Zoological Studies **64:**13 (2025)

Temporal Profile of Histological Changes During the Ovarian Cycle, Secretory Cycle of the Colleteric Gland, and Molt Cycle of the Mantle Cavity in the Mature Externa of the Parasitic Barnacle *Polyascus planus*

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(Received 22 August 2024 / Accepted 5 March 2025 / Published -- 2025)

Communicated by Benny K.K. Chan

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Rhizocephalans are a group of parasitic barnacles that parasitize other crustaceans. The adult parasite consists of an external reproductive sac (the externa), which is connected by a stalk to a system of ramifying rootlets (the interna) that infiltrates the host. The mature externa of *Polyascus planus* undergoes a cycle, with its external color changes from yellow, transiently to yellowish brown as the embryos are developing inside a brood chamber (the mantle cavity), then to brown, and return again to yellow, upon the peak release of larvae. Hence, this cycle of *P. planus* is called the Yellow-Brown cycle after the distinct changes in color; the mature externa typically cycles 3–4 times before it becomes detached from the host. The objectives of the present study are to establish, based on histological observations, temporal profile of three cycles–the ovarian cycle, secretory cycle of the colleteric gland, and the molt cycle of the mantle cavity–that occur concurrently in the

mature externa of *P. planus* and register the changes in chronological order on the timeline of the Yellow-Brown cycle. First, about 2 days after oviposition (1.7 ± 0.3 days post-oviposition, dpo) during the Yellow stage, secondary vitellogenesis begins – a cohort of early vitellogenic oocytes grows in synchrony with significant and rapid accumulation of yolk bodies inside the developing oocytes. Simultaneously, the follicle and muscle cells undergo large-scale spatial rearrangements. By the time the externa is transitioning from the Yellow to Brown stage (7.7 ± 0.8 dpo), mature follicles with fully developed oocytes tightly enveloped by a single layer of follicle cells are formed and the muscle cells embedded in the inter-follicular tissue. In the colleteric gland, secretory activity of the epithelia begins 3-4 days after oviposition (3.5 ± 0.5 dpo), with the formation of the reticulated inner zone of the ovisac throughout the remainder of the Yellow stage and into the Brown stage, followed by the beginning of the secretion of the outer zone when the externa reaches the mid-Brown stage (9.8 ± 0.5 dpo). Finally, the molt cycle of the mantle cavity is initiated later than the other two cycles, entering early pre-molt (D_1) when apolysis–separation of the cuticle from the underlying epidermis–first becomes visible about 5 days post-molting (4.7 ± 0.3 days post-molting, dpm), and reaches late pre-molt (D_2) with deposition of new cuticle during the transition from the Yellow to Brown stage (8.8 ± 0.8 dpm). By the time the externa returns to the Yellow stage (0 day post-peak release of larvae, dppr), ovarian follicles are rupturing, ovisacs showing signs for imminent detachment, and cuticles in extensive apolysis (very late pre-molt, $D_{3\text{-}4}$). Subsequently, within a span of about three days after the externa reaches the Yellow stage, the mantle cavity molts (1.4 ± 0.2 dppr), followed by ovulation and ovisac detachment (2.5 ± 0.3 dppr) and finally deposition of the ovulated ova (oviposition) (3.7 ± 0.3 dppr) into the mantle cavity. Probable modes of endocrine regulation of these cycles are discussed in detail.

Keywords: Parasitism, Rhizocephala, ovarian maturation, molt cycle, ovisac formation

Citation: Chen HY, Liu HC, Toullec JY, Lee CY. 2025. Temporal profile of histological changes during the ovarian cycle, secretory cycle of the colleteric gland, and molt cycle of the mantle cavity in the mature externa of the parasitic barnacle *Polyascus planus*. Zooll Stud **64:**13.

BACKGROUND

Rhizocephala, an infraclass of Cirripedia, comprises a group of highly evolved and specialized parasitic barnacles; they parasitize other crustaceans, mainly decapods (Walker 2001; Høeg et al. 2020). The parasitic stage begins when female larvae that settle on a potential host inoculate a cluster of embryonic cells or a discrete vermiform body (the vermigon) into the host (Glenner and Høeg 1995; Høeg and Lützen 1995; Glenner et al. 2000; Walker 2001). Studies of very early stages of the inoculated parasitic materials showed that the parasite has differentiated into an epithelium-enclosed tumor containing a mass of cells (the nucleus) (Høeg and Lützen 1995). During the period of internal growth, the epithelium of the initially inoculated parasite develops into a ramifying internal root system, the interna, which serves to absorb and store nutrients from the host (Høeg and Lützen 1995; Glenner 2001; Bresciani and Høeg 2001) and possibly plays important roles in host control with specialized structures of the rootlets invading the nervous system of the host (Lianguzova et al. 2021; Lianguzova et al. 2023; Miroliubov et al. 2020). The nucleus develops into the visceral mass and mantle of the future externa, which will eventually emerge through the integument of the host (Høeg and Lützen 1995). The newly emerged externa is then invaded by male cyprids, which are dwarf males residing in and receiving nourishment from a specialized female tissue (the male receptacle) for sperm production; in many species externae not receiving male implantation in due time will degenerate (Høeg and Lützen 1995; Walker 2001).

It is well documented that rhizocephalans influence the morphology, behavior, and physiology of their hosts (see for review Reinhard 1956; Høeg and Lützen 1995); the fact that various aspects of the host are altered or controlled by rhizocephalan parasitism attests to the point that these barnacles are highly successful in adapting to the parasitic mode of life. These host controls include at least anecdysis of externa-carrying hosts (Reinhard1956; Hartnoll 1967; Lützen 1984; O'Brien and Van Wyk 1985; O'Brien and Skinner 1990; Takahashi and Matsuura 1994; Chen et al. 2022), feminization of male hosts (Veillet and Graf 1959; Hartnoll 1967; Nielsen 1970; Rubiliani et al. 1980; Rubiliani-Durozoi et al. 1980; Kristensen et al. 2012; Waiho et al. 2017; Chen et al. 2022; Toyota et al. 2023), parasitic sterilization (Hartnoll 1967; Nielsen 1970; Rubiliani et al. 1980;

Rubiliani-Durozoi et al. 1980; Fazhan et al. 2020; Chen et al. 2022), suppression of immune responses (Payen et al. 1979; Payen et al. 1981; Bresciani and Høeg 2001;Goddard et al. 2005; Bortolini et al., 2008; Hsiao et al. 2016; Waiho et al. 2017; Rowley et al. 2020), altered metabolic profile (Uglow 1969; Shirley et al. 1986; Powell and Rowley 2008; Hsiao et al. 2016; Waiho et al., 2017). While these observations regarding control of the hosts have been long documented (see Høeg 1995; Høeg and Lützen 1995) and there were studies attempting to address these issues (Andrieux 1969; Zerbib et al. 1975; Andrieux et al. 1976; Chassard-Bouchaud and Hubert 1976; Rubiliani and Payen 1979; Rubiliani-Durozoi et al. 1980; Andrieux et al. 1981; Waiho et al. 2020; Chen et al. 2022; Zatylny-Gaudin et al. 2023), the detailed mechanism underlying these host control has remained obscure.

The externa is the reproductive organ of rhizocephalans, which is typically located on the ventral surface of the abdomen and connected to the interna via a stalk. Anatomical and functional observations of the externa have been described in many species and summarized (see for review Høeg 1995; Høeg and Lützen 1995). Briefly, the externa consists of the visceral mass and an enclosing muscular mantle. It is currently considered that ova are produced and mature in the ovarian tissue of the visceral mass; however, a recent report provides interesting data indicating that female germ cells might be originated from the interna (Nesterenko and Miroliubov, 2022). After being ovulated from the follicles, mature ova enter the ovisac, the secretory product of the paired colleteric glands, and are deposited along with the enveloping ovisac into the mantle cavity where the deposited ova are fertilized by spermatozoa (Lange, 2002). The male germ cells are produced by the male spermatogenic tissue residing inside the male receptacle and released into the mantle cavity through the receptacle ducts. The fertilized ova and ensuing embryos develop inside the mantle cavity until the stage of naupliar larvae, which are then expelled into the water through the mantle opening (see Høeg and Lützen 1995 for review; for more recent studies, Korn et al. 2004; Lützen and Takahashi 2005; Alvarez et al. 2010; Nour Eldeen et al. 2019; Fazhan et al. 2020; Golubinsykaya et al. 2021; Chen et al. 2022; Arbuzova et al., 2023).

In a previous study (Chen et al. 2022) we have, using *Polyascus planus* Boschma, 1933 parasitizing the shore crab *Metopograpsus thukuhar* Owen, 1839 as a study system, described the development of the externa, from newly emerged virgin externa to sexually mature externa. Color

of the externa changes along the development; in particular, the sexually mature externa (the Yellow and Brown stages) cycles several times before it becomes degenerated and detached from the host (Chen et al. 2022). During the cycle of the mature externa, the Yellow-Brown (Y-B) cycle after the distinctive change of the color, molting of the mantle cavity, ovarian maturation, ovulation and oviposition, fertilization and embryogenesis, and larval release occur. Thus, during this reproductive phase of the externa, there are several processes taking place, which would require close and intricate coordination. In this study, the detailed histological changes of three cycles that occur concurrently in the sexually mature externa are described. These include (1) the ovarian cycle, in particular with regard to vitellogenesis and folliculogenesis, (2) the secretory cycle of the colleteric gland, with respect to ovisac formation and detachment, and (3) the molt cycle of the mantle cavity. Using the histological characteristics, each of these cycles is staged according to established criteria and the stages are registered on the developmental time-line of the mature externa. Probable endocrine pathways for the regulation of these cycles are discussed in the context of relevant knowledge of Malacostraca or Cirripedia crustaceans. It is expected that the temporal profile established in this report with stage annotation would serve as a basis for future studies of the mechanism of control and coordination of these cycles.

MATERIALS AND METHODS

Animals and tissue sampling

Collection and rearing of the host animals *Metopograpsus thukuhar* followed those described by Hsiao et al. (2016). Briefly, crabs with sexually mature externa, identifiable by their yellow or brown coloration (Chen et al. 2022), were collected from the Da'an Estuary in Tai Chung, Taiwan. These crabs were kept in seawater tanks with a salinity of 30‰ at a temperature of 25°C ± 2°C under a 10L/14D light cycle with continuous aeration. The animals were monitored twice daily (at 9 a.m. and 5 p.m.), with an additional checkup at 9 p.m. when experimental animals were subjected to closer inspections for externa sampling.

Experimental animals bearing one brown externa were chosen and inspected closely for peak release of larvae (accompanied by a rapid change of the color of the externa from brown to yellow), molting of the mantle cavity (as evidenced by the presence of an extruded exuvia), oviposition (as evidenced by a plump externa, after its once vacated mantle cavity had been filled by deposited ova or eggs), and a change of the color of the externa from yellow to brown. These events occurring along the Y-B cycle (see Fig. 1; Chen et al. 2022) are used as reference time points for histological descriptions. Thus, the day when the peak release of larvae was recorded is taken as 0 day post-peak release (dppr), which is also the beginning of the Yellow stage of the externa. The day when the mantle cavity molted was recorded is taken as 0 day post-molting (dpm). The day when oviposition was recorded is taken as 0 day post-oviposition (dpo). The day when the color of the externa turns brown (the Brown stage) after a one-day transit from yellow was also recorded.

Externae for histological processing were collected, using a pair of sterilized fine scissor (Fine Science Tools, Inc), from animals anesthetized with cold seawater at the following time points: five animals at 0 dppr, two at 1 dppr, two at 2 dppr, two at 3 dppr, five at 0 dpm, three at 0 dpo, and at least 3 animals each day from 1 to 12 dpo. Histological changes of the molt cycle are presented using 0 dppr or 0 dpm as reference points, whereas those of the ovarian cycle and secretory cycle using 0 dppr or 0 dpo as reference points.

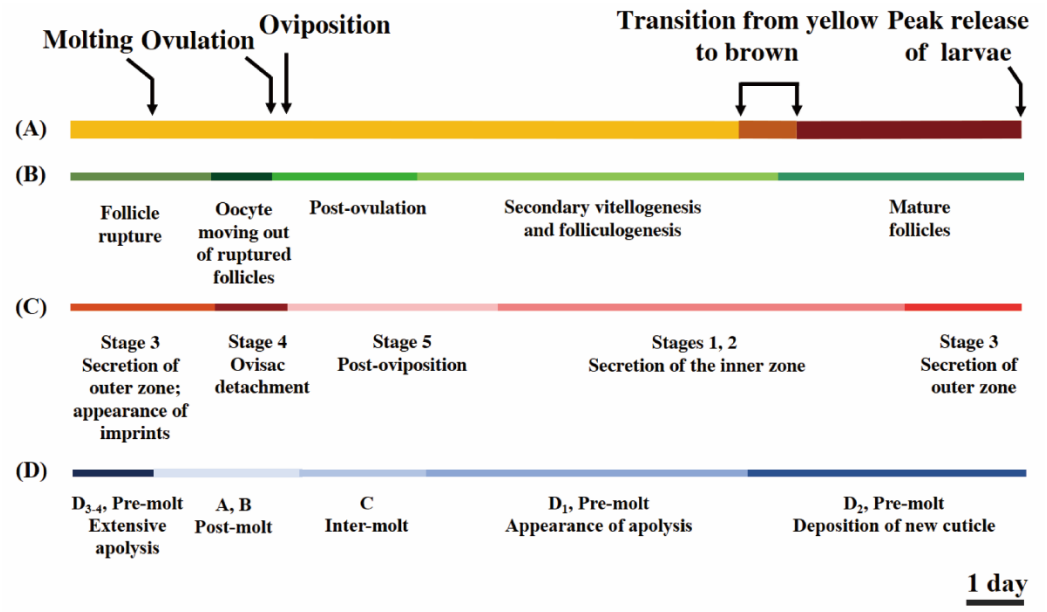

Fig. 1. Progression of three cycles occurring in the mature externa of *Polyascus planus* parasitizing the shore crab *Metopograpsus thukuhar*. During the development of mature externa (A,

the Yellow-Brown cycle), the color of the mature externa changes from yellow, transiently into yellowish brown, then to brown, and returns again to yellow upon the peak release of larvae. Histological changes of (B) the ovarian cycles (see Figs. 3, 4), (C) secretory cycle of the colleteric gland (see Fig. 5), and (D) molt cycle of the mantle cavity (see Fig. 6), are indicated on the timeline of the cycle of the mature externa, in relative to events that can be visualized with unaided eye from the outside of the externa, including molting of the mantle cavity cuticle, oviposition of ova into the mantle cavity, transition of the color of the externa from yellow to brown, and peak release of larvae (which is accompanied by a rapid change of the color of the externa from brown to yellow) (Chen et al. 2022).

Histology, histological observations, and statistical analysis

Tissue processing, sectioning, and staining were performed at the Rapid Science Co. Ltd (Taiwan) as described previously (Chen et al. 2022). Briefly, excised externae were fixed in 10% neutral buffered formalin (NBF) for 48 hours, dehydrated through a graded series of alcohol, embedded in paraffin, sectioned longitudinally at 3 μm, mounted on glass slides, and stained with hematoxylin and eosin Y. Histological observations were performed and images taken using a light microscope (DM 500, Leica) with a software platform Application Suite X (Leica). Histological observations were used to demarcate the period of secondary vitellogenesis and folliculogenesis of the ovarian cycle according to criteria as described by Charniaux-Cotton (1985), and to stage the molt cycle and secretory cycle according to criteria adopted by Skinner (1985) and Lange (2002), respectively (see Fig. 1). Note that indication of ovulation in figure 1 is also based on histological observations.

For the measurement of the diameter of parasite's oocytes, the diameter of 150 most developmentally advanced oocytes in randomly selected visual fields/animal was measured and the mean individual oocyte diameter calculated. Data are expressed as mean values ± S.D. Differences in the oocyte diameter among externae of various developmental stages (*i.e.*, days post-oviposition) were analyzed using one-way ANOVA (with post hoc Tukey’s pairwise comparison) (SigmaStat v. 3.5).

RESULTS

During the reproductive phase of mature externa of *Polyascus planus* parasitizing *Metopograpsus thukuhar*, color of the externa changes from yellow, transiently into yellowish brown, then to brown, and return again to yellow upon the peak release of larvae; mature externa typically cycles 3-4 times before it becomes detached from the host (Fig. 1). Thus, the cycle of the mature externa of *P. planus* is called the Yellow-Brown cycle (the Y-B cycle), after the distinctive color changes of the mature externa of this species (Chen et al. 2022).

Histological changes of three cycles, including the ovarian cycle, secretory cycle of the colleteric gland, and the molt cycle of the mantle cavity, which progress concurrently over a Y-B cycle of the mature externa, are given below and registered on the timeline of the Y-B cycle, in relative to events that are visually identifiable from the outside of the externa (Fig.1). Histological sections of mature externae are presented showing the anatomical structures which are mentioned below (Fig. 2).

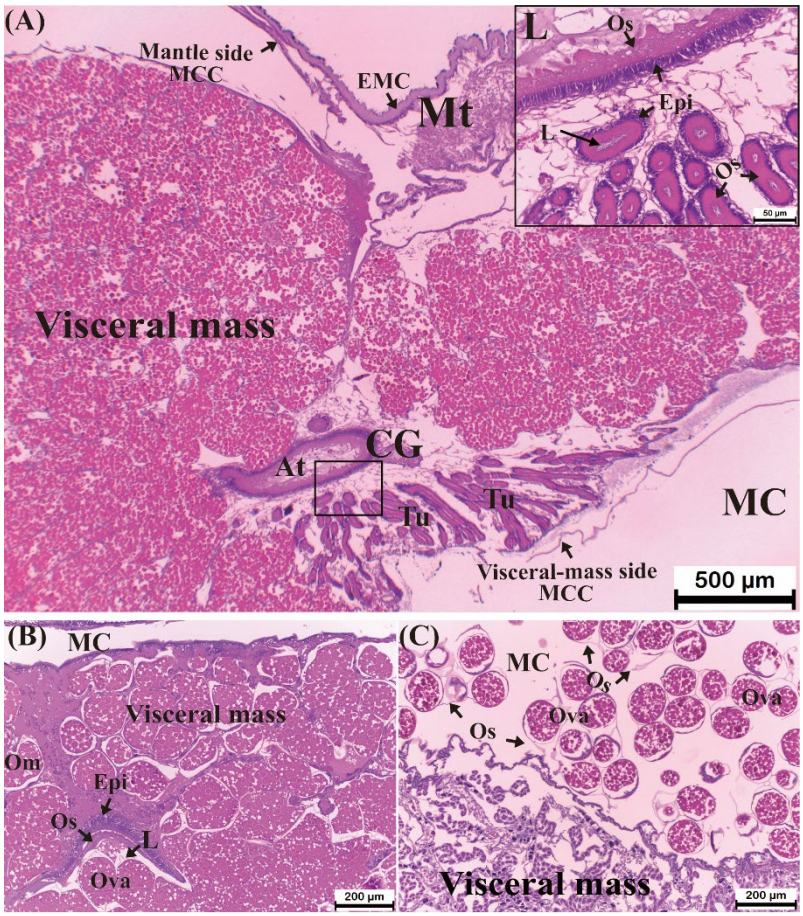

Fig. 2. Histological sections of the mature externa of the *P. planus* highlighting its anatomical structures. (A) A pre-ovipository externa showing the mantle, visceral mass, colleteric gland, and mantle cavity. The mantle cavity cuticle, with a mantle side and a visceral-mass side, and the

external mantle cuticle are pointed by arrows. (Inset in A) An enclosed area of the colleteric gland is magnified showing the detailed structures of the collecteric gland, including the epithelial cells, lumens, and secreted ovisacs of the atrium and branching tubules. (B) A pre-ovipository externa showing that the matured oocytes in the visceral mass are being ovulated and the ovulated ova are entering the lumen of the atrium. Note that the ovisac is partially detached from the secretory epithelium. (C) A post-ovipository externa showing that ova deposited into the mantle cavity are wrapped inside the ovisac. At, atrium; CG, colleteric gland; EMC, external mantle cuticle; Epi, epithelial cell; L, lumen; MC, mantle cavity; MCC, mantle cavity cuticle; Mt, mantle; Om, mature oocyte; Os, ovisac; Tu, tubule.

The ovarian cycle: vitellogenesis, folliculogenesis, and ovulation

Two major ovarian events occur in the visceral mass during the cycle: development and maturation of a new batch of follicles and ovulation of the mature follicles (Figs. 1, 2).

Shortly after oviposition (see below), development of a new cohort of follicles commences, which lasts throughout the remainder of the Yellow stage. On the day when oviposition occurs (i.e., 0 day post-oviposition, 0 dpo), the ovarian lobules, which was once stretched by voluminous mature follicles, now become slackened, clearly revealing the presence of oogonia, pre-vitellogenic oocytes (with a strongly hematoxylin-stained cytoplasm), early vitellogenic oocytes (with a hematoxylin-stained cytoplasm containing a few yolk bodies), and follicle cells within the lobules (Fig. 3A); distribution of these germ cells of various developmental stages and follicle cells in the lobules appears random without regional demarcation according to the type or development stage of the cells (Fig. 3A). Clusters of muscle cells, which were once found in the tissues among individual mature follicles before oviposition, now scatter around the slackened ovarian lobules (Fig. 3A). One to two day after oviposition (1.7 ± 0.3 dpo; $n = 6$), secondary vitellogenesis begins to progress in synchrony so that in the next few days, the early vitellogenic oocytes, as well as their yolk bodies, become increasingly larger in size (Fig. 3B-E). Simultaneously, follicle cells migrate towards and begin to envelope the maturing oocytes; the clusters of muscle cells become disintegrating and these muscle cells are found filling the space among the developing follicles (Fig. 3B–E). About the time when the externa is transitioning from Yellow stage to Brown stage (7.7 ± 0.8 dpo; $n = 3$), the ovarian lobules have contained tightly packed follicles, with individual mature oocytes being enclosed by a thin layer of follicle cells; tissues of elongated muscle cells are tightly embedded in

the inter-follicular tissue (Fig. 3F). Over a period of nine days, from 0 to 8 dpo, the mean oocyte diameter increases significantly about 11 folds; the mean diameter does not increase significantly after 8 dpo (Fig. S1).

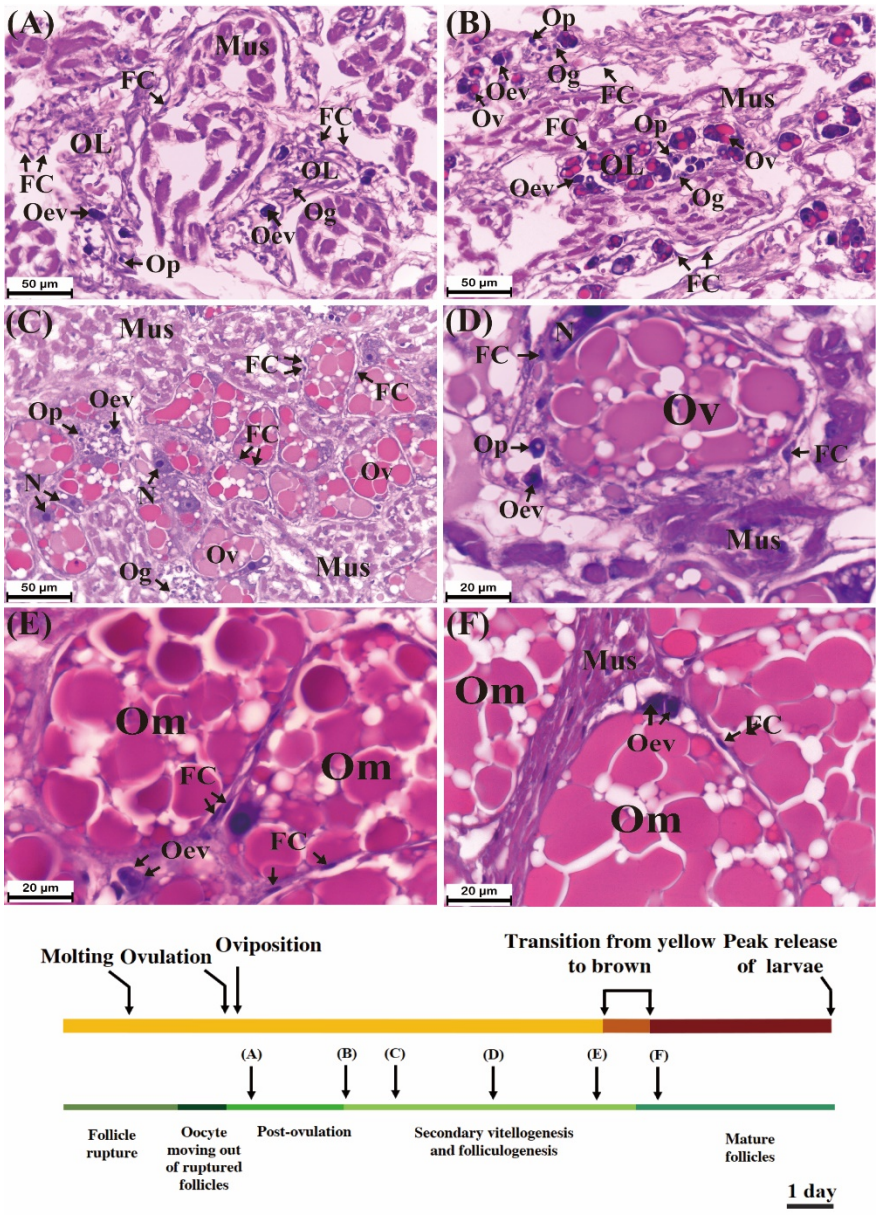

Fig. 3. Histological profile of the ovarian cycle in the mature externa of *P. planus* I. Representative images of the ovarian tissues highlighting the process of vitellogenesis and folliculogenesis over the cycle are shown. A, B, C, D, E, and F: 0, 2, 3, 5, 7, and 8 days post-oviposition (dpo), respectively (0 dpo is the day of oviposition). Note that the slackened ovarian lobes (A) become increasingly organized with the follicle cells migrating towards the oocytes and enveloping them as the latter are undergoing secondary vitellogenesis, illustrating the temporal coincidence between the progression of secondary vitellogenesis and folliculogenesis (B–E). Mature follicles are fully formed when the externa is transiting from the Yellow stage to the Brown stage (F). Also note that germ cells of various developmental stages, from oogonia up to early

vitellogenic oocytes, are present throughout the cycle (A–F). FC, follicle cell; Mus, muscle; N, nucleus; Oev, early vitellogenic oocyte; Og, oogonia; OL, ovarian lobe; Om, mature oocyte; Op, previtellogenic oocyte; Ov, vitellogenic oocyte. The lines of the Yellow-Brown cycle and ovarian cycle from Fig. 1 are given below the micrographs. Parenthesized letters above the line of the ovarian cycle point to the time points of the events illustrated in the micrographs.

The process of ovulation, which would be completed within four days after the externa returns to the Yellow stage, becomes visible at the beginning of that stage (Fig. 4A). Indications of the first step of ovulation (i.e. follicle rupture) are now apparent – follicles become clearly separated from each other with space among neighboring follicles readily visible and follicle cells once encircling oocyte are in the process of becoming separated from it (Fig. 4B). Shortly afterwards (2.5 ± 0.3 days post-peak release (dppr) of larvae; $n = 4$), many mature oocytes are moving out the ruptured follicles (Fig. 4C), which is the second step of ovulation. Ovulated ova are found entering the atrium of the colleteric glands (Fig. 4D).

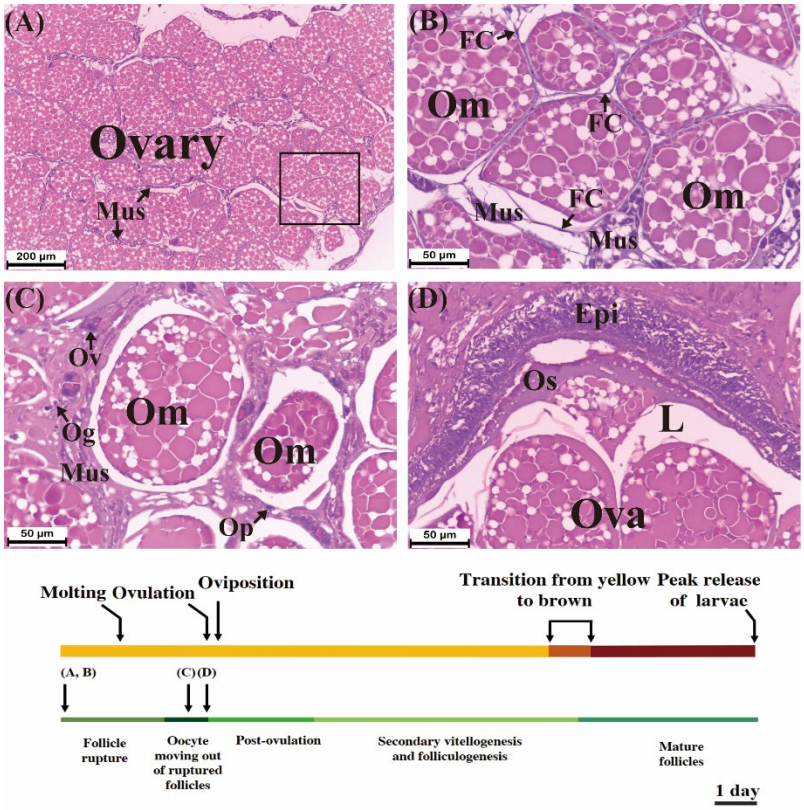

Fig. 4. Histological profile of the ovarian cycle in the mature externa of *P. planus* II. Representative images of the ovarian tissues highlighting the process of ovulation are shown. (A, B, C, D: 0, 0, 3, 3, days post-peak release (dppr) of larvae, respectively (0 dppr is the day when the externa returns to the Yellow stage after the peak release). Note that mature follicles are visibly separated from each other (A) with the follicular cells peeling off from the oocytes (B, enclosed area in A), and that oocytes are moving out of the ruptured follicles (C) and eventually enter the

atrium of the colleteric gland, the ovisac of which is detaching (D). Epi, epithelial cells of the atrium; FC, follicle cell; L, lumen of the atrium; Mus, muscle; Og, oogonia; Om, mature oocyte; Op, previtellogenic oocyte; Os, ovisac; Ov, vitellogenic oocyte. The lines of the Yellow-Brown cycle and ovarian cycle from figure 1 are given here below the micrographs. Parenthesized letters above the line of the ovarian cycle point to the time points of the events illustrated in the micrographs.

The secretory cycle in the colleteric gland: ovisac formation and oviposition

Ovisac is formed by the secretory epithelia of the colleteric gland (Fig. 2). Oviposition occurs three to four days after the externa returns to the Yellow stage (3.7 ± 0.3 dppr, $n = 3$) (Fig. 1). On the day when oviposition occurs, the ovisac is absent from the lumen of the atrium of the colleteric gland; some of the cell debris generated during ovisac detachment can be seen (Fig. 5A). Epithelial secretion typically starts three to four days after oviposition (3.5 ± 0.5 dpo; $n = 2$). Secretion, which is reticulated and called the inner zone, is visible on the apical surfaces of the epithelial cells and continues to grow throughout the rest of the Yellow stage and into the Brown stage (Fig. 5B, C). Approximately the time when the externa reaches the mid-Brown stage (9.8 ± 0.5 dpo; $n = 4$), a thin, dense outer zone of the ovisac appears (Fig. 5D). When the externa returns to the Yellow stage, the outer zone becomes much thicker. Distinctively, hole-like imprints appear in the basal region of the outer zone (Fig. 5E). Approximately three days afterwards (2.5 ± 0.3 dppr; $n = 4$), detachment of the ovisac from the secretory epithelium becomes very prominent (Fig. 5F). Degradation of the cytoplasmic extensions of the epithelial cells is ongoing, generating cellular debris that are found in the space between the ovisac and epithelium; parts of the cytoplasmic extension are still found inserted onto the outer zone (Fig. 5F). Eventually, oviposition occurs, i.e., ova escaped from the ruptured follicles enter the ovisac (see Fig. 4D) and push the detached ovisacs down into the mantle cavity.

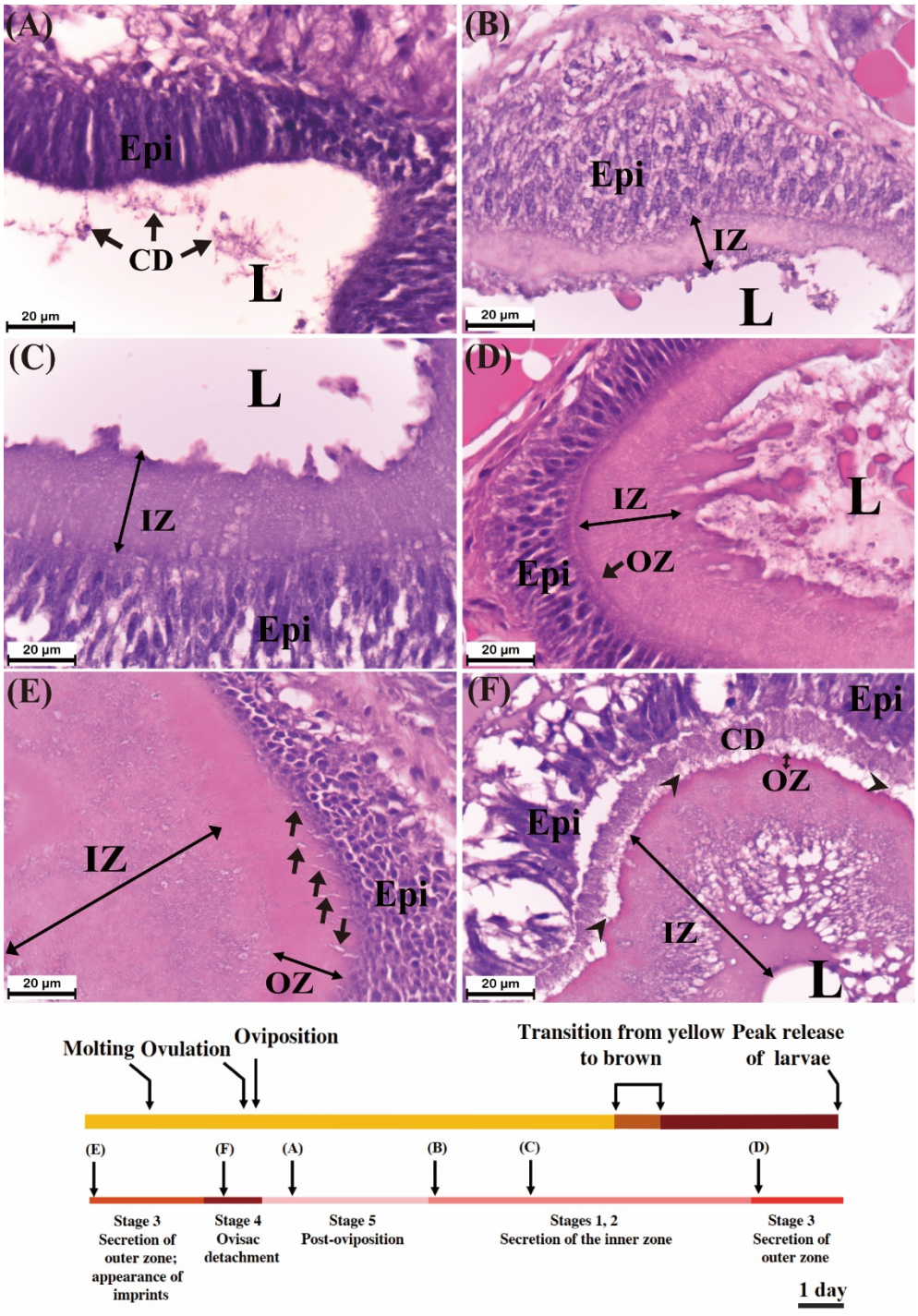

Fig. 5. Histological profile of the secretory cycle of the colleteric gland in the mature externa of *P. planus*. Representative images of the colleteric gland highlighting the process of ovisac formation and detachment. A, B, C, D: 0, 3, 5, 10 days post877 oviposition (dpo), respectively; E, F: 0, 3 days post-peak release (dppr) of larvae, respectively. Note the beginning (B) and continued (C) growth of the inner zone, the appearance of a thin outer zone at the mid-Brown stage (D), the continued growth the outer zone and the appearance of hole-like imprints (E), and the detachment of the ovisac from the epithelium and the presence of cellular debris, derived from degradation of the cytoplasmic extension, between the epithelium and the detaching ovisacs (F). Arrows (↑) in E: hole-like imprints; equilateral arrowhead (➤) in F: the portions of the cytoplasmic extension that are inserted onto the ovisac surface. CD, cellular debris; Epi, epithelial cells; IZ, inner zone; L, lumen;

OZ, outer zone. The lines of the Yellow-Brown cycle and secretory cycle from Fig. 1 are given herebelow the micrographs. Parenthesized letters above the line of the secretory cycle point to the time points of the events illustrated in the micrographs. Histological characteristics are used for stage annotation: Post-oviposition, devoid of ovisac (A), Stages 1, 2, secretion of the inner zone (B, C); Stage 3, secretion of the outer zone (D, E), and Stage 4, ovisac detachment (F).

The molt cycle of the mantle cavity

The mantle cavity is lined by a continuous cuticular layer, the mantle cavity cuticle, with a mantle side (the inner mantle cuticle) and a visceral-mass side (the visceral mass cuticle) (Fig. 2). The mantle cavity cuticle usually molts within two days (1.4 ± 0.2 dppr; $n = 5$) after the externa reaches the Yellow stage (Fig. 1). On the day of molting (*i.e.*, 0 day post-molting, 0 dpm), the cuticular layer overlying the epidermis is relatively thin (Fig. 6A, B). In the next few days, the cuticle becomes increasingly thicker (Fig. 6C, D); approximately 5 dpm (4.7 ± 0.3 dpm; $n = 3$), the sign of apolysis–separation of the cuticle from the underlying epidermis–can be readily seen (Fig. 6E, F). By the time the externa is transitioning from Yellow stage to Brown stage (8.8 ± 0.8 dpm; $n = 4$), a layer of new cuticle has been deposited (Fig. 6G, H); when the externa returns to the Yellow stage (0 dppr; $n = 5$), the extent of apolysis is extensive, occurring in many parts of the mantle cavity (Fig. 6I, J).

The external side of the mantle facing the external environment is also lined by a layer of cuticle (Fig. 2). This cuticle, the external mantle cuticle, never molts during the cycle of mature externa.

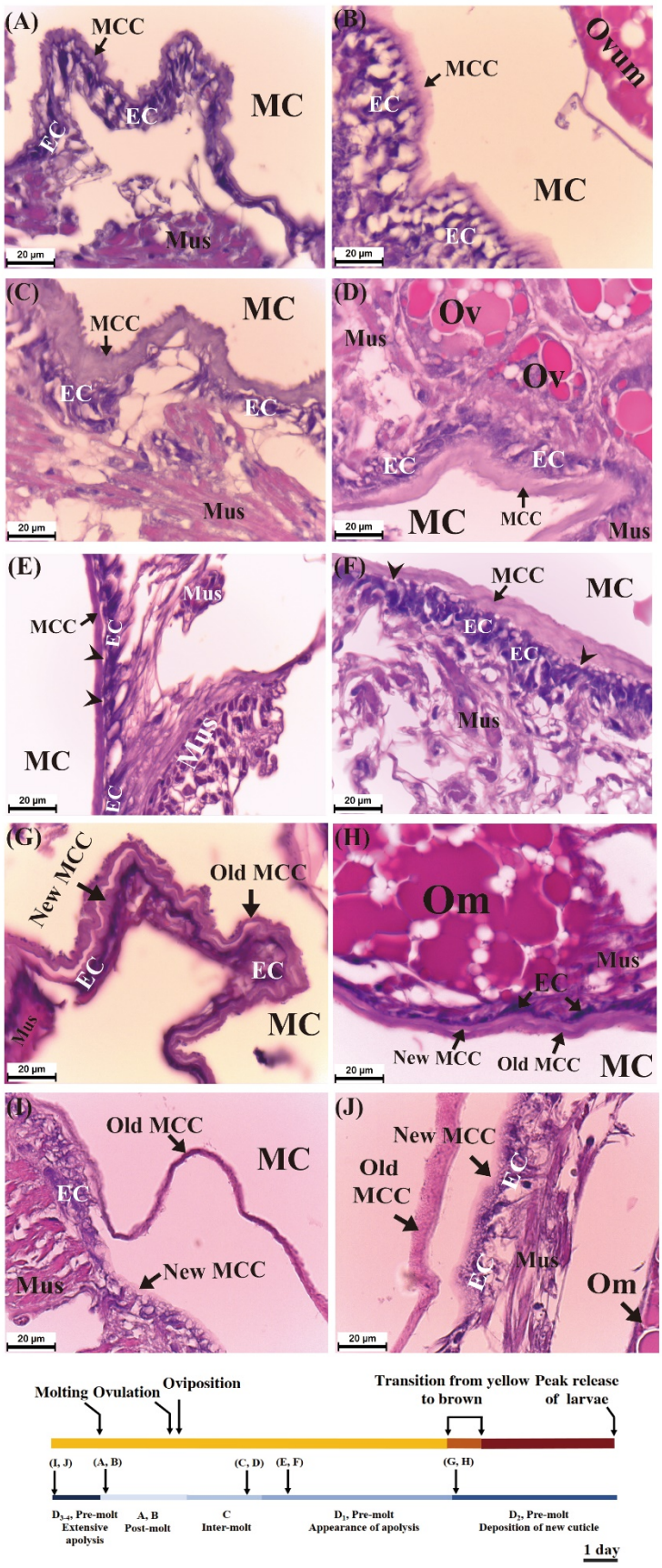

Fig. 6. Histological profile of the molt cycle in the mantle cavity in the mature externa of *P. planus*. (Continued) Representative images of the mantle side (A, C, E, G, I) and the visceral-mass side (B, D, F, H, J) of the mantle cavity highlighting cuticular changes over the cycle are shown. (A,

B), (C, D), (E, F), and (G, H): 0, 4, 5, and 9 days post-molting (dpm), respectively (0 dpm is the day of molting); (I, J): 0 day post-peak release (dppr) of larvae. Equilateral arrowheads in E, F: space between the detached cuticle and epidermis. EC, epidermal cells; MC, mantle cavity; MCC, mantle cavity cuticle; Mus, muscle; Om, mature oocyte; Ov, vitellogenic oocyte. The lines of the Yellow-Brown cycle and molt cycle from figure 1 are given here below the micrographs. Parenthesized letters above the line of the molt cycle point to the time points of the events illustrated in the micrographs. The subdivisions of the pre-molt, D1, D2, and D3-4, are recognized by the occurrence of apolysis (E, F), deposition of new cuticle (G, H), and extensive apolysis indicating that molting is imminent (I, J), respectively. Note that, given histological data currently available, it is not possible to judge when the inter-molt stage begins; thus, the division between the post-molt and inter-molt stage is arbitrarily set. Also note that there is no difference in terms of the stage of cuticular changes between the mantle side and the visceral-mass side (A-J).

DISCUSSION

In this study, we described temporal profile of three individual cycles that occur concurrently in the mature externa of the parasitic barnacle *Polyascus planus* parasitizing the shore crab *Metopograpsus thukuhar*. Histological changes of the ovarian cycle, secretory cycle of the colleteric gland, and molt cycle of the mantle cavity are registered in chronological order on the timeline of the Y-B cycle, in relative to events that can be visualized with unaided eye from the outside of externa (Fig. 1).

It is suggested that the three cycles are intricately coordinated by a network of control pathways, with the cycles being initiated at various time points during the Yellow stage, progressing through the remainder of the stage and the Brown stage so that, when returning to the next Yellow stage, the following events occur consecutively within a span of 3 days – first, the mantle cavity molts, refreshing the brood chamber, then mature follicles ovulate and the ovulated ova are deposited into the mantle cavity to be fertilized (see Fig. 1).

It is further suggested that the presumptive network consists of intertwined control pathways of the host and perhaps also those of the parasite. The notion that physiology of the parasitized host is altered or controlled in many ways by rhizocephalans through manipulating the host's endocrine system to fulfill the requirements of the parasites has often been advocated and widely accepted, although none of the detailed mechanism involved in these manipulations has ever been fully

elucidated (see Høeg 1995; Høeg and Lützen 1995). For example, it has been suggested that induction of vitellogenin synthesis in parasitized males of *M. thukuhar*, a feminized feature, for the ovarian maturation of the *P. planus*, is a result of suppressing the hormonal activity of the host's androgenic gland (Chen et al. 2022); a recent proteomic study of *Carcinus maenas* parasitized by *Sacculina carcini* has suggested that the parasite may inhibit molting and reproduction in parasitized hosts by decreasing the levels of methyl farnesoate, the crustacean equivalent of insect juvenile hormone (Zatylny-Gaudin et al. 2023). It is conceivable that the host's endocrine pathways had been incorporated into the presumed network regulating and coordinating these cycles of *P. planaus* (see below for more specific discussion). On the other hand, there is hardly any evidence supporting the existence of an endocrine system in adult rhizocephalans, which are known for their extremely reduced morphology (see Høeg 1995). However, it is our contention that the lack of relevant evidence probably reflects the scarcity of related study in this regard but not the absence of endogenous neural or endocrine control system in adult rhizocephalans.

Like many other rhizocephalans (see Høeg 1995), the mantle cavity of the mature externa of *P. planus* molts after completing larval release, creating a brood chamber for the forthcoming batch of mature ova (eggs) to be deposited in the chamber, which is now lined by a new layer of cuticle and freed of unfertilized eggs, dead embryos from the previous brood. The cuticular changes of the mantle cavity of *P. planus* (see Figs. 1 and 6) in general agree with those originally described by Drach and adopted by others for staging crustacean molt cycle (Drach 1939; Skinner 1962; Drach and Tchernigovtzeff 1967; Skinner 1985). Endocrine regulation of molting in rhizocephalans remains to be studied. Nonetheless, it has been suggested that the molt cycle of cirripedes is driven by ecdysteroids (Clare 1978), like other groups of arthropods (Lafont 2007; Lafont et al. 2021). The suggestion is based mainly on the combined data derived from works done in several thoracican species. These studies showed that ecdysone and 20-hydroxecdysone (20E) are present in the tissues of *Balanus balanoides* (Bebbington and Morgan 1977), 20E accelerates the molt cycle of *B. balanoides* (Tighe-Ford and Vaile 1972), and ecdysone or 20E stimulates *in vitro* molting activity in isolated tissues of *B. amphitrite* and *B. eburneus* (Cheung and Nigrelli 1974; Freeman and Costlow 1979; Freeman 1980).

Given the assumption that rhizocephalan molting is driven by ecdysteroids, it is interesting to

speculate on where the responsible steroidogenic tissue is located. It is entirely probable that rhizocephalans are capable of producing ecdysteroids that drive the molt cycle of the mantle cavity. However, a steroidogenic tissue has yet to be identified for any cirripedes (see Clare 1978). It is also possible that rhizocephalans had delegated the steroidogenic capacity to their hosts during the course of adapting to a parasitic mode of life. Therefore, an alternative source of ecdysteroids would be from the parasitized host, most likely the Y-organ, the molting gland of decapod crustaceans (see Mykles 2021). However, the notion that parasitized hosts are blocked in an anecdysial status as a result of parasite-induced regression of the Y-organ (hence, its impaired steroidogenic capacity) (Chassard-Bouchaud and Hubert 1976; Zerbib et al. 1975; Andrieux et al. 1976) does not agrees well with the suggestion that the Y-organ is capable of synthesizing ecdysteroids driving the molt cycle of the mantle cavity. Contradicting data nonetheless exist showing that the structural integrity of the Y-organ of externa-bearing hosts could still remain intact (Andrieux 1969) and that there are no significant differences in the levels of ecdysteroids in hemolymph, Y-organ, and integument between unparasitized and parasitized hosts (Andrieux et al. 1981). The contradiction may stem in part from the lack of detailed information in these studies regarding the parasitization status of the host, other than parasitized or not parasitized, with or without externa. To fully address the issue of whether the Y-organ is functional in parasitized hosts, providing more information with respect to the developmental stage of the parasite (*e.g.*, the molt stage of the externa) would be essential.

During the Y-B cycle of the mature externa, the ovary undergoes an ovarian cycle, beginning with the formation of a new batch of mature follicles and ending with ovulation (see Fig. 1). It is noted that oogonia, pre-vitellogenic oocytes, and early vitellogenic oocytes are present in the ovary throughout the cycle (see Fig. 3), suggesting that oogenesis up to the stage of early vitellogenesis is a continuous process. On the other hand, one to two days after oviposition, a batch of early vitellogenic oocytes begins to develop in synchrony and by the time the externa is transitioning to the Brown stage the ovary is packed with fully mature follicles. The different phases of vitellogenesis observed in the mature externa of *P. planaus* resemble those of vitellogenesis in Malacostraca crustaceans, which consists of a primary vitellogenesis and a secondary vitellogenesis (Charniaux-Cotton 1985; Charniaux-Cotton and Payen 1985). The primary vitellogenesis is a

continuous and endogenous process replying on intra-oocyte synthesis of the proteinaceous yolk, whereas the secondary vitellogenesis takes place during reproduction when developing oocytes grow in synchrony into fully mature ones through endocytotic uptake of exogenous yolk protein precursor, vitellogenin, synthesized by extra-ovarian tissues (Meusy 1980; Charniaux-Cotton and Payen 1985; Jaglarz et al. 2014). In this light, it is interesting to point out that vitellogenin synthesized by both host tissues (the hepatopancreas, ovary, or both depending on the sex of the animal) and externa contributes to the formation of the yolk proteins deposited in the ovary (the externa) of *P. planaus* (Chen et al. 2022). Thus, it is probable that, for the ovarian maturation of *P. planus* and perhaps other rhizocephalans, vitellogenin synthesis by the externa (i.e., endogenous synthesis) takes place throughout the ovarian cycle, whereas the synthesis by host tissues (i.e., exogenous synthesis) becomes active during the rapid and synchronous growth of the oocytes, the secondary vitellogenesis.

Studies of vitellogenesis in Malacostraca crustaceans showed that secondary vitellogenesis is under neuroendocrine control (see Meusy 1980; Charniaux-Cotton and Payen 1985; Jaglarz et al. 2014). In decapods, it has been well established that secondary vitellogenesis is negatively regulated by a peptide hormone, vitellogenesis-inhibiting hormone (VIH), which is synthesized and released by a neuroendocrine complex in the eyestalk ganglia, the X-organ/sinus gland (see Charniaux-Cotton and Payen 1985; Chen et al. 2020). In addition, it has long been suggested that vitellogenesis-stimulating factor(s) are present in the central nervous system, in particular the brain and thoracic ganglia; however, identity of any vitellogenesis-stimulating hormone has yet to be determined (see Nagaraju 2011; Jayasankar et al. 2020). Evidence exists that red-pigment concentrating hormone and serotonin function as neurotransmitters in regulating the release of putative vitellogenesis-stimulating factor (Sarojini et al. 1994; Fingerman 1997; Tinikul et al. 2008; Zeng et al. 2016; Chen et al. 2018; Tomy et al. 2016). These vitellogenesis-regulating signals of host origin could be parts of the control network regulating the secondary vitellogenesis in the externa. Recent ultrastructural studies of hosts parasitized by *Peltogaster paguri*, *Peltogasterella gracilis* (Miroliubov et al. 2020), *S. pilosella* (Lianguzova et al. 2021), and *Polyascus polygeneus* (Lianguzova et al. 2023) identified specialized structures in the rootlet-invaded nervous tissues that represent host-parasite interaction sites where the parasites may alter the activity of the host's

nervous system via humoral factors.

In addition to secondary vitellogenesis, there are large-scale tissue rearrangements that occur after oviposition in the ovarian tissue of the externa. The rearrangements involve two somatic cell types, the follicle and muscle cells. The post-ovipository ovarian lobules of *P. planaus* appear not well organized, containing oogonia, pre-vitellogenic oocytes, early vitellogenic oocytes, and follicle cells, which are more or less randomly distributed within the slackened tissue. Folliculogenesis proceeds soon after oviposition; the follicle cells begin migrating towards developing oocytes, which are simultaneously undergoing secondary vitellogenesis. Importantly, the early vitellogenic oocytes, as well as the yolk bodies inside, begin to significantly grow in size when the follicle cells are establishing close contact with the oocytes, (see Fig. 3). The temporal coincidence suggests the importance of the follicle cells for the synchronous phase of secondary vitellogenesis; most likely these cells play the role of aiding the transportation of exogenous vitellogenin into the developing oocytes, similar to what has been confirmed during the secondary vitellogenesis of Malacostraca crustaceans (Charniaux-Cotton 1985). Interestingly, it is considered that the maturation of oocytes in cirripedes does not require the involvement of nurse cells (see Anderson 1994), and a study of the ovaries of two thoracicans (*B. amphitrite* and *B. eburneus*) concluded that there is no indication of the presence of the follicle cells around the oocytes (Fyhn and Costlow 1977). Obviously, whether the presence of ovarian follicle cells is a distinctive feature of parasitic barnacles among cirripedes needs more comparative studies; but the nurturing roles of the follicle cells may be essential for the developing oocytes in rhizocephalans, which rely heavily on resources supplied by their hosts, including vitellogenin (Chen et al. 2022).

While folliculogenesis is taking place, the muscle cells are also being displaced towards the developing oocytes; these muscle cells become elongated and eventually embedded in the inter-follicular tissues at the Brown stage. These ovarian muscle cells are believed to be essential in providing contractile forces to expel mature oocytes into the mantle cavity (Alvarez et al. 2010; Nour Eldeen et al. 2019). The mechanism of ovulation and the regulation thereof receive relatively few studies in crustaceans in general, and in rhizocephalans in particular. Comprehensive review of related studies concludes that the process of ovulation in animals involves at least two steps, first enzymatic degradation and rupture of the follicle, and then the actual movement of the oocyte out of

mature follicle (Schroeder and Talbot 1985). In the lobster *Homarus americanus*, collagenase induces *in vitro* ovulation in mature ovaries, which can be blocked by cysteine, a collagenase inhibitor; the collagenase activity was suggested to be involved in inducing follicle rupture (Talbot 1981a). Furthermore, the muscle cells of the ovarian wall extend into the follicular regions of the ovary; these contractile muscle cells likely play a role of moving the oocytes out of the ruptured follicles (Talbot 1981b).

The secretory product of the colleteric gland is an elastic, transparent sac, which envelop the ovulated eggs as they are deposited into the mantle cavity, where the eggs coalesce into egg masses and are fertilized (Lange 2002). The observations made in the colleteric gland of *P. planus* allow us to stage its secretory cycle (see Figs. 1, 5), including formation of the inner and outer zone, and ovisac detachment, according to those established by an ultrastructural study of the gland of *S. carcini* and *Heterosaccus dollfusi* (Lange 2002). Notably, there are distinctive hole-like imprints found in the outer zone of the ovisac in *P. planus* immediately before the ovisac begins detaching from the secretory epithelium. The rhizocephalan ovisacs resemble in many ways the ovisac formed by the oviductal gland of thoracicans (see Lange 2002). Studies of the ovisacs of both groups showed that cytoplasmic extensions of the epithelial cells make imprints in the outer zone of the sacs, and that ovisac detachment involves degradation of the cytoplasmic extension, leaving cellular debris in the space between the epithelium and detached ovisac (Walker 1980; Lange 2002); subsequent degradation of the portions of the cytoplasmic extension left inside the imprints creates holes on the surface of the ovisac, which are to be used as passages for spermatozoa to pass through the ovisac and fertilize the ova inside (Walker 1977; Klepal et al. 1977; Walker 1980). Therefore, the hole-like imprints we observed in the ovisac of *P. planus* are closely related to the process of ovisac detachment and could be taken as a histological marker forecasting imminent ovisac detachment and oviposition. Knowledge concerning the regulation of the secretory activity of the colleteric/oviductal gland and oviposition in cirripedes is scarce, except several studies revealed that environmental factors, *e.g.*, photoperiod, food availability, and temperature, affect ovisac formation and oviposition in *S. balanoides* (Walley 1965; Crisp and Lewis 1984; and see Clare 1987), the effects of which are most likely to be transduced by neuroendocrine signals. In this respect, perhaps one should look for neuroendocrine pathways of the parasite, rather than that of the host's, as

decapod crustaceans do not possess a pair of secretory glands homologous to the cirripede oviductal/colleteric glands.

CONCLUSIONS

The parasitism and the interaction between rhizocephalans and their decapod host present intriguing opportunities as well as challenging obstacles for interested biologists and physiologists wishing to understand the underlying mechanism of host control manipulated by these parasitic barnacles. In the mature externa of *P. planus*, occurrence of molting, ovulation, oviposition in an orderly manner within a short span of 3 days, which is essential for successful fertilization and hence eventual production of naupliar larvae, strongly suggests that these cycles are highly regulated and intricately coordinated. The temporal profile of these cycles described here would serve as a basis for studies of detailed mechanism underlying the interaction between *P. planaus* and its parasitized host during the reproductive phase of the parasite.

Acknowledgments: The authors thank Ms. Pei-Chen Tsai, Ms. Yu-Hsuan Chen, Ms. River Min Sung, Mr. Yao-Feng, Tsao of the Biodiversity Research Center, Academia Sinica for assistance in animal collection. This research was funded by the National Science and Technology Council (NSTC), Taiwan (111-2311-B-018-001 and 112- 2311-B-018-001 to C-YL; post-doctoral fellowship was provided to HYC under grants 111-2811-B-018-002 and 112-2811-B-018-001).

Authors' contributions: HYC, investigation, data analysis, resources; YC, data analysis; HCL, resource acquisition, comments on the manuscript; JYT, resource acquisition, comments on the manuscript; CY, funding acquisition, data analysis, writing.

Competing interests: The authors have no competing interests.

Availability of data and materials: The data that support the findings of this study are available on request from the corresponding author, CYL.

Consent for publication: All authors contributed to the article and approved the submitted version.

Ethics approval consent to participate: The animal study was reviewed and approved by Experimental protocols involving animals employed by the present study were approved by the Review Committee of National Changhua University of Education, in full accordance with the recommendations (Guidelines for Management and Use of Experimental Animals) set by the Council of Agriculture, Taiwan.

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Supplementary materials

Fig. S1. Changes in the oocyte diameter during the ovarian cycle in the mature externa of *P. planus*. *** indicates significant differences from the mean oocyte diameter at 0 day post-oviposition ($p < 0.001$, Tukey's pairwise comparison; $n = 3$ for each group). (download)