p > 0.05$, $n = 8$, paired t-test). Red bars denote average copulation percentage for each condition. The dashed lines indicate chance level of 50%.


A
No odor
Food odor
♂1: WT
♂2: Ir84a
Cumulative copulation (%)
Time (min)
B
Copulation Rate
C
No Food
Food
Total copulation (%)
p = 0.051
t-test
Copulation percentage of ♂1
p = 0.010
t-test
CS ♀
D
♂2: Or47b
E
F
p = 0.025
t-test
p = 0.96
t-test

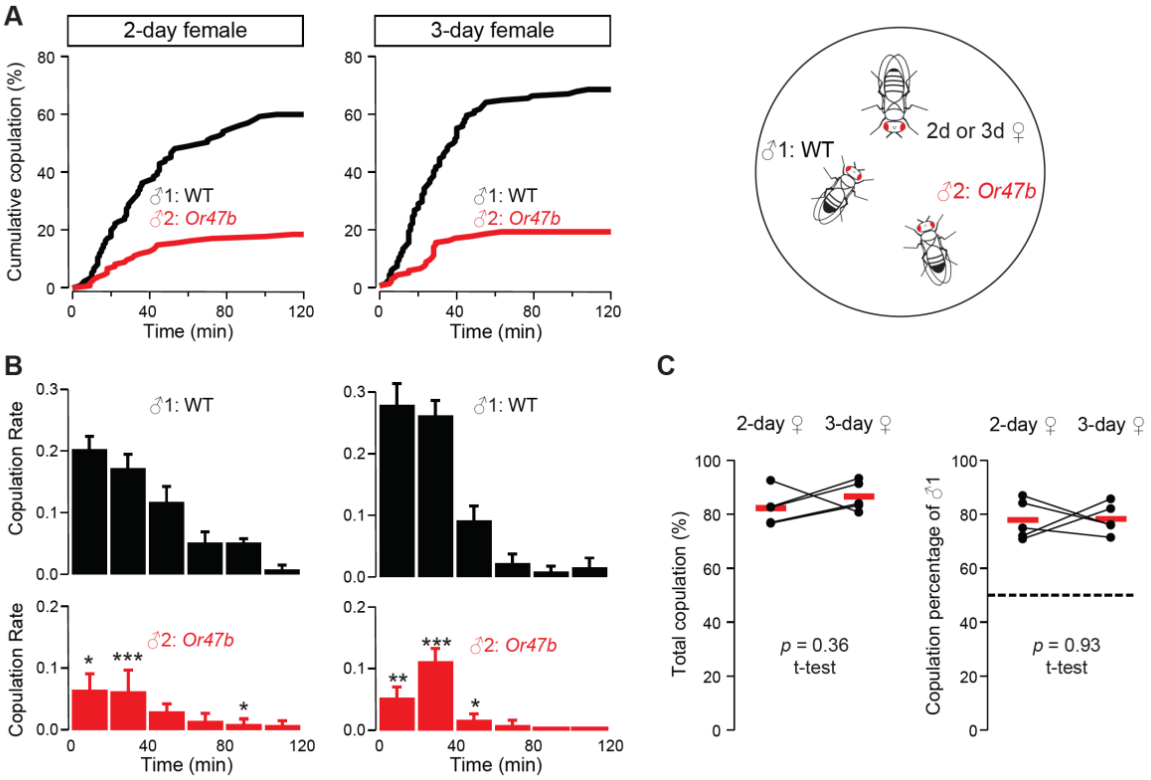


Figure 2. The Age of the Female Fly Does Not Significantly Affect Male Courtship Behavior.

(A) Courtship competition assay – one virgin Canton S female and one male each from the Berlin wild type (WT) and *Or47b*$^{2/2}$ genotypes (right column) – was used to measure male courtship behavior. Cumulative copulation rates are plotted against time for each male genotype with the 2-day-old (left column) and 3-day-old (middle column) females. (B) The copulation distributions with 2-day-old (left) and 3-day-old (right) females binned into twenty-minute segments is plotted for each male genotype. Significances are indicated on the figure (paired t-test). (C) The overall copulation rates observed for the two female fly ages are shown on the left, with lines connecting parallel experiments. There is no significant difference between the overall copulation rates between the two female ages ($p > 0.05$, $n = 5$, paired t-test). Copulation percentages of the wild type males are shown on the right, in which parallel experiments are connected by lines. The wild type male had a significant advantage over the mutant male with both ages of females and there was no significant difference between the two conditions (2-day female: $p = 7.04 * 10^{-9}$, $n = 135$, chi-squared test; 3-day female: $p = 8.90 * 10^{-10}$, $n = 134$, chi-squared test; $p > 0.05$, $n = 5$, paired t-test). Red bars denote average copulation percentage for each condition. The dashed lines indicate chance level of 50%.

advantage, we compared the results of the courtship competition assay using 2-day-old and 3-day-old females (Figure 2). We found no significant differences in either the copulation percentage of the wildtype males or the total copulation. As there appears to be an insignificant upward trend in total copulation, we tested if this trend is significant with a larger age difference by comparing the courtship competition assay's results using 2-day-old and 7-day-old females (Figure 3). We found no significant differences in the copulation percentage of the wildtype males or the total copulation, though we did observe the insignificant upward trend in total copulation again. We did not test females older than one week as female receptivity to courtship begins to decrease after this age (Manning, 1967). Both these experiments demonstrate that the increasing proportion of cuticular palmitoleic acid with age is not enough to create a significant change in male courtship, indicated by the lack of change in the wildtype copulation percentages. These experiments suggest that female age does not significantly affect male courtship. It is possible that, due to assay design, any effects of female age on male courtship are not detected by this assay, potentially due to limitations on the female's ability to terminate courtship (Gromko & Markow, 1993).

Larger Chambers Reduce Courtship

All previous experiments on the Or47b receptor's influence on mating in this assay used 2 cm diameter chambers. As the chamber walls limit the ability of female flies to reject courtship by decamping (Gromko & Markow, 1993), we performed the courtship competition assay in larger 4 cm diameter chamber as well as the 2 cm diameter chambers to determine if limiting female decampment biased the results in favor

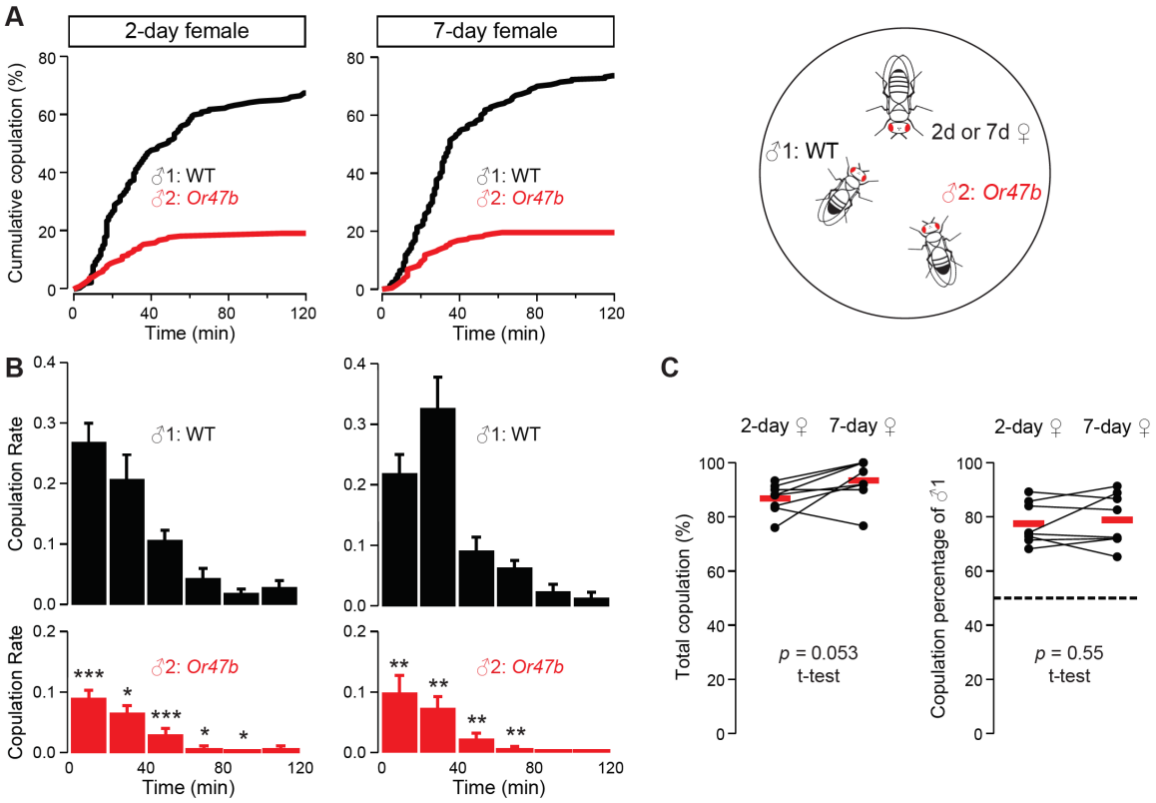


Figure 3. The Age of the Female Canton S Fly Does Not Significantly Affect Male Courtship Behavior.

(A) Courtship competition assay – one virgin Canton S female and one male each from the Berlin wild type (WT) and *Or47b*$^{2/2}$ genotypes (right column) – was used to measure male courtship behavior. Cumulative copulation rates are plotted against time for each male genotype with the 2-day-old (left column) and 7-day-old (middle column) females. (B) The copulation distributions with 2-day-old (left) and 7-day-old (right) females binned into twenty-minute segments is plotted for each male genotype. Significances are indicated on the figure (paired t-test). (C) The overall copulation rates observed for the two female fly ages are shown on the left, with lines connecting parallel experiments. There is no significant difference between the overall copulation rates between the two female ages ($p > 0.05$, $n = 8$, paired t-test). Copulation percentages of the wild type males are shown on the right, in which parallel experiments are connected by lines. The wild type male had a significant advantage over the mutant male with both ages of females and there was no significant difference between the two conditions (2-day female: $p = 3.66 * 10^{-14}$, $n = 213$, chi-squared test; 7-day female: $p = 9.46 * 10^{-17}$, $n = 220$, chi-squared test; $p > 0.05$, $n = 8$, paired t-test). Red bars denote average copulation percentage for each condition. The dashed lines indicate chance level of 50%.

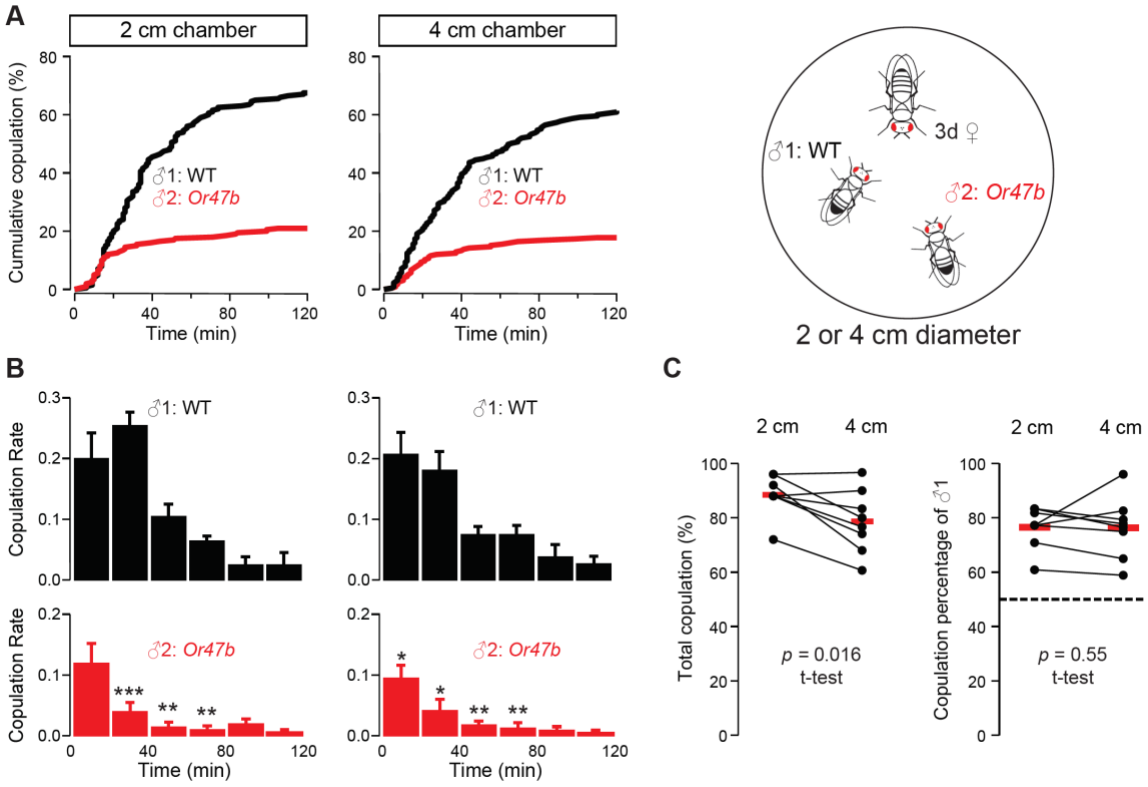


Figure 4. Larger Chambers Significantly Decrease Male Copulation.

(A) Courtship competition assay – one three-day-old virgin Canton S female and one male each from the Berlin wild type (WT) and *Or47b*$^{2/2}$ genotypes (right column) – was used to measure male courtship behavior. Cumulative copulation rates are plotted against time for each male genotype in the 2 cm (left column) and 4 cm (middle column) chambers. (B) The copulation distributions in the 2 cm (left) and 4 cm (right) chambers binned into twenty-minute segments is plotted for each male genotype (paired t-test). (C) The overall copulation rate observed for the two chamber sizes are shown on the left, with lines connecting parallel experiments. There is a significant decrease in overall copulation from 2 cm to 4 cm chambers ($p < 0.05$, $n = 8$, paired t-test). Copulation percentages of the wild type males are shown on the right, in which parallel experiments are connected by lines. The wild type male had a significant advantage over the mutant male in both chamber sizes and there was no significant difference in copulation percentage between the two conditions (2 cm chamber: $p = 2.74 * 10^{-12}$, $n = 200$, chi-squared test; 4 cm chamber: $p = 2.05 * 10^{-13}$, $n = 225$, chi-squared test; $p > 0.05$, $n = 8$, paired t-test). Red bars denote average copulation percentage for each condition. The dashed lines indicate chance level of 50%.

of one male line over the other. We observed no change in the advantage of the wild type males over the Or47b mutant males and a significant decrease in overall copulation (Figure 4), leading to the conclusion that the chamber size did not limit the wild type advantage in this assay. The reduction in courtship shows that the smaller chamber did limit the female's ability to terminate courtship by decamping.

The Or47b Receptor Provides an Important Courtship Cue in the Light

While it has been shown that Or47b receptor activation promotes male courtship (Dweck et al, 2015; Lin et al, 2016), it is not clear how this signal interacts with visual input. Studies have shown that visual cues take over as the primary sensory modality used by male flies to track females in the presence of light (Krstic et al, 2009). We therefore carried out experiments to measure the copulation advantage of wild type over mutant males under red light, which isn't visible to flies (Salcedo et al, 1999), and under white light condition. For this experiment, a new Or47b line with the wild type *white* allele (w^+) was generated, referred to as w^+*; Or47b*$^{2/2}$ to avoid any influence of the *white* locus and *mini-white* allele on experiments performed in the light. The overall level of copulation was higher in the light condition, confirming that visual cues promote male courtship (Figure 5). Furthermore, wild type males had a significant advantage in both conditions, but their advantage was lower in the light than in the dark. These results suggest that the Or47b circuit mediates male courtship motivation in the darkness as well as the light. The reduction in the wildtype male's courtship percentage suggests vision-based promotion of courtship attenuates Or47b-mediated promotion of courtship, though it is possible that Or47b's promotion of courtship limits visual promotion of courtship.

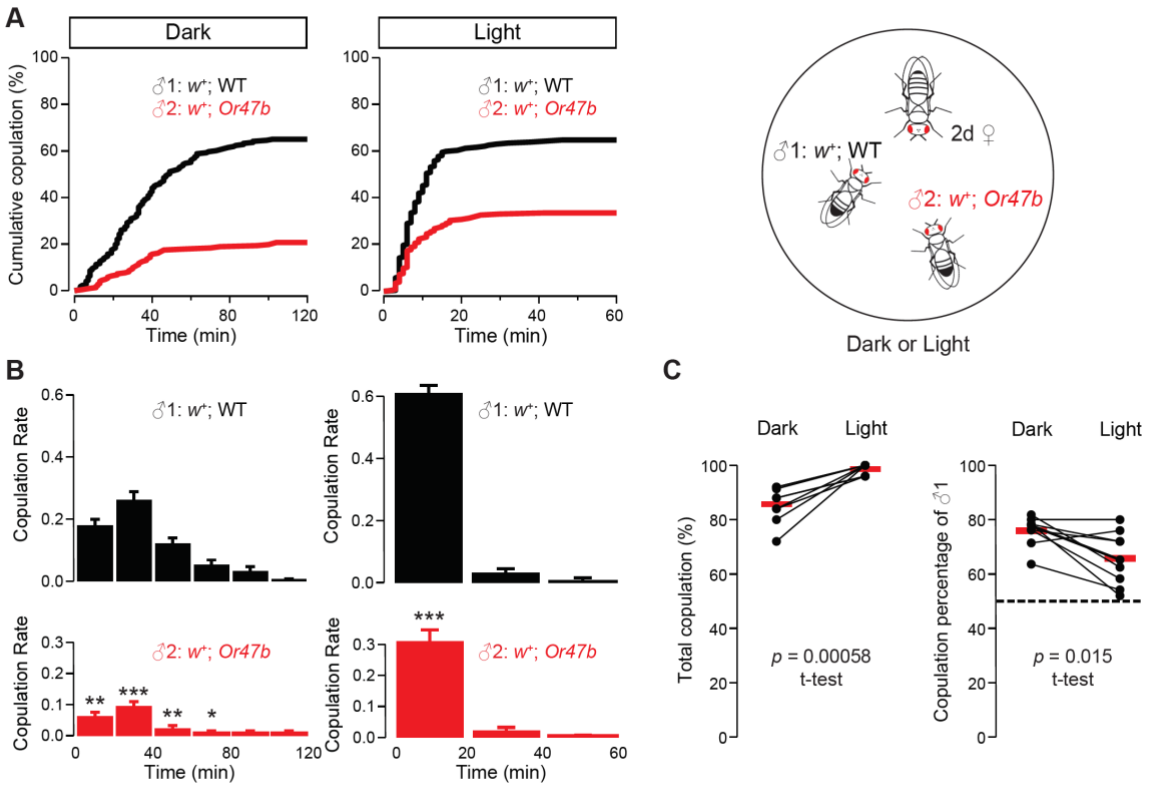


Figure 5. The Or47b Receptor Promotes Male Courtship in the Light and Dark Conditions

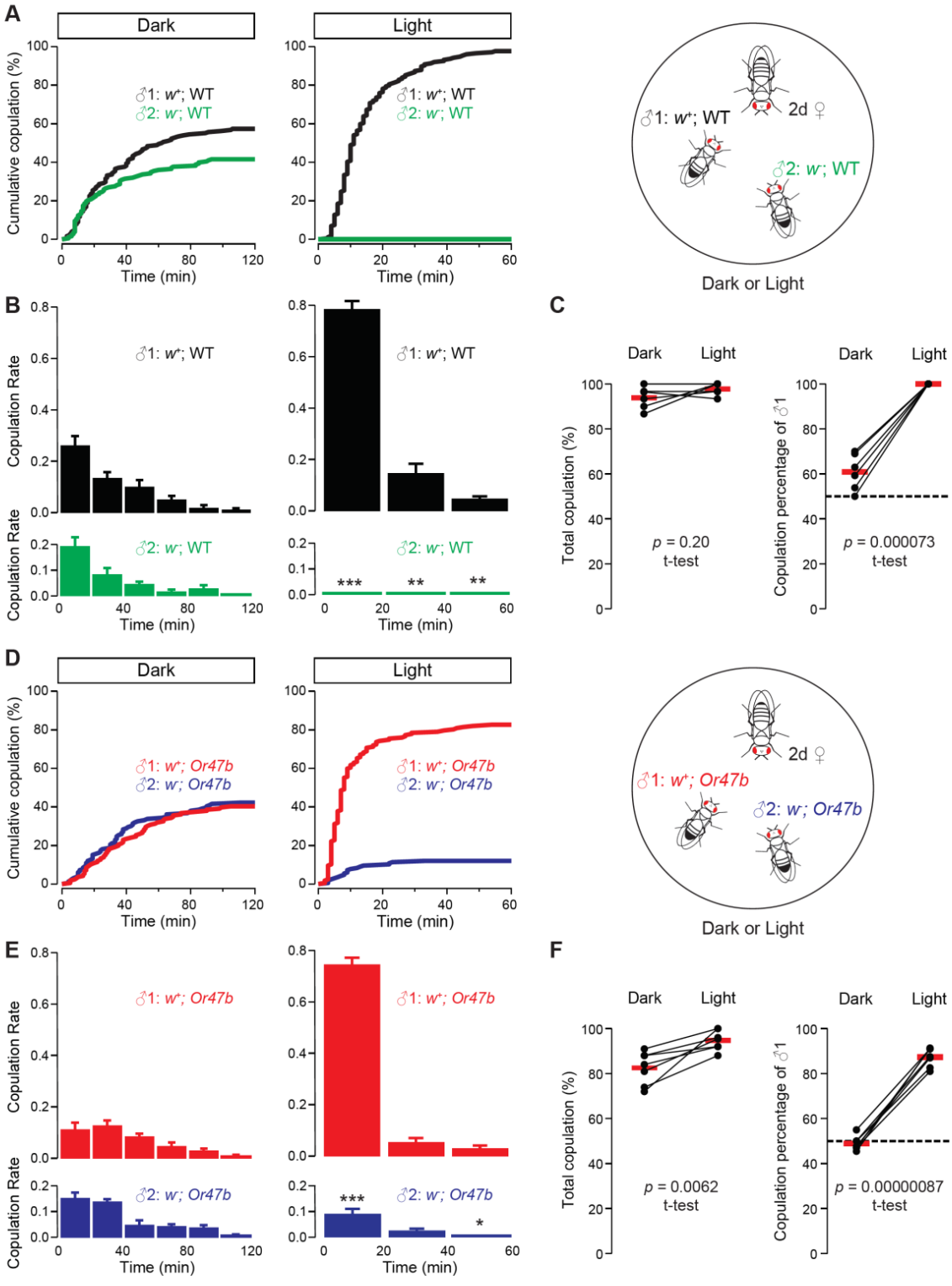
(A) Courtship competition assay – one three-day-old virgin Canton S female and one male each from the Berlin wild type (WT) and w^{+}; *Or47b*$^{2/2}$ genotypes (right column) – was used to measure male courtship behavior. Cumulative copulation rates are plotted against time for each male genotype in dark (left column) and light (middle column) conditions. The assay was shortened to 1 hour long in the light condition as nearly all the chambers had copulation occur within this timeframe. (B) The copulation distributions in the dark (left) and light (right) conditions binned into twenty-minute segments is plotted for each male genotype. Significances are indicated on the figure (paired t-test). (C) The overall copulation rates observed for the two chamber sizes are shown, with lines connecting parallel experiments. There is a significant increase in overall copulation from the dark condition to the light condition ($p < 0.001$, $n = 9$, paired t-test). Copulation percentages of the wild type males are shown on the right, in which parallel experiments are connected by lines. The wild type male had a significant advantage over the mutant male in both lighting conditions and there was a significant decrease in the wild type advantage between the dark and light conditions (dark: $p = 7.87 * 10^{-13}$, $n = 223$, chi-squared test; light: $p = 2.37 * 10^{-6}$, $n = 223$, chi-squared test; $p < 0.05$, $n = 9$, paired t-test). Red bars denote average copulation percentage for each condition. The dashed lines indicate chance level of 50%.

The *White* Gene Affects Male Courtship in the Dark

Previous studies on the effect of *white* mutations on male courtship agree that it impedes courtship in the light condition but have yielded conflicting results on whether it reduces courtship in the dark (Krstic et al, 2013; S. Reed & E. Reed, 1950; Xiao et al, 2017). To address this issue, we performed the courtship competition assay in the light and dark conditions using w^+ and w^- wild type males. We found that the w^+ wild type males had an advantage in both light and dark conditions, with the advantage being much greater in the light (Figure 6A-C). We then investigated whether *white* mutation affected courtship in the dark in Or47b mutants to ensure previous results using this line were not impacted. In contrast to the wildtype results, for the $Or47b^{2/2}$ mutant males, carrying the w^+ or w^- alleles does not affect their copulation success in the dark, despite a dramatic impact under light condition (Figure 6D-F). This may be due to the *mini-white* allele in the $Or47b^{2/2}$ flies rescuing the extra-retinal functions of the w^+ allele. It could also be that the extra-retinal effects of *white* locus mutations on courtship are dependent on Or47b signaling, suggesting that the *white* gene plays a role in the Or47b signaling pathway.

Figure 6. The *White* Gene Reduces Male Copulation in a Light-Dependent Manner

(A) Courtship competition assay – one three-day-old virgin Canton S female and one male each from the Berlin wild type (w^+) and w^- (Berlin) genotypes (right column) – was used to measure male courtship behavior. Cumulative copulation rates are plotted against time for each male genotype in dark (left column) and light (middle column) conditions. The assay was shortened to 1 hour long in the light condition as nearly all the chambers had copulation occur within this timeframe. (B) The copulation distributions in the dark (left) and light (right) conditions binned into twenty-minute segments is plotted for each male genotype. Significances are indicated on the figure (paired t-test). (C) The overall copulation rates observed for the two chamber sizes are shown on the left, with lines connecting parallel experiments. There is no significant change in overall copulation between the two conditions ($p > 0.05$, $n = 6$, paired t-test). Copulation percentages of the wild type males are shown on the right, in which parallel experiments are connected by lines. The wild type male had a significant advantage over the mutant male in both lighting conditions and there was a significant increase in the wild type advantage between the dark and light conditions (dark: $p = 4.19 * 10^{-3}$, $n = 178$, chi-squared test; light: $p = 7.40 * 10^{-39}$, $n = 174$, chi-squared test; $p < 0.0001$, $n = 6$, paired t-test). Red bars denote average copulation percentage for each condition. The dashed lines indicate chance level of 50%. (D) Courtship competition assay – one three-day-old virgin Canton S female and one male each from the w^+; $Or47b^{2/2}$ and $Or47b^{2/2}$ (w^-) genotypes (right column) – was used to measure male courtship behavior. Cumulative copulation rates are plotted against time for each male genotype in dark (left column) and light (middle column) conditions. The assay was shortened to 1 hour long in the light condition as nearly all the chambers had copulation occur within this timeframe. (E) The copulation distributions in the dark (left) and light (right) conditions binned into twenty-minute segments is plotted for each male genotype. Significances are shown on the figure (paired t-test). (F) The overall copulation rates observed for the two chamber sizes are shown on the left, with lines connecting parallel experiments. There is a significant increase in overall copulation between the two conditions ($p < 0.01$, $n = 7$, paired t-test). Copulation percentages of the wild type males are shown on the right, in which parallel experiments are connected by lines. The wild type male had a significant advantage over the mutant male only in the light condition and there was a significant increase in the wild type advantage between the dark and light conditions (dark: $p = 7.98 * 10^{-1}$, $n = 166$, chi-squared test; light: $p = 6.14 * 10^{-21}$, $n = 167$, chi-squared test; $p < 0.0001$, $n = 7$, paired t-test). Red bars denote average copulation percentage for each condition. The dashed lines indicate chance level of 50%.


A
Dark
Light
Cumulative copulation (%)
♂1: w+; WT
♂2: w-; WT
Time (min)
B
Copulation Rate
♂1: w+; WT
♂2: w-; WT
***
**
**
Time (min)
C
♂1: w+; WT
2d ♀
♂2: w-; WT
Dark or Light
Dark
Light
Total copulation (%)
p = 0.20
t-test
Copulation percentage of ♂1
p = 0.000073
t-test
D
Dark
Light
Cumulative copulation (%)
♂1: w+; Or47b
♂2: w-; Or47b
Time (min)
E
Copulation Rate
♂1: w+; Or47b
♂2: w-; Or47b
***
*
Time (min)
F
2d ♀
♂1: w+; Or47b
♂2: w-; Or47b
Dark or Light
Dark
Light
Total copulation (%)
p = 0.0062
t-test
Copulation percentage of ♂1
p = 0.00000087
t-test

DISCUSSION

This study has shown that the Or47b receptor plays an important role in promoting male courtship under a variety of environmental conditions. Grosjean and colleagues (2011) saw that male wildtype flies had an advantage over Ir84a mutants even in the absence of phenyl acetic acid, the ligand of the Ir84a receptor, which they attribute to cuticular phenyl acetic acid from the fly's diet. We did not observe this effect, possibly due to larger chambers used in our assay, the low volatility of phenyl acetic acid and the larger chamber size used in our assay. Our results did match the results obtained by Grosjean and colleagues when using fly food as the odorant. The independent pathways for the promotion of male courtship by food and pheromonal odorant cues suggests a point of integration of these stimuli multiple synapses downstream of the Or47b and Ir84a olfactory receptor neurons (ORNs).

Our study of the impact of female age on male courtship found that it did not have a significant impact. There is a possibility that limitations on the ability of female flies to end courtship by decamping due to the chamber size may have masked any effects of female age. Use of a larger chamber may allow the female to avoid a courting male for a longer period after decampment. Use of courtship assays that use video recording to analyze male and female behavior would be better to identify how differences in female age may affect male courtship behavior.

Our investigation into the effects of increasing the size of the chamber found that the larger chamber resulted in a reduction in total copulation. This may be due to more

effective termination of courtship by females decamping and the increased difficulty for the males to locate the females. However, this assay is not ideal for studying the influences of specific behaviors such as decampment. Courtship assays using video recording would be better suited to further study of this effect.

The Or47b receptor's promotion of male courtship in the light proves it plays an important role in male courtship even when visual cues are available. More targeted knockdowns of vision-based promotion of male courtship will help better understand how visual cues and Or47b signaling interact to promote courtship.

The investigation of the *white* gene's effect on male courtship in the dark helps resolve some of the conflict between previously observed results. Krstic et al (2013) had observed no difference between wild type and mutant males using a ten-minute-long courtship assay. Our results show that the two lines have similar copulation rates in the first twenty minutes and diverge only after that, explaining why this effect wasn't observed in their study. This observation also highlights the importance of using longer assays and looking both at short-term and longer-term behavior as not all factors affecting behavior will have an immediate impact. Our results show similar copulation percentages to those observed by Reed and Reed (1950) using a 24-hour-long two-male two-female assay. Xiao et al (2017) also found that wild type flies had an advantage over *white* mutants using a single pair assay, but the difference they saw was much greater. This may be due to differences in the condition used in their assay as well as differences in the lines used.

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