Mating success associated with the *white* gene in *Drosophila melanogaster*

Chengfeng Xiao[*,1], **Shuang Qiu**[*] **and R Meldrum Robertson**[*]
[*]Department of Biology, Queen's University, Kingston, Ontario K7L 3N6 Canada

ABSTRACT Characteristics of mating behavior in *Drosophila melanogaster* have been well-described, but the genetic basis for male-female mating success is largely unknown. Here we show that the *white* (*w*) gene, a classical eye color gene, is associated with mating success. 81.3 % of wild-type flies copulated within 60 minutes in the circular arenas, whereas few white-eyed mutants mated successfully. The w^+ allele exchanged to the X chromosome or duplicated to the Y chromosome in the white-eyed genetic background rescued the defect of mating success. Addition of a mini-*white* (mw^+) gene to the white-eyed mutant background rectified the defect of mating success and rescued courting in a dosage-dependent manner. Lastly, male-female sexual experience mimicked the effects of w^+/mw^+ in improving successful male-female mating. These data suggest a strong association between the *w* gene and mating success in *Drosophila melanogaster*.

KEYWORDS the *white* gene; Mating success; Courtship behavior; Male-female sexual experience; *Drosophila melanogaster*

Mating behavior in wild-type *Drosophila* consists of a series of courting activities and subsequent copulation. Successful mating is a consequence of sexual interactions between specific male stimuli and appropriate female responses (Mayr 1946). Mating success is obviously of great importance to the fitness of a population during environmental adaptation. Characteristics of mating behavior in *Drosophila* are well-described (Sturtevant 1915; Spieth 1952). Many of the behavioral components are quantifiable and have been genetically attributed to chromosome arrangement or polymorphism (Miller 1958; Spiess *et al.* 1961; Spiess and Langer 1964; Miller and Westphal 1967; Patty 1975), and even individual genes (Merrell 1949b; Jacobs 1960; Connolly *et al.* 1969; Zhang and Odenwald 1995; Hing and Carlson 1996; Hirai *et al.* 1999; Anaka *et al.* 2008). For example, in *Drosophila persimilis* and *Drosophila pseudoobscura*, the males carrying the commonest gene arrangement on the third chromosome mate rapidly whereas the males with the less frequent arrangement mate slowly (Spiess *et al.* 1961; Spiess and Langer 1964).

The *Drosophila white* (*w*) gene, discovered in 1910 by Thomas Hunt Morgan (Morgan 1910), encodes a subunit of an ATP-binding cassette (ABC) transporter, which loads up pigment granules and deposits the content to pigment cells in the compound eyes, ocelli, Malpighian tubules and testis (O'Hare *et al.* 1984; Hazelrigg 1987). In addition, the White protein transports bioamines, neurotransmitters, metabolic intermediates, second messengers and many small molecules (Anaka *et al.* 2008; Sullivan and Sullivan 1975; Sullivan *et al.* 1979; Borycz *et al.* 2008; Evans *et al.* 2008; Sitaraman *et al.* 2008). White is thus proposed to have housekeeping functions in the central nervous system in addition to its classical role in eye pigmentation (Anaka *et al.* 2008; Borycz *et al.* 2008; Xiao and Robertson 2016). *white* is also involved in selective mating. Sturtevant (1915) found that Morgan's white-eyed male flies (Morgan 1910) were less successful than wild-type males in mating females (Sturtevant 1915). A ratio of mating success is 0.75 for white-eyed male to 1 for wild-type male (Reed and Reed 1950). Such a sexual discrimination against white-eyed males eventually results in the elimination of mutant allele of *w* from a laboratory population (Reed and Reed 1950). Although these findings suggest a role for *w* in mating selection, genetic evidence for the association between the *w* gene and mating success is lacking.

Ectopic expression or intracellular mislocation of White protein from mini-*white* (mw^+) gene induces male-male courtship chaining (Zhang and Odenwald 1995; Hing and Carlson 1996; Anaka *et al.* 2008). However, mw^+ males do not reduce courtship preference for females (Anaka *et al.* 2008; Nilsson *et al.* 2000). Complete lack of White reduces sexual arousal of males in the daylight but not in the dark (Krstic *et al.* 2013), but whether or not reduced sexual arousal affects mating success, more specifically, whether w^+ or mw^+ promotes successful copulation is

doi: 10.1534/genetics.XXX.XXXXXX
Manuscript compiled: Wednesday 31st August, 2016%
[1]Department of Biology, Queen's University, Kingston, Ontario K7L 3N6 Canada.
xiao.c@queensu.ca

still unknown.

In the current study we examined mating success in wild-type CS and white-eyed mutant w1118 strains. We demonstrate that w1118 has greatly reduced mating success in a circular arena. Such a reduction can be recovered by several genetic approaches, including the exchange of w^+ allele from wild-type; w^+ duplication to the Y chromosome; and transgenic insertion of a mw^+. We further show a positive correlation between mw^+ copies and the percentage of mating success of males with w1118 genetic background.

Materials and Methods

Flies

Fly strains used in the current study and their sources are listed in Table 1. Flies were maintained with standard medium (cornmeal, agar, molasses and yeast) at 21-23 °C in a light/dark (12/12 hr) condition. w^{1118}(CS) and w^+(w1118) flies carried different w alleles exchanged between CS and w1118 by serial backcrossing for ten generations (Xiao and Robertson 2015). The w^+ duplication (to the Y chromosome) line w^{1118}/Dp(1;Y)$B^S w^+ y^+$ was derived from Raf^{11}/FM6, l(1)FMa^1/Dp(1;Y)$B^S w^+ y^+$ (Bloomington stock center, BSC #5733) by crossing a single male with three w1118 females, and their male progeny into w1118 stock. The latter cross was repeated at least once (Xiao and Robertson 2016). In this study we generated another w^+ duplication line w^{1118}/Dp(1;Y)$w^+ y^+$ from dwg$^{11\text{-}32}$/Dp(1;Y)$w^+ y^+$/C(1)DX, $y^1 f^1$ (BSC #7060). UAS-hsp26, UAS-hsp27, UAS-hsp70 (#3.2, #4.3, #4.4 and #9.1) and UAS-Httex1-Qn-eGFP (n = 47. 72 or 103) were generated under w1118 genetic background (Wang *et al.* 2004; Xiao *et al.* 2007; Zhang *et al.* 2010). 10×UAS-IVS-mCD8::GFP (at attP2 or attP40, BSC #32185, #32186) and 20×UAS-IVS-mCD8::GFP (attP2, BSC #32194) flies were backcrossed to w1118 for ten generations. Flies with UAS-IVS-mCD8::GFP combinations were generated after the backcrossing.

Fly preparation for mating analysis

Naive males and virgin females were collected within five hours after eclosion. Single-sexed flies were grouped as 10-20 per vial, and aged to 4-7 days before the experiments. We used nitrogen gas to knock down flies during collection time. Tested flies were free of nitrogen exposure for at least three days since the initial collection.

Sexual training of w1118 males was carried out as follows: (1) male-male courtship training by CS males: 18 naive w1118 males and 2 naive CS males were mixed in each vial and raised for four days. Male flies obtain sexual experience by courting naive males (Gailey *et al.* 1982; McRobert and Tompkins 1988; Zawistowski and Richmond 1985). The ratio was determined based on the report that white-eyed males rare or predominant to wild-type males in a mixed population increase mating success with white-eyed females (Ehrman 1966); (2) heterospecific training by CS females: 10 naive w1118 males and 10 virgin CS females were mixed in each vial and raised for four days; (3) conspecific training by w1118 females: 10 naive w1118 males and 10 virgin w1118 females were mixed in each vial and raised for four days. Trained w1118 males were then paired individually with a four-day-old w1118 virgin female for the analysis of mating success.

Analysis of mating success

The apparatus for the analysis of mating success was the same as previously reported (Xiao and Robertson 2015). Briefly, a naive/trained male and a virgin female were gently aspirated into a circular arena (1.27 cm diameter 0.3 cm depth). Sexual behavior was monitored with a digital camera (Webcam C905, Logitech) for 60 minutes or an otherwise indicated duration. Mating success (defined as apparent physical attachment between a male and a female) was post-analyzed. The percentage of mating success for each genotype was examined from a minimum of nine pairs. In many experiments conspecific male and female were used for the analysis in order to avoid sexual reluctance between heterospecific flies (Hoenigsberg *et al.* 1959; Dukas 2004). In some experiments uniform females (e.g. CS females) or heterospecific females were used. Experiments were conducted during the light time and at least three hours away from light-dark transit. Light illumination for experiments was provided with a light box (Logan portaview slide/transparency viewer) with surrounding reflections. Dim red illumination was generated by using a red filter (600 nm long-pass, Roscolux #26, Rosco Canada) on the light box.

Evaluation of male courting activity

The courting activity of male fly before copulation was evaluated by calculating a courtship index (Siegel and Hall 1979) with mild modifications. In general, activities of fly pairs within the first three minutes were sampled once every 10 seconds. A courtship index was calculated as the fraction of observation times (18 times within three minutes) during which any courtship occurs. Clearly observable courtship activities in our settings include orientation, female following, and wing extension. We chose the first three minutes for the evaluation of courting activity because copulation did not occur within this period in most of the tested pairs.

Statistics

Fisher's exact test was used to examine the frequency distribution of mating success between two different strains. The Kruskal-Wallis test with Dunn's multiple comparisons was performed to analyze courtship index among four groups of flies. Two-way ANOVA with Bonferroni post-tests was conducted to examine dynamic courtship index within first 180 seconds. Data with normal distribution are presented as average ± standard error of mean (mean ± SEM). Data with non-normal distribution are illustrated as box-plots. A $P < 0.05$ was considered significant difference.

Results

w mutants displayed a defect of mating success

When a naive CS male (4-day-old) and a virgin CS female (4-day-old) were placed into a circular arena (1.27 cm diameter and 0.3 cm depth), they initiated courtship activities and started copulation within minutes. Such a courtship interaction was highly consistent from one pair to another (Figure 1A). In the w mutant w1118, however, a naive male and a virgin female together rarely initiated typical courtship activities and failed to copulate within 60 min. The failure of copulation in w1118 was consistent between pairs (Figure 1B). The difference of copulation success was clearly observable between two strains. We thus used mating success (referred to successful copulation) as a parameter and explored the contribution of w to the sexual interaction between a male and a female.

In CS flies, the percentage of mating success was 81.3 % (13/16) (Figure 2A and 2E). Average mating duration was 26.7 ±

4.3 min with a latency of 8.7 ± 4.1 min (Figure 2A). In w1118, mating success within 60 min was 0 % (0/16) (Figure 2B and 2E), a level significantly lower than that in CS ($P < 0.05$, Fisher's exact test). Mating success of CS flies in the circular arenas was consistent with the observations that several wild-types had successful copulation rates of 50-90 % within 60 min in varying sized mating chambers (MacBean and Parsons 1967; Merrell 1949a; Spiess 1968). w1118 displayed a defect of mating success in the circular arena within 60 min.

CS pairs completed copulation within 60 min. It is possible that w1118 requires longer than an hour to start copulation. We then examined the sexual activities of flies in the circular arenas for 180 min. CS pairs copulated once with a successful rate of 87.5 % (14/16) and finished copulation within the first 60 min. There was no second copulation during the next 120 min (Figure 2C and 2F). This was consistent with previous reports (Sturtevant 1915; Merrell 1949a; Ehrman 1966), and proved the sufficiency of 1-hr observation for mating success in wild-type. As a contrast, w1118 pairs displayed a complete lack of copulation within 180 min (Figure 2D and 2F). Therefore, the defect of mating success in w1118 was evident within a period of prolonged observation.

The defect of mating success within 60 min was also observed in several additional *w* mutants, including w^1 (0 %, 0/16) ($P < 0.05$ compared with CS, Fisher's exact test), w^a (6.3 %, 1/16) ($P < 0.05$ compared with CS, Fisher's exact test) and w^{cf} (0 %, 0/16) ($P < 0.05$ compared with CS, Fisher's exact test) (Figure 2G). Taken together, *w* mutants displayed a defect of mating success in the circular arena.

Male w1118 displayed a severe defect for mating success

We next examined which sex of w1118 contributed largely to the defect of mating success. The percentage of successful copulation between CS male and w1118 female (66.7 %, 12/18) was comparable to that between CS male and CS female (60.0 %, 6/10) with no significant difference (Figure 3A). However, copulation percentage between w1118 male and CS female, and that between w1118 male and w1118 female were both 0 % (0/10) (Figure 3A). Thus male but not female w1118 displayed a severe defect for mating success in the circular arena.

w^+ was associated with mating success

w1118 carries a null allele of *w* on the X chromosome. We examined whether the *w* gene was associated with mating success. We first tested two different male progenies: w^+/Y (F1) and w^{1118}/Y (F1), produced by the cross between male w1118 and female CS, and the reciprocal cross between male CS and female w1118. Paired with respective w^+/w^{1118} sibling females, w^+/Y (F1) displayed mating success (30.0 %, 3/10) slightly higher than w^{1118}/Y (F1) (0 %, 0/10) ($P > 0.05$, Fisher's exact test). Paired with CS females, w^+/Y (F1) displayed significantly higher mating success (90.0 %, 9/10) than w^{1118}/Y (F1) (10.0 %, 1/10) ($P < 0.05$, Fisher's exact test). Similarly, paired with w1118 females, w^+/Y (F1) displayed higher mating success (80.0 %, 8/10) than w^{1118}/Y (F1) (10.0 %, 1/10) ($P < 0.05$, Fisher's exact test) (Figure 3B). Therefore, w^+-carrying F1 males displayed high mating success compared to w^{1118}-carrying F1 males.

We then examined mating success in w^+/Y (w1118) and w^{1118}/Y (CS) flies in which different *w* alleles were exchanged between CS and w1118 by serial backcrossing for ten generations (Xiao and Robertson 2015). Mating success between w^+/Y (w1118) and conspecific w^+/w^+ (w1118) females (37.5 %, 6/16) was higher than that between w^{1118}/Y (CS) and conspecific w^{1118}/w^{1118} (CS) females (6.3 %, 1/16) ($P < 0.05$, Fisher's exact test) (Figure 3C). Results confirmed that w^+-carrying males displayed increased mating success compared with w^{1118}-carrying males.

w^+ duplicated to Y chromosome rescued the defect of mating success in w1118

To further support the association between *w* and mating success, we examined the copulation percentage of males with a w^+ allele duplicated to the Y chromosome. Mating success between w^{1118}/Dp(1;Y)w^+y^+ males and conspecific w^{1118}/w^{1118} females was 75.0 % (12/16), a level comparable to CS (see Figure 2E) with no significant difference. Similar data was obtained using another duplication line. Mating success between w^{1118}/Dp(1;Y)$B^S w^+y^+$ males and conspecific w^{1118}/w^{1118} females (56.3 %, 9/16) was comparable to CS with no significant difference (Figure 3D). Thus, w^+ duplicated to the Y chromosome rescued the defect of mating success in w1118.

mw^+ rescued the defect of mating success in w1118

Drosophila mw^+ is a miniature form of w^+. mw^+ has been widely used as a marker gene to indicate the successful recombination of a transgene. We explored whether mw^+ rescued the defect of mating success in w1118. We tested UAS lines with mw^+-carrying transposons inserted into the X or autosomal (II or III) chromosome. All these flies were synchronized into the w1118 background. The UAS but not other transgenic flies (e.g. Gal4 lines) were chosen to minimize possible complex effects due to ectopic expression of a transcription factor in addition to mw^+ (Xiao and Robertson 2016).

Paired with CS virgin females, male flies with one copy of mw^+ on the autosome showed 0.0 - 12.5 % mating success (Figure 4A). Males with mw^+ on the X chromosome displayed 6.3 - 62.5 % mating success (Figure 4B). Therefore, males with one copy of mw^+ showed an increase of mating success relative to w1118. The observed variance among different UAS lines would be due to expression abundance of mw^+ or a potential second mutation. Likely, the rescue effect of mw^+ was strong if integrated on the X chromosome, perhaps because of an effect of dosage compensation for genes on the X chromosome (Qian and Pirrotta 1995).

Males carrying two copies of mw^+ (heterozygous for each) on the autosomes displayed 0.0 - 12.5 % mating success (Figure 4C). Males with homozygous mw^+ (two copies) on the autosomes displayed 18.5 - 87.5 % mating success (Figure 4D). Males with two homozygous mw^+ (four copies) on the autosomes showed 15.4 - 56.3% mating success (Figure 4E). These data indicated that mw^+ rescued the defect of mating success in w1118.

Notably, males carrying homozygous mw^+ alleles displayed increased mating success compared with males carrying heterozygous mw^+. Mating success was increased from 6.3 % for heterozygous to 87.5 % for homozygous 10×UAS-IVS-mCD8::GFP (on III) flies (Figure 4F). Similar results was observed as 0 % to 75.0 % in 10×UAS-IVS-mCD8::GFP (on II), 12.5 % to 75.0 % in 20×UAS-IVS-mCD8::GFP (on III), 12.5 % to 56.3 % in 10×UAS-IVS-mCD8::GFP;10×UAS-IVS-mCD8::GFP (on II and III), 6.3 % to 56.3 % in 10×UAS-IVS-mCD8::GFP;20×UAS-IVS-mCD8::GFP (on II and III), 0 % to 43.8 % in UAS-hsp70#4.4 (on III), 0 % to 15.4 % in UAS-hsp27;UAS-hsp26 (on II and III), and 6.3 % to 18.8 % in UAS-Httex1-Q47-eGFP (on III) (Figure

4A and 4C-F). The change of mating success for each UAS line was observed between flies with heterozygous and homozygous mw^+ alleles carried in the same transposon. Hence they would have different levels of White protein with the same expression pattern under identical genetic background. There was a strong positive correlation between copies of mw^+ and the percentage of mating success. These data indicate that mw^+ rescued the defect of mating success in a dosage-dependent manner.

mw$^+$ rescued courtship activity in w1118

A wild-type male usually displays a series of courting activities towards a female mate before copulation (Sturtevant 1915; Spieth 1952). We examined whether mw^+ rescued courtship in white-eyed flies. Courtship indices were evaluated in CS, w1118 and mw^+-carrying males. During the first three minutes, most CS males displayed typical courting behaviors (orientation, female following and wing extension) to CS females, whereas w1118 males showed greatly reduced courting activities. Within three minutes, heterozygous 10×UAS-IVS-mCD8::GFP (III) flies (mw^+/+) displayed sporadic courting, while homozygous flies (mw^+/mw^+) showed strong and persistent courting activities (Figure 5A).

Within three minutes, the median courtship index of CS males was 0.83 (interquartile range (IQR) 0.63–0.94, n = 16), w1118 0.11 (IQR 0.06–0.11, n = 16), heterozygous mw^+ 0.28 (IQR 0.28–0.33, n = 15) and homozygous mw^+ 1.00 (IQR 0.94–1.00, n = 15). Male w1118 showed a markedly reduced courtship index compared with CS ($P < 0.05$, Kruskal-Wallis test with Dunn's multiple comparisons). Heterozygous mw^+ males displayed a slightly increased courtship index compared with w1118 with non-significant difference, but a reduced courtship index compared with CS ($P < 0.05$, Kruskal-Wallis test with Dunn's multiple comparisons). Homozygous mw^+ males showed a courtship index higher than heterozygous mw^+ ($P < 0.05$, Kruskal-Wallis test with Dunn's multiple comparisons) (Figure 5B). Interestingly, Homozygous mw^+ males had a courtship index similar to CS males (with non-significant difference). Therefore, mw^+ rescued courtship activity in white-eyed males and the rescue effect was mw^+ copy number-dependent.

We further examined the dynamic changes of courtship indices in CS, w1118 and mw^+ flies within 180 seconds. The initial courtship index was around 0.6 for CS males and around 0.1 for w1118 males, indicating rapid engagement of courtship with females in most CS flies but extremely slow in w1118. The initial courtship index was around 0.1 for heterozygous mw^+ males and 1.0 for homozygous mw^+ males, thus all homozygous mw^+ males started courtship immediately (within ten seconds) with conspecific females. CS males had an average courtship index of 0.5–0.6 in the first 60 sec and a gradual increase within the next 120 sec, whereas w1118 males had a courtship index of 0.0–0.2 most of the time. Courtship indices of CS males were higher than those of w1118 throughout the observation times ($P < 0.01$, repeated measures ANOVA with Bonferroni post-tests). Heterozygous mw^+ flies displayed increased courtship indices in some of the observation periods (i.e. 90, 160 and 180 sec) compared with w1118, but reduced levels in most of the observations compared with CS (repeated measures ANOVA with Bonferroni post-tests). Homozygous mw^+ males had a persistently high courtship index (0.9–1.0) throughout, and surprisingly, during the first 60 seconds, courtship indices of homozygous mw^+ males were even higher than CS males ($P < 0.05$, repeated measures ANOVA with Bonferroni post-tests) (Figure 5C). These data suggest that mw^+ rescued courtship activity, and that homozygous mw^+ alleles over-rectified the courtship in white-eyed males.

Male-female copulation experience rectified the defect of mating success in w1118

To understand how w1118 males might lose mating success in the circular arena, we attempted to improve copulation percentage of w1118 flies through several approaches: (1) increase of arena size, (2) dim red illumination and (3) sexual training.

In small chambers flies often become irritated in behavior and locomotion (Spieth 1952; Xiao and Robertson 2015). The mating success of w1118 could be severely affected by spatial restriction. In large arenas (3.81 cm diameter) CS and w1118 males reduce locomotion compared with locomotion in small arenas (1.27 cm diameter), suggesting reduced irritation of flies in large arenas. CS pairs in large arenas showed 66.7 % (6/9) mating success, a level similar to that in small arenas (see Figure 2E). However, there was no mating success (0 %, 0/9) in w1118 pairs (Figure 6A). Mating success was higher in CS than w1118 ($P < 0.05$, Fisher's exact test). Thus, increasing the arena size had no effect on mating success in wild-type and failed to promote copulation in w1118.

Daylight illumination reduces sexual arousal in w1118 males (Krstic *et al.* 2013). Diminished light improves mating in ebony and *lozenge*^{3} flies (Jacobs 1960; Stocker and Gendre 1989). It is possible that mating success of w1118 would increase under dim red illumination, a condition mimicking darkness due to poor visual sensitivity to red light (McEwen 1918). With dim red illumination, mating success was 56.3 % (9/16) in CS pairs and 0 % (0/16) in w1118 (Figure 6B). There was a clear defect of mating success in w1118 compared with CS ($P < 0.05$, Fisher's exact test). Dim red illumination did not repair the defect of mating success in w1118.

Mating behavior can be modified by male-male courtship (Gailey *et al.* 1982; McRobert and Tompkins 1988; Zawistowski and Richmond 1985). Specific ratios of white-eyed : wild-type males (i.e. < 40 %, or > 80 %) improve mating success in white-eyed flies if two strains (including white-eyed males and females, and wild-type males and females) are present together (Ehrman 1966). We tested out whether w1118 males would learn to improve mating success from CS males. Naive w1118 males and CS males were mixed at 9:1 and raised for four days. Paired with w1118 virgin females, w1118 males showed no mating success at all (0 %, 0/16) in the circular arena (Figure 6C).

Male-female copulation experience enhances mating behavior and success in wild-type flies (Saleem *et al.* 2014). Male-female copulation training might be more effective than male-male courtship training in improving mating success in white-eyed flies. Naive w1118 males and CS virgin females were mixed with equal numbers and raised for four days. Paired with four-day-old conspecific w1118 females, experienced w1118 males displayed 26.7 % (4/15) mating success. If naive w1118 males were copulation-experienced with conspecific virgin females, mating success in w1118 flies was slightly increased to 50.0 % (8/16) compared with heterospecific training ($P > 0.05$, Fisher's exact test) (Figure 6C), but significantly increased compared with naive w1118 males (see Figure 2E) ($P < 0.05$, Fisher's exact test). Therefore, male-female copulation experience rectified the defect of mating success in w1118.

Discussion

There is a clear involvement of *w* in sexual discrimination and courtship performance in male flies (Sturtevant 1915; Zhang and Odenwald 1995; Hing and Carlson 1996; Reed and Reed 1950; Krstic *et al.* 2013), but whether *w* modulates male-female mating success is unknown. Here we show that loss-of-*w* is associated with a defect of mating success in a circular arena, and that addition of mw^+ into a null background recovers mating success in a manner that is mw^+ copy number-dependent. These data firmly support the conclusion that the *w* gene is associated with mating success in *Drosophila melanogaster*.

Successful copulation requires appropriate and adequate sexual effort from both male and female flies. Mating success would be extremely susceptible to factors that affect sexual abilities of either of the sexes separately or their interaction during sexual maturation. White-eyed males display greatly reduced courting activities. This is consistent with previous studies that reduced mating competition against wild-type males and reduced sexual arousal during the daylight in white-eyed males (Sturtevant 1915; Reed and Reed 1950; Krstic *et al.* 2013). White-eye females are less vigorous in resisting or escaping from courtship requests of males (Sturtevant 1915), but such "sexual advantage" promotes no copulation success with white-eyed males in the circular arenas. Therefore, loss-of-*w* impairs sexual behaviors in both males and females, and likely the sexual behaviors in males are more severely affected than females.

Rapid and persistent courting interactions are evident between wild-type male and wild-type female in the circular arena. In contrast, extremely small frequency of courting interactions is observed between white-eyed male and white-eyed female. Size of small arena (1.27 cm diameter) induces increased locomotion in males of both wild-type and white-eyed mutant (Xiao and Robertson 2015). However, once a virgin female is present, a wild-type male rapidly switches locomotion to courting, as revealed by the high initial courtship index. These suggest that courting behavior has a higher priority than exploratory activity in small arena in wild-type flies. Indeed, mating behavior in wild-type is vigorous and highly resistant to environmental stress such as strong smelling of ether (Sturtevant 1915). We find that the change of arena size does not affect the high mating success in wild-type. On the other hand, white-eyed males do not show rapid and persistent engagement of courting, and fail to copulate with virgin females in the arenas. Thus, a high priority to copulation might have been lost or severely impaired in white-eyed flies.

Addition of w^+ to white-eyed flies by exchanging the allele to the X chromosome or duplicating to the Y chromosome rescues the defect of mating success. These findings indicate a strong association between *w* and mating success. This is further supported by the findings that addition of mw^+ to the *w* null background rectifies the defect of mating success, and that the rectification is mw^+ copy number-dependent.

It seems that the rescue of males by adding w^+ to the Y chromosome is sufficient for normal mating success. However, it is also possible that w^+ duplication males take advantage of reduced sexual rejection of sibling females ("sexual advantage" to male) to achieve a high level of mating success. Similarly, adding homozygous mw^+ alleles to the null background rectifies the defect of mating success. It should be mentioned that wild-type virgin females but not white-eyed virgin females were provided to the mw^+ males for mating analysis. Therefore, addition of w^+ or mw^+ to male is critical for improving mating success.

Addition of homozygous mw^+ alleles to white-eyed flies over-rectifies the courting activities of males. This finding echoes the reports that mw^+ is associated with male-male courtship chaining (Zhang and Odenwald 1995; Hing and Carlson 1996; Anaka *et al.* 2008), and that the mw^+ males display bisexual courting activities towards both females and males (Nilsson *et al.* 2000). Our finding suggests that mw^+ partially recovers mating behavior, an effect similar to the common observation that mw^+ partially rescues eye color. In addition, mating success is rectified to a higher level in flies with homozygous mw^+ than heterozygous mw^+ carried in the same transposon with identical chromosomal location, indicating a dosage-dependent relation between mw^+ expression and mating success.

In addition to genetic manipulation of w^+ or mw^+ alleles for successful copulation, sexual experience of white-eyed males by sibling females increases mating success between trained males and virgin females. This would be at least crucial for maintaining a white-eyed stock over generations. On the other hand, sexual isolation for several days would worsen the impaired mating success in white-eyed flies, but the worsening effect, if any, would be tolerable in wild-type. The effect of sexual training is similar to that of genetic implementation of w^+ or mw^+ into *w* null background. However, wild-type flies do not require male-female sexual training at all, and the successful copulation between sexually isolated male and female can be achieved within minutes. Therefore, the impaired mating success due to loss-of-*w* is largely mitigated by male-female sexual experience.

Male-female copulation experience but not male-male interaction improves mating success in white-eyed flies. Our data are consistent with the observation that male-female copulation confers competitive advantages for mating success in wild-type males (Saleem *et al.* 2014). It is unclear whether group-raised white-eyed males could learn to suppress male-male courtship and increase the sexual drive for male-female courting, and it is similarly unclear whether female-female interaction would improve receptiveness. Nevertheless, one thing is quite clear, there is no mating within three hours between group-raised virgin males and virgin females of white-eyed flies. Therefore, it is male-female copulation experience that mimics the effect of w^+/mw^+ gene to rectify the defect of mating success.

How might w^+ mimic male-female copulation experience in improving mating success? Since the first report more than 100 years ago (Morgan 1910), it has long been believed that the *w* gene primarily controls eye color of *Drosophila* (Nolte 1950; Pirrotta and Bröckl 1984). It was not until 1995 that an extra-retinal neural function of White was proposed (Zhang and Odenwald 1995). Since then it has been demonstrated that the White protein uptakes biogenic amines, second messengers, intermediate metabolites and many small molecules including pigment precursors into vesicles and transports them to appropriate subcellular locations (Anaka *et al.* 2008; Evans *et al.* 2008; Sullivan and Sullivan 1975; Sullivan *et al.* 1979; Nolte 1950). The behavioral performance of wild-type flies supports the proposed neural function of White. Wild-type flies have a fast reaction to volatile general anesthetics (VGAs) (Campbell and Nash 2001), fast and consistent locomotor recovery from anoxic coma (Xiao and Robertson 2016; Haddad *et al.* 1997), and high boundary preference of exploratory activities in the circular arena (Xiao and Robertson 2015). In contrast, the loss-of-*w* mutants show altered and light-dependent sensitivity to VGAs (Campbell and Nash 2001), delayed locomotor recovery from anoxia (Xiao and Robertson 2016), reduced boundary preference of exploration

(Xiao and Robertson 2015), and reduced sexual arousal in the daylight (Krstic *et al.* 2013). Therefore, it is highly plausible that the White protein possesses critical housekeeping functions in maintaining appropriate vesicular contents, transporting many molecular substrates and improving signaling efficacy in the nervous system. These functions of White could be essential for mating success, in addition to an evident role in learning and memory (Sitaraman *et al.* 2008).

Acknowledgments

This work was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) grant (RGPIN 40930-09) to R.M.R.

Literature Cited

Anaka, M., C. D. MacDonald, E. Barkova, K. Simon, R. Rostom, R. A. Godoy, A. J. Haigh, I. A. Meinertzhagen, and V. Lloyd, 2008 The *white* gene of *Drosophila melanogaster* encodes a protein with a role in courtship behavior. J Neurogenet **22**: 243–76.

Borycz, J., J. A. Borycz, A. Kubów, V. Lloyd, and I. A. Meinertzhagen, 2008 *Drosophila* ABC transporter mutants *white*, *brown* and *scarlet* have altered contents and distribution of biogenic amines in the brain. J Exp Biol **211**: 3454–66.

Campbell, J. L. and H. A. Nash, 2001 Volatile general anesthetics reveal a neurobiological role for the *white* and *brown* genes of *Drosophila melanogaster*. J Neurobiol **49**: 339–49.

Connolly, K., B. Burnet, and D. Sewell, 1969 Selective mating and eye pigmentation: an analysis of the visual component in the courtship behavior of *Drosophila melanogaster*. Evolution **23**: 548–59.

Dukas, R., 2004 Male fruit flies learn to avoid interspecific courtship. Behav Ecol **15**: 695–8.

Ehrman, L., 1966 Mating success and genotype frequency in *Drosophila*. Anim Behav **14**: 332–9.

Evans, J. M., J. P. Day, P. Cabrero, J. A. Dow, and S. A. Davies, 2008 A new role for a classical gene: *white* transports cyclic gmp. J Exp Biol **211**: 890–9.

Gailey, D. A., F. R. Jackson, and R. W. Siegel, 1982 Male courtship in *Drosophila*: the conditioned response to immature males and its genetic control. Genetics **102**: 771–82.

Haddad, G. G., Y. Sun, R. J. Wyman, and T. Xu, 1997 Genetic basis of tolerance to O_2 deprivation in *Drosophila melanogaster*. Proc Natl Acad Sci U S A **94**: 10809–12.

Hazelrigg, T., 1987 The *Drosophila white* gene: a molecular update. Trends Genet **3**: 43–7.

Hing, A. L. and J. R. Carlson, 1996 Male-male courtship behavior induced by ectopic expression of the *Drosophila white* gene: role of sensory function and age. J Neurobiol **30**: 454–64.

Hirai, Y., H. Sasaki, and M. T. Kimura, 1999 Copulation duration and its genetic control in *Drosophila elegans*. Zool Sci **16**: 211–214.

Hoenigsberg, H., S. Koref Santibanez, and G. Sironi, 1959 Intraspecific sexual preferences in *Drosophila prosaltans* duda and in *Drosophila equinoxialis* dobzhansky. Cell Mol Life Sci **15**: 223–5.

Jacobs, M. E., 1960 Influence of light on mating of *Drosophila melanogaster*. Ecology **41**: 182–188.

Krstic, D., W. Boll, and M. Noll, 2013 Influence of the *white* locus on the courtship behavior of *Drosophila* males. PLoS One **8**: e77904.

MacBean, I. T. and P. A. Parsons, 1967 Directional selection for duration of copulation in *Drosophila melanogaster*. Genetics **56**: 233.

Mayr, E., 1946 Experiments on sexual isolation in *Drosophila* vii. the nature of the isolating mechanisms between *Drosophila pseudoobscura* and *Drosophila persimilis*. Proc Natl Acad Sci U S A **32**: 128–137.

McEwen, R. S., 1918 The reactions to light and to gravity in *Drosophila* and its mutants. J Exp Zool **25**: 49–106.

McRobert, S. P. and L. Tompkins, 1988 Two consequences of homosexual courtship performed by *Drosophila melanogaster* and *Drosophila affinis* males. Evolution **42**: 1093–7.

Merrell, D. J., 1949a Mating between two strains of *Drosophila melanogaster*. Evolution **3**: 266–268.

Merrell, D. J., 1949b Selective mating in *Drosophila melanogaster*. Genetics **34**: 370–389.

Miller, D. D., 1958 Sexual isolation and variation in mating behavior within *Drosophila athabasca*. Evolution **12**: 72–81.

Miller, D. D. and N. J. Westphal, 1967 Further evidence on sexual isolation within *Drosophila athabasca*. Evolution **21**: 479–92.

Morgan, T. H., 1910 Sex limited inheritance in *Drosophila*. Science **32**: 120–2.

Nilsson, E. E., Z. Asztalos, T. Lukacsovich, W. Awano, K. Usui-Aoki, and D. Yamamoto, 2000 *Fruitless* is in the regulatory pathway by which ectopic *mini-white* and transformer induce bisexual courtship in *Drosophila*. J Neurogenet **13**: 213–32.

Nolte, D. J., 1950 The eye-pigmentary system of *Drosophila*: The pigment cells. J Genet **50**: 79–99.

O'Hare, K., C. Murphy, R. Levis, and G. M. Rubin, 1984 DNA sequence of the *white* locus of *Drosophila melanogaster*. J Mol Biol **180**: 437–55.

Patty, R. A., 1975 Investigation of genetic factors influencing duration of copulation in 'eastern'and 'western' *Drosophila athabasca*. Anim Behav **23**: 344–348.

Pirrotta, V. and C. Bröckl, 1984 Transcription of the *Drosophila white* locus and some of its mutants. EMBO J **3**: 563–8.

Qian, S. and V. Pirrotta, 1995 Dosage compensation of the *Drosophila white* gene requires both the X chromosome environment and multiple intragenic elements. Genetics **139**: 733–44.

Reed, S. and E. Reed, 1950 Natural selection in laboratory populations of *Drosophila*. II. Competition between a white-eye gene and its wild type allele. Evolution **4**: 34–42.

Saleem, S., P. H. Ruggles, W. K. Abbott, and G. E. Carney, 2014 Sexual experience enhances *Drosophila melanogaster* male mating behavior and success. PLoS One **9**: e96639.

Siegel, R. W. and J. C. Hall, 1979 Conditioned responses in courtship behavior of normal and mutant *Drosophila*. Proc Natl Acad Sci U S A **76**: 3430–4.

Sitaraman, D., M. Zars, H. Laferriere, Y. C. Chen, A. Sable-Smith, T. Kitamoto, G. E. Rottinghaus, and T. Zars, 2008 Serotonin is necessary for place memory in *Drosophila*. Proc Natl Acad Sci U S A **105**: 5579–84.

Spiess, E. B., 1968 Courtship and mating time in *Drosophila pseudoobscura*. Anim Behav **16**: 470–9.

Spiess, E. B. and B. Langer, 1964 Mating speed control by gene arrangements in *Drosophila pseudoobscura* homokaryotypes. Proc Natl Acad Sci U S A **51**: 1015–1019.

Spiess, E. B., B. Langer, and C. Li, 1961 Chromosomal adaptive polymorphism in *Drosophila persimilis* III. Mating propensity of homokaryotypes. Evolution **15**: 535–44.

Spieth, H. T., 1952 Mating behavior within the genus *Drosophila*

(diptera). Bull Am Mus Nat Hist **99**: 395–474.

Stocker, R. F. and N. Gendre, 1989 Courtship behavior of *Drosophila* genetically or surgically deprived of basiconic sensilla. Behav Genet **19**: 371–85.

Sturtevant, A. H., 1915 Experiments on sex recognition and the problem of sexual selection in *Drosoophilia*. Journal of Animal Behavior **5**: 351–366, http://dx.*doi*.org/10.1037/h0074109.

Sullivan, D. T., L. A. Bell, D. R. Paton, and M. C. Sullivan, 1979 Purine transport by malpighian tubules of pteridine-deficient eye color mutants of *Drosophila melanogaster*. Biochem Genet **17**: 565–73.

Sullivan, D. T. and M. C. Sullivan, 1975 Transport defects as the physiological basis for eye color mutants of *Drosophila melanogaster*. Biochem Genet **13**: 603–13.

Wang, H. D., P. Kazemi-Esfarjani, and S. Benzer, 2004 Multiple-stress analysis for isolation of *Drosophila* longevity genes. Proc Natl Acad Sci U S A **101**: 12610–5.

Xiao, C., V. Mileva-Seitz, L. Seroude, and R. M. Robertson, 2007 Targeting hsp70 to motoneurons protects locomotor activity from hyperthermia in *Drosophila*. Dev neurobiol **67**: 438–55.

Xiao, C. and R. M. Robertson, 2015 Locomotion induced by spatial restriction in adult *Drosophila*. PLoS One **10**: e0135825.

Xiao, C. and R. M. Robertson, 2016 Timing of locomotor recovery from anoxia modulated by the *white* gene in *Drosophila*. Genetics **203**: 787–97.

Zawistowski, S. and R. C. Richmond, 1985 Experience-mediated courtship reduction and competition for mates by male *Drosophila melanogaster*. Behav Genet **15**: 561–9.

Zhang, S., R. Binari, R. Zhou, and N. Perrimon, 2010 A genomewide rna interference screen for modifiers of aggregates formation by mutant huntingtin in *Drosophila*. Genetics **184**: 1165–79.

Zhang, S. D. and W. F. Odenwald, 1995 Misexpression of the *white* (*w*) gene triggers male-male courtship in *Drosophila*. Proc Natl Acad Sci U S A **92**: 5525–9.

Figures and Tables

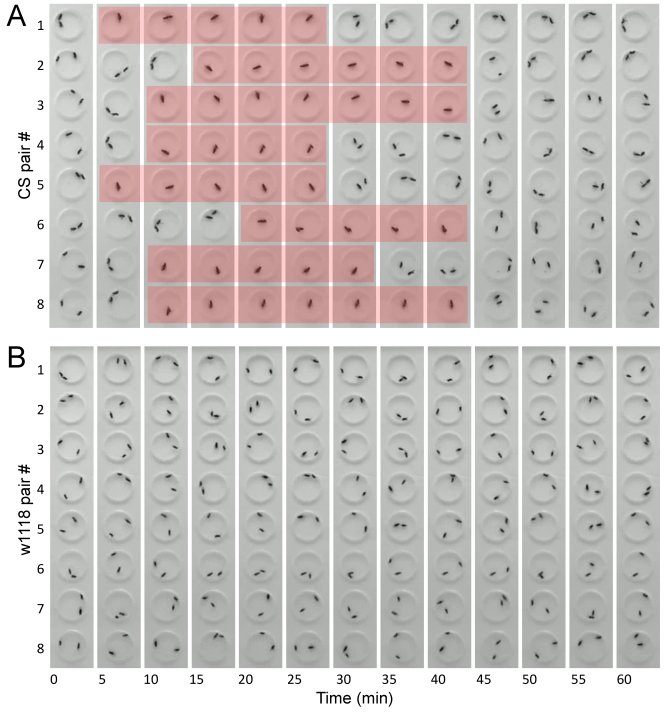

Figure 1 A defect of mating success in w1118. Fly pairs (n = 8) were loaded into the circular arenas (1.27 cm diameter, 0.3 cm depth) and their courting and copulation activities within 60 minutes were examined. Each pair consists of a virgin male (4-6 days old) and a conspecific virgin female (4-6 days old). Successful copulation (color-shaded) was observed in (A) wild-type (CS) but not (B) white-eyed mutant (w118). Shown are the video-frames sampled once per five minutes.

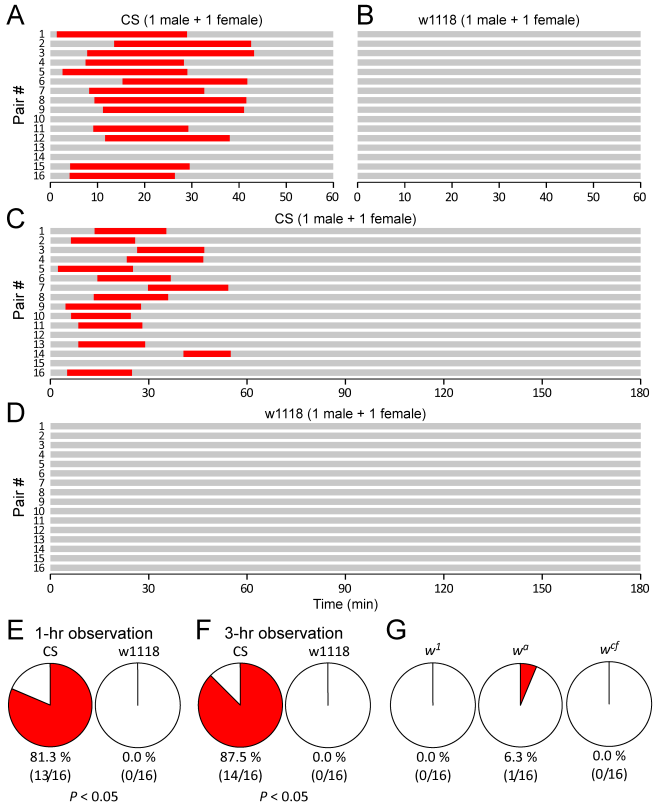

Figure 2 White-eyed mutants displayed greatly reduced mating success. (A, B) Mating success within 60 minutes in CS (n = 16) and w1118 (n = 16) flies. Durations for copulation (red) and non-copulation (grey) are indicated. (C, D) Mating success within 180 minutes in CS (n = 16) and w1118 (n = 16) flies. (E) Percentages of mating success (indicated as red) in one-hour observation in CS and w1118 flies. (F) Percentages of mating success in three-hour observation in CS and w1118 flies. (G) Percentages of mating success within 60 minutes in w^1, w^a and w^{cf} mutants. *P* values from Fisher's exact tests.

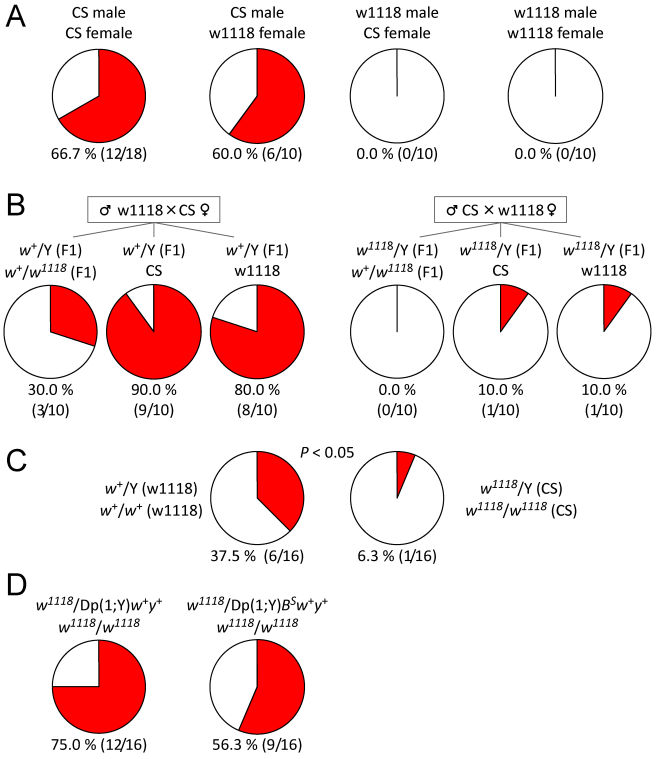

Figure 3 w^+ allele was associated with mating success. (A) Mating success within 60 minutes in different male-female combinations. (B) Mating success between F1 males and varying females (See Materials and Methods). (C) Mating success in w^+(w1118) and w^{1118}(CS) flies in which different *w* alleles are exchanged between CS and w1118 by serial backcrossing. *P* value from Fisher's exact test. (D) Mating success in flies with w^+ duplicated to the Y chromosome.

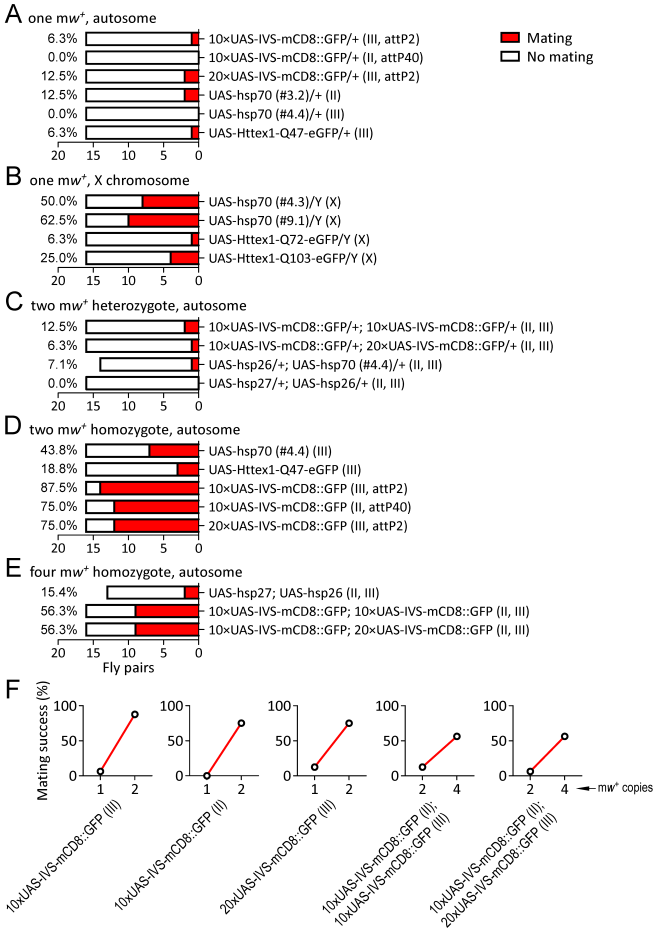

Figure 4 mw^+ rescued mating success in white-eyed flies in a dosage-dependent manner. Mating success in flies with (A) one copy of mw^+ in the autosome, (B) one copy of mw^+ in the X chromosome, (C) two copies of mw^+ (heterozygote) in the autosome, (D) two copies of mw^+ (homozygote) in the autosome, and (E) four copies of mw^+ (homozygote) in the autosome. (F) Correlations of mating success between heterozygous and homozygous flies carrying the same transposons. Numbers indicate mw^+ copies in the autosome. Chromosomal locations of mw^+ are indicated in the parentheses. attP2: site-specific recombination site in the third chromosome; attP40: site-specific recombination site in the second chromosome.

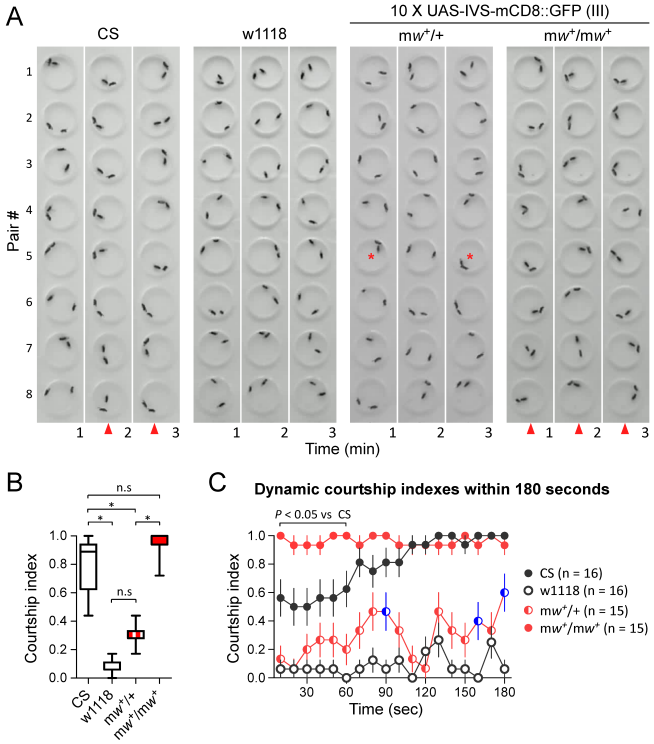

Figure 5 mw^+ rescued courtship activities in white-eyed flies. (A) Courting activities before copulation in CS, w1118 and mw^+ flies. Shown are the video-frames sampled once per minute during first three minutes. There are eight pairs for each genotype. Each pair contains a male and a conspecific female. Red triangles under a column indicate consistent courting in all or most pairs in CS or mw^+/mw^+ flies. Red stars in single arenas denote sporadic courting in mw^+/+ flies. (B) Courtship indices within three minutes in different flies. *, $P < 0.05$ (one-way ANOVA); n.s., non-significance. (C) Dynamic courtship indices within the first 180 seconds in four genotypes. Courtship indices were repeatedly evaluated once every 10 seconds. P value is from two-way ANOVA. Sample sizes are indicated.

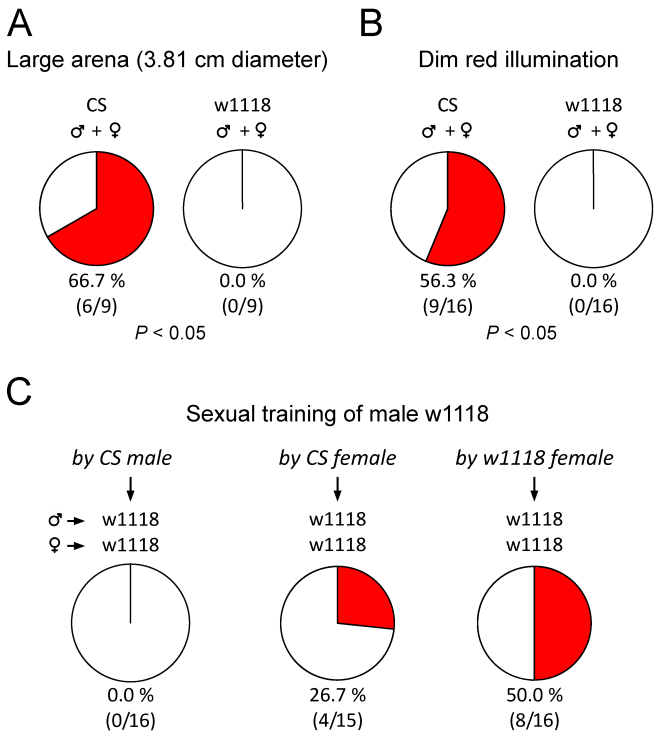

Figure 6 Male-female sexual experience improved mating success in w1118. (A) Mating success of CS and w1118 in large arenas (3.81 cm diameter 0.3 cm depth). (B) Mating success of CS and w1118 under dim red illumination. (C) Mating success between sexually trained w1118 males and virgin w1118 females. Training conditions are indicated (see Materials and Methods for description). *P* values from Fisher's exact tests..

Table 1 Fly strains used in this study and their sources

Strains	Sources
Canton-Special (CS)	BSC[a] #1
w1118	L. Seroude Laboratory
w^{1118}(CS)	(Xiao and Robertson 2015)
w^{+}(w1118)	(Xiao and Robertson 2015)
w^{1}	BSC #145
w^{a}	BSC #148
w^{cf}	BSC #4450
w^{1118}/Dp(1;Y)$B^{S}w^{+}y^{+}$	(Xiao and Robertson 2016)
w^{1118}/Dp(1;Y)$w^{+}y^{+}$	This study
UAS-hsp26 (III)	(Wang *et al.* 2004)
UAS-hsp27 (II)	(Wang *et al.* 2004)
UAS-hsp70 #3.2 (II)	(Xiao *et al.* 2007)
UAS-hsp70 #4.3 (X)	(Xiao *et al.* 2007)
UAS-hsp70 #4.4 (III)	(Xiao *et al.* 2007)
UAS-hsp70 #9.1 (X)	(Xiao *et al.* 2007)
UAS-Httex1-Q47-eGFP	(Zhang *et al.* 2010)
UAS-Httex1-Q72-eGFP	(Zhang *et al.* 2010)
UAS-Httex1-Q103-eGFP	(Zhang *et al.* 2010)
10×UAS-IVS-mCD8::GFP (attP2[b])	BSC #32185
10×UAS-IVS-mCD8::GFP (attP40[c])	BSC #32186
20×UAS-IVS-mCD8::GFP (attP2)	BSC #32194
UAS-hsp27 (II) ; UAS-hsp26 (III)	(Xiao and Robertson 2016)
10×UAS-IVS-mCD8::GFP (attP40); 10×UAS-IVS-mCD8::GFP (attP2)	(Xiao and Robertson 2016)
10×UAS-IVS-mCD8::GFP (attP40); 20×UAS-IVS-mCD8::GFP (attP2)	(Xiao and Robertson 2016)

[a] Bloomington Stock Center
[b] site-specific recombination site on III chromosome
[c] site-specific recombination site on II chromosome