CHAPTER IV

DISCUSSION

1. Male reproductive organs of *Chrysomya. megacephala*

1.1 Testis

The internal male genital organs of *C. megacephala* consist of the various parts commonly found in insects. The testes are located in the posterior abdomen and contain all sperm stages from primary spermatocytes to spermazoa. In almost all other Diptera, the testes are enveloped by two tissue layers consisting of the outer epithelium (or tunica externa) and the inner epithelium (or tunica interna) (Hori, 1960; Snodgrass, 1993). In species where color has been observed in the testes, pigment granules are deposited in the outer epithelium contributing to the apparent visible color (Hori, 1960). As in other Diptera, the TEM micrographs of the testis wall of *C. megacephala* show the outer epithelium is full of the rounded grains containing the pigment, which give the organ its characteristic color. This characteristic has also been observed in the Mexican fruit fly, *Anastrepha ludens* (Valdez, 2001). The coloration of the testes of *C. megacephala* is probably due to the pigment granules being deposited in this layer as maturation progressed. Likewise, the muscle layer in the testis wall between outer epithelium and the inner epithelium, which was first reported in the Mexican fruit fly, *A. ludens* (Valdez, 2001). This feature could be related to the unifollicular structure of this blow fly testis.

Previously, there was report on the color change in the testes in six species of calyptrate muscoids during the pupal and adult stages (Hori, 1960). The change in color of the testes of these flies during the course of pupal life was a progressive change from greenish-yellow through orange to reddish-orange. In muscids, *Ophyra nigra* and *Musca domestica vicina*, the testes continue to change successively from reddish-orange to brown or to fuscous after emergence. However, in *Scopeuma stercorarium, Calliphora grahami, Lucilia sericata* and *Sarcophaga simislis*, the color remained reddish-orange throughout the entire adult stage (Hori, 1960). In this study, the color of the testes of *C. megacephala* changed from pale orange at emergence to a reddish-orange color in 1 old-day flies, and continued to change from reddish-orange to brown or fuscous as the flies aged.

The shape of the testes of the calyptrate muscoids flies have been classified into five types including oval, oblong, fusiform, banana-shape and lamp-shape (Hori, 1960). *C. megacephala* appears to have a pair of oval shaped testes. They maintain their ellipsoidal form for a day or more after emergence then a characteristic constriction occurs at about a third of the length from the base of the testes. It is probably caused by the discharge of spermatozoa into the vas deferens. Daily measurements suggested that this may occur around the seventh day post-emergence. The lower third of the testes of *C. megacephala* seem to function as a pump forcing the developed spermatozoa into the vas deferens. This characteristic change in shape of the mature male testes has been described previously in three other fly genera including: *Musca*, *Lucilia* and *Calliphora* (Hori, 1960). The testis constriction in *C. megacephala* extends apically and changes the form of the testes remarkably as the flies age. Consequently the change of the testes shape of this blowfly species closely

correlates to the age of the fly, and could be used to differentiate the age of individual flies.

Scanning electron microscopy studies of the testes of various insects have shown both similarities and differences of *C. megacacephala.* The morphological characters of testes in four *Plecoptera* species consist of a number of separate follicles, each enclosed by its epithelium and open into the vas deferens (Fausto et al., 2001, 2002). In *Leuctra fusca*, each of the two testes consists of 9 to 10 follicle, almost tubular in shape; *Brachyptera risi*, the follicles are ovoid; *Taeniopteryx stankovichi*, the follicles are more roundish than those *B. risi*; and *T. kuetreiberi* the two testes are united in the median proximal portion, forming a single arc sac. In this current study, SEM investigations of male *C. megacephala* revealed a pair of oval shaped testes with a smooth surface that is occasionally penetrated by tracheoles.

SEM imaging of spermatozoa of *C. megacephala* revealed that they are released in bundles, which are twisted helically and present a very unusual morphology arrangement. The *C. megacephala* presented the elliptical head and wavy long tail of the spermatozoa. In contrast to another insect, SEM micrographs of the spermatozoa of *X. vesparum* showed a conical head and a tail which tapers posteriorly (Dallai et al., 2003). The different morphological characteristics of spermatozoa have been observed by light microscopy, inference contrast microscopy or interferential light microscopy in other insects, for example *Bephratelloides pomorum* (Hymenoptera: Eurytomidae) (Neto et al., 1999); four Plecoptera species (Fausto et al., 2001; Fausto et al., 2002); four species of African platypleurine cicadas (Homoptera: Cicadidae) (Chawanji et al., 2005) and *Drosophila melanogaster* (Diptera: Drosophilidae) (Lopez-Fernandez et al., 2007).

The presence of the primary and secondary spermatocytes observed in the developing spermatozoa of 3 day-old *C. megacephala* males is similar to that observed in the pupal stage of the thrips, *Haplothrips simplex* (Thysanoptera: Phlaeothripidae) (Paccagnini et al., 2006). The ultrastructure of the tail region of spermatozoa of 3 day-old adult *C. megacephala* was not clearly observed in this study and the axoneme pattern was indistinguishable. In an example from another dipteran, Owusu-Daaku et al. (2007) investigated the testes of 4 day-old adult mosquito, *Aedes aegypti* (Diptera: Culicidae), and found multi-organelled spermatozoa with extra tail elements (axonemes and mitochondrial derivatives). Nevertheless, the electron micrograph of the testes of 3 day-old adult *C. megacephala* revealed that the developing axoneme pattern had yet to be transformed into the typical 9+2+2 microtubule pattern.

The tail region of *C. megacephala* spermatozoa consisted of two mitochondrial derivatives, an axoneme and an accessory body. The axoneme of insects are generally regarded as being of the 9+9+2 type, nine outer single accessory tubule, nine doublets and two central single microtubules (Dallai et al., 1993, 2005). The sperm tail of serveral families of Diptera has been examined, the 9+9+2 type was found in *Sarcophaga bullata* (Diptera: Sarcophagidae) (Warner, 1970); *Drosophila melanogaster* (Diptera: Drosophilinae) (Tokuyasu, 1974); *Orfelia* spcies and *Boletina* species (Diptera: Mycetophilidae) (Dallai et al., 1993), whereas in *Culex pipiens* and *Anopheles maculipenis* (Diptera: Culicidae) (Dallai et al., 1993) and Phlebotomine sandfly, *Phlebotomus papatasi* Scopoli (Diptera: Psychodidae) (Ilango, 2005) a type designated as 9+9+1 there is a central rod rather than two microtubules. Nevertheless, the 9+9+0 type has been reported in other Diptera including the mosquito,

Toxorhynchites brevipalpis (Diptera: Culicidae) (Jean-Lou and Xavier, 1988). In other Callipholidae, *Calliphora vomiyoria* presented the 9+9+2 type of the axoneme (Dallai et al., 1993). However, in this investigation, the axoneme pattern of *C. megacephala* was not clearly observed, but there are very similar to the 9+9+2 type.

Changes in the color and shape of the testes of *C. megacephala* are clearly visualized during the adult stages with light microscopy, SEM, and TEM. These changes correlate closely with the age of the male fly. Daily light microscopy measurements showed a progression of testis development and observed changes in length and width combined with the observation of the muscular layer via TEM suggest that first mating may occur about the seventh day after emergence.

1.2 Male accessory glands

This study examined development of the male accessory glands of *C. megacephala* from emergence (0-day-old) until 9-days-old. A significant increase in size of the glands was detected on the third day of adulthood, both in length and width. The increasing size seemed to plateau in around 5 to 6-day-old males, based on the measurement analyses. In the male stalk-eyed fly, *Cyrtodiopsis dalmanni*, the accessory gland size was a critical determination of sexual maturity and male mating frequency (Baker et al., 2003).

The gross morphology of the male accessory glands is very diverse in Calyptrate muscoid flies. According to Hori (1960b), the accessory glands of male muscoid flies constitute six types; (1) no accessory glands, (2) papillary, (3) spherical or ellipsoidal, (4) banana-shaped, (5) coiled, and (6) rod-shaped. Banana-shaped male accessory glands have been observed in many genera of blow flies (*Chrysomya*,

Lucilia, Hemipyrellia, Phormia, Protophormia, Calliphora, Stomorhina, Triceratopyga, Onesia) or flesh flies (*Metopia*) (Hori, 1960). Despite the same banana shape, the slender tubular structure of *C. megacephala* in this study was different from the stout tubular structure observed in *Phormia regina* (Chaiwong et al., unpublished data), indicating the variability in morphology within the group of blow flies. SEM results presented in this study revealed that even in the same species of *C. megacephala*, the sub-apical constriction of male accessory glands in some specimens was noticeable and unexpected. The reason for this sub-apical constriction remains uncertain; variation in the morphology of this organ is questionable.

In the current study of *C. megacephala*, ultrastructural change has been noticed in the accessory gland of 3 -day-old males; in the glandular cells and gland lumen. Specifically in the cells, the presence of numerous rough endoplasmic reticulum and primary and secondary secretory granules was the prominent feature in 3-day-old glands. In *C. megacephala*, whether females can successfully mate with younger or older males has not been fully explored. However, investigation in the fruit fly, *Drosophila pseudoobscura*, suggested that females preferred mating with older males, which may relate to the transfer of larger volumes of sperm and possibly accessory fluids (Avent et al., 2008). Thus, investigation of successful mating related to the development of accessory glands of *C. megacephala* merits study.

In the male accessory glands of insects, there may be cellular diversity, even within a single type (Davey, 1985). Regarding those of *C. megacephala*, the results obtained from this study confirmed the multicellular gland, appearing as a simple tubular structure in morphology. Based on the difference in staining used in the light microscopic study, the glands of *C. megacephala* exhibited one type of cell, but a

difference in functional state. Active glandular cells were predominantly observed, but only a few inactive ones were seen. The former were characterized by bearing large secretory granules within the cytoplasm or at the apical end adjacent to the lumen; while the latter showed no remarkably large secretory granules.

With respect to the structure of the accessory glands of *C. megacephala per se*, they were recognized as ectodermal in origin, on the basis of the gland that opened into the ejaculatory duct (Chapman, 1998b). The simple tubular structure observed in males in this study was different from the multi-chamber organization investigated in females (Chaiwong et al., unpublished data). Interestingly, based on histological examinations, the thick epithelial cells of the accessory glands observed in males were similar to those in females (Bansal and Murad, 1987). Vacuolated cytoplasm of the glandular cells in males was similarly observed in females (Bansal and Murad, 1987c).

At the ultrastructural level, the current study indicated that the glandular cells of the accessory glands of male *C. megacephala* showed typically common structures associating with the production of secretory granules. These included richness in the active phase of rough endoplasmic reticulum, mitochondria and secretory granules, all suggesting high activity in secretory function. Such typical features of cells engaged in high secretory activity has been previously described in the grasshopper, *M. sanguinipes* (Couche and Gillott, 1990); thrips, *F. occidentalis* (Dallai et al., 1997b); triatomine bug, *T. rubrofasciata* (Freitas et al., 2007); and endoparasitoid wasp, *C. vestalis* (Huang et al., 2007). Increment on the secretory activity of male accessory glands was related to maturation (Freitas et al., 2007). In this study, TEM observations presented evidence that the formation of accessory gland secretion

within the lumen, presented in 3-day-old males. However, the precise time of when the fully contained secretion in the lumen formed is unknown. Nevertheless, the formation of accessory gland secretion was accordance with a previous study concerning male triatomine bug, *T. rubrofasciata*, in that the formation of accessory gland secretion was poorly developed in 1-day-old bugs, more developed in 3-day-old ones and highly developed in 5-day-old ones (Freitas et al., 2007).

Regarding the role of male accessory glands in insects, they primarily ensure that the reproductive success has been addressed, including insemination, barriers to reinsemination, contributions to survival and fecundity (Leopold, 1976; Chen, 1984).

In conclusion, this study reports the male accessory glands of *C. megacephala* at the ultrastructural level. This work has expanded on the morphological knowledge of the reproductive organs of this fly species.

1.3 Vas deferent and ejaculatory duct

The vas deferens of *C. megacephala* are simple in structure and connect the testes with the ejaculatory duct. The vas deferens are cylindrical ducts, which do not form seminal vesicles in this species. In some insects, a portion of the vas deferens may be enlarged as the seminal vesicle that serves as a storage reservoir for sperm before it is transferred to the female (Snodgrass, 1993). In other Diptera such as the sandfly, *Phlebotomine perniciosus* (Diptera: Psychodidae) a portion of the vas deferens forms a pear-shaped of seminal vesicle (Fausto et al., 2000).

The shape of the ejaculatory duct of Calyptrate muscoid flies has been divided into three main groups including the N-shaped winding, the short or C-shaped winding and the long or coiled. *Chrysomya* species are considered to be of the short

form of ejaculatory duct (Hori, 1960). The vas deferens in some species of flesh fly, *Sarcophaga*, and muscid, *Musca*, especially in which the ejaculatory duct is short, the right ducts are often longer than the corresponding left ones (Hori, 1960). This study also documented a difference in length between left and right ducts; however, the original anatomical position was not retained during dissection so the result can not make an assumption about how this relates to the actual internal anatomy of the fly.

The ultrastructure of the vas deferens of this blowfly consisted of three layers including the epithelial cell layer with muscle cells, a connective tissue layer and a plasma membrane layer adjacent to the duct lumen. In the sandfly, *P. perniciosus* (Fausto et al., 2000) and the bee *Melipona bicolor bicolor* (Hymenoptera: Apinae) (Dallacqua and da Cruz-Landim, 2003) the epithelial layer was the only layer observed, with muscle cells present in the epithelial layer as was observed here in *C. megacephala*. The most important structures observed in *C. megacephala*: various nucleus shapes, numerous rough endoplasmic reticulum, Golgi complexes, mitochondria and secretions in the plasma membrane, beneath the epithelial cell layer and the connective tissue respectively have not been observed and described in previous studies of the vas deferens of other insects.

The fine structure of the surface of reproductive duct of *C. megacephala* has not been previously studied. Interestingly, SEM micrographs in this investigation showed a pattern consistent with longitudinal muscle on the surface of the ejaculatory duct of *C. megacephala*. However, the transverse sections of the ejaculatory duct of *C. megacephala* did not show evidence of a muscular layer. Conversely, the ejaculatory duct of other Diptera including the house fly, *Musca domestica* (Diptera: Muscidae) (Riemann, 1973) and the lovebug, *Plecia nearctica* (Diptera: Bibionidae)

(Trimble, 1974) demonstrated muscle layers which were visible under transmission electron microscopy. The absence of a muscular layer in the ejaculatory duct has been reported in the flower thrips *F. occidentalis* (Thysanoptera: Thripidae) (Dallai et al., 1997a) and *M. bicolor bicolor* (Hymenoptera: Apinae) (Dallacqua and da Cruz-Landim, 2003).

Previously, the spermatozoa were observed in the vas deferens and/or the ejaculatory duct of thrips, *F. occidentalis* (Dallai et al., 1997a), *P. perniciosus* Newstead (Dipera: Phychodidae) (Fausto et al., 2000), *Allacma fusca* (Collembola: Symphypleona) (Dallai et al., 2000) and *M. bicolor bicolor* (Hymenoptera: Apinae) (Dallacqua and da Cruz-Landim, 2003). However, spermatozoa were not present in either the vas deferens or the ejaculatory duct of *C. megacephala*. The spermatozoa could be found in both ducts in flies older than 3 days-old.

These results also demonstrated the presence of secretory cells in the plasma membrane of the vas deferens and the epithelial cells of ejaculatory duct of *C. megacephala*. Secretory cells are usually associated with the production of secretions that support the functions of accessory reproductive glands, such as spermatopore production, mating plug formation, and sperm activation (Fausto et al., 1997). The function of secretory cells in this species has yet to be investigated.

2. Female reproductive organs of *Chrysomya megacephala*

2.1 Ovary and ovariole

This study examined the development of the ovaries and ovarioles of *C. megacephala* from emergence (0-day-old) until 9-days-old using several methods. The results of this study present a method for age determination of female flies that can be easily adapted for field studies as it is based on practical light microscopy methods. The current study provided the first TEM images of the developing embryonic chorion in this insect which provide interesting insight into the development of the chorion as the egg matures. Measurements of the ovaries also detected significant increases in the size of the ovaries during the period of study, both in length and width based on measurement analyses, which also have potential utility in age determination of female *C. megacephala*. The morphometric characters of ovarian length and width, have been use as a tool in determining the ovarian development of the female Caribbean fruit fly, *Anastrepha suspense* (Diptera: Tephritidae) (Kendra et al., 2006).

The morphological analysis of the ovaries confirmed that it can be used to differentiate the age of females in this fly species. Young blow flies are characterized by a tightly wound and well developed tracheal system surrounding the ovaries and SEM images revealed a thick outer epithelial sheath. In contrast there is a reduction in the extent of the tracheal system in older flies and SEM images revealed a thin ovarian envelope with many holes.

This study is the first to report on the changes in the ovarioles during egg formation in *C. megacephala* which form the basis of the aging technique, as has been

created for other blow flies e.g., *Lucilia cuprina* (Vogt et al., 1974). The cellular changes observed during oogenesis in other calliphorid flies have been documented for *L. cuprina* (Vogt et al., 1974; Beattie and Cheney, 1979), *C. bezziana* (Spradbery and Sands, 1976), *C. hominivorax* (Adams and Reinecke, 1979) and *C. putoria* (Avancini and Prado, 1986). Slight differences occur between studies when comparing the ovarian stages and many of the descriptions vary in the number of ovarian stages present. For example the results showed that in *C. megacephala*, stage I is present at day 0 (just after emergence) of the adult female as was found in *C. putoria* (Avancini and Prado, 1986), however, in *L. cuprina, C. bezziana* and *C. hominivorax* these features occur during the pupal stage.

Another difference was observed in the histology of ovarioles of *C. megacephala* from that described by Bansal and Murad (1987). This study showed that the ovarioles are similar to stage VI (day 8 after emergence) of egg formation. Moreover, this investigation showed that in *C. megacephala*, the second follicles starts to form in the germarium when the first follicle was at stage V or in 7-day-old of female blow flies, while the development of second and third cycles in the Old World screwworm, *C. hominivorax,* was dependent on the removal of the first cycle (Adams and Reinecke, 1979).

In this investigation, *C. megacephala* females completed egg development in eight stages and about ten days under ambient temperature fluctuations (18-27°C). Previous studies have documented the completion of oogenesis in *C. megacephala* in 10-13 days for females reared at 25±2 °C and maintained on raw beef liver (Linhares and Avancini, 1989). The total time for egg development in this study corresponds well with temperature controlled studies which suggests that the differences in the

number and description of stages is largely dependent upon small differences in author interpretation rather than temperature dependent processes.

The development of follicular epithelium of the 3- and 7-day-old blow fly passes through several distinct stages of oognesis. TEM micrographs illustrated the differentiation of the developing chorion layers in young and old adult females. The fine structure of the eggshell of *C. megacephala* showed several layers similar to those observed in *D. melanogaster* (Margaritis et al., 1980). The characteristic changes in the development of the follicular epithelium or the process of chorionogenesis in this blow fly are similar to those described in the stoneflies *Perla marginata* and *P. pallida* (Plecoptera: Perlidae) (Rosciszewska, 1995). The *C. megacephala* oocyte is largely composed of yolk protein, which presented the follicular changes same as other insects in the vitellogenesis (Raikhel and Snigirevskaya, 1998).

The reproductive status or parity of flies can be used to approximate the generation time or the time between egg batches. This information has been used to estimate the population size of the bush fly *Musca vetustissima* (Diptera: Muscidae) (Matthiessen et al., 1986) and is often used in disease surveillance studies of vector mosquitoes (Woodbridge and Walker, 2002). Hence, the stages of ovarian development in *C. megacephala* can be a useful tool for determining the age and approximate generation time and population potential of this blow fly. The light microscopy based age grading system presented herein can also be used to determine the age of flies collected from the field. In addition, the basic information provided by this study can be used to understand the ovarian development and fine structure of the

ovaries and ovarioles of *C. megacephala* which might prove to useful in developing new methods to control this blow fly in the future.

2.2 Female accessory glands

The morphology and histology of the female accessory glands of *C. megacephala* has been previously studied under light microscopy (Bansal and Murad, 1987a); however, the ultrastructure of glands has not been previously studied. Despite a previous light microscope description, there remained a need to provide detailed information on the age of the flies described and the number of flies observed. This result, light microscope studies were also able to document the changes that occur in size during development of the glands from emergence to ten days of age. This is the first study to record the cellular maturation stages present in the accessory glands of the 3-day-old flies using TEM micrographs.

The accessory glands of *C. megacephala* are paired long and tubular glands that open into the dilated anterior genital chamber. Each gland is composed of two distinct regions including an apical bulb and a tubular gland duct which differentiate during maturation of the fly as evidenced by TEM micrographs of 3-day-old flies. This two part accessory gland is similar to those observed in other insect such as the thrips *F. occidentalis* and *Heliothrips haemorrhoidalis* (Thysanoptera: Thripidae) (Dallai et al., 1996; Bene et al., 1998) and the blow fly *C. putoria* (Diptera: Calliphoridae) (Tirone and Avancini, 1997).

The basis or head of the glandular portion of female blow fly accessory glands have been grouped into three types based on overall shape including: (1) oval or

elongate oval, (2) banana shaped or (3) clavate (Hori, 1961). The shape of glandular portion of the female accessory glands of *C. megacephala* was classified as clavate (Hori, 1961). Our SEM micrographs show that the surface of the female accessory glands of this fly are covered in papillae and occasionally penetrated by tracheoles, which is the first report of this morphology in this species. This feature is similar to the globular shape of the cell wall of the female accessory glands of the sandfly *P. perniciosus* (Diptera: Psychodidae) (Fausto et al., 1997).

The results indicated that the two accessory glands did not develop at the same rate but as the complete reproductive system was removed from the fly for study it is, unfortunately, impossible to know what the original anatomical orientation of the fly was and hence which side developed faster. This unequal development in the size of the female accessory glands of *C. megacephala* is consistent with the description given for *C. putoria* (Tirone and Avancini, 1997).

The structure of the epithelial cells of the female accessory gland of *C. megacephala* consisted of both secretory cells and duct forming cells, which is similar to that of many insect ectodermic glands (Dallai et al., 1996). These features have been described in the accessory glands of other insect such as *F. occidentalis*, and *H. haemorrhoidalis* (Thysanoptera: Thripidae) (Dallai et al., 1996; Bene et al., 1998). The cistern cells serving as storage organs for the secretion products of the secretory cells in the production of fluid secretions correspond to that of type 1 insect ectodermal glands, as defined by Noirot and Quennedey (1974; 1991).

Cellular level on the morphology of the 3-day-old female accessory glands in this study revealed that the cistern cells contained transparent electron dense material,

where large amounts of secretions are normally stored, suggesting that was missing or had not yet formed. The tubular duct portion of the gland did have secretions present in the cistern cells. A low volume of secretions was present in the central lumen. These characteristics are similar to those observed in the development of the female accessory gland cells of *Orchesella villosa* (Collembola: Hexapoda) where the epithelial cells of virgin females rarely showed secretions whereas inseminated females showed large amounts of secretions (Dallai et al., 2008).

The function of the secretion of female accessory glands is typically as an adhesive material that serves to cement eggs to the substratum or hold them together in a mass (Chapman, 1998a). The secretions of the accessory glands of *C. putoria* were determined by histochemical test to be composed mainly of a glycoprotein, which is consistent with an adhesive material (Tirone and Avancini, 1997). A cytochemical analysis of the cellular secretions of *C. megacephala* would be necessary to determine with more accuracy the nature and functions of the secretions of this gland.

2.3 Spermathecae

The spermathecae are a sac for the reception and storage of the spermatozoa in a female insect (Hori, 1961; Snodgrass, 1993; Chapman, 1998a). In Diptera the spermathecae vary in number; there are none in family Cloropinae, one in *Anopheles* (Culicidae) and *Simulium* (Simuliidae), two in Drosophilidae, three in *Culex* (Culicidae), *Stegomyia* (Culicidae) and Tabanidae (Hori, 1961). There are three in the blow flies, *L. cuprina* (Diptera: Calliphoridae) (Clift and McDonald, 1973), *C.*

bezziana (Diptera: Calliphoridae) (Spradbery and Sands, 1976) and *C. megacephala* (Basal and Murad, 1987).

The manner of the arrangement of the spermathecae in calyptrate muscoid flies has been divided into three types including 1:2, 0:2 and 1:1:1 (Hori, 1961). This study confirmed that *C. megacephala* has three spermathecae, which are arranged in the 1:2 configuration, with the two of one side loosely bound together and the one on the other side unattached.

This work is the first description of the fine structure of the spermathecae of this blow fly. SEM images showed that tubercles cover the surface of all three of the spermathecae and that each is penetrated by tracheoles. The basal region of spermathecae is connected via a spermathecal duct and longitudinal muscle is clearly visible on surface of the spermathecal duct. The muscle fibers of the spermathecal duct have been investigated under SEM in other Diptera such as the medfly *Ceratitis capitata* (Diptera: Tephritidae), the Caribbean fruit fly, *Anastrepha suspensa* (Diptera: Tephritidae), the phlebotomine sandfly *Phlebotomus papatasi* (Diptera: Psychodidae), four species of *Dysmachus* (*D. fuscipennis, D. picipes, D. praemorsus and D. transcaucasicus*) (Diptera: Asilidae) (Marchini et al., 2001b; Fritz and Turner, 2002; Ilango, 2005; Dallai et al., 2008).

Transmission electron microscope images from this study provide us with detailed information about the epithelial cells and spermathecal lumen and their development of 3- days old. The morphology of the spermatheca of *C. megacephala* has been previously studied by using histological methods, which showed that the epithelial cells consisted of cubiodal secretory cells supported on a basement

membrane. These methods also showed that internally the spermatheca is lined by a very thick layer of dark brown intima which sends out a number of very small processes projecting into the lumen (Bansal and Murad, 1987). Our imaging of the epithelial cells showed that cistern cells are present which store secretions and spermatozoa. The epithelial cells of 3 day-old *C. megacephala*, rarely showed cells with extracellular cisterns and the lumens these cells did not contain spermatozoa, probably suggesting that 3 day-old *C. megacephala* may not yet be inseminated. This situation is similar to that observed in the spermathecae of the *Orchesella villosa* (Colembola, Hexapoda), in which the epithelium of virgin females was not yet differentiated and rarely contained extracellular cistern cells, but appeared to be rich in secretions (Dallai et al., 2008).

The spermathecae of many insects are used for the storage of sperm once the females are inseminated and the spermathecal duct may contain glycogen deposits that can serve as an energy source for the sperm as they pass through to the egg (Chapman, 1998a). TEM images of the cistern cells of *C. megacephala* show secretion materials inside the lumen, this secretion could be use as nutrition for the spermatozoa stored in the spermathecae but this was not examined in the current investigation. The ultrastructure of the spermathecae of *C. megacephala* could be useful for determining the physiological mechanisms responsible for changes in behavior occurring before and during mating and oviposition as well as how fertilization of the eggs occurs in this blowfly species.

2.4 Genital chamber

The genital chamber of *C. megacephala* is a single long tubular organ, which is located in the posterior region of the abdomen of the female in the same orientation that it is generally found in the reproductive tracts of other female insects (Hori, 1961; Snodgrass, 1993; Chapman, 1998a). The histology of the vagina or genital chamber of *C. megacephala* has been studied under light microscopy previously (Bansal and Murad, 1987). However, in the present study a more detailed description of the structure of the genital chamber of *C. megacephala* by using light microscopy, scanning electron microscopy and transmission electron microscopy was presented. This study presents the first measurements of genital chamber length and width and the first SEM and TEM images of genital chamber of *C. megacephala*.

High magnification imaging revealed that the genital chamber consists of a central lumen wrapped by five distinct cell layers, with each layer characterized by a difference in structure and cellular organelles. Interestingly, TEM images showed a muscle layer that was only visible in the third-layer, while longitudinal and circular muscles were visible on the surface in the SEM micrographs. In previous studies muscle layers in the vagina epithelial cells of other arthropods have been observed, including *C. megacephala,* consisting of both circular and the longitudinal muscle (Bansal and Murad, 1987). A similar muscular structure has been observed in the medfly, *C. capitata* (Diptera: Tephritidae) (Marchini et al., 2001a) and the ticks *Ornithodoros (Pavlovskyella) erraticus* (Acari: Argasidae) (Shoura, 1988) and *Ixodes ricinus* (Acari: Ixodidae) (Roshdy, 1969). Additionally, the epithelial cells of the second-layer through the fifth-layer are rich in mitochondria which was not observed in the previous study by Bansal and Murad (1987).

In the medfly, *C. capitata* (Diptera: Tephritidae), a fertilization chamber has been observed as a small protuberance on the ventral surface of the anterior vagina. The tubular vagina of the medfly is also surrounded by a strong muscular sheath (Marchini et al., 2001a). The genital chamber of *C. megacephala* showed evidence of both longitudinal and circular muscle on the surface of the genital chamber, but a fertilization chamber was not observed.

Measurements of the length and width of the genital chamber of *C. megacephala* showed a peak in length on about day seven and a peak in width on day six. These features might be useful in describing the stages of mating, fertilization and parity of this blowfly, when used in conjunction with information about the other reproductive organs in both the female and male.

Results indicating numerous mitochondria in the muscle layer and the lumen lined with cuticle of *C. megacephala*, suggest similarity in function with the genital chambers of other insects, which serve for both copulation and the discharge of eggs, (Snodgrass, 1993). In the medfly, *C. capitata* (Diptera: Tephritidae), sperm bundles can reach the egg micropyle in the fertilization chamber by their own movement in conjunction with the muscle contractions of the chamber pushing the spermatozoa toward the eggs (Marchini et al., 2001a). The muscular layers found in this study suggest that this might be occurred in *C. megacephala* but this was not observed in our study. The ultrastructural features of the genital chamber of this blowfly can be useful to clarify morphology and to understand the function of the reproductive system components.

3. The external genitalia: male and female of *Chrysomya. megacephala*

This study presents the ultrastructure of the genitalia of male and female *C. megacephala*. As for males, the morphology of the genitalia, especially the aedeagus, reveal distinctive characters. The bilobed vesica of *C. megacephala* resemble with those blow flies, *C. pinguis*, *C. defixa* (Senior-White et al., 1940), and *C. bezziana* (Spradbery and Sands, 1976), but are greatly different from other blow fly species such as *Cochliomyia hominivorax*, *Cochliomyia macellaria* (Leite, 1995), or *L. cuprina* (Merritt, 1989). Nevertheless, the appearance of the juxta of *C. megacephala* look like the ventrolateral process found in *C. hominivorax* (Leite, 1995).

Regarding the cercus of male *C. megacephala*, the terminal end is different from those observed in other blow flies, such as *C. hominivorax* (Leite, 1995), *Calliphora fulviceps*, *Calliphora vomitoria*, *Chrysomya albiceps*, *L. cuprina* (Senior-White et al., 1940). Nonetheless, the cercus which is longer than the surstylus in *C. megacephala* resembled those investigated by Senior-White et al. (1940) covering *C. bezziana*, *C. defixa*, *C. phaonis*, *Lucilia porphyrina*. As has been previously observed, the characteristics of the cercus and surstylus are usually utilized in identifications of insects and flies (Senior-White et al., 1940; Leite, 1995).

Observation of the surstylus and cercus of male *C. megacephala* revealed the endowment of sensillae, which is in agreement to other studies, such as blow flies *C. hominivorax* and *C. macellaria* (Leite, 1995); flesh fly, *Parasarcophaga* (*Liosarcophaga*) *dux* (Chaiwong et al., 2007); and bot fly, *Dermatobia hominis* (Fernandes et al., 2004). The dense sensillae on these structures occurs for many fly species; such as blow fly, *C. bezziana* (Senior-White et al., 1940; Spradbery and Sands, 1976), *C. defixa*, *C. phaonis*, *Calliphora vicina*, *C. pattoni*, *Hemipyrellia*

ligurriens, *H. pulchra* (Senior-White et al., 1940); and syrphid fly, *Copestylum alberlena* (Marcos-Garcia and Perez- Banon, 2002). Based on the long bristles, which are similar to the sensilla chaetica and sensilla trichodea, and short bristles, which are similar to sensilla basiconica, found in the surstylus and the cercus of *C. megacephala*, the functions of olfactory sensitivity, mechanosensitivity, dual mechanosensitivity and contact chemosensitivity, or thermosensitivity, have been proposed (Zacharuk, 1985).

Regarding females, the sensillae of the genitalia and/or ovipositors of insects has been recorded for the blow fly species, *P. regina* (Wallis, 1962), *L. cuprina* (Rice, 1976) *C. nigripes* (Ngern-Klun et al., 2007); and face fly, *Musca autumnalis* (Hooper et al., 1972). In the current study, the type, number and distribution of sensillae resemble to those blow fly, *C. nigripes* (Ngern-Klun et al., 2007). These were the sensilla trichodea; the sensilla basiconica and the sensilla placodea.

In regard to *C. megacephala*, males endowed more sensilla trichodea than females, which might explain more mechanosensitive or dually mechanosensitive and contact chemosensitive in function of males than females (Zacharuk, 1985). This is in agreement with the sand fly *Lutzomyia* spp. (Diptera: Psychodidae) (Spiegel et al., 2000). Contrarily, greater densities of sensilla basiconica, sensilla placodea, and sensilla styloconica were found on the genitalia of females than males. A higher number of sensilla were found congregated on the ventral region than the dorsal one, this might imply the more response to hygrosensitivity, chemosensitivity, or mechanosensitivity and chemosensitivity (Zacharuk, 1985). This interpretation agreed with those paired lateral leaflets of *L. cuprina* (corresponding with the cercus in this study) where it has been proposed that the ovipositor of *L. cuprina* plays a role as an

organ of both taste and smell (Rice, 1976). Similar observation of taste sensilla on ovipositor has been found in the moth, *Plutella xylostella* (Qui et al., 1998). Rice and McRae (1976) revealed that sensilla on the ovipositors of insects provide information pertaining to the elicitation of oviposition behavior. Similarly, sensillae on the female terminalia of mosquito, *A. aegypti*, have also been suggested to play a role in copulation and oviposition (Rossignol and McIver, 2005).

The ultrastructural investigation obtained from studying the external sexual organs of *C. megacephala* provides distinctive morphological features of both sexes. Information from this study is not only of taxonomic importance, but may also allow for the better assessment of the functional role that these structures play in the behaviors of both males and females of this fly.

4. Efficacy of the human contraceptive on the male and female reproductive system of *Chrysomya megacephala*

The two main hormones that regulate most of the processes involved in insect reproduction are juvenile hormone (JH) and 20-hydroxyecdysone (20E). In female reproduction, the production of vitellogenins, which are synthesized during the process of vitellogenesis for producing and developing oocytes in the ovarioles, are dependent on both JH and 20E (Rockstein, 1978). Because insect reproductive processes are so dependent on these hormones they have been examined with great interest for their potential to disrupt the reproductive events of pest insect species. The effects of JH and 20E on vitellogenesis and egg development in many insects have been reported such as in the fruit fly *D. melanogaster* (Diptera: Drosophailidae) (Birnbaum and Gilbert, 1990); the mosquito *Culex pipiens* (Diptera: Culicidae) (Khater et al., 1994): the German cockroach *Blattella germanica* (Dictyoptera: Blattellidae) (Cruz et al., 2003); the termite *Reticulitermes flavipes* (Isoptera: Rhinotermitidae) (Elliott and Stay, 2007); and the tick *Amblyomma hebraeum* (Acari: Ixodidae) (Friesen and Kaufman, 2004).

The similarity in the molecular structure of vertebrate hormones with those of insects has led to the suggestion that they might be capable in mimicking the effects that JH and 20E have on the reproductive processes of insects. The effects of vertebrate steroid hormones on the reproductive system of insects have been reported in several studies. Estradiol was found to considerably reduce the oviposition rate of adult female silkworms, *Bombyx mori* (Lepidoptera: Bombycidae) (Ohishi et al., 1985). Three vertebrate hormones (estrogen, testosterone and thyroxine) effected the growth, development and reproduction of the tomato moth, *Lacanobia oleracea*

(Lepidoptera: Noctuidae) (Kirkbride-Smith et al., 2001). The steroids 17-ethinylestradiol (EE) and bisphenol A (BPA) effected the number of egg-ropes produced by the aquatic midge, *Chironomus riparius* (Diptera: Chironomidae) as determined over two generations (Watts et al., 2001, 2003).

This study presents the effects of a human contraceptive, containing levonorgestrel and ethinylestradiol on the development of eggs and the morphology of the reproductive structures themselves in the blow fly *C. megacephala*. In this study, a reduction in egg production and delayed egg maturation were evident in females treated with human contraceptive in all three experiments. Interestingly, these effects were only observed in the first, second and third generations and not observed in the parental generation. It suggests that the human contraceptive can be transferred from the parental flies and is able to persist and potentially accumulate in the subsequent generations. This result is similar to that found in other studies in which the effect of other chemicals with action on the reproductive system of other insects was examined. For example in *Rhodnius prolixus* (Heteroptera: Reduviidae) treated with ^{14}C-pyriproxyfen (juvenile hormone mimic), it was found to be stored in the female fat body, incorporated into the vitellogenin and transferred to the eggs as well (Langley et al., 1990). In another study 17-ethinylestradiol (EE), a synthetic estrogen used as a human female contraceptive effected the number of egg-ropes produced by first generation females of *C. riparius* (Watts et al., 2001).

Juvenile hormone and 20-hydroxyecdysone also have importance in the molting and development of insects in general and thus their effects are mimicked by the class of insecticides known as insect growth regulators (IGR). This class of insecticides has a generalized mode of action that disrupts the hormone levels

associated with the molting cycle and causes the insect to have incomplete development and deformations of culticular structures. Thus IGRs also can affect reproductive processes as well. The IGR, cyromazine was found to inhibit egg production in the sheep blow fly, *L. cuprina* (Diptera: Calliphoridae) and the house fly *Musca domestica* (Diptera: Muscidae) (Friedel and McDonell, 1985; Alam and Motoyama, 2000). The biological activity of neolignans (Yangambin, Burchenllin, Licarin and Grandisin), which are IGRs that mimic JH, on the developmental stages of *C. megacephala* has been studied previously (Cabral et al., 2007a; Cabral et al., 2007b). In the present study of *C. megacephala*, the data showed a significant effect of human contraceptive at 0.072 mg/ml HC on egg production in the first, second and third generations.

Another vertebrate steroid hormone, testosterone, caused a significant decrease in egg production in the tomato moth, *L. oleracea* whereas estradiol was not significantly different from the control (Kirkbride-Smith et al., 2001). The reason for the different results is not clear. The major difference between the tomato moth study and this one is in the method in which the hormone was administered to the insects and in the chemical formulation. In this study adult *C. megacephala* were fed commercial human contraceptive in their drinking water, while in tomato moth, larvae were provided with an artificial diet containing technical grade estradiol (Kirkbride-Smith et al., 2001). However, the effect of the human contraceptive on egg production of *C. megacephala* in this study was present in first, second and third generations and not in the parental generation. Additionally, delayed egg maturation was found in adult *C. megacephala* treated with human contraceptive in all three of the experiments. This result suggests that human contraceptive may specifically effect the

ovaries, especially the germarium, or ecdysone metabolism and consequently, vitellogenesis in *C. megacephala*. This effect is similar to the mode of action suggested for the effect of 17α-ethinylestradiol in delayed molting in *C. riparius* (Watts et al., 2003).

The observed alterations of the reproductive organs suggests that human contraceptive could interfere with the endocrine system or cause the cellular alteration in the testis and ovariole during spermatogenesis and egg development of *C. megacephala*. The alterations observed under SEM in the ovaries of the first generation females showed that the ovarian envelope was thicker than in the control. This morphological change has also been observed in the ovarioles of *C. megacephala* treated with yangambin (Cabral et al., 2007a). However, the present results showed other morphological changes including cracks in the surface of the ovarioles, a wearing away of the ovarian envelope to reveal the chorion of the developing egg, as well as the presence of immature egg.

Developmental changes during the growth and development of larvae and adults were not observed in any of the experiments of this study. Mouthpart deformities have been observed in *C. riparius* larvae recorded at a low exposure concentration of 17α-ethinylestradiol (Watts et al., 2003) but similar effects were not observed in this study.

Although no effect of human contraceptive was observed on egg production in the parental flies *C. megacephala*, the present results show that cellular changes occurred in the testes and ovarioles of parental flies. These morphological changes also persisted into first, second and third generations. This suggests that the human contraceptive can be transmitted from generation to generation.

The effect of the human contraceptive on female *C. megacephala* was shown to cause a decrease in egg production and delayed egg maturation which was confirmed with observations of cellular changes in the ovarioles using electron microscopy techniques. The reproductive structures observed in this study that appeared to be effected by the application of human contraceptive are involved in vitellogenesis and oogenesis in *C. megacephala* similarly to how juvenile hormone and 20-hydroxyecdysone act in insects in general (Rockstein, 1978). In previous studies the effects of JH and 20E on viltellogenesis and oogenesis have been reported in the fruit fly *D. melanogaster* (Birnbaum and Gilbert, 1990); the mosquito *C. pipiens* (Khater et al., 1994): the German cockroach *B. germanica* (Cruz et al., 2003); the termite *R. flavipes* (Elliott and Stay, 2007); and the tick *A. hebraeum* (Friesen and Kaufman, 2004). These results suggest that the effect of human contraceptive on female blow flies might act as a JH or 20E mimic. Generally, JH regulates the formation of new endoplasmic recticulum in the fat body and sequestration of the vitellogenin produced, while 20E regulates the rate of production (Rockstein, 1978; Wigglesworth, 1984).

Additionally, estradiol has been isolated in the ovaries and female fat body of silkworms, *B. mori* (Ohishi et al., 1985; Roy et al., 2007). Ogiso and Ohishi (1986) showed that estradiol is found in the silkworm ovaries and comes from either a biosynthetic pathway in the insect or by transport from the food plant via the hemolymph. The role that estradiol plays in oocyte maturation and embryogenesis in the silkworm, *B. mori* was examined by injecting estrogens into whole pupae but their results were inconclusive as these processes were not affected regardless of whether the application was of additional estradiol or the of an antagonist (Ogiso and Ohishi,

1986). They found that after application of the vertebrate steroid hormones, estone, estradiol-17α and testosterone were transported readily into the ovaries and metabolized (Ogiso and Ohishi, 1986). The observed effects of human contraceptive on delayed egg maturation and the presence of cellular changes in the ovariole or the oocyte of *C. megacephala* suggest that is also transported from the digestive system to the ovaries as in *B. mori*. However, the presence and concentration of the human contraceptive in the reproductive tissues of *C. megacephala* was not explicitly examined in this study.

Unfortunately comparatively little is known about the endocrine regulation of male reproduction with the only well known process being that of the mitotic division rate of spermatogonia, in the formation of spermatocytes. This process is increased by high levels of 20E, but high titers of the JH counter this increase (Rockstein, 1978). However, TEM images of the testes in all of the experiments of the present study showed cellular alterations during spermatogenesis indicated by the presence of the degenerated nuclei of the spermatozoa. It can be suggested that administration of human contraceptive effects spermatogenesis in *C. megacephala* flies in the current generation and in subsequent generations. Sperm production might be a meaningful indicator for determining the effect of human contraceptive on male *C. megacephala*, and a sperm count could be useful in future studies.

The structural characterization of the estradiol steroid isolated from the silkworm, *B. mori* ovaries (Fujimoto et al., 1986) is similar to the structure of human estradiol (Hedge, 1987). Furthermore, estrogen and steroid hormone receptors have been identified in the fruit fly *D. melanogaster* (Thackray et al., 2005). It is possible that these types of receptors are also present in *C. megacephala*. These studies and the

effects of human contraceptive on *C. megacephala* observed in this study suggest that the genetics of this blowfly should be studied to determine the mechanisms and functions of human steroid hormone in this insect.

The results of this study have shown that the human contraceptive reduces egg production, delays egg maturation and causes cellular changes in both the testes and ovarioles of adult *C. megacephala*. Both similarities and differences were noted with respect to previous work in this area. The data presented here suggest that the human contraceptive could be used as part of an integrated control program to reduce reproduction of this blow fly species.