Spring 4-15-2016

Apical Testis Structure and the Effects of Cadmium Treatment on Spermatogenesis in Drosophila

Paulina J. Cardaci
paulina.cardaci@student.shu.edu

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

April 2016

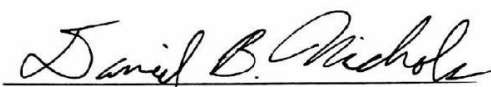Approved By:

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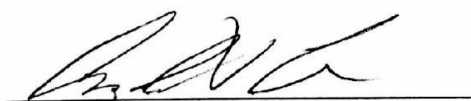Dr. Angela V. Klaus

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Committee Member
Dr. Daniel B. Nichols

_____

Committee Member
Dr. Tin-Chun Chu

_____

Director of Graduate Studies
Dr. Angela V. Klaus

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Chairperson, Biology Department
Dr. Jane L. Ko

was incapable of performing my own research. My lab mates, both undergraduate and graduate, stepped in and assisted with my research; for this I will always be grateful.

My friends and family, especially my grandmother and grandfather, for their much needed and tireless support. Without their support and encouragement I would not have been able to achieve my Master's degree. In the words of my grandfather, "If it was easy, then everyone would do it". This quote haunted me throughout my graduate career but ultimately came to inspire me.

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Abstract

The fruit fly *Drosophila melanogaster* is used extensively as a model for studying molecular, genetic and cellular aspects of human disease and physiology. Our lab has used *D. melanogaster* and related species to study the structure of the testis stem cell niche, as well as other aspects of spermatogenesis. We previously revealed a novel stem cell niche structure in *D. pseudoobscura*, a distant relative of *D. melanogaster.* The signaling center of the *D. melanogaster* stem cell niche has a well-characterized rosette arrangement of fasciclin-positive cells terms the "hub". *D. pseudoobscura,* however, lacks a punctuate hub and instead displays a hemispherical fasciclin-positive zone that fills the apical end of the testis. The first aim of the current work was to characterize the stem cell niche in two additional species based on their evolutionary relationship to *D. melanogaster* and *D. pseudoobscura*: *D. ananassae* and *D. persimilis*. *D. persimilis* is part of the obscura group and is closely related to *D. pseudoobscura; D. ananassae* is part of the melanogaster group. Our work shows that *D. ananassae* has the rosette hub morphology while *D. persiimilis* displays the *D. pseudoobscura* morphology.

The second focus of this project was to examine the effects of cadmium ($CdCl_2$) exposure on spermatogenesis in *D. melanogaster*. Cadmium toxicity is well-studied in mammalian testes and sperm production, but not in Drosophila. In order to assess the effects of $CdCl_2$ on spermatogenesis in *D. melanogaster* we developed two assays: a nuclear staining (DAPI) assay to assess cadmium dosage effects on late spermiogenesis and a Live/Dead assay to assess mature sperm viability. The results of the DAPI assay and the Live/Dead assay both show a detrimental effect by $CdCl_2$ on spermatogenesis in *D. melanogaster.* The goal of the DAPI assay was to examine the number and arrangement of sperm bundles in the basal end of the *D. melanogaster* testis following cadmium treatment. The DAPI assay showed that exposure to increasing

concentration of $CdCl_2$ resulted in an increase in the appearance of abnormal sperm bundles. The Live/Dead assay showed: (1) an increase in the total number of sperm present in the seminal vesicle proportional to increasing amounts of cadmium chloride and (2) an increase in the number of dead sperm proportional to increasing amounts of cadmium chloride.

<center>**Introduction**</center>

I.   **Drosophila Male Reproductive System**

Drosophilidae is a large family of flies with over 3000 species.[1] The male reproductive system is comprised of paired testes, accessory glands, and seminal vesicles, as well as a single ejaculatory bulb and duct (**Figure 1**). Spermatogenesis proceeds from the apical end of the testis towards the basal end, where mature sperm are moved into the seminal vesicles via peristaltic action.[2] Drosophila species have one of two types of testicular morphology: long, coiled testes or ellipsoid testes. The majority of Drosophila species have the long, coiled testicular morphology, except those species in the obscura group which have the ellipsoid morphology. Testes of the long, coiled type have a rounded, free floating apical end that contains the germline stem cells that will eventually develop into sperm.[3-7] Testes of the ellipsoid type are wide and oblong in shape with a broad, hemispherical apical end from which germline stem cells develop into sperm precursors in a wavelike fashion.[8] Regardless of testicular morphology, spermatogenesis proceeds from the apical end of the testis towards the basal end where the seminal vesicle is attached to the testis by a narrow duct. Mature sperm empty from the seminal vesicle into the ejaculatory bulb during mating. Accessory glands attach to the male reproductive tract and produce fluids which contribute to the ejaculate (refer to **Figure 1**).
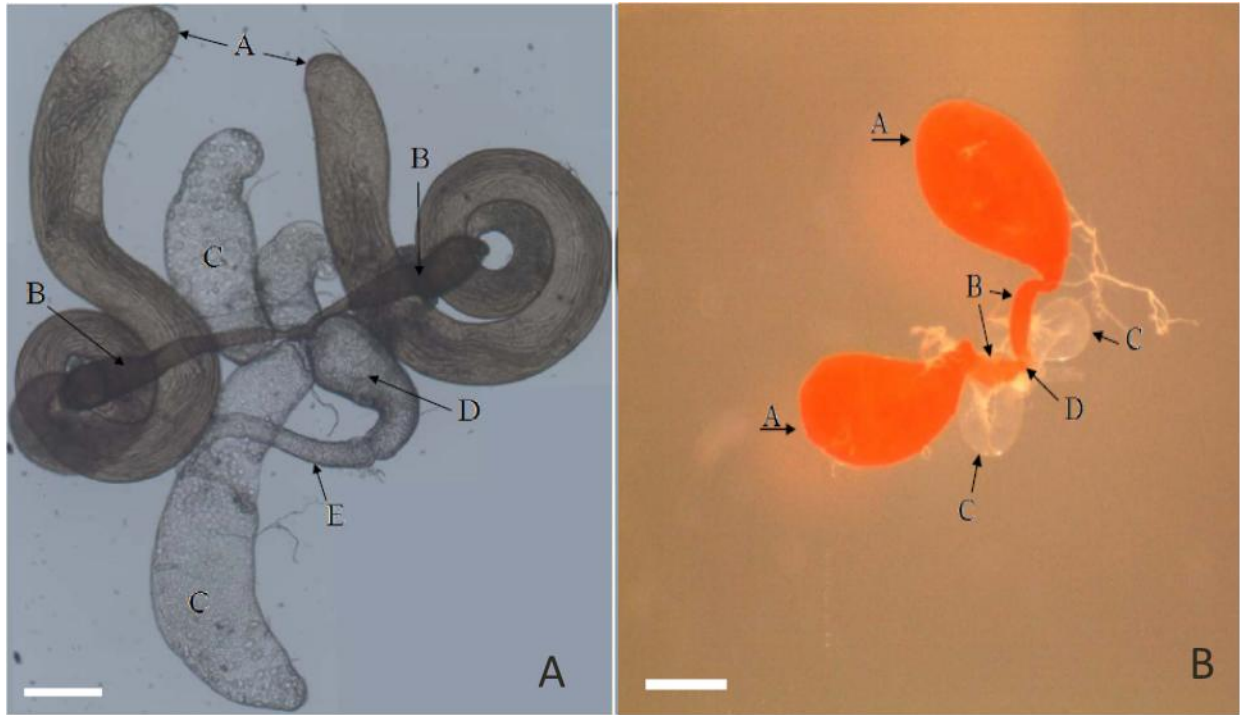
Figure 1: *D. melanogaster* **and** *D. pseudoobscura* **male reproductive systems.** A) Bright field image of *D. melanogaster* testes. A – apical end of the testis. B – seminal vesicles. C – accessory glands. D – ejaculatory bulb. E – ejaculatory duct. The stem cell niche is located in the apical end of the testis. B) Bright field image of *D. pseudoobscura* testes. A – apical end of the testis. B – seminal vesicles. C – accessory glands. D – ejaculatory bulb and duct. Scale bar is 250 μm for both images. (Image from Beaury, 2012).

II. Spermatogenesis

In *D. melanogaster*, spermatogenesis follows an intricately arranged series of steps to yield motile sperm capable of fertilizing eggs. In *D. melanogaster*, spermatogenesis is initiated within the apical end of the testis in a region termed the stem cell niche.[32] This region contains a rosette of somatic cells called the "hub". The hub acts as a signaling center that maintains the stem cell population at a steady level so that sperm can be continually produced in a male fly's lifetime.[1,4,9-10] Germline stem cells and cyst progenitor cells adhere to the hub and give rise to cells that will enter the terminal spermatogenic pathway. Fasciclin III protein is responsible for adherence between the hub cells while Armadillo and DE-Cadherin connect the germline stem cells and cyst progenitor cells to the hub.[11-12] Each germline stem cell will undergo a mitotic division that yields a gonialblast cell that will undergo spermatogenesis and a stem cell that remains attached to the hub.[33] The cyst progenitor cells also undergo mitotic divisions to yield cells that will surround the developing gonialblast throughout the spermatogenic process.[13] (**Figure** 3) The process of spermatogenesis takes place within a capsule called a cyst, which is formed by the union of two cyst progenitor cells.[7,14] The gonialblast inside the cyst undergoes five mitotic divisions that results in 32 diploid clones, now termed spermatogonia.[15] The developing spermatogonia are held together by 1B1, one of several α-Spectrin proteins which make up the fusosome, which forms a cytoplasmic bridge that links the spermatogonia together inside their cyst.[16] The spermatogonia simultaneously enter into a pre-meiotic S-phase and then progress into a G2 phase where they expand their volume by approximately 25-fold. Cells at this stage are referred to as primary spermatocytes.[4,17] Primary spermatocytes undergo the first meiotic division to produce 64 secondary spermatocytes. Secondary spermatocytes undergo the second meiotic division to produce 128 round, haploid spermatids. Haploid round spermatids

process through the post-meiotic stage of spermatogenesis, called spermiogenesis, where they transform into elongated, motile sperm cells capable of fertilization.[18,19] During this process, the nuclei of the spermatids transform into their elongated, terminally differentiated form. Also, during this process the sperm tails are assembled.[19,20] As the developing sperm travel from the apical to the basal end of the testis, the nuclei of the sperm will bunch together to form "arrowhead" structures that are easily distinguishable under confocal microscopy.[19] When the now mature sperm reach the basal end of the testis, they will burst the cyst that has surrounded them for the entire process and enter into the seminal vesicle until they are ejaculated (**Figure 3**).[19,20]

This process has been extensively characterized in *D. melanogaster* but recent work with *D. pseudoobscura* has changed the existing model of spermatogenesis. Recent data suggests that *D. pseudoobscura*, and *D. pseudoobscura* like species of Drosophila, will eclose with a set number of germline stem cells that will differentiate in a wave-like pattern unlike the orderly mitotic divisions of *D. melanogaster* like species.[8]
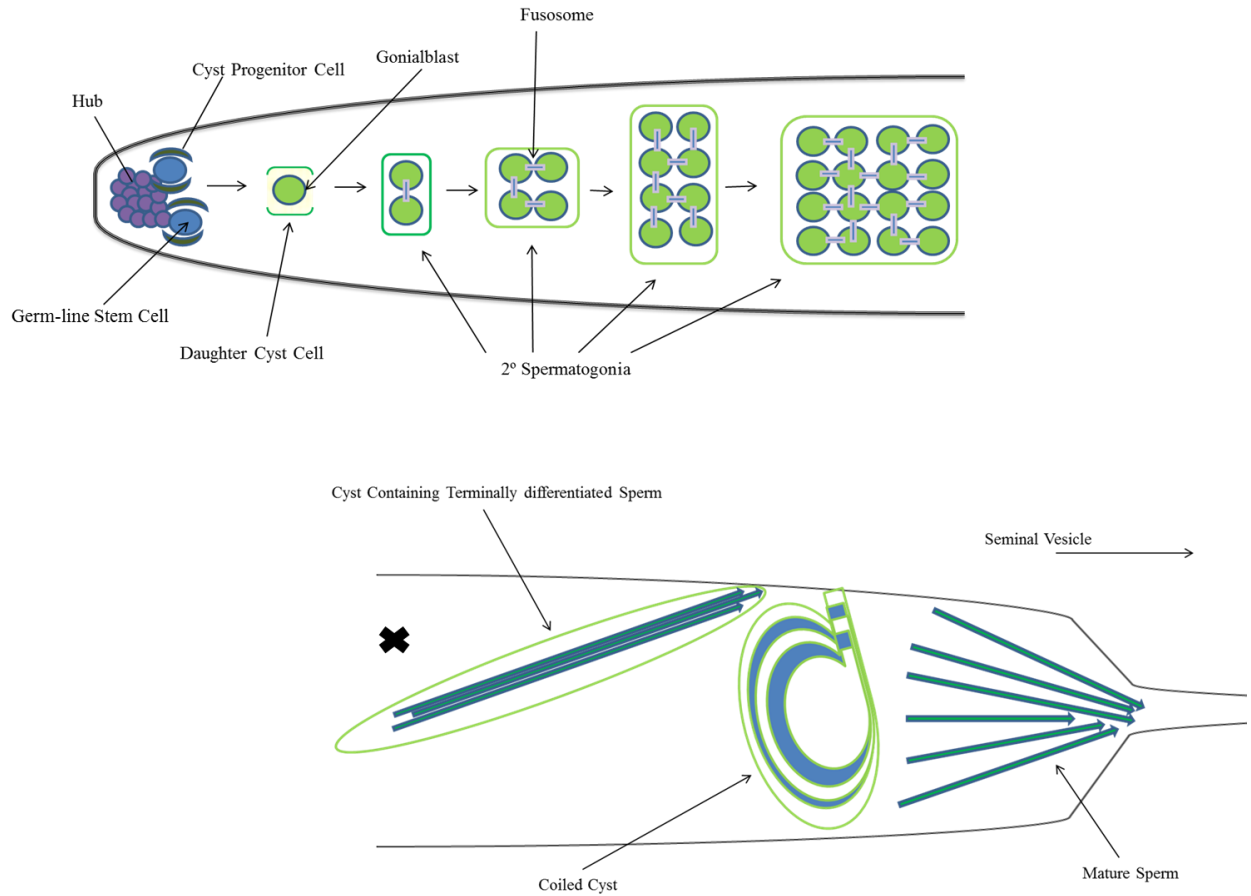
Figure 2: Illustration showing the process of spermatogenesis in *D. melanogaster*. The top of the diagram shows the arrangement of cells in the stem cell niche in the apical end of the testis and the maturation of sperm progressing from gonialblast to secondary spermatogonia. The bottom part of the diagram shows the process of spermiogenesis in the basal end of the testis. It shows the terminally differentiated sperm inside the mature cyst embedded in the epithelium of the basal end of the testis. The cyst will coil around itself and then burst, releasing the mature sperm to travel into the seminal vesicle via peristalsis. The black X on the diagram marks the place in which it is believed $CdCl_2$ disrupts the spermatogenic pathway (Cardaci, 2016).

III. Drosophila Phylogenetic Relationships

The Drosophilidae Family is subdivided into 78 genera. [21] The most commonly studied genus, Drosophila, is the focus of the current work. Over 20 species within the Drosophila genus have their genomes completely sequenced.[22] Specifically within the *Sophophora* subgenus our work will focus on the obscura and the melanogaster groups; *D. pseudoobscura* and *D. persimilis* from the obscura group, *D. ananassae* from the melanogaster group and *D. melanogaster* from the melanogaster subgroup. *D. melanogaster* is the best characterized species of the Drosophila family in regards to testes morphology and the spermatogenic pathway. *D. melanogaster* males have long, coiled testes with a free floating apical end that coils around to a basal end that attaches to the seminal vesicle. *D. ananassae* share the long, coiled testes with *D. melanogaster*. *D. ananassae* is part of the melanogaster group and diverged from the melanogaster subgroup ten million years ago[22] (See **Figure 3**). *D. persimilis* testes have an ellipsoid morphology, characterized by a darker red pigment. *D. persimilis* is part of the obscura group; *D. pseudoobscura* is also a member of the obscura group and shares the ellipsoid testes morphology and red pigmentation of *D. persmilis*. The shared morphology of *D. persimilis* and *D. pseudoobscura* is explained by the fact that they diverged within the obscura group ~500,000 years ago. The melanogaster and obscura group diverged ~30 million years ago, with the melanogaster subgroup diverging ~15 million years ago[23]. This evolutionary divergence explains the drastically different testes morphology seen between the obscura and melanogaster group species (See **Figure 3**).
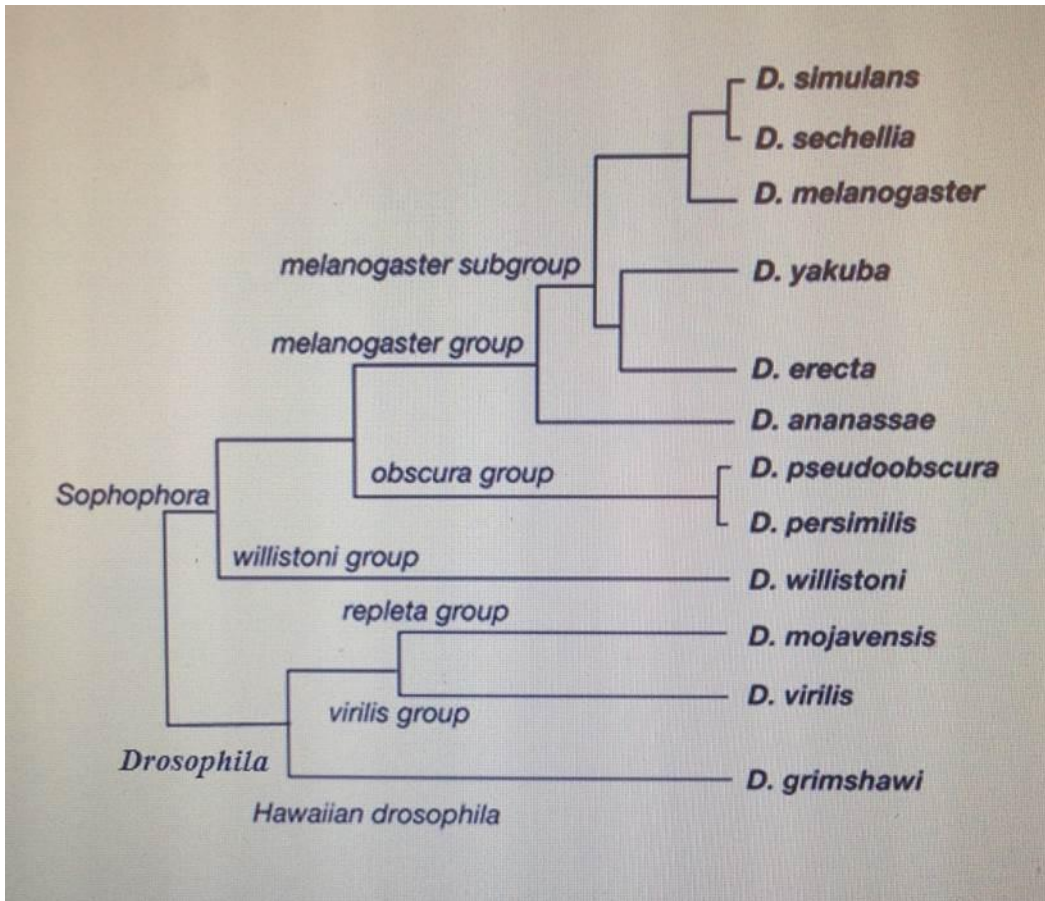
Figure 3: *Drosophila* phylogenies. A phylogenic tree of the original 12 sequences species of *Drosophila* (Cardaci, 2016).

IV. Effects of Heavy Metals on Male Reproduction

Cadmium chloride ($CdCl_2$) is a highly carcinogenic salt that is used extensively in research due to its damaging effects on metabolic processes. Cadmium's damaging effects are due to its ability to produce oxidative stress by depleting glutathione and protein-bound sulfhydryl groups; this results in an increased production of reactive oxygen species such as superoxide ions, hydroxyl radicals, and hydrogen peroxide.[24] These reactive oxygen species will cause lipid peroxidation, DNA damage, membrane damage, altered gene expression, and apoptosis.[24] $CdCl_2$ has been utilized extensively in Drosophila research in order to induce metallotheinein expression.[25,26] $CdCl_2$ is extremely toxic when ingested and has detrimental effects in case of skin contact and inhalation. Exposure to $CdCl_2$ has proven toxicity to the kidneys, blood, liver, mucous membranes, and especially the testes in humans.[24] Cadmium exposure occurs easily as a result from ingestion of certain foods and drinking water, inhalation of contaminated air, tobacco smoke, and ingestion of contaminated soil or dust.[24] There has been extensive research done to examine the effects of cadmium on mammalian male reproduction. Recent work regarding single dose $CdCl_2$ exposure on mouse sperm viability has shown that $CdCl_2$ has a significant short term effect on sperm resulting in abnormal morphology and reduced motility with long term effects (after 35 days) of reduced sperm numbers and reduced sperm motility.[23,27] Also of note, the researchers saw an increase in DNA fragmentation in sperm produced following $CdCl_2$ exposure.[27,28] Another experiment showed that cadmium treatment resulted in increased numbers of apoptotic spermatid and elongate spermatid in seminiferous tubules of rats; they also discovered severe necrosis of the seminiferous epithelium itself.[29]

Spermatogenesis is generally conserved throughout different species and produces structurally similar sperm. Drosophila and *Homo sapiens* will produce very similar sperm, with

exceptions in the shape and length of the sperm head and tails. The process of individualization in the sperm head and the microtubule arrangement of the sperm tail is evolutionary conserved.[13] From this it can be confidently concluded that a detrimental effect on spermatogenesis in Drosophila would translate to a detrimental effect on spermatogenesis in *Homo sapiens.*

Several studies have shown that $CdCl_2$ activates the Metal-responsive transcription factor I (MTF-1) which will bind to the metal responsive elements (MREs) in the promoter region of target genes and upregulates their transcription.[30] Metallothioneins are the most frequently targeted genes for MTF-1 and encodes for small, cysteine rich scavenger proteins that will scavenge heavy metal particles.[30] Two potential MTF-1 interactors in Drosophila have been identified, both related to the regulatory protein Dumpy-30 (Dpy30). Dpy-30L1 is widely expressed in various organs of Drosophila including: larval brain, gonads, imaginal discs, salivary glands and in the brain, testes, ovaries and salivary glands of adult flies. Dpy-30L2 however is selectively expressed in the testes in adult male Drosophila, specifically elongating spermatids. Researchers found that transfection of Dpy-30L1 and Dpy-30L2 inhibited the expression of MTF-1 driven genes; MTF-1 is the only known factor to interact with MREs and inhibits production of metallothionein. They also found that constitutive expression of Dpy-30L1 transgene in flies resulted in elevated sensitivity to both cadmium and zinc (Dpy-30L2 overexpression did not exhibit the same result). Further results of selective gene knockout of Dpy-30L1 and Dpy-30L2: knockout of Dpy-30L1 resulted in viable, fertile adult male Drosophila whereas knockout of Dpy-30L2 results in complete male sterility. They investigated further by examining the sperm motility of Dpy-30L2 knockout inside the female Drosophila reproductive tract and found that motility is almost completely impaired and continue to decrease with aging.[31]

V.   Goals of the Current Work

The current work had two main goals. First, we sought to elucidate the structure of the apical testes in two species related to *D. melanogaster*, one of which displays the coiled tubular testicular morphology (*D. ananassae*) and one which has the ellipsoid morphology (*D. persimilis*). In order to examine the stem cell niche of the four species of interest in the Drosophila genus, the apical testes were examined by utilizing immunofluorescence staining of Fascillin III-positive cells under confocal microscopy. Second, we aimed to examine the effects of cadmium chloride exposure on spermatogenesis. In order to assess the effects of cadmium chloride on spermatogenesis, two assays were developed: (1) a DAPI assay to examine sperm bundles in the basal end of *D. melanogaster* testis and (2) a Live/Dead assay to examine total, live, and dead sperm present in the seminal vesicle of cadmium chloride treated *D. melanogaster* testes.

Materials and Methods

I.   Fly Stock and Cultures

D. melanogaster, D. ananassae, D. pseudoobscura and *D. persimilis* were obtained from the University of California, San Diego, *Drosophila* Species Stock Center. Flies were maintained on a Jazz Mix *Drosophila* media (Fischer Scientific) in plugged vials at 25ºC.

II.   Cadmium Chloride Food Preparation

Solid $CdCl_2$ salt (Sigma Aldrich) was added to deionized water to prepare a 10 mM stock. $CdCl_2$ food was prepared at concentrations of 10 µM, 25 µM, 50 µM, 75 µM, 100 µM, 300 µM, and 500 µM. Food was prepared by adding 10 µL, 25 µL, 50 µL, 75 µL, 100 µL, 300 µL, and 500 µL to 10 mL of cooling JazzMix fly food and allowed to settle overnight. Freshly eclosed flies were added to $CdCl_2$ and control food for 48 hours to allow for mating and egg-laying to occur. After 48 hours, the adult flies were removed from the vials and the larva were allowed to mature. Once the pupa eclosed, the males were separated from the females and allowed to mature on uncontaminated food for 72 hours prior to sacrifice and testes removal.

III. DAPI-staining protocol

Freshly eclosed *D. melanogaster* flies were transferred onto control (those without $CdCl_2$), 10 µM, 25 µM, 50 µM, 75 µM, 100 µM, 300 µM, and 500 µM $CdCl_2$ food vials for 48 hours to allow mating and egg laying to occur. After 48 hours, the adult *D. melanogaster* flies were removed and the eggs allowed to mature into adult flies. Upon eclosure, the adult male flies were transferred to uncontaminated food for 72 hours. On the third day, the virgin male flies were sacrificed and testes removed from the body and the accessory glands removed. The testes were placed in a 4% paraformaldehyde solution for 60 minutes, followed by 3 washes for 2

minutes each in 1X Phosphate Buffer Saline (PBS). The washed testes were placed on microscope slide with a solution of 4', 6-diamidino-2-phenylindole (DAPI) and MOWIOL and imaged using confocal microscopy.

IV. Live/Dead Protocol

Freshly eclosed *D. melanogaster* flies were transferred onto control (those without CdCl$_2$), 10 µM, 25 µM, 50 µM, 75 µM, 100 µM, 300 µM, and 500 µM CdCl$_2$ food vials for 48 hours to allow mating and egg laying to occur. After 48 hours, the adult *D. melanogaster* flies were removed and the eggs allowed to mature to adult flies. Upon eclosure, the adult male flies were transferred to uncontaminated food for 72 hours. On the third day, the virgin male flies were sacrificed and their testes removed. The seminal vesicles were removed from the testes and the isolated seminal vesicles were placed onto a separate dissecting slide with 1X PBS. Sperm were extracted by puncturing the seminal vesicle with an insulin needle and transferring the pool of sperm to 8 µL of 1X PBS on a large coverslip. 1 µL of SYBR® 14 dye was added to the sperm solution and was allowed to incubate in the dark for three minutes. 1 µL of propidium iodide was added to the sperm solution and was allowed to incubate in the dark for one minute. A coverslip was placed over the sperm solution and the image taken within six minutes using confocal microscopy. After the z-stack was collected, a maximum intensity projection image was generated using Fluoview. Using the counting tool in Fluoview, the live and dead sperm were counted (refer to **Figure 4**).

V. Statistical Analysis

Two-tailed Student's t-tests were performed using GraphPad Prism, version 4. A minimum n value of three was used for all datasets. Results were considered statistically significant with p-value > 0.05.
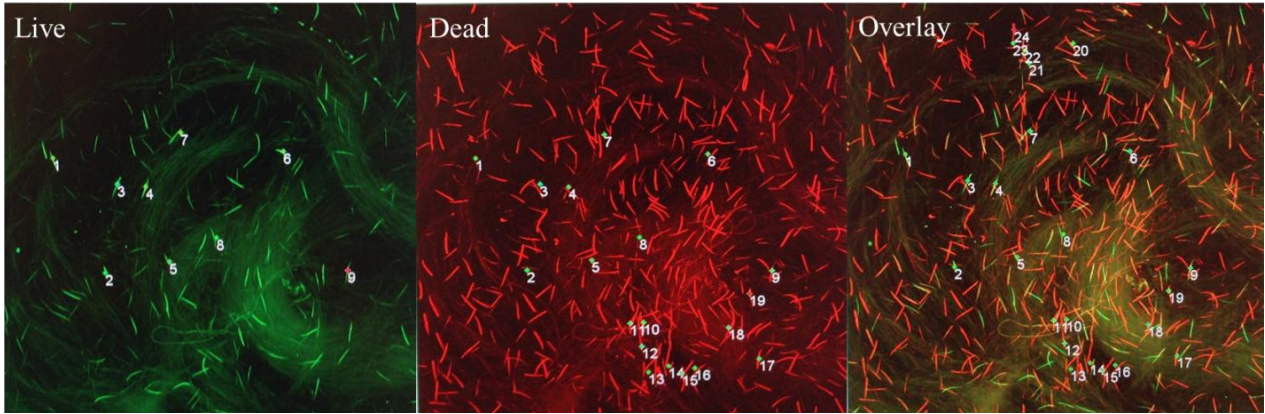
Figure 4: Live/Dead stain quantification protocol. The left panel shows the tagging of the live sperm, stained green with SYBR® 14 dye. The middle panel shows the tagging of the dead sperm, stained red with propidium iodide. The right panel shows the overlay of the live/dead stain.

V. Fasciclin III Antibody Staining

Five or six sets of dissected testes were fixed by placing in ice-cold 1:1 methanol/acetone solution for 20 minutes. Testes were washed in 1X PBS 3 times for 2 minutes each. Testes were then permeabalized in 0.5% PBS (1X PBS containing 0.5% Triton X-100) for 15 minutes on a shaker. Testes were washed 3 times for 2 minutes each in 1X PBS then blocked in 3% bovine serum albumin (BSA) in 1X PBS for 1 hour on a shaker. The testes were then washed 3 times for 2 minutes each in 1X PBS. The testes were stained with a primary antibody using a 1:5 dilution of mouse anti-Fasciclin III (Developmental Studies Hybridoma Bank; University of Iowa, Department of Biology) in 1X PBS solution. Each experimental set was placed in a 4°C refrigerator overnight. At least two control testes were placed in 1X PBS solution lacking the primary antibody overnight as well. After 12 hours the testes were rinsed 3 times for 2 minutes each in 1X PBS. The experimental testes and the control testes were then stained in secondary antibody, Alexa Fluor 488 goat anti-rat, at a 1:100 concentration in a 1X PBS solution and placed on a shaker for an hour. The testes were then washed in 1X PBS 3 times for 5 minutes each. They were then mounted in 25 µL of a MOWIOL and DAPI solution on glass slides, sealed with glass coverslips, and viewed on an Olympus FV 1000 confocal microscope.

VI. Drosophila persimilis immunofluorescence staining

The same staining protocol was used for *D. persimilis* as for *D. anansassae* with a few differences. A set of 5-6 testes was dissected from 5-6 male flies in 1X PBS for each experimental concentration and control. The testes were fixed by being placed on ice-cold 1:1 methanol/acetone solution for 20 minutes. The testes were then washed in 1X PBS 3 times for 2 minutes each. They were then permeabalized in 0.5% PBS (1X PBS containing 0.5% Triton X-

100) for 15 minutes on a shaker. The testes were then washed 3 times for 2 minutes each again in 1X PBS. They were then blocked in 3% BSA in1X PBS blocking solution for 1 hour on a shaker. They were then again washed 3 times for 2 minutes each in 1X PBS. The testes were then initially stained with a primary antibody with a 1:10 dilution of mouse anti-Fasciclin III in 1X PBS solution. Each experimental set was placed in a 4°C fridge overnight. At least two control testes were placed in 1X PBS overnight as well. After 12 hours, they were rinsed again 3 times for 2 minutes each in 1X PBS. The experimental testes as well as the control were then stained in secondary antibody, Alexa Fluor 488 goat anti-rat, at a 1:500 concentration in a 1X PBS solution and placed on a shaker for an hour. They were then washed in 1X PBS 3 times for 5 minutes each. They were then mounted in a MOWIOL and DAPI solution on glass slides with micro-glass cover slips, and viewed under confocal microscopy. The process was repeated twice with a 1:5 dilution of primary as well, with a secondary concentration of 1:500 for the latter two trials.

VII. Confocal microscopy

Samples were imaged on an Olympus FV 1000 confocal laser scanning microscope (CLSM). Image stacks of the prepared Drosophila testes were obtained using 20x, 40x, and 60x objective lenses. A 405 nm laser was used to excite the DAPI nuclear stain, and a 488 nm argon laser was used to excite the secondary antibody, Alexa Fluor 488, as well as the SYBR® 14 stain and a 493 nm laser was used to excite the propidium iodide stain. Saturation levels were set using the hi-low tool available in the color look-up table (LUT). The thickness of the z stacks was optimized depending on the trial and the structure being observed, but the best images were taken at 1024x1024 pixel resolution, with an 8 microsecond/pixel scan rate using the 40X oil immersion lens.

<p style="text-align: center;">**Results**</p>

I.   Anti-Fasciclin Staining of the Apical Testis

In order to elucidate the structure of the apical testis in *D. ananassae* and *D. persimilis* the apical end of the testes was stained with antibodies raised against known *D. melanogaster* and *D. pseudoobscura* stem cell niche proteins. **Figure 5** shows the anti-Fasciclin staining for *D.melanogaster;* the blue signal is the DAPI stain and the green is the Fluor488 which indicates Anti-Fasciclin. A positive staining for the rosette hub is shown in panel B. **Figure 6** shows the results of anti-Fasciclin in *D.pseudoobscura* where blue indicates DAPI stain and green is the Fluor488 which indicates Anti-Fasciclin. A diffuse staining of the hub was shown. **Figure 7** shows the staining results for *D. ananassae*. *D. ananassae* displayed the rosette staining pattern of the hub usually seen in *D. melanogaster*. **Figure 8** shows the staining results for *D. persimilis*. *D. persimilis* displays the wave like staining pattern of the hub previously seen in *D. pseudoobscura*.
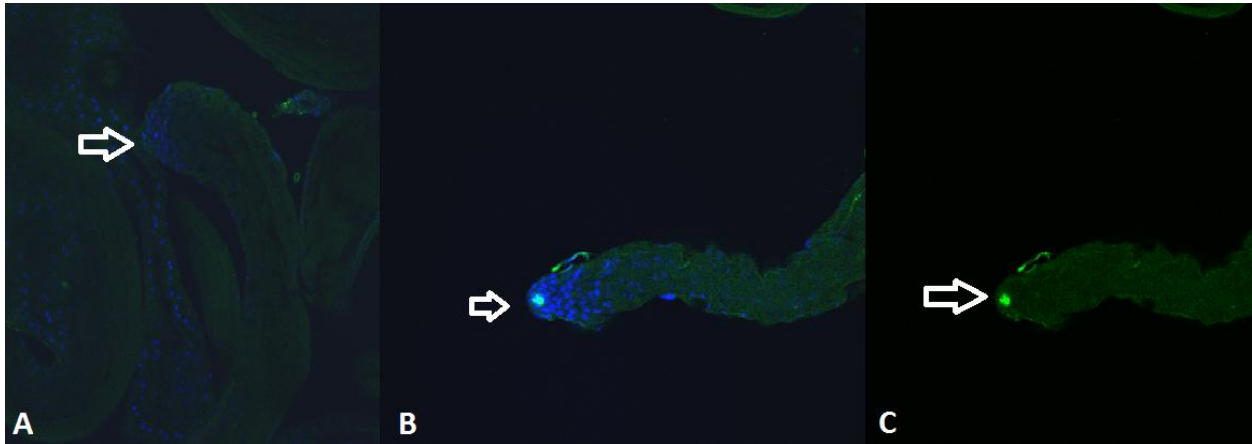
Figure 5: Anti fasciclin (1:50) staining in *D. melanogaster*. (A) 1:50 concentration negative control for *D. melanogaster*. Apical end is indicated by the arrow. The blue is DAPI staining and the green is Fluor488. No signal is seen. (B) 1:50 concentration experimental for *D. melanogaster*. Apical end is indicated by the arrow. The blue signal is DAPI staining and the green signal is Fluor488. There is signal seen in the hub area, the hub is the green rosette. (C) The same image as (B), but with only the Fluor488 channel shown. Arrows indicate the apical end. Images were taken with a 20x oil immersion lens.
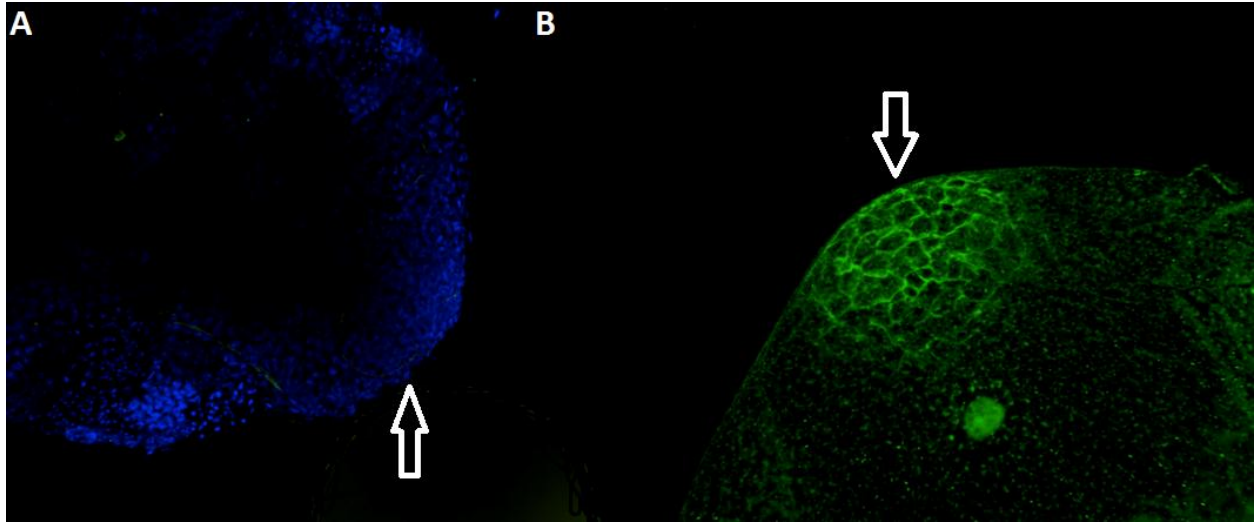
Figure 6: **Anti fasciclin staining in *D. pseudoobscura*.** (A) Apical end of *D. pseudoobscura* control. The apical end is indicated by the arrow. (B) Apical end of *D. pseudoobscura* experimental. Signal is seen. Arrows indicate the hub area. (Images taken from Marenco, 2014.)
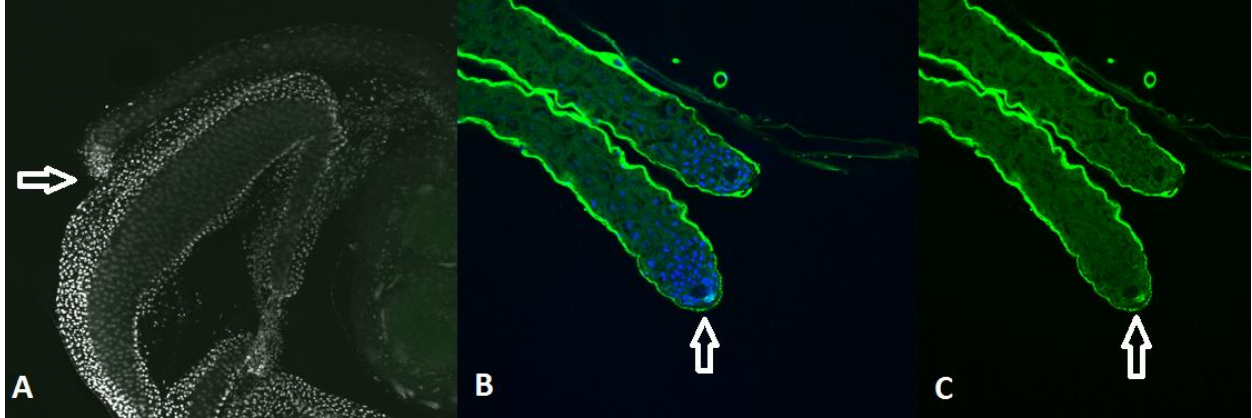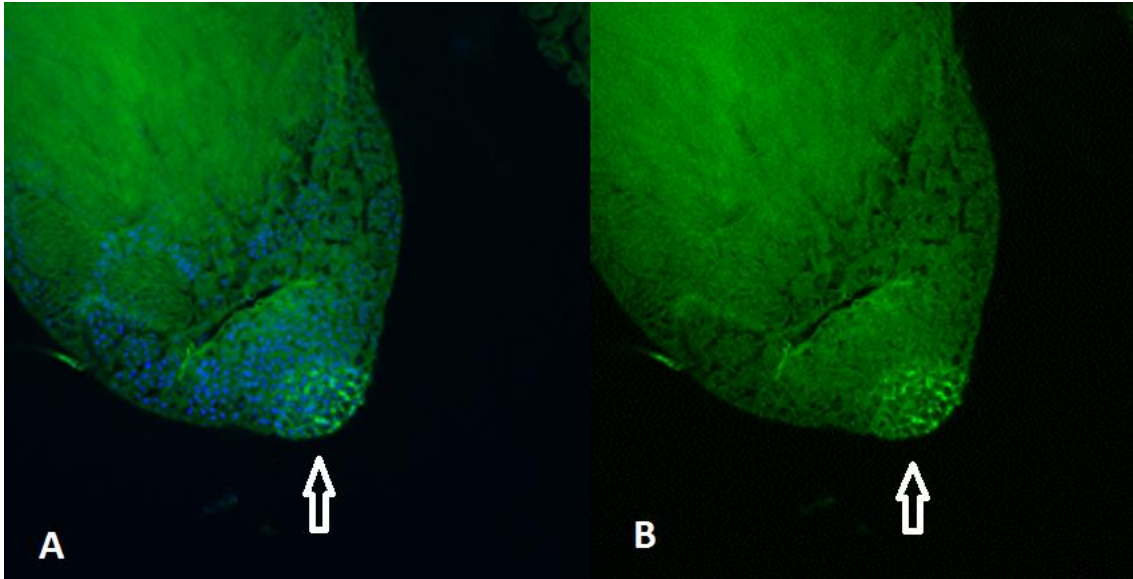
Figure 7: Anti fasciclin (1:5) staining in *D. ananassae*. (A) The 1:5 concentration negative control for *D. ananassae*. The apical end of one testis and one accessory gland is shown. DAPI staining is shown in grey, and Fluor 488 is the faint green. No signal is shown in the control. (B) The 1:5 concentration experimental for *D. ananassae*. The two apical ends are shown of the testes. DAPI is shown in blue, and Fluor488 is shown in green. Signal is seen in the hub area. (C) The same image as (B) with only the Fluor488 shown. The hub is present at the tip. Arrows indicate the apical ends. (A) 20x oil immersion, and (B)-(C) are at 60x.

**Figure 8**: **Anti fasciclin (1:5) staining in *D. persimilis*.** (A) The 1:5 concentration experimental for *D. persimilis*. A testis is shown. The blue is DAPI staining and the green is Fluor488. There is signal shown in the hub area. (B) The same image as (A) with only the Fluor488 channel shown. Arrows indicate the apical ends and hub areas. Images taken with a 40x oil immersion objective lens.

II. Cadmium Chloride Assays

In order to perform the $CdCl_2$ treatment assays, $CdCl_2$ was incorporated into Drosophila JazzMix and *D. melanogaster* larva were cultured until they eclosed. Once eclosed, virgin males were transfered onto uncontaminated JazzMix for 72 hours to allow sexual organs to mature. After 72 hours the testes were removed and mounted and stained with DAPI (for the spermiogenesis assay) or the sperm removed from the seminal vesicle and stained with Live/Dead stain (for the sperm quantification assay). **Figure 9** shows the fly culture vials in which *D. melanogaster* were cultured. There was an obvious decrease in the amount of larva, pupa, and eclosed flies proportional to the increase in $CdCl_2$ exposure. **Figure 10** shows the results of the DAPI assay; testes were examined for the presence of sperm bundles in the basal end of *D. melanogaster* testes. Of note, there was an increase in the presence of abnormal sperm bundles proportional to increasing $CdCl_2$ exposure. **Figure 11** compares the number of normal to abnormal sperm bundles found in each experimental condition. At the highest concentration, the number of abnormal sperm bundles was roughly equal to the number of normal sperm bundles. **Figure 12** has two graphs: the top shows the percentage of normal sperm bundles seen for each experimental condition and the bottom graph shows the total number of sperm bundles shown for each experimental condition. The number of total sperm bundles stayed constant for each experimental condition but the percentage of normal sperm bundles decreased with increasing exposure to $CdCl_2$. **Figure 13** is the confocal images from the Live/Dead assay in which the live sperm are stained green and the dead sperm stained red. **Figure 14** quantifies the total number of sperm seen for each experimental condition; there was an increase in total number of sperm proportional to an increase in $CdCl_2$ exposure. The second graph shows the percentage of live sperm shown for each experimental condition; there was less live sperm shown with an increase in $CdCl_2$ exposure.

Figure 15 shows the comparison of live to dead sperm for each experimental condition; it

shows an increase in the amount of dead sperm proportional to increasing $CdCl_2$ exposure.
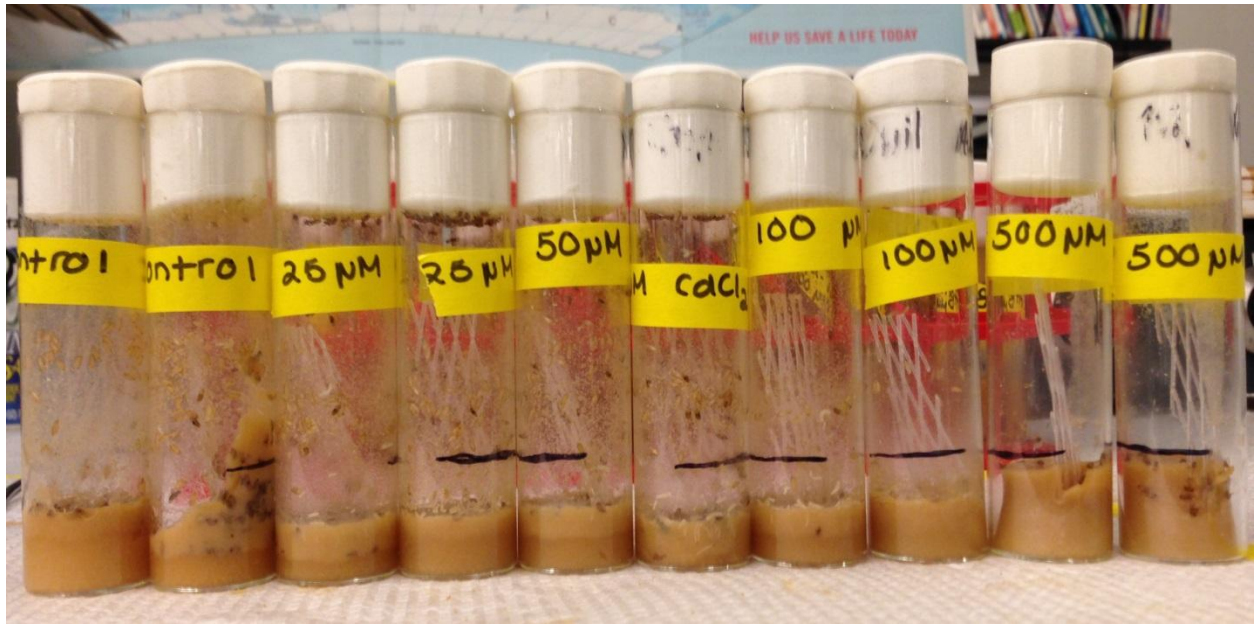
Figure 9: Cadmium Cultures. From left to right: control vials, 25µM CdCl$_2$, 50µM CdCl$_2$, 100µM CdCl$_2$, and 500µM CdCl$_2$ fly culture vials (10µM CdCl$_2$, 75µM CdCl$_2$, and 300µM CdCl$_2$ concentrations were added at a later date). Two vials of each concentration were prepared. Of importance to note is the decreasing number of larva, pupa, and eclosed flies with the increasing concentration of CdCl$_2$ with no larva, pupa or eclosed flies noted in the 300 µM CdCl$_2$ and 500µm CdCl$_2$ vials for every trial.
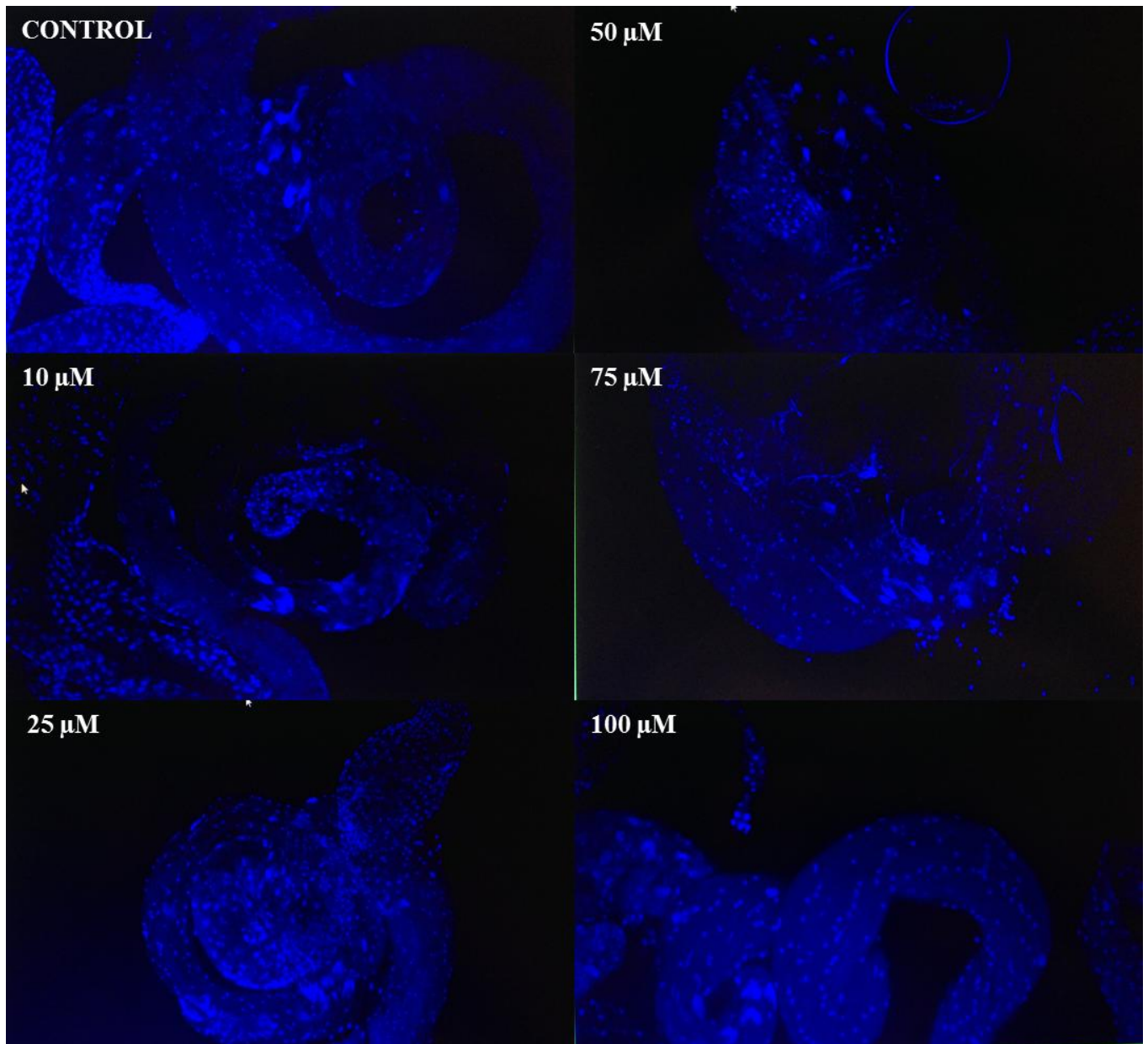
Figure 10: DAPI staining of the basal end of *D. melanogaster* testis. Arrows indicate bundles of developing sperm. DAPI stains nucleic acid; sperm bundles are distinguished by their arrowhead shape appearance. Exposure to increasing concentrations of cadmium chloride showed an increase in the number of abnormal bundles (those with a more diffuse appearance).
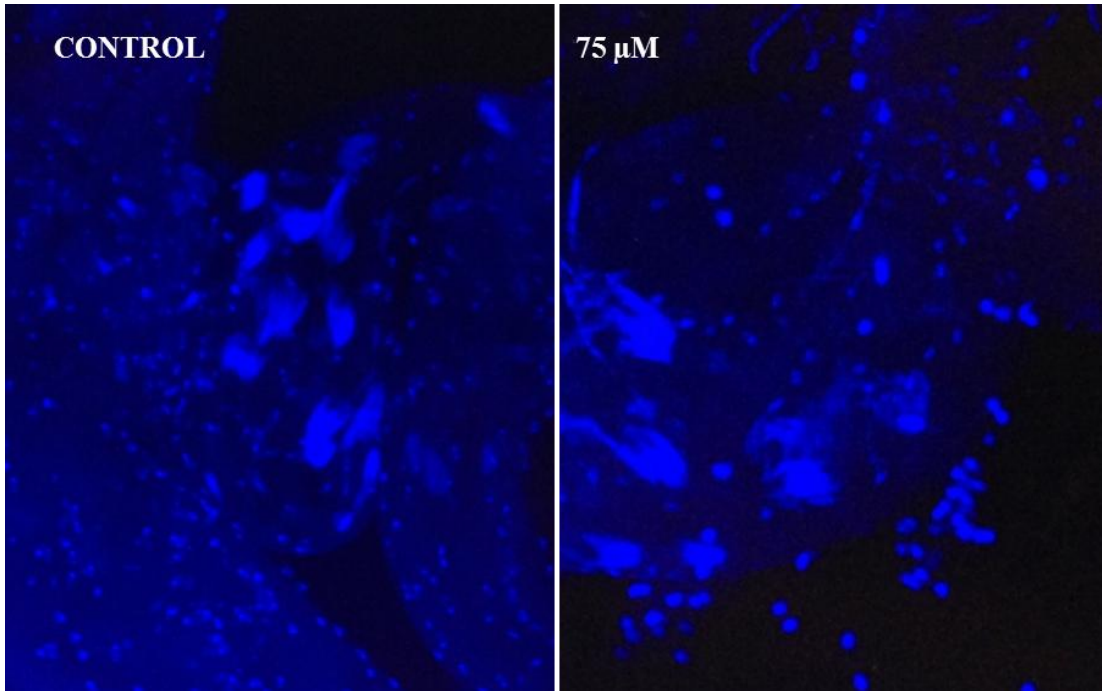
Figure 10, enlargement: DAPI staining of the basal end of *D. melanogaster* testis.
Comparison of control testis with normal sperm bundle formation to 75 µM exposed testis with
abnormal sperm bundles.
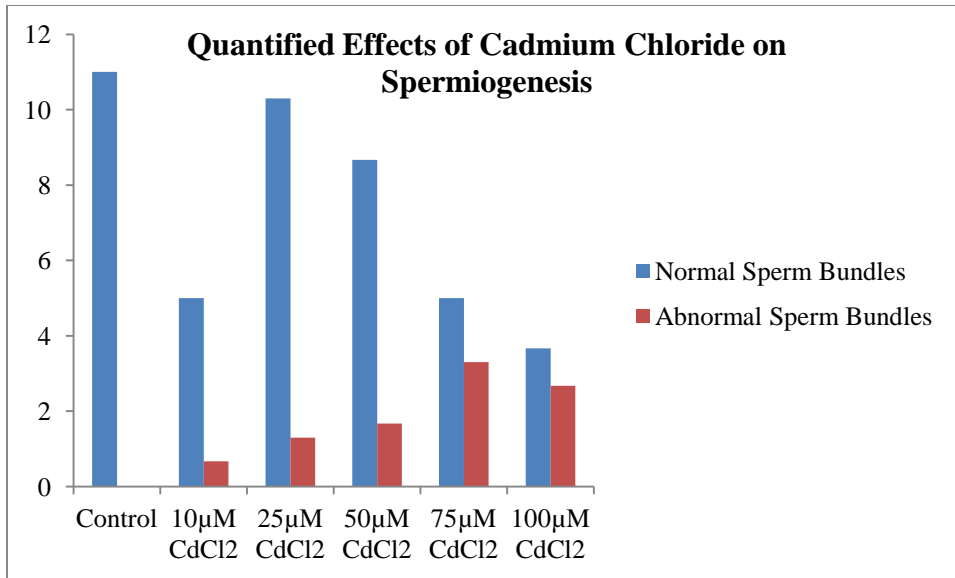
Figure 11: Quantified Effects of Cadmium Chloride on Spermiogenesis. Graph shows the quantification of normal sperm bundles compared to abnormal sperm bundles in the basal end of *D. melanogaster* for each experimental condition. Of note is the overall decrease in the number of total bundles with increasing $CdCl_2$ exposure. See **Figure 12** for statistical analysis.
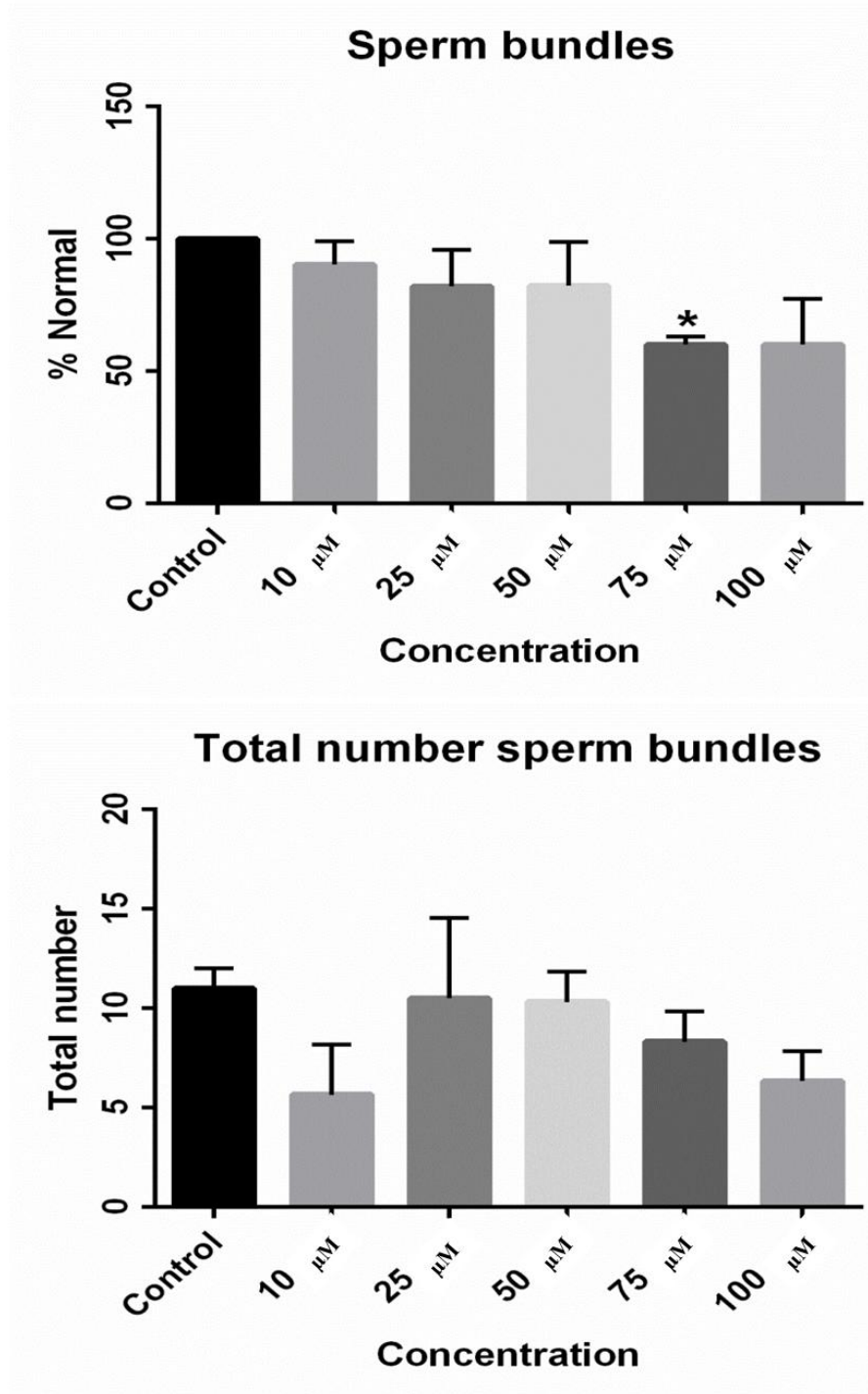
Figure 12: Quantified Effects of Cadmium Chloride on Spermiogenesis. The top graph shows the percentage of normal sperm bundles seen in each experimental condition. Standard error bars are shown and stars indicate $p < 0.05$; n value was at least 3. The bottom graph shows the total number of sperm bundles seen for each experimental condition. Standard error bars are shown.
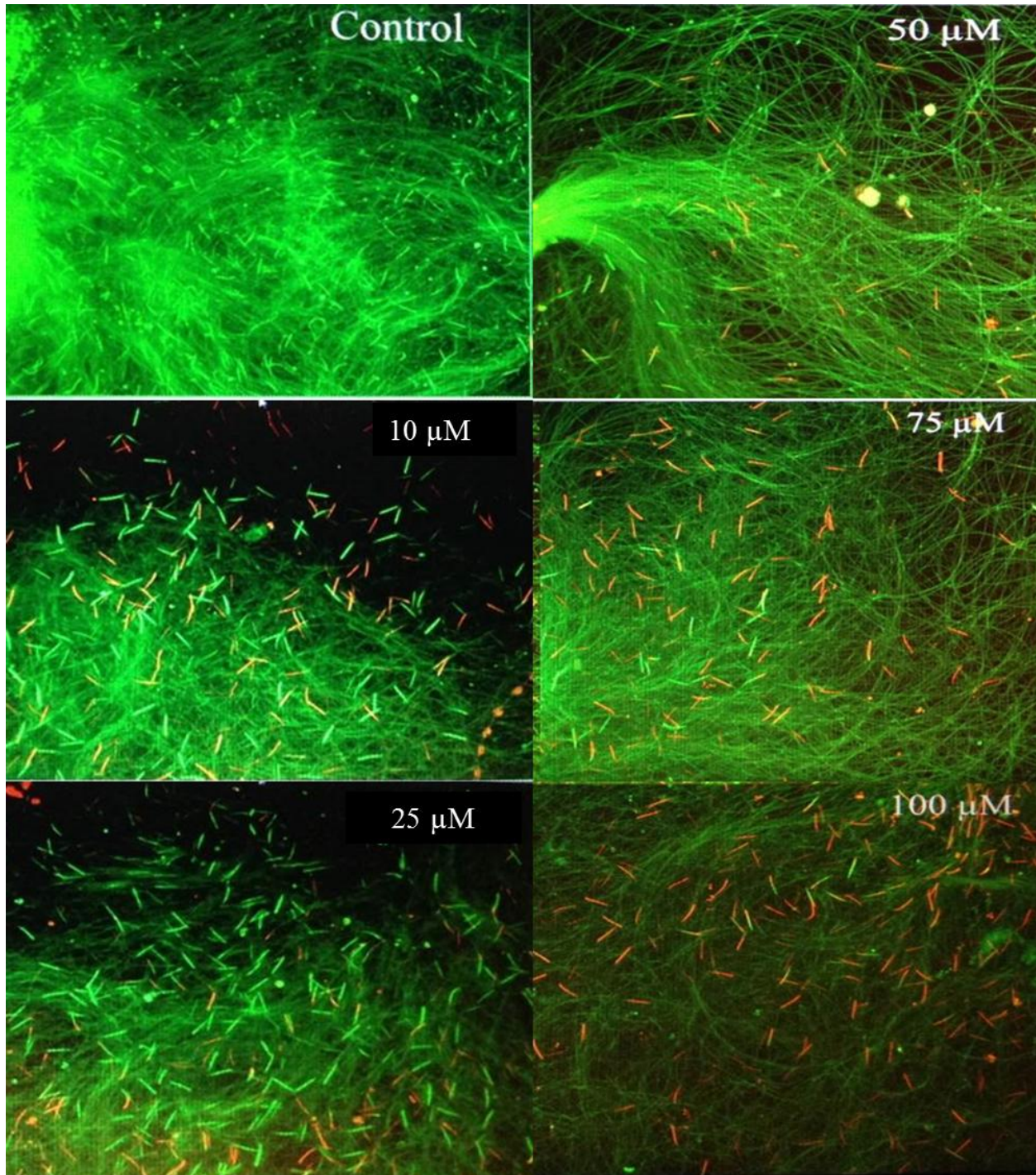
Figure 13: Live/Dead Staining of sperm extracted from the seminal vesicles of *D. melanogaster.* The green stain is SYBR Green which stains live tissue and the red stain is propidium iodide which stains dead tissue. Of note is the increasing amount of sperm stained red proportional to the increasing concentration of cadmium chloride exposure.
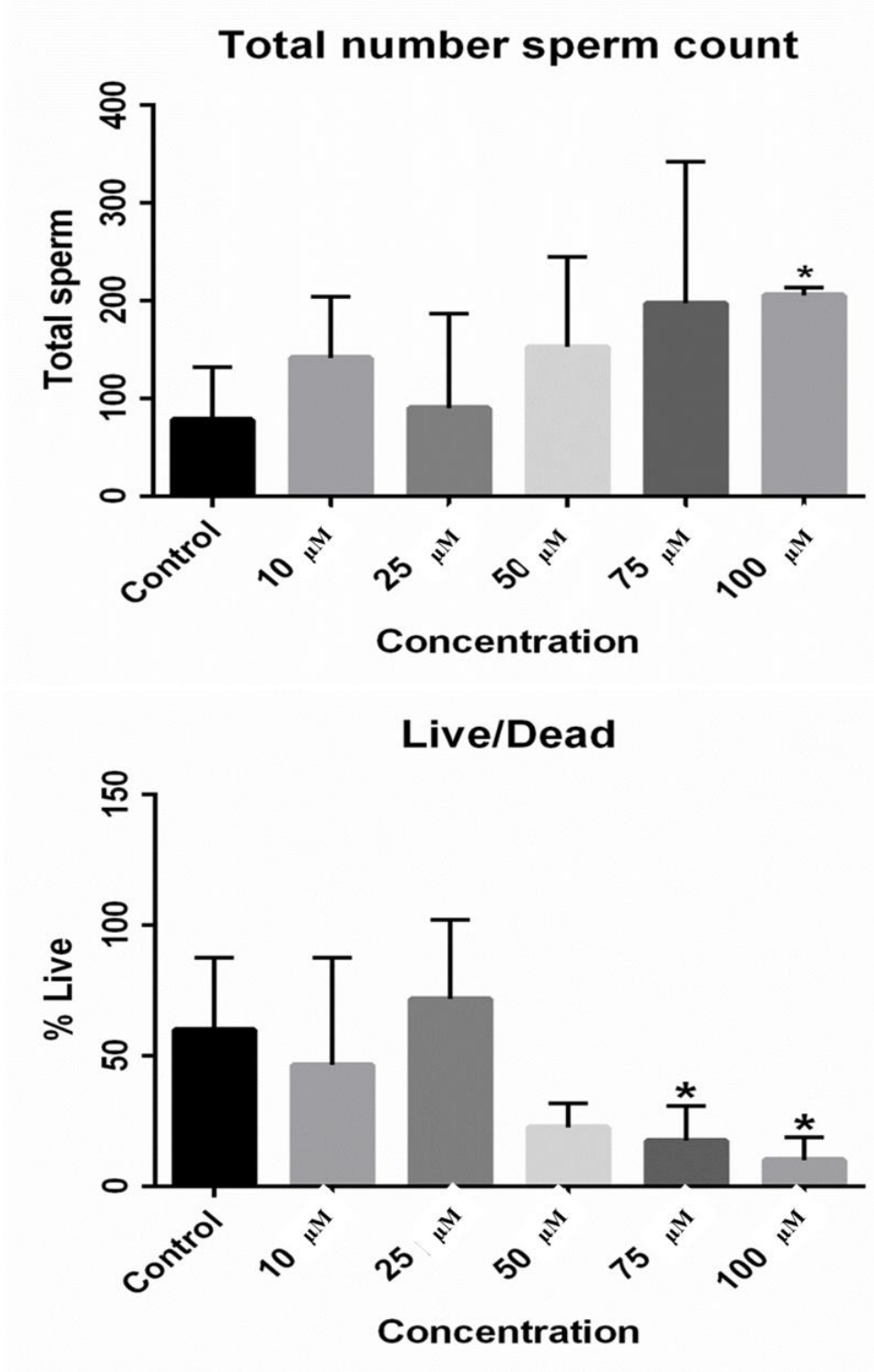
Figure 14: Live/Dead sperm Quantification. The top graph shows the total number of sperm found for each concentration for all data sets collected. Standard error bars are shown and stars indicate p<0.05; n value of at least 3. The bottom graph shows the percentage of live sperm seen for each experimental condition. Standard error bars are shown and stars indicate p<0.05; n value of at least 3.
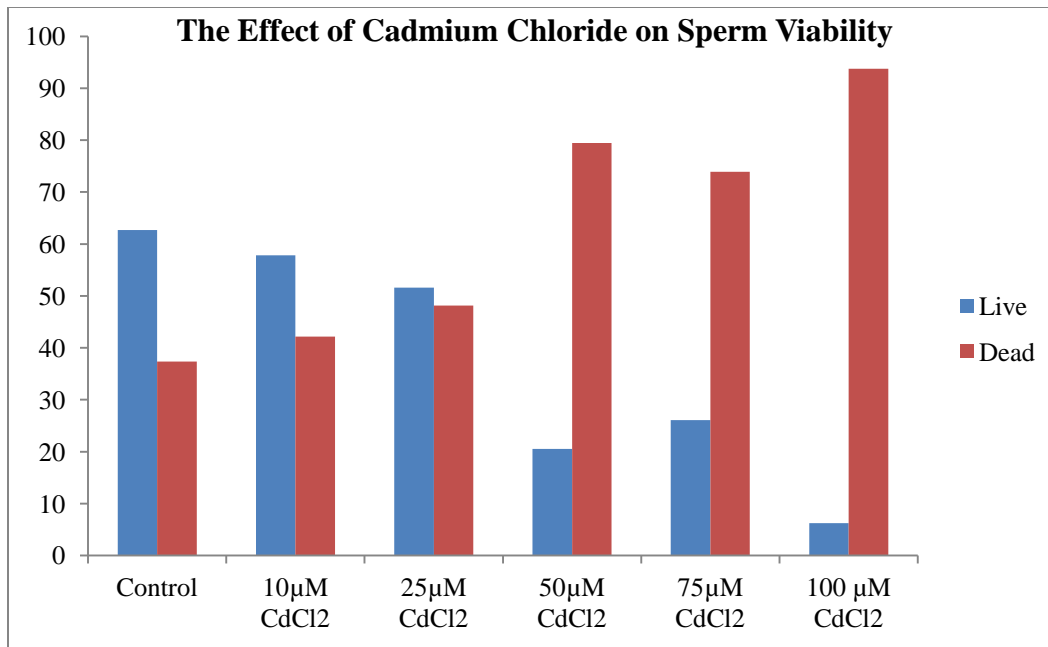
Figure 15: The Effect of Cadmium Chloride on Sperm Viability. Graph showcases the quantification of live, dead, and total sperm in each experimental condition. Numbers of Live versus Dead sperm are displayed as percentages of total number of sperm quantified. See Figure 14 for statistical analysis.

Discussion

I.   Structural Characterization of the Stem Cell Niche

The stem cell niche of *D. persimilis* and *D. ananassae* were successfully stained using antibodies raised against anti-Fasciclin III. *D. ananassae* displayed the rosette staining pattern previously seen in *D.melanogaster* (**Figure 7**). This indicates that the process of spermatogenesis in *D. ananassae* will follow the same sequence of events as in *D. melanogaster*. *D. persimilis* showed the same wave like pattern of the hub shown in *D. pseudoobscura* (**Figure 8**). Recent data from our lab suggests that *D. pseudoobscura*, and *D. pseudoobscura* like species of Drosophila, will eclose with a set number of germline stem cells that will differentiate in a wave-like pattern unlike the orderly mitotic divisions of *D. melanogaster* like species[8]. This suggests that *D. pseudoobscura* is lacking a hub that signals the differentiation of germline stem cells into mature sperm.

II.   Cadmium Chloride Treatment: Sperm Bundle Assay

The results of the DAPI assay demonstrate that cadmium chloride exposure has a detrimental effect on spermiogenesis in Drosophila. **Figure 10** shows the result of the spermiogenesis assay; it shows the appearance of abnormal sperm bundles after exposure to high levels of cadmium as compared to control. **Figure 11** shows the comparison of abnormal to normal sperm bundles for each experimental condition. **Figure 12** shows the percentage of normal sperm bundles (the top graph) and the total number of sperm bundles (the bottom graph) for each experimental condition; following an increasing concentration of cadmium chloride exposure there was an increased incidence of abnormal sperm bundles. Normal sperm bundles have an arrowhead appearance; abnormal sperm bundles appear as diffuse clouds (**Figure 10** enlargement). A possible mechanism for the appearance of the abnormal sperm bundles is that the sperm bundles

are not embedding in the epithelium of the basal end of the testes. It is probable that these abnormal sperm bundles are bursting prematurely in the testes so that a higher number of total sperm is found in the seminal vesicle at higher concentrations of cadmium. Our data indicate that the coiling of the sperm bundles is timed, that is, it appears that one cyst is bursting at a time (refer to **Figure 3**). One sperm bundle should yield 128 mature sperm. As shown in **Figure 14**, the maximum number of sperm produced was 148 in the control flies but 217 in the 100 μM treated group. Recent work has shown that as the cyst embeds in the basal epithelium of the testes, F- actin based processes extend from the head cyst cell to fill the interstitial space of the maturing spermatid bundle. These actin processes appear to hold the sperm heads in place. Disruption of these F-actin based processes resulted in spermatid bundle disassembly and premature release of sperm from the cyst.[19] It is possible that $CdCl_2$ exposure results in a disruption of these F-actin based processes so that the sperm bundles do not form properly and sperm are released prematurely from their cyst.

III.   Cadmium Chloride Treatment: Live/Dead Assay

The results of the Live/Dead assay demonstrate that cadmium chloride exposure has a detrimental effect on the production of viable sperm. **Figure 13** shows the results of the Live/Dead assay where dead sperm are stained red and live sperm stained green; there was an obvious increase in the amount of dead sperm proportional to increasing exposure to higher concentrations of cadmium. Additionally, there is an increasing number of total sperm found in the seminal vesicle that is directly proportional to the cadmium chloride concentration. **Figure 15** shows the comparison of live to dead sperm for each experimental condition; the amount of live sperm decreased with exposure to increasing concentrations of cadmium while the amount of dead sperm increased with exposure to increasing concentrations of cadmium. Work by Zeng

et al (2011) in rats showed that cadmium exposure resulted in increased number of apoptotic and elongate spermatids in seminiferous tubules of rats, as well as necrosis of the seminiferous epithelium.[29] The exact mechanism that results in a high number of dead sperm at high concentrations of $CdCl_2$ is unknown. However, work by Oliveira, et al (2009) showed that $CdCl_2$ exposure resulted in DNA fragmentation in rats. It is possible that nuclear degradation is occurring in the $CdCl_2$ treated Drosophila sperm; this theory is supported by the high number of dead sperm found in the 100μM seminal vesicle. Another possibility is the cadmium treatment induced necrosis in the epithelium of the basal testis, resulting in the head cyst cell being unable to embed and thus abnormal sperm bundle formation. This theory is supported by the data showing that the abnormal sperm bundles yielded a higher number of sperm as a result of premature bursting of the sperm bundles and thus a higher number of dead sperm in the higher concentrations of cadmium chloride exposure. This theory is also supported by work by Hew et al (1993) that showed a failure of sperm release from Sertoli cells in rats following a single intraperitoneal injection of cadmium chloride.[35] Another possible explanation for the disrupted sperm head arrangement in cadmium-treated flies may be that the migration of sperm nuclei in round spermatid cysts is perturbed so that the nuclei never associate properly with the head cyst cell.[20,34]

IV.   Future Directions

Our lab has extensively studied the Sophophora subgenus of Drosophilidae. Future studies will examine the stem cell niche relationship in the Drosophila subgenus. Recent work has shown that two arrangements exist in the Sophophora genus: either a rosette arrangement or a wave-like pattern. We plan further work on the arrangement of the stem cell niche in the

Drosophila subgenus to examine the evolutionary relationship between the various species and its effect on the development of the stem cell niche.

Other future directions include examining the effect of cadmium chloride exposure on spermatogenesis in other members of the Sophophora subgenus. One such possibility is within the obscura group, typified by *D. pseudoobscura* or *D. persimilis* (species that do not exhibit the same hub pattern seen in the melanogaster group). Also of potential interest could be examining the effects of $CdCl_2$ exposure on the retarded development of *D. melanogaster* through the larval, pupal and eclosure stages of development. Lastly, the mechanism of the possible DNA fragmentation in the $CdCl_2$ treated sperm could be examined in order to determine if nuclear degradation is occurring.

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