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Spermiogenesis and in Vitro Culture of Spermatogenic Cysts in Drosophila Pseudoobscura

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SPERMIOGENESIS AND *IN VITRO* CULTURE OF

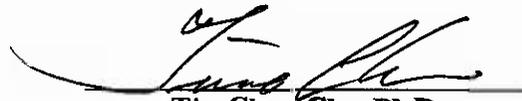# SPERMATOGENIC CYSTS IN *DROSOPHILA*

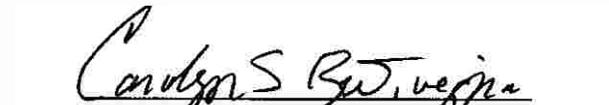# *PSEUDOOBSCURA*

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ABSTRACT

Spermatogenesis is a complex process that involves differentiation and morphological changes of diploid stem cells into highly specialized, haploid, motile germ cells. Many of the transformative processes involved in mammalian spermatogenesis are conserved in flies, and their study would be facilitated by the availability of a reliable *in vitro* culture system. Culture of *Drosophila melanogaster* spermatogenic cysts is limited in usefulness because of early degeneration of cysts, low yield of elongated spermatozoa, and rare occurrences of motile sperm. We have developed a culture system for studying fly spermatogenesis using isolated spermatogenic cysts from the testes of *Drosophila pseudoobscura*. This species offers several advantages over *D. melanogaster*: (1) *D. pseudoobscura* testes can be easily distinguished in pupal stages due to intense red pigmentation, (2) survival of cysts to the elongated, motile form is easily achieved and highly repeatable, and (3) only minimal media is necessary in order to grow sperm to motility. Cysts containing primary spermatocytes isolated from late-stage pupa developed into motile sperm usually within five days of culture. Cysts underwent meiosis, elongation, individualization and coiling just as in *D. melanogaster*. To the best of our knowledge, this is the first report of *Drosophila* sperm cell culture where motility is consistently achieved. This culture system should prove valuable for spermatogenesis studies where cellular transformation processes must be manipulated *in vitro*.

Introduction

Flies of the genus *Drosophila* have been utilized as model organisms for genetic and developmental studies over the last century. These flies possess advantages over other model organisms in that they have a short generation time, great fecundity, hardiness, and they can be cheaply maintained in the laboratory. Additionally, the relative ease of induction of mutations and the availability of fully sequenced genomes make these flies amenable to genetic investigation.

Although *Drosophila melanogaster* has been the species of choice in the bulk of genetic studies, *Drosophila pseudoobscura* has proven more amenable for population genetics, race formation and species differentiation studies (Dobzhansky, 1936; Noor et al., 2001; Machando et al., 2002). Unlike *D. melanogaster*, certain species within the *D. pseudoobscura* group can be crossed and produce fertile offspring (Dobzhansky, 1936). Hence, *D. pseudoobscura* has contributed greatly to hybrid sterility studies. In addition, this group is known to have sperm polymegaly, a form of sperm heteromorphism characterized by production of two different size spermatozoa within the same animal (Snook and Karr, 1998) whose only difference is the head and tail lengths.

Genes expressed in the testes of *D. pseudoobscura* have been reported as showing decreased identity to their *D. melanogaster* orthologs (Richards et al., 2005) even though the overall gene conservation between the two species is high. However, since spermatogenesis is a highly evolutionary conserved process such that insect and mammals exhibit remarkably similar cell morphologies and the basic regulatory pathways controlling spermatogenesis, understanding spermatogenesis in *D. pseudoobscura* should offer a wealth of cellular and genomic knowledge that will be

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widely applied in helping to further understand stem cell maintenance and cell behavior, particularly the coordinated sequence of morphological changes that lead to differentiation of germ cells into highly specialized cells of great complexity.

Spermatogenesis in flies

The process of spermatogenesis is maintained throughout the lifetime of the male organism via regeneration of stem cells. Spermatogenesis in *Drosophila melanogaster* in particular has been extensively studied (Cooper, 1950; Cross and Shellenbarger, 1979; Fuller, 1993) and chronological changes of the germ cells to motile spermatids at the ultrastructral level have been described in great detail (Tokuyasu, 1974; 1975; Stanley *et.al.*, 1972 and 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, 1980; 1982).

Several attempts have been made to isolate and culture *Drosophila* germ line cells. *In vitro* culture in *D. hydei* has been done by Fowler (1973), Fowler and Johannison (1976), and Liebrich (1981 &1982). *In vitro* culture in *D. melanogaster* was done by Cross and Shellenbarger (1979) and partial culture by Noguchi and Miller, (2003). However, none of these attempts have been successful in establishing a stable cell line such as the female germ-line stem cells (fGSCs) cell line established by Niki. *et al.* (2006) in which fGSCs coexists with ovarian somatic stem cells. The above problem is largely due to the low survival rates of the isolated cysts stemming from lack of appropriate media conditioned specifically for spermatogonial cell culture.

Spermatogenesis in *Drosophila* starts at the apical end of the testis where the germinal proliferation center is located (Fig. 1). Within this center are three types of cells: (1) the hub cells that appear to be responsible for the multipotency of the stem cell in the hub, (2) germ-line stem cells (GSCs) and (3) the somatic cyst progenitor cells (SSCs) clustered in a rosette around the hub (Hardy *et al.* 1979). Germ-line stem cell division is asymmetrical, producing a stem cell and a spermatogonium that is displaced laterally from the hub and becomes encapsulated by two daughter cells from two somatic cyst progenitor cells. The rest of the spermatogenesis process occurs within this cyst. The encapsulated spermatogonium undergoes a synchronous series of incomplete mitotic divisions and a growth phase resulting in a series of primary spermatocytes in syncytium within a cyst.

Primary spermatocytes are characterized by a growth phase where the cells increase greatly in volume and high gene expression. Primary spermatocyte cysts then commit to two meiotic divisions resulting in haploid round spermatids and finally differentiation resulting in mature elongated sperm (Fuller, 1993). In *D. melanogaster* there are four mitotic divisions producing a cyst of 16 primary spermatocytes while in *D. pseudoobscura* there are five divisions resulting in a cyst of 32 primary spermatocytes.

After the two meiotic divisions, the cysts have 64 and 128 round spermatids respectively. Since cytokinesis in both mitotic and meiotic divisions is incomplete (Lindsley and Tokuyasu, 1980), the spermatogonia are interconnected by cytoplasmic bridges as they undergo synchronous mitotic and meiotic divisions and produce groups of syncytial spermatids. See Figure 2 for an overview of cyst development in *Drosophila*.

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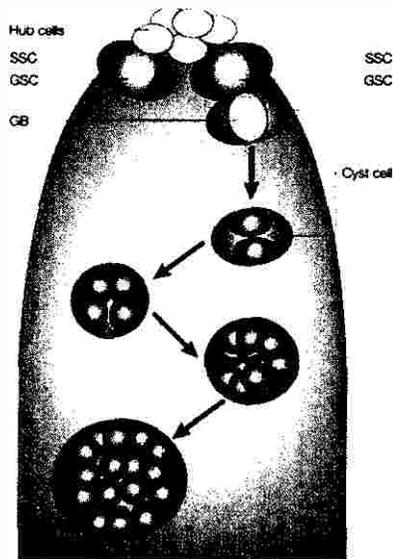
Fig. 1 An illustration of the apical tip of Drosophila testis. The hub cells, germ stem cells (GSCs) and the Somatic stem cells (SSCs) make up the tip. These cells are connected through adherences and gap junctions. The encapsulated gonial blast (GB) undergoes a series of mitotic divisions resulting in a 16 spermatocyte cyst.

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- Apical cells
- Spermatogonia
- Follicular epithelium
- Spermatocytes
- Round Spermatids
- Elongating Spermatids
- Cysted Spermatozoa
- Free spermatozoa
- Basal epithelium

Vas deferens
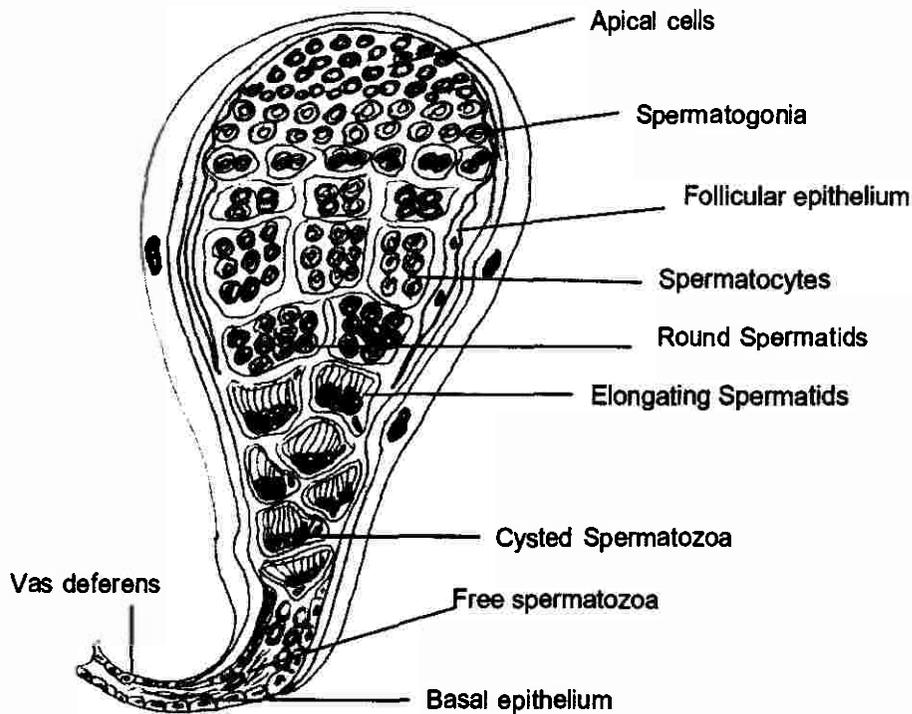
Fig.2 A schematic diagram of *Drosophila pseudoobscura* testis showing germ stem cells transformation into mature spermatozoa. Hand sketched after Valdez, J. M. (2001) Ultrastructure of the testis of the Mexican fruit fly (Diptera: Tephritidae). Ann. Entomol. Soc. Am 94. 251– 256.

The final event in sperm production is the terminal differentiation of the immature spermatids in a process called spermiogenesis. This process involves elongation of the round spermatids, individualization whereby membrane remodeling for each sperm is obtained while the cytoplasmic bridges are disconnected and the coiling process where the differentiated sperm within a cyst are rolled into a disc and eventually burst out of the cyst. During elongation, nuclear transformation in the sperm head occurs where the sperm nucleus is modified from a spherical shape to a condensed and elongated needle-shape. This is achieved by the replacement of histones by linker histone-like proteins which are then replaced by protamines bringing about the transformation from nucleosomal to highly condensed state of the chromatin (Renkawitz-Pohl and Raja, 2005).

During individualization most of the spermatid's cytoplasmic components are expelled as a cystic bulge that traverses the entire length of the cyst initiating at the head to the tail region of the spermatids (Noguchi and Miller, 2003) and eventually collects into a waste bag at the caudal end of the cyst. According to findings by Steller et al. (2003), the individualization process is an apoptosis-like process that results in the expulsion of most of the cytoplasmic components in the spermatids and change in the morphology through caspase activation. Caspase inhibitors prevent cytoplasm removal resulting in sterile males. Bazinet et al. (1998) and Noguchi and Miller (2003) reported that actin polymerization is responsible for the cystic bulge movement during individualization process. Beckingham et al. (2008) identified the gene *yuri gagarin*, a component of the motile actin cones that individualize the spermatids. *yuri* is a *Drosophila* specific gene. Most of *Drosophila* species including *D. melanogaster* have

one copy of *yuri* except in *D. pseudoobscura* and it close relative *D. persimilis* in which two related *yuri* genes are present. In *yuri* mutant, initialization of actin cones and nuclear behavior are aberrant.

The coiling process is the final event in the differentiation of sperm. *Drosophila* males produce sperm of remarkable length, up to 1.8mm for *D. melanogaster* and 6 cm in *D. bifurca* brought about by specialized actin and microtubules (Beckingham et al., 2008). As a result, drosophilid flies have developed mechanisms such as the sperm-roller to aid in the packaging of mature sperm. Mature sperm are usually coiled bundles that burst from the cysts before they are moved into the seminal vesicles for storage.

In previous studies where cysts containing the germ cells were obtained from pupa aged 24-50 hours, sperm motility was never observed *in vitro* even though some cysts completed the coiling stage (Liebrich, 1981). Cross and Shellenbarger (1979) noted that the cyst development was also atypical. Elongating cysts only achieved a third (~500 µm) of their normal length of about 1800 µm, coiling commenced prior to the termination of individualization stage and the whole duration of *in vitro* development (45hrs) was less than half of in vivo development of 96 hours.

Liebrich (1981) reported that *D. hydei* cysts isolated when their spermatocytes were in prophase I stage differentiated for 2-3 days at 22°C *in vitro*. However, in temperatures lower than room temperature, differentiation was prolonged to approximately 5 days. He further reported that spermatogonial cysts were difficult to cultivate for longer than 3 days as they died before entering metaphase I.

The purpose of the current study was to (1) establish an *in vitro* culture system that would facilitate study of spermiogenesis in *Drosophila* and performance of

experiments requiring manipulation of cells that would otherwise be difficult or impossible *in vivo* and (2) offer more details into the biology of *D. pseudoobscura* by characterizing spermatogenesis in this species which to date is unavailable despite its genome having been sequenced and the wide use of the fly in hybrid sterility studies. It was found that spermatogenesis in *D. pseudoobscura* was very similar to that of *D. melanogaster* but, spermatogenic cysts in *D.pseudoobscura* were more resilient when cultured *in vitro* and they required very minimal media.

MATERIALS AND METHODS

I. Fly stock and cultures

The fly stocks used were obtained from Tucson Drosophila Stock Center (University of Arizona, Tucson) as wild type and cultured in our laboratory on Jazz Mix Drosophila medium (Fisher) at 25°C.

II. Live squash of testis

Adult testes were dissected for comparison of morphology and developmental differences with the cultured testes and cysts. Adult flies were anesthetized by placing them in a vial on crushed ice for 5 minutes. They were then placed on a depression slide with a drop of 1X phosphate buffered saline (PBS) and the testes removed using forceps and a tungsten needle. The testes were transferred to a 20 µl of 1X PBS on a cover slip and a glass slide was gently lowered until the fluid touched the slide sucking up the cover-slip, providing a very gentle squash. The testes were observed on an inverted phase contrast Leica DMIL microscope and images taken with a cooled CCD camera (Leica DFC 300).

III.   Culture conditions.

Three different culture media were tested based on previous studies by Cross and Shellenbarger (1979), Liebrich (1981), and Noguchi and Miller (2003). These included Schneider's media, Grace's insect media (both used as purchased from Invitrogen, USA) and powdered Shields and Sang M3 media with L-glutamine, without potassium

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bicarbonate (Sigma-Aldrich, St Louis, MO). The latter was prepared in our laboratory as per manufacturer's instructions. All three media were supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1X penicillin/streptomycin cocktail (Sigma-Aldrich). The serum was heat-inactivated at 50°C for 30 minutes.

Testes and cysts cultured in Grace's and M3 media remained viable for up to 7 days while those in Schneider's media began to darken and degenerate after 24 hour in culture. However, growth in the Grace media was noticeably retarded. Testes cultured in M3 media showed the best development and viability as compared to development of testes *in vivo*. Hence all the results reported here were those obtained while utilizing Shields and Sang M3 media supplemented as described above.

IV.   *In vitro* culture of testes and isolated cysts

The organ culture of testes was done according to Niki *et al.* (2008) with some modifications. Pupae were used instead of larvae and only some testes were punctured before culturing. The pupae were soaked in 70% alcohol for 10 minutes and then rinsed with distilled water. They were then dissected in 1X phosphate buffered saline (PBS) on a clean bench sterilized with 70% alcohol for 15 minutes. The testes were washed twice in 1X PBS and once in culture media with 1X penicillin/streptomycin. Finally, three pairs of testes were inoculated per well in 500 µl of supplemented media in a 24-well plate.

For cyst culture, the testes were obtained as described above. The cysts were isolated according to Noguchi and Miller (2003). After two washes in 1X PBS, the testes were transferred into a drop of M3 media with 1% penicillin/streptomycin. The basal part of the testes was held with forceps and the apical end was punctured with a tungsten

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needle. The cysts were gently squeezed out into the media and the testis coat was discarded. The cysts tended to be held together by a matrix thus teasing them out with tungsten needles was necessary and resulted in less shearing of the cysts. Isolated cysts were then transferred with a pipette into a sterile 24-well plate. Each well contained 450 μl of M3 media with 10% fetal bovine serum and 1% penicillin/streptomycin cocktail and 50 ul of cysts. The cysts were cultured at room temperature (22°C) without CO_2.

Cysts were monitored daily for morphology changes and images were collected using an inverted, phase contrast Leica DMIL microscope and Leica cooled CCD camera.

IV. Quantification of cyst growth

Cysts were quantified by manually counting of viable cysts present at each developmental stage after 24, 48, 72, 96 hours and 1 week in culture. Normal cysts were defined as those that had normal morphology and lacked any kind of degeneration such as bursting of the cysts. The cysts were categorized in developmental stages based on the number of cells per cyst, morphology and size of the cyst.

Cyst length was calculated by first acquiring images of mature cysts (those that were at individualization and early coiling stages) from both in vitro cultures and from testes dissected from adult flies of the same age as the cultured cysts. Cyst length was accomplished using Image J software to measure the length of the cysts and MS Excel spreadsheet to analyze the mean cyst length.

RESULTS

I Comparison between *D. melanogaster* and *D. pseudoobscura* testes

A wide variation in the morphology and appearance of the testes among *Drosophila* fly species exist. Adult testes are pigmented with the color varying depending on the species. *D. melanogaster 's* testes have a light yellow pigmentation while *D. pseudoobscura 's* are bright orange (Fig. 3). The pigments become darker with age and the testes can be seen through the lower abodmen of the fly as early as the pupal stage in *D. pseudoobscura* species (Fig.3b). *D. melanogaster* testes are long, coiled tubes that are closed at the apical end and connected at the basal end to the seminal vesicles via small ducts that parallel the vas deferens in mammals. The *D. pseudoobscura* testes are short and ellipsoidal shaped and are similar to *D. melanogaster* in that they are closed on the apical end and open basally into the seminal vesicles via small ducts. Spermatogenesis in both species begins at the apical end where the stem cells are found and proceeds towards the basal end. However, sperm tails of elongating spermatids grow towards the apex even as the general movement of the entire cyst is in the opposite direction.

A

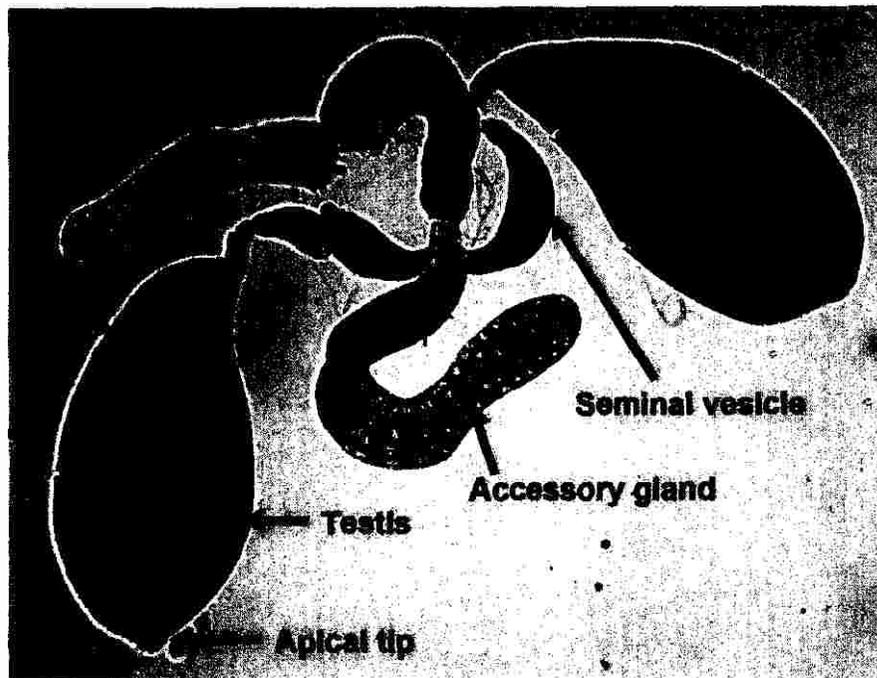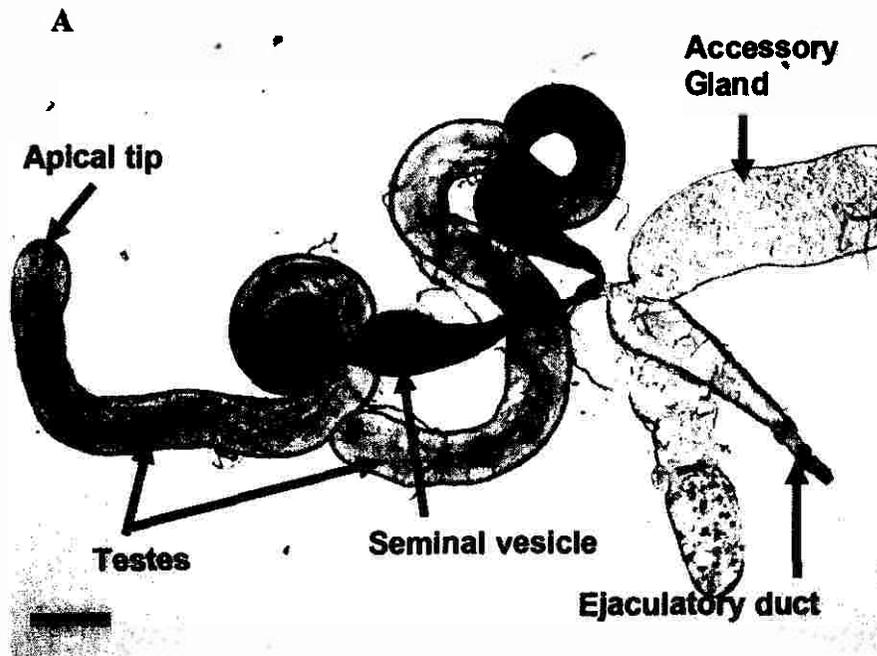Apical tip

Accessory Gland

Testes

Seminal vesicle

Ejaculatory duct

Seminal vesicle

Accessory gland

Testis

Apical tip

Fig. 3. Bright-field images of live squash of (a) *D.melanogaster* and (b) *D. pseudoobscura* testes. Scale bar = 100 µm

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II.   Organ culture of testes from pupa

To test whether the testes of D.pseudoobscura were cultivatable in growth media, testes were obtained from 24-48 hour old pupae and inoculated into 24-well culture plate. At the time of removal the testes are visible at the posterior end of the pupa due to their bright orange pigmentation. This is important because at this developmental stage sexing of flies is easy and also the testes are free from fat body making it easier to isolate them without injury. Earlier stage testes are clear and imbedded in fat which makes them extremely difficult to isolate.

To test whether the testicular walls were essential for the development and differentiation of germ cells, some testes were punctured at the basal end by dissociating the seminal vesicles or puncturing the apical end with forceps hence facilitating the emergence of cysts from the wound sites. The other testes were left intact and monitored for growth in culture media (Fig. 4). Cultures were maintained for as long as the testes appeared healthy, meaning there were no signs of degeneration of cysts emerging from the wound sites, the morphology of the testis was normal and there was no clouding of the media as is indicative of microbial contamination.

Intact cultured testes were observed to have peristaltic contractions for up to 4 days in culture indicating that the testicular walls of *D.pseudoobscura* consisted of smooth muscle layer. Examination of intact testes cultured for up to 7 days revealed mature, motile sperm that were coiled in bundles (Fig. 5a). A majority of the cysts were elongated and any round cysts were virtually non-existent. Testes that had been ruptured on the basal end had similar results as intact testes except that sperm were non-motile and were less organized (Fig. 5b). Testes that were ruptured at the apical end had cysts emerging

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from the wound sites and growth was evident up to elongation stage for the first 3 days (Fig. 4). However, growth ceased thereafter and the testes began to shrink. The experiment was repeated three times with three cultures each time and the results were consistent. The results indicate that the germ cells that were present within the testes at the time of culture underwent both division and differentiation but the stem cell division and maintenance system ceased hence the absence of earlier stage spermatogonial and primary spermatocyte cysts. The different results based on the wound site on testes indicate that the direction of progression of spermatogenesis within the testes is ordered and essential for proper development and maturation of germ cells within the cysts.
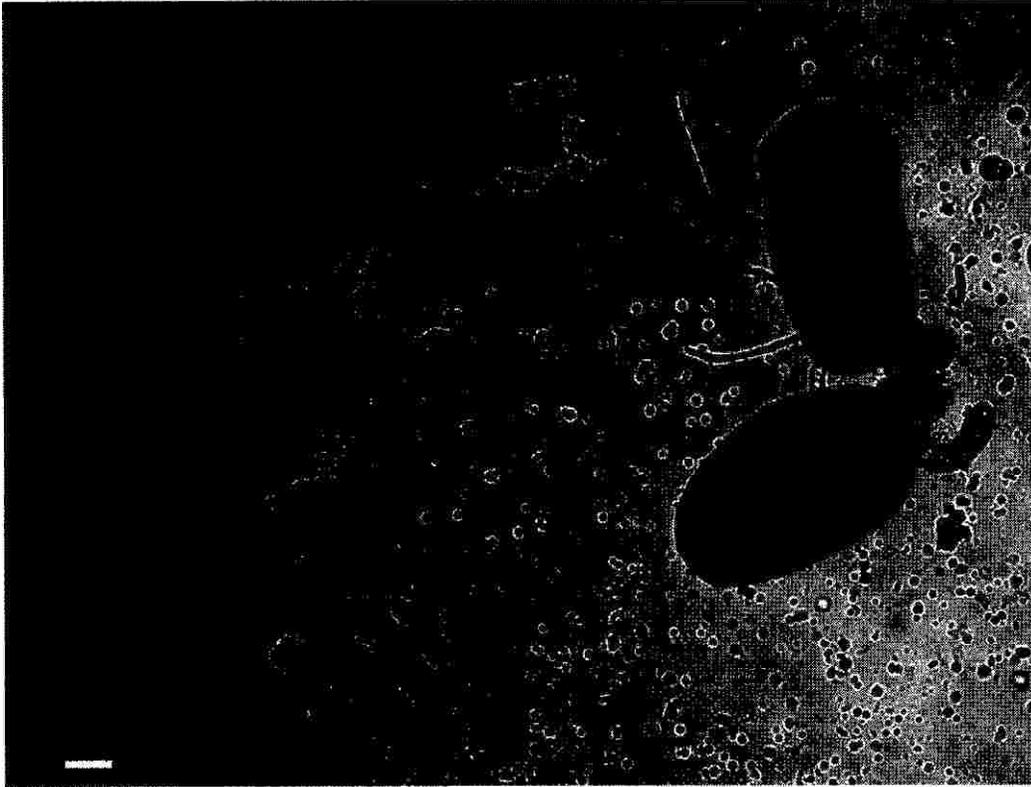
Figure 4. Phase contrast image of cultured pupal testes after 3 days in growth media. Testes that were punctured at the apical end had cysts emerge from the wound and differentiate but after 3 days no more growth was apparent. Scale bar = 100μm
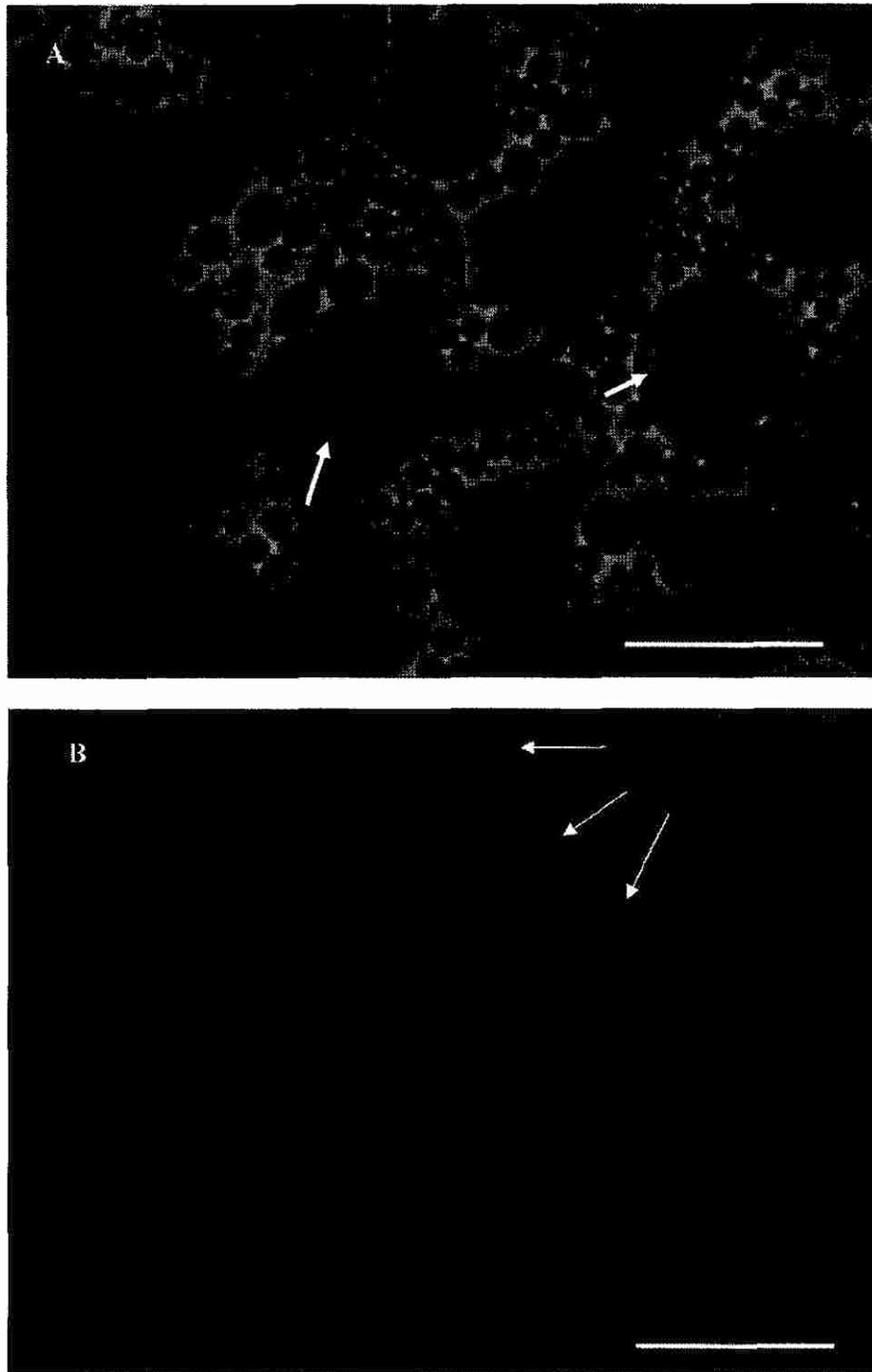
**Figure 5.** Phase contrast images of cellular contents of testes after 7 days in culture, (A) mature, coiled and motile sperm from intact testes (white arrows), and waste bags containing cytoplasm expelled from differentiated spermatids (black arrows). (B) mature, non-motile sperm that are not coiled in bundles (arrows) from testes that were ruptured at the basal end before culturing. Scale bar = 100µm

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III. Culture of isolated cysts

Pupal testes isolated between 24-48 hours after pupation contained predominantly spermatogonia and primary spermatocyte cysts with few spermatid cysts (Fig.6). At this time, none of the cysts were ever observed in advanced stages of differentiation past elongation. Cysts started to differentiate shortly after culturing. After 24 hours in culture, there was a rapid increase in the number of cysts containing late stage primary spermatocytes and differentiating spermatids. However, there was apparent degeneration of earlier stage cysts after 24 hours and by the fourth day most of spermatogonia and primary spermatocyte cysts had degenerated (Fig.7). Staging of cultured cysts was done using the cyst size, the number of germ cells within the cyst, the shape of the germ cell nucleus and mitochondria as well as the general morphology of the cyst.

Spermatogonia that were undergoing mitotic divisions were identifiable based on the number of spermatogonia cells per cyst. Once encapsulated within a cyst, the germ cell in *D. pseudoobscura* undergoes five synchronous mitotic divisions resulting in a cyst with 32 spermatogonial cells that are interconnected via cytoplasmic bridges (Sharer et al., 2008). The cysts then undergo a growth phase and the cells increase in size but they are all equal (Figs.8a and 8b). At this time, the cysts are about 75 μm long but they tend to have irregular shape.

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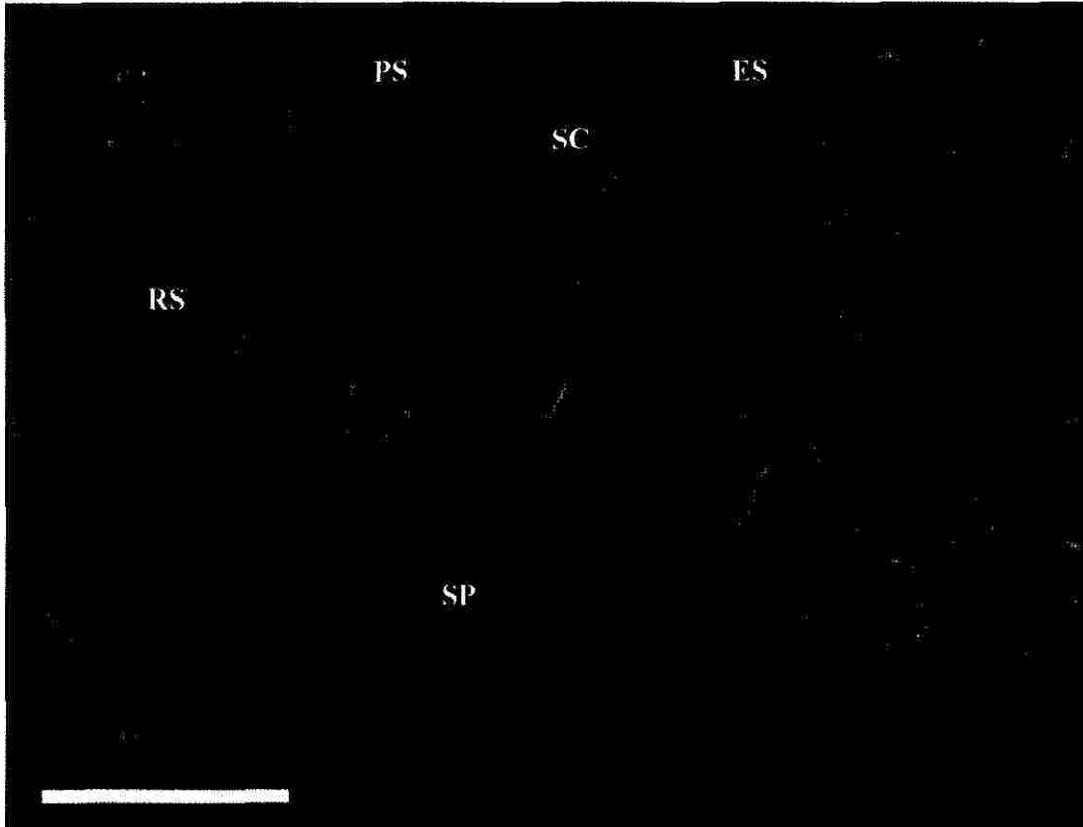
Figure 6. Differentiating isolated cysts after 24 hours in culture.
(SC) Single cells and individual spermatogonia, (SP) mitotic spermatogonia cyst, these include cysts with 2-16 spermatogonia cells per cyst, (PS) primary spermatocytes cyst, these includes cysts with 32 and 64 spermatocytes per cyst (RS) Round spermatids cyst containing 128 spermatids per cyst and (ES) Elongating spermatids cyst. Scale bar, 100μm
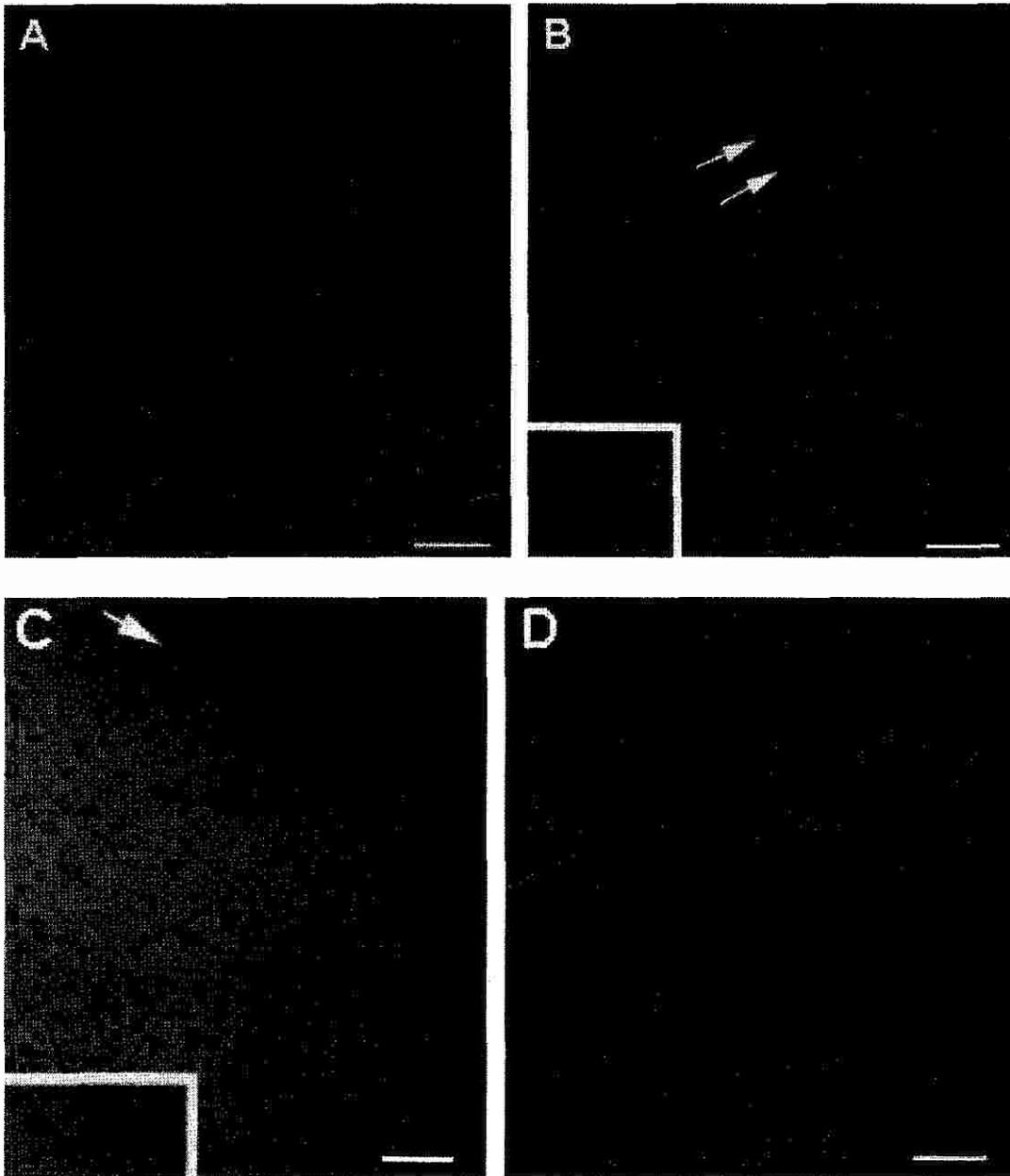
Figure 7. Differentiating isolated cysts over 4 days in culture (a) Fresh culture shortly after culture initiation (b) Cysts after culture overnight, note there are more elongating cysts and spermatogonia degeneration is apparent (arrows and inset) (c) 2 days culture. Most elongated cysts have a cystic bulge (arrow and inset) indicative of individualization stage and some are at early coiling stage. (d) 4 days culture. Most of spermatogonia and primary spermatocytes are dead. Only post meiotic cysts are still visible. Scale bar = 100 μm

A photographic record of the dynamics of spermiogenesis in *D. pseudoobscura in vitro* is presented in Figures 8A through 8M. Since the cysts were only semi-adherent to the culture dish, it was not possible to follow a single cyst to maturation and hence, Figure 8 is a composite of several different cysts. Before meiosis, each cyst contained 32 spermatocytes surrounded by two cyst cells. The spermatocytes had large spherical nuclei surrounded by the mitochondria giving them the appearance of being hollow (Figs. 8a and 8b). After two rounds of meiosis, the cysts contained round spermatids that were much smaller than spermatocytes. Their nuclei were smaller and the mitochondria had congregated into a sphere next to the nucleus (Figs. 8c - 8e). This is referred to as the onion stage (Fuller, 1993) and the transformation results in the cyst having a more regular shape.

The end of onion stage signified the initialization of the elongation phase. This phase was characterized by elongation of both the nucleus and mitochondria from sphere shaped to spindle shaped. The nuclei also migrated within the cyst to one region giving the cyst a droplet-like shape (Fig. 8f). As elongation commenced, the cyst assumed an ellipsoidal shape and the spermatids within it were twisted (Fig.8g). Noguchi and Miller (2003) showed that in *D. melanogaster* the process of elongation of the spermatids' nuclei and the cyst as the sperm tails are formed is achieved by the presence of microtubules surrounding the nuclei and also extending the length of the spermatids. At the end of elongation, the nuclei are fully elongated and the DNA is condensed. Fully elongated cysts then underwent individualization. This phase was characterized by the presence of a cystic bulge somewhere along the length of the cyst (Fig. 8i and 8j). After individualization the cysts were narrower with the head region becoming more pointed. The cyst development culminated with the cyst coiling into a disc. In some cysts, there was no further development but sperm-whipping was

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recorded within the cysts indicating sperm motility in these cysts. In other cysts, sperm

wound into a bundle burst out the cyst cells (Fig. 8k-8m).
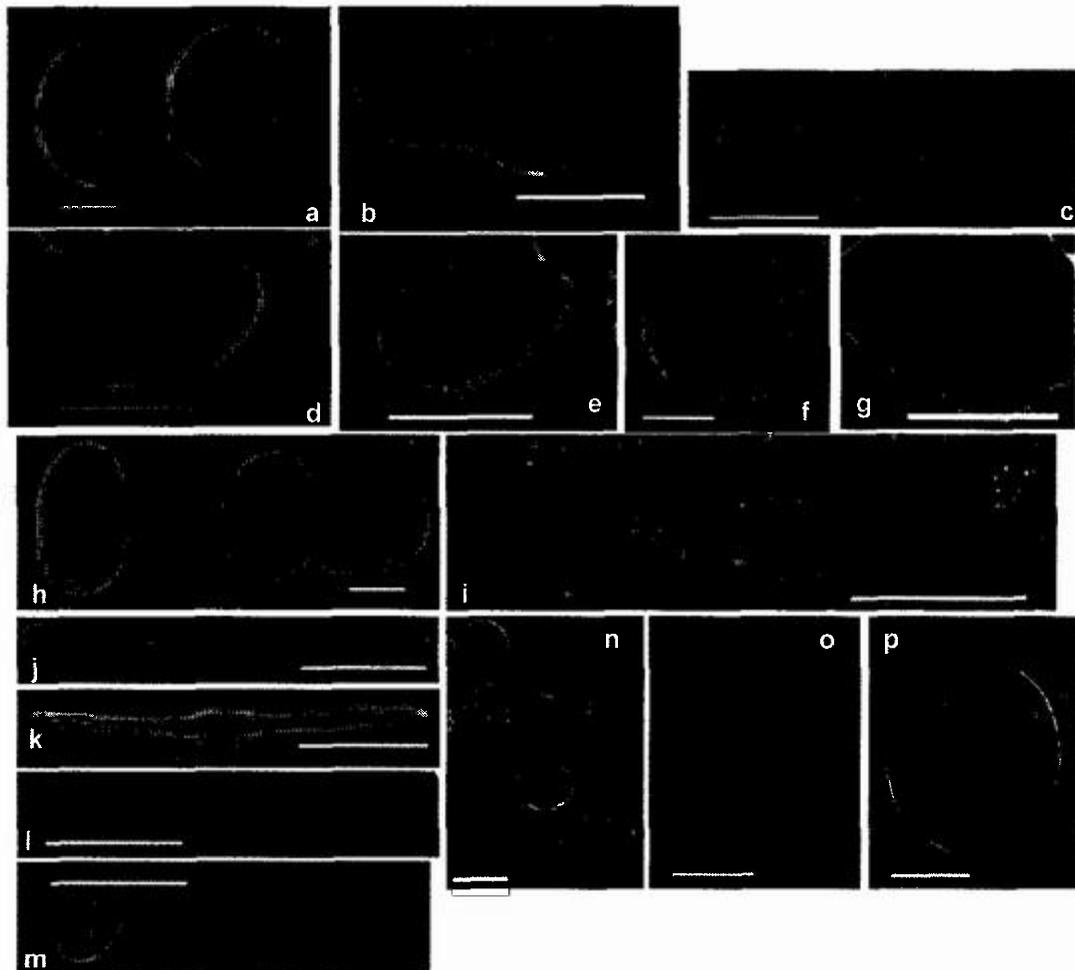
Figure 8. Phase contrast micrographs showing the sequence of *in vitro* spermiogenesis in isolated cultured cysts of *D. pseudoobscura*. (a) 16-cell primary spermatocytes (b) 32-cell primary spermatocyte cyst prior to meiosis. (c) decapsulated 32-cell primary spermatocyte cyst. All cells are interconnected via cystoplasmic bridges. (d) post-meiotic cyst containing 128-round spermatids (e) post-meiotic cyst prior to elongation (f) nuclei migration and rearrangement to one region in a round-spermatid cyst (g) squash preparation of a round spermatid cyst at higher magnification, the nuclei appear white in phase contrast while the mitochondria appear black (h) droplet-like shaped cyst as elongation commences, the more elongated cyst is ellipsoid (i) squash preparation of elongating cyst from culture, cyst cells have burst and the twisted spermatids tails can be seen. (j) fully elongated cyst (k) individualizing cyst, sperm individualization takes place in cystic bulge region as the bulge moves in head to tail direction (l) Beginning of coiling at the apex of the cyst. The cystic bulge has almost terminated (arrow) (m) cyst shortens as coiling progresses (n) coiling is almost complete and (o) cyst cells degenerate revealing mature individualized sperm (p) sperm are packed in a coiled bundle as spermatogenesis culminates. Scale bars (a, f, h =25 μm, b- e, g, n– p = 50 μm, i- m =100 μm)

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Quantification of cyst growth

Cyst maturation was defined as development of the cyst up to the individualization stage which has the characteristic cystic bulge somewhere along the length of the cyst. The survival rates of cysts were quantified based on how many viable cysts were present at each stage; spermatogonia and primary spermatocytes, round and elongating spermatids, individualizing and coiling stage between 24 hours and 7 days in culture per experiment. The results of the series of culture shown in Fig. 7 are summarized in Table 1.

Spermatogonial and primary spermatocyte cysts had low survival rates (25%, 38/150 by day 4 of culture) due to the fact that their cysts cells burst and numerous grape-like clumps of cells were apparent in culture by the third day. However, late-stage primary spermatocytes cysts undergoing meiotic divisions albeit few, were still visible in culture for up to 7 days.

Post-meiotic cysts containing spermatids had higher success rates (67%, 210/313) of developing to maturation. In the series shown in Table 1, of the 313 post meiotic cysts present after 24 hours in culture, 42 cysts had developed to individualization stage and 168 to the coiling stage by day 4 of culture. On the other hand, a significant number of cysts were observed to have arrested development during elongation and consequently degenerated. There were no viable elongated cysts evident after a week in culture and those that had fully coiled for some time were also degenerating. It took about 48 hours for cysts to differentiate from round spermatids to fully coiled cysts.

The length of mature cultured cysts was also compared to length of mature cysts obtained from adult males (*in vivo*). In order to quantify *in vitro* vs. *in vivo* mature cyst length, images of cysts undergoing individualization and those with the characteristic hook at the apical end indicating the beginning of coiling were taken from both *in vitro* and *in vivo* samples. Cyst measurements were taken using Image J software and analyzed using MS Excel spreadsheet. The results (Fig.9) indicate that there was no significant length difference between *in vitro* and *in vivo* cysts. *In vivo* cysts had a mean cyst length of $334.9 \pm 16.7\mu m$ while *in vitro* mean cyst length was $331.9 \pm 14.5\mu m$. It is important to note that in addition to sperm length variation between flies, *D. pseudoobscura* exhibits sperm polymegaly, a form of sperm heteromorphism characterized by production of sperm of two different lengths and this could explain the large standard deviations in the means.

	24 hrs	3days	4days	7days
Spermatogonia and Primary spermatocytes cysts*	150	76	38	18
Post meiotic Round spermatids cysts	178	186	84	6
Elongating spermatids cysts	120	240	96	-
Individualizing spermatids cysts	12	35	42	12
Coiled spermatids cysts	3	96	168	72

Table 1. Quantification of development of cysts into advanced stages *in vitro*. *includes 8-32-cell cysts. There were no individualizing or coiled cysts at the initiation of the culture.

TABLE 2. The length of fully elongated cyst *in vitro* compared to *in vivo* cysts (N = the number of males donating the cysts)

	N (cysts)	Mean length ± SE (μM)
In vivo cysts	5 (32)	334 .9 ± 16.7
In vitro cysts	10 (45)	331.9 ± 14.5
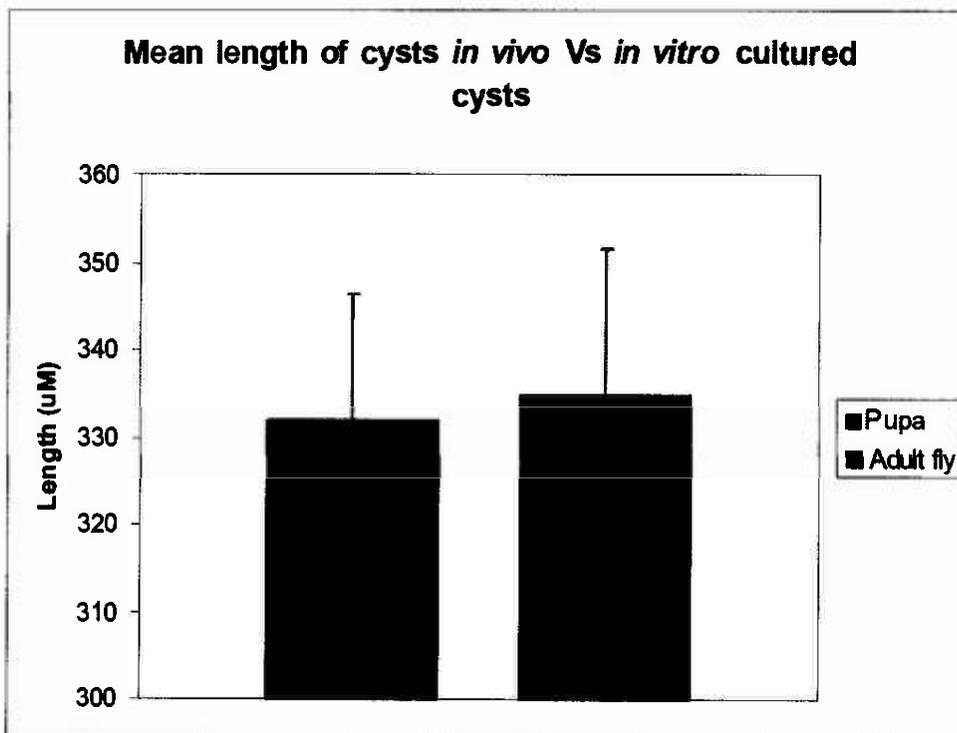
Figure 9. Comparison of cyst length *in vivo* vs. *in vitro*. Results indicate overlapping means showing that there was no significant difference between the cysts.

Discussion

Our results indicate that *D. pseudoobscura* is an excellent species for potentially studying spermatogenesis *in vitro* based on the high rates of success of both organ and cyst culture. Additionally, only minimal medium was necessary to produce mature spermatozoa for both systems *in vitro*.

The testis of *Anastrepha ludens,* a Mexican fruit fly, whose morphology is very similar to *D. pseuodoobscura,* has been reported as having peristaltic contractions similar to those observed on *D. pseudoobscura* testis (Valdez, 2001). The large and wide single saclike morphology of these testes makes it necessary to have a muscular testicular wall in order to move the developing cysts from the apical tip to the basal end of the testis unlike in long slender tubular testis where proliferation and consequent growth of cysts enable them to push each other along the testis length.

The epithelial cells at the basal end of *Drosophila* testes are reported to have structures such as endoplasmic reticulum, mitochondria, vacuoles and a multifolded distal surface (Valdez, 2001), all which are indicative of an active synthesis and/or secretion function. These epithelial cells were thought to be like the Sertoli cells in the mammals having a nutritive function for the spermatozoa as individual spermatozoa are found imbedded in their distal folds (Schärer *et al.*, 2008) but, the fact that mature spermatozoa are released here from the encapsulating cysts may suggest that these cells are secreting a substance that degenerates the cyst cells freeing the sperm for transportation into the seminal vesicle. However, this explanation is contradicted by the fact that some of the isolated cysts had their cyst cells degenerate, and packed sperm bundles were released.

Our organ culture results suggest that the testicular walls are important for the proper development and progression of spermatogenesis in *D. pseudoobscura* since intact testes yielded mature and motile sperm while those punctured at the basal end had mature but non-motile sperm with the apically ruptured testes yielding no sperm at all.

Phase contrast images of developing cysts suggested that germ cell development and differentiation in *D. pseudoobscura* resembled that of *D. melanogaster* in all the phases previously reported during *in vitro* culture. Once a spermatogonium is committed to differentiation, the daughter cells of all subsequent mitotic and the two meiotic divisions remain interconnected by intercellular bridges. During individualization, the individual sperm are released from syncytium by the coordinated action of actin cones (Noguchi and Miller, 2003) that traverse the entire cyst length from the head to the tail region expelling excess cytoplasm into a cystic bulge and eventually the waste bag. This process is similar to the mammalian spermatogenesis with the exception of lack of the encapsulating cyst cells and the fact that in mammals the spermatids are held in syncytium by the neck region residual bodies that contain excess cytoplasm and are themselves interconnected by cytoplasmic bridges.

One of the most unique transformations brought about by the process of spermiogenesis is the elongation of nucleus and the concurrent chromatin compaction. This transformation in mammals has been shown to render the DNA transcriptionally inactive (Ward and Zalensky, 1996). Since spermiogenesis is regulated by genes and chromatin condensation occurs early in spermiogenesis before the sperm are completely differentiated, RNA synthesis must occur prior to the conclusion of elongation stage. RNA synthesis in *Drosophila* terminates in the primary spermatocyte stage and the

transcripts are stored in the cytoplasm for later use (Gould-Somero and Holland, 1974). Furthermore, transcription is reactivated in a few loci specifically in mid-elongation spermatid cysts (White-Cooper et. al, 2008). In soti mutants, one of the genes expressed at this period, elongating cysts fail to individualize. This event coincides with the nuclear decondensation and recondensation during elongation stage (Cenci et. al., 1994). This might help explain why a significant number of cysts became arrested in the elongation stage in the current work. It could be that the nuclear decondensation and recondensation in these cysts was abnormal resulting in lack of expression of mid-elongation genes and hence, the consequent arresting of the cysts.

The extensive bursting of spermatogonial and primary spermatocyte cysts was unaccounted for although Cross and Shellenbarger (1979) reported that repeated exposure to light caused abnormal morphology and bursting of cysts. Other possible explanations could be that the absence of the hub cells caused subsequent absence of essential signals that maintain the spermatoganial cells as well as maintaining the stem cell system. Kiger et al, (2001) reported that activation of the JAK-STAT pathway by unpaired ligand (Upd) secreted by the apical hub cells is essential for the maintenance of germ-line stem cells as well as the somatic cyst progenitor cells. It is also possible that the culture media lacked essential ingredients or that there was an intertesticular signal missing in isolated cysts that directs the cysts sequential transitions from mitotic to meiotic divisions. In the pre-published work of Ueishi et al, (2009), they report that GSC maintenance, spermatogonial proliferation as well as the spermatocyte growth before meiosis may be under direct control of insulin like proteins (ILPs) that have only been reported in neural-

secretory cells in male *Drosophila*. All these are possible points of future investigations in the effort to improve the culture media.

The overall success of the culture media affirmed *D. psuedoobscura* as an excellent model for studying spermatogenesis *in vitro* in *Drosophila*. Although it may never replace *D. melanogaster* as the animal model for genetic and developmental studies, the fact that its genome has been fully sequenced makes it an ideal species for comparative studies. *D. pseudoobscura* offers an easy and consistent *in vitro* culture system for studies where cellular transformation processes must be manipulated *in vitro*. Not only did cysts develop fully to motility consistently, the cysts also grew to their normal length as those *in vivo*.

In vitro culture of *Drosophila melanogaster* spermatogenic cysts is limited in its usefulness because of early degeneration of cysts, atypical development of cysts and rare occurrences of motile sperm. On the other hand, *Drosophila pseudoobscura* offers several advantages over *D. melanogaster*. *D. pseudoobscura* testes can easily be distinguished in pupal stages due to intense red pigmentation and the survival of cysts to the elongated, motile form is easily achieved and highly repeatable with only minimal media necessary for culture maintenance. Cysts containing primary spermatocytes isolated from late-stage pupa developed into motile sperm usually within five days of culture. Cysts underwent meiosis, elongation, individualization and coiling just as in *D. melanogaster*. To the best of our knowledge, this is the first report of *Drosophila* sperm cell culture where motility is consistently achieved. This culture system should prove valuable for spermatogenesis studies where cellular transformation processes must be manipulated *in vitro*.

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