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# Characterization of the Stem Cell Niche in Drosophila Testes

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# CHARACTERIZATION OF THE STEM CELL NICHE IN *DROSOPHILA* TESTES

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Submitted in partial fulfillment of the requirements for the Degree of Master of Science in Biology from the Department of Biological Sciences of Seton Hall University

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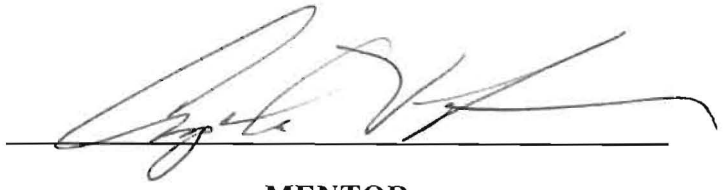
**APPROVED BY**



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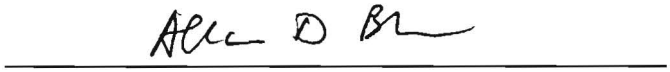

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## ABSTRACT

Fruit flies (Genus *Drosophila*) are widely distributed geographically and commonly used in genetics, cell biology, biochemistry, neuroscience, medicine and developmental biology studies. *Drosophila melanogaster* is the most familiar experimental fruit fly species. Our lab is focused on characterizing spermatogenesis in twelve *Drosophila* species whose genomes have been sequenced. This includes the characterization of the stem cell niche – a region of the fly testis that maintains a steady pool of stem cells that continuously give rise to mature spermatozoa throughout the adult life of the animal.

*D. simulans* is *D. melanogaster*'s closest evolutionary relative. Therefore, we are specifically interested in characterizing the stem cell niche, spermatogenesis and testicular architecture in this species. Also, our lab has developed a culture system that will enable us to study spermatogenesis in *D. pseudoobscura in vitro*, so we are interested in studying the stem cell niche in this species as well. Spermatogenesis in flies occurs within cysts that are contained within the blind-ended, tubular or ellipsoid testes. Spermatogenic stem cells are enclosed within cysts at the apical end of the testes, and sperm development proceeds as the cysts are pushed down the testis towards the basal end. The work we report here involves the use of immunofluorescence staining, confocal imaging, and three-dimensional reconstruction to localize cells of the stem cell niche in *D. simulans* and *D. pseudoobscura*. Comparing the stem cell niche in *D. simulans* and *D. pseudoobscura* with *D. melanogaster* may offer important insights into spermatogenesis and evolution in this model organism.

# INTRODUCTION

## Overview

The fruit fly, *Drosophila melanogaster,* is a holometabolous insect, meaning it has egg, larval and pupal stages prior to the adult stage and matures through complete metamorphosis. *Drosophila* flies are used as model organisms for genetic, cellular, developmental, and evolutionary studies because of the vast number of species in the genus, small size, easy attainability, short generation time and ease of maintenance in the laboratory. For evolution studies, another reason these species are considered advantageous over other model organisms is because the males of this genus are known to have widely varying sperm length, as well as the longest sperm cells of any organism on earth, including one species, *D. bifurca*, that have sperm that are 5.8 cm long (Pitnick *et al.*, 1995). Sperm length is an excellent trait for studying species differences as it is species specific and is the most rapidly evolving trait in insects (Joly *et al.*, 1989, 1991).

A comprehensive understanding of the development, reproduction and physiology is already known about *D. melanogaster,* but not much is known about *D. simulans* and *D. pseudoobscura*. Although *D. melanogaster* has been the species of choice in genetic and developmental biological studies, *D. simulans* can also provide useful cellular information that can be applied in helping researchers further understand cell behavior and stem cell maintenance. *D. simulans* is the closest evolutionary relative to *D. melanogaster*, (**Fig. 1**). Also, *D. pseudoobscura* has been used more extensively than *D. melanogaster* for studies of population genetics, race formation, and species differentiation (Dobzhansky, 1936; Noor *et al.*, 2001; Machando *et al.*, 2002). Hence, comparison of both these species with *D. melanogaster* is

expected to offer further important insights into the biology of this model for experimental purposes, for the study of spermatogenesis, and for studies of evolution.

**Spermatogenesis in flies**

*Drosophila* spermatogenesis offers an excellent system for studying the genetics of the differentiation of germ cells from germline stem cells to mature, motile sperm. Specific areas of interest include regulation of germ cell proliferation, meiosis, mitochondria derivative formation, flagella formation and individualization (Castrillon *et al.*, 1993; Fuller, 1993; Kemphues *et al.*, 1980). Spermatogenesis is a complex process that involves the production of fully functional mature sperm from the primordial germ cell. This entire developmental process is maintained throughout the male organism's lifetime by regeneration of stem cells. Spermatogenesis in *Drosophila melanogaster* was studied as early as 1907 by N. M. Stevens (Metz, 1926) and extensively studied by others in later years (Cooper, 1950; Cross and Shellenbarger, 1979; Fuller, 1993). The differentiation of the germ cells to motile sperm at the ultrastructural level has been described in great detail (Tokuyasu, 1974, 1975a, 1975b; Stanley *et al.*, 1972; Tates, 1971). However, only limited studies have been done on other *Drosophila* species, and have mainly focused on *D. hydei* (Fowler, 1973; Fowler and Johannisson, 1976; Liebrich, 1981; 1982).

The molecular mechanisms that control stem cell behavior and how stem cells relate to their stem cell niche may be facilitated by studying *Drosophila* testes where they provide good insights into this processes (Singh and Hou, 2008). The testes of the *D. melanogaster* male are blind-ended, coiled tubes. The testis tissue is composed of muscle and pigment cells on the outside, while the stem cells, supporting somatic cells, and spermatogenic cells are contained within the lumen of the testis (**Fig. 2**; White-Cooper, 2008). When unrolled, the testes can reach

a remarkable length of 68 mm (Joly *et al.*, 1995; Pitnick, 1996). In the apical tip of the *D. melanogaster* testis, 7-10 germline stem cells (GSCs) and 14-20 somatic cyst progenitor cells (SSCs) are directly attached to a somatic signaling center called the hub. The putative hub cells (approximately 12) are arranged in a rosette and are responsible for maintaining the germline and cyst progenitor in an undifferentiated state (Fuller, 1993; Gonczy and DiNardo, 1996; Hardy *et al.*, 1979; Kiger and Fuller, 2001; Lindsley and Tokuyasu, 1980; Yamashita *et al.*, 2003).

Spermatogenesis in *D. melanogaster* occurs within cysts at the apical end of the blind ended testis (**Fig. 3**). There are three types of cells within the testis: (1) hub cells, which are responsible for maintaining stem cells in an undifferentiated state (2) germline stem cells and (3) somatic cyst progenitor cells that are in a rosette arrangement around the hub (Hardy *et al.*, 1979). Stem cells are unique because they are able to renew themselves and are able to produce cells that will travel on a pathway to differentiated sperm. The testicular tip contains stems cells that will mature into spermatozoa, and stem cells that will encapsulate the developing sperm. At the mid embryonic stage the male GSCs (mGSCs) are established in the embryonic gonads (Le Bras & Van Doren, 2006). To ensure that the niche develops in the anterior section of male embryonic gonads it requires a receptor tyrosine kinase known as sevenless (Kitadate *et al.*, 2007). In *D. melanogaster*, the niche contains a cluster of 12 somatic cells called the hub and is in contact with an average of five to nine mGSCs distributed in a characteristic rosette arrangement to maintain spermatogenesis (Gonczy *et al.*, 1996; Fuller, 1998; Hardy *et al.*, 1979; Kiger & Fuller, 2001). The mGSCs divide to produce one daughter cell that remains adjacent to and in contact with the hub and retains its stem cell identity while the other daughter cell leaves the putative hub and initiates spermatogenesis ((Hardy *et al.* 1979; Yamashita *et al.*, 2003, 2007; Kawamato *et al.*, 2008).

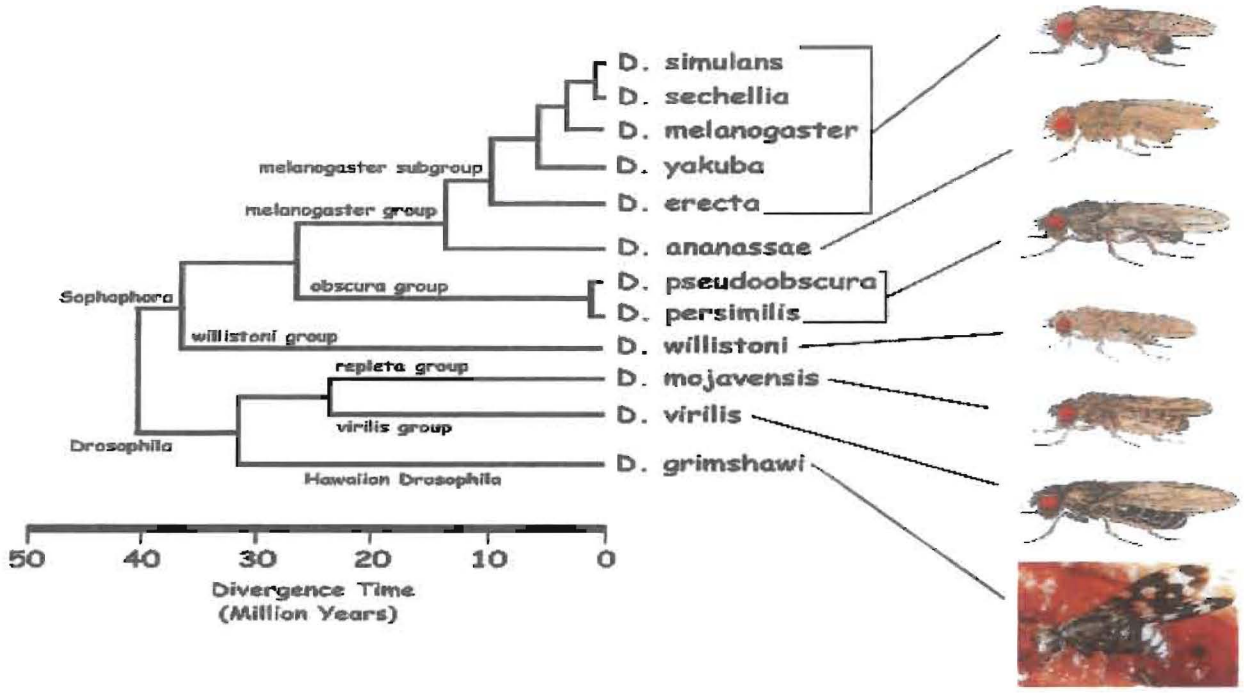


**Fig. 1** This phylogeny depicts the evolutionary relationships among the genera in the family *Drosophilidae*. The 12 *Drosophila* species shown all have had their genomes sequenced. *D. melanogaster's* closest evolutionary relative is *D. simulans*.

Figure modified from: http://insects.eugenes.org/DroSpeGe/ and Gilbert (2007).

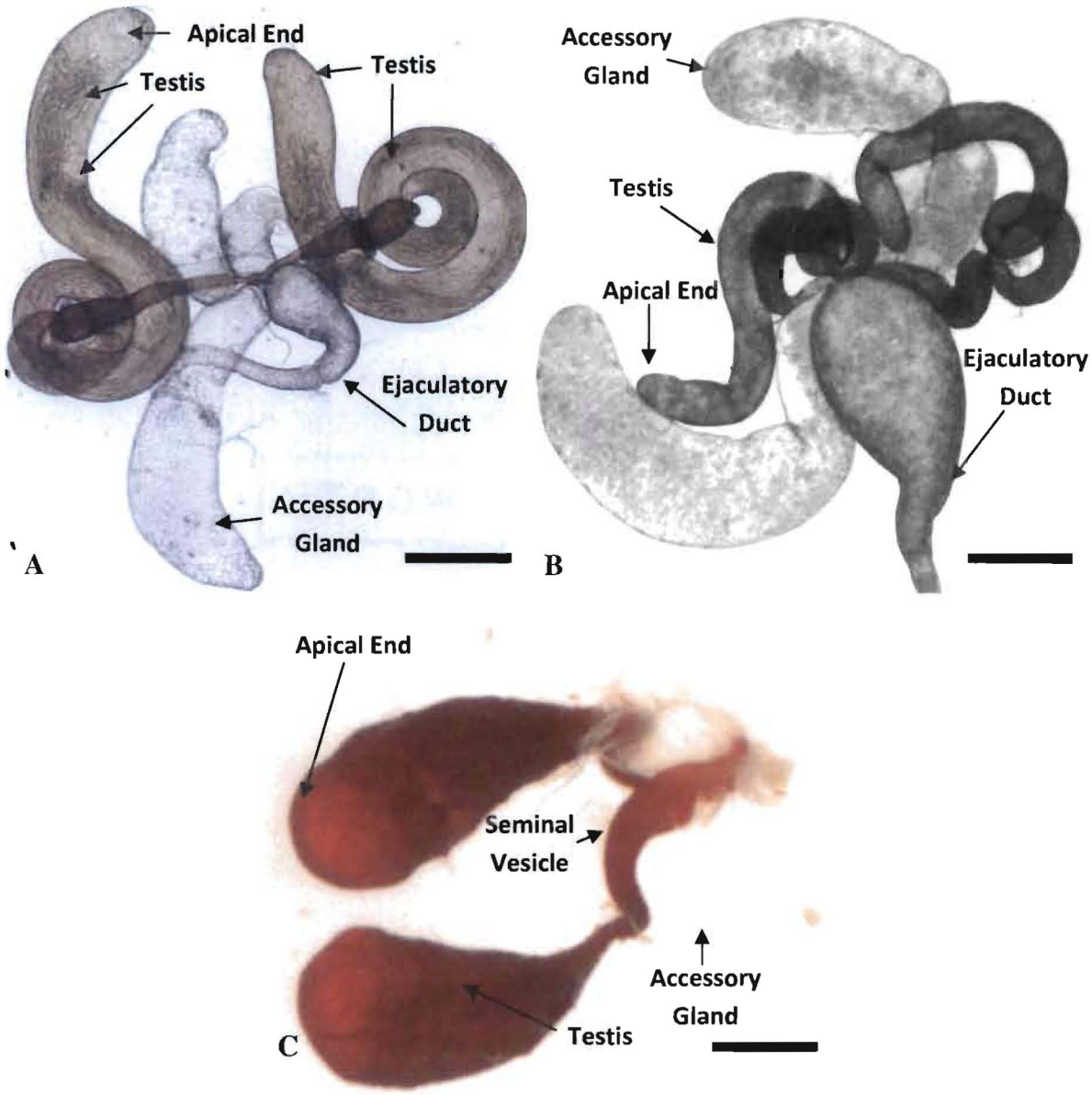


**Fig. 2** Brightfield micrographs of whole testes dissected from adult flies. (A) *D. melanogaster,* image montage (B) *D. simulans*, image montage (C) *D. pseudoobscura*. Scale bar = 100 μm

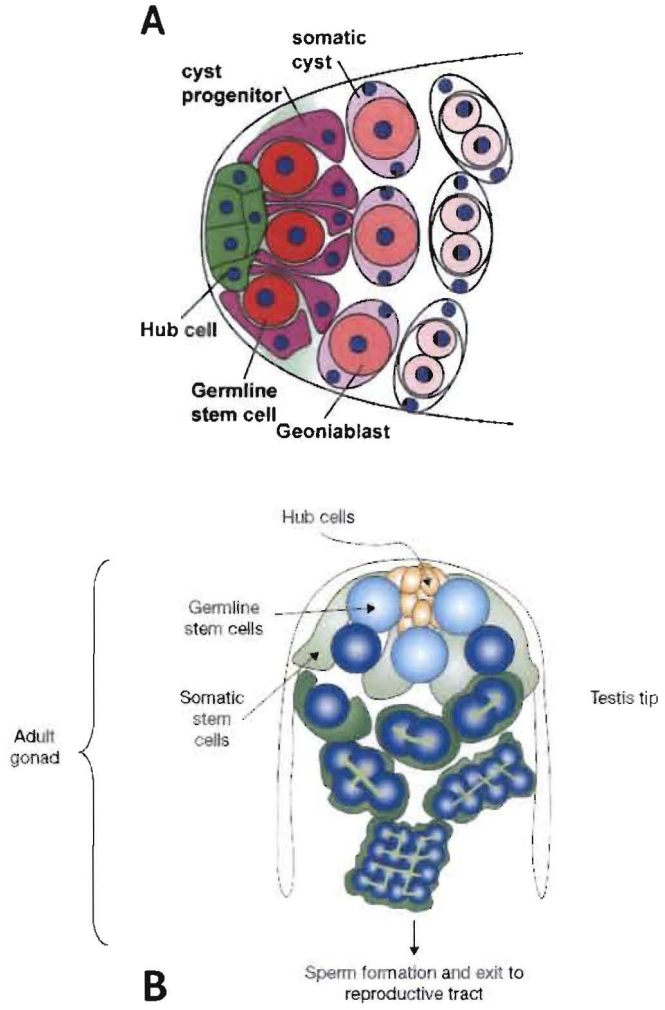


**Fig. 3** Two different depictions of the stem cell niche in *D. melanogaster*. (A) (Green) putative hub cells and (Purple) cyst progenitors function as a niche to maintain GSCs, allowing (Pink) germ cells outside the niche to differentiate. From Xie, T. (2008). (B) Another representation of the apical tip of *D. melanogaster* testis. The stem cell niche within the *Drosophila* testis is created by a group of somatic stem cells known as the putative hub, which is located at the tip of the testis. The putative hub regulates a population of (Light Blue) germline and (Light Green) somatic stem cells that produce undifferentiated progeny required for spermatogenesis. Within the embryonic gonad the putative hub develops which forms as (Yellow) germ cells joined together with clusters of (Green) somatic gonadal precursors. The gonialblast will undergo a series of mitotic divisions which result in 16 spermatocyte cysts. From Van Doren, M. (2007).

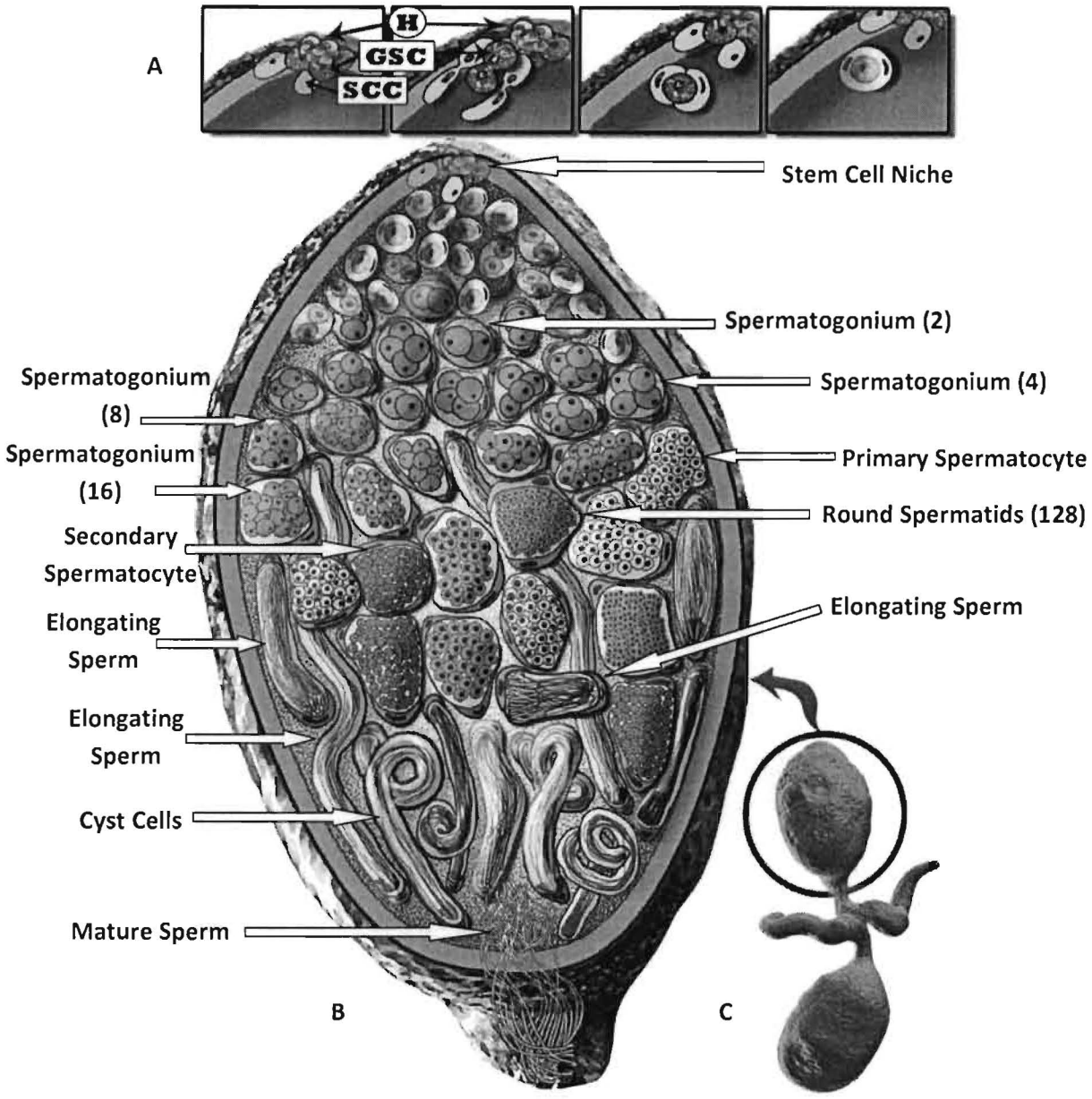


**Fig. 4.** A diagram of *D. pseudoobscura* testis. Illustrated by Kenji Nakai. (A) Visualization of the putative hub cells, germ stem cells and somatic cyst progenitor cells. (B) The internal structures showing spermatogonia, primary spermatocytes, secondary spermatocytes, round spermatids, elongating sperm and mature sperm. (C) Representation of the whole testis. From Njogu, M., Ricketts, P-G., Klaus, A.V. (2010).

When stem cells are removed from their natural environment many stem cells will lose their ability to self-renew, which suggests that the stem cells niche is imperative in controlling and maintaining stem cell behavior (Yamashita *et al.*, 2005). In the *Drosophila* apical testis, the stem cell niche appears to release signals responsible for stem cell renewal, survival, and maintenance. Additionally, cell-cell adhesion between stem cells and putative hub cells anchors stem cells within the niche and the physical relationship of stem cells and niche cells to each other results in the orientation of stem cell mitotic spindles so that division occurs in a plane perpendicular to the putative hub (Xie and Spradling, 2000; Song *et al.*, 2002; Kiger *et al.*, 2001; Tulina and Matunis, 2001).

The discussion of the stem cell niche first began for the utilization of the human hematopoietic system (Trentin, 1970). A "homing" process is used where the niche can retain its stem cells by recruiting more stem cells (Whetton and Graham, 1999). Once the GSC niches are depleted of their GSCs, the niche will still maintain their stem cell features and even continue signaling more somatic stem cells to take up the empty space (Kai and Spradling, 2003). To maintain the stem cell identity and appropriate number of stem cells there needs to be a localized signaling between stem cells and the niche (Fuchs *et al.*, 2004). With high levels of *Drosophila* E-cadherin (DE-cadherin) and β-cadherin at the boundary between the putative hub and GSCs there is a tight bond of GSCs to their niche (Yamashita *et al.*, 2003). The stem cell populations rely heavily on signals that the putative hub produces allowing for proper differentiation and maintenance. Within the putative hub cells are an unpaired ligand (*upd*) where *upd* acts as a signal to activate janus kinases and signal transducers and activators of transcription (JAK-STAT) to control GSCs/SSCs self-renewal in the *Drosophila* male testes (Brawley and Matunis, 2004; Kawase *et al.*, 2004; Kiger and Fuller, 2001; Schulz *et al.*, 2004; Shivdasani and Ingham, 2003;

Tulina and Matunis, 2001). When *upd* is over expressed it can lead to an accumulation of somatic cells in testes, which suggests that *upd* can also control the maintenance of SSCs (Kiger *et al.*, 2001; Tulina *et al.*, 2001). Glass bottom boat (*gbb*) and decapentaplegic (*dpp*), which are part of the BMP pathway, are expressed in the putative hub and somatic stem cells regulate the proliferation of spermatogonial cells (Matunis *et al.*, 1997). Although, with the dpp signaling pathway there is a restriction of the proliferation of the differentiated daughter cells (Lin, 1998). The *Drosophila* heat shock protein *hsp83* gene plays an essential role for Raf-mediated signaling (Van Der Straten *et al.*, 1997), tyrosine kinase signaling (Cutforth and Rubin, 2004) and spermatogenesis (Yue *et al.*, 1999). Through the Raf/MAPK (mitogen-activated protein kinase) pathway the *Drosophila* EGFR (DER) functions in SSCs (Singh & Hou, 2008). In order for the signal of the SSC to GSC to occur is with the presence of the DER/Raf/MAPK, which helps support the differentiation of GSCs and restrict its self-renewal. When the signal is not sent appropriately to the GSCs, the daughters of the GSCs will be able to retain their stem cell identity (Singh & Hou, 2008).

Insulin signaling also plays an essential role in *Drosophila* spermatogenesis for cell division in male GSCs to produce new germline cysts and for GSCs to renew (Ueishi *et al.*, 2009). Within the cyst cells there is an activation of the epidermal growth factor receptor signaling pathway, which can prevent the proliferation of GSCs or help differentiate once the germ cells leave the stem cell niche (Kiger *et al.*, 2000; Matunis *et al.*, 1997; Schulz *et al.*, 2002; Tran *et al.*, 2000). In addition, cell to cell communication is essential for the survival and differentiation of the spermatogonia and cyst cell component (Leatherman and Dinardo, 2008; Gilboa, 2008).

Within the *Drosophila* testis the GSC number and spermatogenesis activity will undergo age related changes where it will start to decline (Boyle *et al.*, 2007; Wallenfang *et al.*, 2006). During the aging process the division rate of GSCs slows down significantly where this slowing correlates with a reduction in the number of somatic putative hub cells that contribute to the stem cell niche (Wallenfang *et al.*, 2006). The putative hub cells within the testes of older males present reduced expression of E-cadherin and *upd* transcription, which correlates with an overall decrease in stem cell number in each niche (Boyle *et al.*, 2007).

Brawley and Matunis (2004) reported that in the male germline, 4-cell and 8-cell spermatogonial cysts break down to form individual cells that re-initiate self-renewing divisions. Among *Drosophila* species the number of spermatogonial divisions differs and its control may be a target of evolution (Fuller, 1998). *D. melanogaster* and *D. simulans* undergo four rounds of mitotic division, which will produce 16 primary spermatocytes that are surrounded by two cyst cells, whereas *D. pseudoobscura* undergoes five rounds and contains cysts containing 32 primary spermatocytes. In *D. melanogaster* testes, the number of mitotic divisions is limited by *bam* expression which appears to initiate spermatogonial differentiation into primary spermatocytes (Yamashita, 2005). The primary spermatocytes undergo a growth phase where the cells increase gene expression as well as increase in volume and size. The primary spermatocyte stage lasts for approximately 90 hours and during this time the cells grow enormously, with a 25-fold volume increase (White-Cooper, 2008). Primary spermatocyte cysts will then commit to two meiotic divisions resulting in haploid round spermatids and finally post-meiotic differentiation, which results in mature elongated sperm (Fuller, 1993). Once the meiotic divisions are completed and the round spermatids have undergone the changes that transform them into fully elongated cells, the resulting sperm cells in *D. melanogaster* are roughly 2 mm in length (White-Cooper, 2008).

It takes approximately 130 hours to produce 64 spermatids after the initiation of spermatogenesis *in vivo*; 16-cell spermatogonia take 96 hours to differentiate into mature spermatids *in vivo* (Tokuyasu, 1975a).

In *Drosophila*, one of the late stages of spermatogenesis is individualization, which results in remodeling and deposition of the plasma membrane around each individual sperm. It involves an actin cone structure and many other components which help regulate the actin dynamics to deposit the membrane along the length of the sperm (Xiao & Yang, 2007; Noguchi and Miller, 2003; Fabrizio *et al.*, 1998). The final event in the differentiation of sperm is when the mature sperm coil, burst from the cyst and finally pass into the seminal vesicle for storage. Typically, each of the paired testes opens into the sperm duct, part of which is dilated to form the seminal vesicles (Demerec, 1950), and which unite to connect with the ejaculatory duct (Joly *et al.*, 2003). When *Drosophila* mate the mature sperm move into the ejaculatory duct. The sperm will be mixed with fluids produced by the accessory glands and finally released into the female so that the life cycle can occur continually.

**Current Approach**

In the current work, we sought to test the efficacy of commercially available primary antibodies (originally generated against *Drosophila melanogaster* testes antigens) in two species of interest, *D. simulans* and *D. pseudoobscura*. The overall goal was to characterize the stem cell niche and testicular architecture in *D. simulans* and *D. pseudoobscura* as compared to *D. melanogaster*. We also did some preliminary staining of *D. persimilis* testes as this species is the sister to *D. pseudoobscura*. We used two primary antibodies, anti-fasciclin III and anti-Vasa. Fasciclin III is a cell adhesion molecule that is expressed on a subset of neurons and axons which

will stain the hub cells in *Drosophila* (Grenningloh *et al.* 1991). Vasa is a protein that acts as a marker of the germline. It is part of the DEAD-box family of genes where it can encode ATP dependent RNA helicase and helps in assembling the pole plasm (Hay *et al.*, 1988). The anti-vasa antibody specifically stains GSCs and all germ cells. MitoTracker Red CMXRos selectively stains mitochondria within the testes (Poot et al., 1996). According to Ichihara *et al.*(2007), the highest amount of mitochondria is found in the hub cells, which are located at the apical tip of the testes. Using these probes, we attempted to visualize the stem cell niche in three dimensions using confocal laser scanning microscopy (CLSM). The optical sectioning of the CLSM provides allows for highly detailed and accurate collections of the morphological structures of interest. Using this approach, we were able to definitively localize the hub for *D. similans* using anti-fasciclin III, but not for *D. pseudoobscura*. Anti-vasa staining was inconclusive for all species analyzed. Staining with MitoTracker Red CMXRos showed multiple staining sites within the apical testes for all species tested. It is not clear which of these sites might represent the hub. Our results indicate that anti-fasciclin III works well in *D. simulans* with the fixation and staining protocol chosen for this work, however, further investigation is needed to show efficacy in other species.

# MATERIALS AND METHODS

## I. Fly stock and cultures

The fly stocks for *D. melanogaster, D. simulans* and *D. pseudoobscura* were obtained from Tucson *Drosophila* Stock Center (University of Arizona, Tucson). They were cultured in our laboratory and given Jazz Mix *Drosophila* medium (Fisher) at 25°C.

## II. Dissection of *Drosophila* testes from adult flies

Males and females can be readily sexed using a dissecting microscope, or with the naked eye. The adults can be distinguished from each other in the following ways: (1) size of adult: the female is generally larger than the male, (2) shape of abdomen: the female abdomen curves to a point whereas the male abdomen is round and much shorter, (3) markings on the abdomen: alternating dark and light bands can be seen on the entire rear portion of the female, the last few segments of the male are fused, (4) appearance of sex comb: only males have a sex comb, a fringe of black bristles on the forelegs, (5) external genitalia on abdomen: located at the tip of the abdomen, the ovipositor of the female is pointed. The claspers of the male are darkly pigmented, arranged in circular form, and located just ventral to the tip. The testes were dissected from adult males as follows: Flies were transferred from an active culture to an empty vial and anesthetized on ice or with the use of a $CO_2$ gun. A depression slide was placed onto a dissecting microscope and an adult male was selected from the vial and placed on a small drop of 4% formaldehyde in PBST (1X PBS, 0.05% Triton X-100) on the depression slide. The head of the fly was gently removed with a pair of fine dissecting needles and head pushed away from the working space. The anterior end of the fly was held with one dissecting needle and the second one was placed on the posterior tip of the fly abdomen. The internal organs were gently pulled from

the fly by moving the second dissecting needle away from the fly body. The paired testes were then separated from the rest of the internal organs without rupturing them. The testes of *D. melanogaster* and *D. simulans* are naturally pigmented yellowish/greenish, whereas *D. pseudoobscura* are pigmented bright orange.

### III. MitoTracker Red CMXRos staining

A depression slide was placed onto a dissecting microscope and adult testes were removed from the fly as described above. Five to ten *Drosophila* testes were dissected and fixed in 4% formaldehyde in PBST. The testes were fixed inside a humidified chamber for 20 minutes at room temperature in 4% formaldehyde in PBST to keep the testes moist. The testes were then rinsed twice with 1X PBS for 5 minutes each. In order to label the mitochondria, the testes were placed in a spot plate well and incubated with 200 nM Mitotracker Red CMXRos (Invitrogen) in PBS for 20 minutes at room temperature, followed by rinsing three times at room temperature in PBST, 10 minutes each rinse in a different well on the spot plate. The testes were mounted in Mowiol medium supplemented with 5 ug/mL DAPI on a glass slide and a coverslip was gently placed over the testes. The mounting media was allowed to set overnight and the specimen was imaged on an Olympus Fluoview 1000 confocal laser scanning microscope (CLSM).

### IV. Immunohistochemical staining

Two primary antibodies were used for each of the *Drosophila* species, anti-fasciclin III (Developmental Studies Hybridoma Bank, DSHB) and anti-vasa (DSHB). The dilutions used are summarized in summarized in Table 1. Testes were dissected in 4% formaldehyde in PBST and fixed for 20-40 minutes in a humidifier chamber. The testes were then rinsed two

times in PBS for 5 minutes each. The testes were placed into a spot plate where they were blocked in 10% bovine serum albumin (BSA) overnight at 4°C in a humidified chamber. The next day they were incubated in primary antibody overnight at 4°C. After the incubation period, the testes were washed three times in PBST for 15 minutes each. The testes were incubated in secondary antibody for 2 hours at room temperature in a spot plate. The following secondary antibodies were both used at 1:200: Alexa Fluor 546 donkey anti-mouse (Invitrogen) and Alexa Fluor 488 rabbit anti-rat (Invitrogen). The testes were counterstained mounted in Mowiol supplemented with 5 ug/ml DAPI. A coverslip was placed over the testes and then viewed on the CLSM.

## V. Confocal microscopy

Samples were imaged on an Olympus FV-1000 CLSM. We visualized all of our samples by collecting thin optical sections, known as a z-series, from thick specimens. Samples were initially located using the wide-field fluorescence imaging mode at 10X before switching to the 40X or 60X oil immersion lens for data collection. Light exposure was limited in order to avoid bleaching the dyes. The reporter dyes are summarized in Table 2 (Alexa Fluor 488, Alexa Fluor 546, DAPI and MitoTracker). For each dye, the high voltage and the offset were set to appropriate levels. Using the High/Low look-up table (LUT), the high voltage was either increased or decreased until only a few red pixels (corresponding to pure white) were present in the image. The offset was similarly set so that no blue pixels (pure black) were present in the image before collecting the data. Confocal image stacks (optical sections) were collected by manually setting the upper and lower volume limits and setting the scan rate to 8 microsceonds/pixel.

## VI. Image processing, 3-D reconstruction and movie generation

Using Imaris 7.1 (Bitplane AG, Zurich, Switzerland) software, optical image stacks were reconstructed into 3-D volume renderings of the specimen. Image analysis and maximum intensity projections (MIPs) were done using Imaris. The 3-D rendering and image processing programs were run on a PC visualization workstation. Movie generation was performed by capturing real-time motion of rendered models from the ImarisSlice and ImarisSurpass modules using Hypercam version 2.16.01 (Hyperionics technology, Murrysville, PA).

| Antibody | Cell Type | Species raised in | Suggested Dilution | Source |
|---|---|---|---|---|
| Fasciclin III | Hub | Mouse | 1:50 | DSHB |
| Vasa | GSCs and all germ cells | Rabbit | 1:5000 | DSHB |

**Table 1**. Primary antibodies specifically used for labeling hub and male germline stem cells and their progeny.

| Dyes | Excitation (nm) | Emission (nm) |
|---|---|---|
| DAPI | 358 | 461 |
| MitoTracker Red | 579 | 599 |
| Alexa Fluor 488 | 495 | 519 |
| Alexa Fluor 546 | 554 | 570 |

**Table 2**. List of excitation and emission wavelengths of dyes utilized in fluorescence.

# RESULTS

## I. MitoTracker CMXRos Red staining

As noted earlier, mitochondria have been reported to be more prevalent in hub cells versus other cell types within the apical testes in *D. melanogaster* (Ishihara et al., 2007). We tested this in *D. simulans* and *D. pseudoobscura* to try to determine the morphology and location of hub cells by utilizing MitoTracker Red CMXRos which specifically stains mitochondria in fixed tissue. *D. melanogaster* was used as a positive control. In *D. melanogaster* (**Fig. 5A**) multiple localized regions in the apical end were stained. One large region was evident (arrow) and was surrounded by smaller stained regions. In. *D. simulans* (**Fig. 5B**) a similar staining pattern was detected. In *D. pseudoobscura* (**Fig. 5C and 5D**) multiple areas were also stained, but the regions were more scattered than those found in either *D. simulans* or *D. melanogaster*. Additionally, in *D. pseudoobscura* did not appear to have a larger region surrounded by smaller satellites (**Fig. 5D**).

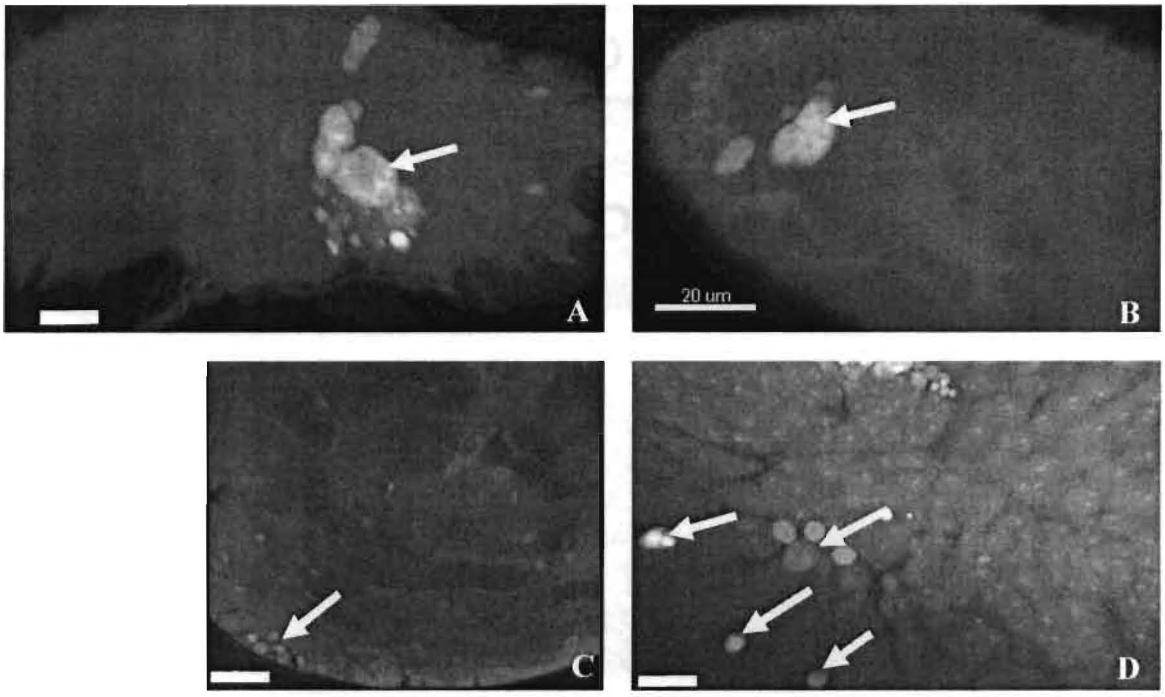


**Fig. 5** MitoTracker Red CMXRos specifically stains mitochondria. (A) *D. melanogaster* (40x oil) apical tip stained with MitoTracker Red CMXRos. (B) *D. simulans* (40x oil) apical tip stained with MitoTracker Red CMXRos. Micron marker 20 μm. (C) *D. pseudoobscura* (40x oil) apical end stained with MitoTracker Red CMXRos. (D) Enlarged view of *D. pseudoobscura* (40x oil) apical end stained with MitoTracker Red CMXRos. All of the images are maximum intensity projections of data collected by confocal microscopy. Scale bar = 50 μm

## II. Immunohistological staining of *Drosophila* testes (anti-Fasciclin III)

We also attempted to detect the hub in *D. simulans* and *D. pseudoobscura* using an antibody against the fasciclin III protein. The positive control, (**Fig. 6B**) shows a bright signal (arrow) in the apical region of the testes for *D. melanogaster*. *D. simulans* (**Fig. 6D**) showed a similar bright signal as *D. melanogaster*, indicated by the white arrow. *D. pseudoobscura* (**Fig. 6F & Fig. 7**) showed many bright signals indicated by the white arrows.

There are two species that are a part of the obscura group and are sibling species, *D. pseudoobscura* and *D. persimilis*. As we saw previously, *D. persmilis* (**Fig. 8B**) showed multiple putative hubs as we saw with *D. pseudoobscura*.

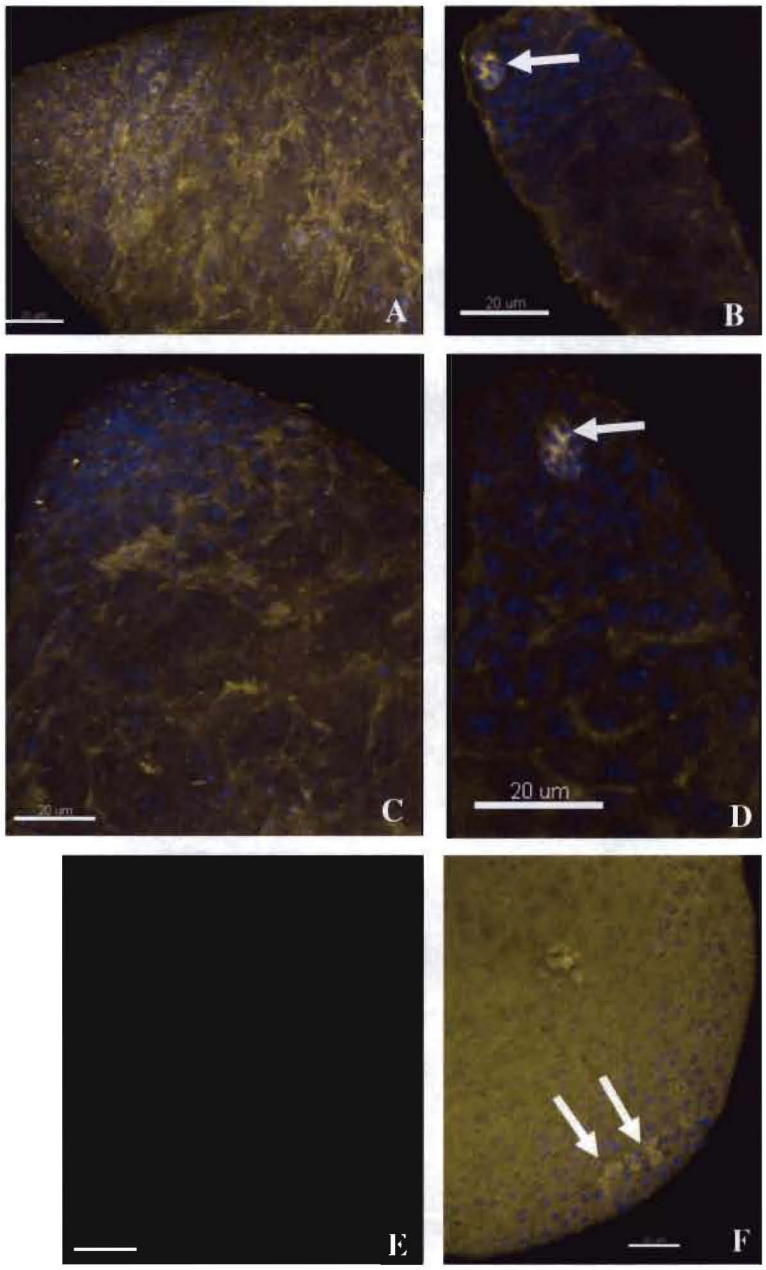


**Fig. 6** Anti-fasciclin (fas) III (yellow) antibody stained with DAPI (blue) to label DNA. Anti-fas III specifically labels the putative hub cells (white arrows). (A) Control of *D. melanogaster* apical tip stained with no primary antibody (anti-fas III). (B) *D. melanogaster* stained with primary and secondary antibody and counterstained with DAPI. (C) Control of *D. simulans* apical tip stained with no primary antibody and conjugated with secondary antibody. (D) *D. simulans* stained with primary and secondary and counterstained with DAPI. (E) Control of *D. pseudoobscura* apical end stained with secondary antibody only. (A-E) All were (40x oil). (F) *D. pseudoobscura* (60x oil) stained with primary and secondary antibodies and counterstained with DAPI. All micron markers are 20 μm.

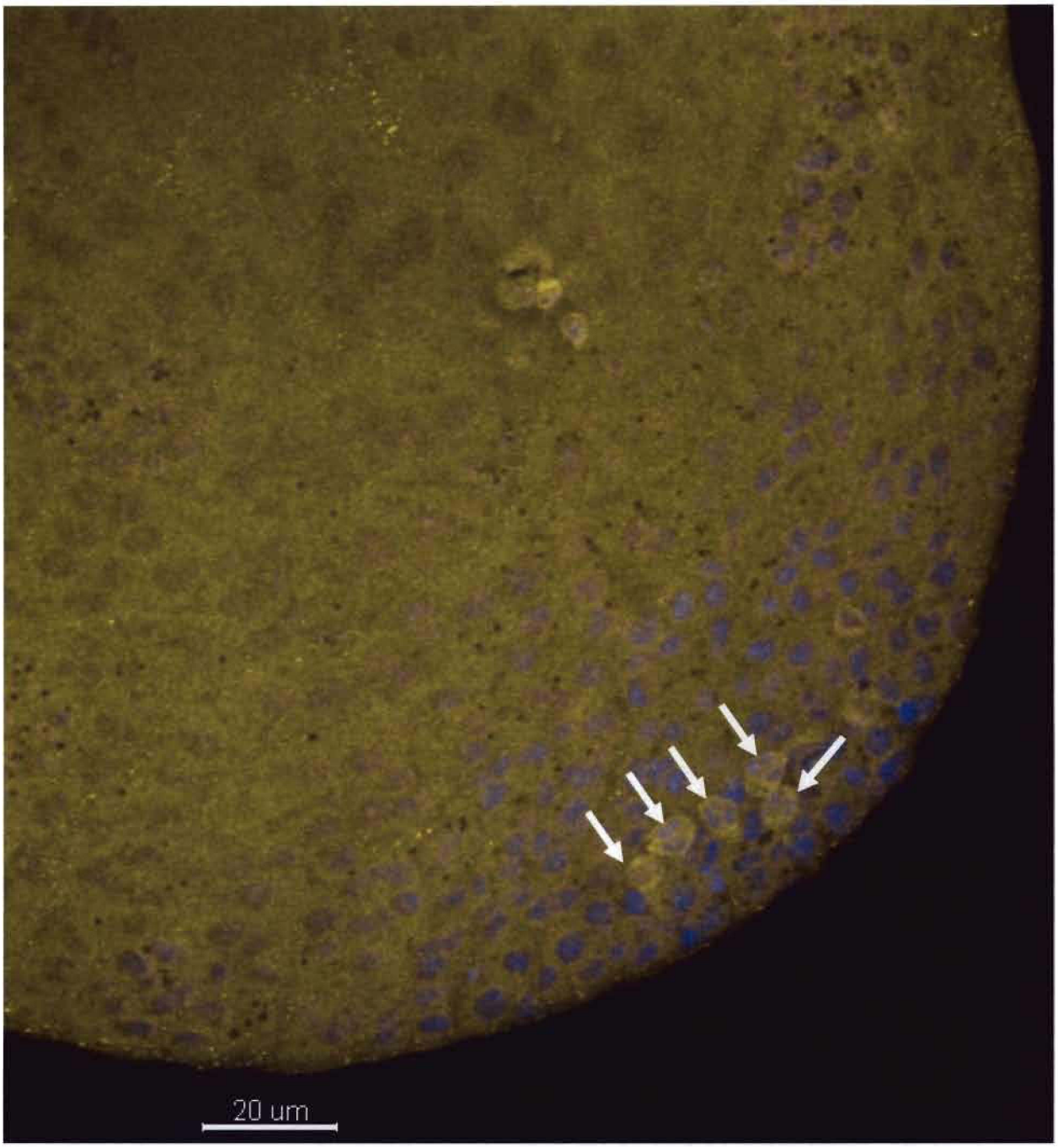


**Fig. 7** Enlargement of (**Fig. 6F**) showing an array of putative hub cells in *D. pseudoobscura.* The white arrows indicate structures that resemble the hub regions seen in *D. melanogaster* and *D. simulans*.

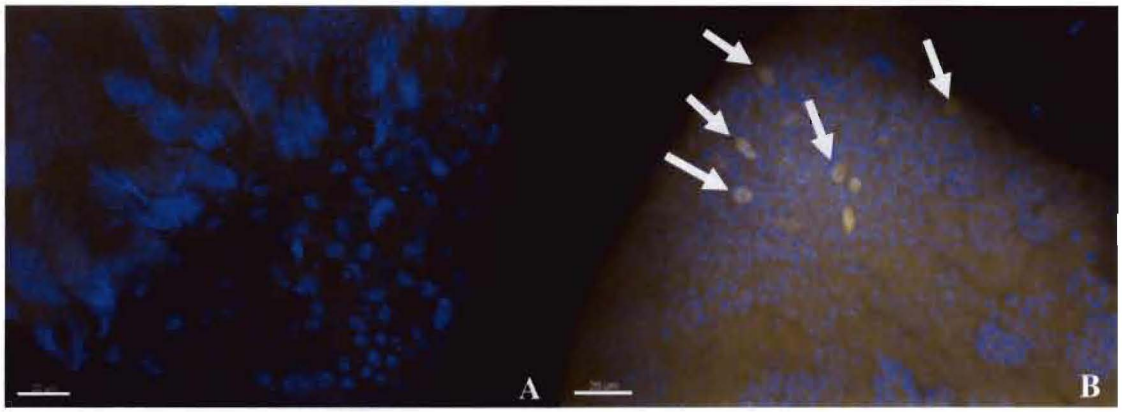


**Fig. 8** Anti-fasciclin (fas) III (yellow) antibody stained with DAPI (blue) to label DNA. Anti-fas III specifically labels the putative hub cells (white arrows). (A) Control of *D. persimilis* (60x oil) apical end incubated secondary antibody only and counterstained with DAPI. (B) *D. persimilis* (60x oil) incubated with primary and secondary antibodies and counterstained with DAPI. All of the images are maximum intensity projections of data collected by confocal microscopy. Both micron markers are 20 μm.

## III. Immunohistological staining of *Drosophila* testes (anti-Vasa)

In order to test for the location of the GSCs and all germ cells within the testes we used an antitbody to Vasa protein. With this antibody we were able to visualize the germ cells in the three species at the apical end of the testes. Although some staining was achieved, no conclusive patterns emerged in the positive control (*D. melanogaster*), or the other species of interest (*D. simulans* and *D. pseudoobscura*). **Fig 9A and 9B** show the control and experimental staining patterns obtained for *D. melanogaster*. Similar results were obtained for *D. simulans* (**Figs. 9C and 9D**) and *D. pseudoobscura* (**Figs. 9E and 9F**). In some cases, scattered structures similar to the structures seen with Mitotracker Red staining were detected in both control and experimental samples (arrows, **Fig 10**) in *D. pseudoobscura*.

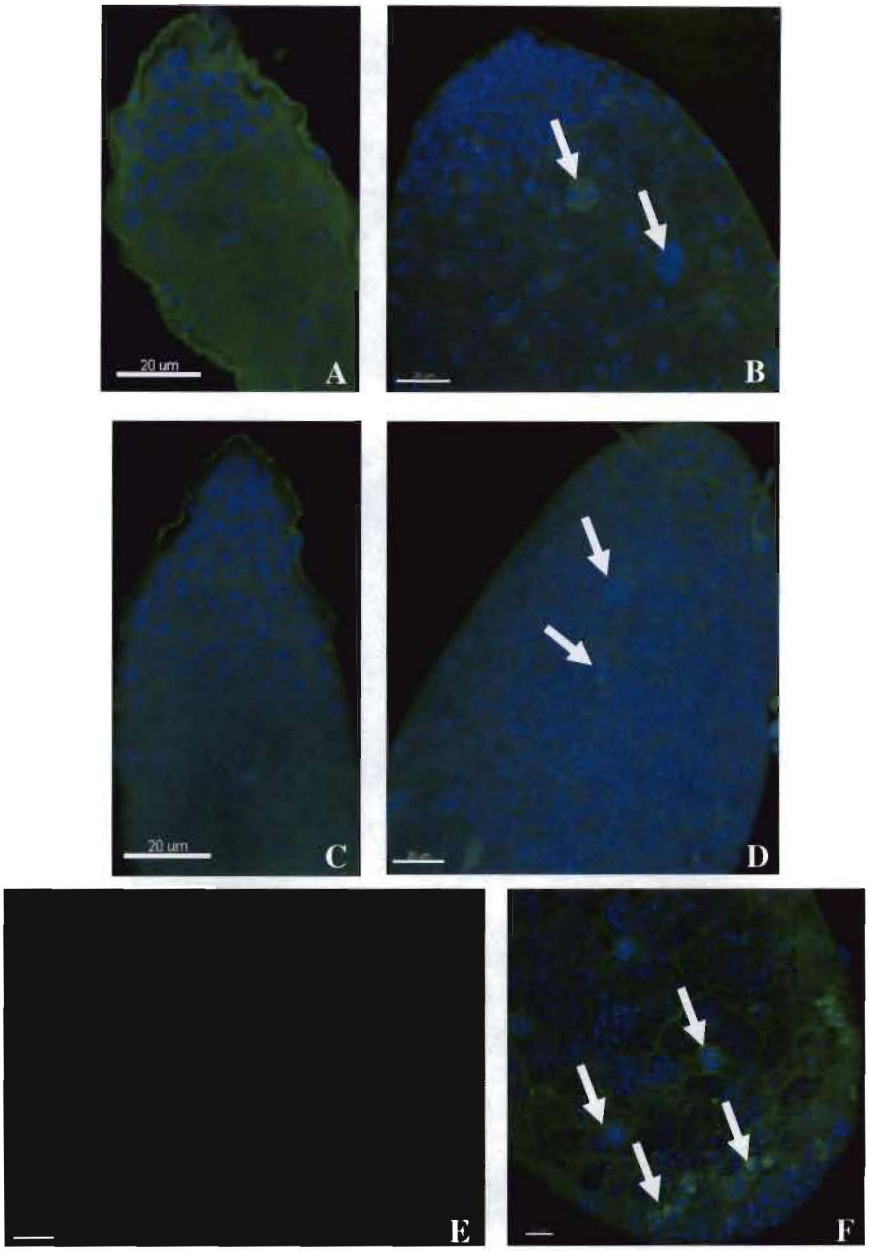


**Fig. 9** Anti-vasa antibody (green) stained with DAPI (blue) to label DNA. Anti-vasa specifically labels GSCs and all germ cells. (A) Control of *D. melanogaster* apical tip stained with no primary antibody (anti-vasa) and conjugated with secondary antibody donkey anti-rat conjugated to Alexa-488. (B) *D. melanogaster* stained with primary and secondary antibody and counterstained with DAPI. (C) Control of *D. simulans* apical tip stained with no primary antibody and secondary antibody conjugated to a different fluorophore. (D) *D. simulans* stained with primary and secondary and counterstained with DAPI. (E) Control of *D. pseudoobscura* apical end stained with no primary antibody and conjugated with secondary antibody. (A-E) All were (40X oil). (F) *D. pseudoobscura* (600x oil) stained with primary and secondary antibodies and counterstained with DAPI. All of the images are maximum intensity projections of data collected by confocal microscopy. All micron markers are 20 μm.

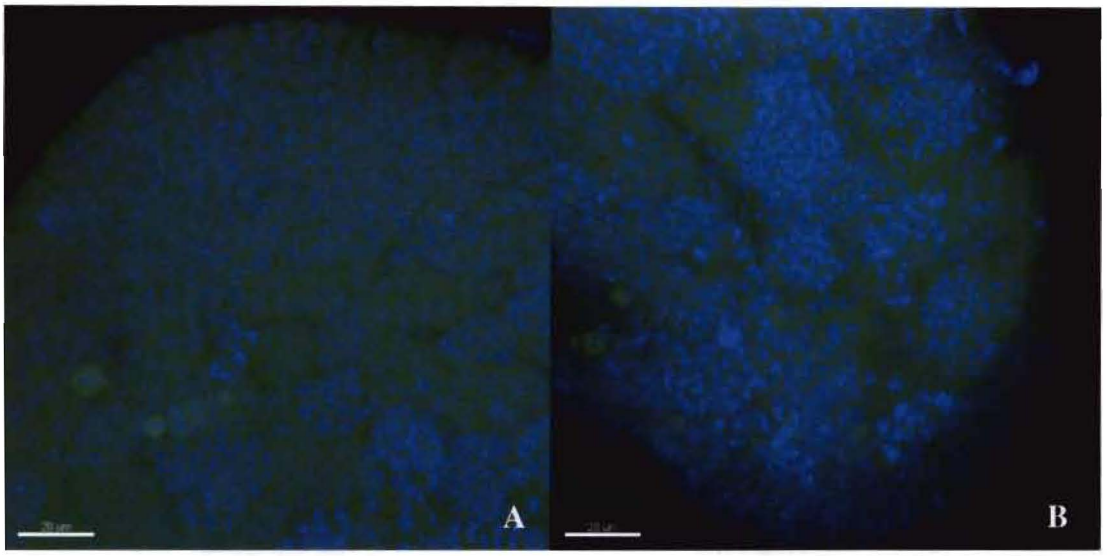


**Fig. 10** Anti-vasa antibody (green) stained with DAPI (blue) to label DNA. Anti-vasa specifically labels GSCs and all germ cells. (A) Control of *D. pseudoobscura* (40X oil) apical end stained with no primary antibody (anti-vasa) and conjugated with secondary antibody donkey anti-rat conjugated to Alexa-488. (B) *D. pseudoobscura* (60x oil) stained with primary and secondary antibodies and counterstained with DAPI. All of the images are maximum intensity projections of data collected by confocal microscopy. Both micron markers are 20 μm.

## DISCUSSION

Our results indicate that it may be possible that there are multiple hub locations in *D. pseudoobscura*. We were able to locate a single hub cell region in both *D. melanogaster* and *D. simulans* with the ant-fasciclin III staining. Anti-fasciclin staining of *D. pseudoobscura* is still inconclusive, but multiple putative hub cell regions seemed to be detected in *D. pseudoobscura*. To support this theory we did a preliminary staining with *D. persimilis* (**Fig. 8**) to determine if we could produce similar results to *D. pseudoobscura*. Similar staining patterns were obtained in this species, which may mean that multiple hub cell regions are present. We suggest the possibility of multiple hubs in both *D. pseudoobscura* and *D. persimilis* due to their different testicular morphology as compared to *D. melanogaster* and *D. simulans*. Mitotracker Red CMXRos stains mitochondria within the testes and the regions containing the highest amount of mitochondria are known to be the putative hub cells. In (**Fig. 5C & 5D**) there are multiple locations where mitochondria are being stained in *D. pseudoobscura*, which may support the multiple hub theory. However, multiple regions were also detected in *D. melanogaster* and *D. simulans* using Mitotracker, both of these species have only one hub region as indicated by anti-fasciclin staining.

According to Voog (2008) there has not been any exploration in the precise relationship between somatic cyst cells (SSCs) and hub cells and proposed that SSCs may serve as a source of cells that contribute to the apical hub and consequently the stem cell niche. The SSCs may be a potential source of cells that contribute to the formation of putative multiple hubs.

When comparing *Drosophila* spermatogenesis to mammalian spermatogenesis they are both astonishingly similar. About 61% of all *Drosophila* genes are similar to humans (Lander *et al.*; 2001) and 59% of human genes implicated in disease have identifiable homologues in the *Drosophila* genome (Chien *et al.*, 2002; Reiter, *et al.*, 2001). The stem cell niche is of vital

importance because of the housing and protection it provides for the resident stem cells to support normal development for the differentiated cells. The niche activates many different signals to allow the cells to self renew so it acts as an excellent model to study niche development. Improper regulation of stem cell behavior is known to lead to cancer formation, tissue degeneration, and premature aging (Singh & Hou, 2008).

Since the stem cell niche is of great importance, future studies in this area can lead to possible therapeutic agents to treat cancer, help slow down degenerative diseases and the aging process and even come up with regenerative medicine. To the best of our knowledge, this is the first study to characterize the stem cell niche in both *D. simulans and D. pseudoobscura* in comparison with *D. melanogaster*. A better understanding of how stem cell niches are established and regulated in mammalian systems could facilitate modulation of the niche to enhance transplantation of stem cells in regenerative medicine (Adams, GB, *et al.*; 2007). Perhaps there is a pathway in *D. pseudoobscura* that allows fresh hub cells to differentiate and become detached to form separate, multiple hubs. The data shown here is valuable because in the end we will be able to understand the spermatogenic processes and also have a better understanding of how cell behavior, cellular differentiation, and even stem cell maintenance functions in different *Drosophila* species.

## Continuing Work

In our attempt to characterize the architecture of the testis in *D. pseudoobscura*, we will use different fixation protocols such as acetone-methanol or freezing to try to confirm results obtained in the current work. Also different antibodies known to stain hub cells, such as those against Armadillo and DE-Cadherin, will be tested. Since stem cells start to decrease as the testis ages we will try to repeat staining with *D. pseudoobscura* at known ages (Pupae, 1 day, 2 day, and 1 week adults). Lastly, our lab will try a dual stain with both anti-fasciclin III and anti-vasa to be able to differentiate the hub cells, germ line stem cells and all germ cells in the same *Drosophila* testis. The comparative knowledge gained from these different *Drosophila* species will allow us to reveal the defining mechanisms of the hub cells and germline stem cells in future studies. In the end, knowing the important signals the stem cell niche supplies can help with future potential applications such as therapeutic agents, degenerative diseases, aging and regenerative medicine.

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