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Confocal Characterization of the Apical Testes and Ultrastructural Analysis of the Seminal Visicles of Drosophila pseudoobscura

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Michael W Beaury

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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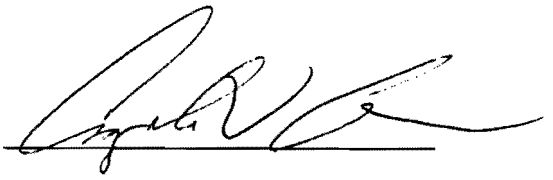
Approved By



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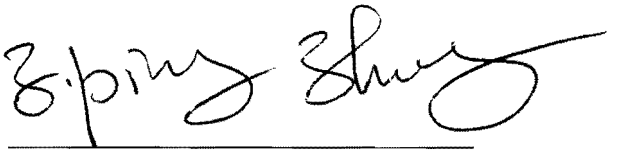

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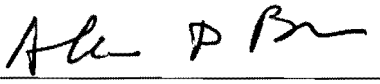

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Abstract

The *Drosophila* genus contains thousands of different species. *Drosophila melanogaster* is the most popular *Drosophila* species used in biological research. *Drosophila pseudoobscura* is the model *Drosophila* species used in our laboratory and is a distant relative to *D. melanogaster*. Both of these species' genetic codes have been completely sequenced and are very different from each other genetically and morphologically. One major morphological difference between these two species is their testes structure. The testes of *D. melanogaster* are long, coiled, thin tube-like structures; *D. pseudoobscura* testes are wide and ellipsoidal. The focus of the current work was to characterize the apical end of *D. pseudoobscura* testes (which plays a pivotal role in the initiation of spermatogenesis) using antibodies available for the *D. melanogaster* testes. Using immunofluorescence techniques, we have characterized *D. pseudoobscura* adherent proteins in a specific region termed "the hub" responsible for maintaining the cells associated with the stem cell niche, using antibodies raised against the hub proteins in *D. melanogaster*. The hub proteins probed were fasciclin III, DE-cadherin and armadillo. Fasciclin III is a glycoprotein that is associated with adhering cells together within the central nervous system and other tissues, including the testis hub. DE-cadherin and armadillo are specifically associated with the stem cell niche of *Drosophila* species and serve to adhere the cells within the structure. In addition, we have characterized the ultrastructure of the seminal vesicles of *D. pseudoobscura* and have attempted to interpret the processes that occur in the storage and post-spermatogenesis maturation of *Drosophila* sperm cells.

Introduction

I. Overview

The most popular fly species model used in biology is *D. melanogaster. D. melanogaster* belongs to the *melanogaster* group of the *Drosophila* genus. Its genetic code has been completely sequenced and is often used to compare the genetic variation of other species of *Drosophila*. In addition to *D. melanogaster,* eleven other *Drosophila* species (chosen based on evolutionary distance from *D. melanogaster*) genomes have been sequenced (Figure 1; Gilbert, 2007). One of these species, *D. pseudoobscura*, is of particular interest to our lab as we have developed a system for culturing the spermatogenic cysts isolated from the testes (Njogu et al, 2010; Ricketts et al, 2011). Additionally, we have characterized the sperm DNA binding proteins, called protamine-like proteins, involved in chromatin compaction during spermatogenesis (Alvi et al, 2012). In the current work, we sought to characterize the region of the *D. pseudoobscura* testes called the stem cell niche. The stem cell niche is the region of the testes where spermatogenic stem cells proliferate for the life of the adult fly. The stem cell niche is well characterized in *D. melanogaster* (Hardy et al, 1979; Singh et al, 2010; Sheng et al, 2011). However, the structure of the niche has not been explored in any other *Drosophila* species. As described below. *D. pseudoobscura* is interesting because the morphology of the testes is very unusual as compared to other *Drosophila* testes. We hypothesized that the structure of the stem cell niche may also exhibit significant variation as compared to *D. melanogaster.*

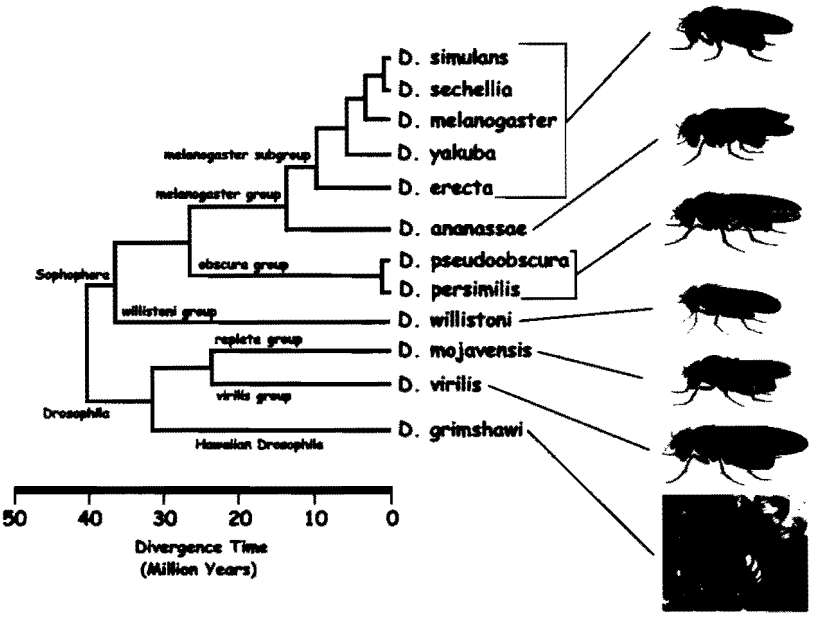


Figure 1; A phylogenic tree of *Drosophila* species whose genomes have been completely sequenced. (Modified from Gilbert, 2007)

II. Testes Structure of *Drosophila* Species

As shown in Figure 2, the *D. melanogaster* testes are long, thin tube like structures. The apical end is isolated while the basal end of the testes coils around the seminal vesicles. The color of the testes is a light yellow-green. As shown in Figure 3, the *D. pseudoobscura* testes are large, oblong shaped structures. The apical ends of the testes are large and wide and the basal end thins out and is directly connected to the seminal vesicles without coiling around them. The color of the testes is a bright orange-red. Because of the striking difference in morphology, we speculated that the arrangement of spermatogenic cells and structures within the lumen of the testis may also be different.

III. Spermatogenesis in *Drosophila*

Spermatogenesis is a complex process where sperm cells develop from stem cells within the testes. Germ line stem cells (GSCs) and cyst progenitor cells (CPCs) located in the stem cell niche at the apical end of the testes receive signals from a cluster of somatic cells known as the hub. After the stem cells divide; one of the daughter cells remains connected to the niche and remains a stem cell, the other cell differentiates into mature sperm. The germ line stem cell differentiates to a gonialblast cells and the progenitor cyst cell differentiates to a cyst cell (Gonczy et al. 1996). The gonialblasts are encased in two cyst cells and are pushed through the length of the testes from the stem cell niche to the basal end of the testes as they undergo a series of incomplete mitotic divisions which will result in spermatogonia. There are four mitotic divisions that occur in D. melanogaster testes, five mitotic divisions that occur in *D. pseudoobscura* testes (Scharer et al. 2008). A growth phase occurs so the spermatogonia differentiate to spermatocytes. Once the growth phase of spermatocytes is

complete, two meiotic divisions occur to develop spermatid cells. These spermatid cells undergo a series of transformations via a process called spermiogenesis and are transferred from the basal end of the testes to the seminal vesicles where they are stored (Tokuyasu et al. 1972, 1974, 1975). The arrangement of cell types found with the fly testis is shown in Figure 4.

The focus of current work was to study the apical end of the testes in *D. pseudoobscura*, specifically the stem cell niche. The stem cell niche is at the tip of the structure and contains three major types of cells; germ-line stem cells, cyst progenitor cells, and a cluster of somatic cells known as hub cells (Sheng et al. 2011). The hub cells are concentrated together to form the structure called the hub. The hub directs signals to the germ-line stem cells and cyst progenitor cells that allow them to divide and differentiate when necessary (Riparbelli et al. 2005).

IV. Characterization of the Apical *Drosophila* Testes

The apical end of the *D. melanogaster* testes was first characterized using transmission electron microscopy by R.W. Hardy in 1979. The characterization showed a detailed image of the proliferation center of the testes known as the hub, and the germ line stem cells and cyst progenitor cells attached to the hub. Now known as the stem cell niche, this area in the testes is the starting point of spermatogenesis in fruit flies.

Because the testes of *D. pseudoobscura* are very different from the testes of *D. melanogaster*, we hypothesized that the structure of the apical end of the testes including the stem cell niche may also be quite different.

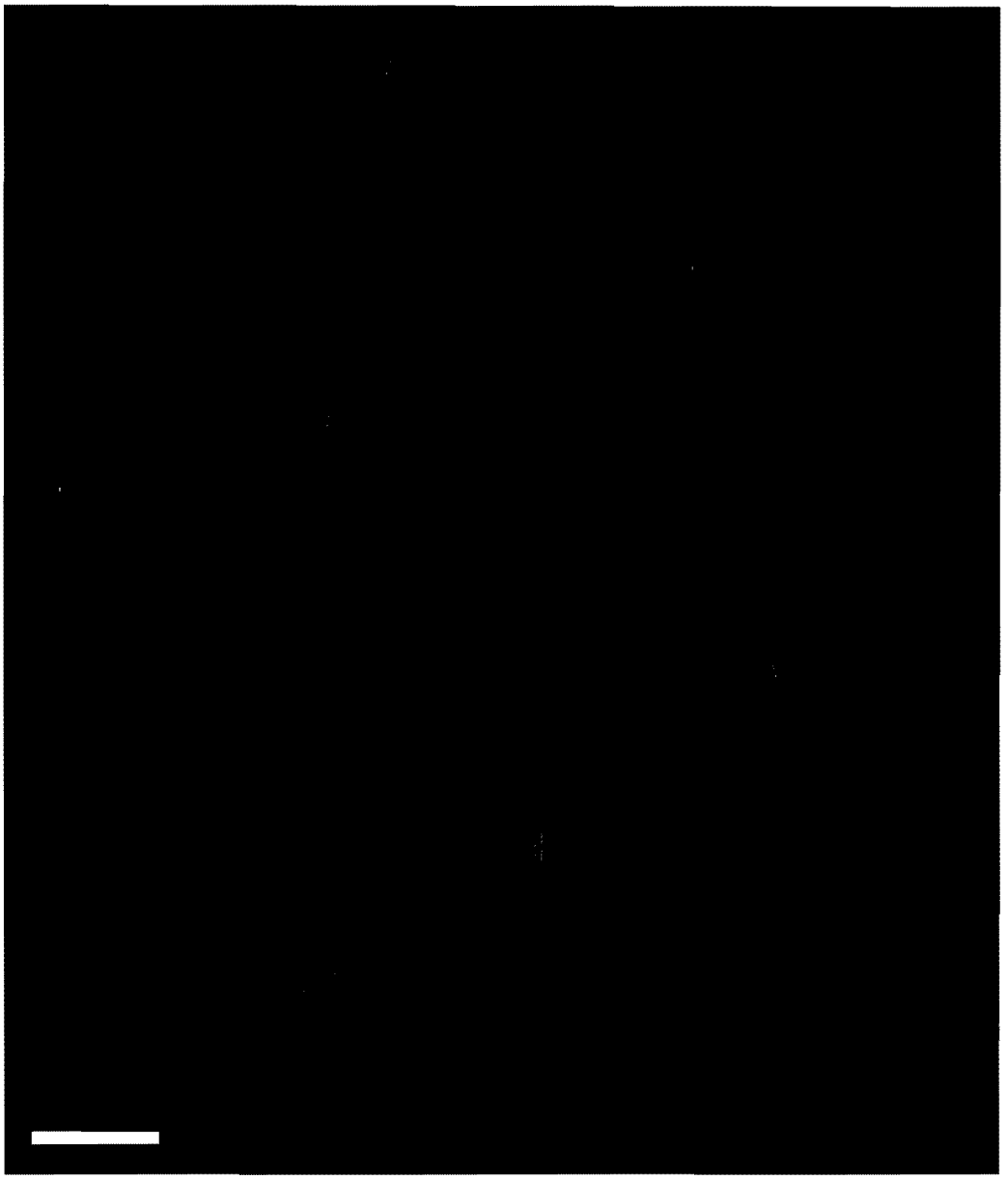


Figure 2; Bright field image of *D. melanogaster* testes. A – apical end of the testes. B – seminal vesicles. C – accessory glands. D – ejaculatory bulb. E – ejaculatory duct. The stem cell niche is located in the apical end of the testes. Scale bar is 250 μm

Figure 3; Bright field image of *D. pseudoobscura* testes. A – apical end of the testes. B – seminal vesicles. C – accessory glands. D – ejaculatory bulb and duct. Scale bar is 250 μm

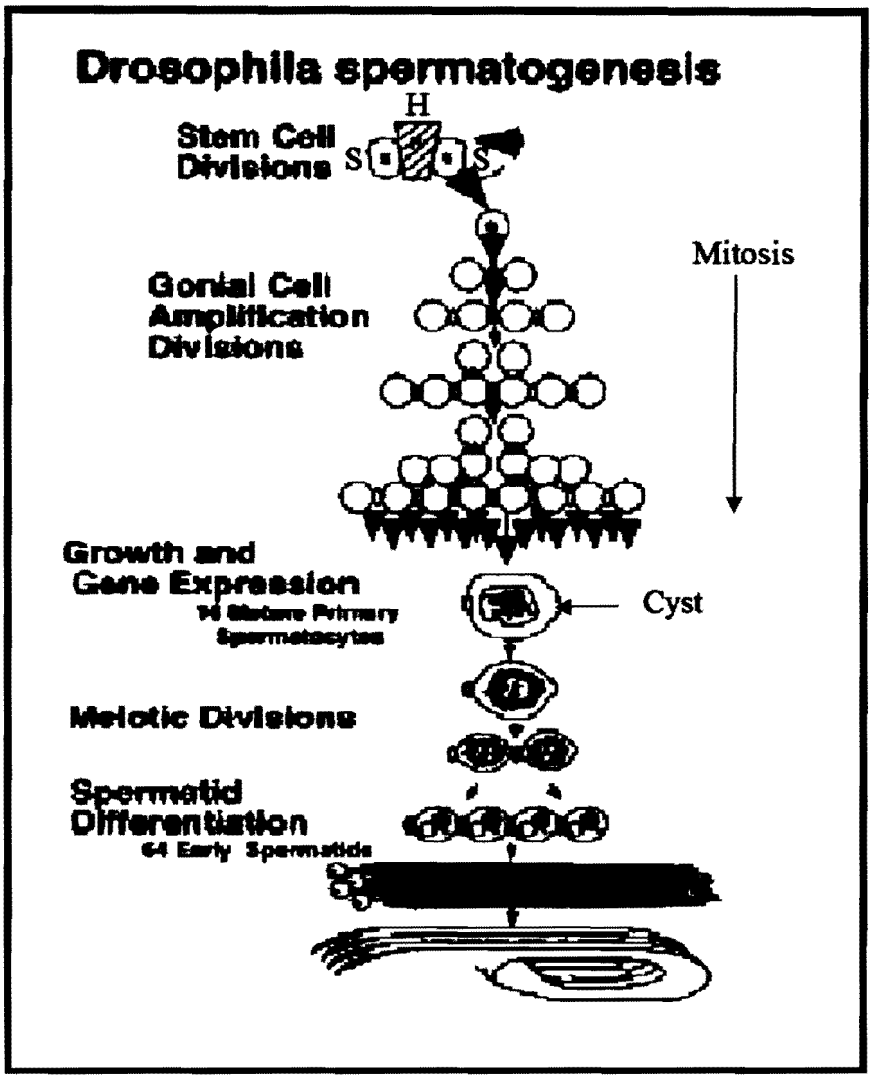


Figure 4; A simplified depiction of *Drosophila* spermatogenesis, showing the stem cell divisions, gonial amplification and spermatid differentiation. H – cluster of somatic cells known as the hub. S – stem cells (GSCs and CPCs) associated with the hub. Mitotic divisions of proliferating GSCs known as gonial cells are shown. Proliferated CPCs known as cyst cells encase the dividing gonial cells. Adapted from Fuller, 1998.

Our goal was to use immunofluorescence techniques to characterize proteins of the hub in the stem cell niche of *D. pseudoobscura* with antibodies raised against the same proteins in *D. melanogaster* (Sheng et al. 2011). Proteins DE-cadherin and armadillo act as adherent molecules to keep the hub cells together and keep germ line stem cells and cyst progenitor cells attached to the hub to prevent them from differentiating (Myster et al. 2003). *Drosophila* fasciclin III is a glycoprotein that plays a role in the adhesion of cells within the central nervous system and other tissues, including the stem cell niche in the testes (Snow et al. 1989). These three proteins are known to be located in the stem cell niche and have been well-characterized in *D. melanogaster.*

Several studies have employed the use of these antibodies to investigate processes associated with the stem cell niche of *D. melanogaster.* The stem cell niche was identified previously in *D. melanogaster*, when investigators were identifying the somatic stem cells associated with it with the utilization of anti-fasciclin III and DE-cadherin antibodies (Voog et al, 2009). The adherent junction, which consisting of the adherent proteins DE-cadherin and armadillo, plays a major role in the polarizing of embryonic epithelial cells in female *D. melanogaster* (Huang et al, 2011). In studying the dynein light chain 1 in somatic cyst cells, the investigators used the antibodies for the DE-cadherin and armadillo proteins to view the loss of their function when the cell signaling is disrupted (Joti et al, 2011).

V. Ultrastructure of the *Drosophila* Seminal Vesicles

High resolution details of the tissue morphology of fruit fly testes is available for very few species, and only *D. melanogaster* has been characterized in a systematic manner, at least for the testes. Previous work has characterized the ultrastructure of testes of several

fruit fly species using transmission electron microscopy. Hardy and Tokuyasu are well known for their work in describing the cellular processes of *D. melanogaster* testes (Hardy et al; 1979. Tokuyasu et al; 1972, 1974, 1975). The structure of the stem cell niche and all of the cells associated with the structure have been described in detail using transmission electron micrographs of the apical end of *D. pseudoobscura* (Hardy et al; 1979). Processes of spermiogenesis including the individualization of sperm, the coiling process, nuclear transformation, and head to tail alignment of the sperm in the testes of *D. melanogaster* were described using transmission electron microscopy (Tokuaysu et al; 1972, 1974, 1975). The morphology of the testes of the Mexican fruit fly, *Anastrepha ludens,* has similar characteristics to *D. pseudoobscura* testes and has been described ultrastructurally (Valdez et al, 2001). In the current work, we characterized the ultrastructural morphology of the seminal vesicles of *D. pseudoobscura* using transmission electron microscopy. The walls of the testes and seminal vesicles are composed of an outer pigment layer and an inner smooth muscle layer, similar to what has been reported for *D. melanogaster* and *Anastrepha ludens* (Sarno, et al. 2011). The epithelium of the seminal vesicles in *D. pseudoobscura* has a convoluted morphology that is similar to what has been reported in *D. bifurca* (Pitnick et al, 1995). This is interesting because these species are very distantly related and the gross morphology of their seminal vesicles is very different.

Materials and Methods

I. Fly Stock and Cultures

Fly stocks were attained from the University of California San Diego *Drosophila* Species Stock Center. Fly species were cultured separately in our laboratory in plugged vial containing Jazz Mix *Drosophila* media (Fisher Scientific) at 25°C

II. *Drosophila* Dissection for Immunofluorescence

Adult flies 24 – 48 hours in age were anesthetized by placing plugged vials containing the flies on ice for about 5 minutes. Male flies were removed from the vial and placed on a depression slide in 1X phosphate buffer saline (PBS). Testes were removed from the abdomen by carefully cutting the end of the abdomen using very fine probes and pushing the testes out.

III. Immunofluorescence Staining of *Drosophila melanogaster* Testes

D. melanogaster testes were dissected in 1X PBS and then placed in 4% formaldehyde in PBST (1X PBS, 0.1% Triton X-100) for 40 minutes. Testes were then rinsed in PBST 3 times for 2 minutes each and placed in a 1:10 concentration of bovine serum albumin (BSA) in PBST and left overnight in 4°C. After 12 hour incubation at 4°C, the testes were rinsed in PBST 3 times for 15 minutes each. They were then placed in a primary antibody – PBST solution (1:50 anti-fasciclin III, 1:50 DE cadherin, 1:50 armadillo). All primary antibodies were obtained from the Developmental Studies Hybridoma Bank. The testes were incubated at 4°C for 12 hours. After the second incubation, the testes were washed in PBST 3 times for 15 minutes each. They were then placed in their respective secondary antibody – PBST

solution; 1:1000 Alexa-fluor 488 goat anti-mouse for anti-fasciclin III and armadillo treated testes and 1:1000 Alexa-fluor 488 rabbit anti-rat for DE cadherin treated testes. All secondary antibodies were obtained from Sigma-Aldrich. They were incubated for 12 hours in 4°C.Control samples were processed in the exact same way as the experimental samples except that the primary antibody was eliminated from the protocol.

After the third incubation, the samples were washed again in PBST 3 times for 15 minutes each. A slide was prepared with a droplet of MOWIOL mounting medium containing 5 µg/ml DAPI to counterstain the nuclei. The testes were then placed in the mounting solution and covered with a coverslip. The samples were then viewed on an Olympus FV 1000 confocal laser scanning microscope (CLSM).

IV. Immunofluorescence Staining of *Drosophila pseudoobscura* Testes

D. pseudoobscura testes were dissected in 1X PBS and placed in ice cold methanol and stored in 4°C for 30 minutes. Immediately after the 30 minute incubation, the testes were transferred to ice cold acetone and stored in 4°C for 15 minutes. The testes were washed for 2 minutes in PBST 3 times. They were placed in a 1:10 bovine serum albumin (BSA) - PBST solution and stored in 4°C for 12 hours. After 12 hours, the testes were rinsed in PBST 3 times for 15 minutes each. They were then placed in a primary antibody - PBST solution (1:10 anti-fasciclin III, 1:10 DE cadherin, 1:10 armadillo). They were left in 4°C for 12 hours. After the second incubation, the testes were washed in PBST three times for 15 minutes each. They were then placed in their respective secondary antibody - PBST solution; 1:500 Alexa-fluor 488 goat anti-mouse for anti-fasciclin III and armadillo treated testes and 1:500 Alexa-fluor 488 rabbit anti-rat for DE cadherin treated testes (DSHB). They were

incubated for 12 hours in 4°C. Control samples were processed in the exact same way as the experimental samples except that the primary antibody was eliminated from the protocol.

After the third incubation, the samples were washed again in PBST three times for 15 minutes each. A slide was prepared with a droplet of MOWIOL mounting medium containing 5 µg/ml DAPI. The testes were then placed in the mounting solution and covered with a coverslip. The samples were then viewed on an Olympus FV 1000 CLSM.

V. BLAST Analysis and T-Coffee Alignment of Hub Proteins

BLAST searches of the three hub proteins of interest (DE-cadherin, fascicilin III, and armadillo) were performed using NCBI BLASTp (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome). The amino acid sequences for *D. melanogaster* DE-cadherin, fasciclin III, and armadillo were selected and chosen to undergo a BLAST analysis against the genome of *D. pseudoobscura* species. The top result for each protein BLAST contained the same or a similar amino acid sequence of the protein in *D. pseudoobscura* based on the protein chosen from *D. melanogaster.* The E value and the percent coverage of the top match were recorded.

Once the BLAST analyses were completed, the entire amino acid sequence of each *D. melanogaster* hub proteins and the top matched result corresponding to the same protein in *D. pseudoobscura* were aligned using T-Coffee software (http://tcoffee.crg.cat/apps/tcoffee/do:regular) to show the exact corresponding amino acid matches for each of the three hub proteins analyzed.

VI. Confocal Imaging and Visualization of *Drosophila pseudoobscura* Image Stacks

An Olympus FV1000 CLSM was used to obtain the high resolution images of *D. melanogaster* and *D. pseudoobscura* testes. The testes were imaged using a 40X oil immersion lens. A 488 argon laser was used to excite the Alexa-fluor 488 secondary antibodies present in the sample. A 405 nm laser was used to excite the DAPI stain within cell nuclei. Image stacks of the entire testis were collected by assigning points of depths of the testis image to be recorded and the thickness of sections were optimized to ensure full detail of the area of interest. Sections of the tissues were collected at a sampling speed of 10 microseconds per pixel and at a resolution of 1024 x 1024. Recorded image stacks visualized using the computer program Imaris (Bitplane Inc.).

VII. Transmission Electron Microscope Preparation of *Drosophila pseudoobscura* Testes, Seminal Vesicles, and Accessory Glands

Adult *D. pseudoobscura* flies aged 24 - 48 hours were anesthetized on ice for five minutes. Male flies were then transferred to a depression slide containing a 2.5 % glutaraldehyde in 0.1 M cacodylate buffer. The testes were removed from the abdomen using fine probes. Once the testes were removed from the fly, syringe needles were used to accurately cut and separate the testes, seminal vesicles, and accessory glands. The samples were incubated in the glutaraldehyde fixative solution for one hour at room temperature and then rinsed with 0.1 M cacodylate buffer three times for five minutes each. Under a fume hood, samples were then post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hour at room temperature. Samples were then rinsed with 0.1 M cacodylate buffer three times for five minutes each under the fume hood.

Samples were removed from buffer and dehydrated in an ethanol series. Samples were placed in 50% ethanol (EtOH) for 15 minutes. After 15 minutes in 50% EtOH, samples were placed in 70% EtOH for another 15 minutes. Samples were then placed in 95% EtOH for 20 minutes (two changes), followed by placement in two changes of 100% EtOH for 20 minutes each.

Samples were then infiltrated with plastic resin by removing the samples from 100% EtOH and placing in 100% acetone for 20 minutes. After 20 minutes, the testes were placed in a 1:1 resin medium - acetone solution for 1 hour. After 1 hour, samples were transferred from the 1:1 solution to a 2:1 resin medium - acetone solution and allow them to incubate overnight at room temperature on a rotator. The resin used was Embed-812 (Electron Microscopy Sciences).

The following day, samples were embedding in resin by removing the samples from the 2:1 solution and placing them in 100% resin for 1 hour on a rotator. After an hour, samples were transferred to fresh 100% resin and allowed to incubate on the rotator for 1 - 6 hours. Samples were then transferred from the 100% resin to molds that were partially filled with a resin - accelerator solution. Samples were positioned so they were located at the center of the tip of the mold. The remainder of the mold was filled with resin - accelerator solution and placed in an oven overnight at 60°C.

Once hardened, the sample blocks were removed from the molds, sectioned on an ultramicrotome, stained with uranyl acetate, and viewed on an FEI Tecnai Spirit transmission electron microscope located at the College of Staten Island.

Results

I. Immunofluorescence Staining Overview

Staining the stem cell niche of *D. melanogaster* was successful using all three of the antibodies that were raised against the proteins for this species. However the fixation used for the *D. melanogaster* testes was not successful for the staining of the stem cell niche of the *D. pseudoobscura.* Using a methanol/acetone fixation, the antibody for the fasciclin III protein successfully stained the proteins in the hub in *D. pseudoobscura.* However, regardless of fixation method, we were unsuccessful in staining for DE-cadherin and armadillo in *D. pseudoobscura.* The staining and fixation protocols tested are summarized in Table 1.

II. Immunofluorescence Staining of *Drosophila melanogaster* Testes

Using known antibodies raised against the proteins located in the hub of *D. melanogaster* testes, the stem cell niche was characterized and served as a positive control for the experimental protocols for staining the stem cell niche of *D. pseudoobscura.* Anti-fasciclin III antibodies respond to glycoproteins that allow the somatic cells of the hub adhere to one another. The confocal images in Figures 5A and 5B show *D. melanogaster* testes with a small circular structure - the hub - located at the apical end of the testes. The blue DAPI stain counterstains the nuclei of the cells within the area. The DE-cadherin antibody responds to adherent molecules that keep hub cells clustered together and maintains the interaction of GSCs and CPCs to the hub. The images shown in Figures 5C and 5D represent the the apical end of *D. melanogaster* testes where positive staining of DE-cadherin in the hub is seen. Armadillo staining of the hub cells in D. melanogaster is shown in Figures 5E and 5F. Staining of the hub was not seen in negative controls (not shown).

III. Immunofluorescence Staining of *Drosophila pseudoobscura* Testes

i. Anti-fasciclin III

The staining of the hub in the apical end of the *D. melanogaster* testes using the three raised against the known proteins within the structre indicated that the immunofluorescence staining of the hub was successful in *D. melanogaster* to serve as a positive control for using these antibodies in *D. pseudoobscura* testes.

Anti-fasciclin III was the primary antibody that was successful in staining the hub-like structure of *D. pseudoobscura.* The structure stained in the testes of *D. pseudoobscura* is very large formation that fills a large portion of the depth and area of the apical end. Figure 6B shows a side view of the testes stained with the anti-fasciclin III antibody. Figures 6C, 6D and 6E are also side view images of the testes stained with anti-fasciclin III antibody. The DAPI stain was omitted to highlight the hub-like structure and the depth it takes up within the apical end of the testes. Figure 6F is a top view of the apical end of the testes that depicts the area that the hub structure encompasses. Figure 6A is a *D. pseudoobscura* control testis that was not exposed to the anti-fasciclin III primary antibody but was treated with the secondary antibody. The hub was not stained in control samples.

ii. DE-cadherin and armadillo antibodies

Unlike anti-fasciclin III, the primary antibodies against DE-cadherin and armadillo did not successfully stain the hub-like structure within the apical end of the *D. pseudoobscura* testes that we saw with anti-fasciclin staining.

Species	Fixation	Antibody	Successful
Dmel	4% Formaldehyde	Anti-Fasciclin III	Yes
Dmel	4% Formaldehyde	DE Cadherin	Yes
Dmel	4% Formaldehyde	Armadillo	Yes
Dpse	4% Formaldehyde	Anti-Fasciclin III	No
Dpse	4% Formaldehyde	DE Cadherin	No
Dpse	4% Formaldehyde	Armadillo	No
Dpse	Methanol/Acetone	Anti-Fasciclin III	Yes
Dpse	Methanol/Acetone	DE Cadherin	No
Dpse	Methanol/Acetone	Armadillo	No

Table 1; A depiction of which antibodies and fixations were able to result in a successful immunofluorescent staining of the stem cell niche in the apical end of the *Drosophila* testes. 4% formaldehyde fixation was unsuccessful at staining the stem cell niche of *D. pseudoobscura* testes. Methanol/acetone fixation was successful in staining the stem cell niche with anti-fasciclin III antibody.

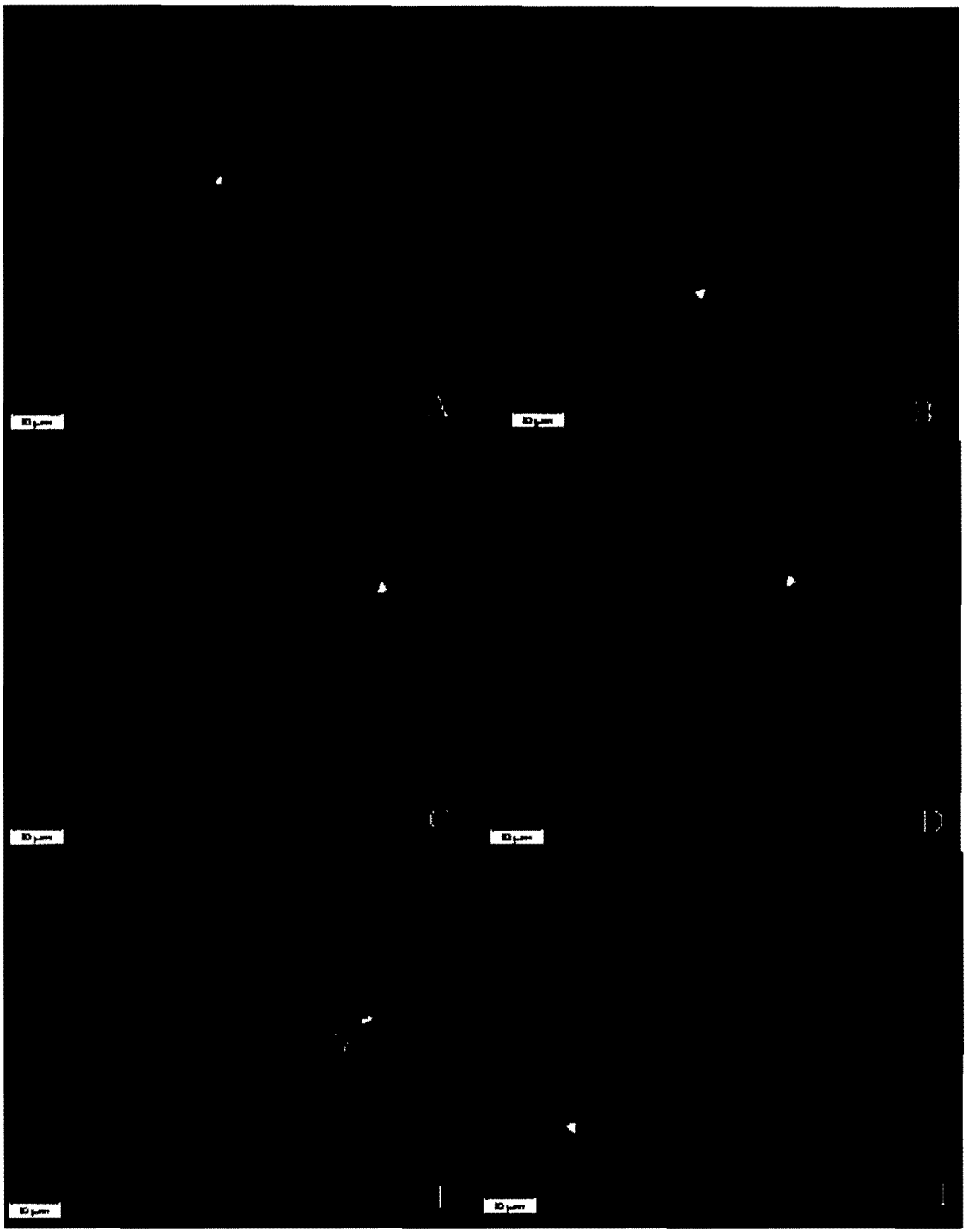


Figure 5: *D. melanogaster* testes stained with anti-fasciclin, DE-cadherin and armadillo. (A and B) The hub of the stem cell niche of the *D. melanogaster* testes that have been stained with the anti-fasciclin III primary antibody. The nuclei are stained blue with DAPI. (C and D) The hub of the *D. melanogaster* stained with the DE-cadherin primary antibody. (E and F) The hub of the *D. melanogaster* stained with the armadillo primary antibody. Control samples were not exposed to any of the primary antibodies and showed no staining of the hub structure (not shown). Scale bars for all panels = 20 μm. All structures were recorded under same magnification.

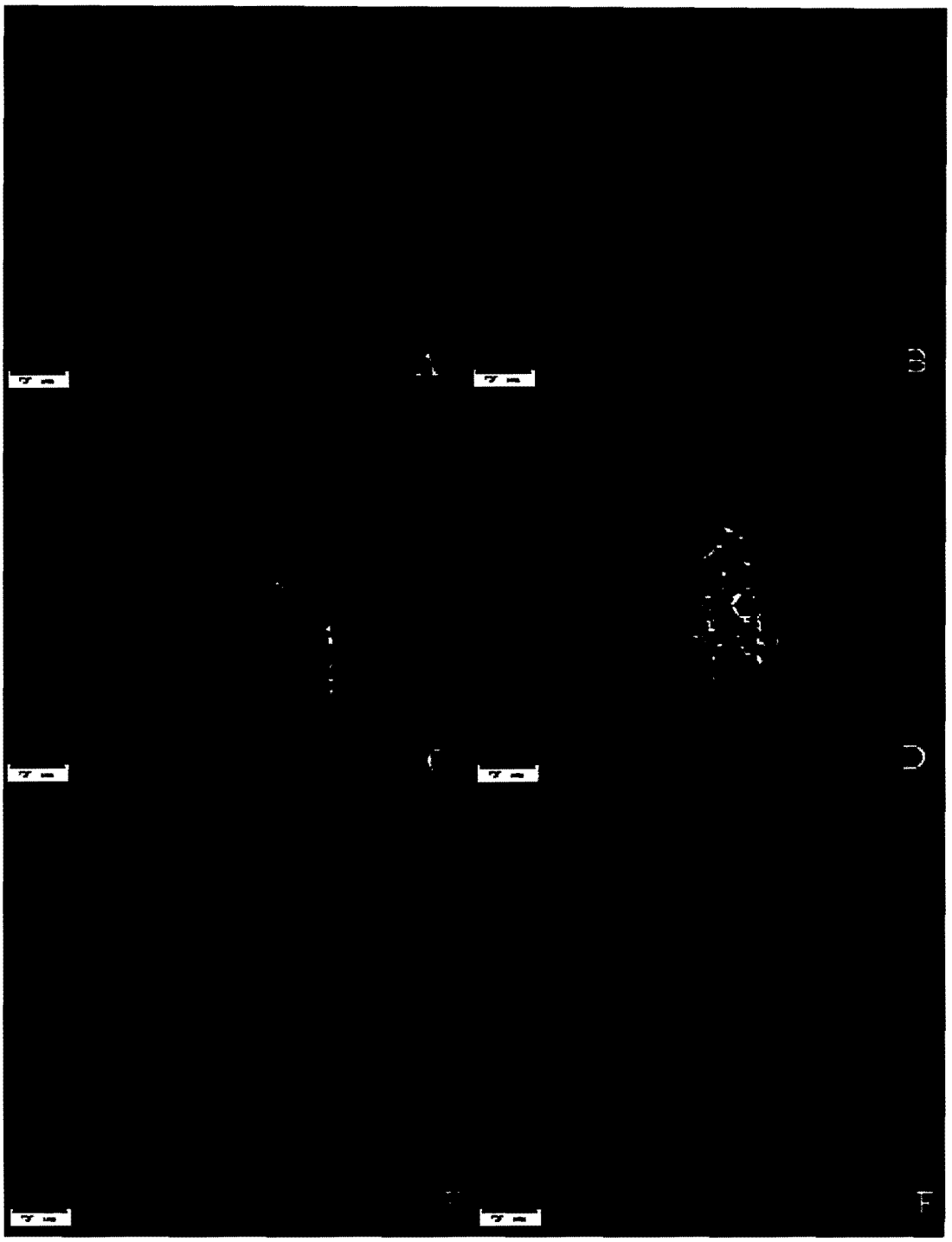


Figure 6: *D. pseudoobscura* testes stained with anti-fasciclin. (A) Apical end of a *D. pseudoobscura* testis that was not exposed to anti-fasciclin III primary antibodies (control). The nuclei are stained blue with DAPI. (B) Side view of a *D. pseudoobscura* testes showing the hub structure. (C - E) Side views of the apical end of *D. pseudoobscura* testes with the hub of the stem cell niche stained with the anti-fasciclin III antibody. The DAPI emission has been omitted to show the hub more clearly. (F) Top view of the apical end of a *D. pseudoobscura.* Scale bar in all panels = 20 μm. All structures were recorded at the same magnification.

Figures 7A and 7B show side view images of the apical end of *D. pseudoobscura* testes that were treated with the DE-cadherin primary antibody. The hub-like structure found in the anti-fasciclin treated samples was not seen in these samples. Figures 7C and 7D show side view images of the apical end of *D. pseudoobscura* testes that were treated with the armadillo primary antibody. Similar to the samples treated with DE-cadherin, the hub-like structure was not detected within these samples.

IV. Transmission Electron Microscopy of the Seminal Vesicles of *Drosophila pseudoobscura*

Figure 8A, 8B and 8C are transmission electron micrographs of the tissue layers that make up the seminal vesicles of *D. pseudoobscura.* The outer layer is a thick pigment layer that gives the testis its bright orange-red color. A thick smooth muscle layer is present for the use of peristalsis and the movement of mature sperm down the seminal vesicle. The next layer is composed of epithelium that probably plays a role in the maintenance of those cells. Figure 8D is a detailed image of a septate junction that connects two epithelial cells together. Figure 8E shows a cross sectional view of epithelial layer. The dark thin structure between the epithelium layer and the area containing sperm cells may be microvilli or stereocilia cross sections which suggests that the epithelial layer interacts with the sperm cell environment. Figure 8F is a low magnification view of the area containing mature sperm in the seminal vesicle. The large structures within the area may be waste bags; however it is possible that these structures are involved in the maintenance of the mature sperm cells within this environment.

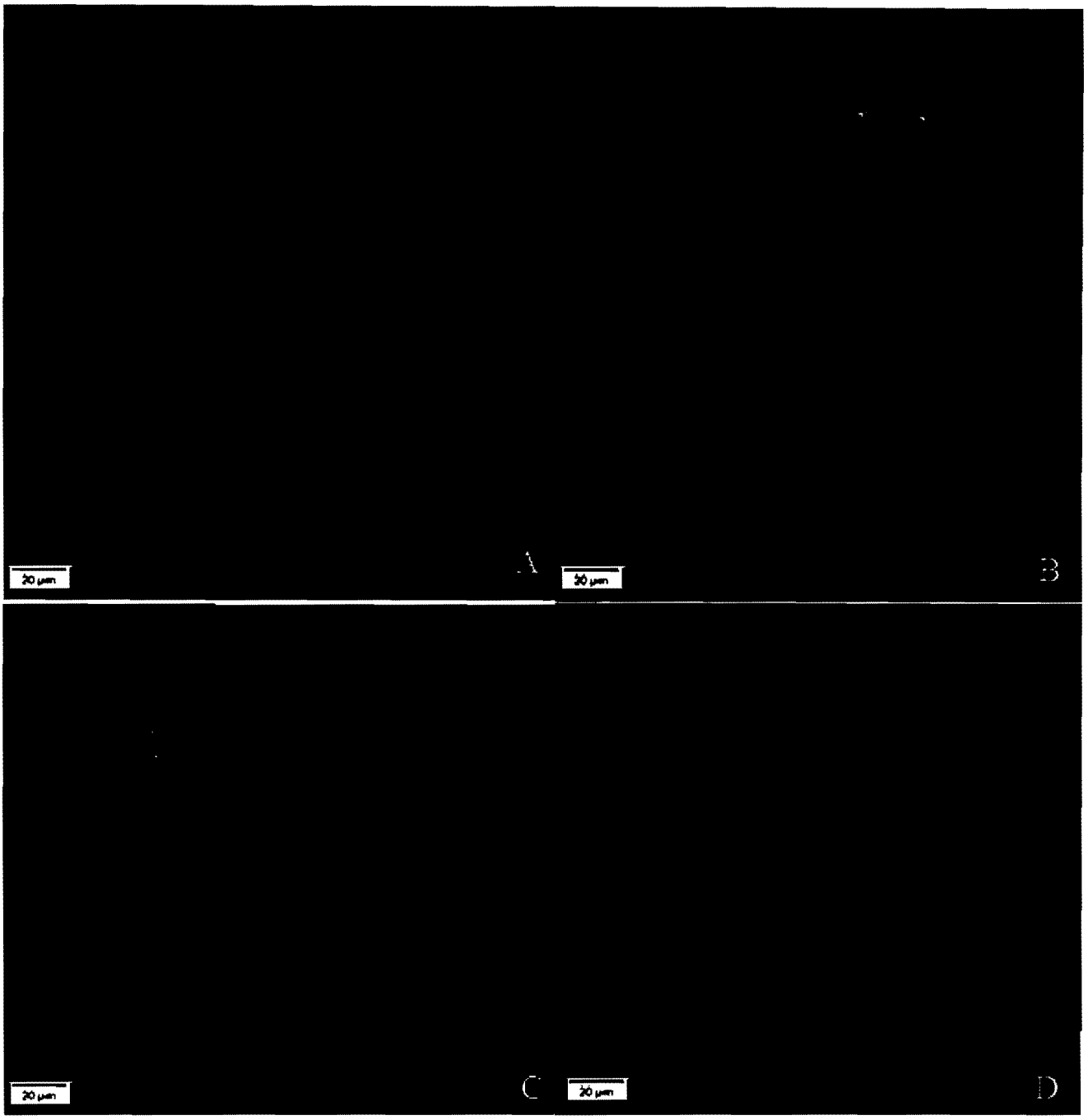


Figure 7: *D. pseudoobscura* testes stained with DE-cadherin and armadillo. (A and B) Apical ends of *D. pseudoobscura* testes that were exposed to DE-cadherin primary antibody; however the hub cells were not detected. The nuclei are stained blue with DAPI. (C and D) Apical ends of *D. pseudoobscura* testes that were exposed to armadillo antibody; however the hub cells were not detected. The nuclei are stained blue with DAPI.

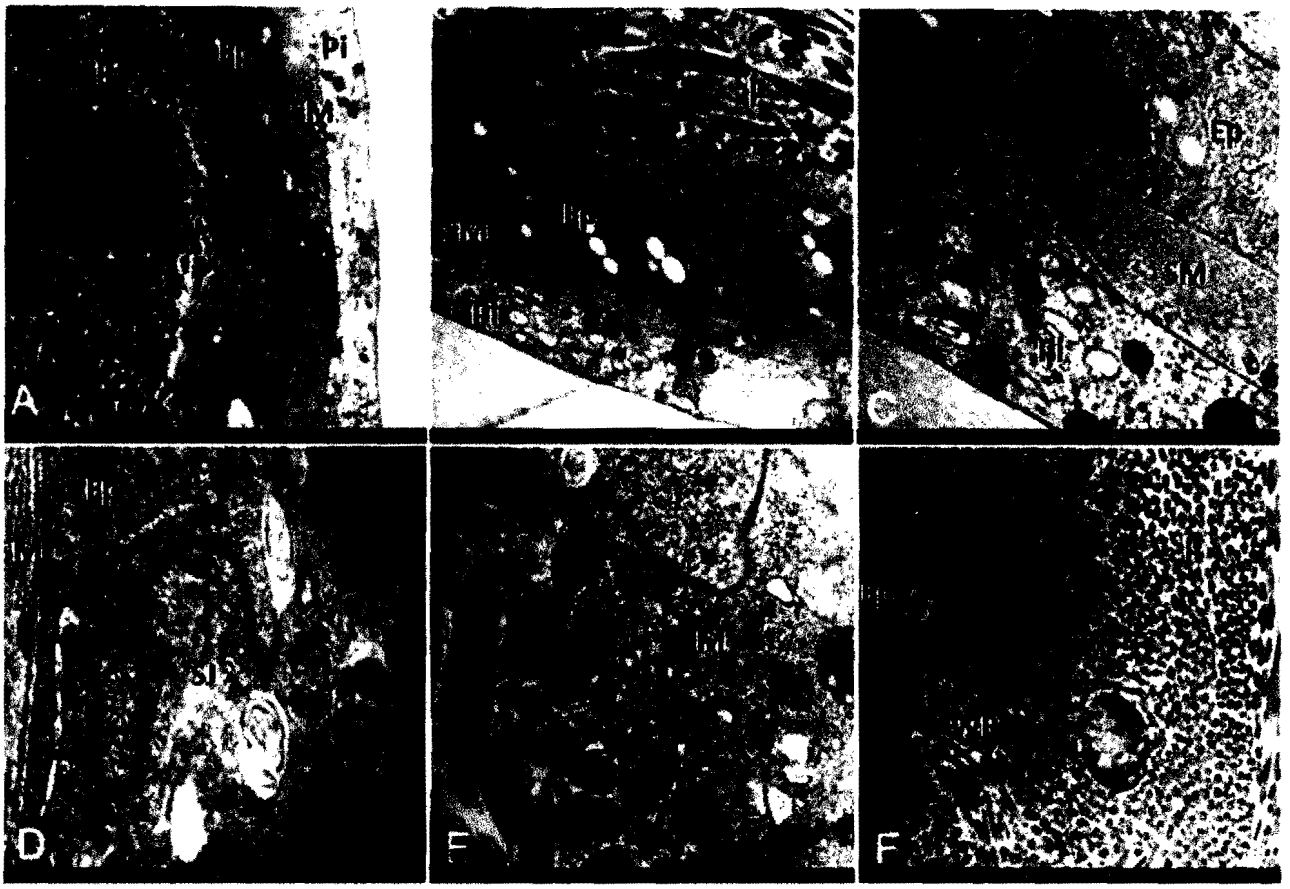


Figure 8: Transmission electron micrographs of the seminal vesicles of *D. pseudoobscura.* (A) Low magnification view of pigment layer (Pi), smooth muscle layer (SM), epithelium (Ep) and mature sperm (Sp) (from right to left). (B) High magnification of the wall layers shown in (A). (C) Pigment layer, muscle layer and epithelium. (D) Septate junction (SJ) between two epithelial cells. (E) Cross sectional view of probable microvilli (M) of the seminal vesicle epithelium. (F) Low magnification view of mature sperm in the seminal vesicle, possible waste bags (WB) located within the mass of sperm.

Discussion

I. Immunofluorescence of the Stem Cell Niche in the *Drosophila melanogaster* Testes

The stem cell niche of the apical end in *D. melanogaster* was successfully stained using all three of the antibodies raised against each protein. The hub in *D. melanogaster* takes up a small part of the tip of the apical end of the testes. In the testes that were exposed to the antibodies anti-fasciclin III and armadillo, the hub appears to be a small, circular orb of several cells which means that these specific proteins are highly concentrated within this cellular area. In the testes that were exposed to DE-cadherin antibodies, the cells of the hub appear to be not so clustered together. This result may be interpreted to mean that DE-cadherin proteins are expressed in a dispersed pattern within the stem cell niche. The staining patterns reported here for *D. melanogaster* are consistent with previous reports on staining of the hub cells using anti-fasciclin III (Snow et al, 1989), anti-armadillo (Myster et al, 2003) and anti-DE-cadherin (Voog et al, 2009) antibodies.

Because all three of the protein-antibody interactions were successful in *D. melanogaster*, these results can be utilized as the positive control when associating these antibodies to the same proteins in a different species, in this case *D. pseudoobscura.*

II. Immunofluorescence of the Stem Cell Niche in the *Drosophila pseudoobscura* Testes

i. Anti-fasciclin III

BLAST analysis and T-Coffee alignments of the DE-cadherin, armadillo, and fasciclin proteins of *D. pseudoobscura* reveal very close or nearly exact matches for the same proteins in *D. melanogaster* (Figures 9–14); therefore we hypothesized that the antibodies and fixation techniques could be used to fluoresce the hub cells of the stem cell niche in *D.*

pseudoobscura. However using the exact methodology used for *D. melanogaster* was not successful for any of the antibodies used for *D. pseudoobscura.*

Ice cold methanol and ice cold acetone fixation yielded results for anti-fasciclin III of *D. pseudoobscura* hub cells. In *D. pseudoobscura,* the hub appears to be a very large structure that is wide enough to fill the apical end and extends deep into the structure of the testes. This is a major difference when compared to the hub of the *D. melanogaster* testis. The *D. melanogaster* hub is a small concenetrated set of cells localized is a small area of the apical end, whereas the *D. pseudoobscura* hub takes up a majority of the apical end and the cells are not as concentrated in a single area, rather there appears to be many more cells present in the hub that extend through the depth of the apical testis. To the best of our knowledge, this is the first report on the structure of the stem cell niche in a species other than *D. melanogaster.* The unusual structure of the hub in *D. pseudoobscura* is consistent with the unusual morphology of the testes themselves.

ii. DE-cadherin and armadillo

We were able to stain the hub in *D. pseudoobscura* testes using one of the antibodies, anti-fasciclin III, however we were unsuccessful in staining the hub using either DE-cadherin and armadillo primary antibodies. The reason why the two antibodies did not fluoresce the hub is unknown. Unlike previous complications with the 4% formaldehyde fixation step between the two species of *Drosophila,* we were able to successfully stain the

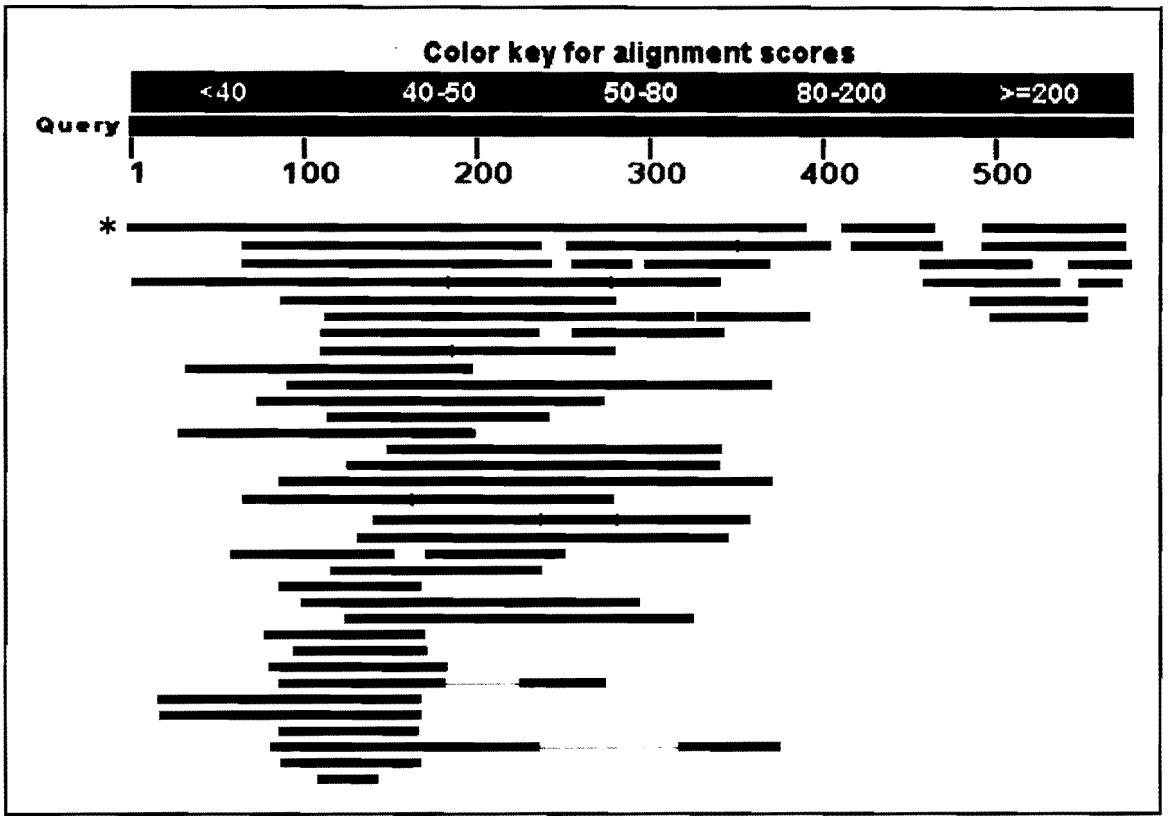


Figure 9: Blast analysis of fasciclin III protein in *D. pseudoobscura* compared to *D. melanogaster*. The query sequence is *D. melanogaster*. The best match for *D. pseudoobscura* is the first row (*). Result: 91% identical

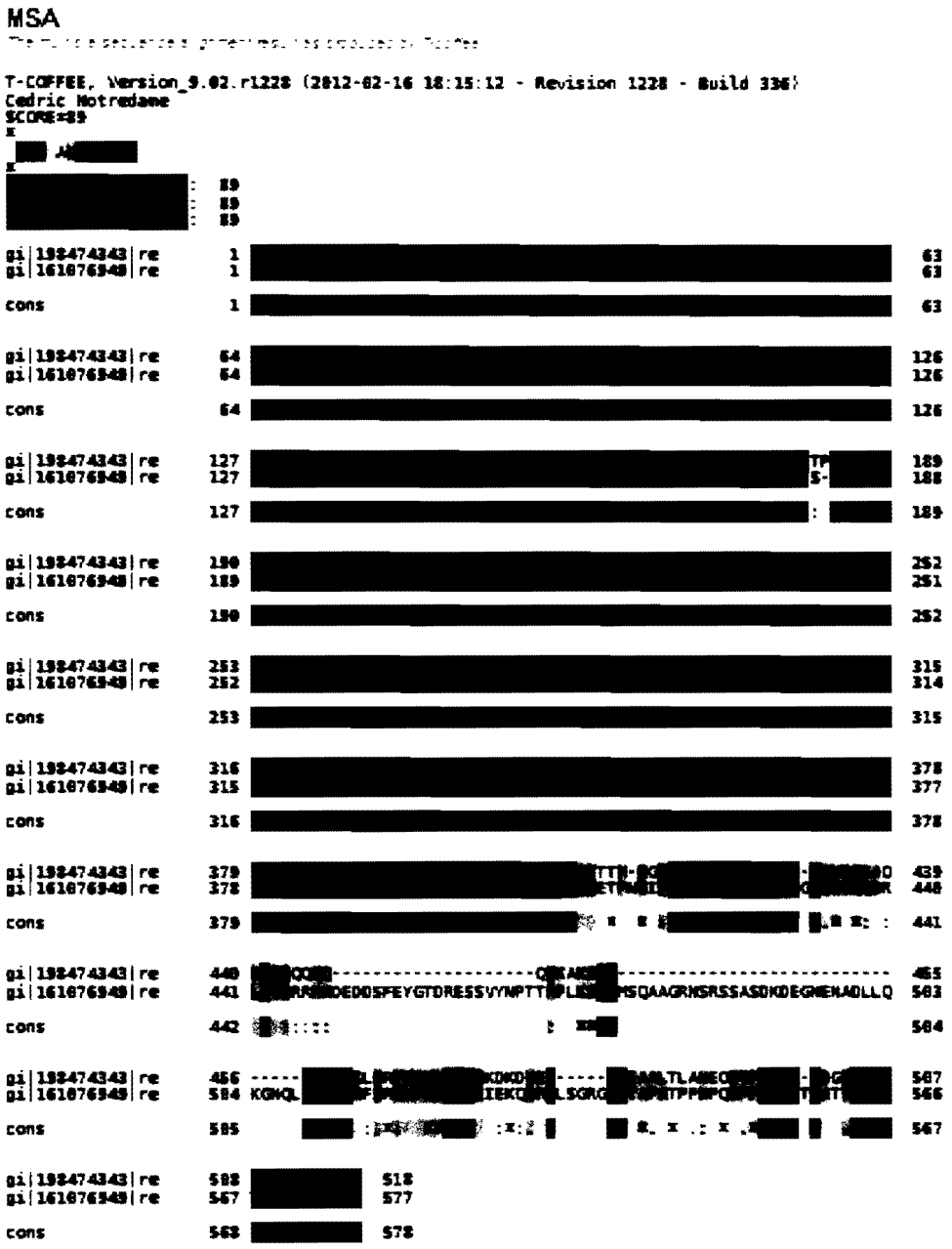


Figure 10: T-Coffee alignment of Fasciclin III protein sequence comparison from *D. pseudoobscura* and *D. melanogaster*.

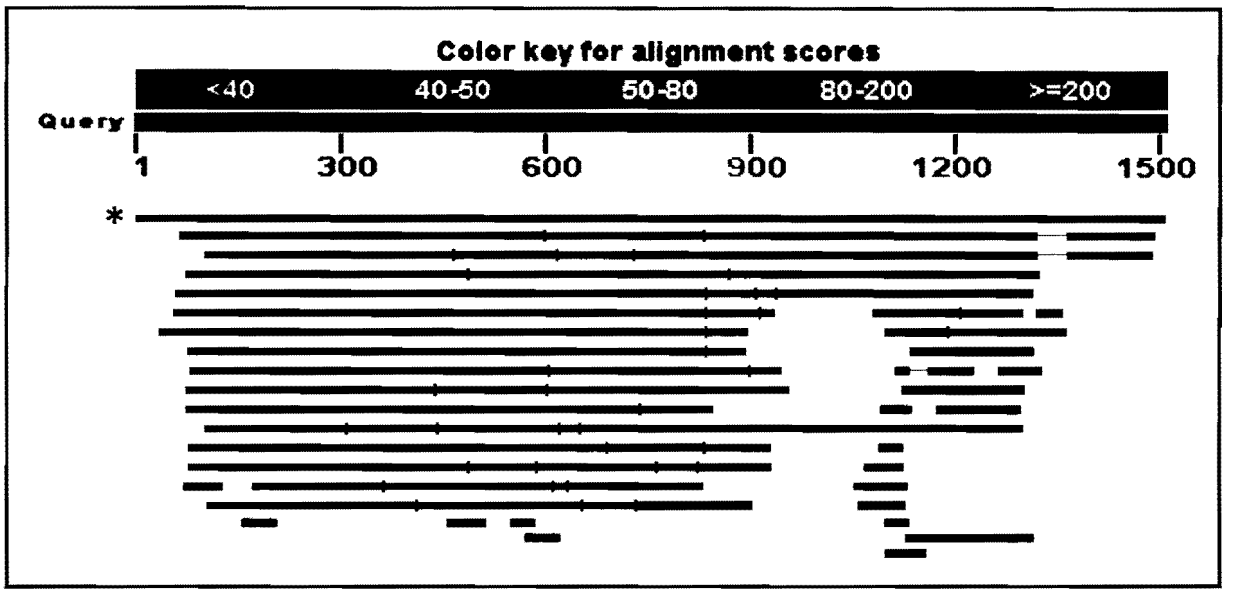
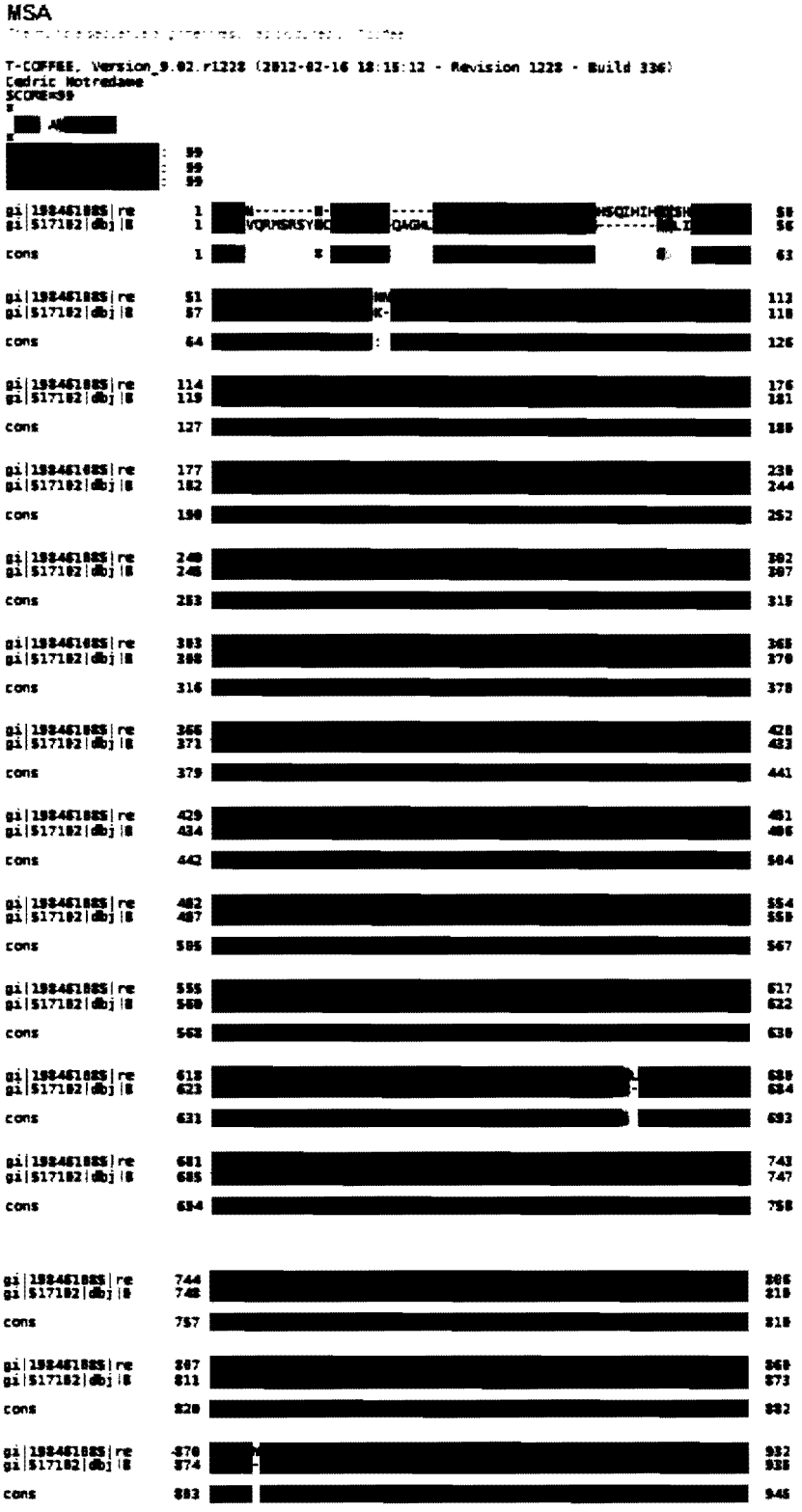


Figure 11: BLAST analysis of DE cadherin protein in *D. pseudoobscura* compared to *D. melanogaster*. The query sequence is *D. melanogaster*. The best match for *D. pseudoobscura* is the first row (*). Result: 99% identical

MSA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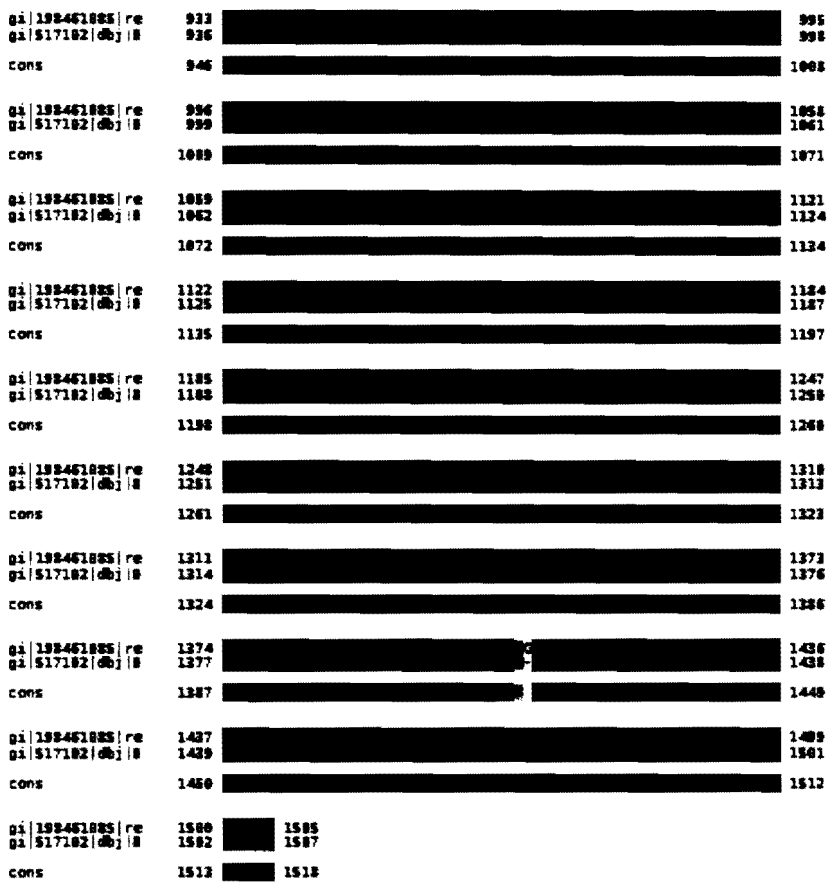

Figure 12: T-C-offee alignment of DE-cadherin protein sequence comparison from *D. pseudoobscura* and *D. melanogaster*.

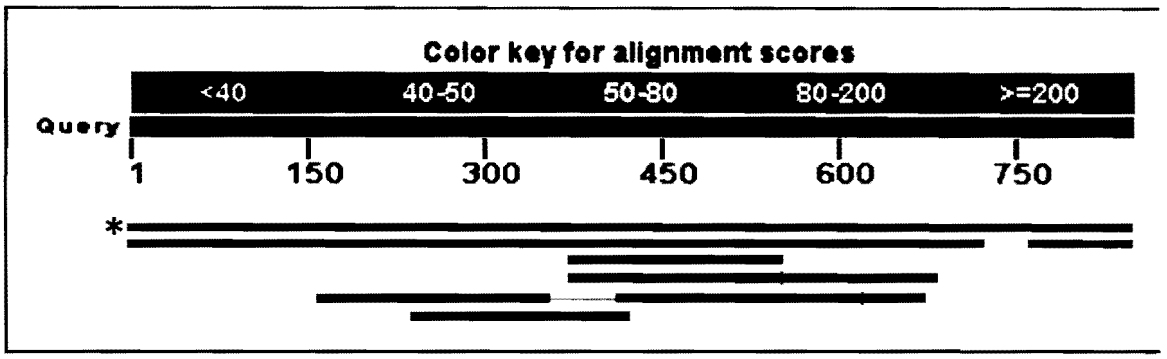

Figure 13: BLAST analysis of armadillo protein in *D. pseudoobscura* compared to *D. melanogaster*. The query sequence is *D. melanogaster*. The best match for *D. pseudoobscura* is the first row (*). Result: 100% identical.

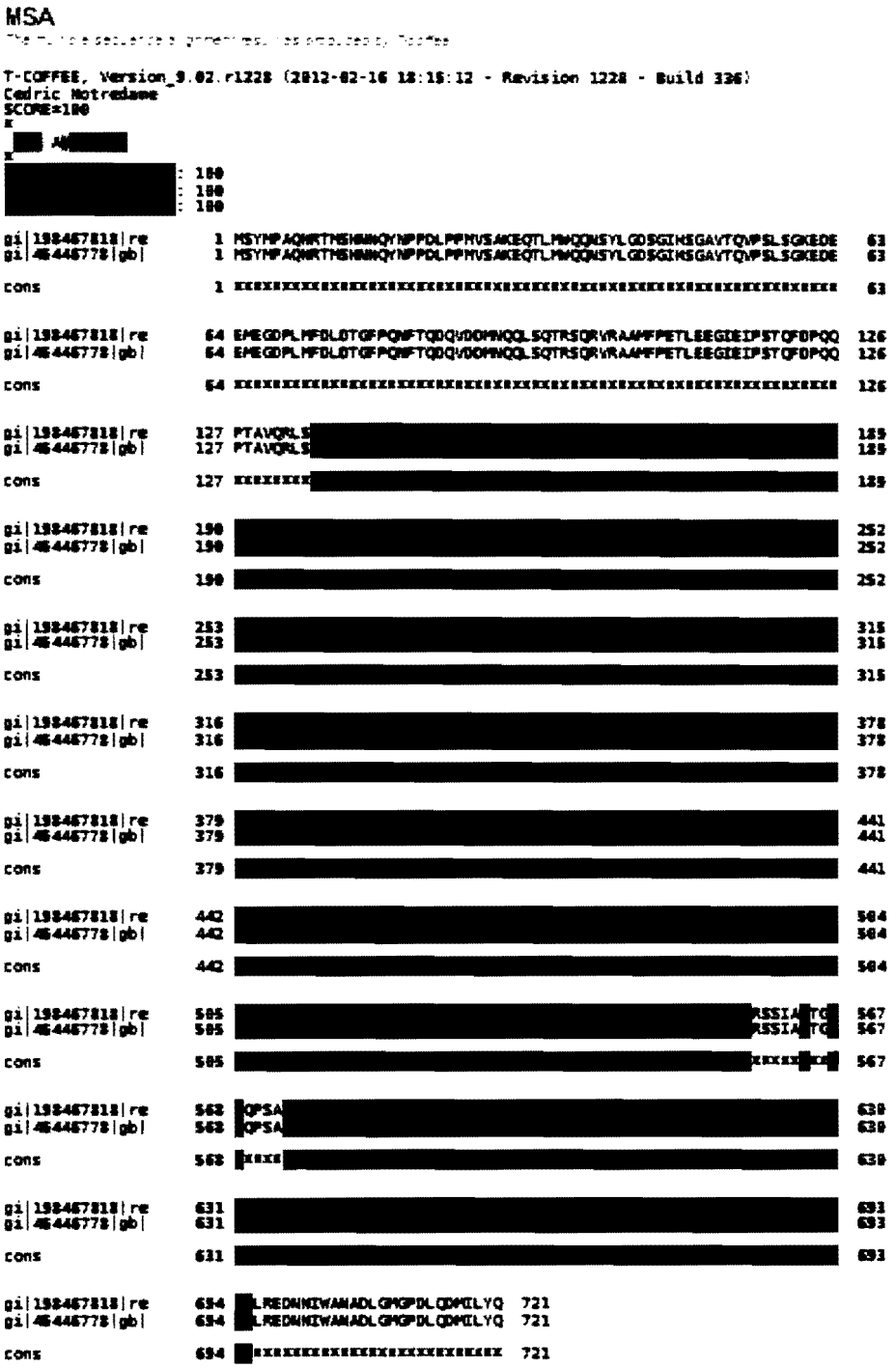

Figure 14: T-Coffee alignment of armadillo protein sequence comparison from *D. pseudoobscura* and *D. melanogaster*.

hub with anti-fasciclin III using the methanol and acetone fixation but not with the DE-cadherin and armadillo antibodies.

III. Transmission Electron Microscopy of the Seminal Vesicles of *Drosophila pseudoobscura*

Our results show ultrastructural features of *D. pseudoobscura* seminal vesicles not previously reported. To the best of our knowledge, the ultrastructure of the seminal vesicle in the genus *Drosophila* has only been reported for *D. bifurca* – a distant relative of *D. pseudoobscura* (Joly et al., 2003).

The tissue layers of the seminal vesicle begins with a large pigmented layer that gives the organ the orange-red color which is similar to the characteristics of the Mexican fruit fly, *Anastrepha ludens* (Valdez et al. 2001). The next layer is a large smooth muscle layer. The smooth muscle layer found in our study of *D. pseudoobscura* appears to be thicker and more well-developed than that found in *D. bifurca*. The smooth muscle allows for slight movement of the mature sperm through the seminal vesicles via peristalsis which allows for sperm to enter and be stored in the seminal vesicles. The even larger epithelial layer followed the smooth muscle layer of the seminal vesicle. The epithelium of the seminal vesicle is bound together by highly convoluted septate junctions. These junctions have been previously reported in a specific structure found in an unusual organ in *D. bifurca* called the sperm roller (Joly et al., 2003) which is continuous with the seminal vesicle. However, the convoluted nature of the junction, and the junction itself, has not been specifically reported for the seminal vesicle of any other *Drosophila* species.

In the epithelium layer closer to the mature sperm cells, there appear to structures which may represent microvilli or stereocilia that may interact with the mature sperm cells. Interstingly, the mammalian sperm storage organ called the epididymis, which may be considered to be analogous to the fly seminal vesicle, is also lined with epithelium that have numerous microvilli projections (Primiani et al, 2007). The epididymis is known to play a role in post meiotic maturation of mammalian cells (Nath et al, 2012); however it is unknown if the seminal vesicles serve a similar function in fly sperm maturation. The similarity in morphology may suggest the epithelium in the fly seminal vesicles plays a similar role. In one study using rat epididymus, researchers have observed membrane bound vesicles around stereocilia within the epithelium that contain various enzymes which are believed to maintain the environment of the sperm cells present (Fornes et al, 1995).

In the area of the seminal vesicles where the mature sperm are stored, we noted large structures which may be waste bags. Waste bags are structures that form as a result of one of the final stages of spermiogenesis where excess cytoplasm and organelles are eliminated from the sperm cells in the testes (Metzendorf et al, 2010). Similar to the septate junctions between epithelium cells, the apparent waste bags seen in the seminal vesicles in the current study have not been previously reported. The function of these structures is unknown. If these structures are not waste bags, it is possible that their function is to maintain the environment of the stored sperm cells.

IV. Future Directions

After a successful immunofluorescence staining of the presumed hub in the apical end of the testes of *D. pseudoobscura* using anti-fasciclin III, the goal is to stain this structure again

using at least one of the antibodies for DE-cadherin and armadillo that are raised against the proteins of the hub structure using protocols that will be further adjusted. Staining of the hub with additional antibodies would allow us to confidently claim that the structure of the stem cell niche in the apical end of *D. pseudoobscura* testes is significantly different from the apical end of *D. melanogaster* testes.

Transmission electron microscopy can be used to understand the ultrastructure of the reproductive organs and the processes that occur within them, and can be used to describe the differences between *D. melanogaste*r and other species. We will continue to characterize the ultrastructure of the reproductive organs of *D. pseudoobscura* using TEM.

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