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Research

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Research

Reproductive anatomy and embryogenesis of a viviparous, phonotactic, parasitoid fly

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Ormia ochracea (Bigot 1889) is a phonotactic parasitoid fly that targets a variety of field cricket species as hosts for their developing young. Female flies locate their hosts by tracking a male cricket's courtship song. Viviparous larvae are then deposited on or near the host which they then pierce and penetrate, completing larval development within the cricket's abdomen. Here, we describe *Ormia* male and female reproductive tracts exposed through micro-surgery and imaged with a variety of microscopic techniques. We also describe for the first time, both embryonic and early larval development that takes place within the female flies' "uterus." Special note is made of the phenomenon of intra-uterine embryonic growth, and prospects for accessing gametes or germ-cells for molecular engineering are discussed.

Keywords: parasitoid, fly, embryo, development, uterus

Introduction

Ormia ochracea is a phonotactic parasitoid fly that parasitizes a variety of field crickets (Wineriter and Walker 1990, Broder et al. 2023). Reproduction in *Ormia ochracea* depends on the ability of gravid female flies to locate suitable host crickets by eavesdropping on male cricket calling songs (Cade 1975). Upon detection and recognition of calling songs (Lee et al. 2019, Jirik et al. 2023), *O. ochracea* will engage in flying (Müller and Robert 2001) and walking phonotaxis (Mason et al. 2005, Lee et. al. 2009) to home in on the source location (Fig. 1A). While extensive research has been conducted on their specialized hearing and parasitic behavior (reviewed in Robert 2005, Lakes-Harlan and Lehmann 2015), little work has been published on their reproductive anatomy, oogenesis, or embryology, partly due to the difficulty in maintaining a laboratory culture (Dominguez et al. in press). Most members of the *Tachinid* family are oviparous and parasitize other insects with females laying eggs on or near their host (Vincent and Bertram 2010). Hatching larvae then penetrate the cuticle of their host and feed as endoparasites.

Ormia is somewhat unusual among *Tachinid* parasites, in that it is larviparous, a mature female brooding her embryos within a "uterus" until they hatch as fully developed first instar larvae before being deposited on the cricket host. More specifically, they appear to exhibit adenotrophic viviparity as the developing larvae appear to grow within the uterus, deriving nourishment from some maternal source (Benoit et al. 2015). In this way, *Ormia* more closely resembles flies of the family, *Sarcophaga*, or "flesh-flies" and *Calliphorids*, or "blow-flies" (Meier et al. 1999). The planidia larva pierces the cricket host integument in the delicate cuticle between the more chitinous cuticular plates, and enters the host (Fig. 1B). Within the cricket host, larvae feed first on hemolymph, and later on muscle and fat body, creating a "respiratory funnel" in association with the host's abdominal wall (Adamo et al. 1995). After 6 to 10 d, larvae emerge from the host (which is killed) and pupate (Fig. 1D–F). Pupae develop outside the host for ~16 to 20 d before adult eclosion. Adults typically survive 2 to 3 wk, females longer than males (Wineriter and Walker 1990). In this work, we characterize the male and female reproductive tracts of *O. ochracea* using a variety of microscopic techniques providing details of embryogenesis and early larval development in utero.

Fig. 1. A) Female *Ormia ochracea* and a male domestic cricket, *Acheta domesticus*. Scale bar = 10 mm. B) Larvae deposited on soft tissue between thorax and abdomen of a female cricket. Scale bar = 1 mm. C) Scanning electron microgram of the larval head showing the "scalpel" that helps penetrate the host cuticle of larvae as it penetrates host tissue. Scale bar = 30 µm. D) Dead cricket one week later with a larvae emerging from the cricket. Scale bar = 5 mm. E) Wandering larvae preparing to pupate. Black respiratory tubercles mark the posterior. Scale bar = 2 mm. F) Pupa. Scale bar = 2 mm.

Materials and Methods

Animal Care

Crickets

Freshly molted adult *A. domesticus* were acquired from The Big Co. (Ham Lake, MN) and kept at ~21 °C over the course of the experiment. They were housed individually in 3.25 oz sauce cups with perforated lids to allow for respiration. We provided the animals with water and food (Purina Complete alfalfa rabbit feed pellets) ad libitum. Containers were checked daily for fly larval emergence, pupation, and animal deaths.

Flies

Donor *O. ochracea* were reared in temperature, humidity, and light-controlled environmental chambers (Power Scientific Inc, model DROS52503, Pipersville, PA) set to a 12 h light/12 h dark cycle at 75% humidity, and provided with butterfly nectar (The Birding Company, MA) ad libitum. Eclosed F1 flies were kept in the same sauce cup container as the cricket they parasitized. Flies were fed butterfly nectar via a cotton stick that was changed daily.

Manual Parasitizations

Manual parasitizations were performed by extracting larvae from freshly dispatched donor flies. This involved dissecting the abdomen of a fly and spreading out larvae contained in the fly reproductive tract on a petri dish lined with moist filter paper. Freshly extracted larvae move around in the petri dish and often stand on their posterior ends and move their anterior ends in a wave-like motion. On a piece of damp filter paper, larvae are most active within the first 2 h after removal from the gravid fly but can potentially survive for 7 to 8 h (Beckers et al. 2011). In this state, larvae can easily attach to a wooden probe. Once attached to the probe, we placed individual larva on top of the articular sclerite located just above the anterior margin of the cricket's thorax and directly underneath the pronotum (Fig. 1B). Larvae from each of the donor flies were used to infest several hundred *A. domesticus*.

The parasitized cricket was then housed in a 3.25 oz sauce cup. Twelve gravid female flies were dissected for this work, as well as 6 "virgin" females and 6 males.

Microscopy

Gravid and nongravid *O. ochracea* were dissected using an Olympus SZ60 stereo dissecting microscope with a brightfield/darkfield base after sedation on ice. Best results were obtained by dissecting the abdomen on the ventral side, followed by excising the genital disc. The hindgut and reproductive tract are attached to the genital disc. The hindgut was then removed, leaving just the reproductive tract, with the genital disc attached. Samples were hydrated using Grace's insect media (G8142, Sigma-Aldrich). Samples intended for observation of embryonic development were treated with Hoechst 33258 (Molecular Probes), 2-µg/mL final concentration, mixed in 19-µL Grace's Media, for 5 min. One drop of Grace's insect media was placed on samples to dilute stain and maintain sample hydration. Samples were observed on an Olympus (BX40) fluorescence microscope and photographed with a DP70 digital camera.

Results

Female reproductive anatomy

Ovaries

Twin ovaries discharge the unfertilized eggs into a pair of lateral oviducts, which join into a common oviduct (Figs 2 and 3). Hoechst staining suggests that the nuclei of the oviduct are likely diploid in nature, in that nuclei of the oviduct are the same size as those of the egg (Fig. 3E). The common oviduct then joins the uterus near where the female accessory glands, seminal receptacle, and spermathecae are attached ((Fig. 3). We refer to this as the reproductive junction.

Oogenesis

Oogenesis is comparable to that described for both *Drosophila melanogaster* and for *Exorista sorbillans*, another *Tachinid* (Boregowda 1994, McLaughlin 2015). Each ovary contains numerous ovarioles with follicle cells surrounding the developing egg (Fig. 3C). Flies (in general) possess a meroistic ovary, one in which nurse cells: nutritive cells connected to the developing oocyte, synthesize and supply both RNA and proteins to the oocyte throughout follicle development via cytoplasmic bridges. In polytrophic ovarioles, (as seen in *Drosophila* and most flies), nurse cells remain with the developing oocyte as the follicle develops (Büning 1994).

We observed early follicles appearing to "bud" off from the germarium (Fig. 4A and B). A complete sheath of follicle cells is established early on. The oocyte grows within the enclosed follicle from 20 to 150 µm in length. A cuboidal layer of follicle cells form a sheath around the growing oocyte and appear to secrete chorion material as the follicle elongates from a sphere to an ellipsoid (Fig. 4C and D). Once the oocyte reaches a length of ~100 to 150 µm, the cuboidal follicle cells become squamous and degenerate (Fig. 4E). The death of nurse cells and later the follicle cells during oogenesis in flies has been richly studied as a model for programmed cell death (McCall 2004, Bolobolova et al. 2020). At this time, a distinctive micropyle (a channel through the chorion) can be easily observed (Fig. 4F). In other insects, it has been noted that sperm enter the egg through this micropyle. Its anterior location was confirmed by observation of the micropyle in fully developed larvae with recognizable tagma and segments. The loss of nurse cells early in development and degeneration of the surrounding follicle cells seem significant in light of observations that the oocyte continues to grow from 20 to 150 µm within the ovary, and postfertilization embryos quadruple in size during their passage through the uterus. This latter observation suggests that the developing embryo receives maternal nourishment in utero.

The "Reproductive Junction"

Spermathecae

Three distinctive brown ovoid organs form the spermathecae (Fig. 5A, E). Sperm delivered at the vaginal opening swim as far as 3 cm through the coiled uterus before arriving at the 3 ducts that connect with these organs. The spermathecae are reportedly utilized for long-term sperm storage (Pitnick et al. 1999, Qazi et al. 2003). They appear functional in this species in that we observed active flagellar beating of sperm within the 3 spermathecae in gravid female flies (Supplementary material movie 1).

Seminal receptacle

The seminal receptacle is a blindly ending tube arising as an outpocket from the anteroventral portion of the uterus, and folded back

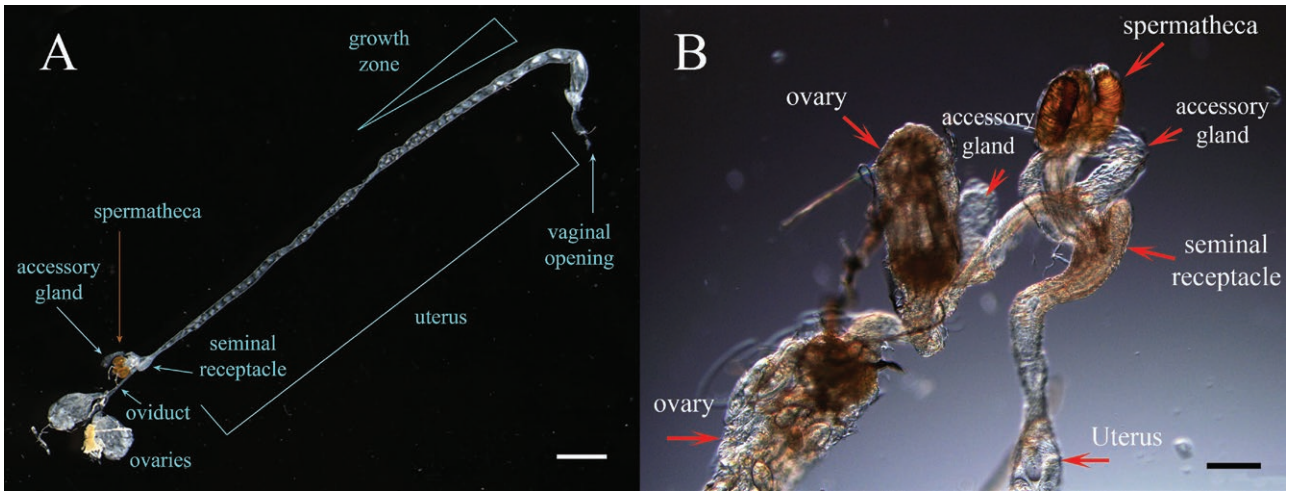

Fig. 2. A) Darkfield view of a female reproductive tract dissected from a gravid *O. ochracea* female. Eggs are present throughout the fly uterus, with embryos increasing in size as they advance from oviduct to ovipositor, especially over the distal-most segment ("growth zone"). Scale bar: 500 µm. B) The "reproductive junction" where the oviduct meets the uterus, attended by accessory glands, seminal receptacle, and spermathecae. Scale bar = 100 µm.

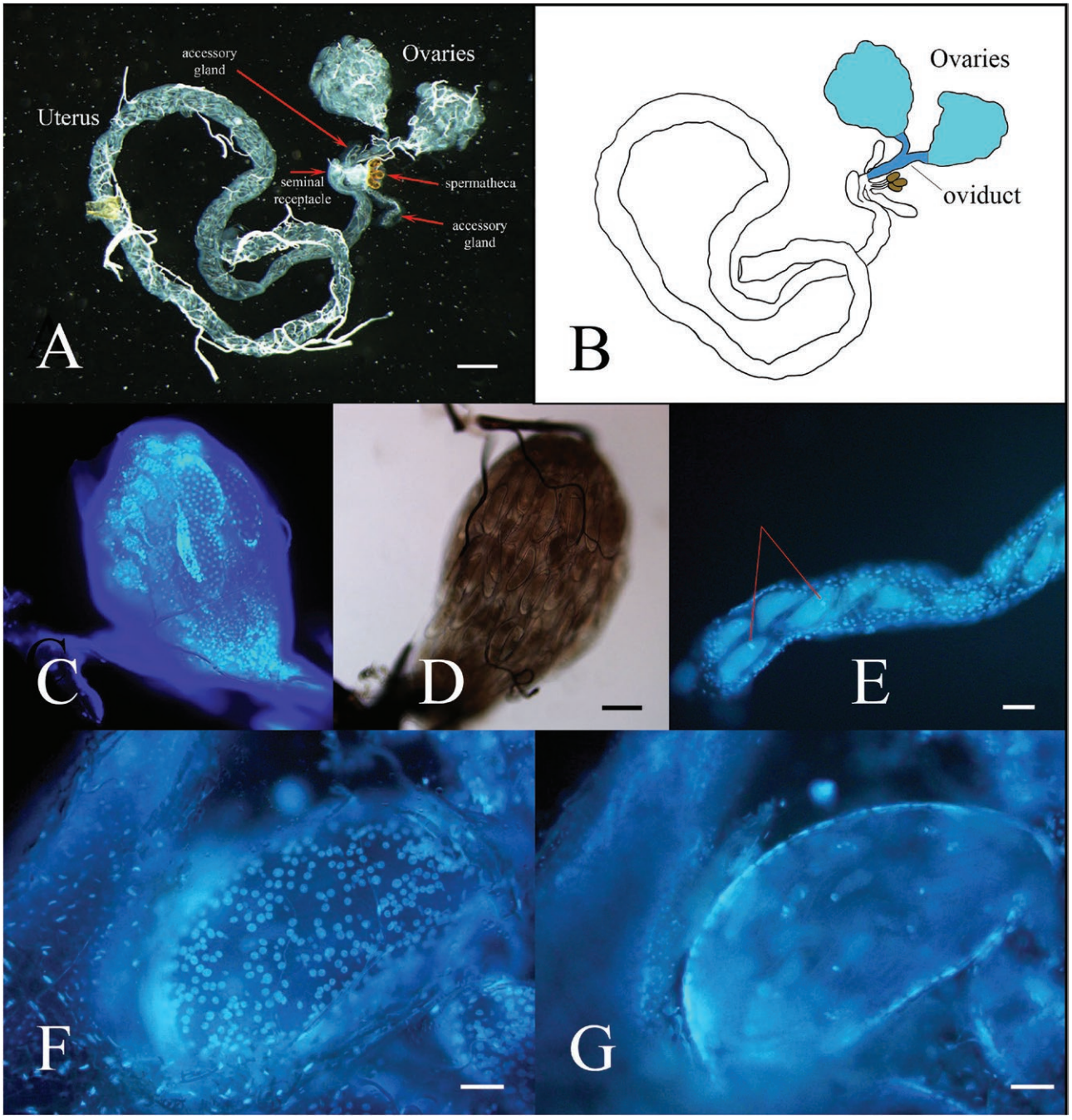

Fig. 3. A) Darkfield view of dissected female reproductive tract, scale bar = 200 µm. B) Diagram of image in Fig. 3A for reference. C) Developing ovary with Hoechst stain to reveal nuclei of follicle cells. D) Mature ovary with eggs, ready for ovulation. Scale bar = 50 µm. E) Uni-nucleate oocytes traveling down oviduct (Hoechst stain, pointers). Note: oocyte nuclei appear the same size as oviduct epithelium. Scale bar = 50 µm. F) Hoechst labeling of ovarian epithelium nuclei. G) Deep focus of same ovary seen in Fig. 3F showing singular diploid nuclei of the premeiotic oocytes. Scale bars = 50 µm.

toward the ovaries (Fig. 5F) (Nonidez 1920). It has been suggested that the seminal receptacle may provide short term storage and more directly delivers the sperm that fertilize eggs that pass from oviduct to uterus (Pitnick et al. 1999). It has also been suggested that the seminal receptacle arose as a later, secondary evolutionary innovation, possibly serving a redundant function to the spermathecae.

Female accessory glands

Directly posterior to the spermathecae are the female accessory glands (Fig. 5C). There are 2 such glands, structured as sacs of single-layered glandular cells with large vacuoles. A large duct through the center of the glands connects to the uterus. The exact role of the accessory glands is uncertain, though it may provide nutrients upon which developing embryos can survive or secretions that promote egg motility.

Uterus

From reproductive junction to vaginal opening, the uterus can extend up to 3 cm in length (Figs 2 and 6). Sperm from a mating partner travel this length before being stored in the spermathecae or

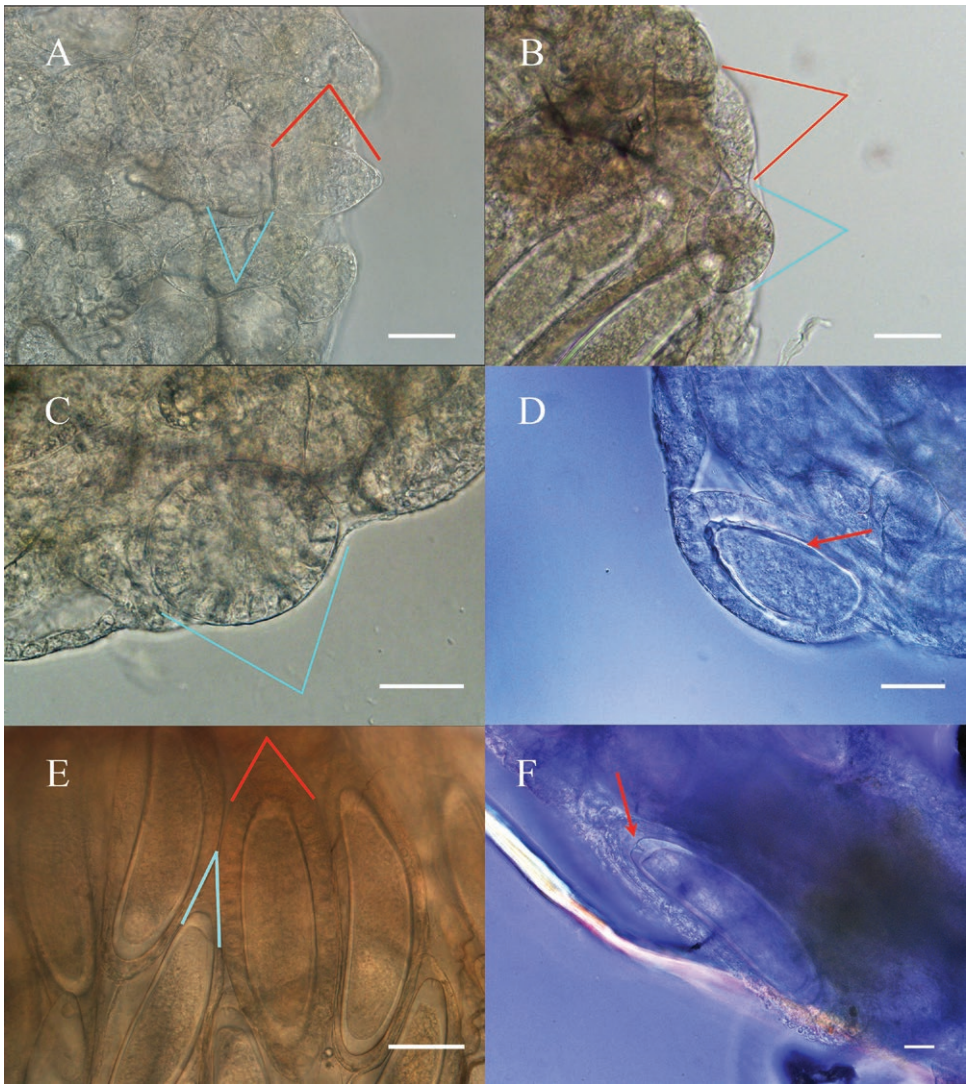

Fig. 4. Developing follicles. A) Early stage exhibiting a cell-lined spherical capsule (arrows) attached to an acorn-shaped cellular mass (pointers). This "acorn-shaped mass" likely represents the germarium. Scale = 20 µm. B) A follicle (pointers) appears to be separating from the germarium (arrows). Scale = 20 µm. C) After separating from the germarium, the follicle continues to grow. Scale = 10 µm. D) One sees evidence of chorion-deposition (highly refractile material under DIC) by the cuboidal follicle epithelium (pointer). Scale = 10 µm. E) As eggs grow and mature, follicle cells (upper pointers) appear to degenerate (lower pointers). Scale = 20 µm. F) A mature egg showing a distinct micropyle (arrow). Scale = 20 µm.

seminal receptacle. The eggs travel this same length as the embryos grow and develop into fully mature first instar larvae. In gravid females, dozens of mature first instar larvae can be seen, stored in the more distal stretch of the uterus. We observed loose, shed chorions from only the latest larvae that had hatched in utero, and were ready for deposition on the cricket host.

Embryogenesis

In *Drosophila melanogaster*, a fly in which embryogenesis has been richly studied (Foe and Alberts 1983, Foe 1989, Leptin 1999, Kotadia et al. 2010), the zygotic nucleus undergoes multiple mitotic divisions without cell division within a centro-lecithal syncytium. Again, in *Drosophila*, the early nuclear divisions (1 to 3) occur without cell divisions deep within the yolky anterior cytoplasm. Later (divisions 4 to 6), the nuclei spread throughout the central yolk mass, and after divisions 8 to 10, most nuclei migrate uniformly to the egg cell cortex creating the syncytial blastoderm, while others remain in the yolky interior, the so-called yolk nuclei. These latter typically undergo DNA endoreduplication (Foe and Alberts 1983). After 4 more syncytial nuclear divisions, cellularization takes place creating the cellular blastoderm. It is noteworthy, that at the time of nuclear migration, the *Drosophila* nuclei are distributed uniformly across the anterior–posterior, as well as dorsal–ventral axes.

In *O. ochracea*, the early cortical nuclei (following nuclear migration) are not distributed uniformly across the egg cortex but appear confined to a cortical island in the antero-ventral portion of the egg (Fig. 7A). We refer to this as the "germ island" in the developing embryo. The germ island undergoes a posterior expansion, forming a germ band along the ventral side of the embryo, eventually reaching the posterior end of the egg (Fig. 7B). We were unable to discern when cellularization occurs. The germ band continues to expand, with the posterior cells/nuclei arching over the dorsal surface in a process known as germ band extension (red curve, Fig. 7C). During germ band extension, segmentation begins to occur. The germ band retracts, as segmentation proceeds (Fig. 7D).

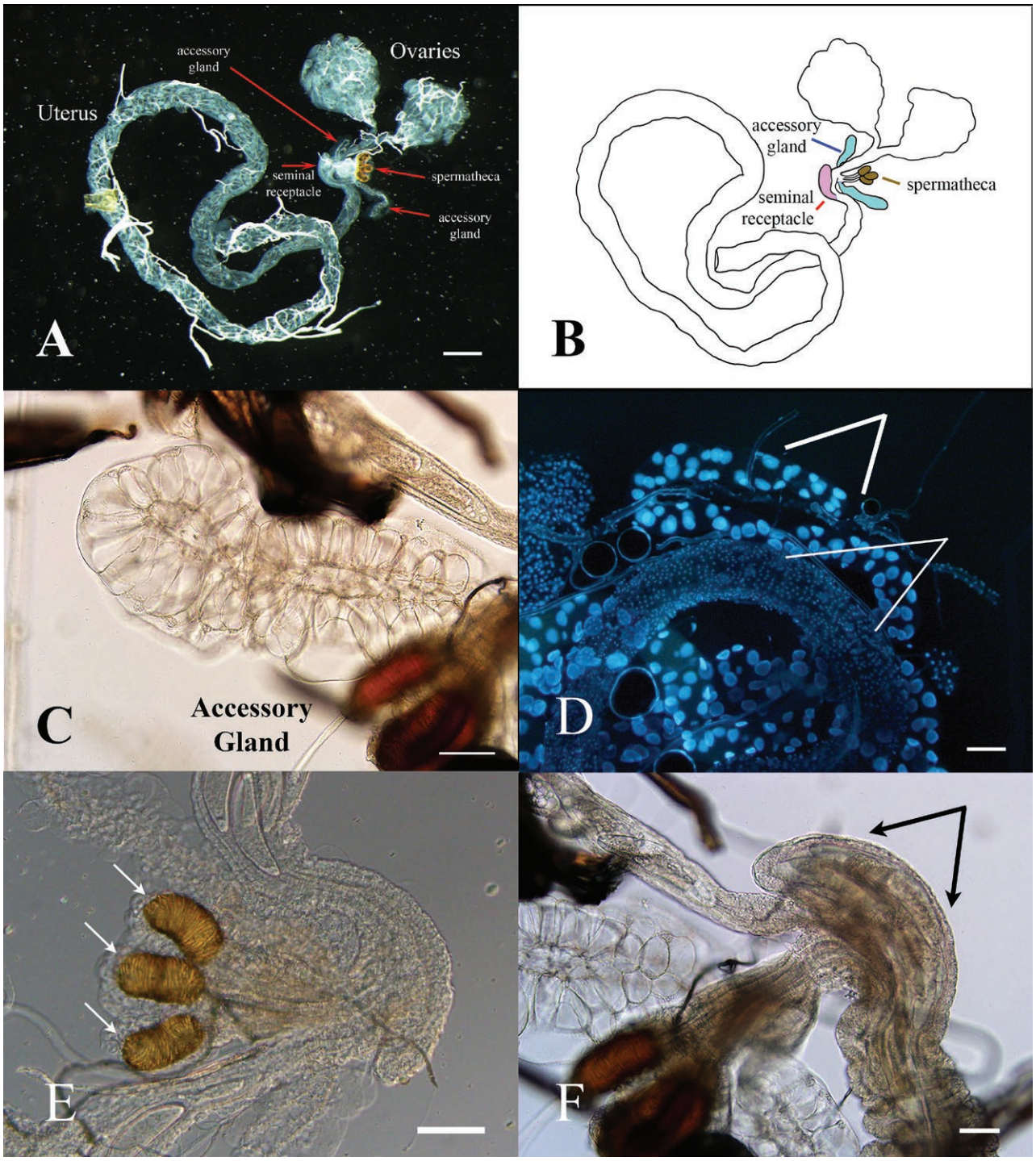

Fig. 5. The Reproductive junction. A) Darkfield view of a dissected female reproductive tract. Scale bar = 200 µm. B) Diagram of image in Fig. 5A highlighting the "reproductive junction." C) Female accessory gland. Scale bar = 50 µm. D) Female accessory gland stained with Hoechst showing endoreduplicated nuclear material (thick pointers) compared with nuclei of neighboring oviduct epithelium (narrow pointers). Scale bar = 100 µm. E) Three spermathecae (arrows). Scale bar = 100 µm. F) Seminal receptacle (arrows). Scale bar = 50 µm.

After germ band extension and retraction, the embryo undergoes "dorsal closure" in which the lateral margins of germ band spread dorsally, eventually sealing in the central, yolky mass (Figs 7E and 8A). The dorsal larval cuticle begins to darken following dorsal closure, at which time the larvae begin to exhibit motility and light-responsiveness (Fig. 8B).

Freshly ovulated eggs are ~167 µm in length. Fully developed embryos prior to pigmentation reach a length of ~600 to 650 µm. Fully pigmented larvae are ~550 µm (Fig. 9). This indicates a significant change in total egg volume over the course of development. It seems clear that the parent fly provides nutrients in utero, nutrients which enter the egg, possibly through the micropyle or via some other, trans-chorion transport.

An unexpected observation was that virgin females (collected and isolated just as they emerged from their puparium) undergo ovulation and the resultant unfertilized eggs proceeded to grow

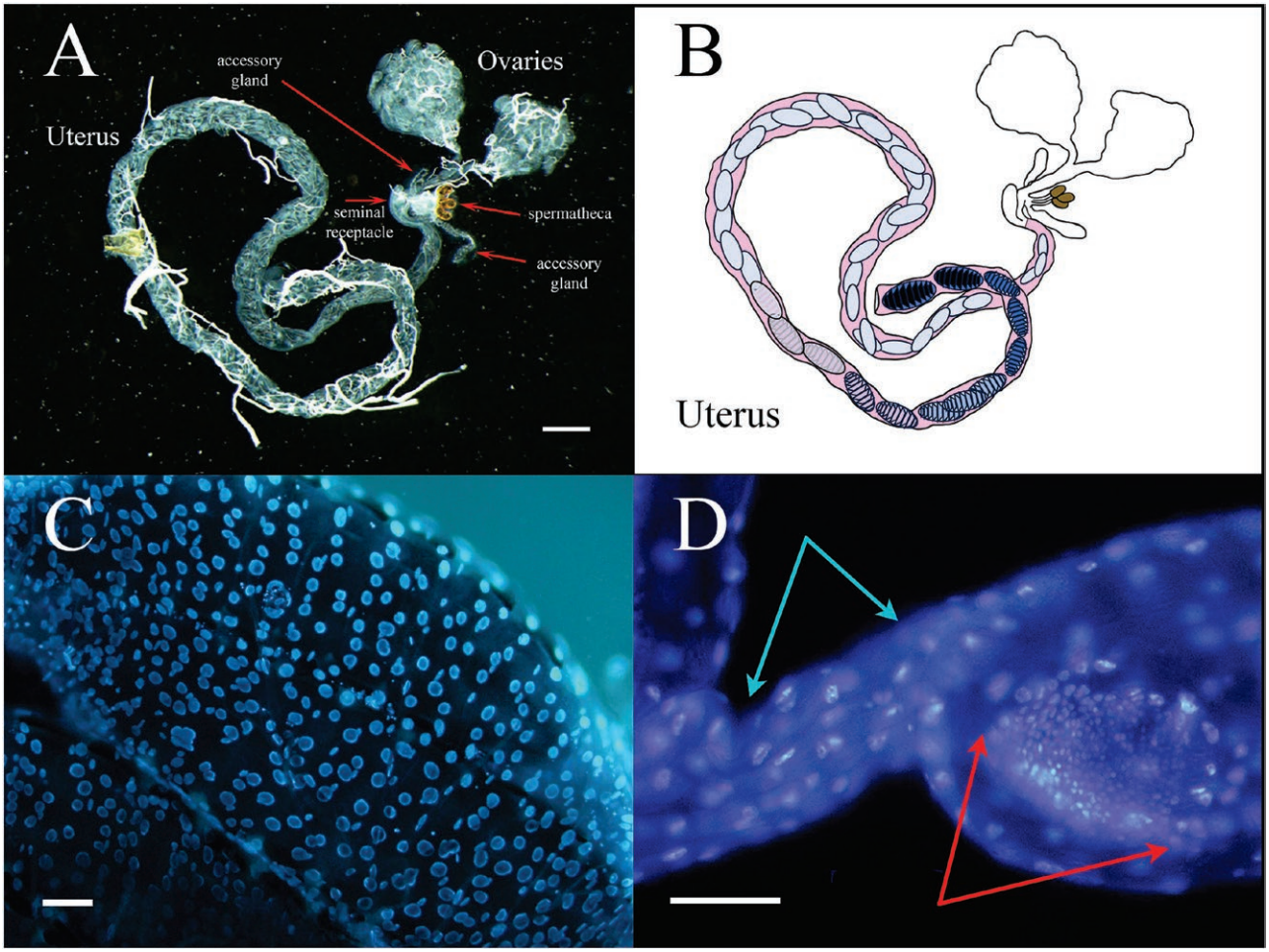

Fig. 6. A) Darkfield view of dissected female reproductive tract, scale bar = 200 µm. B) Diagram of image in Fig. 6A, highlighting the uterus (in pink) and developing embryos. C) Hoechst-labeled nuclei of the distal uterine epithelium encompassing a fully developed 1st instar larvae. Note enlarged nuclei suggesting high transcriptional activity. Scale bar = 20 µm. D) Hoechst-labeled uterus focusing inside the embryo to show relative size of presumably diploid embryonic nuclei (red arrows), vs larger nuclei of the uterine epithelium (blue arrows). Uterine nuclear endoreduplication suggests high synthetic activity. Scale bar = 20 µm.

and undergo rudimentary parthenogenetic development (Fig. 10). Infertile eggs reached a final maximum size of about 650 µm. Hoechst staining revealed that nuclei within these unfertilized embryos divided yielding at least 32 to 64 nuclei. Curiously, these nuclei appeared to be in a range of sizes (reflecting various degrees of genetic endoreduplication).

Infertile embryonic nuclei occupying the dorso-lateral cytoplasm appear enlarged, while those occupying the anterior-ventral cytoplasm (the presumptive germ island cytoplasm) appear small. We hypothesize that differences in nuclear size (revealed by Hoechst staining) reflect differences in relative degrees of endoreduplication, distinguishing future "yolk nuclei" from potential somatic nuclei as seen in fertilized *Drosophila* embryos (Foe and Alberts 1983).

Within the uterus of fertilized female flies, larvae appear to hatch from the chorion and are ready to be delivered on or near the host cricket. (It is possible that "hatching" might also be coincident with the mechanical process of larval deposition). Mature larvae exhibit 9 pigmented dorsal tergites and lateral pleurites, and 11 sets of ventral denticle bands. Curiously, the larvae exhibit intense autofluorescence especially on the ventral side (fluorescence observed using a filter with a 377-nm optimal excitation wavelength, and emitting at 447 nm) (Fig. 11 C–F).

Male Reproductive Anatomy

The male reproductive tract appears relatively conventional, possessing a pair of testes and male accessory glands, and delivering mature sperm through the ejaculatory duct to the ejaculatory bulb, and finally to an elaborate, chitinous intromittent organ. Hoechst staining suggests that cells of the male accessory glands have also undergone endoreduplication (the nuclei appear larger than in neighboring tissues, Fig. 12D), and mature sperm cells appear to be 80 to 90 µm in length (Fig. 12E). Curiously, upon exposure to the medium (3 to 5 min), the spermatozoa coil into tight ringlets (Fig. 12F). Though almost certainly artifactual, it may be worth noting so that these are not mistaken for the natural form of the sperm in future studies.

Discussion

The Enigma of Embryonic Growth In Utero

The nurse cells that directly support early oogenesis by providing both RNA and proteins degenerate prior to ovulation. Similarly, follicle cells that ensheath the oocyte degenerate upon deposition of the chorion and prior to ovulation. In *Drosophila*, where egg maturation and sperm storage have been richly studied, permeability of the egg envelope to small molecules decreases substantially during

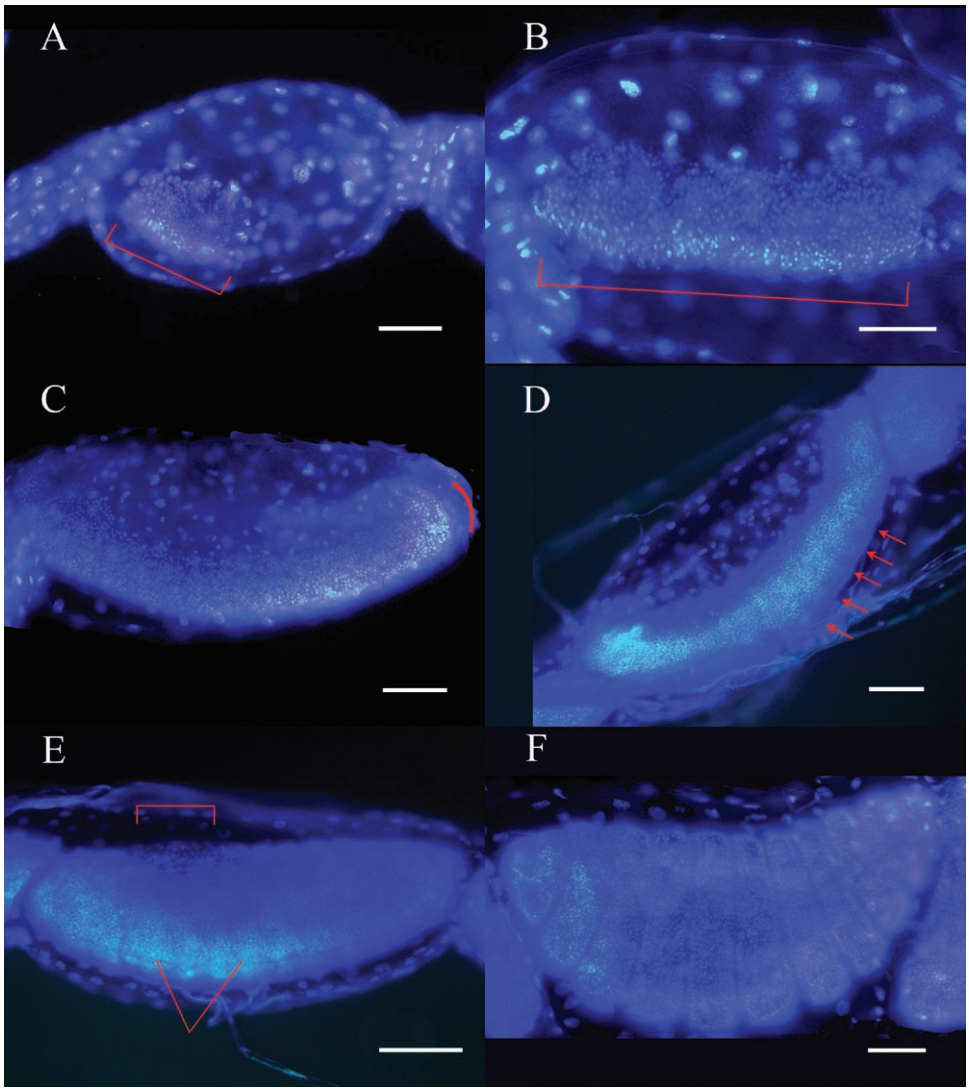

Fig. 7. Hoechst staining of developing embryos observed within the *Ormia* uterus. A) Early formation of "germ island" of nuclei (brackets). Scale bar = 20 μm. B) Expansion of the germ island into a germ band (within bracket). Scale bar = 20 μm. C) Germ band extension, (curved line). Scale bar = 20 μm. D) Segmentation (marked by red arrows) following germ band retraction. Scale bar = 20 μm. E) Dorsal closure. Bracket indicates final dorsal gap being covered by epiboly. Pointers indicate dark "groove" indicating ventral furrow. Scale bar = 40 μm. F) A fully developed embryo. Scale bar = 40 μm. Embryos are oriented (when possible) with anterior to the viewer's left.

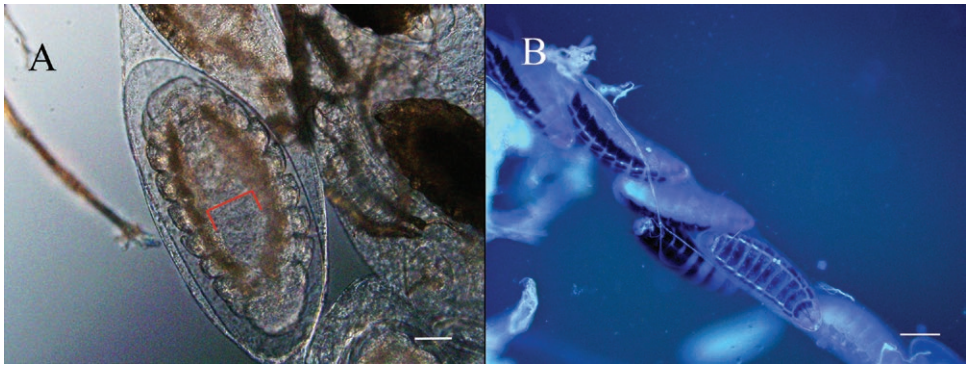

Fig. 8. A) Developing embryo undergoing dorsal closure (brackets). Scale bar = 25 μm. B) Mature larvae lined up within the distal portion of the uterus exhibiting autofluorescence. Scale bar = 100 μm.

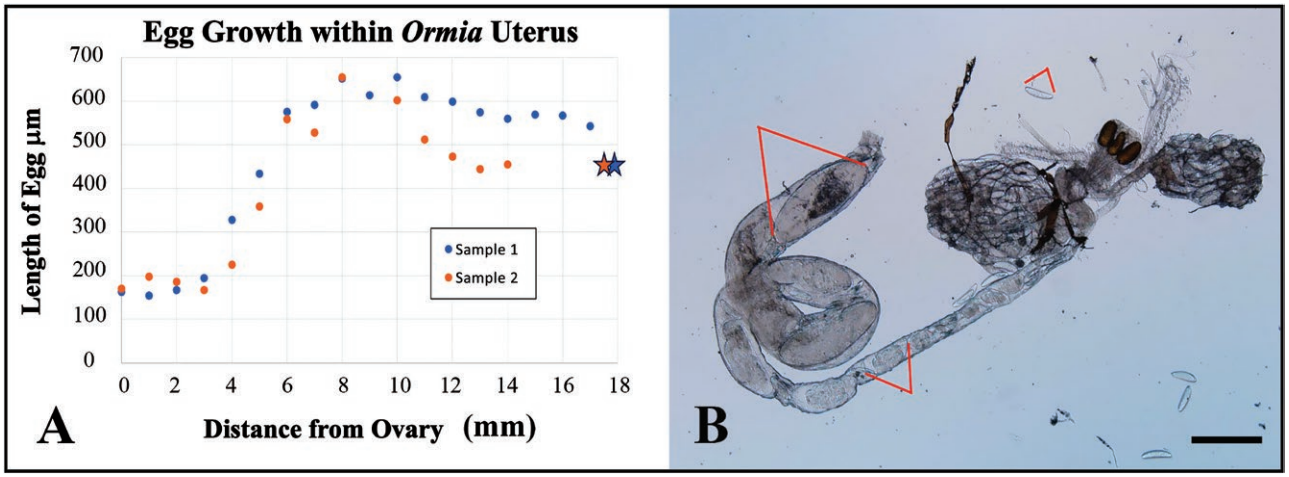

Fig. 9. A) The length of an egg-enclosed embryo within the uterus, as a function of distance from the ovary (mm). Embryo exhibits growth (quadrupling in size) in the segment of uterus 4–8 mm distal to ovary. Embryos shorten as they become fully mature larvae (10–14 mm distal to the oviduct). Stars indicate size of fully mature larvae. (Data from two specimens are shown). B) A portion of the *Ormia* ovary showing the region of egg growth. Scale bar = 400 µm.

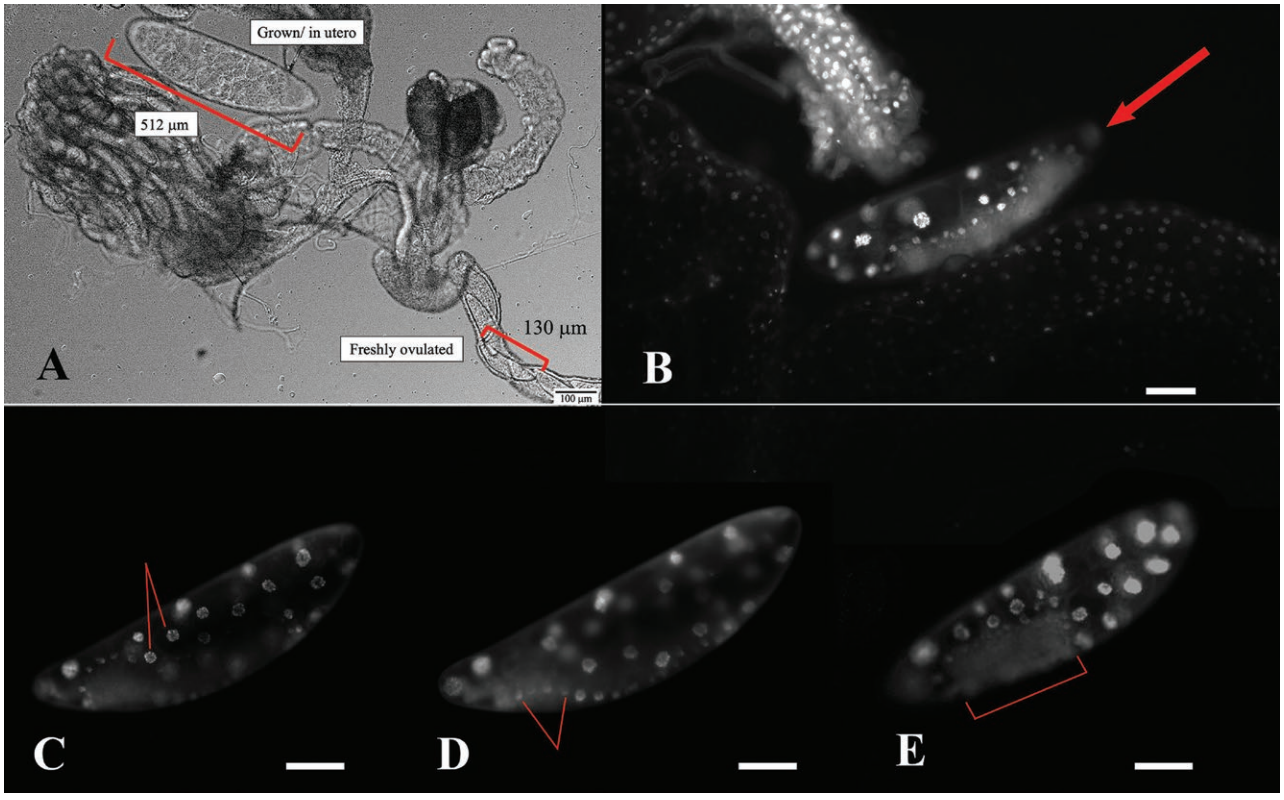

Fig. 10. Parthenogenetic development in virgin females. A) The female reproductive tract dissected from a virgin female *Ormia ochracea*. Note the increase in egg size following ovulation. B) An egg isolated from the uterus showing multiple nuclei (of varying size/ploidy). C, D) The same unfertilized egg exhibiting large nuclei that have presumably undergone endoreduplication, along dorso-lateral margins (arrows in C), and smaller nuclei on the anterior ventral region that would form the germ island (arrows in D). E) Another unfertilized egg showing an opaque patch of cytoplasm flanked by small nuclei (bracket). This region is presumably where the germ island would normally form. Scale bars = 100 µm.

ovulation (Heifetz et al. 2001, Qazi et al. 2003). If the same is true of *O. ochracea*, we are posed with an interesting question. Embryos quadruple in size during postfertilization development and passage down the long uterus. Without accessory cells to provide the embryo with nutrients, and while enclosed in a chorion of uncertain permeability, how do these ovo-larviporous *Tachinid* embryos grow in utero? We hypothesize that the uterine fluids provide nutrients to the developing embryos and that the embryos imbibe these uterine nutrients through the micropyle: the one opening that allows embryonic tissues access to the surrounding medium. When labeling embryos with Hoechst dye, we witnessed the dye first entering the embryonic space through the micropyle, labeling anterior nuclei first before diffusing throughout the rest of the embryo. This is an unusual situation, in that most members of the *Tachinid* family are

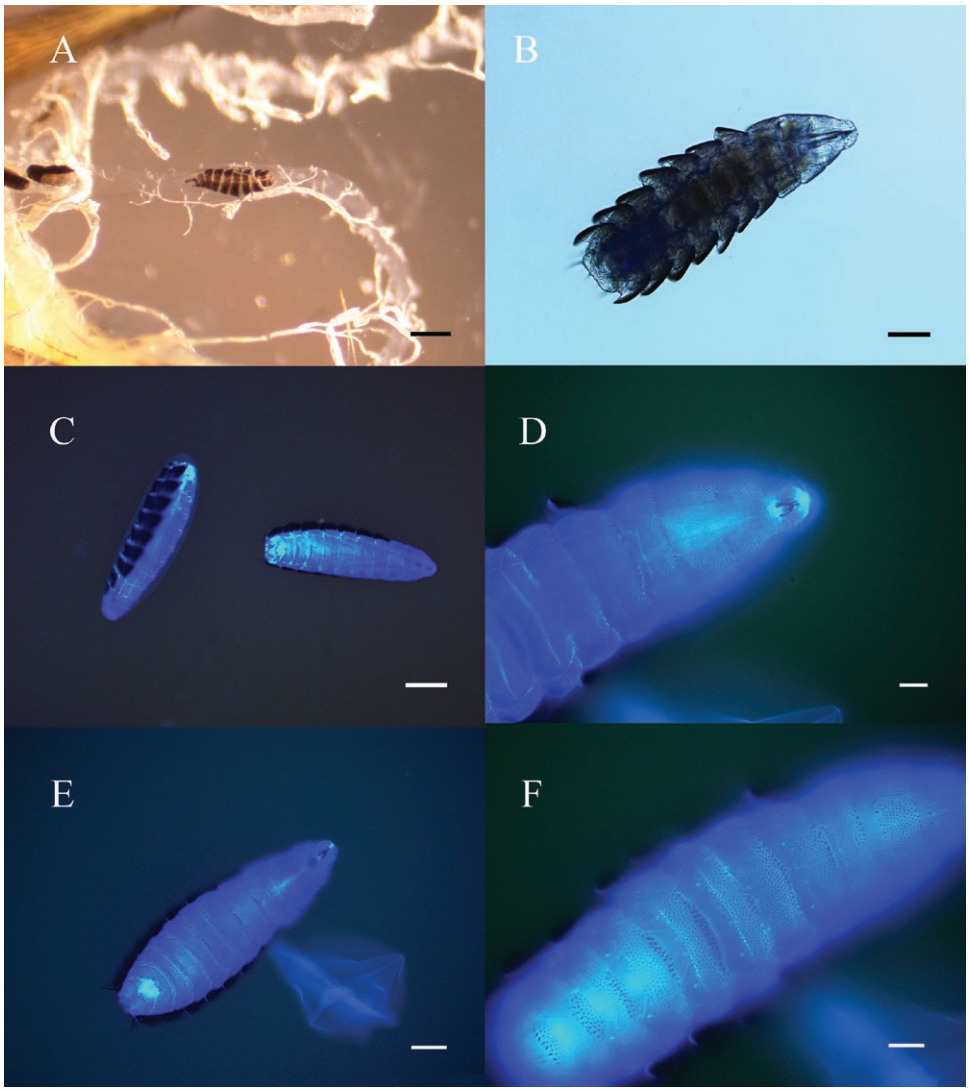

Fig. 11. A) Male *Ormia ochracea* reproductive tract viewed with darkfield microscopy. Scale bar = 100 mm.B) Sketch of Fig. 11A highlighting testes and accessory glands. C) Close-up of testis and accessory gland from Fig. 11A. Scale bar = 50 mm. D) Hoechst stain of accessory gland highlighting large (endoreduplicated) nuclei. Scale bar = 50 mm. E) Spermatozoa and spermatocytes from ruptured testis. Scale bar = 20 mm. F) Ring-forms that spermatozoa adopt upon exposure to Grace's medium. Scale bar = 20 mm.

oviparous, depositing and attaching fertilized eggs onto their host species, eggs which will hatch and develop outside the mother's body. Few tachinids exhibit larvipary as seen in *O. ochracea*.

An unrelated, ovo-larviparous fly offers a similar conundrum with a highly specialized solution that may represent a reproductive analogy. The African tsetse fly, *Glossina sp*. [*Diptera*, *Glossinidae*] develops one solitary egg at a time. The embryo hatches within the uterus, where it passes all 3 larval instars, nourished by a specialized "milk gland." The milk gland appears to be a highly hypertrophied version of the female accessory gland in close association with the fat body. It provides both lipid and protein to the developing, single larva (Tobe et al. 1973, Ma et al. 1975, Attardo et al. 2020). It may be significant, that in *O. ochracea*, both uterine epithelium, and the female accessory gland nuclei appear to have undergone some form of DNA endoreduplication, suggesting that they are engaged in biosynthetic activities that could play a role in supplying intra-uterine larval nourishment. Further work is needed to confirm this.

A related problem is that, as the intra-uterine larvae grow, the chorion must expand to keep up. The chorion is initially secreted during oogenesis by the follicle cells. Since follicle cells are gone prior to fertilization and ovulation, how does the chorion expand to accommodate embryonic growth?

Partial Parthenogenesis

A surprise discovery was that virgin female flies ovulate, and their unfertilized eggs undergo at least early stages of "embryonic" development. This includes intra-uterine growth, syncytial nuclear divisions, and even an apparent differentiation of diploid somatic nuclei (in proximity to what would have become the germ island) from syncytial "yolk nuclei" that appeared to have undergone DNA endoreduplication. (We did not have the facilities to measure actual ploidies, so this was based on observed differences in nuclear size and fluorescence intensity using Hoechst dye). There are many examples of partial parthenogenetic development among flies (reviewed by Sperling and Glover 2023). Among 40 *Drosophila* species screened, most are capable of initiating parthenogenetic development (Markow 2013). A final note on *Ormia* parthenogenetic development: the "embryos" that developed from unfertilized eggs,

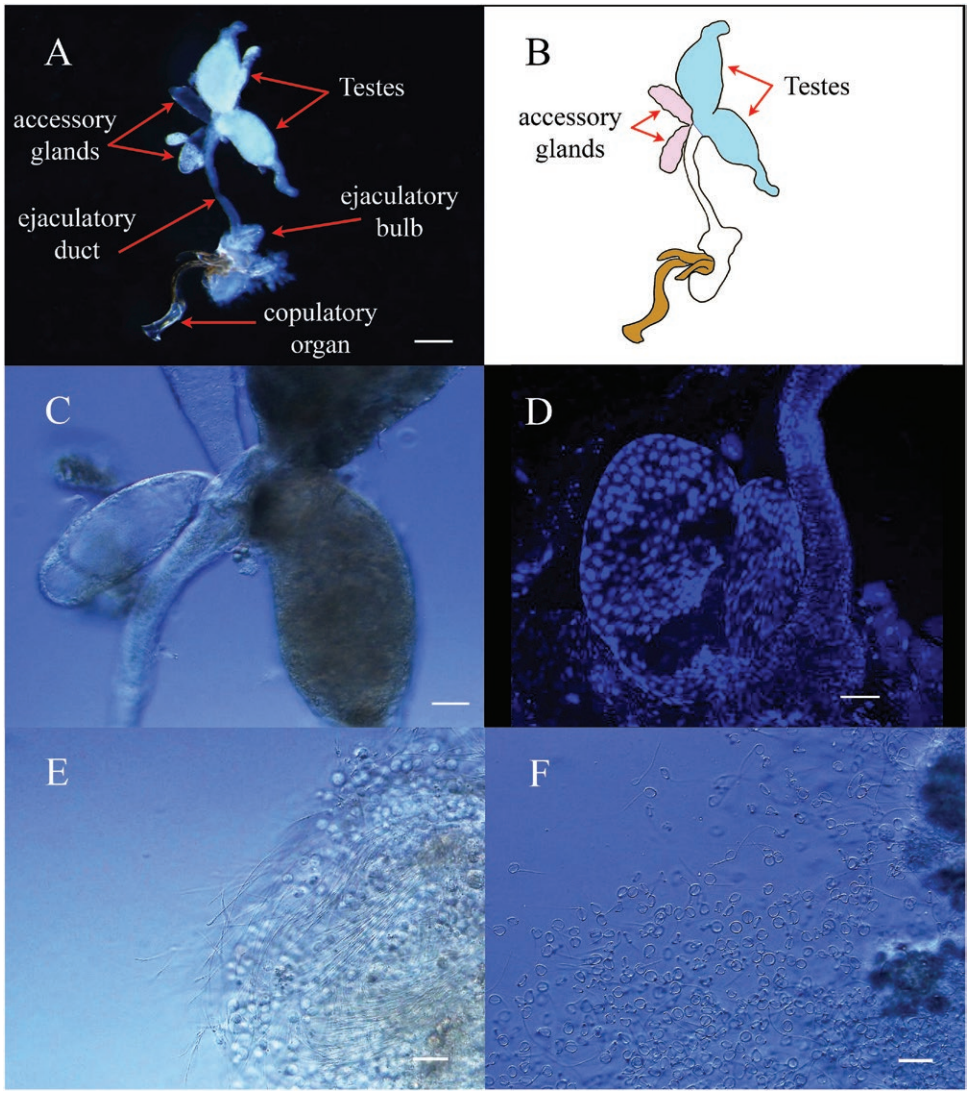

Fig. 12. A) Male *Ormia ochracea* reproductive tract viewed with darkfield microscopy. Scale bar = 100 µm. B) Sketch of Fig. 10A highlighting testes and accessory glands. C) Close-up of testis and accessory gland from Fig. 10A. Scale bar = 50 mm. D) Hoechst stain of accessory gland highlighting large (endoreduplicated) nuclei. Scale bar = 50 mm. E) Spermatozoa and spermatocytes from ruptured testis. Scale bar = 20 mm. F) Ring-forms that spermatozoa adopt upon exposure to Grace's medium. Scale bar = 20 mm.

though enlarged, did not show larval features (cellularization, segmentation/dorsal closure/cuticle development). Rather they seemed highly vacuolated. Possibly these large vacuoles contain whatever nutrients (lipids?) the embryos received during intrauterine growth. These might be collected for future analysis.

The Potential for Constructing Transgenic *Ormia*

As *O. ochracea* is a model system for understanding the neuroethological basis of auditory perception of host cricket calling songs (Mason et al. 2001, Lakes-Harlan and Lehmann 2015, Lee and Mason 2017, Gray et al. 2019, Mason 2021, Jirik et al. 2023, Wikle et al. 2025), it is attractive to think about developing genetic tools. Our study reveals that for *O. ochracea*, this presents an especially daunting task. Conventional molecular genetic methods applied to *Drosophila* rely almost exclusively on the ability to micro-inject gene constructs into the "pole plasm" of the early oocyte. That this is possible is due to the fact that shortly after fertilization, *Drosophila* lay their eggs making them easily accessible for manipulation/micro-injection. In *Ormia*, ovulation and fertilization occur deep within the reproductive tract, and stages comparable to those of *Drosophila* embryogenesis require surgery, manipulation of eggs within the uterus, and subsequent recovery of the fly so that larval development can proceed in utero. At this time, the most promising avenue would seem to be to manipulate sperm cells rather than eggs, potentially delivering the transgenic sperm to the distal region of the uterus via an attempt at artificial insemination. Genetic modification of insect sperm has been achieved in several species including the silk moth, *Bombyx mori* (Shamila and Mathavan 1998, Li et al. 2003) and the honey bee, *Apis mellifera* (Robinson et al. 2000). The prospect of developing *Ormia* into a model for molecular genetic modification appears to be a fair way off.

Supplementary material

Supplementary material is available at *Annals of the Entomological Society of America* online.

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