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Regulation of oogenesis in honey bee workers via programed cell death

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ABSTRACT

Reproductive division of labour characterises eusociality. Currently little is known about the mechanisms that underlie the 'sterility' of the worker caste, but queen pheromone plays a major role in regulating the reproductive state. Here we investigate oogenesis in the young adult honey bee worker ovary in the presence of queen pheromone and in its absence. When queen pheromone is absent, workers can activate their ovaries and have well-developed follicles. When queen pheromone is present, even though workers have non-activated ovaries, they continually produce oocytes which are aborted at an early stage. Therefore, irrespective of the presence of the queen, the young adult worker ovary contains oocytes. By this means young workers retain reproductive plasticity. The degeneration of the germ cells in the ovarioles of workers in the presence of queen pheromone has the morphological hallmarks of programmed cell death. Therefore the mechanistic basis of 'worker sterility' relies in part on the regulation of oogenesis via programmed cell death. Our results suggest that honey bees have co-opted a highly conserved checkpoint at mid-oogenesis to regulate the fertility of the worker caste.

1. Introduction

In a social insect colony, the queen has reproductive hegemony over her sterile daughters. However there is a spectrum in the degree of 'worker sterility', even in bees. At one extreme there is the stingless bee genus *Frieseomelitta* where worker ovaries completely degenerate during development (Boleli et al., 1999). At the other extreme is the stingless bee genus *Melipona* where worker ovaries are well developed and workers contribute to male production (Tóth et al., 2004). The honey bee, *Apis mellifera*, is intermediate between these two extremes. The ovaries of honey bee workers are much reduced relative to those of queens, but remain functionally competent (Snodgrass, 1956).

The honey bee worker has a high degree of reproductive plasticity. Queen mandibular pheromone suppresses ovary activation in workers (Hoover et al., 2003). In the absence of a queen the workers (which cannot mate) have the potential to activate their ovaries and lay haploid male-destined eggs. Conversely, if workers with activated ovaries are moved from a queenless colony to a queenright colony their ovaries regress (Malka et al., 2007). Workers also activate their ovaries on a seasonal basis, in particular during swarming season when colonies are temporarily queenless after the old queen has departed and the new queen has not begun to lay eggs (Holmes et al., 2013; Woyciechowski and Kuszewska, 2012). Another group of pheromones, emitted by the brood also contribute to suppressing ovary activation in workers (Alaux et al., 2010; Traynor et al., 2014).

The ovarioles comprise the structural and functional units of the honey bee ovary. The major reproductive difference between the two female castes is that workers have 2–12 ovarioles per ovary whereas queens have 160–180 (Snodgrass, 1956). It is important to note that the term 'ovariole' is used to describe both the outer layer of cells of the ovariole (epithelial sheath, otherwise known as the tunica externa) and the inner, non-cellular membrane (tunica propria). Inside the tunica propria are both somatic cells and germ cells. The somatic cells encapsulate and support the germ cells. A germ cell gives rise not only to an oocyte but also to nutritive nurse cells, which remain directly connected to the oocyte to allow the transport of nutrients into the developing oocyte (Büning, 1994). The honey bee ovary is therefore classified as being polytrophic meroistic.

The majority of studies on the adult honey bee ovary have focused on oogenesis in the queen (Berger and Abdalla, 2005; Patrício and Cruz-Landim, 2002; Paulcke, 1901; Snodgrass, 1956) with only one study examining early oogenesis in queenright and queenless workers (Tanaka and Hartfelder, 2004). Here, we systematically investigate oogenesis in the young adult worker ovary in the presence or absence of queen pheromone in a controlled environment without the confounding factor of brood pheromones. We were expecting no oocytes in the ovarioles of workers in the presence of queen pheromone and that oocytes would be

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present in the ovarioles of workers in the absence of queen pheromone. Instead we show that queen pheromone alters oocyte development in the worker, thereby leading to 'worker sterility'.

2. Materials and methods

2.1. Biological material

Age-matched adult workers of Australian commercial stock derived from *A. m. ligustica* were obtained by incubating overnight combs of emerging brood. The brood was from a single queenright colony which minimises the genetic and environmental heterogeneity among experimental treatments. The following day, the emerged workers were placed in four laboratory cages ($n \approx 150$ per cage) each fitted with a 5×5 cm section of natural honey comb. Two of the cages contained a strip (0.5 queen equivalents) of queen mandibular pheromone (Phero Tech Inc., Canada) to simulate queen presence, whereas the other two cages contained no queen pheromone. The caged workers were provided with honey, ground pollen and water *ad libitum*. Workers were collected at 14 days of age and samples immediately frozen on dry ice.

2.2. Tissue dissection

We dissected a subset ($n = 77$) of the workers in cold PBS under a dissecting microscope (Olympus SZ40): workers exposed to queen pheromone ($n = 41$) and not exposed to queen pheromone ($n = 36$). The tergal surface was opened and the gut, fat body and first four tergites removed, leaving the abdominal cavity containing the paired ovaries (anchored to the last tergite) and the venom gland.

During the dissection, the ovaries were scored as: non-activated (transparent, thread-like ovaries) or activated (opaque, swollen ovaries) (Vergoz et al., 2012). In cages containing queen pheromone, no workers had activated ovaries, whereas in cages with no queen pheromone 12.5% of workers had activated ovaries.

2.3. Tissue preparation

The ovaries were prepared as if for *in situ* hybridisation (Dallacqua and Bitondi, 2014; Zimmerman et al., 2013). Four abdominal cavities with their connected ovaries were placed in a vial containing heptane fixative (1 mL heptane, 80 μL HEPES/HEM buffer, 100 μL of 8% (w/v) paraformaldehyde and 20 μL DMSO) and shaken for 30 min. The samples were rinsed in PBS and then dehydrated with an increasing concentration of ethanol (25%, 50%, 75% and 100% v/v). These were stored overnight at −20 °C. The samples were rehydrated with a decreasing concentration of ethanol (75%, 50% and 25% v/v) and then PBS-Tween 20. Samples were fixed in triton fixative (89.01% PBS, 0.09% Triton X-100, 0.9% of 16% (w/v) paraformaldehyde and 10% DMSO v/v), shaken for 20 min and washed in PBS-Tween 20. The samples were incubated in proteinase K solution (approximately 20 μg/mL) for 15 min, then twice washed in glycine (1% w/v) and PBS-Tween 20. The samples were refixed in triton fixative and shaken for 20 min and washed in PBS-Tween 20. Nucleic acid was stained with SYTOX Blue Nucleic Acid Stain (Invitrogen) (0.002% v/v) for 20 min and washed in PBS-Tween 20. The paired ovaries were then dissected away from their abdominal cavity in PBS-Tween 20, immersed in glycerol (70% v/v) and mounted on individual slides.

2.4. Imaging

Paired ovaries were observed immediately under a laser scanning confocal microscope (Leica TCS SP5 II). SYTOX Blue was excited by a 458 nm laser and emitted light was collected between 465 and 484 nm. Red autofluorescence was excited by 561 nm and 633 nm lasers and emitted light was collected between 580–650 nm or 650–700 nm, respectively.

3. Results

The ovariole of the adult honey bee worker shows successive developmental stages of oogenesis from the tip to the base. The ovariole can be divided into regions depending on cell type (somatic or germ) and stage of germ cell development. These regions are the terminal filament, the germarium, the vitellarium, and the pedicel (which is sometimes referred to as the basal stalk) (Fig. 1A).

Workers not exposed to queen pheromone can have activated ovaries and their ovarioles have well-developed oocytes. The developing oocytes cause the epithelial sheath to bulge (Fig. 1A) producing the defining feature of the activated ovary phenotype. The basal end of the tunica propria of an activated ovary is 71 μm ± 6 μm ($n = 5$) in diameter. The terminal filament, a stack of aligned disc-shaped cells that are orientated transversely (Fig. 1B), is located at the apical end of the tunica propria. This filament contains cells that are mainly of somatic origin but germline stem cells are also present (Tanaka and Hartfelder, 2004). In the early-germarium stage, there are undifferentiated germ cells known as oogonia (Fig. 1C). In this region, individual cells are hard to differentiate. In the mid-germarium stage, the early oocyte is centrally located and is differentiated by size from its surrounding cells that develop into trophocyte nurse cells (approximately 39–59 per oocyte (Büning, 1994)) (Fig. 1D). The nucleus of the oocyte, known as the germinal vesicle, is relatively large and contains a small black dot indicative of the chromatin beginning to condense. In the late-germarium stage, the oocytes are now located at the basal end of their associated nurse cells (Fig. 1E). The germinal vesicle by this point appears as an unstained dark structure due to the condensed chromatin inside, known as the karyosome. The vitellarium stage of development is where self-contained follicles are present (Büning, 1994). At the vitellarium stage the oocyte is enlarged and encapsulated by a monolayer of columnar follicle cells (Fig. 1F), which are somatic in origin. In the nurse chamber the nurse cell size follows a gradient; those cells closest to the oocyte are the largest (Fig. 1A and F). The nurse cells have very large nuclei relative to the cytoplasm as well as bright staining around their nuclei (Fig. 1F) which is indicative of high levels of transcription in these cells (Gutzeit et al., 1993). Each ovariole terminates in a structure called the pedicel (Fig. 1G), which is somatic in origin. The pedicel acts as a plug to the oviduct opening and degrades when an egg is ready to be laid (Büning, 1994). Late oogenesis is characterised by developmental programmed cell death of the nurse cells (Fig. 1H). The basal nurse cells have a rounded appearance, condensed chromatin and an absent cytoplasm (Fig. 1H) due to the 'dumping' of their cytoplasmic contents into the oocyte (Cavaliere et al., 1998).

Sometimes the ovarioles of workers not exposed to queen pheromone show what we term a 'transition phenotype' between non-activated ovaries and activated ovaries (Fig. 1I). In these ovarioles the germarium region has both healthy and dead oocytes. The developing oocytes in the middle of the tunica propria are healthy, as indicated by chromatin condensing in the germinal vesicles (Fig. 1I). However, at the basal end of the tunica propria the oocytes are dead as evidenced by the absence of staining (Fig. 1I). The oocytes' nurse cells are dying, as evidenced by the pycnotic nuclei (central and highly compact, the condensed chromatin stains heavily) (Fig. 1I).