INFLUENCE OF GENETIC VARIATION ON STEM CELL DIVISION FREQUENCY IN *DROSOPHILA MELANOGASTER*

by

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(Under the Direction of CORDULA SCHULZ)

ABSTRACT

The *Drosophila melanogaster white* gene encodes for half of an ATP-Binding Cassette (ABC) Transporter. With its binding partner, Brown or Scarlet, it functions as an amine transporter in the central nervous system. The *white* gene was the first isolated genetic mutant in *Drosophila.* This mutation is now a widely used genetic selection marker. As of yet, the entirety of phenotypic effects caused by *white* mutations has yet to be determined. Thus, the objective of this study was to determine whether *white* gene mutations affect the regulation of germline stem cell (GSC) proliferation in adult testes. For this purpose, a mating assay was conducted using *white* mutants, w^{1118}, and Canton S. & Oregon R. (wildtype controls). It was found that when placed in isolation, w^{1118} males displayed aberrant mitotic indices of GSCs than normal. These results demonstrate that *white* mutations have an indirect effect on GSC frequency regulation.

INDEX WORDS: *Drosophila melanogaster*, germline stem cell, mitotic index, *white*, mating assay

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DEDICATION

This thesis is dedicated to my family, friends and especially my loving, and supportive mother, without whose support I would not have realized my potential and be where I am today.

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CHAPTER 1

INTRODUCTION & LITERATURE

Brief Stem Cell Review

It is widely understood that stem cells are the foundation of all human and animal development, self-maintenance, and reproduction. These undifferentiated cells can give rise to an indefinite number of progeny and play a multitude of roles in the organism. They have the dual ability to self-renew or differentiate into mature cells with a more specific function, such as blood cells, brain cells, heart muscle or bone depending on environmental molecular cues. No other cell type in the body has the natural ability to generate new cell types. Embryonic (pluripotent) stem cells come from blastocyst, or embryos that are around three to five days old (Martin, 1981). These stem cells can divide into more stem cells or can become any type of cell in the body (Geijsen *et al.*, 2004; Suda *et al.*, 1987). Adult stem cells are found in adult tissue and contribute to tissue homeostasis due to natural causes, disease, and injury. Compared to embryonic cells, adult stems cells have a more limited ability in that they do not naturally create all cell types (de Cuevas & Matunis, 2011). A further specialized stem cell, called germline stem cells (GSCs), give rise to gametes for reproduction, and therefore are the source of human and animal reproduction (de Cuevas & Matunis, 2011; Li & Xie, 2005; Nayernia *et al.*, 2006; Shivdasani & Ingham, 2003).

A small change in the frequency of germline stem cell divisions can dramatically affect the number of resulting differentiated cells. For example, a single GSC division in

Drosophila ultimately becomes sixty-four spermatids after amplification and differentiation (de Cuevas & Matunis, 2011; M T Fuller, 1993; Nayernia et al., 2006). In addition, mutated stem cells have been implicated in playing a crucial role in tumor initiation and development, and could provide vital insights for medical advancement (Hime *et al.,* 2007; Reya *et al.,* 2001).

Understanding the underlying mechanisms that regulate this system, in combination with currently available technologies for manipulation, would be an invaluable asset to the science community. Stem cell research has profound potential to contribute to the advancement of multiple aspects of society, with many applications such as: contraceptive discovery, cell therapy, regenerative medicine, fertility treatments, as well as provide guiding principles for new and innovative research.

Spermatogenesis in Male Drosophila Testis

For this investigation we used adult male *Drosophila melanogaster* as the model organism. *Drosophila* males have two testes that contain GSCs, which produce gametes for reproduction. These cells are in direct contact with a small group of terminally differentiated stromal cells located at the apical tip of the testes, referred to as the hub (Hardy *et al*., 1979) (Figure. 1.2.B, C). Each GSC is enclosed by two cyst stem cells (CySCs), which have cytoplasmic extensions that also are in contact with the hub (Figure. 1.3). This contact allows for the passage of molecular signals that maintain the CySC's identity, via the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT), and Hedgehog signaling pathway (Leatherman & Dinardo, 2010; Schulz, Wood, Jones, Tazuke, & Fuller, 2002; Tulina & Matunis, 2001). JAK/STAT is a cytokine

induced signaling pathway that regulates stem cell populations, among other essential developmental processes. JAK activation stimulates cell migration, proliferation, differentiation, and apoptosis (Decotto & Spradling, 2005; Gregory, Came, & Brown, 2008; Ihle & Kerr, 1995; Ihle, 1995; Matsuda et al., 1999). In *Drosophila* testes, this pathway is activated when Unpaired (Upd), a secreted extracellular protein encoded by the *unpaired* gene, binds to the receptor Domeless (Dome), encoded by the *Domeless* gene (Brown, Hu, & Hombría, 2001). Upd binding induces a conformational change that brings together JAK proteins Hopscotch (Hop) (Binari & Perrimon, 1994). Hop is a tyrosine kinase of the JAK family that is encoded by the *Hopscotch* gene and is located on the intracellular domain of the receptor (Livnah *et al.,* 1999). This conformational change allows the JAK proteins to cross phosphorylate each other as well as the Domeless receptor (Harrison *et al.,* 1998; Zeidler *et al.,* 1999). This also creates a binding site for the transcription factor STAT, encoded by *STAT92E,* which is in turn phosphorylated by JAK (Hou *et al.*. 1996; Yan *et al.,* 1996). Once phosphorylated, STAT proteins form dimers which translocate to the nucleus where they act to regulate transcription (Hombría & Brown, 2002; Schindler & Darnell, 1995).

GSCs maintain their identities through signaling from their associating CySCs, via the Transforming Growth Factor β (TGF-β) signaling pathway, as well as receive essential maintenance signals. The TGF-β ligand is expressed on the cyst stem cells and when bound to its receptor acts to suppress the expression of Bam protein. Bam is necessary for the cells to undergo differentiation and without it they remain stem cells (Dansereau & Lasko, 2008; Leatherman & Dinardo, 2010; Shivdasani & Ingham, 2003).

The stem cell niche is located at the apical tip of the testis, and consists of the hub, which is immediately surrounded by GSCs, and their two accompanying CySC (Figure 1.3). GSCs undergo asymmetric division, in which one cell remains a GSC and the other is displaced from the hub and becomes a gonialblast (Hardy *et al*., 1979)(Figure 1.2A). The gonialblasts undergo four rounds of transit amplification divisions with incomplete cytokinesis, at which point they become spermatogonia (Figure 1.2A, & Figure 1.3). The incomplete cytokinesis creates stable cytoplasmic bridges linking sister germline cells intercellular (de Cuevas & Matunis, 2011; White-Cooper, 2010) (Figure 1.3). These sixteen spermatogonia increase in size and become spermatocytes after completion of the S-phase, and initiate distinct gene expression. Next, the spermatocytes undergo two rounds of meiotic division, after which they differentiate into elongated spermatids. The spermatids then shed their cytoplasmic bridges and develop separate plasma membranes (Figure 1.2A). The spermatids then develop into mature sperm, which are then transported to the seminal vesicle, where they are stored until needed (de Cuevas & Matunis, 2011; M T Fuller, 1993; Hardy et al., 1979; Tates, 1971; White-Cooper, 2010).

Throughout the proliferation process, the gonialblast are continuously enclosed by two somatic cyst cells, which originate from an asymmetric division of the CySCs that form the microenvironment of the originating GSC. Simply put, the GSC and its accompanying CySCs divide to create the germline daughter cell (i.e. gonialblast) and its accompanying somatic cyst cells. The somatic cyst cells do not undergo further divisions after enclosing the gonialblast; instead they grow in size to increase their cell surface area in order to sustain enclosure of the gonialblast and its progeny (Hardy et al., 1979).

Drosophila melanogaster as a Model

The *Drosophila* testis are among the most well documented stem cell niches, and have become an ideal model for investigating the biology of adult stem cells *in vivo*. Organization of the niche makes identifying structures, imaging, and manipulation much more convenient than other models (de Cuevas & Matunis, 2011; Tates, 1971). Development of techniques utilizing molecular markers allows for clear distinction of GSCs from differentiating daughters. There are also a wide array of research tools available that allow for genetic manipulation with extremely high temporal and spatial resolution (Duffy, 2002; M T Fuller, 1998; Margaret T Fuller & Spradling, 2007; Phelps & Brand, 1998). In addition, this model provides convenience of genetic tractability, fast generation cycles, and has a well-documented life cycle.

Most importantly, many of the mechanisms guiding the processes in *Drosophila* testes are considered essential for the function of tissues in other organisms, such as gametogenesis in mammals. *Drosophila* stem cell populations are remarkably similar to vertebrates with roughly 77% functional orthologs of human disease genes. This allows for subsequent discoveries to be more easily translated for applicability (Croop et al., 1997; Hime et al., 2007; Reiter, Potocki, Chien, Gribskov, & Bier, 2001).

The white Gene

Function & Description

The *white* gene, along with *brown* and *scarlet*, belong to the ATP-Binding Cassette (ABC) Transporter Superfamily. The *white* gene encodes for White protein, which acts as half of the heterodimer for an ABC transporter. White plays a role in

transportation of molecules in the brain. Its designated binding partner, *brown* or *scarlet* gene products, determines what it transports. Based on evidence from early studies, it has been implied that White/Brown and White/Scarlet protein complexes form transporters responsible for the translocation of guanine and tryptophan, respectively into the cells (Ewart *et al.*, 1994; O'hare, Levis *et al.*, 1983; David T. Sullivan *et al.*, 1979). However, other studies suggest that White/Brown and White/Scarlet protein complexes form a transporter to mediate transportation of red (drosopterins) and brown (xanthommatin) pigments respectively (Mackenzie *et al.*, 2000). So rather than transporting tryptophan into cells, the White/Scarlet complex imports 3-hydroxykynurenine, a necessary ingredient for a xanthommatin metabolic intermediate, through the membrane of the pigment granule within the cell. Evidence has shown that the White/Scarlet complex does not localize to the plasma membrane, but instead, to the pigment granule membrane within the pigment cell, further down stream of the tryptophan transportation stage. This alternate theory is still consistent with previous observations; rather there is a difference of interpretation based on localization data. Additionally, no evidence has shown that White localizes to the plasma membrane of the cell (Mackenzie et al., 2000; Sullivan *et al.*, 1980).

An ABC transporter complex is composed of two transmembrane domains made up of six alpha helices, and two ATP-binding regions. Each transporter protein White, Brown, and Scarlet contain one transmembrane domain and one ATP-binding region which hetero-dimerize to form a complete functional transporter (Dreesen, Johnson, & Henikoff, 1988; Ewart *et al.*, 1994; Tearle *et al.*, 1989).

Tryptophan is an essential amino acid and it is taken up through the animals diet. It's the biosynthetic precursor for the monoamine neurotransmitter, and hormone, serotonin (5-hydroxytryptamine, 5-HT). The end product of Tryptophan- Kynurenine (TRY-KYO) pathway is the brown eye pigment, ommochrome (Mackenzie *et al*., 2000; Oxenkrug, 2010). TRY is an amino acid necessary for biosynthesis of proteins and methoxyindoles (serotonin and melatonin). The red pigments, drosopterins, also contribute to *Drosophila's* eye pigmentation. Drosopterins are synthesized from guanine and brown pigments called ommochromes, which are created from TRY (Mackenzie et al., 1999; Oxenkrug *et al*., 2007). TRY 2,3-dioxygenase 2 (TDO) is an intracellular enzyme that is made from TRY when the indole ring is cleaved. This is a rate-limiting enzyme of TRY metabolism and is commonly manipulated in studies trying to understand the nature of the pathway. The cleavage of TRYs indole ring also forms formylkynurenine, and in turn, kynurenine (KYN). Because TDO is an intracellular enzyme, TRY must enter the cell to be available as a substrate for KYN formation (Howells & Ryall, 1975; Howells, Summers, & Ryall, 1977; Kudo, Boyd, Sargent, & Redman, 2001). Pigment cells possess the necessary machinery to catalyze the conversion of intracellular tryptophan to kynurenine by tryptophan oxygenase, as well as the conversion of kynurenine to 3-hydroxykynurenine by kynurenine hydroxylase. This supports the idea that White may not transport tryptophan into the cell. (Mackenzie *et al.*, 2000; Mackenzie *et al.*, 1999)

Origin & Application

A key aspect of genetic tractability in *Drosophila* is using a phenotypic marker that enable a researcher to track offspring genotypes. The *white* gene was in fact the first genetic mutant to be isolated in *Drosophila* (Morgan, 1910). Mutations in *white* give rise to white-eyed flies, which is the most obvious of its phenotypes. This in turn, led to widespread popularity of utilizing *white* as a genetic marker, and is now universally used in laboratories.

White Mutational Effects

ABC Transporter. The TRY-KYN pathway is the first step of eye pigment synthesis (R. Tearle, 1991). The *white* mutant is deficient in both the White/Scarlet and the White/Brown ABC transporters in pigment cells. This causes the white eye color (lack of pigmentation) phenotype (Sullivan *et al.,* 1980).

Bioamines. Accurately measuring bioamine levels in flies has proven to be a difficult task due to the unstable nature of hormones. It has also been reported that *Drosophila* undergo naturally occurring fluctuations of amine levels, depending on age, and sex (Anaka *et al.,* 2008; Borycz *et al.,* 2008; Hardie & Hirsh, 2006). In consequence, very general interpretations have been made on the subject due to conflicting evidence. For example, in *white* mutants, flies have been shown to have a reduced uptake of tryptophan. However, in such cases serotonin levels did not appear to be affected (Sullivan & Sullivan, 1975). In contrast, a more recent study, utilizing a different technique for quantification, showed dopamine levels in the heads of *white* mutants are measured to be 33% higher than previously reported. In the same study, serotonin was

measured to have 365% higher levels than data previously reported (Hardie & Hirsh, 2006). Differences may result from varying techniques, or variations of genotypes. Until a consensus technique is incorporated for this type of research, results are consistent with the conclusion that *white* mutations alter bioamine levels in flies.

Behavioral Abnormalities. The mushroom body is a region of the brain that contains serotonergic neurons and is associated with learning, memory and courtship behavior. Reported evidence suggests that there is White-Scarlet ABC transporter activity in this region of the brain, which contribute to the reported phenotypes of *white* mutants displaying learning and memory abnormalities (Ewart & Howells, 1998).

In *Drosophila*, it has been discovered that courtship is a learned behavior that is incorporated into the animal's memory when it begins to court. The naïve males learn by mistakenly courting other males and getting rejected. Eventually they learn that their advances are futile and only mate females, a process called courtship conditioning (Anaka et al., 2008). The wildtype males quickly learn how and whom to court without problems, but *white* mutants have been reported in undergoing male-male courting exceeding the traditional time frame for courtship conditioning (Zhang & Odenwald, 1995).

Scientific Approach

As previously stated, *Drosophila* stem cells and their properties have been studied extensively. A very clear understanding of stem cell microenvironments, the stem cell niche, and developmental pathways regulating cell fate decisions has been discovered (de Cuevas & Matunis, 2011; Li & Xie, 2005). However, recent observations provide

evidence that adult *Drosophila* GSCs have a significantly higher rate of division in response to mating, compared to that of non-mated animals ($p<0.05$ Fisher's test; Parrott & Schulz, unpublished data). These findings suggest that GSCs are capable of adjusting their productivity based on environmental cues. Meanwhile, no mechanisms have been identified to explain how GSCs sense and respond to this increase in demand for gametes during mating. The *white gene* has been shown to be more versatile than previously suspected, so investigations were performed in an attempt to further uncover the unknowns of *white*.

For this study, a series of mating assays and analytical tools were designed to investigate the question: Do *white* gene mutations alter pathway(s) that regulate GSCs division frequency in response to mating?

The mating assays were designed as such to permit evaluation of the dynamics of germline stem cell division rates *in vivo*. We utilized immunostaining techniques with antibodies raised against well-characterized cell-specific cytoplasmic markers to identify stem cells in the niche. Immunofluorescent microcopy allowed for quantification of GSC mitotic activity, which is reported as the mitotic-phase index of GSCs (MI^{GSC}; percentage of GSCs with mitotic activity, divided by the total number of GSCs observed) (figure.1.2C). Using this information, in combination with the newfound evidence that GSCs respond to mating, we were able to assess the effects of genetic variability on the mitotic activity of GSCs *in vivo*.

CHAPTER 2

METHODOLOGY

Drosophila Stocks and Genetics

All fly stocks in this study were maintained on standard medium containing, cornmeal, molasses, yeast, agar, Tegosept (a mold inhibitor) and propionic acid (a bactericide). Fly following stocks were obtained from the Bloomington Stock Center (www.flystocks.bio.indiana.edu/): *white*^{1118}, two wild-type lines: Oregon R. and Canton S., and the fruitless mutant: *fru*^{3}-ry^{506} P{PZ}*fru*^{3}/MKRS. *fru*^{1} (In(3R)*fru*), the null allele *fru*$^{P1.LexA}$, and *fru*$^{\Delta tra}$ were a gift from Dr. Bruce Baker (Howard Hughes Medical Institute, Janelia Farms Research Campus). *fru*$^{\Delta tra}$ exhibits abnormal mating behavior primarily in females (Demir & Dickson, 2005), thus for our purposes, this allele was used as a genetic background control.

Generation of fly Stocks

fru^{1} and *fru*^{3} females were crossed with *fru*^{lexA} and *fru*$^{\Delta tra}$ males to generate the following transheterozygous combination of fly stocks: *fru*^{1}/*fru*^{lexA}, *fru*^{1}/*fru*$^{\Delta tra}$, *fru*^{3}/*fru*^{lexA}, and *fru*^{3}/*fru*$^{\Delta tra}$. Lines generated by crossing *fru*^{1} and *fru*^{3} females to *fru*^{lexA} and *fru*$^{\Delta tra}$ males.

Mate Exposure Assays

All mate exposure assays were performed in a 26.5 °C incubator with day/night-light cycle using Oregon R. virgin females for mating. Experimental males and females were collected as virgins and housed separately. Male flies were between 1 and 10 days old at the start of the experiment. Animals were placed in bottles with apple juice plates (apple juice, agar, and water) and yeast paste for 24 hours prior to being placed in experimental conditions to ensure proper nutrition.

The following experimental conditions were designed as such to test how germline line stem cell mitotic indices respond to the presence of other males as mating competitors and to test the effects of mating. Two conditions were used: isolation and mass. For the isolation condition, an isolation chamber was designed that allowed the execution of daily maintenance with minimal disruption to the animals' environment, similar to that of the Mass groups (Figure 2.1A). The chambers contained perforations at the bottom that allowed for air and CO_2 influx, for anesthetizing the animals when replacing the virgin females (Figure 2.1C, E). Apple juice agar with yeast was placed in the lid (Figure 2.1B). Isolation groups contained one male fly, housed either alone in an isolation chamber (Iso), or with three other males (Celibate-Iso), or with three virgin females (Mated-Iso) (Figure 2.1D-E). The next experimental environment was Mass group. These groups were housed in food bottles of either 100 or 400 animals. The mass bottles contained either only the experimental male line with no female contact (Celibate-Mass), or males and females (Mated-Mass). All celibate or mated groups outlined in this paper were placed at a 1:3 ratio; experimental group: female/male environment, depending on desired environment.

Isolated conditions allowed for assessment of the animals when no other animals were present which can be assumed as the baseline measurement. Mated-Iso conditions allowed us to assess how flies respond when no male competition is present. Lastly, Mass groups allow us the opportunity to assess the effect of male competition.

For the mating assay, males were placed in their allotted environments and animals were fed an apple juice agar plate with yeast paste. After 24 hours, flies were anesthetized with CO_2 and females were sorted out and replaced with fresh virgin females, new apple juice plates, and yeast paste. The male-only cohort and isolated males received new apple juice plates and yeast paste only. This process was repeated every 24 hours for two days. At the conclusion of the third day, testes were dissected and stained for analysis.

Histochemistry and Immunofluorescent Microscopy

The tissue was dissected in Testis Isolation Buffer (TIB: 10 mM Tris-HCl, 47mM NaCl, pH 6.8, 183 mM KCl) and fixed with 70µl of 37% formaldehyde solution and 500µl of 1x PBT buffer (1x PBS with 0.1 % Triton X-100, pH 7.5; 1x PBS: 3 mM NaH_2PO_4, 7 mM Na_2HPO_4, 130 mM NaCl, pH 7.4) for 30 minutes while rocking at room temperature. Next the tissue was rinsed three times to stop the fixing process. To rinse, the PBT/formaldehyde mixture was removed from the tissue and new 1x PBT was added. Replacing the 1x PBT and placing the sample at room temperature while rocking for 20 minutes then washed the tissue. This wash was repeated two times. Afterwards the tissue was incubated overnight with primary antibodies at 4 °C rocking. The tissue was then rinsed three times and washed three times for 20 minutes with 1x PBT on a rocker at

room temperature. Next, fluorochrome coupled secondary antibodies were added to the tissue and incubated for 2 hours at room temperature rocking in the dark. The tissue was again rinsed three times and washed three times for 20 minutes 1x PBT in the dark. Finally, the tissue was embedded on glass slides using Vectashield embedding medium (Vector Laboratories).

The following hybridoma/monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD, and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242: mouse α-FasciclinIII 7G10 (1:10) developed by C. Goodman. Goat α-Vasa (1:150) was obtained from Santa Cruz Biotechnology, and Millipore supplied the rabbit α-phosphorylated Histone-H3 (1:500). The following fluorescence-coupled secondary antibodies (Molecular Probes) were used at 1:1000: donkey α-mouse (568), donkey α-goat (488), and donkey α-rabbit (568). Tissues were observed using a Zeiss Axiophot microscope in fluorescent microscopy. Images were taken with a CCD camera using an Apotome and Axiovision Rel Software.

Germline stem cells are located at the apical tip of the testes in direct contact with a group of terminally differentiated cells called the hub (Figure 1.2B-C). To obtain the images of the germline stem cells, the hub, stained with primary antibody mouse α-FasciclinIII and secondary antibody donkey α-mouse (568), was located on the microscope slide in the DsRed channel using 40x objective. The germline cells, stained with primary antibody goat α-Vasa and secondary antibody donkey α-goat (488) were visible via the GFP channel. Thus, the germline stem cells were identified by Vasa-positive cells in direct contact with FasciclinIII-positive cells. Z-stacks images of 1μm

per stack were taken at 40x to ensure clear visualization of the maximum number of stem cells per testis. Cells undergoing mitotic division were identified via phosphorylated Histone-H3 staining, stained with primary rabbit α-phosphorylated Histone-H3 and donkey α-rabbit (568).

Data Analysis

All stem cell division results were obtained at a steady state via fixed cells using molecular markers and reported as mitotic indices. The mitotic index is the percentage of germline stem cells undergoing mitotic division and is calculated by dividing the number of germline stem cells in division (more specifically, Phosphorylated-Histone H3 positive staining in red that is completely encompassed by a Vasa positive cell in green), by the total number of germline stem cells (Figure 1.2C). The cells were then counted and the mitotic index was calculated. P-values were calculated using Independent–samples Student's *t*-test, and Two-way Analysis of Variance (ANOVA) test, in Microsoft Excel (Version 14.1.0) and SigmaPlot (Version 12.5) software.

CHAPTER 3

RESULTS

The method of data quantification used is designed to permit evaluation of the variations of germline stem cell division rates *in vivo* via immunofluorescent microscopy. We determined the percentage of GSC undergoing mitotic division (M-phase index, or MI) at a steady state via tissue fixing and molecular markers (Refer to Figure 1.2). Previous data from our lab has established that mating significantly increases MI^{GSC} in *Drosophila* males compared to celibate flies ($p<0.005$, Parrott & Schulz, data unpublished). We utilized this knowledge as a basic standard for further understanding the practical details that govern the regulation of GSC division frequencies in relation to *white* mutants (w^{1118}).

w^{1118} Displays Deviant MI^{GSC} Compared To Wildtype Males

Under mating assay conditions, w^{1118} animals show a deviant MI^{GSC} when compared to wildtype animals. More specifically, w^{1118} males placed in isolated conditions exhibited a statistically elevated MI^{GSC} in comparison to w^{1118} animals placed in celibate conditions, but no difference to males placed in mating conditions ($p<0.005$, and $p>0.05$, respectively, Figure 3.1). This is distinct from Oregon R. wildtype males, which exhibit no significant increase in MI^{GSC} of mated versus celibate males. Although when placed in mated conditions with virgin females, both wildtype and w^{1118} showed a

significant increase in MI^{GSC} compared to the celibate males housed exclusively in an all-male population ($p<0.005$, Figure 3.1).

By repeating these experiments using w^{1118} and wildtype lines from other laboratories, the previous phenotype of both lines was reinforced. Laboratory stocks are maintained in isolated environments and reproduced over many generations. Under these conditions inbreeding, population bottlenecks, and environmental differences can potentially cause genetic shifts. As such, it is possible for animals of the same genetic origin to result in two genetically divergent populations when maintained in different laboratories (Cohan, 1984; Whitlock & Fowler, 1999). Thus, to ensure that the unique phenotypes that were observed were not solely limited to our *white* mutant and wildtype Oregon R" animals, and to further substantiate our findings, we included a second line of *white* mutants and wildtype (w^{1118} and Canton S.) contributed by the laboratory of Dr. Ping Shen (University of Georgia).

Wildtype lines (Oregon R. and Canton S.) have mitotic indices that display similar overall patterns. Canton S. flies show a significant increase in MI^{GSC} of mated groups in comparison to celibate and isolated groups ($p<0.0001$, & $p<0.005$, respectively, Figure 3.2). In addition, no significant difference is observed in indices between celibate and isolated males ($p>0.05$, Figure 3.2). Similarly, in Oregon R. flies, mated animals show a marked increase compared to isolated ($p>0.01$, Figure 3.2) and the differences between celibate and isolated mitotic indices is not significant, although just barely with a p-value of $p=0.0527$ (Figure 3.2). In contrast, w^{1118} exhibited a higher MI^{GSC} when placed in isolated conditions than is seen in the celibate group ($p<0.0001$, Figure 3.2), a marked

difference not seen in wildtype lines. These findings suggest that mutations to the *white* gene have an indirect effect on the regulation of germline stem cell proliferation.

CHAPTER 4

DISCUSSION

Mutations to the *white* gene alter a pathway in *Drosophila* male GSCs that modifies division frequency in response to mating. Preliminary data shows that adult *Drosophila* GSCs have a significantly higher rate of division in response to mating compared to that of non-mated animals ($p<0.05$ Fisher's test; Parrott & Schulz, unpublished data). When previously conducting unrelated mating assays, using w^{1118} as a selective control, the results were consistently uncharacteristic of wildtype animals (data not shown). Also, we have supporting evidence that courtship alone is not sufficient to cause this hallmark response seen in MI^{GSC} after mating.

The mating assays outlined in this report were designed to allow for the interpretation of how MI^{GSC} of w^{1118} animals respond to various mating environments compared to that of wildtype. Environmental factors controlled for include, population density and the presence of males and/or females. To ensure mated males had an adequate opportunity to mate, they were placed at a 1:3 (male : female) ratio and virgin females were replaced daily. To further control for the effects of population density, the non-mated males (celibate groups) were also placed at a 1:3 ratio.

w^{1118} Flies Placed in Isolation Show Unusually High MI^{GSC}

We are confident that *white* mutations have an effect on MI^{GSC}. However, the question of how are mutations to *white* affecting MI^{GSC}, is more ambiguous. When

interpreting the presented results, it is important to note that isolated flies received no interaction from other animals during the assays. Thus, one can assume that the mitotic index indicated from isolated groups serves as a, non-stimulated, reference for comparison to its corresponding groups. However, due to lack of eliminating evidence, one can equally determine a more complex explanation of the interaction between MI^{GSC} and their environment. With this in mind, the results can be interpreted in multiple ways.

In accepting that the MI^{GSC} of the isolation conditions serve as a non-stimulated reference for comparison, this data suggests that *white* mutants have a higher MI^{GSC} without introducing any excitatory stimuli, like in the mated condition, when compared to wildtype. This suggests that w^{1118} GSCs divide at a hyperactive-rate. In addition, one can say that they have a dramatic response associated to conditions that include the presence of other males and the lacks females (seen in Figure 3.1 & 3.2). Mated-Mass groups included other male flies without showing a dramatic effect. Similarly, Mated-Iso groups were composed of only three females and the MI^{GSC} pattern remained similar to wildtype.

One can also speculate that, if one removes the assumption of the isolation group acting as a non-stimulated reference, w^{1118} respond within the range of normality in all aspects, except when placed in isolation. This new interpretation could suggest that *white* is acting on a redundant pathway that is similarly influenced by stimuli from the presence of other animals via pheromones, auditory, or visual cues, and that the evidence of the mutants dysfunction is more apparent in the absence of outside stimuli. Many broad speculations can be made from this data but further testing is needed to answer the many new questions that these results inspire. Such as, does hyperactivity create more sperm for reproduction? Is there an overlooked phenotype associated with the over-proliferating

cells? Does the MI^{GSC} of w^{1118} respond when exposed to isolated aspect of courtship such as, pheromones, or visual cues only...etc. We can only say for sure that this data suggests that the mutation found in w^{1118} causes abnormal MI^{GSC} by creating a phenotype consisting of an unusually high MI^{GSC} when the mutant is placed in an isolated environment.

CHAPTER 5

CONCLUSION

Future Directions & Conclusion

I will now briefly touch on a broader interpretation of the results in relation to the literature and how they may relate underlying causes of the phenotype observed in w^{1118}.

Reported data indicates that misexpression of the *white* causes male-male courtship in that mating is a learned behavior, which are mediated in the mushroom body by the monoamine serotonin. Serotonin is converted from its precursor tryptophan, and *white* is a crucial component of the tryptophan transporter (Ames, 1986; Anaka et al., 2008; Ewart & Howells, 1998; Hardie & Hirsh, 2006; Sitaraman et al., 2008; David T. Sullivan et al., 1979).

Additionally, we have new evidence that flies that have mutations to the *Fruitless* gene (*fru*), a transcription factor in the sex determination hierarchy that mediates courtship behavior, display similar abnormal MI^{GSC} phenotypes to w^{1118} (data not shown). Fruitless plays a significant role in the induction of male-specific serotonergic neurons (in abdominal ganglion) that innervate parts of the internal reproductive organs, mate choice, as well as the development of reproductive organs, such as the Mol of Lawrence. Previous studies show that mutant *fru* animals display male-male courtship behavior, abnormalities in the structure of serotonergic-abdominal ganglion neurons, and have abnormal levels of serotonin. (Arthur & Magnusson, 2005; Becnel *et al.*, 2011; Demir & Dickson, 2005; Heinrichs, Ryner, & Baker, 1998; Lee & Hall, 2001; Mellert *et al.*, 2010;

Villella et al., 1997) Importantly, the *fru* lines directly observed to display the w^{1118} phenotype, have a genetic background of wildtype or *rosy*, and did not have a *white* mutation.

Furthermore, the Becnel lab (2011) found that activity of serotonin receptor 7 in *Drosophila* (5-HT^{7}Dro) is essential for normal courtship and mating behaviors. It mediates levels of interest in both males and females, and is essential for normal courtship and mating. This is the first reported evidence of direct involvement of a particular serotonin receptor subtype in courtship and mating in the fly (Becnel *et. al*., 2011). To investigate the growing theory of a correlation between serotonin and GSC division frequency, we performed an RT-PCR on wildtype testis tissue. Interestingly, we discovered that at least four of five serotonin receptors found in *Drosophila* are expressed in wildtype testes (data not shown).

We've concluded that the *white* gene can have an indirect effect on the pathway(s) that regulate germline stem cell proliferation in *Drosophila* testis. In short, this universally used genetic marker is not fully understood, particularly in combination with other genetic mutations. Novel findings in this report provide new awareness to *white* mutational effects, and provide new insight for analytical interpretations of *white*. In addition, the collective body of knowledge presented may suggest that serotonin has a more profound effect on GSC regulation than previously expected. Direct connections have yet to be made between serotonin and GSC proliferation, but the findings presented provide support of a potential relationship between the two. There is also an adequate amount of literary evidence further supporting a link between serotonin and phenotypic

similarities observed between *white* and *Fruitless* mutants. I strongly believe that pursuing this novel line of investigate will prove to be, dare I say…*Fruit*-ful?

Figure 1.1: *Immunofluorescent Labeling of Whole Testis Anatomy in Wildtype Drosophila.* (Apical tip) Hub, indicated by asterisk, cluster of terminally differentiated stromal cells; GSC, germline stem cell; GB, gonialblast. Vasa, expressed in germline cells; Dapi, stains cell nuclei.

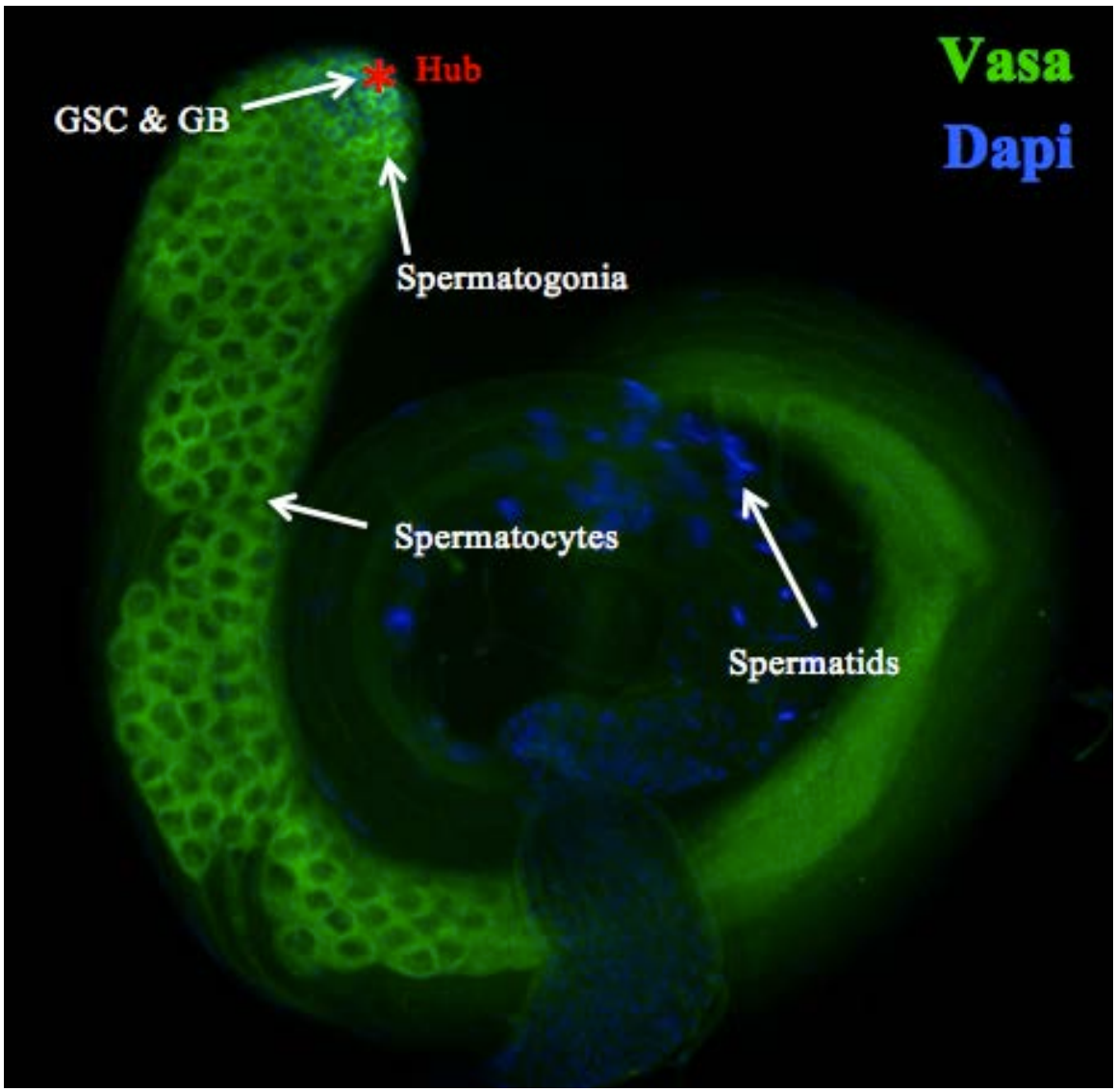

Hub
GSC & GB
Spermatogonia
Spermatocytes
Spermatids
Vasa
Dapi

Figure 1.2: *The Organization & Proliferation Process of Germline Stem Cells in Drosophila Wildtype Testes.* (A) Graphical depiction of germline stem cell proliferation process (beginning: top to end: bottom) GB, gonialblast; GSC, germline stem cell; TA-divisions, transit amplification divisions. (B) Graphical depiction of the germline stem cell niche at the apical tip of the testes. Hub, cluster of terminally differentiated stromal cells; GSC, germline stem cells; CySC, cyst stem cells. (C) Immunofluorescent labeling of a wildtype germline stem cell niche. Vasa, expressed in all germline cells; FasIII, FasciclinIII (staining circled in white) expressed by the hub cells; pHH3, phosphorylated Histone-H3 (shown inside of the furthest GSC to the right) labels exposed Histone-H3 of uncoiled DNA, i.e. cells undergoing mitotic division. GSCs are identified as vasa positive cells in direct contact with FasIII positive cells. Germline stem cell mitotic index (MI^{GSC}) is calculated by dividing the number of germline stem cells in division (Phosphorylated-Histone H3 positive staining in red that is completely encompassed by a Vasa positive cell in green), by the total number of germline stem cells.

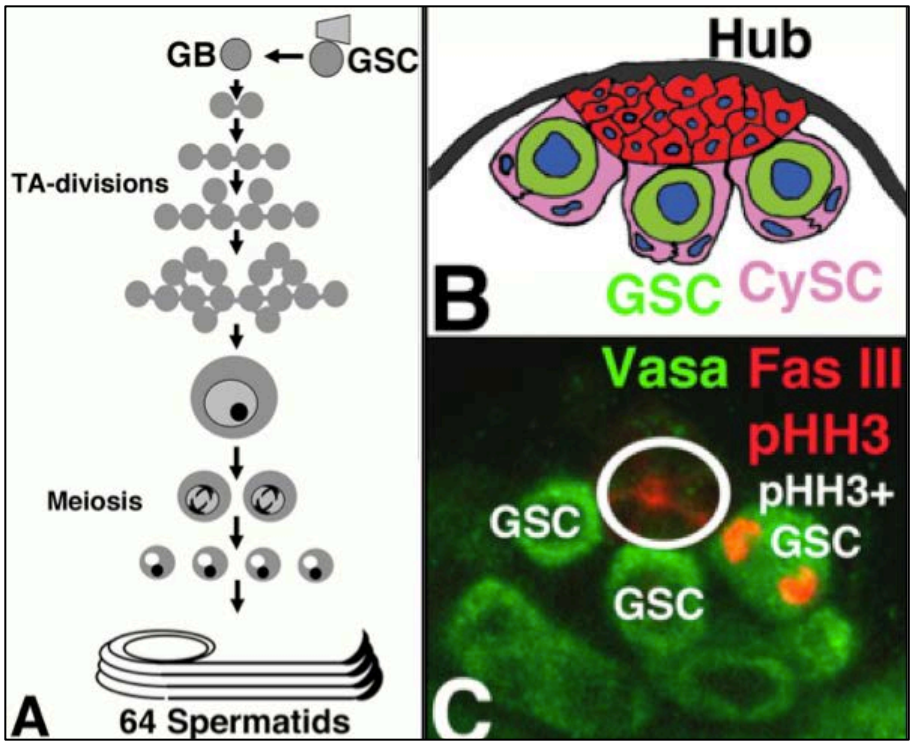

GB
GSC
TA-divisions
Meiosis
A
64 Spermatids
Hub
B
GSC
CySC
Vasa
Fas III
pHH3
pHH3+
GSC
GSC
GSC
C

Figure 1.3: *Graphical Depiction of the Germline Stem Cell Proliferation Process in Drosophila Wildtype Testes.* (Beginning: top, to end: bottom) CySC, cyst stem cell; Hub, cluster of terminally differentiated stromal cells; GSC, germline stem cell; GB, gonialblast; CC, cyst cell; SG, spermatogonia; SC, spermatocyte.

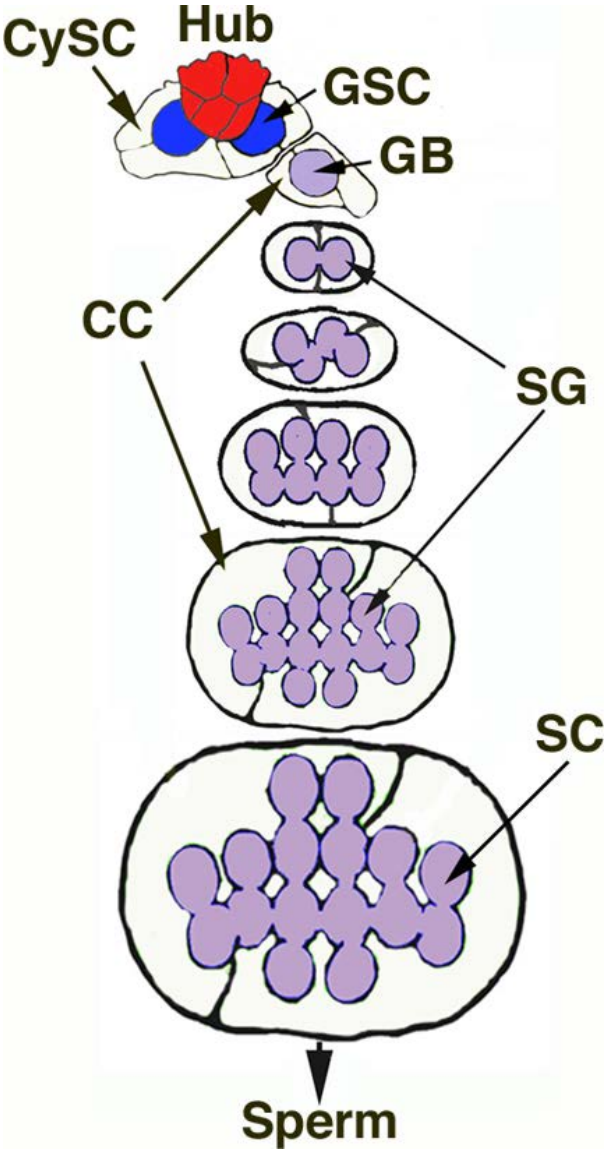

CySC
Hub
GSC
GB
CC
SG
SC
Sperm

Figure 2.1: *Isolation Mating Chamber.* An apparatus built to house the isolation (Iso) groups during mating assays. (A) The entirety of a mating container for one group. It contains 100 individual chambers. (B) The lid of the chamber that holds the apple juice agar and yeast to feed the animals and seal the individual chambers. (C) View of the bottom of the chamber. This allows necessary oxygen to enter and is placed over a CO_2 pad for anesthetizing the animals. (D) Top view of container looking down in to the chambers. (E) View of the bottom perforated portion of an individual chamber that has been removed from the container. (F) Side view of individual chamber removed from the container. The container is made from a 1000µl pipette tip box. The tips are cut and melted onto a small piece of wire screen and trimmed. The tips are then glued into the container for easy use. Putters are used to place and remove animals.

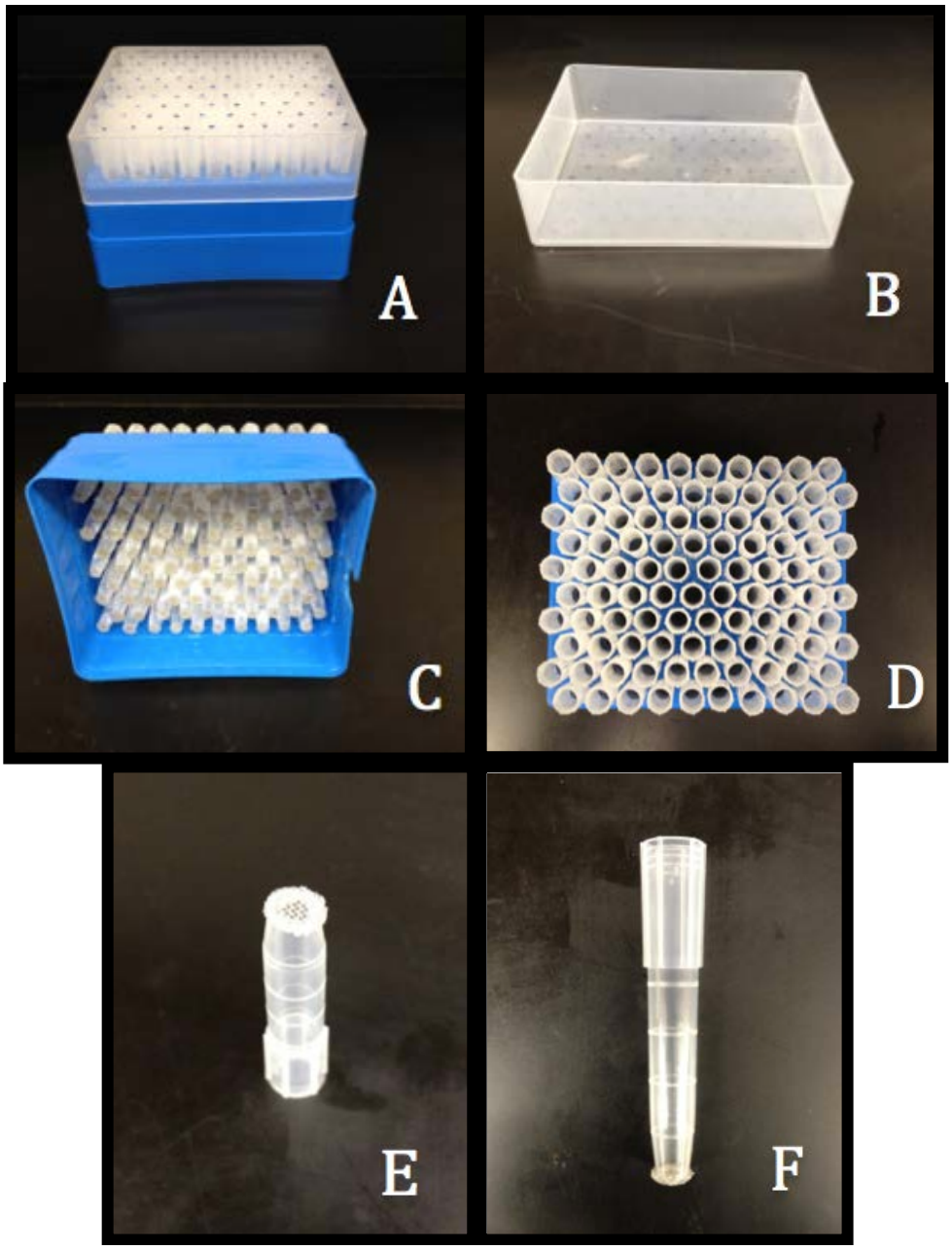

A
B
C
D
E
F

Figure 3.1: *Assay to Compare Mating Conditions MI^{GSC} of w^{1118} Males and Wildtype (Oregon R.).* The four mating conditions of this assay include: Mated-Mass: 25 virgin males were placed with 75 virgin females. Mated-Iso: single virgin males placed with 3 virgin females. Celibate-Mass: 25 virgin males placed with 75 other virgin males. Isolated: males housed alone. All celibate or mated groups were placed at a 1:3 ratio; experimental males to the desired mating group, respectively. All p-values were calculated using Independent–samples Student's *t*-test, and Two-way Analysis of Variance (ANOVA) test.

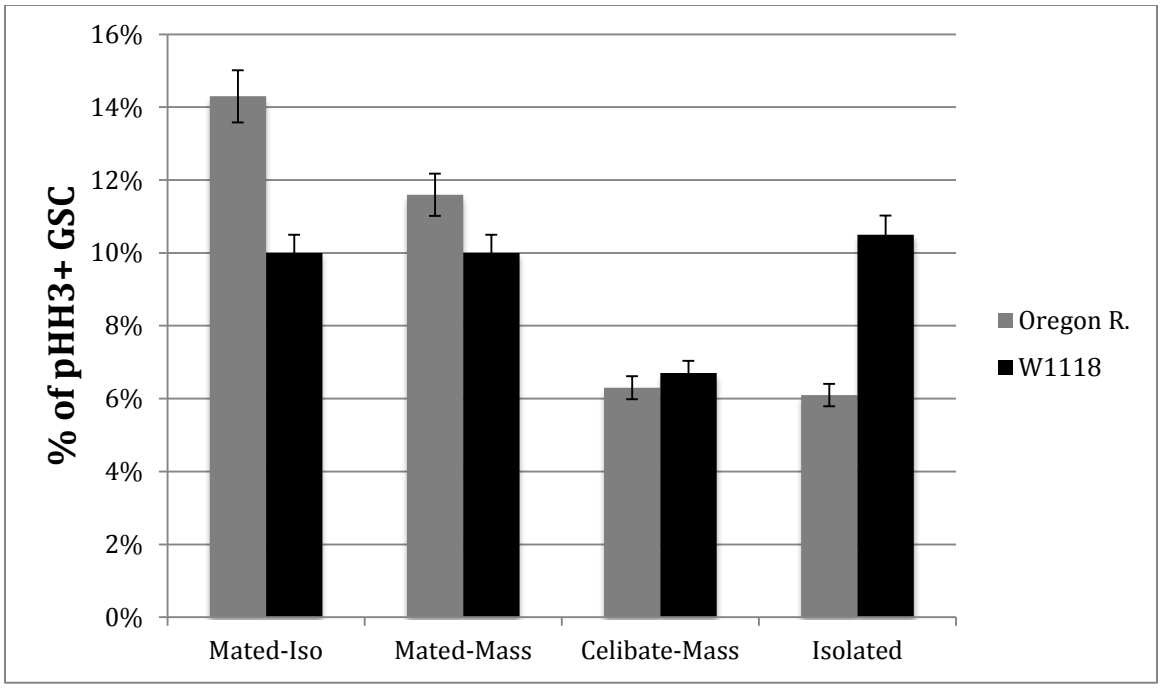
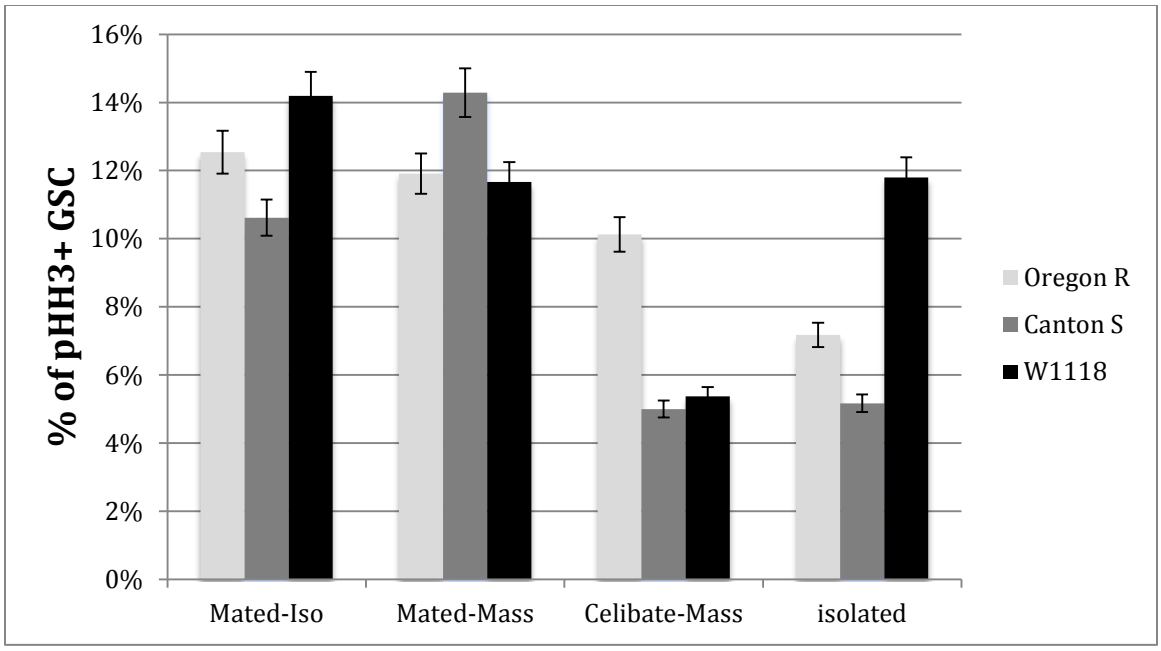

16%
14%
12%
10%
8%
6%
4%
2%
0%
% of pHH3+ GSC
Mated-Iso
Mated-Mass
Celibate-Mass
Isolated
Oregon R.
W1118

Figure 3.2: *Mating Assay to Compare MI^{GSC} of w^{1118} Males and Wildtype Lines*. The four mating conditions of this assay include: Mated-Mass: 25 virgin males were placed with 75 virgin females. Mated-Iso: single virgin males placed with 3 virgin females. Celibate-Mass: 25 virgin males placed with 75 other virgin males. Isolated: males housed alone. All celibate or mated groups were placed at a 1:3 ratio; experimental males to the desired mating group, respectively. All p-values were calculated using Independent–samples Student's *t*-test, and Two-way Analysis of Variance (ANOVA) test.


% of pHH3+ GSC
16%
14%
12%
10%
8%
6%
4%
2%
0%
Mated-Iso
Mated-Mass
Celibate-Mass
isolated
Oregon R
Canton S
W1118

REFERENCES:

Ames, G. F.-L. (1986). The basis of multidrug resistance in mammalian cells: Homology with bacterial transport. *Cell*, *47*(3), 323–324. doi:http://dx.doi.org/10.1016/0092-8674(86)90585-4

Anaka, M., MacDonald, C. D., Barkova, E., Simon, K., Rostom, R., Godoy, R. A., … Lloyd, V. (2008). The white gene of Drosophila melanogaster encodes a protein with a role in courtship behavior. *Journal of neurogenetics*, *22*, 243–276. doi:10.1080/01677060802309629

Arthur, B., & Magnusson, M. (2005). Microanalysis of Drosophila Courtship Behaviour. (L. Anolli, S. Duncan Jr., M. S. Magnusson, & G. Riva, Eds.)*EMERGING COMMUNICATION*, 100–107. Retrieved from http://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:Microanalysis+of+Drosophila+Courtship+Behaviour#0

Becnel, J., Johnson, O., Luo, J., Nässel, D. R., & Nichols, C. D. (2011). The serotonin 5-HT7Dro receptor is expressed in the brain of Drosophila, and is essential for normal courtship and mating. *PloS one*, *6*(6), e20800. doi:10.1371/journal.pone.0020800

Binari, R., & Perrimon, N. (1994). Stripe-specific regulation of pair-rule genes by hopscotch, a putative Jak family tyrosine kinase in Drosophila. *Genes & development*, *8*, 300–312. doi:10.1101/gad.8.3.300

Borycz, J., Borycz, J. a, Kubów, A., Lloyd, V., & Meinertzhagen, I. a. (2008). Drosophila ABC transporter mutants white, brown and scarlet have altered contents and distribution of biogenic amines in thBorycz, J., Borycz, J. a, Kubów, a, Lloyd, V., & Meinertzhagen, I. a. (2008). Drosophila ABC transporter mutants white, brown and sc. *The Journal of experimental biology*, *211*(Pt 21), 3454–66. doi:10.1242/jeb.021162

Brown, S., Hu, N., & Hombría, J. C. (2001). Identification of the first invertebrate interleukin JAK/STAT receptor, the Drosophila gene domeless. *Current biology : CB*, *11*, 1700–1705. doi:10.1016/S0960-9822(01)00524-3

Cohan, F. M. (1984). Can uniform selection retard random genetic divergence between isolated conspecific populations? *Evolution*, *38*, 495–504. doi:10.2307/2408699

Croop, J. M., Tiller, G. E., Fletcher, J. A., Lux, M. L., Raab, E., Goldenson, D., … Wu, R. L. (1997). Isolation and characterization of a mammalian homolog of the Drosophila white gene. *Gene*, *185*, 77–85. doi:10.1016/S0378-1119(96)00633-6

Dansereau, D. a, & Lasko, P. (2008). The development of germline stem cells in Drosophila. *Methods in Molecular Biology*, *450*, 3–26. doi:10.1007/978-1-60327-214-8_1

De Cuevas, M., & Matunis, E. L. (2011). The stem cell niche: lessons from the Drosophila testis. *Development (Cambridge, England), 138*(14), 2861–9. doi:10.1242/dev.056242

Decotto, E., & Spradling, A. C. (2005). The Drosophila ovarian and testis stem cell niches: similar somatic stem cells and signals. *Developmental cell*, *9*, 501–510. doi:10.1016/j.devcel.2005.08.012

Demir, E., & Dickson, B. J. (2005). fruitless splicing specifies male courtship behavior in Drosophila. *Cell*, *121*(5), 785–94. doi:10.1016/j.cell.2005.04.027

Dreesen, T. D., Johnson, D. H., & Henikoff, S. (1988). The brown protein of Drosophila melanogaster is similar to the white protein and to components of active transport complexes. *Molecular and cellular biology*, *8*, 5206–5215. doi:10.1128/MCB.8.12.5206.Updated

Duffy, J. (2002). GAL4 system in Drosophila: a fly geneticist's Swiss army knife. *genesis*, *34*(1-2), 1–15. doi:10.1002/gene.10150

Ewart, G. D., Cannell, D., Cox, G. B., & Howells, A. J. (1994). Mutational analysis of the traffic ATPase (ABC) transporters involved in uptake of eye pigment precursors in Drosophila melanogaster. Implications for structure-function relationships. *The Journal of biological chemistry*, *269*, 10370–10377.

Ewart, G. D., & Howells, A. J. (1998). ABC transporters involved in transport of eye pigment precursors in Drosophila melanogaster. *Methods in enzymology, 292*, 213–24. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9711556

Fuller, M T. (1993). Spermatogenesis, in The Development of Drosophila (Bate, M. and Martinez-Arias, A., eds.). *Cold Spring Harbor Laboratory Press*, 71–147.

Fuller, M T. (1998). Genetic control of cell proliferation and differentiation in Drosophila spermatogenesis. *Semin Cell Dev Biol*, *9*, 433–44.

Fuller, Margaret T, & Spradling, A. C. (2007). Male and female Drosophila germline stem cells: two versions of immortality. *Science (New York, N.Y.), 316*, 402–404. doi:10.1126/science.1140861

Geijsen, N., Horoschak, M., Kim, K., Gribnau, J., Eggan, K., & Daley, G. Q. (2004). Derivation of embryonic germ cells and male gametes from embryonic stem cells. *Nature*, *427*, 148–154. doi:10.1038/nature02247

Gregory, L., Came, P. J., & Brown, S. (2008). Stem cell regulation by JAK/STAT signaling in Drosophila. *Seminars in cell & developmental biology, 19*, 407–13. doi:10.1016/j.semcdb.2008.06.003

Hardie, S. L., & Hirsh, J. (2006). An improved method for the separation and detection of biogenic amines in adult Drosophila brain extracts by high performance liquid chromatography. *Journal of Neuroscience Methods, 153*, 243–249. Retrieved from <Go to ISI>://000238101700010

Hardy, R. W., Tokuyasu, K. T., Lindsley, D. L., & Garavito, M. (1979). The germinal proliferation center in the testis of Drosophila melanogaster. *Journal of Ultrastructure Research, 69*(2), 180–190. Retrieved from http://www.sciencedirect.com/science/article/pii/S0022532079901084

Harrison, D. A., McCoon, P. E., Binari, R., Gilman, M., & Perrimon, N. (1998). Drosophila unpaired encodes a secreted protein that activates the JAK signaling pathway. *Genes & development, 12,* 3252–3263. doi:10.1101/gad.12.20.3252

Heinrichs, Volker; Ryner, Lisa C.; Baker, B. S. (1998). Regulation of sex-specific selection of fruitless 5' splice sites by transformer and transformer-2. *Molecular and cellular biology, 18*(1), 450–8. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=121514&tool=pmcentrez&rendertype=abstract

Hime, G. R., Loveland, K. L., & Abud, H. E. (2007). Drosophila spermatogenesis: insights into testicular cancer. *International journal of andrology, 30,* 265–274; discussion 274. doi:10.1111/j.1365-2605.2007.00767.x

Hombría, J. C.-G., & Brown, S. (2002). The fertile field of Drosophila Jak/STAT signalling. *Current biology : CB, 12*(16), R569–75. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12194841

Hou, X. S., Melnick, M. B., & Perrimon, N. (1996). Marelle acts downstream of the Drosophila HOP/JAK kinase and encodes a protein similar to the mammalian STATs. *Cell, 84*, 411–419. doi:10.1016/S0092-8674(00)81286-6

Howells, A. J., & Ryall, R. L. (1975). A biochemical study of the scarlet eye-color mutant of Drosophila melanogaster. *Biochemical genetics*, *13*, 273–282. doi:10.1007/BF00486022

Howells, A. J., Summers, K. M., & Ryall, R. L. (1977). Developmental patterns of 3-hydroxykynurenine accumulation in white and various other eye color mutants of Drosophila melanogaster. *Biochemical genetics*, *15*, 1049–1059. doi:10.1007/BF00484496

Ihle, J. N. (1995). Cytokine Receptor Signaling. *Nature*, *377*, 591–594.

Ihle, J. N., & Kerr, I. M. (1995). Jaks and Stats in signaling by the cytokine receptor superfamily. *Trends in genetics : TIG*, *11*, 69–74. doi:10.1016/S0168-9525(00)89000-9

Kudo, Y., Boyd, C. A. R., Sargent, I. L., & Redman, C. W. G. (2001). Tryptophan degradation by human placental indoleamine 2 , 3-dioxygenase regulates lymphocyte proliferation. *Journal of Physiology*, *535*, 207–215.

Leatherman, J. L., & Dinardo, S. (2010). Germline self-renewal requires cyst stem cells and stat regulates niche adhesion in Drosophila testes. *Nature cell biology*, *12*, 806–811. doi:10.1038/ncb2086

Lee, G., & Hall, J. C. (2001). Abnormalities of Male-Specific FRU Protein and Serotonin Expression in the CNS of fruitless Mutants in Drosophila. *J. Neurosci.*, *21*(2), 513–526. Retrieved from http://www.jneurosci.org/content/21/2/513.long

Li, L., & Xie, T. (2005). Stem cell niche: structure and function. *Annual review of cell and developmental biology*, *21*, 605–31. doi:10.1146/annurev.cellbio.21.012704.131525

Livnah, O., Stura, E. A., Middleton, S. A., Johnson, D. L., Jolliffe, L. K., & Wilson, I. A. (1999). Crystallographic evidence for preformed dimers of erythropoietin receptor before ligand activation. *Science (New York, N.Y.)*, *283*, 987–990. doi:10.1126/science.283.5404.987

Mackenzie, S M, Howells, A. J., Cox, G. B., & Ewart, G. D. (2000). Sub-cellular localisation of the white/scarlet ABC transporter to pigment granule membranes within the compound eye of Drosophila melanogaster. *Genetica*, *108*, 239–252. doi:10.1023/A:1004115718597

Mackenzie, Susan M, Brooker, M. R., Gill, T. R., Cox, G. B., Howells, A. J., & Ewart, G. D. (1999). Mutations in the white gene of Drosophila melanogaster affecting ABC transporters that determine eye colouration. *Biochimica et biophysica acta*, *1419*(2), 173–85. doi:10.1016/S0005-2736(99)00064-4

Martin, G. R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proceedings of the National Academy of Sciences*, *78*(12), 7634–7638. doi:10.1073/pnas.78.12.7634

Matsuda, T., Nakamura, T., Nakao, K., Arai, T., Katsuki, M., Heike, T., & Yokota, T. (1999). STAT3 activation is sufficient to maintain an undifferentiated state of mouse

embryonic stem cells. *The EMBO journal*, *18*, 4261–4269. doi:10.1093/emboj/18.15.4261

Mellert, D. J., Knapp, J.-M., Manoli, D. S., Meissner, G. W., & Baker, B. S. (2010). Midline crossing by gustatory receptor neuron axons is regulated by fruitless, doublesex and the Roundabout receptors. *Development (Cambridge, England)*, *137*(2), 323–32. doi:10.1242/dev.045047

Morgan, T. H. (1910). Sex-Limited Inheritance in Drosophila. *Science*, *32*, 120–122. doi:10.1126/science.32.812.120

Nayernia, K., Li, M., & Engel, W. (2006). Spermatogonial stem cells. *Current Opinion in Cell Biology*, *10*, 259–282. Retrieved from http://linkinghub.elsevier.com/retrieve/pii/S0076687906190114

O'hare, K., Levis, R., & Rubin, G. M. (1983). Transcription of the white locus in Drosophila melanogaster. *Proceedings of the National Academy of Sciences of the United States of America*, *80*, 6917–6921. doi:10.1073/pnas.80.22.6917

Oxenkrug, G F, Sablin, S. O., & Requintina, P. J. (2007). Effect of methylene blue and related redox dyes on monoamine oxidase activity; rat pineal content of N-acetylserotonin, melatonin, and related indoles; and righting reflex in melatonin-primed frogs. *Annals of the New York Academy of Sciences*, *1122*, 245–252. doi:1122/1/245 [pii]\r10.1196/annals.1403.017

Oxenkrug, Gregory F. (2010). The extended life span of Drosophila melanogaster eye-color (white and vermilion) mutants with impaired formation of kynurenine. *Journal of neural transmission (Vienna, Austria : 1996)*, *117*(1), 23–6. doi:10.1007/s00702-009-0341-7

Phelps, C. B., & Brand, A. H. (1998). Ectopic gene expression in Drosophila using GAL4 system. *Methods (San Diego, Calif.)*, *14*, 367–379. doi:10.1006/meth.1998.0592

Reiter, L. T., Potocki, L., Chien, S., Gribskov, M., & Bier, E. (2001). A systematic analysis of human disease-associated gene sequences in Drosophila melanogaster. *Genome research*, *11*(6), 1114–25. doi:10.1101/gr.169101

Reya, T., Morrison, S. J., Clarke, M. F., & Weissman, I. L. (2001). Stem cells, cancer, and cancer stem cells. *Nature*, *414*, 105–111. doi:10.1038/35102167

Schindler, C., & Darnell, J. E. (1995). Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. *Annual review of biochemistry*, *64*, 621–651. doi:10.1146/annurev.biochem.64.1.621

Schulz, C., Wood, C. G., Jones, D. L., Tazuke, S. I., & Fuller, M. T. (2002). Signaling from germ cells mediated by the rhomboid homolog stet organizes encapsulation by somatic support cells. *Development (Cambridge, England)*, *129*, 4523–4534.

Shivdasani, A. A., & Ingham, P. W. (2003). Regulation of Stem Cell Maintenance and Transit Amplifying Cell Proliferation by TGF-β Signaling in Drosophila

Spermatogenesis. *Current Biology*, *13*(23), 2065–2072. doi:10.1016/j.cub.2003.10.063

Sitaraman, D., Zars, M., Laferriere, H., Chen, Y.-C., Sable-Smith, A., Kitamoto, T., … Zars, T. (2008). Serotonin is necessary for place memory in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(14), 5579–84. doi:10.1073/pnas.0710168105

Suda, Y., Suzuki, M., Ikawa, Y., & Aizawa, S. (1987). Mouse embryonic stem cells exhibit indefinite proliferative potential. *J Cell Physiol*, *133*, 197–201. doi:10.1002/jcp.1041330127

Sullivan, D T, Bell, L. A., Paton, D. R., & Sullivan, M. C. (1980). Genetic and functional analysis of tryptophan transport in Malpighian tubules of Drosophila. *Biochemical genetics*, *18*, 1109–1130. doi:10.1007/BF00484342

Sullivan, D T, & Sullivan, M. C. (1975). Transport defects as the physiological basis for eye color mutants of Drosophila melanogaster. *Biochemical genetics*, *13*, 603–613. doi:10.1007/BF00484918

Sullivan, David T., Bell, L. A., Paton, D. R., & Sullivan, M. C. (1979). Purine transport by Malpighian tubules of pteridine-deficient eye color mutants of Drosophila melanogaster. *Biochemical Genetics*, *17*(5-6), 565–573. doi:10.1007/BF00498891

Tates, A. D. (1971). Cytodifferentiation during spermatogenesis in Drosophila Melanogaster: an electron microscope study. Retrieved from http://catalog.hathitrust.org/Record/000708807

Tearle, R. (1991). Tissue specific effects of ommochrome pathway mutations in Drosophila melanogaster. *Genetical research*, *57*, 257–266.

Tearle, R. G., Belote, J. M., McKeown, M., Baker, B. S., & Howells, A. J. (1989). Cloning and characterization of the scarlet gene of Drosophila melanogaster. *Genetics*, *122*, 595–606.

Tulina, N., & Matunis, E. (2001). Control of stem cell self-renewal in Drosophila spermatogenesis by JAK-STAT signaling. *Science (New York, N.Y.)*, *294*, 2546–2549. doi:10.1126/science.1066700

Villella, A., Gailey, D., Berwald, B., Ohshima, S., Barnes, P. T., & Hall, J. C. (1997). Extended reproductive roles of the fruitless gene in Drosophila melanogaster revealed by behavioral analysis of new fru mutants. *Genetics*, (147), 1107–1130. Retrieved from http://www.genetics.org/content/147/3/1107.short

White-Cooper, H. (2010). Molecular mechanisms of gene regulation during Drosophila spermatogenesis. *Reproduction*, *139*, 11–21.

Whitlock, M., & Fowler, K. (1999). The Changes in Genetics and Environmental Variance with Inbreeding in Drosophila melanogaster. *Genetics*. Retrieved from http://www.genetics.org/content/152/1/345.short

Yan, R., Small, S., Desplan, C., Dearolf, C. R., & Darnell, J. E. (1996). Identification of a Stat gene that functions in Drosophila development. *Cell*, *84*, 421–430. doi:10.1016/S0092-8674(00)81287-8

Zeidler, M. P., Perrimon, N., & Strutt, D. I. (1999). Polarity determination in the Drosophila eye: a novel role for unpaired and JAK/STAT signaling. *Genes & development*, *13*, 1342–1353. doi:10.1101/gad.13.10.1342

Zhang, S. D., & Odenwald, W. F. (1995). Misexpression of the white (w) gene triggers male-male courtship in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(12), 5525–5529. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=41728&tool=pmcentrez&rendertype=abstract