EXPRESSION OF *YELLOW* IN THE CENTRAL NERVOUS SYSTEM AS A COURTSHIP INDICATOR AND ITS EFFECTS ON THE REGULATION OF MALE MATING SUCCESS IN *DROSOPHILA MELANOGASTER* AND *DROSOPHILA VIRILIS*[1]

by

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[1] Title has been changed to convey new findings.

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ABSTRACT

The *Drosophila melanogaster* males perform a courtship ritual consisting of a fixed six-step routine. The *yellow* gene is required for normal male courtship behavior and subsequent male mating success. This study examined the effects of *yellow* (*D. melanogaster and D. virilis*) on successful male courtship behavior in adult male *D. melanogaster yellow* mutants. It also investigates the regulation of the Yellow protein in the genetic makeup of the *Drosophila* fly. Results from the courtship experiments found that *D. melanogaster yellow* mutant harboring a *D.melanogaster yellow* transgene restores the courtship behavior of the *D.melanogaster yellow* mutants to normal levels, however, the *yellow* transgene from *D. virilis* does not restore the courtship behavior of the *D. melanogaster yellow* mutants to its normal levels.

On the other hand, at the current stage of this study, no significant differences have been found between the *yellow* expression patterns of the adult brains in wild type *D. melanogaster* flies harboring a *D. virilis yellow* mating success enhancer (MRS) – GFP transgene and in wild type *D. melanogaster* flies harboring a *D. melanogaster yellow* mating success

enhancer (MRS) – GFP transgene. Imperative experimentations will continue to progress, and previous literature depicting such experiments on third instar larvae will be further researched on to devise a more promising method to determine *yellow* expression in the adult central nervous system.

Overall, this study suggests that *yellow* has indeed the ability to restore defective courtship behavior in adult males, which supports past literature. However, it is plausibly cis-regulated and not conserved throughout different *Drosophila* species. Further studies should be conducted to reveal the possibly many other currently unknown roles *yellow* play in normal *Drosophila* courtship behavior.

INTRODUCTION

Neurobiology is the biological study of the nervous system. The nervous system is an organ system containing a network of specialized cells called neurons. These neurons coordinate the actions of an animal and transmit signals between different parts of its body through their synapses. In most animals the nervous system consists of two parts – central and peripheral. The central nervous system contains the brain and spinal cord, while the peripheral nervous system consists of sensory neurons, clusters of neurons called ganglia, and nerves connecting them to each other and to the central nervous system. In this paper, we shall be focusing on the central nervous system, specifically, the control of the central nervous system over the reproductive mechanism in the animal.

Reproduction is a common phenomenon present throughout all animals, and it usually involves heterosexual courtship behavior that is central to the divergence and diversity of animal species and is of obvious adaptive significance (Drapeau, Cyran, Viering, Geyer & Long, 2006). In many species the basic program of courtship behavior is innate. These inborn, instinctual behaviors are likely to be the result of gene action during development that establishes the potential for behavior, given the appropriate external stimulation (Baker, Hall & Taylor, 2001). Keeping this in mind, this paper aims to focus on

the effects of the action of an important courtship gene, *yellow*, in the model organism *Drosophila*.

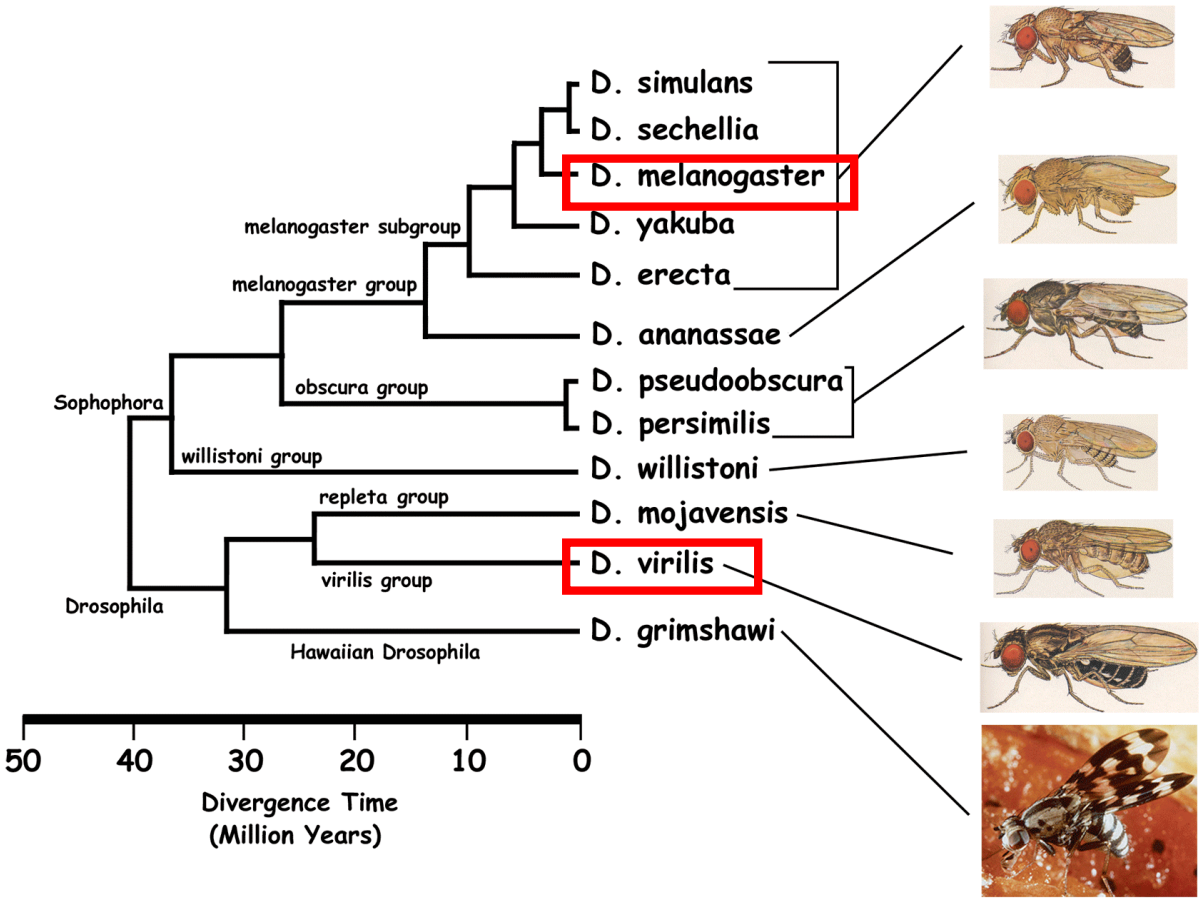


Fig. 1: Drosophila Phylogeny.

This phylogenetic tree indicates the divergence time between different *Drosophila* species. The boxed species shown are *D. melanogaster* and *D. virilis*, and by tracing the path between the two species, it is revealed that their divergence time is at least 40 million years and that they plausibly share the same ancestor before diverging into Sophophora and Drosophila (Russo, Takezaki, & Nei, 1995).

Drosophila is a genus of small flies and belongs to the family *Drosophilidae*. Its members are more commonly known as "fruit flies", which refer to the characteristic of many species to linger around overripe or rotting fruit. The entire genus contains more than 1,500 species and is very diverse in their appearances, behaviors, and breeding habitats (Bächli, 1999-2006). One particular species of *Drosophila*, *D. melanogaster*, has been widely used as a model organism in genetics as well as in evolutionary and developmental biology. In this study, *D. melanogaster* and another species, *D. virilis*, are used to investigate the differences in mating behavior caused by the orthologus *yellow* genes. *D. melanogaster* and *D. virilis* have diverged about 40 million years ago (Russo, Takezaki, & Nei, 1995) (Fig. 1). Despite the fact that the overall genotypic and phenotypic make-up of the two species has a lot of similarities, the long phylogenetic distance between them also suggests significant differences between the two. This makes them ideal subjects for comparing how orthologous *yellow* genes are expressed in the same trans environment and also how they affect male mating success.

In *D. melanogaster*, the *yellow* gene is located on the X chromosome and it is required for the production of melanin (Exploratorium, n.d.). Compared to wild type flies, *D. melanogaster yellow* mutant flies are unable to produce melanin,

which is a black pigment. Hence, in the abdomen, thorax, bristles and wings, *yellow* mutant flies look yellowish as compared to their wild type counterparts. Similarly, *yellow* mutants in other Drosophila species are also unable to produce this black pigment and appear yellowish (Exploratorium, n.d.). This suggests that the overall function of the *yellow* gene is most likely conserved amongst Drosophila species.

Yellow has also been proven to play a role in normal male courtship behavior (Drapeau, Cyran, Viering, Geyer & Long, 2006). Courtship behavior is central to the evolution of animal species, and has been widely studied in *D. melanogaster.* Indeed, *D. melanogaster* is one of the two species with the best-characterized molecular, genetic and cellular mechanisms underlying courtship behavior (Goodwin, 1999). In *D. melanogaster*, the male courtship ritual consists of six steps: orienting, following, horizontal wing extension, wing vibration, genital licking, and attempted copulation (Bastock & Manning, 1955; Bastock, 1967; Hall et al., 1982; Hall, 1994a; Yamamoto et al., 1997; Greenspan & Ferveur, 2000) (Fig. 2). During courtship, wild-type *D. melanogaste*r males "sing" to the females by extending their wings to an angle of 90° and vibrating them to generate a courtship "song" (Drapeau, Radovic, Wittkopp, & Long, 2003). This stimulates the females to become receptive to the males' mating advances. Past studies

have shown that mutations in *yellow* decrease male mating success. More specifically *yellow* mutant *D. melanogaster* males, have a ~50% reduction in normal wing extension levels, and hence a reduction in sound generation (Bastock, 1956; Burnet et al., 1973; Hall, 1994a). It is also known that courtship behavior is a rapidly evolving trait and there are many differences varying across species (Drapeau, Radovic, Wittkopp, & Long, 2003). One of my goals in this study is to measure the differences in male mating success between transgenic *D. melanogaster* flies harboring the native or *D. virilis yellow* transgenes.

Additionally, Yellow protein is shown to be expressed in the central nervous system of the third in-star *D. melanogaster* larvae, and this has been proven be correlated with the courtship behavior in adult male flies (Drapeau, Cyran, Viering, Geyer, & Long, 2006). With this in mind, I hypothesized that changes in *yellow* gene expression in the brains of different Drosophila species may correlate with the behavioral differences amongst those particular species. Past studies have shown that in *yellow* mutants of *D. melanogaster*, proper *yellow* expression can be restored by a wild type *yellow* gene from the same species (Drapeau, Radovic, Wittkopp, & Long, 2003). With that in mind, one of the purposes of this study is to test whether orthologous *yellow* genes from other Drosophila species can recapitulate *yellow* expression in *D. melanogaster yellow* mutant flies. In particular, I will look at the expression pattern in the brain driven

by *D. virilis yellow* transgene in *D. melanogaster yellow* mutant host flies and compare it to the native *yellow* expression pattern of wild-type *D. melanogaster* flies.

In order to compare the brain expressions of *yellow* from different Drosophila species, namely, *D. melanogster* and *D. virilis*, I used adult *D. melanogaster* flies harboring wild type *D. melanogaster yellow* gene versus wild type *D. virilis yellow* gene. Subsequently, I looked at the male mating success in each transgenic line using the Copulatron (Drapeau & Long, 2000) (Fig. 3). With the Copulatron assay, one can determine the amount of time taken for the female to be receptive to the male's mating advances.

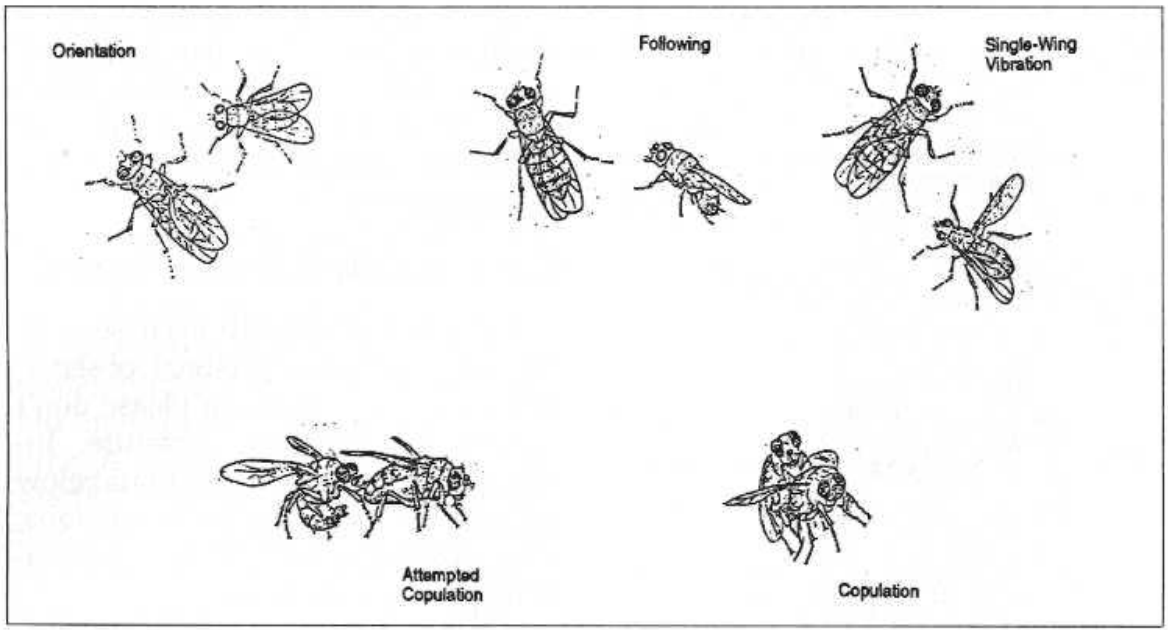


Fig 2. Drosophila Courtship Behavior

Drosophila courtship ritual showing four of six male fly behaviors, beginning from orienting, to following, horizontal wing extension and wing vibration, genital licking and attempted copulation, and finally copulation, which signals successful male courtship behavior.

An interesting point to note for this study is that it studies how Neurobiology and Evolutionary biology can complement each other perfectly to explain how the actions of a gene, in this case *yellow*, can affect the neurology and behavior of an organism. Also, how the evolutionary changes of the gene over time may contribute to the diversification of neurological expression, and thus behavior. With that said, this paper focuses on the important courtship gene, *yellow*, in the model organism *Drosophila*. The goals of this study are: (1) to test whether the orthologous *yellow* genes from *D. virilis* can recapitulate *yellow* expression in *D. melanogaster* mutant male flies; and (2) to find out whether there are any cis- or trans-regulatory differences between *D. melanogaster* versus *D. virilis yellow* genes. Two different *Drosophila* species, *D. melanogaster* and *D. virilis*, were used for this study. For both species, adult male flies were randomly selected for the various experiments. I compared the *yellow* expression patterns in the brain amongst wild type *D. melanogaster* flies harboring a *D. virilis yellow* mating success enhancer (MRS) – GFP transgene, wild type *D. melanogaster* flies harboring a *D. melanogaster yellow* mating success enhancer (MRS) – GFP transgene gene, wild type *D. melanogaster* flies, *D. melanogaster yellow* null mutants, and wild-type *D. melanogaster* harborig a “no enhancer” – GFP transgene. This was done through the processes of dissection, followed by

immunocyto-staining using anti-GFP and anti-Yellow antibodies and confocal microscopy. I also compared the time taken for successful male courtship between randomly selected *D. melanogaster yellow* mutant female flies and each of the following four strains of flies: *D. melanogaster yellow* mutants harboring a *D. virilis yellow* transgene, *D. melanogaster yellow* mutants harboring a *D. melanogaster yellow* transgene, wild type *D. melanogaster* flies, and *D. melanogaster yellow* null mutants. This was conducted using the Copulatron (Fig. 3), while observing by eye. The collected data was analyzed by statistical tests to compare the effects of D. melanogaster versus D. virilis yellow transgenes on mating behavior.

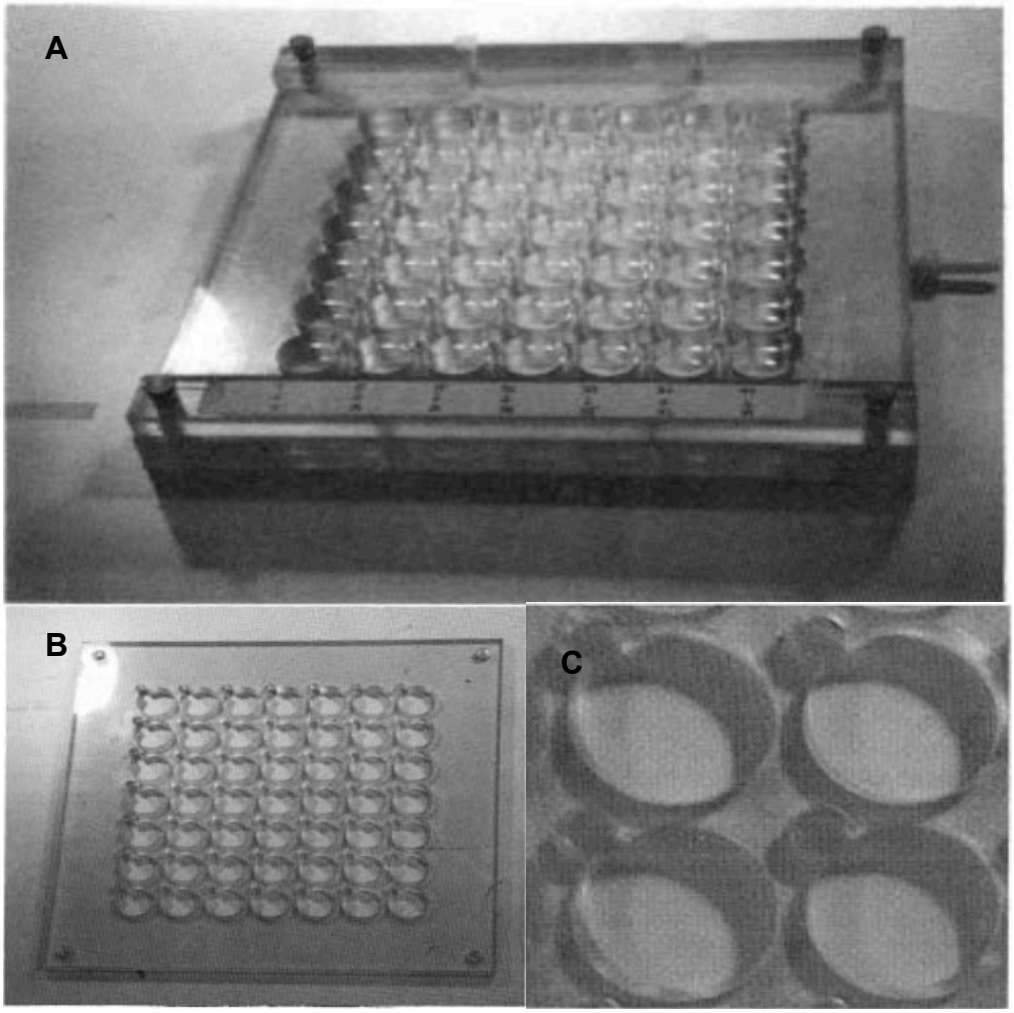


Fig 3. The Copulatron (Drapeau, & Long, 2000)

The Copulatron, measured 11” x 11” square (constructed by Drapeau, & Long, 2000). (A) The fully constructed Copulatron without the top plastic sheet covering. Carbon dioxide is passed from the tube as shown on the right side through the bottom of the set-up. (B) Top view of one of the two pieces of the mating chambers component of the Copulatron. Up to forty-nine pairs of flies can be observed in each experimental set-up. (C) Close-up view of four mating chambers; the four smaller antechambers are used to contain fly media.

METHODS & MATERIALS

Transgenic Fly Stocks and Crosses

For the courtship study, two of the transgenes were constructed by cloning either a *D. melanogaster* or a *D. virilis yellow* gene into a pre-constructed piggybac-attB-3xp3-GFP vector to create the desired piggybac-attB-*yellow*-3xp3-GFP vector, which is then injected into *white D. melanogaster* mutants by Genetic Services, Inc.

Following that, a new balancer chromosome fly line was constructed by crossing two other balancer lines (Lines 4888 X 7197). Both lines were obtained from Bloomington Stock Center. This new balancer line was then used to make the transgenes homozygous. It is important to note here that the fourth chromosome is not considered to contribute significantly to the genetic make-up of the flies in this study. With these in mind, fly line 4888 has the genotype $w^{-}y^{-}/w^{-}y^{-}$; Cyo/Bc[1]; +/Ubi-GFP and line 7197 has the genotype $w^{-}y^{+}$; Cyo/Kr; D^{1}/TM6B. Amongst the F_1 progeny of the 4888 x 7197 cross, the flies with the genotype $w^{-}y^{-}/w^{-}y^{+}$; Cyo/Bc[1]; D^{1}/Ubi-GFP were selected, using their curly-wing, red-eye and glowing-body phenotypes. The selected F_1 flies were then crossed to each other, and *yellow* mutant F_2 flies with the

genotype w^-y^-/ w^-y^-; Cyo/Bc[1]; D^1/Ubi-GFP were selected using their curly-wing, red-eye and glowing-body phenotypes (Fig. 4).

This new balancer line was then crossed to transgenic flies with different genotypes: (1) transgenic *D. melanogaster white* mutant flies harboring a *yellow* transgene from either *D. melanogaster* or *D. virilis* (w^-y^+; *yellow* transgene/+; +/+); (2) white mutant *D. melanogaster* flies without the transgene (w^-y^+; +/+; +/+). For the first cross, F_1 flies with the genotype w^-y^-/w^-y^+; Cyo/*yellow* transgene; Ubi-GFP/+ were selected and crossed to each other. F_2 male flies of the genotype w-y-; *yellow* transgene/*yellow* transgene; +/+ were selected by their glowing-eye phenotype, and these were used in the experiment measuring courtship behavior as described in the next section (Fig. 5). For the second cross, F1 flies with the genotype w^-y^-/w^-y^+; Cyo/+; Ubi-GFP were selected, and either backcrossed to the male parent fly to obtain a wild type fly (w^-y^+; +/+; +/+), or crossed to each other to produce a *yellow* mutant fly (w^-y^-; +/+; +/+), both of which were also used in the courtship experiment as controls for comparison with the courtship behavior of the transgenic flies (Fig. 6). It is important to point out now that at the later of part of this study, it was found that the transgenic flies contained an extra wild type *D. melanogaster yellow* gene, therefore I will take this into account during the analysis of the results at the later part of this paper.

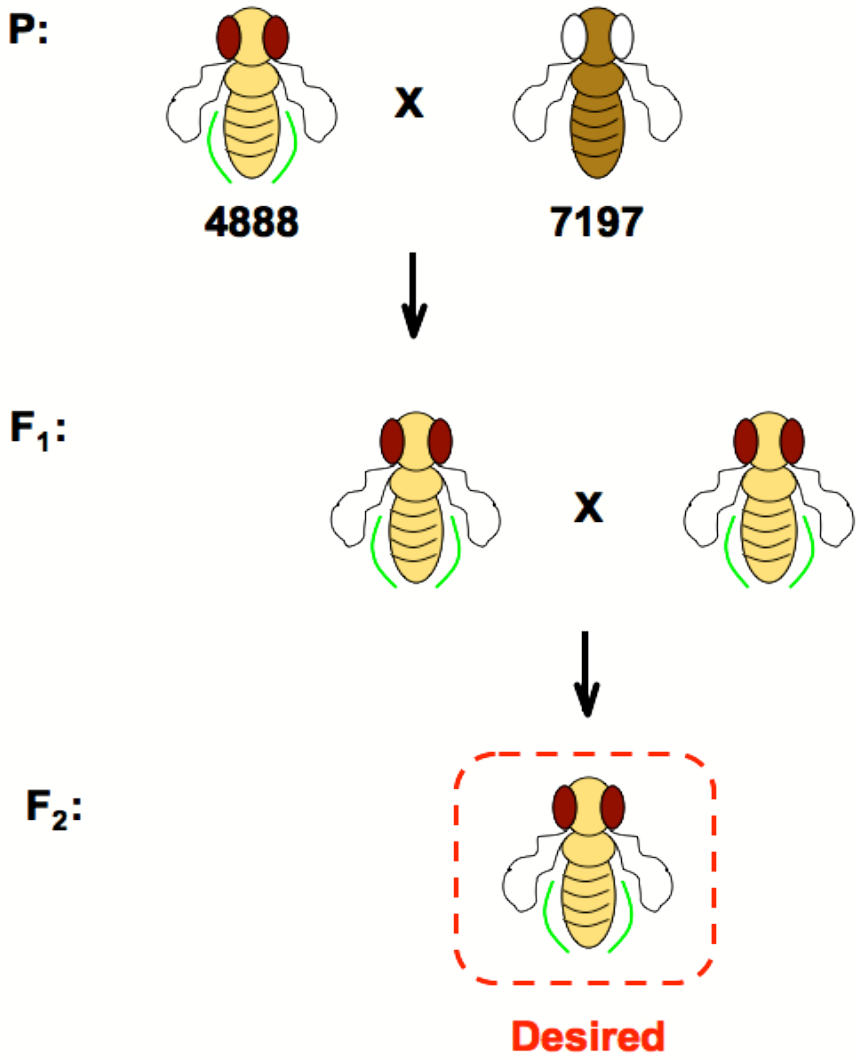


Fig 4. Fly Cross to Create Balancer Chromosome Line

The parental cross was between line 4888 female flies and line 7197 male flies. F_1 progeny with chromosomes $w^{-}y^{-}/w^{-}y^{+}$; Cyo/Bc[1]; D^{1}/Ubi-GFP were selected for. They were distinguished phenotypically by their curly wings, red eyes and glowing bodies. The selected F_1 flies were then crossed to each other, and *yellow* mutant F_2 flies with chromosomes $w^{-}y^{-}/ w^{-}y^{-}$; Cyo/Bc[1]; D^{1}/Ubi-GFP were chosen. They were also distinguished by their curly wings, red eyes and glowing bodies phenotypes. The selected F_2 flies were then used to cross with the transgenic fly lines.

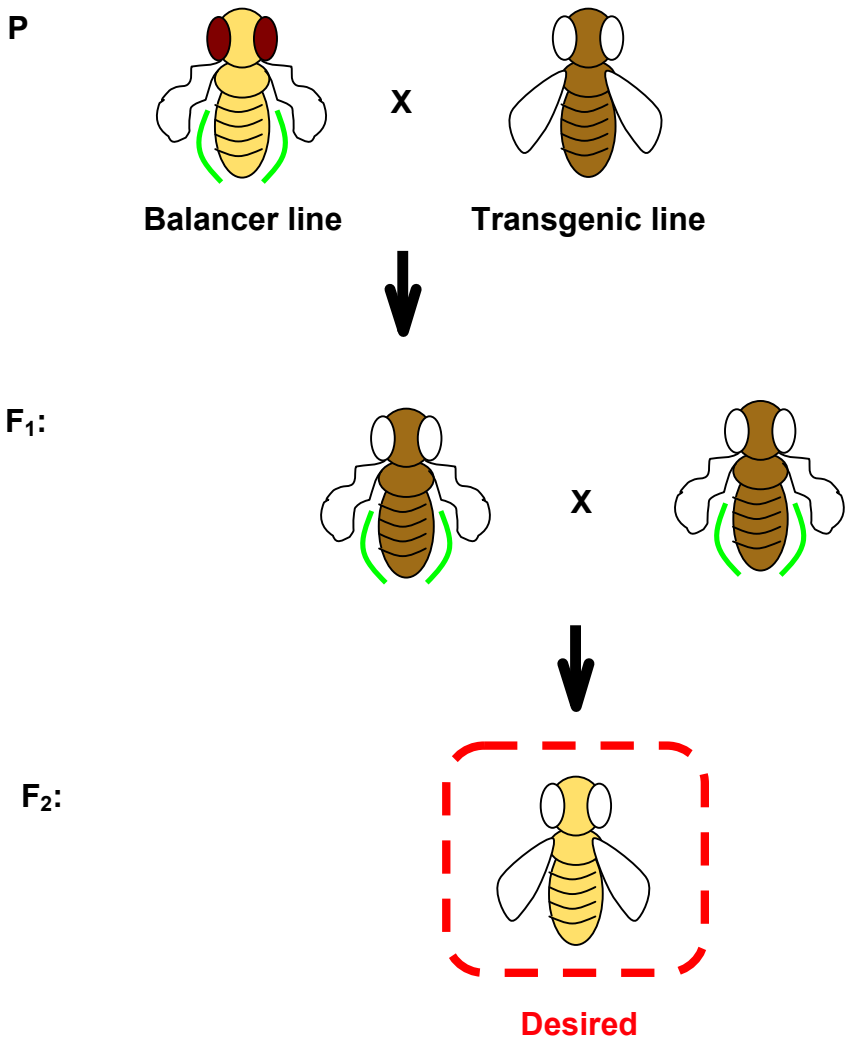


Fig 5. Fly Cross to create homozygous transgenic lines

The initial parental cross was between a female balancer fly (Fig. 4) and male transgenic fly. F_1 progeny with genotype $w^{-}y^{-}/w^{-}y^{+}$; Cyo/*yellow* transgene; Ubi-GFP/+ were selected and crossed to each other, yielding F_2 male flies of genotype w-y-; *yellow* transgene/*yellow* transgene; +/+. These were selected by their glowing eyes phenotype, and were used in the experiment measuring their courtship behavior.

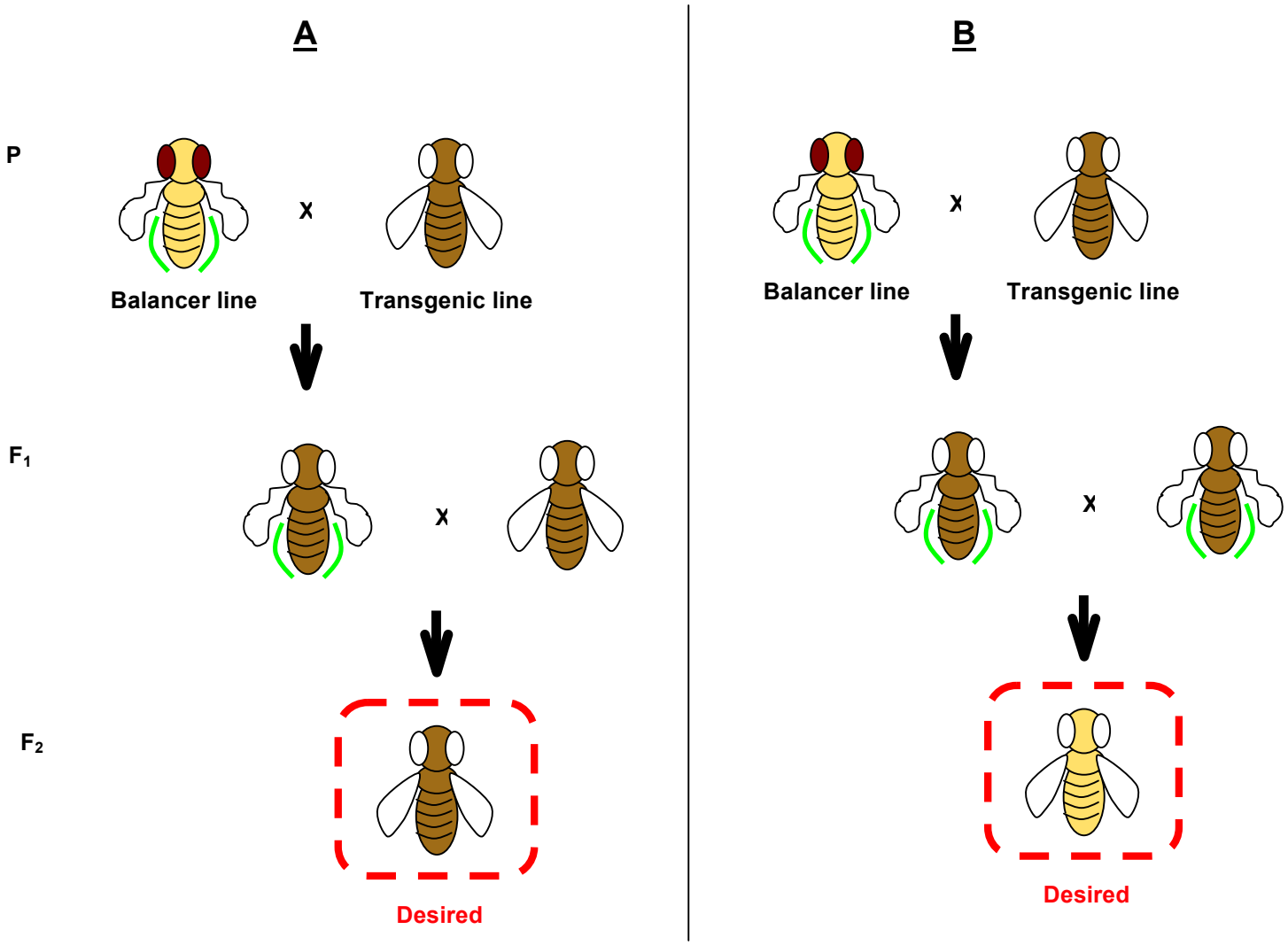


Fig 6. **Fly Cross to create homozygous wild type and *yellow* mutant flies**

The parental cross was between a female balancer fly (Fig. 4) and a wild type *D. melanogaster* male in which the transgens was not successfully injected. F1 flies with genotype $w^{-}y^{-}/w^{-}y^{+}$; Cyo/+; Ubi-GFP were selected, and either (A) backcrossed to the male parent fly to yield a wild type fly, or (B) crossed to each other to produce a *yellow* mutant.

In order to obtain the brain expression patterns of orthologous *yellow* genes, male flies randomly chosen from each of the transgenic lines harboring one of the following transgenes were used: “5’upstream of *D. melanogaster yellow* – nuclearGFP”, “5’upstream of *D. virilis yellow* – nuclearGFP”, and “nuclearGFP”. These transgenes were cloned into piggyBac-attB vector and injected into *w D. melanogaster* attp40 line. Injections and transformant screenings were done by Genetic Services, Inc.

Courtship Behavior

Behavior assays were performed using the Copulatron, a multi-chambered apparatus for observing large numbers of uncompetitive insect courtship behavior simultaneously (Drapeau and Long, 2000) (Fig. 3). Previously, Drapeau et al. has shown that correct wing extension in males has led to females becoming more receptive to courtship, and thus a shorter time taken for successful male courtship. Therefore, for this study, efficient courtship behavior was determined by the time taken for the male flies to successfully court and copulate with their female counterparts, measured from the time the plastic film separating the pair of flies was removed.

Flies were placed in the Copulatron using brief CO_2 anesthesia and a recovery time of 30 minutes before the start of an assay. Males and females

were separated by a piece of lightly oiled plastic, and fresh fly media was placed in a small adjoining chamber within each chamber for the flies to eat during this 30 minutes lag time, and at any other point during the assay. Single in-bred adult males 3 to 4 days post-eclosion (see previous section on fly stocks for details) were observed by eye in chambers with single adult in-bred *yellow* mutant females, also 3 to 4 days post-eclosion.

Four male genotypes were analyzed for this assay. In order to create these genotypes, virgin hybrid balancer female flies (see previous section for details on crossing of balancer lines 4888 and 7197 to obtain hybrid balancer females) were crossed to both transgenic and non-transgenic virgin male flies (see previous section for details of crosses) to obtain both target fly genotypes as well as control flies. Flies were crossed *en masse* in plastic food vials in groups of about 5 males and 5 females, and females were allowed to lay eggs.

The mating assay was performed twice a day – at 1pm and at 4pm – initially for a week to obtain a sample size of n=139 for each genotype tested. However, initial results showed that the mating efficiency of flies decreased tremendously at 4pm compared to at 1pm, thus the experiment was revised, and the assay was performed once a day at 1pm. Each assay had a time limit of one hour, and the indication for successful male courtship (in which the time taken was recorded) was when the male curled his abdomen and

attempted copulation without the female rejecting him by moving away. For each set of experiment, adult male and female flies were obtained from randomly selected bottles, to isolate any effects on behavior due to developmental or environmental conditions rather than genotype. The four different male genotypes tested were: *D. melanogaster yellow* mutants harboring a *D. virilis yellow* transgene, *D. melanogaster yellow* mutants harboring a *D. melanogaster yellow* transgene, wild type *D. melanogaster* flies, and *D. melanogaster yellow* null mutants. 12 to 13 males of each genotype were tested for their male courtship efficiency in each assay, giving a total of 139 individuals for each genotype at the end of about 2 weeks.

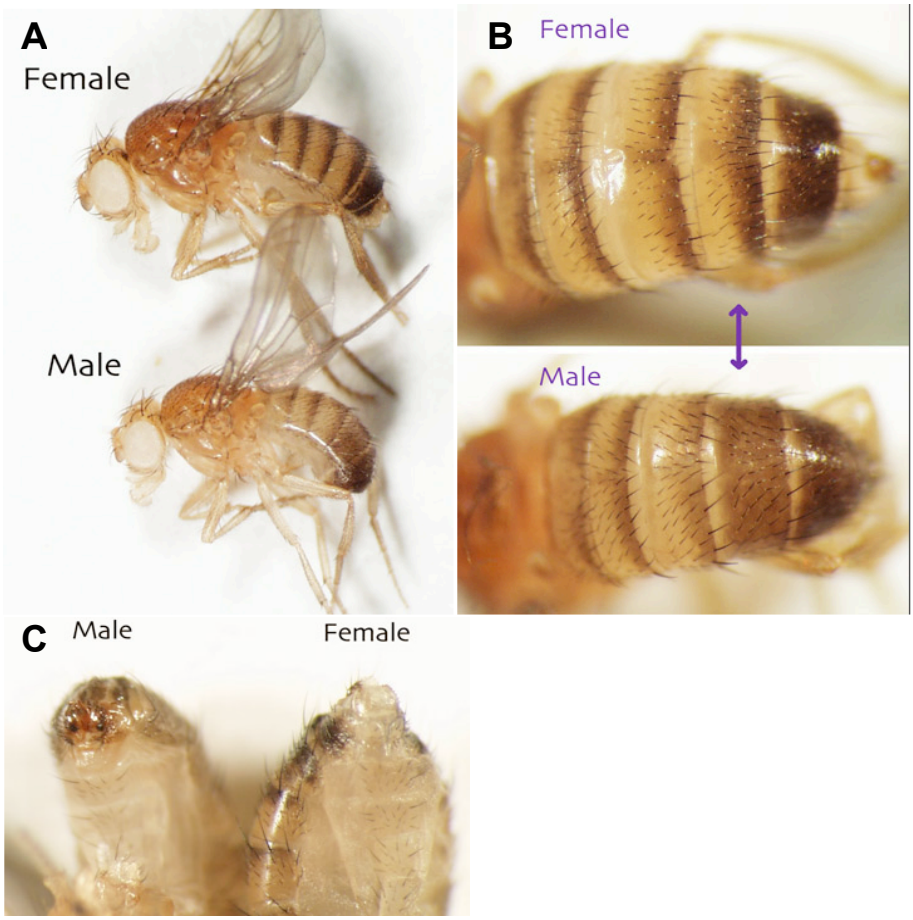


Fig 7. Drosophila Sexing

(A) Female is generally larger than the male; (B) The male's A5 segment is pigmented in the anterior half, and female's A5 is not (arrows); (C) Genitalia (ventral view, posterior is up). Note the circle of darkly pigmented parts in the male. In contrast, the tip of the female's abdomen is lightly colored and pointed (Edwards, n.d.).

Adult Dissection and Immunocytochemistry

Adult flies were sexed based on the presence of male genitialia as they are the easiest and most reliable character to use in determining sex, and males were selected for dissection (Fig. 7). This is because the target was to determine the effects of *yellow* on the efficiency of courtship behavior, thus males were selected for this imaging study to determine their brain expression patterns of the *yellow* gene. The transgenic flies contain cytoplasmic 3xp3-GFP inserted in their genome as the transgenic marker, which is expressed in the eye related neuron cells in the brain (Horn, Jaunich, & Wimmer, 2000). This blocks the visualization of nuclear-GFP, which was the reporter of *yellow* expression in the brain. Hence, in the later part of the study, wild type and *yellow* null mutant *D. melanogaster* flies were also dissected and assayed using anti-Yellow antibody to show the areas in which *yellow* is expressed in the brain. Subsequently, these images were used to compare with that of the transgenic flies to see the brain expression patterns of *yellow* in these flies.

The adult male fly brains were dissected in Ringer's solution and processed by a modified immunocytochemistry protocol from Dr Orie Shafer (Appendix A). For the wild type and *yellow* mutant *D. melanogaster* flies, the primary antibody cocktail contains Rat anti-Yellow (1:50; made by Dr Trisha Wittkopp), Mouse anti-Synorf 1 (1:10; obtained from Invitrogen) in Phosphate

Buffered Saline with added Triton X-100 (PBS-T) to make a total of 30μl of primary antibody cocktail. The secondary antibody cocktail contains Alexa 488 Goat anti-Rat (1:1000; obtained from Invitrogen) and Alexa 568 Goat anti-Mouse (1:1000; obtained from Invitrogen) in PBS-T to make a total volume of 100μl. For the transgenic flies, the primary antibody cocktail contains Rabbit anti-GFP (1:500; obtained from Invitrogen) and Mouse Elav-9F8A9 antibody (1:100; obtained from Invitrogen) in PBS-T to make a total volume of 1000μl. Similar to the other set of dissections, the secondary antibody cocktail contains Alexa 488 Goat anti-Rabbit (1:1000; obtained from Invitrogen) and Alexa 568 Conjugated Goat anti-Mouse (1:1000; obtained from Invitrogen) in PBS-T to make a total volume of 100μl.

The imaging was done using confocal microscopy (Olympus Fluoview FV 1000, model number BX61WI) from Dr Orie Shafer's laboratory. The magnification used for imaging the wild type flies, *yellow* mutant *D. melanogaster* flies and transgenic flies are UPlanSApo 20X (numerical aperture 0.75). The program used for imaging is Fluoview, and the intensity of the images was adjusted using the lookup tables. The images will be presented in the results section, and further discussed in the discussion section.

Data Collection and Analysis

In this study, the only factor subjected for comparison was the type of *yellow* gene or *yellow* enhancers harbored by the *D. melanogaster yellow* mutants (*D. melanogaster* vs. *D. virilis*). For the courtship behavior experiment, successful male courtship was defined as the time taken from when the plastic sheet separating the pair of flies was removed, to the time when the male copulates with the female. This courtship behavior was observed and determined by the naked eye, and all time values were measured using a Traceable® Big Digit 4-Channel Timer. The time limit to still be considered successful male courtship was 60 minutes, and the time values collected for successful male courtship were measured to the nearest second. For males that managed to court the female at and after the 60-minute limit, or those that did not manage to court the females at all were considered unsuccessful. The relationship between *D. melanogaster yellow* mutants harboring *yellow* transgene from *D. melanogaster* and that from *D. virilis* were analyzed using One-Way Analysis of Variance (ANOVA) test.

For the brain expression patterns experiment, the dissected brains were imaged using Olympus Fluoview FV 1000 (model number BX61WI) confocal microscope under UPlanSApo 20X (numerical aperture 0.75)

magnification, and were viewed on Fluoview in Dr Orie Shafer's laboratory, and the intensity of the images was adjusted using the lookup tables. It was initially planned that the targeted brain areas – those that show *yellow* expression – will be enlarged and imaged at a higher resolution so that the neurons expressing *yellow* can be pinpointed. However, it was later found that the laboratory fly stocks used contain a cytoplasmic 3xp3-GFP that is expressed in the eyes, implying that the whole brain will express GFP, most of which are due to 3xp3 than to *yellow* MRS enhancer, hence the dissected brains were not imaged under high resolution. I will be further discussing this in the results and discussion sections in the later part of this paper.

RESULTS

Measure of Successful Male Courtship

Firstly, it is important to note here that the various abbreviations are applied to the different *D. melanogaster* lines used in this study: *D. melanogaster yellow* mutants harboring a wild type *D. virilis yellow* gene (abbreviated DV trans), *D. melanogaster yellow* mutants harboring a wild type *D. melanogaster yellow* gene (abbreviated DM trans), wild type *D. melanogaster* flies (abbreviated DM y+), and *D. melanogaster yellow* null mutants (abbreviated DM y-). By looking at the scatter plot (Fig. 8), the trend shows that a majority of *D. melanogaster yellow* mutants harboring a wild tyoe *D. melanogaster yellow* gene successfully courted the females within 15 minutes of starting the experiment. This is consistent with past literature that *yellow* plays a role in normal male courtship behavior, and its presence restores the normal function of male courtship behavior in a *yellow* mutant. For *D. melanogaster yellow* mutants harboring a wild type *D. virilis yellow* gene, majority successfully courted the females within 30 minutes of starting the experiment, which may either imply that the *D. virilis yellow* gene plays a role in restoring the normal function of male courtship behavior in a *D.*

melanogaster yellow mutant, or it might also be due to the extra copy of wild type *D. melanogaster yellow* present in both the transgenic flies. Wild type *D. melanogaster* flies and *D. melanogaster yellow* mutants appear to have a wider spread in the time taken to achieve successful courtship, however, there is a significant number of wild type *D. melanogaster* flies that managed to successfully court the females within 15 minutes from the start of the experiment. The implications of this finding will be further discussed in the following paragraphs of this section.

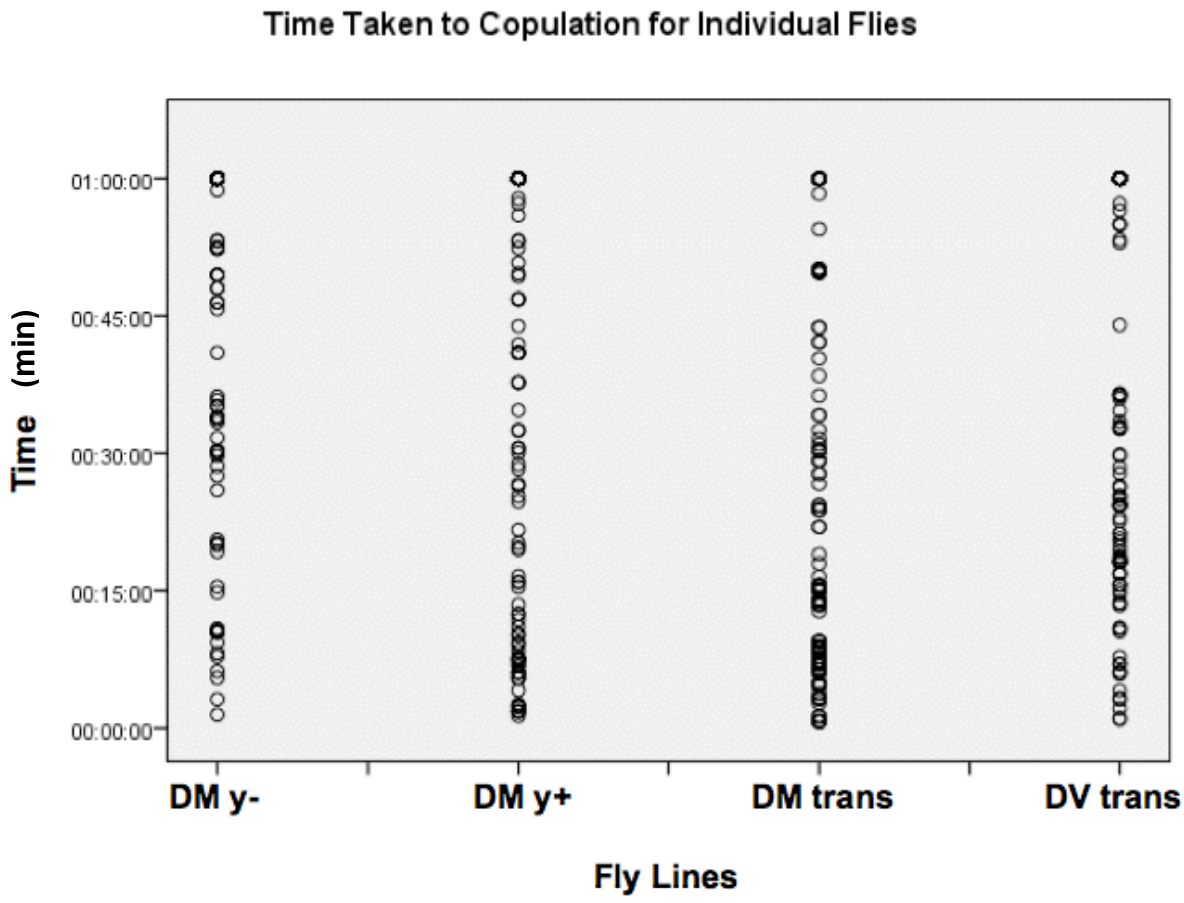


Fig 8. Scatter Plot Showing Individual Time Taken to Copulation

It is very clear that a majority of *D. melanogaster yellow* mutants harboring *D. melanogaster yellow* transgene take between 0 – 15 min to copulate. For *D. melanogaster yellow* mutants harboring *D. virilis yellow* transgene, the mode is set between 15 – 30 min. Wild type *D. melanogaster* flies and *D. melanogaster yellow* mutants give larger variation in the time taken to copulation, however, wild type *D. melanogaster* flies appear to achieve copulation earlier in the 0 – 15min time range, but is not significant enough to make it significantly different from that of *D. melanogaster yellow* mutants harboring *D. virilis yellow* transgene (Fig 11).

The general trend in Fig. 8 can also be seen in the bar chart depicting the mean time taken for a fly line to achieve successful male courtship (Fig. 9). *D. melanogaster yellow* mutants harboring *D. melanogaster yellow* gene took the shortest mean time of 27 minutes, compared to *D. melanogaster yellow* mutants, which took the longest mean time of 49 minutes. As mentioned earlier, this supports past findings that *yellow* restores defective male courtship behavior to its normal levels. In addition, the mean time taken for *D. melanogaster* wild type flies and *D. melanogaster yellow* mutants harboring *D. virilis yellow* gene are about the same. However, the time taken for copulation by wild type *D. melanogaster* flies (σ = 22 min 41 s) vary more than *D. melanogaster yellow* mutants harboring *D. virilis yellow* gene (σ = 20 min 57 s). This is supported by Fig. 10 and Fig. 11, in which the percentage of successful male courtship is higher in *D. melanogaster yellow* mutants harboring *D. virilis yellow* gene than in *D. melanogaster* wild type flies (59.29% DV trans versus 54.29% DM y+ for within individual fly lines, and 26% DV trans versus 24% DM y+ for between the fly lines).

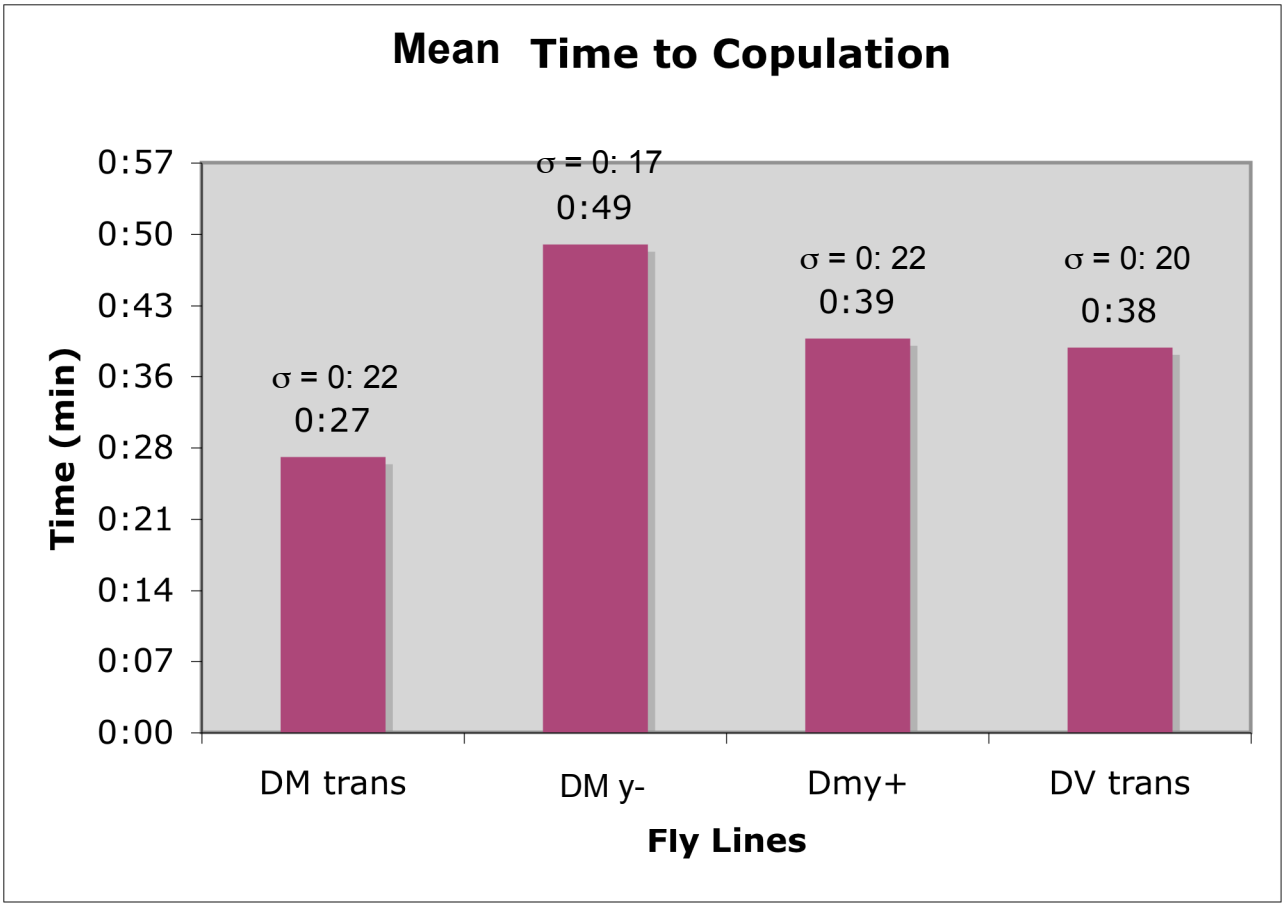


Fig 9. Bar Chart Showing Mean Time to Copulation

The shortest mean time taken to copulation are *D. melanogaster yellow* mutants harboring *D. melanogaster yellow* transgene (27 min), suggesting that *yellow* indeed restores normal courtship function of *yellow* mutants. On the other hand, *D. melanogaster yellow* mutants take the longest mean time to achieve copulation (49 min), supporting *yellow* as a gene regulating courtship behavior. The mean time taken for *D. melanogaster* wild type flies and *D. melanogaster yellow* mutants harboring *D. virilis yellow* transgene are almost the same, implying that *yellow* is not conserved throughout all *Drosophila* species, and that plausibly presence of the specific *yellow* gene is able to restore the courtship behavior of a specific fly line to normal levels.

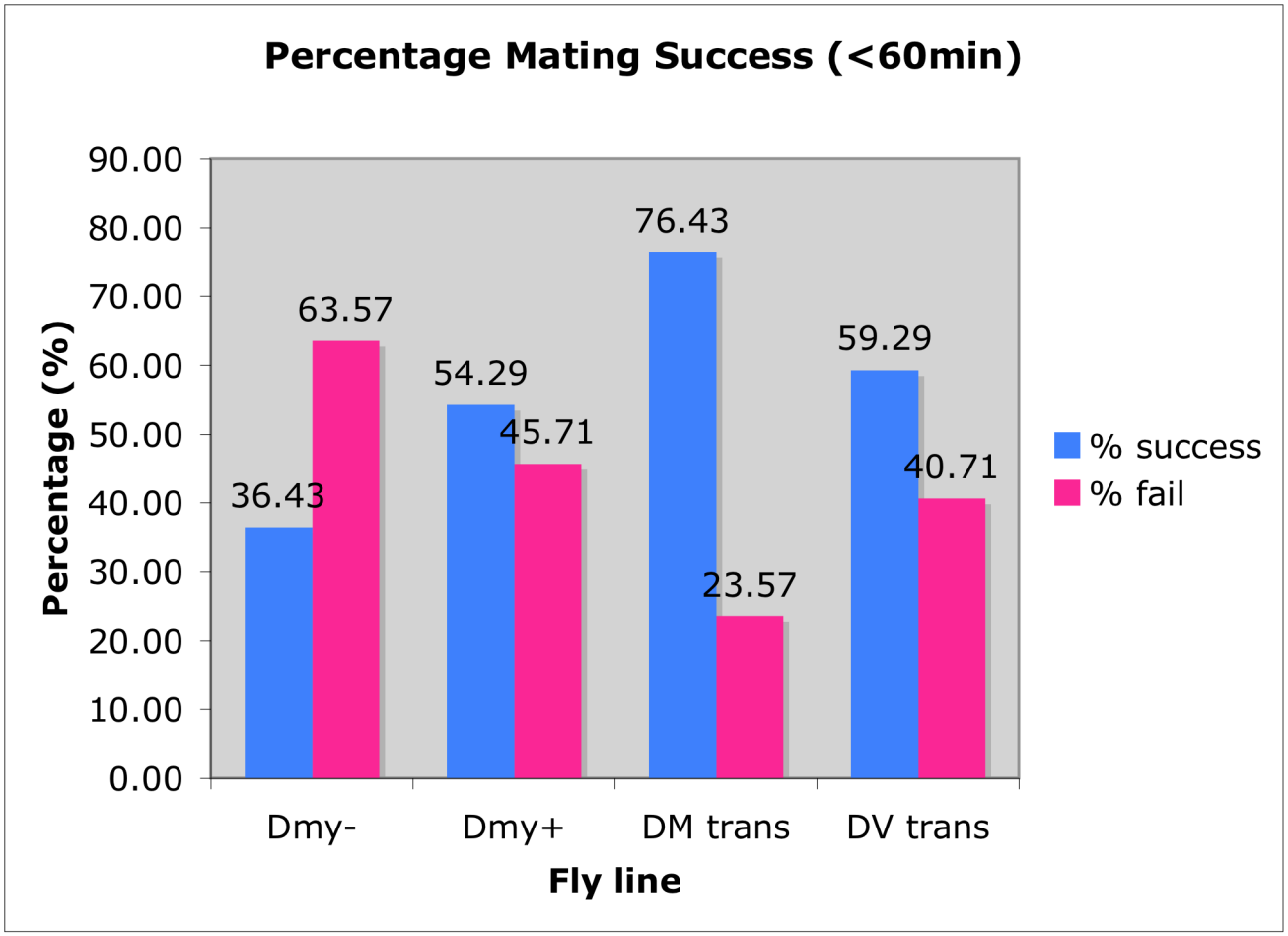


Fig 10. Bar Chart Showing Percentage Mating Success and Failure Within the Same Fly Line

Successful male courtship is most prevalent in *D. melanogaster yellow* mutants harboring *D. melanogaster yellow* transgene (76.43% success versus 23.57% failure), while the least successful is *D. melanogaster yellow* mutants (36.43% success versus 63.57% failure). For *D. melanogaster yellow* mutants harboring *D. virilis yellow* transgene and *D. melanogaster* wild type flies, the success and failure rates between the two gr oups are quite similar, but it can be seen that *D. melanogaster yellow* mutants harboring *D. virilis yellow* transgene have a higher success rate (59.29% versus 54.29% for *D. melanogaster* wild type flies).

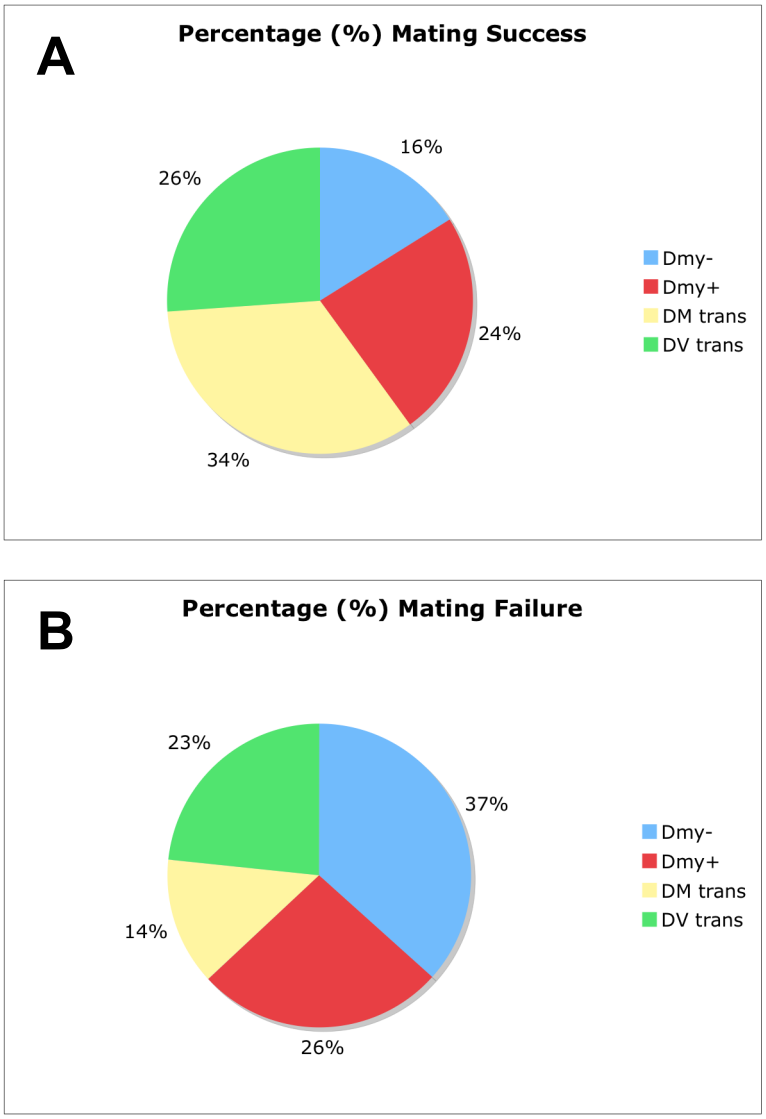


Fig 11. Pie Chart Showing Percentage Mating Success and Failure Between Different Fly Lines

(A) Percentage mating success chart. The highest percentage mating success is *D. melanogaster yellow* mutant haboring *D. melanogaster yellow* transgene (34%). Next is *D. melanogaster yellow* mutant haboring *D. virilis yellow* transgene (26%), followed closely by wild type *D. melanogaster* flies (24%) and lastly, *D. melanogaster yellow* mutants (16%); (B) Percentage mating failure chart. It is a reciprocal of (A). Highest failure is *D. melanogaster yellow* mutants (37%), followed by wild type *D. melanogaster* flies (26%), *D. melanogaster yellow* mutant haboring *D. virilis yellow* transgene (23%), and *D. melanogaster yellow* mutant

Statistical analysis was also carried out, and both the Kolmogorov–Smirnov and Shapiro-Wilk normality tests gave a significance of 0.000 for all four fly lines (Fig 12). This implies that the null hypothesis is rejected ($p<0.05$), and that data collected from all the fly lines do not follow a normal distribution. Despite that, a one-way Analysis of Variance (ANOVA) test was chosen as it is best suited to test the significance of the differences between the four lines (Fig. 13) in this case. Post-hoc Bonferroni and Games-Howell correction were used, with Games-Howell chosen to support Bonferroni correction as the variance was different for each of the four lines. Results from the ANOVA test show that the time to copulation for each line is significantly different from each other ($p< 0.05$) with the exception of the difference between *D. melanogaster yellow* mutant harboring *D. virilis yellow* transgene and wild type *D. melanogaster* flies ($p= 1.000$ for Bonferroni post-hoc and $p=0.982$ for Games-Howell post-hoc, both values of $p>0.05$). This coincides with the results in Fig. 9, which shows that the average time taken by *D. melanogaster yellow* mutant harboring *D. virilis yellow* transgene and wild type *D. melanogaster* flies are about the same.

Descriptive Statistics

	N	Range	Minimum	Maximum	Mean		Std. Deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
DMy1	139	0:58:31.000	0:01:29.000	1:00:00.000	0:49:22.806	0:01:28.702	0:17:25.776	1093647.737
DMy-	139	0:58:40.000	0:01:20.000	1:00:00.000	0:39:53.460	0:01:55.519	0:22:41.954	1854917.714
DMy+								
DMtrans	139	0:59:17.000	0:00:43.000	1:00:00.000	0:27:54.626	0:01:52.777	0:22:09.625	1767902.743
DVtrans	139	0:59:01.000	0:00:59.000	1:00:00.000	0:38:54.669	0:01:46.669	0:20:57.607	1581575.368
Valid N (listwise)	139							

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
DMy1	139	100.0%	0	.0%	139	100.0%
DMwt	139	100.0%	0	.0%	139	100.0%
DVtrans	139	100.0%	0	.0%	139	100.0%
DMtrans	139	100.0%	0	.0%	139	100.0%

Descriptives

			Statistic	Std. Error
DMy1	Mean		0:49:22.806	0:01:28.702
	95% Confidence Interval for Mean	Lower Bound	0:46:27.416	
		Upper Bound	0:52:18.196	
	5% Trimmed Mean		0:51:12.136	
	Median		1:00:00.000	
	Variance		1093647.737	
	Std. Deviation		0:17:25.776	
	Minimum		0:01:29.000	
	Maximum		1:00:00.000	
	Range		0:58:31.000	
	Interquartile Range		0:19:00.000	
	Skewness		-1.438	.206
	Kurtosis		.650	.408
DMwt	Mean		0:39:53.460	0:01:55.519
	95% Confidence Interval for Mean	Lower Bound	0:36:05.044	
		Upper Bound	0:43:41.877	

Descriptives (Continued from previous page)

			Statistic	Std. Error
	5% Trimmed Mean		0:40:53.008	
	Median		0:52:26.000	
	Variance		1854917.714	
	Std. Deviation		0:22:41.954	
	Minimum		0:01:20.000	
	Maximum		1:00:00.000	
	Range		0:58:40.000	
	Interquartile Range		0:44:36.000	
	Skewness		-.534	.206
	Kurtosis		-1.431	.408
DVtrans	Mean		0:38:54.669	0:01:46.669
	95% Confidence Interval for Mean	Lower Bound	0:35:23.752	
		Upper Bound	0:42:25.586	
	5% Trimmed Mean		0:39:44.251	
	Median		0:36:24.000	
	Variance		1581575.368	
	Std. Deviation		0:20:57.607	
	Minimum		0:00:59.000	
	Maximum		1:00:00.000	
	Range		0:59:01.000	
	Interquartile Range		0:41:21.000	
	Skewness		-.234	.206
	Kurtosis		-1.584	.408
DMtrans	Mean		0:27:54.626	0:01:52.777
	95% Confidence Interval for Mean	Lower Bound	0:24:11.631	
		Upper Bound	0:31:37.621	
	5% Trimmed Mean		0:27:38.181	
	Median		0:22:00.000	
	Variance		1767902.743	
	Std. Deviation		0:22:09.625	
	Minimum		0:00:43.000	
	Maximum		1:00:00.000	
	Range		0:59:17.000	
	Interquartile Range		0:46:10.000	
	Skewness		.416	.206
	Kurtosis		-1.445	.408

Extreme Values

			Case Number	Value
DM y-	Highest	1	51	1:00:00.000
		2	52	1:00:00.000
		3	53	1:00:00.000
		4	54	1:00:00.000
		5	55	1:00:00.000[a]
	Lowest	1	1	0:01:29.000
		2	2	0:03:11.000
		3	3	0:05:30.000
		4	4	0:06:14.000
		5	5	0:07:52.000
DM y+	Highest	1	76	1:00:00.000
		2	77	1:00:00.000
		3	78	1:00:00.000
		4	79	1:00:00.000
		5	80	1:00:00.000[a]
	Lowest	1	1	0:01:20.000
		2	2	0:01:47.000
		3	5	0:01:52.000
		4	4	0:01:52.000
		5	3	0:01:52.000
DVtrans	Highest	1	82	1:00:00.000
		2	83	1:00:00.000
		3	84	1:00:00.000
		4	85	1:00:00.000
		5	86	1:00:00.000[a]
	Lowest	1	1	0:00:59.000
		2	2	0:01:07.000
		3	3	0:02:09.000
		4	4	0:03:09.000
		5	5	0:03:20.000
DMtrans	Highest	1	107	1:00:00.000
		2	108	1:00:00.000
		3	109	1:00:00.000
		4	110	1:00:00.000
		5	111	1:00:00.000[a]
	Lowest	1	2	0:00:43.000
		2	1	0:00:43.000
		3	3	0:00:45.000
		4	6	0:00:46.000
		5	5	0:00:46.000[b]

a. Only a partial list of cases with the value 1:00:00 are shown in the table of upper extremes.

b. Only a partial list of cases with the value 0:00:46 are shown in the table of lower extremes.

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
DM y-	.376	139	.000	.658	139	.000
DM y+	.273	139	.000	.779	139	.000
DVtrans	.267	139	.000	.815	139	.000
DMtrans	.186	139	.000	.843	139	.000

a. Lilliefors Significance Correction

Fig 12. Descriptive Statistics Showing Mean, Standard Deviation, Variance, and Tests of Normality

Both tests of normality show a significance of 0.000 for all fly lines. As p-values are less than 0.05, null hypothesis is rejected and that the data collected from the four fly lines do not follow a normal distribution.

One-Way ANOVA

Test of Homogeneity of Variances

Time

Levene Statistic	df1	df2	Sig.
14.802	3	552	.000

ANOVA

Time

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.159E8	3	38618296.232	24.527	.000
Within Groups	8.691E8	552	1574510.891		
Total	9.850E8	555			

Multiple Comparisons

Dependent Variable:Time

	(I) Species	(J) Species	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Bonferroni	1	2	0:09:29.345*	0:02:30.515	.001	0:02:50.811	0:16:07.880
		3	0:21:28.180*	0:02:30.515	.000	0:14:49.645	0:28:06.714
		4	0:10:28.137*	0:02:30.515	.000	0:03:49.602	0:17:06.671
	2	1	-0:09:29.345*	0:02:30.515	.001	-0:16:07.880	-0:02:50.811
		3	0:11:58.835*	0:02:30.515	.000	0:05:20.300	0:18:37.369
		4	0:00:58.791	0:02:30.515	1.000	-0:05:39.743	0:07:37.326
	3	1	-0:21:28.180*	0:02:30.515	.000	-0:28:06.714	-0:14:49.645
		2	-0:11:58.835*	0:02:30.515	.000	-0:18:37.369	-0:05:20.300
		4	-0:11:00.043*	0:02:30.515	.000	-0:17:38.578	-0:04:21.509
	4	1	-0:10:28.137*	0:02:30.515	.000	-0:17:06.671	-0:03:49.602
		2	-0:00:58.791	0:02:30.515	1.000	-0:07:37.326	0:05:39.743
		3	0:11:00.043*	0:02:30.515	.000	0:04:21.509	0:17:38.578
Games-Howell	1	2	0:09:29.345*	0:02:25.646	.001	0:03:12.734	0:15:45.957
		3	0:21:28.180*	0:02:23.481	.000	0:15:17.192	0:27:39.167
		4	0:10:28.137*	0:02:18.731	.000	0:04:29.479	0:16:26.794
	2	1	-0:09:29.345*	0:02:25.646	.001	-0:15:45.957	-0:03:12.734
		3	0:11:58.835*	0:02:41.442	.000	0:05:01.546	0:18:56.123
		4	0:00:58.791	0:02:37.235	.982	-0:05:47.639	0:07:45.221
	3	1	-0:21:28.180*	0:02:23.481	.000	-0:27:39.167	-0:15:17.192
		2	-0:11:58.835*	0:02:41.442	.000	-0:18:56.123	-0:05:01.546
		4	-0:11:00.043*	0:02:35.232	.000	-0:17:41.287	-0:04:18.800
	4	1	-0:10:28.137*	0:02:18.731	.000	-0:16:26.794	-0:04:29.479
		2	-0:00:58.791	0:02:37.235	.982	-0:07:45.221	0:05:47.639
		3	0:11:00.043*	0:02:35.232	.000	0:04:18.800	0:17:41.287

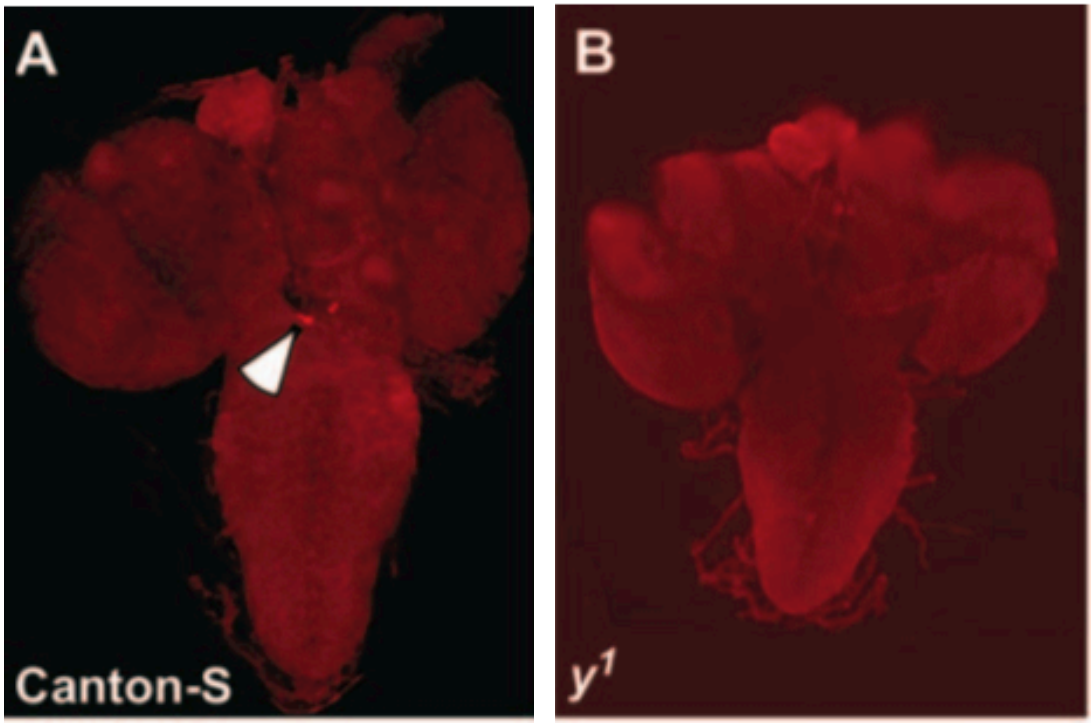
*. The mean difference is significant at the 0.05 level.

Fig 13. One-way Analysis of Variance (ANOVA) Test, generated using SPSS.

Legend: 1 – DM y-; 2 – DM y+; 3 – DM trans; 4 – DV trans

As of now, results from the imaging experiments yielded no significant differences between the brains of adult male *D. melanogaster* transgenic flies with *D. melanogaster yellow* 5' upstream enhancer and *D. melanogaster* transgenic flies with *D. virilis yellow* 5' upstream enhancer. Fig. 14, taken from Drapeau et al. (2006, their Figure 5) showed the presence of Yellow expression in third in-star larval central nervous system, and this is proven to affect adult male courtship behavior. However, no Yellow expression was found in the male adult central nervous systems of wild type *D. melanogaster* flies, *D. melanogaster yellow* mutants, and *D. melanogaster yellow* mutants with no enhancer (Fig. 15). In all three images, Rat anti-Yellow was used as the primary antibody (1:50; made by Dr Trisha Wittkopp). Due to the lack of results as shown in Fig. 15, it was implausible to focus at high resolution on likely areas of Yellow expression in the brains of adult male *D. melanogaster* transgenic flies with *D. melanogaster yellow* 5' upstream enhancer and *D. melanogaster* transgenic flies with *D. virilis yellow* 5' upstream enhancer to determine Yellow expression. A cytoplasmic 3xp3-GFP marker for the transgenic flies interferes with any possible nucleic Yellow 5' upstream enhancer-GFP expression in the brain, as shown in Fig. 16. The green

fluorescence indicates the binding of Rabbit anti-GFP to the GFP proteins. 3xp3-GFP is cytoplasmic and is expressed in the eyes, which translates to being expressed in the optic lobes which is likely to further extend its expression throughout the whole brain due to the vast neuronal connections made from the optic lobes. This interferes with the expression of *yellow*,-enhancer-nuclearGFP transgene which is only expressed in the nucleus of the neurons as compared to the cytoplasmic expression driven by 3xp3.



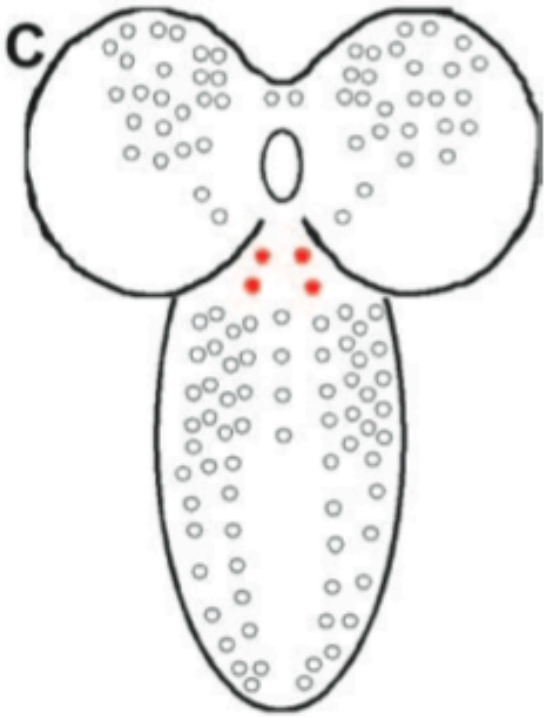


Fig 14. Presence of Yellow protein in third in-star central nervous system (CNS) neural cells (Drapeau et al., 2006, Figure 5)

(A) Canton-S wild type positive control CNS, showing anti-Yellow staining in four CNS cells; (B) The y1 loss-of-function negative control CNS, showing no anti-Yellow staining; (C) Schematic of the third in-star CNS, after Truman et al. (1993, their Figure 7), showing approximate locations of dividing neuroblasts at this developmental stage. The Yellow cells may be the four neuroblasts in red (see also Drapeau et al. 2003).

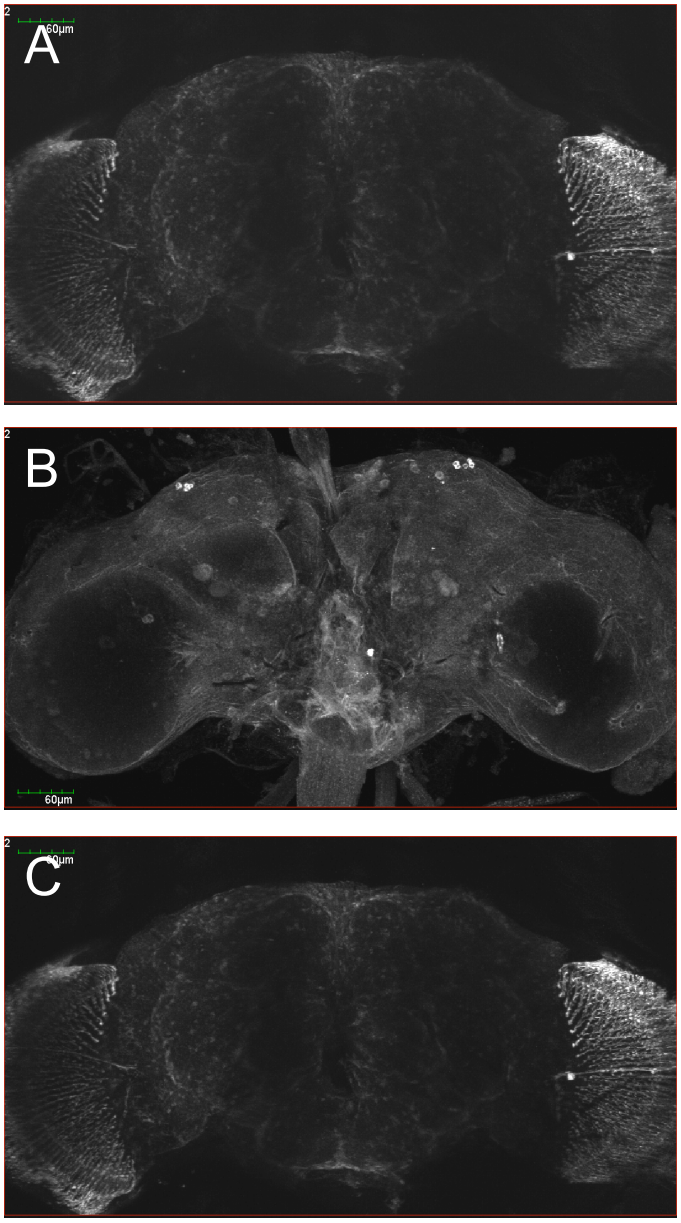


Fig 15. Adult control male central nervous system

(A) *Canton S*; (B) *D. melanogaster yellow* null mutant; (C) *D. melanogaster* with no enhancer-GFP. Stained with Rat anti-Yellow and anti-Synorf 1 primary antibodies. These appear to have no significant difference among them, suggesting that the there might not be any *yellow* expression, or the possibility that the primary antibody Rat α-Yellow may have degraded over the years.

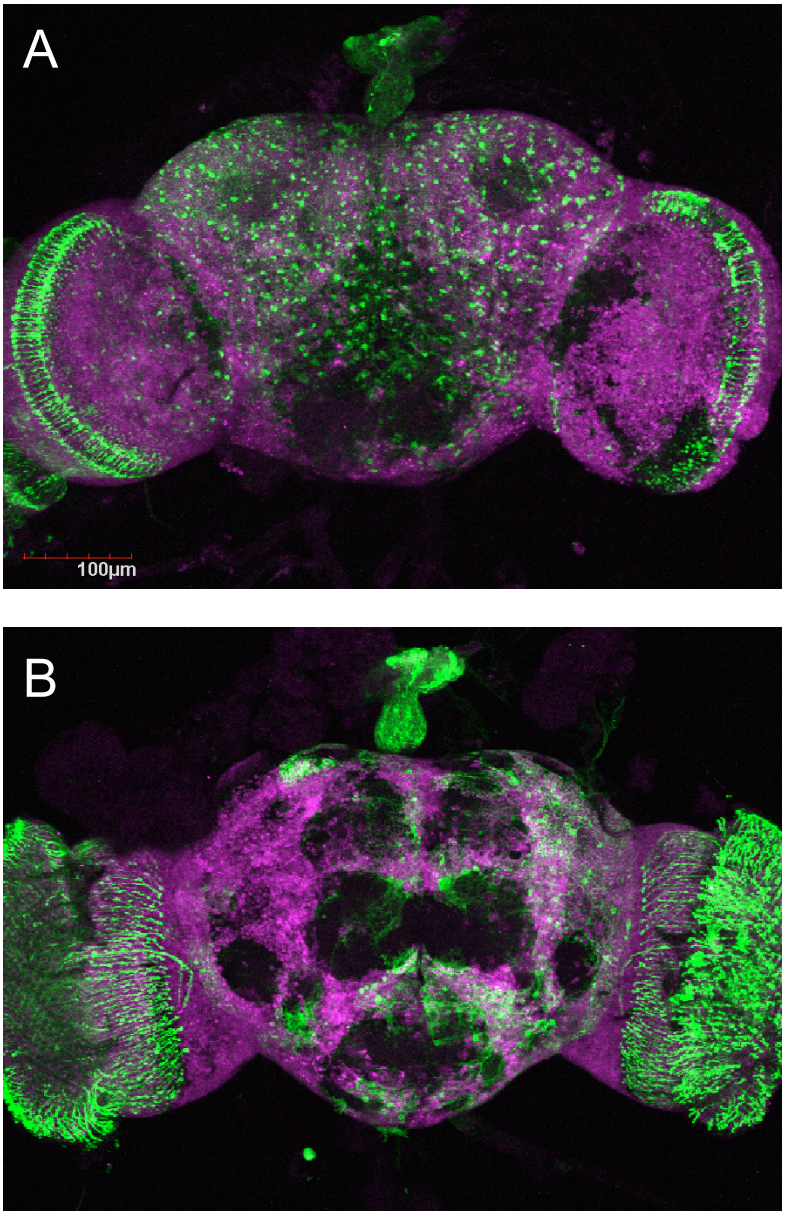


Fig 16. Adult transgenic male central nervous system

(A) Transgenic *D. melanogaster* with *D. melanogaster yellow* 5' upstream enhancer; (B) Transgenic *D. melanogaster* with *D. virilis yellow* 5' upstream enhancer. Stained with Rabbit anti-GFP and Mouse Elav-9F8A9 primary antibodies. Green fluorescence is due to GFP expression of both 3xp3 and plausibly *yellow*. Magenta fluorescence acts as a control marker to show that immunocytochemistry worked.

DISCUSSION

Setbacks

A major setback in this study was the initial inability to clone Attachment-B (AttB) sequence into the pre-constructed Hermes-*D. melanogaster yellow*-GFP and Hermes-*D. virilis yellow*-GFP vectors obtained from Dr Trisha Wittkopp. The attB sequence in the transgenic vectors allow these constructed vectors to bind to the same sites (attP site) on the *Drosophila* genome. This cloning problem may be because the vector was constructed a significant number of years ago, causing the DNA to degrade significantly This put a strain on the time limit of this study (2 semesters) and hence another method was adopted as an attempt to resolve the problem. Instead of using the pre-constructed Hermes vector, *yellow* (from *D melanogaster* and *D. virilis*) was cloned into a piggybac-attB-3xp3-GFP vector. While it is supposedly more difficult to clone *yellow* as compared to attB into a vector due to its much larger size, it turned out to be a better option than the previous one.

Another obstacle faced during the data collection for this thesis was that our laboratory only found out nearing the end of the courtship experiment that some of our laboratory transgenic flies, including my transgenic lines used for the courtship study (*D. melanogaster yellow* mutants harboring a *D.*

virilis yellow transgene, and *D. melanogaster yellow* mutants harboring a *D. melanogaster yellow* transgene) has a wild type *D. melanogaster yellow* gene next to the attP insertion site in the genome. This complicated my study, as my question was on the effects of orthologous *yellow* genes on courtship behavior in flies, thus my results from the experiment could not be easily interpreted. For instance, if successful courtship was observed in one of the transgenic lines I have used (*D. melanogaster yellow* mutants harboring a *D. virilis yellow* gene, and *D. melanogaster yellow* mutants harboring a *D. melanogaster yellow* gene), one cannot distinguish whether this rescue is coming from the *yellow* transgene or the *yellow* that already existed in the genome of the transgenic host. Moreover, the *yellow* gene in the transgenic host makes it harder to interpret a difference observed between the two transgenic lines used for the courtship study. I therefore am only able to speculate that any difference in their courtship behavior (i.e. time taken for successful male courtship) may be due to the number of copies of *yellow* in their genetic make-up. *D. melanogaster yellow* mutants harboring a *D. virilis yellow* transgene have a copy of the *D. virilis yellow* gene and a copy of the *D. melanogaster yellow* gene, *D. melanogaster yellow* mutants harboring a *D. melanogaster yellow* transgene have two copies of the *D. melanogaster*

yellow genes, wild type *D. melanogaster* flies have a copy of the *D. melanogaster yellow* gene, and *D. melanogaster yellow* null mutants have no copies of the *D. melanogaster yellow* gene. That said, if the function of *yellow* is conserved, the average time taken for successful male courtship should be the same for *D. melanogaster yellow* mutants harboring a *D. virilis yellow* gene and *D. melanogaster yellow* mutants harboring a *D. melanogaster yellow* gene. On the other hand, if it is not conserved, the average time taken for *D. melanogaster yellow* mutants harboring a *D. virilis yellow* gene should be about the same as that for the wild type *D. melanogaster* flies.

Imaging of the adult fly brains for expression of Yellow also posed problems for this study. Firstly, transgenic fly stocks in our laboratory contain a cytoplasmic 3xp3-GFP marker, which is expressed in the eyes of the flies. The implication of this is that this marker is expressed in the majority of the brain, making it difficult to distinguish the *yellow* enhancer driven nuclear GFP gene. Additionally, previous immunocytochemistry experiments showed Yellow brain expression only on Drosophila larvae despite researching the courtship behavior in adult flies. In order to further support and to build on the results of past experiments, for this study, adult fly brains were selected to see if they have similar levels and patterns of *yellow* expression as that of

their larval counterparts, So far imaging of the adult fly brains showed no expression of *yellow*. This may be due to the primary anti-Yellow antibody used, as it has been made by Dr Trisha Wittkopp a significant number of years ago, and may have degraded over the years. However, it was the only stock in our laboratory, and it appeared to have been pre-diluted with an unknown dilution factor. Regretfully, due to time constrains of this paper, I have not been able to characterize the brain expression pattern of yellow in adult flies. Nevertheless, I will be doing further research on this subject after the submission of this paper.

Limitations

Behavioral effects due to the environmental conditions of the Copulatron and the laboratory could serve as a confounding factor for successful male courtship. Among these environmental factors, the few more important ones include temperature, lighting, living space, time of the day when the experiment was conducted and weather conditions.

The developmental period of the Drosophila fly varies with temperature, similar to many other ectoderms. Temperatures above ideal decrease development times, whereas temperatures below ideal increase development time (Ashburner et al., 2005; Ashburner et al., 1978). In

crowded conditions, development time increases, and the emerging flies turn out smaller than average due to competition for nutrition (Bakker, 1961; Chiang, 1950). The levels of lighting also affect the visibility of the male flies – the brighter the environment, the faster adult males can locate adult females and enter into the courtship routine (Burnet, & Connolly, 1973). In addition, it has been noted during this study that different times of the day when the experiment is conducted and external weather conditions can lead to significant changes in the courtship behavior – as the day progresses, the number of successful courtships decrease significantly. The same happens when the external temperature is lower (snowy day versus clear day).

Several measures have been taken to reduce the effects of these factors – the flies were kept in as similar conditions from each other as possible. Experimental repeats were always conducted at the same time of the day and the number of flies in each vial was kept constant. Moreover, this part of the study was modified such that only one set of experiment is run per day. Any other environmental effects were assumed to be constant between different samples assayed. Hence, the results of the courtship experiment are assumed to be unaffected from any environmental conditions that we were unable to control.

Future Directions

Investigations of the *yellow* expression patterns in adult brain in *D. melanogaster* transgenic fly lines (*D. melanogaster* hosts harboring a *D. virilis yellow* 5' upstream enhancer versus *D. melanogaster* hosts harboring a *D. melanogaster yellow* 5' upstream enhancer) will continue to progress, so that *yellow* expression in the adult brains can be mapped and compared to that of the third in-star larval brain expression I expect that the results of this experiment will either support previous studies or provide new insights into the expression of *yellow* in the brain and its possible effects on male courtship behaviors.

It would also be worthwhile to investigate the effects of manipulating *yellow* brain expression on courtship behavior through the UAS/Gal4 system. This can be done by crossing UAS-yellow to a number of Gal4 lines (for example, pan-neuronal elav-Gal4 and the BG380-Gal4) that turn on the Gal 4 activator protein in different brain expression patterns. In this case, one can again compare *yellow* expression in the third in star larvae to that of adult males. Ultimately, one can look at the correlation between the changes of yellow expression in the brain and male courtship behaviors. For any Gal4 line that affect behavior, the expression of the Gal4 protein will be further examined by crossing a fly expressing Gal4 to a fly carrying UAS-GFP. The

progeny of this cross will express UAS-GFP in cells where Gal4 is expressed. One can obtain the brain expression patterns of GFP in a particular Gal4 line using anti-GFP and subsequently correlate this differences in male courtship behavior. This can give us a better idea of the roles *yellow* play in male courtship behavior. It is possible that *yellow* has effects on fly behaviors other than male courtship. Characterizing and manipulating *yellow* expression in the brain can give some insights about these other possible behavioral effects. Hence, *yellow* can be understood better not just as a pigmentation gene but also as one that plays a role in proper fly behaviors.

In conjunction with the molecular studies, behavioral tests including placing the flies in a competitive courtship setting, and under stress conditions by manipulating the environmental factors would be helpful for interpretation of current results. It would also be meaningful to expand the findings to compare between-species differences in courtship behavior. This could be done first by comparing the courtship behavior between *D. virilis yellow* mutants harboring a *D. virilis yellow* transgene and wild type *D. virilis* flies to determine if *yellow* restores courtship behavior in flies to support the results of the *D. melanogaster yellow* gene rescue experiments. If the results are potentially positive, another courtship experiment to compare between *D.*

virilis yellow mutants harboring a *D. virilis yellow* transgene and *D. virilis yellow* mutants harboring a *D. melanogaster yellow* transgene could be carried out.

Last but not least, gender is a variable that has often been downplayed in studies. The focus of *Drosophila* courtship studies have always been on adult males, and females' receptivity to male courtship advances have always been overlooked. Thus, in my opinion it is worthwhile to design studies based on female courtship behavior. It would be interesting to see if *yellow* has any effect on the females' receptivity that may contribute to successful male courtship behavior. A major expansion of this thesis seeks to allow greater understanding of the relationship between gene expression and behavior in living organisms. Further experimentation would also open new possibilities for gene rescues on defective behavior due to gene mutation in the future.

APPENDIX A

Modified immunocytochemistry protocol

1. Dissect brains, starting from the eye area and working towards the center of the head, carefully exposing the brain and temporarily storing in Ringer's solution.
2. Remove Ringer's solution and replace with fix. Rotate at room temperature for 1 hr.
3. Wash twice with PBS and re-dissect the brains to get rid of any remaining debris.
4. Incubate brains in a mixture of 30μl normal goat serum and 970μl PBS-T on a rotator at room temperature for 1 hr to enable non-specific binding of proteins.
5. Replace PBS-T with 40μl of primary antibody (***include recipe***) and incubate at 4°C on a rotator for 2 nights.
6. Remove the primary antibody and wash 5 times, 15 minutes per wash, with PBS-T on a rotator at room temperature.
7. Incubate brains in 100μl secondary antibody (***include recipe***) and rotate at 4°C on a rotator overnight.
8. Repeat washing process as in Step (6), then rinse twice with PBS.

9. Mount the brains on a polylysine-coated cover slip, then dehydrate and clear the brains by adding 30%, 50% and 70% glycerol in sequence for 5 minutes each.

10. On a microscope slide, attach 2 uncoated cover slips at the sides using nail polish (see figure 1).

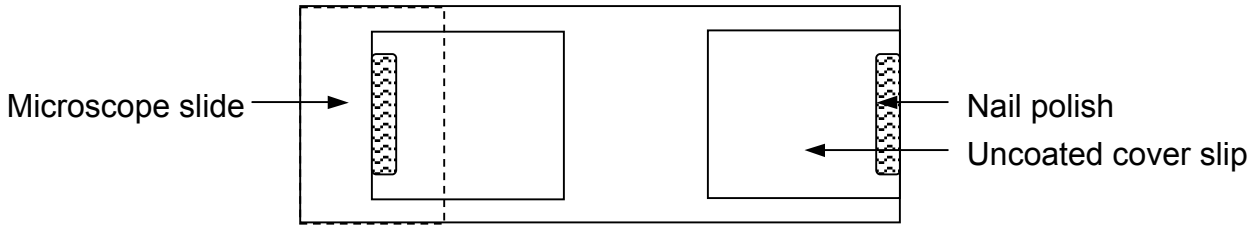
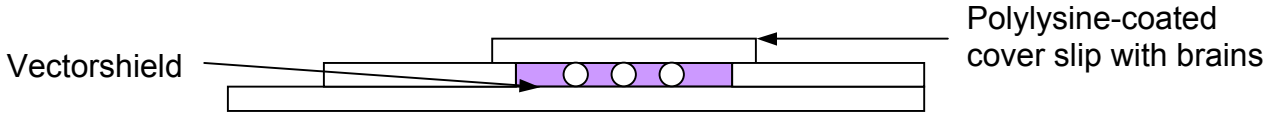


Figure 1. Microscope slide with uncoated cover slips

11. Then, put 4-5 drops of Vectorshield in the well created by the uncoated cover slips and carefully mount the brain over it, taking care to not create any air bubbles.



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