CELL DEATH IN THE GERMLINE – MECHANISMS AND CONSEQUENCES FOR REPRODUCTIVE PLASTICITY IN SOCIAL BEES

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ABSTRACT

Its serial architecture makes the insect ovary an interesting playground to study the regulation of cell death and identify critical check points along the apical-basal axis of the ovarioles. In *Drosophila melanogaster*, cell death is observed at two points: (1) in the germarium, where entire germ cell clusters may die in response to environmental conditions, and (2) as an obligatory event at the end of oogenesis, when nurse cells dump their cytoplasm into the oocyte and, subsequently, when the follicle epithelial cells form a chorion. The social organization of bees, wasps and ants depends on the monopolization of reproduction by a queen. This has marked consequences on the ovary phenotype of queens and workers. The role of programmed cell death in larval ovary development and in adult ovary function is best studied in honey bees. During larval development, workers loose over 90% of the ovariole primordia. This cell death is induced by a low juvenile hormone titer causing breakdown of the actin cytoskeleton in germ cell clusters. The actin cytoskeleton also plays a major role in the control of cell death in the ovary of adult bees, where many TUNEL-labeled and pycnotic nuclei are detected in a germarial region rich in actin agglomerates. This suggests that common mechanisms may regulate cell death in the ovaries of bees, both during the shaping of the caste-specific ovary phenotypes during larval development, and during the tuning of reproductive activity in adult bees.

Key words: Actin, *Apis mellifera* juvenile hormone, ovariole, social insect

INTRODUCTION

The production of eggs presents a high investment for any female and the number of offspring produced is subject to intense selection. Regulation of ovary activity to avoid investing valuable resources in eggs when there is little chance that they result in viable and fertile offspring, thus, is an important fitness component. Consequently, cell death is a common phenomenon in the germline of most if not all animal phyla [48]. It has been reported in *Caenorhabditis elegans* [28], in *Hydra* [51], in sponges [22,30,83], in mice [58] and in many insect species, including flies, mosquitoes and bees [7,14,53,55,69].

The ovary of insects is composed of several ovarioles. These represent a serial organization of functional units with a clear proximal-distal axis along which oocytes develop from stem cells to fully chorionated eggs. This axis is also coincident with the anterior-posterior axis of the embryo [9]. Because of this highly structured architecture, the ovarioles of insects are an interesting system to study and investigate regulatory mechanisms in oogenesis. At the distal end of the germarium, germline stem cells undergo differential mitotic divisions generating germ cells committed to proceed through all steps of oogenesis, until they reach the follicle growth zone where oocytes take up large amounts of yolk protein precursor (vitellogenin) from the hemolymph, before the surrounding follicle cells lay down an outer protective egg shell, the chorion. The structural details of the insect ovary and its different types of ovarioles have been meticulously compiled by Büning [9], and the physiological, especially hormonal control mechanisms underlying vitellogenesis have been extensively reviewed by Raikhel *et al.* [64], so that the present review

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can concentrate on the role of programmed cell death (PCD). This aspect is only now beginning to emerge as a mechanism that may play a major role in tuning reproductive activity and which is capable of generating reproductive plasticity and alternative ovary phenotypes, particularly in the female castes of social insects. We will discuss the role of PCD in the ovary under two headings, first, its occurrence and putative function during oogenesis in adult female insects, and second, the role of PCD in larval development and its effect on ovary phenotype.

Programmed cell death in the fly ovary - brief overview

The mechanisms underlying cell death in *Drosophila melanogaster* are very similar to those described in mammals [35,72,86]. Seven caspases have been annotated in the *Drosophila* genome, three belonging to the class of initiators (Dredd/Dcp-2, Dronc and Strica/Dream) and four to the class of effector caspases (Dcp-1, Drice, Decay and Damm/Daydream) [42]. Besides the caspases, five further genes play a major role in PCD in *Drosophila*, three positive regulators, *reaper (rpr)* [92], *head involution defective (hid)* [26] and *grim* [13], and two negative regulators, *DIAP-1* and *DIAP-2* [34]. The mRNAs of *rpr, hid, grim, DIAP-1* and *DIAP-2* have all been detected in the *Drosophila* ovary, but interestingly, *rpr, hid* and *grim* are not necessary to induce cell death in nurse cells [23].

Oogenesis starts with the asymmetric division of a germline stem cell generating a cystoblast that undergoes four mitotic divisions with incomplete cytokinesis. In this cluster of 16 cells, one is determined to become the oocyte, while the others assume the nurse cell fate. During all further stages of oogenesis, these 16 cells remain connected by large cytoplasmatic bridges known as ring canals. Fly oogenesis is well characterized and has been divided into 14 stages [40]. PCD is a prominent feature observed in nurse cells in stage 11-13 follicles [23], after these cells dumped all their cytoplasm into the oocyte shortly before the chorion is laid down. At the end of this cytoplasm dumping event, which lasts about 30 min [43], the nuclei of the nurse cells start to show signs of cell death and the nurse cell residues are phagocytosed by cells of the follicle epithelium. An actin network retains the nuclei in the nurse cell chamber and prevents them from being dumped into the oocyte together with the cytoplasm [16,60]. The massive transfer of cytoplasm into the oocyte has been attributed to a myosin-based contraction of the subcortical actin network of nurse cells [91]. Nurse cells in *quail* and *singed* mutants do not show this actin network that retains the nucleus and do not undergo PCD. In these flies, the nucleus drifts into the ring canal and blocks the transport of cytoplasm into the oocyte [11]. Apoptosis of nurse cells at the end of oogenesis is, thus, a fundamental phenomenon in the polytrophic meroistic ovary and has also been described in other higher dipterans, such as *Dacus oleae* and *Ceratitis capitata* [53,55], suggesting that a genetically conserved mechanism governs nurse cell PCD [55].

An interesting question is how the oocyte is protected from undergoing apoptosis as well in this syncytial structure, and some studies indicate that nurse cell apoptosis may be quite distinct from cell death in somatic tissues [49]. First of all, PCD in nurse cells is of independent *reaper, hid* or *grim* expression [23]. Second, the over expression of DIAP-1 did not inhibit PCD in stage 11-13 nurse cells, while it prevented cell death in other tissues and also in earlier stages of oogenesis (stage 8, for example), suggesting a caspase-independent pathway for PCD in nurse cells [59].

Shortly after the nurse cells have dumped their cytoplasm, the somatic follicle epithelium cells also undergo apoptosis. The last function of these cells is the massive synthesis of chorion proteins, which form the protective egg shell [46,47,84]. The follicle epithelium cells die at stage 14, and at stage 14b the egg is released into the lateral oviduct by a contraction of the epithelial sheath at the base of the ovariole [40,44]. This contraction is thought to provide the mechanical force required to remove the remnants of the follicle epithelial cells from the chorion before they are phagocytosed by the epithelium of the lateral oviduct [54]. Even though apparently trivial, removal of the follicle epithelium cell remnants is an important step, since their persistence can block the passage of the egg into the lateral and from there into the common oviduct [54].

Besides this normal cell death at the end of oogenesis, entire follicles can undergo apoptosis in response to poor nutrition of the female or to other

adverse conditions [49]. In these cases, PCD has been evidenced in the germarium (oogenesis stages 2a/b) and about half way through oogenesis (stage 8). This cell death was observed in response to nutritional protein deprivation, blocking of ecdysone signaling, chemical treatment, or anomalous development of individual follicles [12,18,20,25,52]. PCD in stage 8 results in the degeneration of entire follicles, including follicle cells, nurse cells and the oocyte. At this stage, cell death is not accompanied by changes in the actin cytoskeleton [12,52]. The sequence of cell death events at defined steps in the oogenesis program is, therefore, essential not only for the normal progression of oogenesis but also for its fine tuning with nutritional and environmental conditions. Not surprisingly, PCD also provides an interesting means of reducing fecundity in parasitized mosquitoes [37].

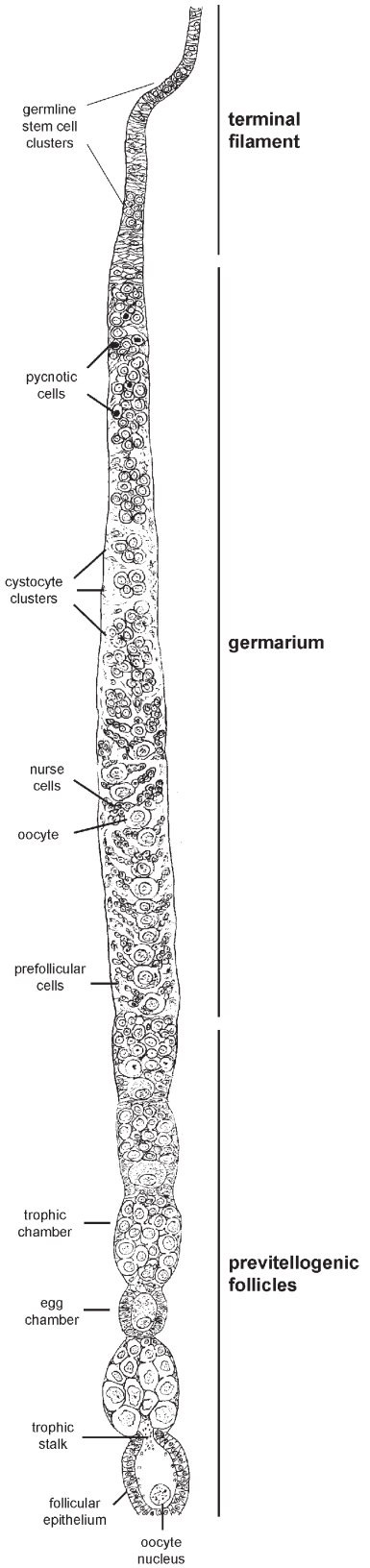
Programmed cell death and differential fertility in honey bee queens and workers

In highly eusocial bees, wasps and ants, the queen is the reproductively dominant female in the colony, while the workers are mostly functionally sterile. The honey bee queen exerts this dominance via her pheromones. Nevertheless, one in a thousand workers in a honey bee colony - which may contain 10,000 to over 50,000 workers - may not succumb to this pheromonal repression and may start to lay eggs [65,88]. These few workers can produce a significant portion (i.e., 7% in certain cases) of the haploid eggs, but only a very small proportion of these eggs will actually give rise to males [65,87,89]. Most of the worker-produced eggs are removed by other workers in a process termed policing [3,6,65,66,67]. The proportion of workers with activated ovaries varies significantly between the different species of the genus *Apis*. In a sample of 800 workers of the dwarf honey bee, *Apis florea*, not a single worker showed signs of ovary activation [29]. This stands in contrast with *Apis cerana,* where up to 5% of the workers had activated ovaries [56].

The ovary of honey bees is of the polytrophic-meroistic type, like that of *Drosophila melanogaster*, but its ovarioles are much elongated and numerous follicles in all stages of development can be found along its proximal-distal axis (Fig. 1). This organization makes it possible that a queen, who possesses approximately 200 of these serial units in each of her ovaries, is capable of producing up to 2,000 eggs per day. In contrast, workers generally possess only 1-12 ovarioles in each ovary and these usually do not show signs of progressive oogenesis as long as a queen is present in the colony. These dramatic differences in fertility obviously require tight tuning of individual reproduction to colony conditions. Recent studies therefore investigated at which time point(s) oogenesis may be blocked in the ovary of adult queen and worker honey bees [4,5,57,82]. In histological sections we detected germ cells with pycnotic nuclei in the upper portion of the germarium, that is, in the region where cystocytes undergo sequential mitotic divisions (Fig. 2a), and also in the region where nurse cells and the oocyte form a comet-like arrangement, just before getting surrounded by follicular epithelium cells and separating basally from the germarium. Such indications of PCD were detected in both castes, yet, the incidence of pycnotic nuclei in these regions was considerably higher in ovarioles dissected from workers kept in the presence of the queen, than in queenless workers or in queens.

While queens start to lay eggs at high rates soon after mating, possibly after transfer of male factors [15], but not while unmated, the initial stages of oogenesis appeared to proceed normally also in virgin queens, and to follow similar dynamics, independent of mating status. Oogenesis in queens, thus, seems to be largely controlled at the late stages, primarily at initiation of vitellogenesis by enhanced vitellogenin synthesis in the fat body [2]. This is in accordance with the detection of cellular disintegration in previtellogenic follicles of virgin queens. The mechanism of resorption of previtellogenic follicles has been investigated in *Drosophila melanogaster*, where it correlates with an elevated ecdysteroid titer [79]. Radioimmunossay analyses of the ecdysteroid titer in honey bees revealed a small peak of ecdysteroids around day 4 after bees emerged from the brood cells. The physiological function of this hormone peak in bees is still a mystery, but its timing - it precedes the initiation of the queen's mating flights - is consistent with an involvement in follicle degeneration in virgin queens.

A closer look at the ovarioles of workers reared in the presence of the queen revealed a high incidence



of disintegration of the fusomal cytoskeleton in the regions were pycnotic nuclei were preferentially encountered. By fluorescence-labeled phalloidin staining we detected disorganized early fusomes and large actin agglomerates in the region where fusomes are gradually transformed into ring canals (Fig. 2c). In the larval stages, the maintenance of fusome integrity is dependent on an elevated juvenile hormone (JH) titer [76,77]. In adult bees, however, other regulatory mechanisms will have to be considered, since the JH titers in egg-laying queens and workers are much lower than in older, non-laying workers [32,73], due to repression by an elevated vitellogenin titer [27].

The presence of the queen is a critical factor in the progression of oogenesis in the worker ovary, and it is especially the queen's pheromones that are thought to exert a repressor action [17,41,50,93]. For this reason we investigated whether workers reared in the absence of the queen since the embryonic stage differ from workers reared in the presence of the queen with respect to PCD incidence along the ovarioles. TUNEL labeling revealed foci of cell death in the upper germarial regions, coincident with the occurrence of pycnotic nuclei (Fig. 2b) and with actin cytoskeleton disintegration [82]. In addition

Figure 1. Diagram of a single ovariole of an adult honey bee queen. The drawing is a reconstruction from 3 μm histological resin sections that were stained with methylene-blue/basic fuchsin [31]. It starts with the proximal portion of the terminal filament, just above the germarium. The germinal filament consists of stacked disc-shaped somatic cells, but also contains interspersed groups of putative germline stem cells [82]. The apical part of the germarium shows the mitotic divisions of germline cells, followed by the condensation of fusomes in the central region of the cystocyte clusters, and further below, the zone of transformation of the fusomes into ring canals, accompanied by a structural reorganization of the clusters into comet-shaped arrangements. The latter exhibit a clear trophocytes/oocyte polarity and are gradually invested and separated from one another by prefollicular cells. Follicles consisting of nurse cell and egg chambers are sequentially pinched off from the basal region of the germarium. The most basal follicle in this diagram shows the trophic stalk through which the nurse cells release their products into the oocyte. This stalk contains many membranous inclusions and mitochondria. Note that investing membranes of the ovariole had been removed before fixation.

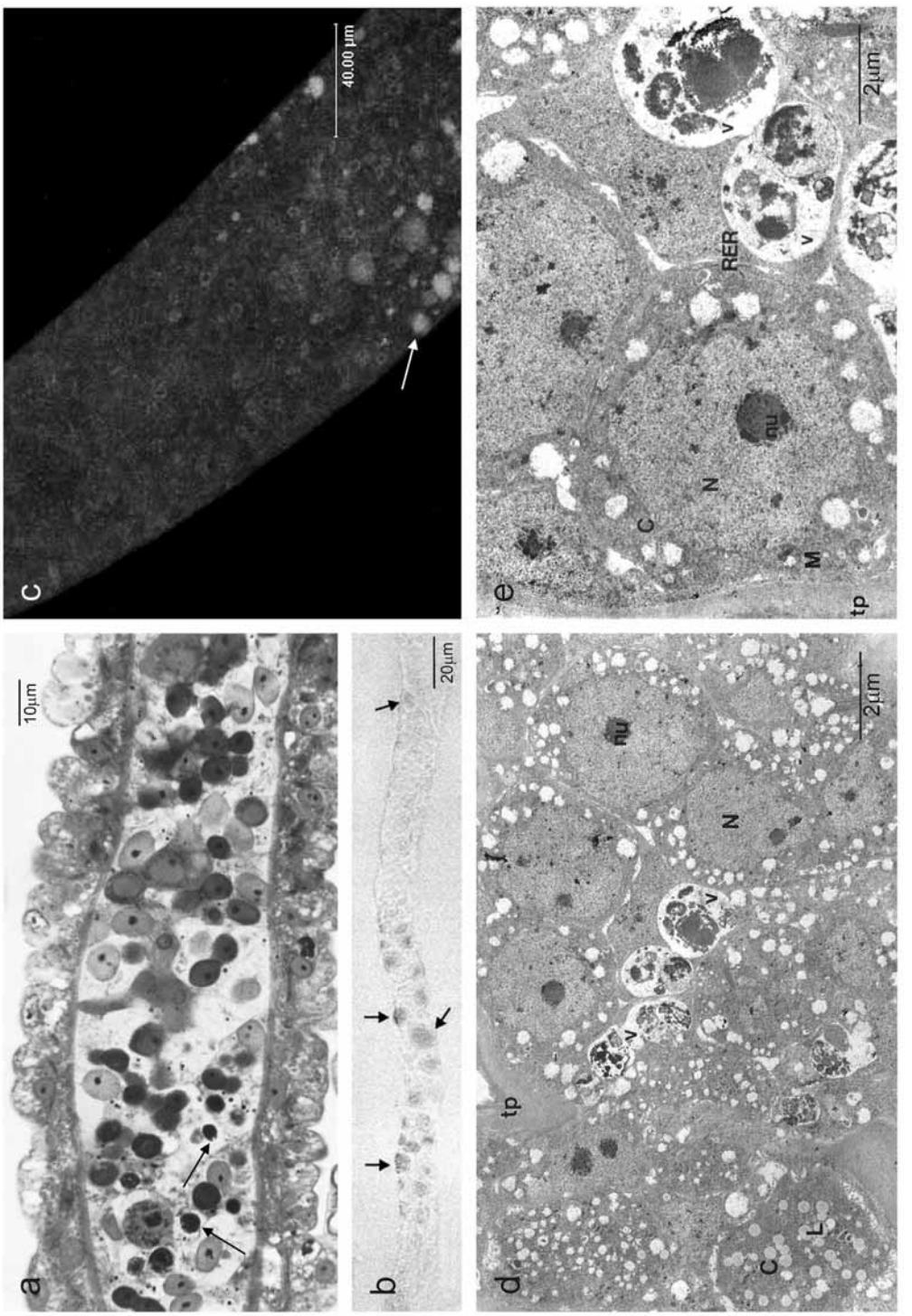


Figure 2. Programmed cell death in the ovary of adult honey bee workers kept under normal colony conditions (queen present). **A.** Histological section (3 µm) of a worker ovariole showing pycnotic nuclei (**arrows**) in the germarial region where germ cells undergo mitotic divisions. **B.** DNA fragmentation evidenced by TUNEL-labeled nuclei (**arrows**) is frequently detected in this same region of the germarium. **C.** TRITC-phalloidin staining reveals the formation of actin agglomerates (**arrows**) just below the germarial region where fusomes are transformed into ring canals. **D** and **E.** Ultrastructure of the germarium of guard bee ovarioles showing many cells in the final stages of programmed cell death; The cytoplasm of prefollicular cells (somatic cells) and also of germline cells contains large autophagic vacuoles; **C** – cystocyte, **L** – lipid vacuole; **M** – mitochondria, **N** – nucleus, **nu** – nucleolus, **RER** – rough endoplasmatic reticulum, **tp** – tunica propria, **V** – vacuole.

we detected TUNEL-labeled cells in the terminal filament, in the trophic chamber of growing follicles and in follicle epithelial cells. The principal region of cell death in the ovary of worker bees corresponds to region 2a/2b in the *Drosophila* ovary, where Drummond-Barbosa and Spradling [20] evidenced a local control of PCD, suggesting a conserved mechanism regulating PCD in the early stages of oogenesis. Workers reared in the presence of the queen showed a higher incidence of PCD signs in the germarium than orphan workers, especially those that were never exposed to the queen's pheromones.

With progressive age and the transition of a worker from the nurse bee to the forager stage, cell death signs in the ovary become ever more pronounced. Ultrastructure analyses revealed that the germarium in ovarioles of guard bees and foragers is severely reduced and shows the typical figures of advanced autophagy (Figs. 2d-e). In older foragers it is only the delimiting *tunica propria* and the peritoneal sheath that are left of the ovarioles.

Analyses of ovariole structure have also been performed in stingless bees (Meliponina), the sister group of the honey bees (Apina), in particularly in *Scaptotrigona postica, Tetragonisca angustula* and *Frieseomelitta xanthopleura,* as well as in other species of bees [75]. Pycnotic nuclei were detected in the ovaries of all species, indicating that cell death is a common phenomenon in the worker caste ovary. It is generally associated with advanced age and the change in function from the nurse bee to the forager stage. There is considerable variability, however, in ovary function and worker egg-laying in the nurse bee stage of stingless bees, so that the regulation of cell death may play a role in the species-specific patterns of worker reproduction in honey bees and in stingless bees. The employment of cell death in the control of worker reproduction has been taken to the extreme in *Frieseomelitta varia*, where the ovary is completely degraded during the pupal stage and is transformed into a storage organ-like structure [7].

Programmed cell death and the establishment of alternative ovary phenotypes in eusocial bees

The degeneration of the entire ovary during postembryonic development in the stingless bee genus *Frieseomelitta* marks one extreme in ovary phenotype development in highly eusocial bees [80,81]. The other extreme are stingless bee genera where workers and queens do not differ in basic ovary structure, that is, ovariole number and ovary functionality [21], or the primitively eusocial bumble bee workers in the competition phase of the colony cycle [24]. An intermediate situation characterized by a high level of phenotypic and developmental plasticity is the ovary of the honey bee. There is not only a considerable variation in ovariole number between the different species in this genus (Table 1), but also is the high ovariole number of queens and the strongly decreased ovariole number in workers [78], a result of a gradual and sequential decision process. This has been shown in experiments where queens were reared from worker larvae transferred to queen cells at different ages [90]. We noted a significant reduction in ovariole number when these queens had been reared from worker larvae that had already passed the second larval instar before being transferred [19].

Like all the other morphological and anatomical caste characters of honey bees, this caste-specific development of ovary phenotypes is a result of differential feeding of the larvae in the early stages, and of a marked nutritional switch in the fourth and early fifth larval instar. This differential nutrition causes a caste-specific activation of the corpora allata [62] and of the prothoracic gland [31], resulting in a high JH titer in young queen larvae and an earlier increase in the ecdysteroid titer in fifth instar queen larvae, when compared to workers [63,70].

Investigation of the dynamics of ovary development in the honey bee began ninety years ago [94]. Both castes start out with a similar number of ovariole primordia (150 and more) in each ovary, yet while this high ovariole number is maintained in queens, workers loose over 90% of these until pupa-

Table I. Variation in ovariole numbers in queens and workers of different honey bee species and in two subspecies of *Apis mellifera*. Indicated are mean numbers of ovarioles per ovary (reported ranges in parentheses). Data from Velthuis [85] and Ruttner [74].

Apis species or subspecies	queen ovariole number	worker ovariole number
Apis mellifera ligustica	173 (155-190)	3.3 (1-24)
Apis mellifera mellifera	162 (127-183)	5.3 (1-12)
Apis cerana	73	8.6 (4-21)
Apis dorsata	130	33 (17-60)

tion. A detailed analysis of ovariole ultrastructure in the larval stages has revealed a large number of degenerating germline cells in worker ovarioles at the beginning of the fifth larval instar [33,69]. TUNEL labeling of larval ovaries confirmed the ultrastructural evidence of PCD in the worker caste [76]. Apoptotic cells are first detected in late feeding-staged larvae in the central region of the ovariole primordia (Fig. 3b), when germline cells start to undergo the incomplete mitotic divisions leading to the formation of germ cell clusters. During the subsequent phases of the fifth instar, the number of TUNEL-labeled cells increases and reaches also the apical and basal regions of the ovarioles, leading to the entire disintegration of most ovarioles. In queen ovaries, cell death is a rare and sporadic event during the larval stages. This guarantees that the high number of ovariole primordial established in the early larval stages is maintained throughout metamorphosis and until emergence of the adult queen. Complementary labeling of S-phase nuclei by incorporation of the bromodeoxyuridine (BrdU) showed that cell death in worker ovarioles in the fifth larval instar is preceded by mitotic activity [76]. The passage through a mitotic cycle before a worker bee's germ cells initiate a cell death program can be interpreted as part of a conserved mechanism, as it has also been encountered in mammalian tissues [1]. It may represent a mechanism in cell death induction that is characteristic for eukaryotic cells that die prior to having reached a state of terminal differentiation.

The massive cell death detected by TUNEL labeling in late feeding stage worker larvae is only the final manifestation in a series of PCD events, since in our ultrastructure analyses we could detect cells exhibiting advanced stages of degeneration already in ovarioles of early fifth-instar worker larvae (Fig. 3a). Sporadic cell death in early larval stages has also been reported by Reginato and Cruz Landim [68]. The temporal coincidence of PCD events in worker larvae with the appearance of numerous cystocyte clusters as a consequence of incomplete cytokinesis in germ cells led us to investigate the cytoskeleton structure in developing bee ovaries. TRITC-phalloidin labeling revealed that F-actin constitutes a major component of the polyfusomes that start to form in the germ cell region of fourth larval instar ovaries in both castes. While the number and size of polyfusomes markedly increases in fifth-instar queen ovaries (Fig. 3c), the F-actin distribution in worker ovaries changes dramatically, with many cells exhibiting apparently unorganized actin agglomerates in their cytoplasm (Fig. 3d). In terms of timing, the manifestation of a disorganized actin cytoskeleton, thus, precedes the onset of massive cell death that was detected by TUNEL labeling, and in terms of functional significance, this observation resembles the situation described in the ovaries of adult bees, where actin agglomerates were also detected in ovariole regions that showed a high incidence of pycnotic nuclei (see Fig. 2).

The timing of PCD events in the differentiation of the alternative ovary phenotypes in honey bees suggested that the caste-specific differences in the hemolymph JH titer may be critical for the decision whether PCD is initiated or not in the germ cell region of the developing ovary. When we applied JH to fourth-instar worker larvae, a protocol frequently employed in studies on caste development in honey bees and stingless bees [10,61,71], we observed that an artificially elevated JH titer diminished the mitotic activity in the worker ovary during the critical phase of PCD induction and also prevented disintegration of the fusomal and cortical actin cytoskeleton [76,77]. Such a parallel action of JH on DNA synthesis and cytoskeleton organization has also been observed in salivary glands of the fruit fly, *Drosophila melanogaster*, and the tobacco hornworm, *Manduca sexta* [38,39].

FINAL CONSIDERATIONS

Comparing PCD characteristics observed in the larval and adult ovary of honey bee workers suggests that maintenance of the fusomal actin cytoskeleton plays an important role in preventing the induction of cell death in the early stages of oogenesis. This early decision of whether to keep a germ cell cluster alive or drive it to apoptosis via cytoskeletal degradation has primarily been detected in the ovaries of social bees. It has not been described in such detail in other insects, including *Drosophila*, where it has been reported to be limited to the narrow 2a/b zone [20].

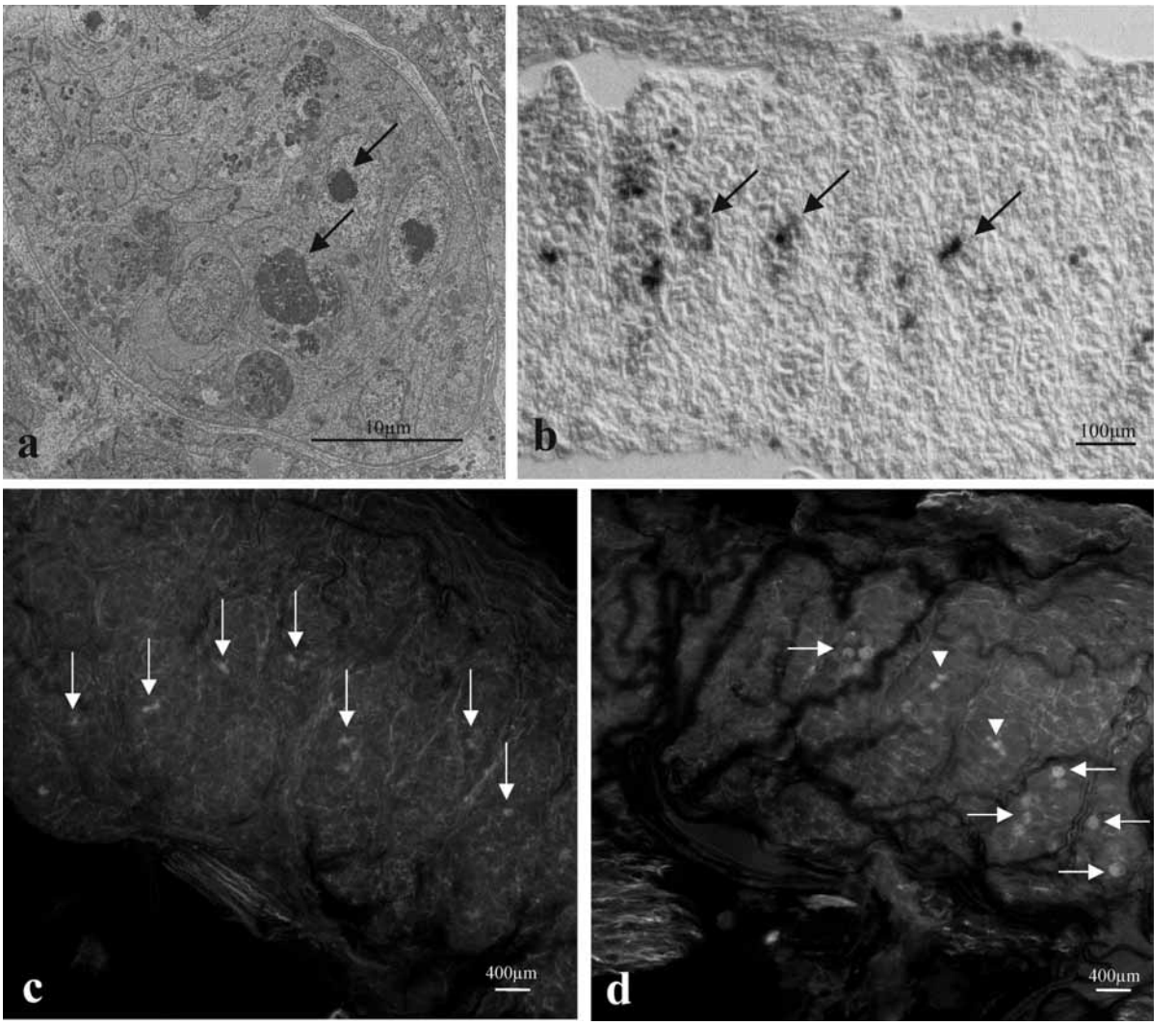


Figure 3. Programmed cell death in the ovary of honey bee larvae. **A**. Ultrastructure of germ cell cluster in the ovary of early 5th instar worker larva, arrows indicate degenerating nuclei. **B.** DNA fragmentation in the ovary of late 5th instar worker larva, the arrows point to some of the TUNEL labeled nuclei. **C.** TRITC-phalloidin staining in the ovary of early 5th instar queen larva, F-actin is organized and forms the central material of fusomes (**arrows**). **D.** TRITC-phalloidin staining in the ovary of early 5th instar worker larva, the F-actin cytoskeleton is disorganized and shows agglomerates (**arrows**), a few well organized fusomes are still visible (**arrowhead**).

The long germarium of the honey bee ovary with its numerous germline cysts developing in sequence (Fig. 1) facilitates the detection of these events. Also in the context of ovariole number determination, the honey bee ovary is an interesting playground for investigations into this important fitness parameter, which is not easily studied in other insects due to a much reduced natural variation in ovariole number. There is variation, however, in many insect species, and we know very little about the determinants of such variation. Studies on ovariole number determination in *Drosophila* have revealed both genetic factors and environmental influence, primarily nutrition, to affect this fitness character [36,89]. In honey bees, larval nutrition is clearly the major determinant of ovariole number [19], yet recently, we could detect also a genetic component. This was revealed by a microsatellite analysis of worker genotype, which

showed a strong correlation between paternal genotype of a worker with the number of ovarioles in her ovaries and the facility with which these can become activated [45]. We expect this genetic component to be part of an apoptosis-preventing mechanisms that allows the survival of more than an average number of ovarioles past the critical larval stages.

The genetic architecture and proximate mechanisms that induce or prevent PCD in the insect ovary have been mainly studied in *Drosophila*. Even though investigations into mechanisms underlying PCD in bee ovaries have only just begun, they already added a new facet to the picture, namely the role of JH in regulating the proliferation rate of larval germ cells and in the maintenance of the fusomal and cortical actin cytoskeleton in germline cysts. It is important to note in this context that JH probably plays this protective role primarily in the larval stages. Based on measurements of JH titers in adult bees, this hormone, however, is probably not involved in preventing PCD in ovaries of adult queens and workers.

At this point it is interesting to speculate which factors may actually be involved in the local control of PCD induction in the oogenesis progression in adult bees. Since nutrition, especially a protein-rich diet is of general importance for insect fertility, it could be possible that the ancient pathway of insulin signaling [8] has been remodeled into a control function for oogenesis in the alternative ovary phenotypes of bees. With the release of version 3.0 of the honey bee genome (http://hgsc.bcm.tmc.edu/projects/honeybee) such factors, as well as other candidate genes, are now becoming amenable to expression studies in the honey bee castes, as a model for large-scale PCD in the insect germline.

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