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Development of the larval ovary in the moth, *Plodia interpunctella*

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Abstract

The morphogenesis of ovaries and the organization of germ cells within them were visualized during the larval stages of the moth, *Plodia interpunctella*. The germ cells were observed by utilizing confocal microscopy coupled with immuno-fluorescent staining for the α-crystallin protein 25 (αCP25). The αCP25 was previously shown to be specific to germ cells of pupae and adults, and this study shows that αCP25 is present in larval germ cells as well. A cluster of 28 germ cells that stain for αCP25 was found in the gonads of newly hatched first instar larvae. The founding germ cells became segregated into four clusters, most likely by somatic cell intrusion, around the beginning of the second instar. Division of the primary germ cells began by the end of the second instar and the formation of all cystoblasts appeared to be completed within the four ovarioles by the end of the third instar. Within the ovarioles of third instar larvae, the germ cells were organized with a distal cap of seven germ cells which was segregated from the majority of the germ cells. The main body of germ cells was arranged around a central germ cell-free core as a spiral. Divisions of the cystoblasts to form cystocyte clusters were nearly completed during the fourth (last) larval instar. These features suggest that the strategy to produce follicles in moths is fundamentally different from the fruitfly, *Drosophila*. It appears that during the initial stages of ovary development in *P. interpunctella*, the primary germ cells undergo stage-complete divisions that are completed prior to the onset of the next set of divisions, which results in a complete complement of follicles available by the time of adult eclosion, while in *Drosophila* the primary germ cell divisions are initiated in the adult stage, and follicles are produced individually as resources are available.
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Keywords: Ovary; Cystoblast; Cystocyte; Ovariole; Immuno-fluorescence; Confocal microscopy; Indian meal moth

1. Introduction

In many families of Lepidoptera, the completion of egg development occurs during the late larval and pupal stages as an adaptation to a non-feeding, short-lived adult stage where the female must be capable of laying eggs soon after eclosion (Ramaswamy et al., 1997). This life style requires that follicle development and consequently ovarian organogenesis take place during earlier developmental stages. Examination of ovarian development confirms the early maturation of the gonads in some Lepidoptera. At adult eclosion, the ovaries of the Indian meal moth, *Plodia interpunctella*, contain follicles that are completing choriogenesis and will be competent for fertilization and deposition within 7 h after eclosion (Zimowska et al., 1995). The development of follicles was described during pupal/pharate adult development by utilizing immuno-staining for the α-crystallin protein 25 (αCP25) (Zimowska et al., 1991). The αCP25 has been shown to be present in the germ cells of pupal and adult ovaries and testes but not the somatic tissues of these organs (Zimowska et al., 1991; Shirk and Zimowska, 1997; Shirk et al., 1998). At the larval–pupal molt, the ovarioles were found to contain germ cells in eight-cell cystocyte complexes but without the presence of a surrounding follicular epithelium

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(Zimowska et al., 1991). Separation and enclosure of the individual cystocyte complexes by follicle cells began concomitantly with the initiation of adult development following the major peak of ecdysteroids that occurs 24 h after pupation (Zimowska et al., 1991; Shaaya et al., 1993).

Even though the later stages of follicle development have been intensively examined in Lepidoptera, little is known about the development of the ovaries and germ cells between the embryo and the beginning of pharate adult development. In the silkworm *Bombyx mori*, it was suggested, based on cell size and structure from electron micrographs, that the germ cells formed in a lateral position in the embryo (Miya, 1958, 1959). Subsequently, it was definitively shown that the germ cells form as a single cluster of cells in the lateral and ventral region associated with the localization of the *vasa* transcript (Nakao, 1999) rather than posteriorly as is found in *Drosophila* (Hay et al., 1990; Lasko and Ashburner, 1990). Similar observations were made in the tobacco hornworm *Manduca sexta* although the germ cells could only be detected after germ band formation because the immuno-staining of the germ cells with mAb 3B11 only occurs after cellularization (Nardi, 1993). The large germ cells were observed as a loose aggregate that collected on the midline of the *M. sexta* embryo near the posterior pole. However, as in *B. mori*, the location of germ cell formation in *M. sexta* does not appear to be directly posterior as found in *Drosophila*. After segmentation of the *M. sexta* embryo, the germ cells were initially distributed between three abdominal segments and then segregated laterally into two groups. Subsequently, the germ cells formed two tight clusters of 18–30 germ cells localized within the fifth abdominal segment (Nardi, 1993).

To determine the course of germ cell development and ovarian organogenesis in a lepidopteran, we examined gonads from the larval stages of *P. interpunctella* utilizing immuno-fluorescent staining for αCP25 to provide a means of visualizing the germ cells. We found that the organization of the ovarian structures occurs at very early larval stages and that the course of germ cell divisions in Lepidoptera is different than that reported for *Drosophila*.

2. Materials and methods

The *Plodia interpunctella* colony was reared according to Silhacek and Miller (1972) in a 16 h light : 8 h dark cycle at 30 °C and 70% relative humidity. The *P. interpunctella* colony has been in continuous culture since 1972 and has four larval instars instead of the five larval instars found in wild populations.

2.1. Immuno-histochemistry

Larvae at various stages of development were dissected in lepidopteran saline (Weevers, 1966) so that the gonads could be removed. Prior to fixation, the gonads were dissected from the surrounding sheaths which were found to be nearly impermeable to fixatives (Fig. 1A). Following dissection, the gonads were made permeable by immersing the tissues in methanol briefly and then fixing them in (4% w/v) depolymerized paraformaldahyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4 °C. The gonads were washed five times for

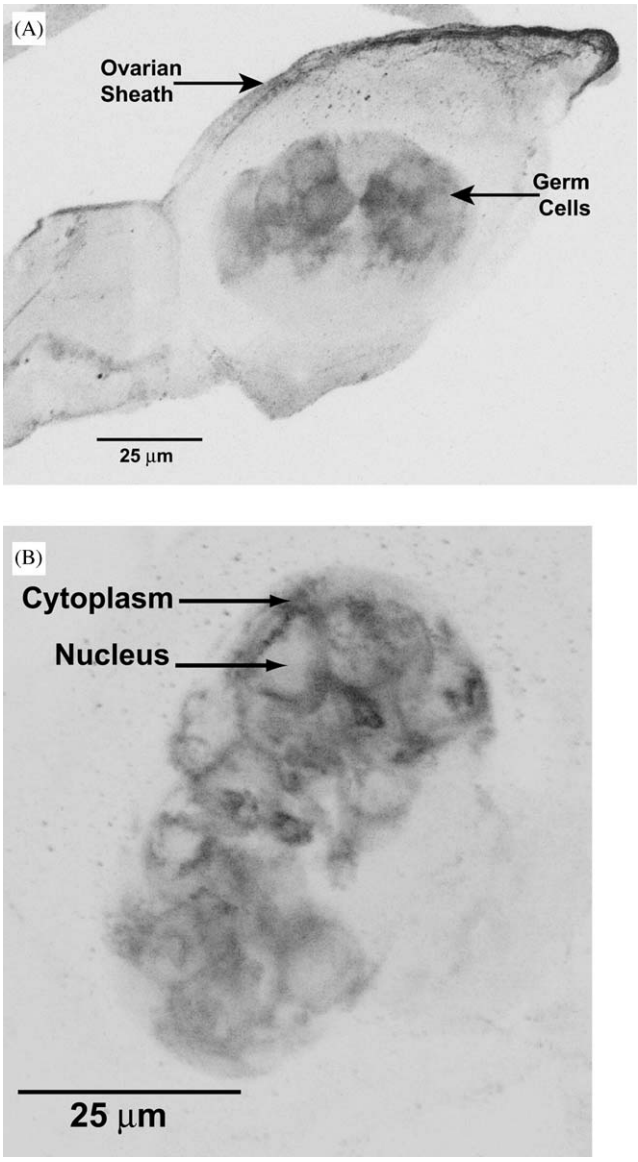

Fig. 1. Ovary from first instar *Plodia* larvae. Panel A shows the ovary after immuno-fluorescent staining without removing the ovary from the sheath. Panel B shows the immuno-fluorescent staining of the ovary after it was removed from the ovarian sheath. The confocal images are compilations of optical sections through the entire specimens.

1 h in 0.1 M phosphate (pH 7.4), 0.1% azide, and 1% Triton-X 100 (PBAT) and washed extensively in PBAT for up to 72 h at 24 °C. The gonads were blocked with 2% non-reactive goat serum in PBAT at 24 °C and then incubated with the following sera: polyclonal rabbit αCP25 antiserum (Zimowska et al., 1991), preabsorbed polyclonal rabbit αCP25 antiserum (Shirk et al., 1998), or non-reactive serum diluted 1:250 in PBAT at 24 °C overnight. The gonads were washed in PBAT five times for 1 h each and then incubated with anti-rabbit goat IgG conjugated with fluorescein isothiocyanate (GAR-FITC) diluted 1:40 in PBAT with 2% non-reactive goat serum at 24 °C overnight. The gonads were washed in PBAT five times for 1 h each at 24 °C. The gonad preparations were dehydrated and cleared in 80% glycerol diluted with 50 mM carbonate buffer (pH 9.4). Whole mounts of gonads (in 80% glycerol diluted with 50 mM carbonate buffer, pH 9.4, and 0.1% N-propyl gallate) were examined with an Olympus BHS microscope equipped with a BH2-RFL reflected light fluorescence attachment with an HQ Fluorescence filter (Chroma Technology Corp., Brattleboro, VT). Subsequently, the gonad preparations were examined using an MRC600 confocal laser scanning imaging system (BioRad, Hercules, CA). The individual optical sections and the compiled optical section images were analyzed.

3. Results

Based on the selective immuno-staining of germ cells with αCP25 antiserum (Zimowska et al., 1991; Shirk and Zimowska, 1997), ovaries of various stages of *Plodia* larvae were immuno-fluorescently stained for αCP25. In larval stages 1 and 2, there are no observable morphological markers that permitted discrimination of the two sexes nor could visible differences in the structure of the gonads be discerned. Therefore, the sex and gonad type of early larval instars could not be determined prior to dissection. When the ovaries from newly hatched larvae were dissected without removing the surrounding sheaths, the immuno-staining was incomplete (Fig. 1A). By removing the ovarian sheaths and treating the exposed ovaries with methanol to make the tissues more permeable, staining of the germ cells was complete (Fig. 1B). The αCP25 accumulated primarily within the cytoplasm of the germ cells which permitted the counting of the germ cells based on the appearance of clear nuclei within the cluster of germ cells (Fig 1B). Typically, 28 primary germ cells were observed within each ovary of first instar larvae as determined from the reconstructions of the serial optical sections through the immuno-fluorescently stained gonads.

At the end of the first instar, there were still the same numbers of αCP25 staining cells within each ovary, which indicted that the germ cells had not undergone any mitotic divisions (Fig. 2). However, the germ cells were being segregated into four separate groups as grooves appeared in the cluster of primary germ cells leaving approximately seven cells per cluster (Fig. 2). The segregation may be the result of somatic cell activities because there were no visible cell processes extending from the germ cells which would suggest migration by these cells.

In early second instar larvae, the segregation of the primary germ cells was complete and the ovary were divided into four major lobes (Fig. 3). There was an increase in the number of germ cells found within the ovaries, which indicated that the primary germ cells had initiated mitotic divisions. By the end of the second

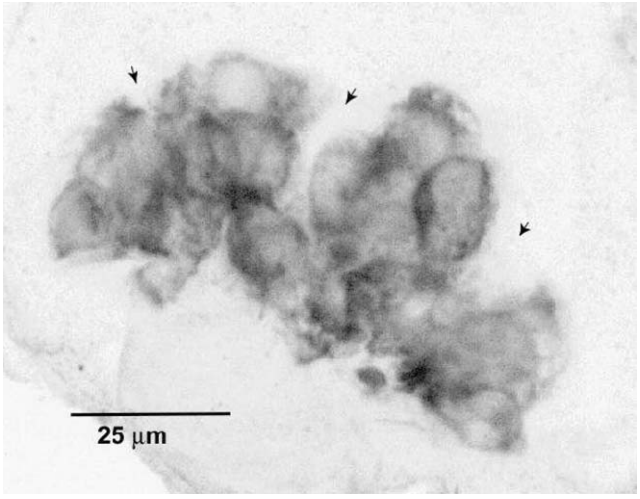

Fig. 2. An ovary from a late first instar *Plodia* larva. Somatic tissue segregation of the founding germ cells into separate ovarioles was apparent (marked by arrowheads). The confocal image is a compilation of optical sections through the specimen.

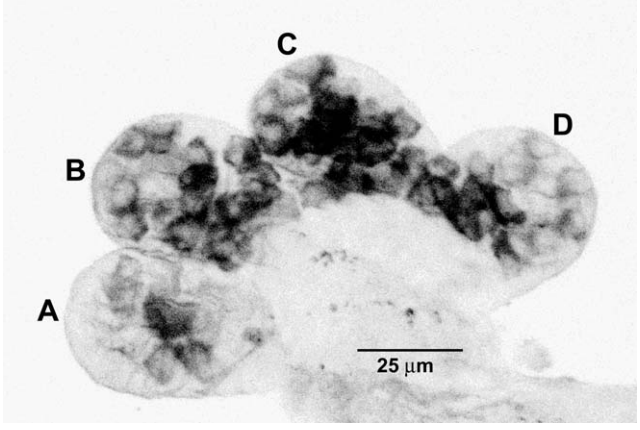

Fig. 3. A gonad (ovary) from an early second instar *Plodia* larva. Segregation of the ovary into four ovarioles (A, B, C and D) was complete and the number of cystoblasts had increased from the earlier stages. The confocal image is a compilation of optical sections through the specimen.

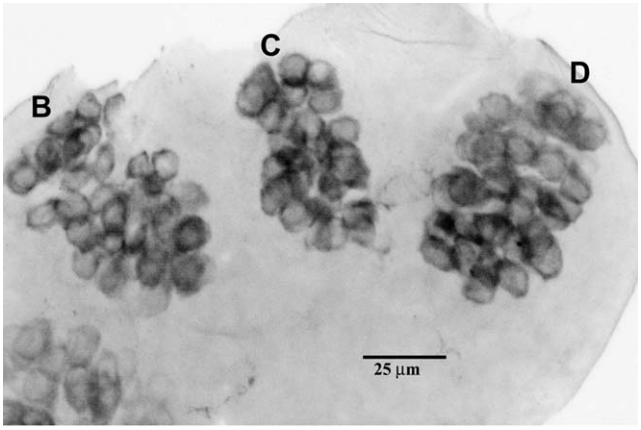

Fig. 4. An ovary from a late second instar *Plodia* larva. The three visible ovarioles were labeled B, C and D. The number of cystoblasts has increased from the early second instar. The cystoblasts become organized within the ovarioles with a separation between the distal-most cluster of germ cells and the other germ cells present in each ovariole. The confocal image is a compilation of optical sections through the specimen.

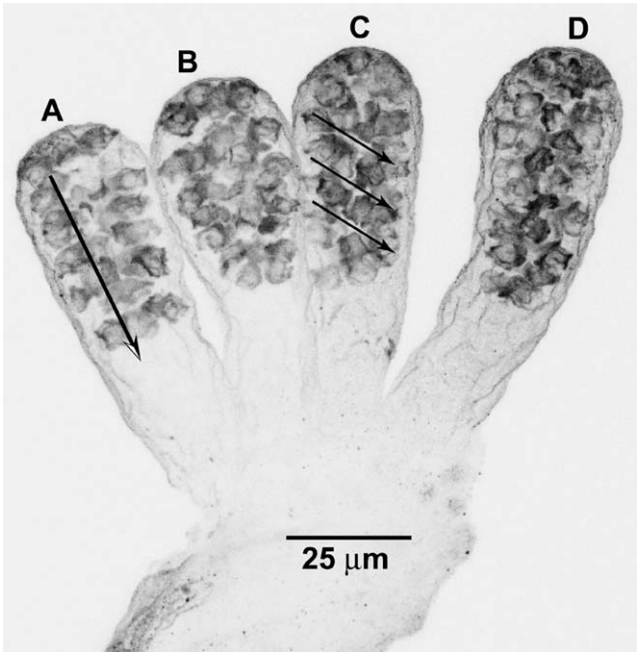

Fig. 5. An ovary from a late third instar *Plodia* larva. The number of cystoblasts had increased and the cells showed three levels of organization within the ovarioles. The confocal image is a compilation of half the optical sections through the specimen. The separation between the distal cluster of germ cells and the other germ cells within each ovariole is maintained. In ovariole A, a central space can be observed that does not contain immuno-fluorescently stained germ cells (paralleled by the half-open headed arrow). In ovariole C, a diagonal alignment of the germ cells can be seen (paralleled by the three arrows).

larval instar, the distinctive morphology of the ovarioles was apparent. There was also a greater level of organization within the four ovarioles with a ring of seven germ cells at the most anterior region of the ovariole with a second cluster with more cells aligned along and around a central axis (Fig. 4).

At the end of the third (penultimate) instar, the four ovarioles were clearly structurally defined within the ovary (Fig. 5). Very distinct morphological arrangements of the germ cells within the ovarioles were also apparent. A ring of seven cells was located at the most anterior region of each ovariole as observed in the late second instar. In the more posterior region of the ovarioles, there were large numbers of germ cells that were arranged around a central core. There were approximately 55–57 germ cells in the posterior cluster in each ovariole. The central region did not contain αCP25 antigen (Fig. 6A, ovariole C), which demonstrates that the central core of the ovariole is not occupied by germ cells. The nature of the material within the central core was not determined in this study. It was most notable that the germ cells were arranged in a spiral around the central core (Fig. 5, ovariole C). The spiral arrangement of germ cells was confirmed by the construction of a physical model.

Ovaries in newly molted white pupae, i.e., before sclerotization and melanization of the cuticle, had many more germ cells per ovariole than were present at the end of the previous instar (Fig. 7). The length of the ovarioles increased over threefold from 100 μM in the late third instar to over 350 μM. An overall spiral organization of germ cell clusters continues to be observed before could not be discerned. The most posterior terminal clusters of germ cells, shown in the

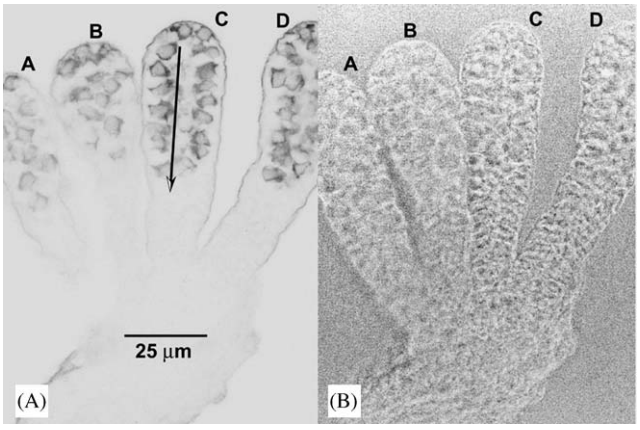

Fig. 6. An ovary from a late third instar *Plodia* larva. The image in panel A is a single confocal optical section from the ovary. The image in panel B is the same section viewed with transmitted light. The non-staining central area of the ovarioles can be seen in ovariole C (paralleled by the half-open headed arrow).

arrowed expanded insert, had eight cells per cluster. Such a cluster of eight cells is indicative of a group of cystocytes that has completed the three cystocyte divisions which represent the germ cell component of a

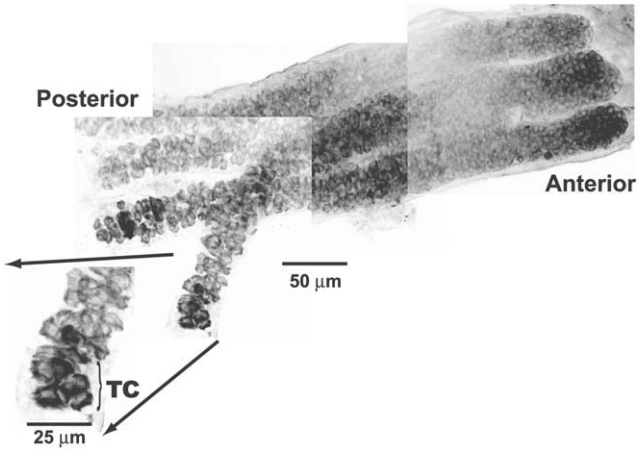

Fig. 7. An ovary from a newly pupated *Plodia*. The image is a reassembly of three scanning regions that covered the entirety of the ovary. Each region is the compilation of the optical sections through the region. The arrows show a projection of the most posterior or terminal clusters of cystocytes (TC).

follicle. Each cystocyte cluster was related to the other clusters in a spiral fashion.

4. Discussion

The morphogenesis of the ovary in *P. interpunctella* follows a developmental path somewhat different both temporally and structurally from that observed in the fruitfly, *Drosophila melanogaster*. At the completion of embryogenesis of *Drosophila*, each gonad contains from 8 to 32 germ cell primorida (Demerec, 1950). During the larval stages, the germ cells undergo limited mitotic divisions but do not become organized within ovarioles until metamorphosis (King et al, 1968; King, 1970). At around 12 h after ecdysis to the third larval instar, formation of the terminal filaments begins under the influence of the *bab* gene and continues until pupariation (Godt and Laski, 1995). Just after pupariation, from 13–20 terminal filaments are present in the ovary and it is these structures that establish the position of the ovarioles.

Subsequently, two to three primary germ cells become associated with each of the terminal filaments (Wieschaus and Szabad, 1979). Division of the primary germ cells results in the formation of the daughter cystoblasts which are contained within the germarium. The cystoblasts undergo four divisions with incomplete cytokinesis, which results in a cluster of 16 cystocytes that share intercellular connections. The cystocyte cluster is then surrounded by follicle cells to complete the follicular structure for maturing the oocyte. Rapid formation of the follicles begins after the peak of juvenile hormone that occurs shortly after eclosion and continues throughout most of the adult stage when sufficient food resources are available (Bownes, 1986).

Unlike *Drosophila*, *P. interpunctella* has a short-lived adult stage (approximately 5–7 days) which requires that the female complete much of oocyte development before eclosion of the adult. From this study, we found that the paired ovaries in the first instar larvae of *P. interpunctella* each contain a cluster of approximately 28 primary germ cells. By the end of the first instar, the germ cells within the gonads of *P. interpunctella* were segregated into four group of cells. The segregation of the germ cells is most likely directed by somatic cells. The germ cells did not show any characteristic irregular cellular shapes or processes that are associated with cell mobility at the time when the segregation occurred. Whether the developmental organization of the ovarioles is dependent on the activity of the *bab* gene as in *Drosophila* (Godt and Laski, 1995) or is dependent upon another mechanism has not been determined at this time. Once the four ovarioles are formed, the number of germ cells increased in the ovarioles, demonstrating that the mitotic divisions of the germ cells had begun.

By the end of the second instar, typical ovariole structure could be distinguished. At the anterior tip of each ovariole, there was a ring of seven germ cells that were separated from the other germ cells within the ovariole (see Figs. 4, 5 and 6). Whether these cells are the founding primary germ cells of the ovariole could not be determined in this study. We hypothesize that they are the original primary germ cells and that this structurally defines the germ cell niche (Lin, 2002) where they are dividing to give rise to the population of cystoblasts within the ovarioles.

The morphology of the ovarioles in the late third instar is consistent with this hypothesis. First, there are seven germ cells at the anterior tip of each ovariole separated from the other germ cells. Second, the germ cells are arranged around a central core in a spiral. At the end of the third instar, there are approximately 56 germ cells in each ovary, which would suggest that each of the seven anterior germ cells had undergone eight mitotic divisions to give rise to cystoblasts. Thus, by the end of the third instar all of the divisions for the cystoblasts appear to have taken place, and the ovaries contain the cystoblasts necessary to form all of the follicles/eggs that the female can produce as an adult.

In the fourth larval instar, the number of germ cells in the ovarioles increases dramatically (compare Figs. 6 and 7). This suggests that each cystoblast present at the end of the third instar undergoes three divisions to form the cystocyte clusters during the last (fourth) instar. This is most clearly observed in the posterior portion of each ovariole where the terminal cluster of germ cells is comprised of eight cells (Fig. 7 insert), indicative of an oocyte and seven nurse cell cluster. At the beginning of the pupal stage all of the mitotic divisions of the germ

cells appear to have taken place, and the ovary is prepared to complete follicle formation and vitellogenesis following the initiation of pharate adult development (Zimowska et al., 1991; Shaaya et al., 1993). However, the anterior ring of seven of germ cells was still observed after pupation as seen in Fig. 1A Zimowska et al. (1991), which suggests that there is potential for production of additional follicles even after this stage.

The developmental timing of when the cystocyte clusters are formed occurs even earlier in *B. mori* as cystocyte clusters without the surrounding follicular epithelium were observed in early last instar larvae (Miya et al., 1970). That ovarian development occurs in *B. mori* at earlier stages than in *P. interpunctella* is further supported by the observation that vitellogenesis and choriogenesis are complete for most oocytes before eclosion of the adult in *B. mori* (Legay, 1979) while these are completed just after eclosion in *P. interpunctella* (Zimowska et al., 1995).

Although the follicles in *P. interpunctella* share similar structural features with those in *Drosophila*, there are considerable differences in ovarian, ovariole and germ cell morphogenesis between the two species. Even though the end product, i.e., the follicle, is structurally nearly the same, the production strategy is quite different. In *Drosophila* follicles are produced singularly as resources are available in the adult stage (Bownes, 1986; Drummond-Barbosa and Spradling, 2001), whereas in *P. interpunctella* follicles are mass produced during the larval stages when resources are abundant as an adaptation to the short non-feeding adult stage. The difference in the production strategy results in the completion of the formation of all cystoblasts before initiating the cystocyte divisions in *P. interpunctella*. Mitotic divisions to produce the cystoblasts from primary germ cells appear to occur by the end of the third instar and before the initiation of cystocyte divisions during the fourth instar in *P. interpunctella*, while cystoblast and cystocyte divisions take place continuously and independently of the formation of other follicles throughout the adult stage in *Drosophila*.

The majority of ovarian organogenesis occurs much earlier in development in this moth than it does in *Drosophila*. Segregation of ovarioles begins in the first larval instar of *P. interpunctella* whereas ovariole formation takes place just after pupariation in *Drosophila*. Divisions to produce the majority of the cystocyte nurse cell–oocyte complexes occur by the end of the last larval instar in *Plodia*. Follicle formation and completion of follicle formation begins within the ovarioles concurrent with the initiation of pharate adult development (Zimowska et al., 1991). Female *P. interpunctella* can mate within 2 h after eclosion and choriogenesis of the terminal follicles is complete within 7 h after eclosion allowing the female to begin laying eggs within 8 h after eclosion (Zimowska et al., 1995). On the other hand, adult female *Drosophila* initiate follicle development after eclosion, and begin laying eggs within 48 h after eclosion (Postlethwait and Shirk, 1981), with follicle formation occurring continuously throughout the adult stage in *Drosophila* (Bownes, 1986).

The physiological strategy to produce eggs in *Drosophila* is to form individual follicles as resources are available to the adult. However, in *P. interpunctella*, each stage of the process is completed during the larval stage before initiating the next step so that in the end there are large numbers of follicles ready for fertilization in the adult soon after eclosion. These differences provide an excellent opportunity for comparative studies in determining the pattern of ovarian development in these two orders in Lepidoptera and Diptera.

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