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Original article

Ovariole structure and oogenesis in queens and workers of the stingless bee *Melipona quadrifasciata* (Hymenoptera: Apidae, Meliponini) kept under different social conditions*

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Abstract – The high variability in the reproductive biology of stingless bees makes them very amenable for comparative studies with other eusocial bee taxa. We investigated the structural organization of the ovaries of *Melipona quadrifasciata* queens and workers kept under different social conditions by analyzing their general histology, mitotic activity, and microfilament organization. The overall dynamics of ovarian activity were similar in the two castes, and at emergence their ovarioles contained a previtellogenic follicle. Stingless bees and honey bees differ in the structural organization in the lower germarium, but they have in common synchronized mitotic activity and putative germ line stem cells in the terminal filament. Unlike honey bees, stingless bee workers lay trophic eggs in addition to reproductive eggs. The overall similarities in oogenesis between the two taxa suggest that the decision to form trophic eggs should only occur in the late stages of oogenesis.

worker reproduction / follicle development / actin cytoskeleton / ovary histology

1. INTRODUCTION

Whether stingless bees (Meliponini) and honey bees (Apini) are sister taxa within the corbiculate bees is still a matter of debate (for an up-to-date overview of phylogenetic hypothesis see Kawakita et al., 2008). It is, however, unquestionable that these two taxa have reached the apex in the evolution of sociality in the Apidae. It is equally unquestionable that the honey bee, *Apis mellifera* L., is one the best studied insect species, but there are difficulties for understanding the evolution of sociality in the Apini because this tribe (Michener, 2000) is composed of a single genus of ten species, all of which show many derived features within the corbiculate bees (such as extreme levels of polyandry, extreme differences in ovariole number between queens and workers, progressive feeding of the larvae on a diet of primarily glandular origin, and vertical combs and buzzing dances; Winston, 1987).

Comparative studies within stingless bees and with honey bees have a long history and are increasing in frequency (for reviews see Michener, 1974; Sakagami, 1982; Nogueira-Neto, 1997; Roubik, 2006; Rasmussen and Camargo, 2008) Many of these studies are putting emphasis on the reproductive process, especially the role of the cell provisioning/oviposition process (POP) in colony integration (Sakagami, 1982; Zucchi et al., 1999; Cepeda, 2006) and on differential reproductive activity of queens and workers (Engels and Imperatriz-Fonseca,

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1990; Hartfelder et al., 2006). Since differential reproduction lies at the core of insect sociality, understanding oogenesis and its intrinsic control mechanisms in the castes becomes a crucial factor. Theoretically, queens should either manipulate workers to forego reproduction, or the workers should accept the superior reproductive potential of the queen as a fact in their own interest and, supposedly, such decisions should ultimately be contingent on mating systems and genetic relationships between colony members. Surprisingly, however, stingless bees are a bazaar of diversity in worker reproductive options, despite very minor differentiation in their mating systems (Peters et al., 1999; Toth et al., 2004; Velthuis et al., 2005). This variability makes stingless bees, and especially their reproductive system biology, an ideal object for comparative studies, including comparisons with the "eccentric" honey bees.

In the genus *Apis*, the main morphological difference between the queen and worker caste resides within the ovary. In queen ovaries, the number of serial functional units, the ovarioles, varies between 180 and 200 per ovary, whereas the standard number in workers is around 2–12 ovarioles per ovary (Snodgrass, 1956). The reduced number of ovarioles in workers is the outcome of an autophagic cell-death process taking place during the fifth larval instar (Hartfelder and Steinbrück, 1997). Crucial endogenous components of this caste divergence are the juvenile hormone titer (Schmidt Capella and Hartfelder, 1998), TOR signaling (Patel et al., 2007), and the genome-methylation status (Kucharski et al., 2008), superposed on or acting in concert with genotype determinants for worker ovariole number (Amdam et al., 2006; Makert et al., 2006). Except for the anarchistic mutant phenotype (Montague and Oldroyd, 1998), workers are functionally sterile and activate their ovaries only when a colony has lost its queen (Winston, 1987). Under such conditions, oogenesis in the worker ovary follows the same pathway as in queens (Tanaka and Hartfelder, 2004).

In contrast to the honey bee, and as in most stingless bees, queens and workers of *Melipona quadrifasciata* have four ovarioles in each ovary (Cruz-Landim, 2000). In queens, these ovarioles are more elongated than in workers. As they become active egg layers, the ovarioles extend enormously in length under progressive oogenesis activity, causing the abdomen to swell and the queen to become physogastric (Sakagami, 1982). Quite different from the honey bees, *Melipona* workers at a certain age are active egg layers in the presence of the queen (Engels and Engels, 1977; Engels and Imperatriz-Fonseca, 1990; Cepeda, 2006).

In Meliponini, these workers can produce two types of eggs: trophic eggs and reproductive eggs. This phenomenon is frequently observed in various species of stingless bees, and in the case of reproductive eggs, these may make a considerable contribution to a colony's male production (Beig, 1972; Machado et al., 1984; Peters et al., 1999; Drummond et al., 2000; Paxton et al., 2003; Toth et al., 2004). Workers laying reproductive eggs usually do this right after the queen has laid her egg and while sealing the brood cell (Engels and Imperatriz-Fonseca, 1990; Koedam et al., 2007). Trophic eggs, in contrast, are laid as the queen inspects provisioned brood cells right before she lays her egg. They differ from reproductive eggs in size and shape (Koedam et al., 1996) and they have been considered as more immature than reproductive ones (Cruz-Landim and Cruz Höfling, 1971).

While worker reproduction – including almost simultaneous production of trophic and reproductive eggs – seems to be the most commonly followed strategy in stingless bees, workers of some species do not oviposit in the presence of the queen even to supply trophic eggs to the queen (for review see Hartfelder et al., 2006). In *Frieseomelitta varia*, worker reproduction is even brought to a complete halt, as the ovary primordia in the worker caste undergo complete degeneration during metamorphosis (Boleli et al., 1999).

How oogenesis becomes turned on in queens once they have mated (Melo et al., 2001) and how it proceeds in egg-laying queens and reproductive workers is therefore a major issue in stingless bee sociobiology. Using a comparative analysis with the dynamics of the processes occurring in honey

bees, we attempt to shed light on the common principles and differences between the two major groups of highly eusocial bees. In this study, we start out with a general histological analysis of *Melipona quadrifasciata* ovarioles dissected from virgin queens, egg-laying queens, and workers of different ages and reproductive status. For a better understanding of the dynamics of oogenesis, especially its control at the early stages of follicle formation, we also performed BrdU-labeling experiments to detect mitotic cells and labeling of the actin cytoskeleton by TRITC-phalloidin for fluorescence microscopy.

2. MATERIALS AND METHODS

2.1. Bees

Queens and workers were collected from colonies of *Melipona quadrifasciata anthidioides* Lepeletier kept at the Departamento de Genética, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Brasil. To obtain queens and workers of known age, brood combs containing older pupae were retrieved from the colonies and transferred to an incubator (28 °C, 80% r.h.). Ten newly emerged workers were dissected and their ovaries were fixed immediately (see below). Other newly emerged workers were paint-marked and then kept under different social conditions. A first set of workers was introduced into an observation colony headed by an egg-laying queen. These workers were then collected as they either reached a definite age (5, 7, 10, 15, 20, or 30 days) or when they were behaviorally identified as either nurses or foragers.

The second set of workers was kept under queenless conditions. In this setup, groups of 10 workers were established in Petri dishes and were kept in an incubator (28 °C, 80% r.h.) until reaching a defined age (5, 7, or 10 days). In the Petri dishes, they were supplied daily with a sucrose solution, a fermented pollen mixture (Silva and Zucoloto, 1990), and water.

Newly emerged queens were collected and were kept individually in Petri dishes, each receiving five young hive bees as companions. The queens were also collected for ovary analysis when they were 5, 7, or 10 days old. Egg-laying, physogastric queens were obtained from normally managed colonies.

2.2. General histology

Ovaries were dissected in honey bee saline, and the ovarioles were individualized in honey bee culture medium (Rachinsky and Hartfelder, 1998) before tracheae and the peritoneal sheath were manually removed as far as possible with the aid of number 5 watchmaker forceps (Dumont). The individualized ovarioles were fixed for 2 h in a glutaraldehyde/paraformaldehyde (2%/2%) mix in cacodylate buffer (50 mM, pH 7.4). Subsequently, they were postfixed for 15–30 min in osmium tetroxide (0.5%) to facilitate their visualization during embedding and sectioning. After dehydration in an ascending ethanol series, they were embedded in methacrylate resin (Historesin, Leica). Sections of 2–3 μm thickness were stained with methylene blue/basic fuchsin, as previously described (Hartfelder and Steinbrück, 1997).

2.3. TRITC-Phalloidin staining of actin

For actin visualization, ovarioles were dissected as described above, fixed for 2 h at 4 °C in 4% PBS-buffered paraformaldehyde, and rinsed several times in PBS (10 mM phosphate buffer pH 7.4, 0.9% NaCl) before permeabilization for 5 min in PBS-T (10 mM PBS plus 0.2% Triton X-100). Subsequently, the ovarioles were incubated for 24 h at 4 °C in TRITC-phalloidin (Sigma) at a final dilution of 1 mg/mL in PBS. The stained and rinsed ovarioles were embedded in glycerol-propylgalate (90% glycerol, 10 mM PBS, 0.1% sodium azide, 3% n-propylgalate). The whole-mount ovary preparations were analyzed by conventional epifluorescence microscopy in an Axioskop II (Zeiss) before selected ovarioles were documented on a laser confocal system (Leica TCS SP2-SE).

2.4. BrdU immunocytochemistry

Ovaries were rapidly dissected in honey bee saline and then immediately incubated for 3 h at 34 °C in 5-bromo-2'deoxy-uridine (BrdU) labeling reagent (Roche) at a final concentration of 50 mM in honey bee tissue culture medium (Rachinsky and Hartfelder, 1998) supplemented with 0.1% DMSO. After two brief rinses in fresh medium, the ovarioles were individualized and cleared of trachea and peritonial sheath before fixation (overnight at 4 °C) in acidic alcohol (70% ethanol in 50 mM

glycine buffer, pH 2.0). Detection of cells that were in S-phase during the incubation period was performed with a BrdU immunocytochemistry kit (HCS24, Oncogene Research products), which consists of a biotinylated-BrdU-antibody/streptavidine-peroxidase system with DAB as substrate. The stained ovarioles were mounted in glycerol for differential interference contrast (DIC) microscopy in an Axioskop II system (Zeiss).

3. RESULTS

3.1. Oogenesis in *Melipona quadrifasciata* queens and workers – histological analysis

Even as they emerged from the brood cells, both castes had ovarioles showing a clear separation between the germarial region and the region of follicular growth. In the germarium, all of the early stages of oogenesis were identifiable, starting with the formation of cystocyte clusters in the apical region (Fig. 1A; apical is to left) to initial follicle formation in the region where the germarium widens and forms a transition zone to the region of previtellogenic follicles (Fig. 1A). As is characteristic for this stage, the ovarioles of workers contained a single basal previtellogenic follicle in each ovariole.

In older workers, these basal follicles became vitellogenic. Initial stages of vitellogenesis were observed in 5 day-old workers kept in the absence of a queen (Fig. 1B), and vitellogenesis continued independent of whether a queen was present or not, as seen in 10 day-old workers kept in the presence of the queen (Fig. 1C) and in nurse workers of undetermined age collected from an observation colony (Fig. 1D). With progressing vitellogenesis, differentiation processes were seen in the nurse chamber, where intercellular bridges were visible between nurse cells (arrows in Fig. 1C), followed by a change of the rounded nucleus to a more irregular shape (Fig. 1D). Fully grown follicles were observed in 30 day-old workers kept in the presence of a queen (Fig. 1E).

Programmed cell death (PCD) was first detected within ovarioles of 15 day-old workers collected from the observation colony. Isolated PCD events could be seen in the germarium and more strongly in previtellogenic follicles where some trophic chambers appeared completely degraded (Fig. 1F). Foragers collected when returning to the hive with a pollen load, as well as some of the 30 day-old workers collected according to their age labeling, also showed signs of PCD in their ovarioles. In the basal ovariole stalks of foragers, we noted extensive cell degeneration (Fig. 1G), in part possibly resulting from previous ovulations, such as the presence of corpus luteum-like structures and peritoneal sheath remnants.

Queen ovarioles were longer than those of workers. In newly emerged and virgin queens, this occurred especially the germarial region, which was much extended in comparison to workers. Moreover, they showed a series of cystocyte rosettes lined up in sequence (arrows in Fig. 2A), followed by several oocyte/trophocyte complexes (Fig. 2B). Also in distinction to workers, several previtellogenic and early vitellogenic follicles were seen in alignment within the ovarioles of 5–7 day-old virgin queens (Fig. 2C).

In general aspects, *Melipona quadrifasciata* workers are thus much more similar to queens in their ovary structure than are *Apis mellifera* queens and workers. Nevertheless, there are also common aspects shared between the two species. As in honey bees, the ovarioles of stingless bees were characterized by a much elongated terminal filament, clearly evidencing two cell types (Fig. 3A). The flattened highly chromogenic cell type represented the typical stack-of-coins somatic cells generally found in insect terminal filaments (Büning, 1994), whereas the weakly stained more rounded cell type resembled a similar cell type detected in the honey bee terminal filament which we and others tentatively consider as germ line stem cell (Gutzeit et al., 1993; Tanaka and Hartfelder, 2004). A second aspect where *M. quadrifasciata* ovaries were similar to those of *A. mellifera* is the presence of somatic cells (arrows in Fig. 3B) interspersed between the trophocytes in the nurse chamber (Ramamurty, 1977; Tanaka and Hartfelder, 2004).

The two species, however, clearly differ from one another in the architecture of the oocyte/trophocyte arrangements in the basal

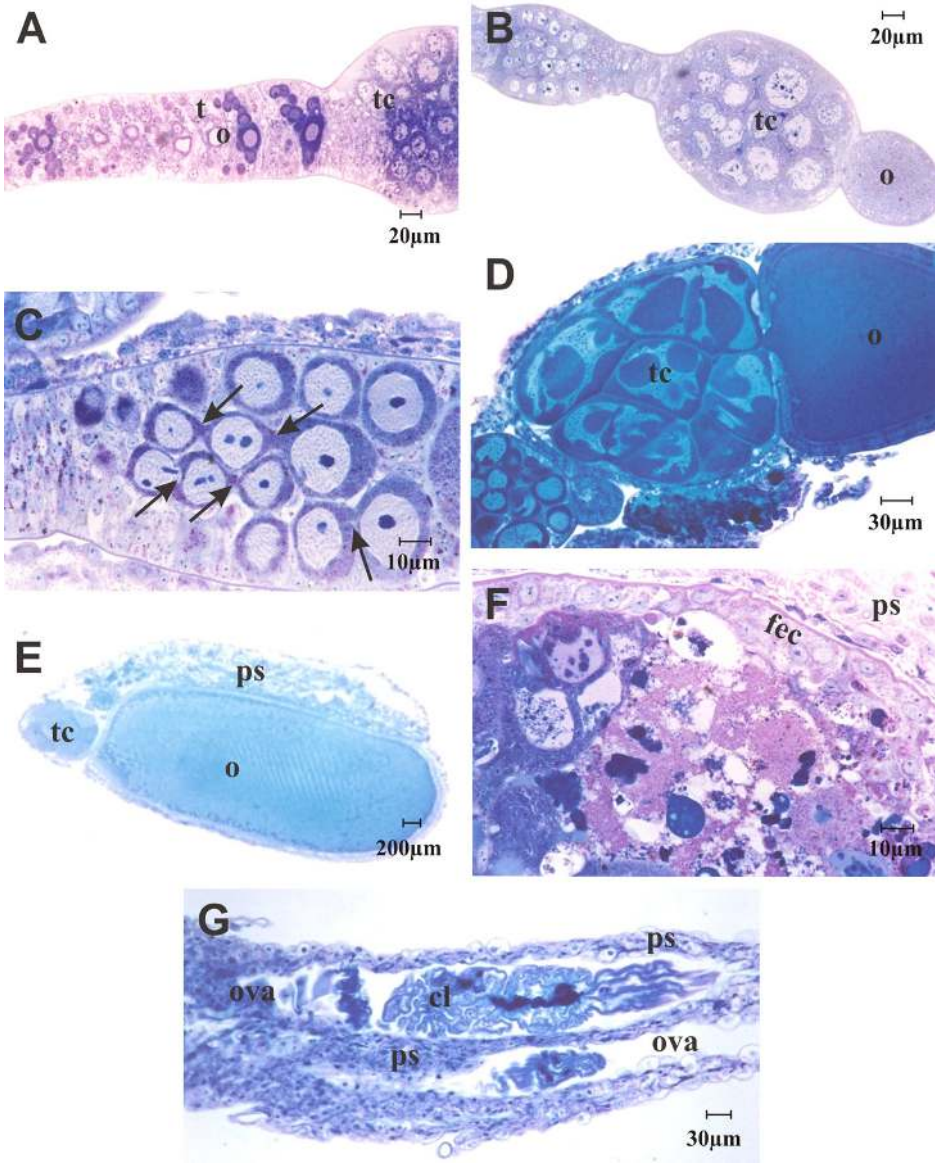

Figure 1. Histological sections of ovarioles of *Melipona quadrifasciata* workers kept under different social and maintenance conditions; 2–3 μm sections were stained with methylene blue/basic fuchsin. (A) ovariole of newly emerged worker evidencing the separation between the germarial region and the region of follicular growth containing a single basal follicle; (B) 5 day-old worker kept in an incubator, showing advanced development of the basal previtellogenic follicle and an arrested subsequent follicle (upper left corner); (C) previtellogenic follicle of a 10 day-old worker kept in observation colony, evidencing intercellular bridges (arrows) between trophocytes within trophic chamber; (D) early vitellogenic follicle of nurse bee of undetermined age illustrating the shape change in trophocyte nuclei from a rounded to irregular shape; (E) ovariole of 30 day-old worker containing a fully developed follicle; (F) 15 day-old worker kept in observation colony and (G) forager showing signs of programmed cell death in follicles and the basal ovary region, respectively. cl, *corpus luteum*; fec, follicle epithelial cells; ova, ovariole remnants; o, oocyte; ps, peritoneal sheath; t, trophocyte; tc, trophic chamber.

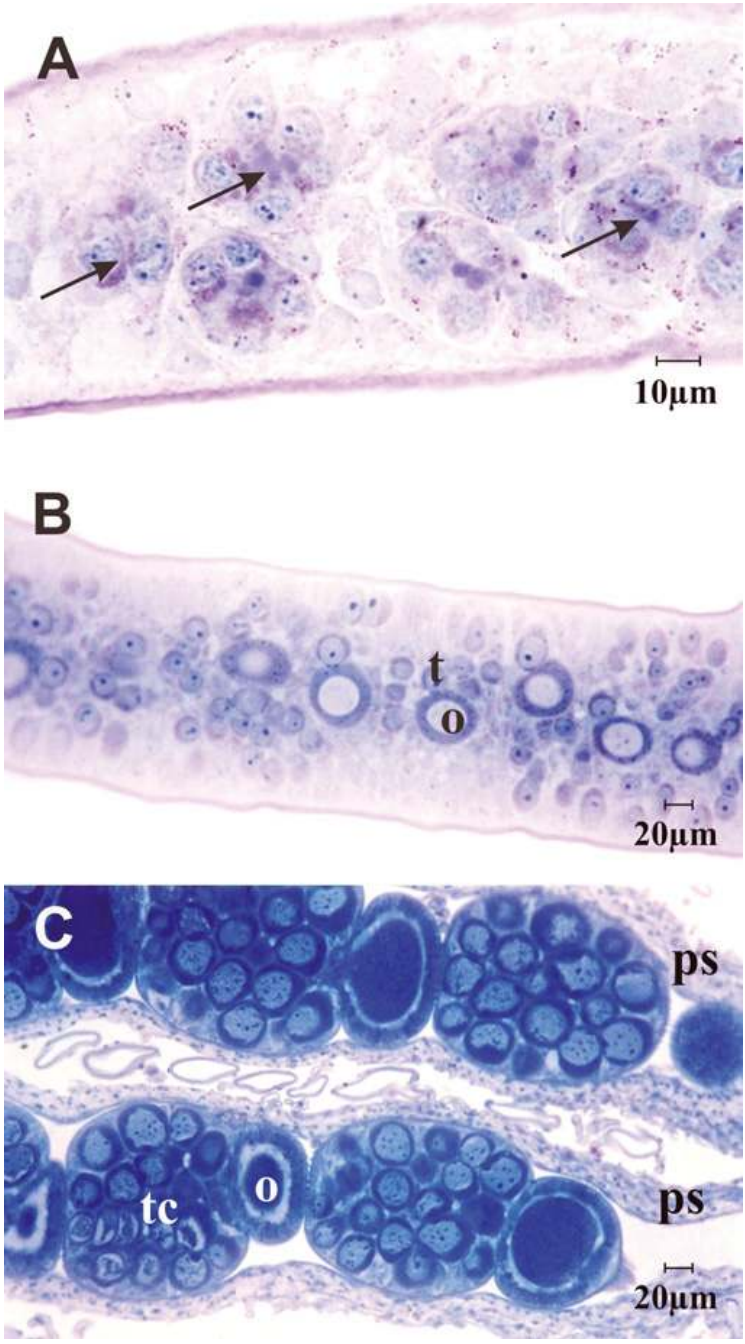

Figure 2. Histological sections of ovarioles of *Melipona. quadrifasciata* virgin queens kept in an incubator with a small group of accompanying workers. (A) upper germarial region of 7 day-old queen showing a sequence of cystocyte rosettes (arrows); (B) lower portion of germarium of 5 day-old queen showing a series of oocytes, each surrounded by trophocytes; (C) two ovarioles of 7 day-old queen sectioned in the region of early vitellogenic growth of the follicles. o, oocyte; ps, peritoneal sheath; t, trophocyte; tc, trophic chamber.

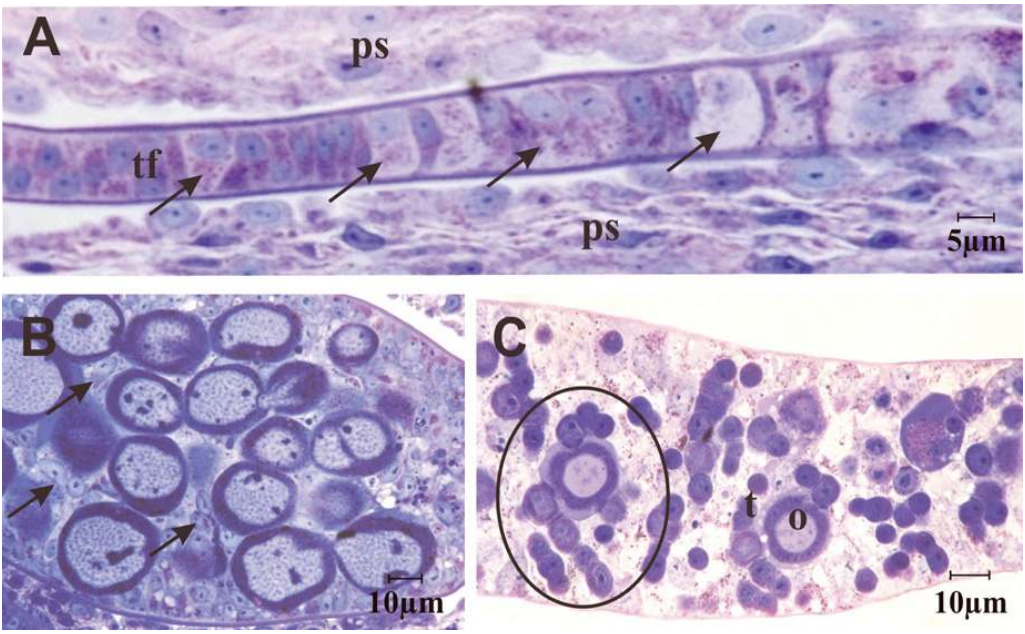

Figure 3. Histological sections of ovarioles of *Melipona quadrifasciata*. (A) terminal filament of ovariole of 15 day-old worker showing the structural difference between the typical flat and strongly chromogenic somatic cells and the weakly staining second cell type; (B) trophic chamber of previtellogenic follicle of 7 day-old worker evidencing the presence of intertrophocytic cells (arrows) distributed among nurse cells; (C) lower germarial region of ovariole of newly emerged worker showing how each oocyte is surrounded by a basket of trophocytes (circle). o, oocyte; ps, peritoneal sheath; t, trophocyte; tf, terminal filament.

region of the germarium. In the honey bee, this is a highly organized and polarized comet-like arrangement consisting of a basal oocyte and long lateral branches of trophocytes. In the stingless bee ovary, the architecture of this complex is less polarized, showing a more centrally located oocyte surrounded by a basket of future nurse cells (circle in Fig. 3C).

3.2. Mitotic activity during oogenesis

As was the case for the general ovariole organization, patterns of mitotic activity within the ovarioles were also little affected by social conditions and maintenance conditions in both castes. For this reason, we present the results for queens and workers together. We generally observed BrdU-labeled cells in the terminal filament (Fig. 4A), at the top of the germarium (Figs. 4B, E) and in the more basal portion of the germarium (Figs. 4C, G). In the terminal filament and in the upper germarium, we noted expressive levels of synchrony in mitotic events, with some parts showing high levels of BrdU incorporation, while others were essentially free of any labeled cells (Figs. 4B, D, E, F). In the more basal region of the germarium, BrdU labeling appeared spatially more restricted, indicating mitotic cycle synchrony within, but not between cystocyte clusters (Fig. 4C).

As the follicles separate from the germarium and enter the previtellogenic growth phase, the synchrony becomes further reduced, as only individual trophocyte nuclei incorporated BrdU during the incubation period (Fig. 4H). As trophocytes do not divide any more after follicles are formed, we interpret this BrdU labeling as representing polyploidization events.

These analyses of cell-cycle activity within the ovarioles further underline the similarity between queens and young workers, as

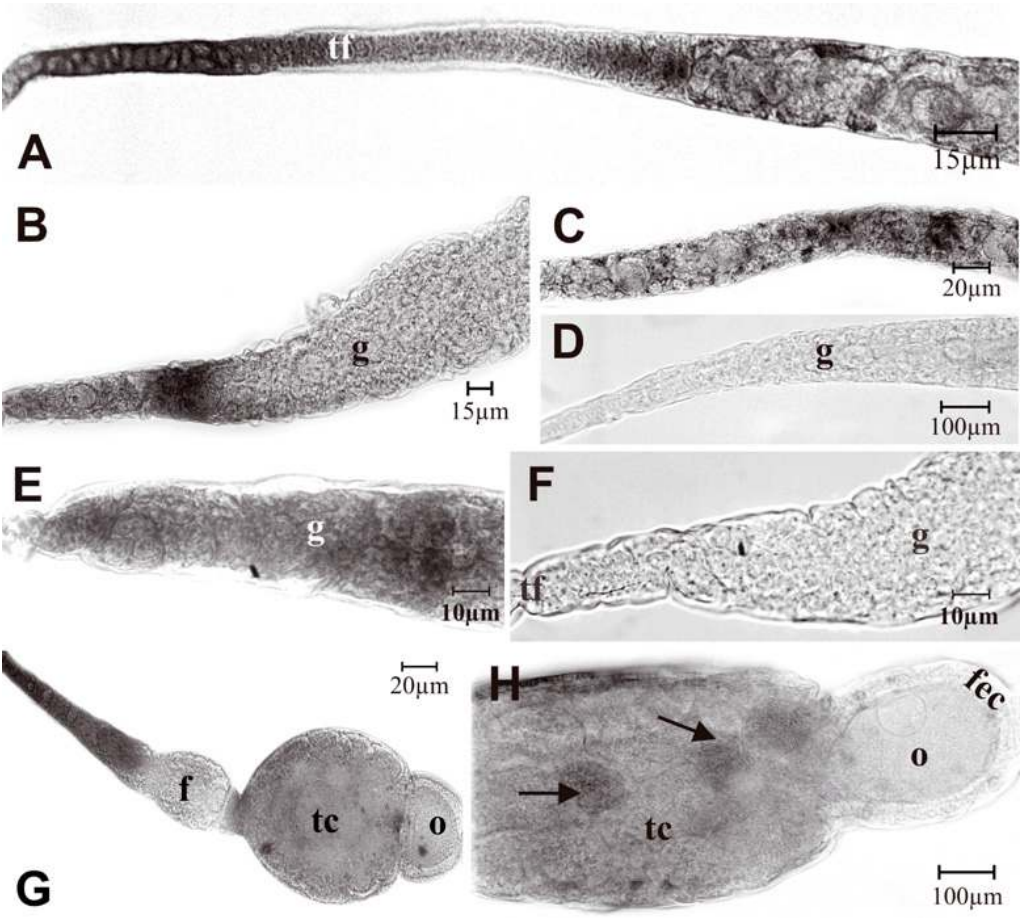

Figure 4. BrdU-detection of mitotically active cells in whole-mount preparations of *Melipona quadrifasciata* ovarioles. (A) ovariole of newly emerged worker showing strong BrdU-labeling in the terminal filament and the upper germarium; (B) BrdU-positive S-phase cells at the terminal filament/upper germarium border of 7 day-old worker kept in incubator; (C) locally concentrated BrdU labeling in germarium of 10 day-old worker kept in incubator; (D) absence of BrdU-labeled cells in germarium of 15 day-old worker kept in observation colony; (E and F) synchrony of proliferative activity revealed by locally concentrated (E) and absence of (F) BrdU labeling in germarium of physogastric queen; (G) overview of BrdU staining in ovariole of 7 day-old worker kept in incubator; (H) isolated polyploidization events revealed by BrdU-labeled trophocyte nuclei in trophic chamber of 15 day-old worker kept in observation colony. f, follicle; fec, follicle ephitelial cells; g, germarium; o, oocyte; tc, trophic chamber; tf, terminal filament.

previously noted in the histological analyses. High levels of BrdU labeling were observed in queens and in young workers, whereas ovarioles dissected from 20 or 30 day-old workers did not show BrdU-labeling, indicating gradual cessation of ovarian activity in accordance with the histological observations.

3.3. The ovarian actin cytoskeleton

The actin cytoskeleton represents an important marker characterizing progressive stages of oogenesis in insect ovarioles (Warn et al., 1985; Cooley and Theurkauf, 1994; Lin et al., 1994; Rübsam and Büning, 2001). We

employed this marker to study ovarioles of the stingless bee *M. quadrifasciata* and noted very striking similarities, not only among queens and workers, but also with ovarioles of the honey bee, which we had investigated in a previous study (Tanaka and Hartfelder, 2004).

F-actin appeared in different structural configurations along the entire ovariole. In the terminal filament section, the stack-of-coins-like somatic cells exhibited a much more strongly labeled actin cytoskeleton than the second cell type which only showed backgound staining (Fig. 5A). At some positions, spot-like F-actin configurations were visible, either interspersed between the somatic terminal filament cells or just adjacent to the border between the terminal filament and the germarial cap region (Fig. 5B). Spot markings of similar size were visible in the upper germarium, where they appeared in single file (Figs. 5B, C). These spots were very similar to those observed in the honey bee ovary, where they were identified as fusomal actin agglomerates (Tanaka and Hartfelder, 2004). As the germarium gradually enlarged in queens, the fusomal actin spots lost their single-file arrangement and spread out first into two columns before they then spread out over the entire germarial diameter (Fig. 5D). This region coincides with the one where we observed the strongest mitotic activity, as evidenced by BrdU labeling (Fig. 4E). This is also the region of gradual expansion of the polyfusomes, marking the center of each mitotically growing cystocyte clusters (Fig. 5F). Subsequently, the polyfusomes dissolve and the fusomal actin cytoskeleton becomes reorganized into ring canals (Fig. 5E) that connect the future trophocytes to each other and to the still hardly distinguishable oocyte.

As mentioned above, these transition steps in the organization of the germ line-associated actin cytoskeleton are very similar in the queen and worker ovary and, in the latter, we could find it identically organized under all social and maintenance conditions analyzed in this study. This finding underlines the observation previously made in honey bees that the early steps in oogenesis make their appearance independent of caste and associated reproductive potential (Tanaka and Hartfelder, 2004). The only apparent difference between queens and workers lies in the length of the respective ovariolar portion, as can be seen when comparing Figure 5C, showing an ovariole from a 10 day-old worker, with Figure 5D, showing a virgin queen ovariole with a much longer and wider zone of cystocyte clusters.

The difference between queen and worker ovarioles becomes more pronounced in the lower germarium, where queen ovarioles showed a much longer file of prefollicular arrangements (Fig. 5G). Noticeably in this basal germarial region, where the basket-like prefollicular arrangements were detected in histological sections (Fig. 3C), it is the cortical actin cytoskeleton which becomes more prominent, forming an especially thick lining at the oocyte cortex. The spot or bar-like thickenings in the cortical actin layer between trophocytes probably represent ring canal borders.

The cortical actin cytoskeleton remains a prominent feature in previtellogenic follicles (Fig. 5H). In workers, it is only a single basal follicle which becomes vitellogenic, the following one being arrested in an early previtellogenic stage. This stands in contrast with queens where all sequential stages of previtellogenic and vitellogenic follicles can be found. An interesting observation was the detection of an F-actin spot inside early previtellogenic oocytes (arrow in Fig. 5H). Judging from its position, this spot seems to be associated with the oocyte nucleus.

4. DISCUSSION

The differences in reproductive options for honey bee and stingless bee queens and workers (Engels and Imperatriz-Fonseca, 1990) are reflected in structural differences in their reproductive organs. A major disparity between the two taxa is the way by which workers provide their queen with nutrients to enable her to constantly produce a large number of eggs. In many stingless bees, including *M. quadrifasciata*, workers produce trophic eggs in addition to a few reproductive eggs. The former are eaten by the queen and represent a rich source of vitellogenin and other high energy

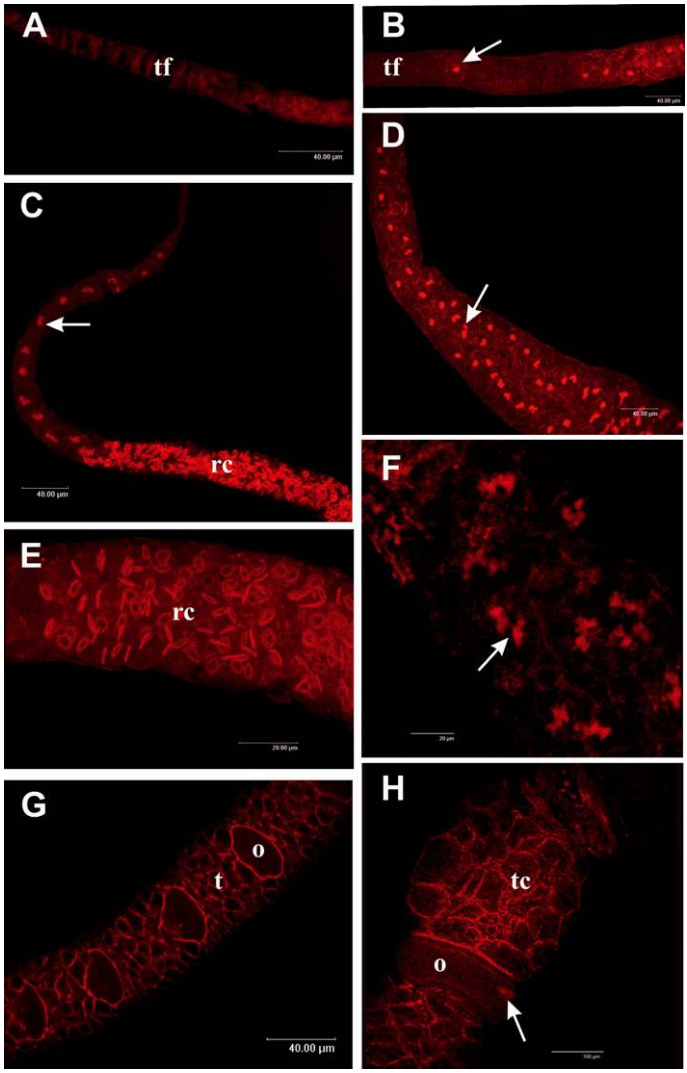

Figure 5. Confocal microscopy of TRITC-phalloidin labeled actin cytoskeleton in *Melipona quadrifasciata* ovarioles. (A) terminal filament of 7-day old worker kept in incubator, showing differential staining for the two terminal filament cell types; (B) transition region from terminal filament to germarium in ovariole of newly emerged queen, showing punctated concentration of F-actin; (C) germarium of 10 day-old worker kept in incubator, exhibiting concentrated TRITC-phalloidin labeling in single file of polyfusomes (upper left corner) and in ring canals; (D) upper germarium of newly emerged queen revealing concentrated F-actin in early (upper left corner) and in branched polyfusomes (lower right corner); (E) detail view of TRITC-phalloidin labeled ring canals in lower germarium of 5 day-old worker kept in incubator; (F) detail view of TRITC-phalloidin labeled branched polyfusomes of physogastric queen; (G) lower germarium of 5 day-old virgin queen kept in incubator, showing strong cortical actin staining in oocytes and somewhat lesser also in trophocytes; punctate staining may represent intercellular bridges; (H) early previtellogenic follicle in ovariole of physogastric queen showing TRITC-phalloidin-labeling for cortical actin in the oocyte and in the nurse cells of its trophic chamber; the punctate actin concentration in the oocyte may be associated with the karyosome. o, oocyte; rc, ring canal; t, trophocyte; tc, trophic chamber; tf, terminal filament. Scale bars represent 20 μm in E and F, 40 μm in A, B, C, D and G, 100 μm in H.

yolk components to sustain the queen's egg production rate.

Already at emergence from the brood cells, the ovarioles of *M. quadrifasciata* contain a basal follicle in a large previtellogenic stage, reflecting the future egg-laying capacity of these workers. Since workers are capable of producing two type of eggs almost coincidentally, and because trophic eggs differ from reproductive ones in size and shape (Koedam et al., 1996), we decided to investigate if possible trophic-egg production by young workers may reside in differences in oogenesis (for example, cell division, cystocyte rosette formation, or previtellogenic follicle structure) when compared to reproductive eggs produced by queens. Our results showed that early stages of the oogenesis in young workers and queens are highly similar at the cellular level, indicating that divergence in egg types occurs probably only late in follicle development.

Queen ovarioles are more elongated than those of workers, mainly because of a much extended terminal filament. In the upper germarium of queen ovarioles, the apparent difference between *M. quadrifasciata* queens and workers is the larger number of cystocyte rosettes in the former and, consequently, the larger number of developing previtellogenic follicles in a linear arrangement. This difference explains the higher rates of egg production in stingless bee queens when compared to workers. In this respect, our results are in accordance with those of Martins and Serrão (2004a) showing that virgin queen ovarioles have several previtellogenic follicles in the vitellarium, even though this region is not yet enlarged.

In conclusion, our results indicate that the overall dynamics of ovarian activity in young queens and workers are quite similar. Furthermore, oogenesis in workers appears to be more related to age and to be relatively independent of social conditions (presence or absence of the queen), group size, and general maintenance conditions.

In terms of general ovariole architecture, the following features deserve attention. Like in honey bees, the terminal filaments in *M. quadrifasciata* ovarioles are also composed of two cell types – the more rounded cell type with a large nucleus and an apparently undifferentiated cytoplasm that is atypical for insects. The detection of this cell type, together with the presence of actin spots and mitotic events in the terminal filament, has led us to hypothesize that the terminal filament of honey bees may contain niches of germ line stem cells, in addition to the typical flattened somatic cells (Tanaka and Hartfelder, 2004). The similarity between honey bees and stingless bees in this character underlines the possible importance of this structurally unusual ovariole character for oogenesis in social bees. Thus the question is: does this character make its appearance as the queens of social Hymenoptera increase their oogenesis rates, or is it already present in solitary ancestors, representing part of the ovary ground plan of this group?

In the germarium, we detected strong labeling of polyfusomes by TRITC-phalloidin, evidencing also in stingless bees the presence of F-actin in polyfusomes. The fusomes is a germ line-specific organelle that contains cytoskeletal proteins (Lin et al., 1994). When polyfusomes are transformed into ring canals, F-actin becomes allocated to the walls of the ring canals. A similar pattern of actin localization was observed in *A. mellifera* (Tanaka and Hartfelder, 2004) and other hymenopterans (Jablonska and Bilinski, 2001; Jablonska and Kisiel, 2002). An interesting difference between honey bees and stingless bees, however, seems to reside in the timing of the appearance of F-actin within the fusomes. In the honey bee, F-actin is only detected in older, branched polyfusomes (Tanaka and Hartfelder, 2004); in *Melipona*, it makes its appearance in the smallest fusomes at the very tip of the germarium. This indicates a difference between the two species (or taxa) in how actin may become recruited to the set of fusomal proteins (Lin et al., 1994).

After oocytes become distinguishable from trophocytes in the median-basal region of the germarium, another difference between the two bee species becomes apparent. In honey bee ovarioles, the oocyte of each germ cell cluster comes to occupy a basal position and is surrounded by linear branches of trophocytes, giving the whole prefollicular arrangement a

comet-like appearance (Tanaka and Hartfelder, 2004). In *M. quadrifasciata,* ovaries with such a distinct architecture were not observed. Instead, oocytes remained in a much more central position and were surrounded by baskets of trophocytes. It looks as if the original cystocyte rosette configuration first seen in more apical regions of the germarium is maintained until separation of the previtellogenic follicle from the germarium. A similar oocyte-trophocyte assembly as the one observed in the germarium of *M. quadrifasciata* ovarioles has also been described in solitary bees (Martins and Serrão, 2004b), indicating that the comet-like arrangement is a derived feature of highly eusocial honey bees. The role of these oocyte-trophocyte arrangements is still unknown.

A decision that has to be made next, as the follicle undergoes previtellogenic and vitellogenic growth, is whether its fate will be that of a trophic or a reproductive egg. Since the two egg types differ in size and morphology (Koedam et al., 1996; Chinh et al., 2003) and are not only a result of worker behavior – that is, where and when during the Provisioning-Oviposition Process (POP) an egg is laid – this decision has to be made during follicle growth. We observed that young workers of *M. quadrifasciata* usually have a single previtellogenic follicle in their ovarioles. This basal follicle is always much more advanced than the subsequent one, which appears to be arrested in development right after it became separated from the germarium. Such a strong divergence in development between the basal and the next follicle was also noted in ovarioles of solitary bees (Martins and Serrão, 2004b), and in fact, this seems to be an ancestral feature which these bees share with dipterans. The latter also have polytrophic meroistic ovaries and in many species development of the penultimate follicle is hormonally blocked until the basal follicle is released (Bylemans et al., 1994; De Loof et al., 1995).

In *M. quadrifasciata,* most of these developing follicles found in the young workers should probably become trophic eggs, and Cruz-Landim (2000) proposed that rapid follicle growth of the future trophic eggs may be due to precocious yolk deposition in the oocyte and that the stimulatory effect for the production of trophic eggs may come from the continuous contact that these young workers have with the queen. In our study, however, we observed essentially similar timelines of follicle development for workers that were in contact with a queen, for orphan workers that did not have any contact with a queen, and also for worker that were over 20 days old. Our results thus suggest that the egg fate should be determined in the final stages of oogenesis, probably only during or shortly before choriogenesis, and that trophic egg production may be a worker-specific oogenesis program that is independent of the queen's presence. In this sense, trophic egg production in stingless bees, like *Melipona quadrifasciata*, may, in fact, simply be an adaptation of the archetypal oogenesis program of solitary bees. While a dominant queen would normally tend to suppress worker ovarian activity, stingless bee workers may have exploited the queen's need to maintain her super-stimulated reproductive activity by offering trophic eggs and, via this worker ovarian activity in the queen's interest, also gain in individual fitness by the occasional production of a reproductive egg.

In conclusion, stingless bees, represented here by *M. quadrifasciata*, are an interesting group for comparative studies on reproductive system functions in the context of social evolution. With respect to ovariole number and presence of previtellogenic basal follicles in both castes at emergence, the stingless bees are clearly more similar to solitary and primitively eusocial bees than to honey bees (Martins and Serrão, 2004b). Yet other structural features, such as the occurrence of a second cell type in the terminal filament, possibly representing germ line stem cells (Tanaka and Hartfelder, 2004), and a long file of prefollicles in the germarium, especially apparent in ovarioles of queens, are characters that stingless bees share with the honey bees. An interesting finding, possibly even related to the decision of whether an egg is to become a trophic or a reproductive one, is the association of F-actin with the oocyte nucleus in developing follicles, since in addition to nuclear cytoskeleton functions (Kumaran et al., 2008) nuclear actin is now also known to play gene regulatory roles (Percipalle and Visa, 2006) and

in oocytes of insects it has been described as a component of the karyosome (Rübsam and Büning, 2001).

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Structure des ovarioles et ovogenèse chez les reines et les ouvrières de l'abeille sans aiguillon, *Melipona quadrifasciata* (Hymenoptera, Apidae, Meliponini) en fonction des conditions sociales.

ovaire / histologie / reproduction des ouvrières / follicule / développement / cytosquelette / actine

Zusammenfassung – Oogenese und Ovariolenstruktur bei Königinnen und Arbeiterinnen der Stachellosen Biene *Melipona quadrifasciata* (Hymenoptera: Apidae, Meliponini), die unter unterschiedlichen sozialen Bedingungen gehalten wurden. Die Tatsache, dass Stachellose Bienen nicht nur hocheusocial sind, sondern auch sehr variabel in ihrer Reproduktionsbiologie, macht sie zu interessanten Studienobjekten für vergleichende Untersuchungen, insbesondere mt der Honigbiene, *Apis mellifera*. In der vorliegenden Arbeit analysierten wir die strukturelle Organisation der Ovariolen und die Dynamik der Ovogenese bei der Stachellosen Biene *Melipona quadrifasciata*, in Königinnen und Arbeiterinnen unterschiedlichen Alters und unter verschiedenen sozialen Haltungsbedingungen.
Im Hinblick auf die generelle Histologie der Ovariolen (Abb. 1–3) fanden wir nur wenige Unterschiede zwischen Königinnen und Arbeiterinnen, ausser dass erstere ein wesenlich längeres Germarium aufweisen, in dem sich eine grössere Zahl an Follikeln sequentiell entwickelt. Ein zweiter Unterschied liegt in der Zahl der prävitellogenen Follikel, die bereits beim Schlupf aus der Brutzelle angelegt sind. Während die Ovariolen von Arbeiterinnen lediglich einen prävitellogenen Follikel aufweisen, sind dies bei Königinnen stets mehrere. Diese Befunde stehen in Einklang mit der Eiproduktion junger *M. quadrifascata* Arbeiterinnen, die neben Nähreiern auch funktionelle Eier ablegen können, aus denen sich Männchen entwickeln.
Auch im Hinblick auf die Dynamik der Mitoseprozesse, die durch BrdU-Inkubation und Detektion in Ovariolen sichtbar gemacht wurden (Abb. 4), unterschieden sich Königinnen und Arbeiterinnen nur wenig. Sowohl im Terminalfilum als auch im Germarium zeigte sich eine regionale Synchronie im Zellzyklus, die sich im weiteren Verlauf der Oogenese sukzessiv verliert. In diesem Punkt sind sich Stachellose Bienen und Honigbienen sehr ähnlich.
F-Aktin wurde mittels TRITC-Palloidin-Markierung und Laserkonfokalmikroskopie sichbar gemacht (Abb. 5). Bereits die frühesten Oogenesestadien wiesen F-Aktin in den Fusomen auf, das mit der Auflösung der verzweigten Polyfusome im weiteren Verlauf der Oogenese in die Ringkanäle übergeht. Erst mit der Differenzierung von Oocyten und Nährzellen im unteren Bereich des Germariums und dann nach Abtrennung der Follikel aus dem Germarium weisen die Nährzellen und insbesondere die Oocyten ein stark markiertes corticales Aktin-Cytoskelet auf.
Eine interessante Parallele in der Ovariolenstruktur von Honigbienen und Stachellosen Bienen bildet das Vorkommen eines zweiten Zelltyps im Terminalfilum. Interkaliert zwischen den typischen abgeplatteten somatischen Zellen, die wie in einem Münzstapel angeordnet sind, fanden sich auch bei *M. quadrifasciata* abgerundete Zellen, die ein nur schwach färbbares Cytoplasma und kein ausgeprägtes Aktin-Cytoskelett aufwiesen. Es könnte sich hierbei wie auch bei Honigbienen um Stammzellen der Keimbahn handeln.

Arbeiterinnenreproduction / Follikelentwicklung / Aktin-Cytoskelett / Ovarhistologie

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