Regular Paper

EFFECT OF TOPICAL APPLICATION OF JUVENILE HORMONE ON THE OCCURRENCE OF CELL DEATH IN THE LARVAE OF HONEYBEE (*Apis mellifera)* *

William Fernando Antonialli Jr[1] and Carminda da Cruz-Landim[2]

[1]State University of Mato Grosso do Sul (UEMS) at Dourados, Dourados, MS,
[2]Department of Biology, Institute of Biosciences at Rio Claro, Paulista State University (UNESP), Rio Claro, SP, Brazil.

ABSTRACT

Workers and queens of *Apis mellifera* present marked dimorphism evidenced by the great development of queen ovaries and atrophy of worker ones as a result of differential diets given by the nurse workers to the developing larvae. The present paper reports the results of an investigation on how the administration of juvenile hormone (JH) to worker larvae affects the cell death in their ovaries. Larvae from 2nd and 3rd instars received topical application of JH III diluted in hexane in the concentration of 1µg/µL. The ovaries of the treated and non-treated larvae (control) were collected in the 3rd, 4th and 5th instars and prepared for cell death observation by staining with Hoechst and Propidium Iodide and by labeling with TUNEL reaction. The results showed that the ovaries of the control larvae present higher rates of cell death, in all ages, than those found on the JH-treated larvae ovaries and that the effect of the aforementioned treatment was independent of the larvae stage to which it was applied being thus the same for the 2nd and 3rd instars. The results suggest that the hormone application partially inhibited the cell death in the workers ovaries thus affecting the key characteristic essential in the differentiation of larvae fated to be workers and queens. The exogenous administration of JH to workers larvae reproduces the diet effect responsible for castes differentiation in *Apis mellifera* indicating a close relationship between the quality of larval nourishment and juvenile hormone production, which ultimately evidences the role of the diet in the expression of genes linked to JH synthesis.

Key words: *Apis mellifera,* cell death, female castes, juvenile hormone, ovary

INTRODUCTION

Cell death has long been observed during development and throughout the entire lifetime of living organisms. The type of cell death is, in general, classified according to its occurrence, morphology or biological function. Glucksmann [14] and Hurle [18] classified the cell death according to its biological role in: 1. phylogenetic death, when responsible by the loss of vestigial structures or organs during the development; 2. metamorphic death, when taking place during tissues and organs remodeling in organisms suffering metamorphosis; and 3. histogenic death, when occurring during differentiation and maturation of the organism. Saunders [33] referring to these physiological cell death used the expression "death clock" meaning that each cell type has an innate determined lifespan and are, therefore programmed from the beginning to death.

Henceforth, as well established since long ago, the programmed cell death has an important role in the organism modulation and functioning during the embryonic development, morphogenesis and metamorphosis as it has been observed in some organisms as the holometabolous insects. Besides, the programmed cell death is important in regulating the size of cell population during the growth and maturity phases, by exerting a force equal and opposite to mitosis. This kind of programmed cell death, generally called apoptosis, occurs without causing inflammatory reactions in the tissues, and is morphologically and physiologically well defined [5,17,19,23,28,35,36].

Correspondence to: Dr. William Fernando Antonialli Junior
Universidade Estadual do Mato Grosso do Sul (UEMS), Cidade Universitária de Dourados, Rodovia Dourados-Itahum, KM 12, CEP 79804-970, Dourados, MS, Brasil. E-mail: antonialli@uems.br
*Research performed in the laboratories of the Biology Department of Biosciences Institute (UNESP)

Although the cells are programmed to die at a certain time, several exogenous factors, others than non-physiological stimulation, such as virus infections or other causes of illness [2,4,10,21,37], may unchain the cell death programming such as hormones, cytokines, interleukins, and so on. The presence or absence of specific growth factors, the increase or decrease of hormonal titers may promote or suppress the activation of the programmed cell death [8,13]. In addition, the same stimulus may produce opposite effects in different cell types [1].

During the occurrence of the apoptosis, the DNA is cleaved by the endonucleases activation, preferentially at the internucleosomal sites, leaving free ends that can be identified by TUNEL reaction (Terminal-transferase dUPT Nick end Labeling). The DNA fragments under electroforesis in agarose gel gives a characteristic ladder pattern [37].

In eusocial bees such as *Apis mellifera* in which the ovaries present a differential development in the female castes, these organs constitute a good model for studying the role of cell death in the modulation of the castes fertility. Both castes, the queens - the fertile females, and the workers - the sterile females, originate from the same kind of egg, undergo the same embryonic development, only diverging during their larval development due to the differential type of nutrition that they receive. The castes differentiation in *A. mellifera* is, therefore, determined by their larval diet [3,22,34].

While 2 to 3-day-old larvae fated to be queens are fed with royal jelly, the same aged larvae fated to be workers receive a less rich diet from the nurse workers, what downregulates the rates of juvenile hormone (JH) production and, consequently, its titer in the hemolymph. The hormones titers, mainly the JH but also the ecdysteroids are, therefore, directly responsible for the changes that occur in the post-embryonic determination of castes in bees [7,9,15,25,27,32]. Hartfelder *et al.* [16] found that the titers of this hormone affect the ovarian development during lifetime. Nevertheless, the morphological features of the mechanisms causing the atrophy that takes place in the workers ovaries are poorly known. The differential nutrition and its eventual implication on JH titers is the unchaining factor of the expression of the genes that control the castes differentiation. How this expression manifests itself in the ovaries is the scope of this work. Therefore, this work's intent is to verify the effect of extra doses of JH in the death of ovary cells when these doses are topically applied to workers larvae of *Apis mellifera* in the 2nd and 3rd instars or more precisely between 48 and 72 h after larvae eclosion.

MATERIAL AND METHODS

Brood combs isolated by wire screen were made available of a queen with a good performance of posture, for a period of approximately 6 h. The comb containing the eggs was, afterwards, transferred to a queenless colony where they were left to develop. The 48 h-old- (2nd instar) and the 72 h-old- (3rd instar) ecloded larvae were separated in two groups. One group received a topical treatment with 1 µL of JH III (Sigma) diluted in hexane in the concentration of 1 µg/µL and the other was the non-assisted control group. The application was performed directly on the comb by using an automatic micropipette Gibson P². The development observed in the assisted larvae was compared to the one observed in the non-assisted larvae of the control group.

The developmental instar of the larvae was determined by measuring the width of the cephalic capsule and by applying the Dyar law [11]. The results showed that each instar lasts 24 h. Therefore, samples of the JH-treated and JH-non-treated larvae were collected at each 24 h after the hormone administration, a procedure which was maintained up to the 6th day after larvae eclosion (fifth instar). Afterwards, the larvae were anesthetized by cold and had their ovaries dissected. Three larvae, or six ovaries of each larval age were studied.

Ovaries staining

The ovarioles of the dissected ovaries from both the JH-treated larvae and the control larvae were released by the extraction of the outer capsule of the organ, and prior to fixation the ovarioles were incubated in a solution of one drop of Hoechst at 0.001% in sterile distilled water during 10 min, followed by incubation in Propidium Iodide (2.5 µg/µL) during 5 min. Immediately after staining the ovaries were examined and photographed under a fluorescence microscope (Leica DMLB) using a UV excitation filter of 365 nm and a barrier filter of 400 nm.

TUNEL labeling

After releasing and dissecting the ovarioles as described above, the ovaries of both experimental and control larvae groups were fixed in phosphate buffered 4% paraformaldehyde and afterwards they were examined using the *in situ* TUNEL technique to detect cell death according to the POD-1684-817 kit instructions. The negative control was performed without the treatment with T-d-T/labeled-deoxinucleotide.

RESULTS

The staining of the fresh ovaries by the combination of Hoechst and Propidium Iodide treatments allows the distinction between health and damaged cells because the Hoechst, a supravital stain, stains the nuclei of health cells while the Propidium Iodide only stains the ones with changed membrane permeability. The ovaries of 48 h-old-larvae (2nd instar) treated with JH (Fig. 1A-D) presented a smaller number of cells stained by the Propidium Iodide, in all of the subsequent instars, than the one observed in the control group larvae at the same development stage (Fig. 1H-L). The same was observed on the 72 h-old larvae (3rd instar) which were treated with hormone (Fig. 1E-G), when comparing them with the control group larvae. However, no visible differences in the number of stained cells were observed as a result of the hormone administration being applied either at the 2nd or at the 3rd instars.

Similarly, the results obtained with the TUNEL technique showed less labeled nuclei in the ovaries of the JH-treated larvae (Fig. 2A-G) than in the ones of the control (Fig. 2H-L). Nevertheless, with this technique it was observed that the treatment in the 2nd instar was more effective in preventing cell death in the subsequent instars (Fig. 2A-D). The negative control of the test, does not show labeled nuclei proving the validity of the results (Fig. 2M-O).

DISCUSSION

When a colony of *A. mellifera* looses the queen the workers produce new queens by feeding 2 to 3-day-old larvae with just royal jelly. Older than 2 to 3-day-old larvae fed in the same way will not result in a well developed queen. One of the differences between the queen and the worker is on their ovary size. While each queen ovary consists of 180 to 200 long ovarioles, the ovary of the worker has only 2 to 12 short ones. Therefore, although the worker ovary is capable of producing eggs the potential fertility of this caste is several times much lower than that of the queen and, in addition, the workers cannot mate. It becomes clear that the differentiation between both castes is a post-embryonic event due to a less rich nutrition received by the larvae fated to be worker from the 2nd or 3rd day on, after eclosion. It is believed that the worker inflicts itself a kind of nutritional castration which generates a drastic reduction in the number and length of their ovarioles.

The JH prevents cell death in several tissues of insects [8]. Knowing that there are differences in the endogenous hormones titers between the two castes and that this was correlated with their differentiation, the possibility of manipulating their hemolymph contents by reprogramming the tissues and organs differentiation at the molecular and cellular levels exists.

Both Hoechst and Propidium Iodide methods used in this study showed that cell death takes place at a great rate in the ovaries of the control larvae. Although the stains here used do not inform the type of death affecting the cells they are useful to differentiate intact, healthy cells stained by the Hoechst, from the ones with impaired membrane permeability stained by the Propidium Iodide [20,31].

In accordance with previous results [29,30], the present study shows that the cell death was observed in the control ovaries since the 3rd instar. The high rates of cell death found in the 3rd, 4th, and 5th instars of the control ovaries, ought to be due to the lower titers of JH present in worker larvae hemolymph [6,7,15,27], which were partially diminished in the case of the experimental larvae due to the topical application of JH. The hormone concentration applied was chosen because previous experiments done with similar objective showed its effectiveness in provoking changes in the physiology of some organs of bees [1,12,24].

At the 5th instar the rates of cell death increased even in the ovaries of the treated larvae. In this late instar the titers of ecdysteroids were already increasing while the JH was almost vanished thus increasing the general rates of cell death [13,16,25, 26,27], a phenomenon typical of the beginning of the metamorphosis. In the pre-pupae queen an increase of the JH titer [25,26] prevents the induction of cells death in the ovaries guaranteeing the survival of the ovarioles. Therefore, although the extra dose of JH applied in the 2nd or 3rd instar is enough to partially prevent cell death during the larval phase in the treated workers it does not prevent the death that takes place in late last larval instars and pupation because the JH is not produced in worker larvae, as it occurs in queen.

The labeling with TUNEL evidenced a lower number of dead cells than what was observed when the Propidium Iodide procedure was used. A possible explanation is that the Propidium Iodide

labels any kind of damaged cells while the TUNEL reaction marks exclusively the cells where DNA fragmentation occurs, which on the other hand, is not the only cell death type occurring in the ovaries [30]. In fact, the Propidium Iodide stains nuclei of every cells with disturbed membrane permeability, an event that precedes cell death.

In conclusion, the present study showed that the exogenous application of juvenile hormone prevents cell death in the worker ovaries of *Apis mellifera* honeybees during their larval development hence simulating the functional role of the special enriched nutrition received by the queen larvae. Actually, the nutrition acts by regulating the hemolymph titers of JH in the castes, supposedly by modulating the expression of JH-synthesis linked genes. The results presented here also confirm that apoptosis is not the only type of programmed cell death occurring in bees ovaries [30].

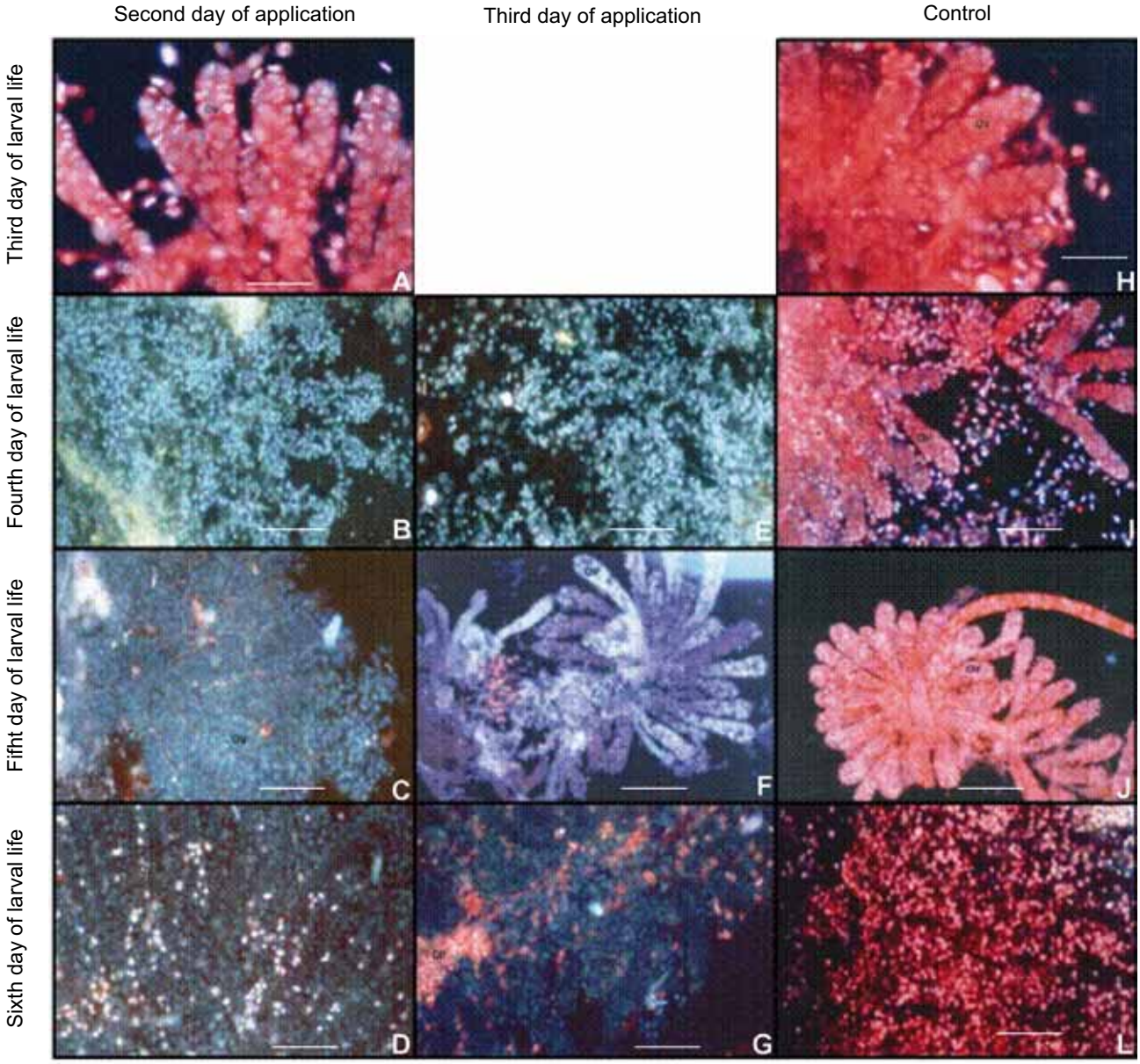

Figure 1. Ovarioles from 2 and 3 days old worker larvae of *A. Mellifera* treated with topical application of 1µL of JH (**A**-**G**), and control without treatment (**H**-**L**). The health cells have the nuclei stained in blue (Hoechst). The nuclei of the dying are stained in red by the Propidium Iodide. **ov**-ovarioles, **ce**-ovary capsule. **A**, **B, H,** Bars = 30 µm; **C**, **D**, **E**, **G, I**, **L,** Bars = 50 µm; **F**, **J,** Bars = 80 µm. (Panels F and J, after Cruz-Landim et al., 2006, Braz. J. Morphol. Sci. 23:27-42).

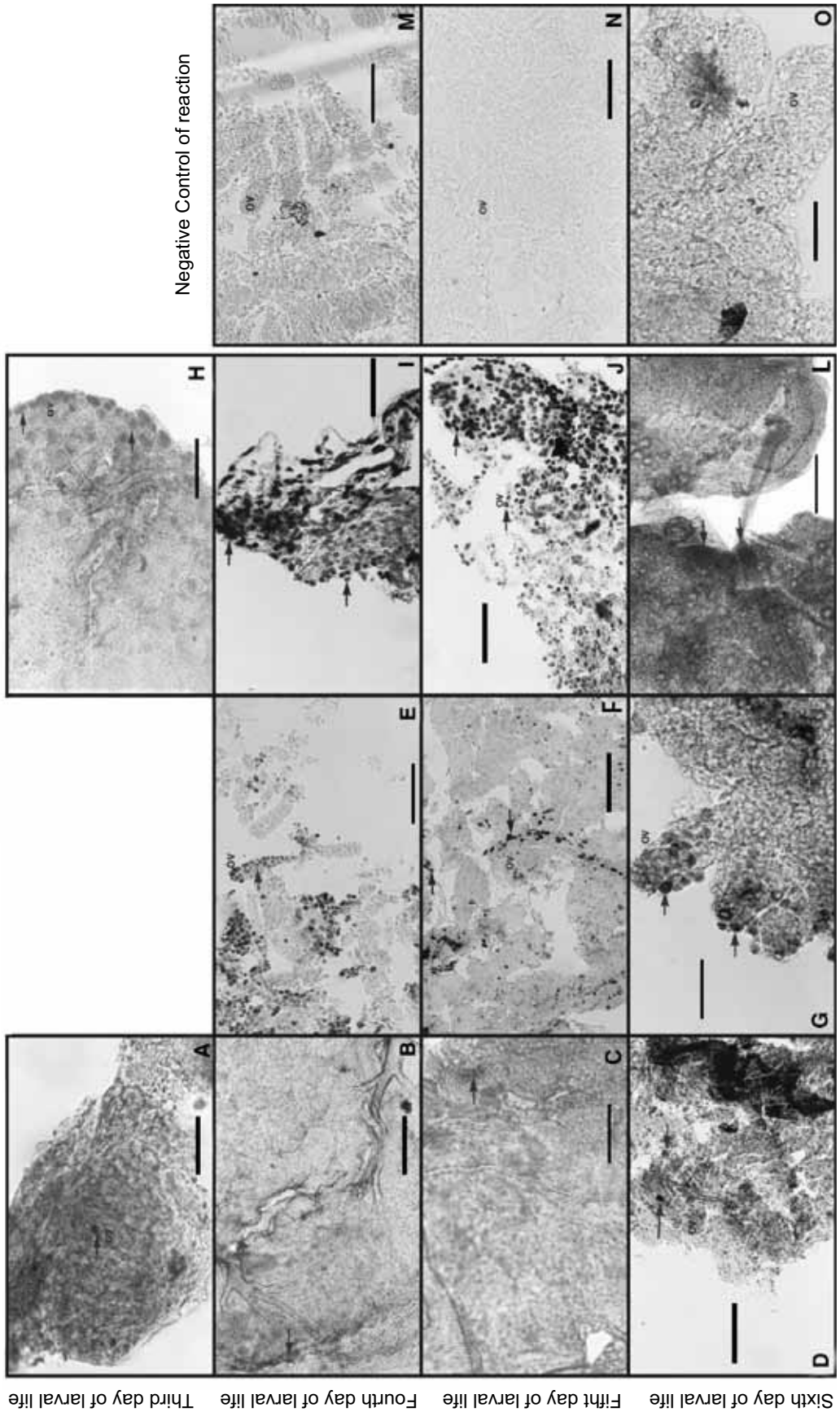

Figure 2. Ovarioles of experimental (**A-G**), control larvae (**H-L**) and negative control (**M-N**) of *A. mellifera* 2 and 3 days old labeled with TUNEL showing positive reaction to cell death (arrows). **A, G,** Bars = 20 µm, **B, C, H, L, O,** Bars = 35 µm; **D, E, F, I, J, M, N,** Bars = 50 µm.

ACKNOWLEDGMENTS

Financial support for this study was provided by the *Fundação de Amparo à Pesquisa do Estado de São Paulo* (FAPESP), grant # 98/15057-6. (The State of São Paulo Research Foundation) and by the *Conselho Nacional de Desenvolvimento Científico e Tecnológico*/CNPq (National Council for Scientific and Technological Development) and to Prof. Dr. Elza da Costa Cruz Vasconcellos for language correction.

REFERENCES

1. Abdalla FC, Gracioli LF, Salles HC, Cruz-Landim C (2001) Effect of topical application of juvenile hormone (JH) in honeybee worker larvae on the development of Dufour's and Koschewnikow's glands. *Sociobiology* **37**, 185-191.
2. Arends MJ, Wyllie AH (1991) Apoptosis: mechanism and roles in pathology. *Int. Rev. Exp. Pathol.* **32,** 223-254.
3. Asencot M, Lensky Y (1976) The effect of soluble sugars and juvenile hormone on the differentiation of the female honeybee larvae (*Apis mellifera L.*) to queens. *Life Sci.* **18,** 693-699.
4. Bowen ID, Bowen SM (1990) *Programmed Cell Death in Tumours and Tissues.* Chapman and Hall: London.
5. Bursch W, Kleine L, Tenniswood M (1990) The biochemistry of cell death by apoptosis. *Biochem. Cell Biol.* **68**,1071-1074.
6. Capella ICS, Hartfelder K (1998) Controle hormonal do desenvolvimento pós-embrionário dos ovários de *Apis mellifera*. In: *Anais do 3 Encontro sobre Abelhas,* Ribeirão Preto (SP), Brazil. p. 207-211.
7. Copijn GM, Beetsma J, Wirtz P (1979) Queen differentiation and mortality after application of different juvenile hormone analogues to worker larvae of the honeybee (*Apis mellifera* L.). *Proc. K. Ned. Akad. Wed. Ser- C* **82**, 29-42.
8. Dai JD, Gilbert LI (1998) Juvenile hormone prevents the onset of programmed cell death in the glands of *Manduca sexta. Gen. Comp. Endocrinol.* **109,** 155-165.
9. Dietz A, Herman HR, Blum MS (1979) The role of exogenous JH-I, JH-II, JH-III and anti-JH (Precocene II) on the queen induction of 4.5 day-old-worker honey bee. *J. Insect Physiol.* **25**, 503-512.
10. Doi T, Nishida K, Matsuo M,Yoshida A, Murakami T, Inoue H (2002) Evidence of oncotic cell death and DNA fragmentation in human hypertrophic chondrocytes in chondro-ostehyte. *Osteoarthritis Cartilage* **10**, 270-276.
11. Dyar HG (1890) The number of molts of lepidopterous larvae. *Psiche* **5,** 420-422.
12. Farinha EK, Torneiros LM, Silva-de-Moraes RM, Cruz-Landim C (1988) Influência hormonal sobre o conteúdo de DNA nas células da glândula salivar larval de *Melipona quadrifasciata anthidioides* Lep. (Apidae, Meliponinae). I. Hormônio juvenil. *Naturalia* **13,** 75-83.
13. Fujiwara H, Ogai S (2001) Ecdiysteroid-induced programmed cell death and cell proliferation during pupal wing development of silkworm, *Bombyx mori*. *Dev. Genes Evol.* **211,** 118-123.
14. Glücksmann A (1951) Cell deaths in normal vertebrate ontogeny. *Biol. Rev. Cambridge Philosoph. Soc.* **26,** 59-86.
15. Hartfelder K, Engels W (1998) Social insect polymorphism: hormonal regulation of plasticity in development and reproduction in the honeybee. *Curr. Top. Dev. Biol.* **40**, 45-77.
16. Hartfelder K, Köstlin K, Hepperle C (1995) Ecdysteroid-dependent protein synthesis in caste-specific development of the larval honey bee ovary. *Roux Arch. Dev. Biol.* **205**, 73-80.
17. Huppertz B, Frank HG, Kaufmann P (1999) The apoptosis cascade -- morphological and immunohistochemical methods for its visualization. *Anat. Embryol.* **200**, 1-18.
18. Hurle JM (1988) Cell death in developing systems. *Methods Achiev. Exp. Pathol.* **13,** 55-86.
19. Kerr JFR, Willie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wider ranging implications in tissue kinetics. *Br. J. Cancer* **26,** 239-257.
20. Locke SJ, Peng YS, Cross NLA (1990) Supravital staining technique for honey bee spermatozoa. *Physiol. Entomol.* **15**, 187-192.
21. Majno G, Joris I. (1995) Apoptosis, oncosis, and necrosis. An overview of cell death. *Am. J. Pathol.* **146,** 3-19.
22. Michener CD (1974) *The Social Behavior of Bees*. Harvard University Press: Cambridge.
23. Osborne BA, Schwartz LM (1994) Essential genes that regulate apoptosis. *Trends Cell Biol.* **4**, 394-398.
24. Paes-de-Oliveira VT, Cruz-Landim C (2001) Experimental control of the extra doses of juvenile hormone on bee development: the case of the wax glands of *Apis mellifera* (Hymenoptera: Apidae). *Sociobiology* **38,** 513-521.
25. Pinto LZ, Hartfelder K, Bitondi MM, Simões ZLP (2002) Ecdysteroid titers in pupae of highly social bees relate to distinct modes of caste development. *J. Insect Physiol.* **48**, 783-790.
26. Rachinsky A, Engels W (1995) Caste development in honeybees (*Apis mellifera)*: juvenile hormone turns on ecdysteroids. *Naturvissenchaften* **82,** 378-379.
27. Rachinsky A, Strambi C, Strambi A, Hartfelder K (1990) Caste and metamorphosis: hemolymph titers of juvenile hormone and ecdysteroids in last instar honeybee larvae. *Gen. Comp. Endocrinol.* **79**, 31-38.
28. Raff MC, Barres BA, Burne JF, Coles HS, Tshizaki Y, Jacobson MD (1993) Programmed cell death and control of cell survival: lessons from the nervous system. *Science* **262**, 695-700.

29. Reginato RD, Cruz-Landim C (2001) Differentiation of the worker's ovary in *Apis mellifera* L. (Hymenoptera, Apidae) during life of the larvae. *Invert. Reprod. Develop.* **39,** 127-134.
30. Reginato RD, Cruz-Landim C (2002) Morphological characterization of cell death during the ovary differentiation in worker honey bee. *Cell Biol. Intern.* **26,** 243-251.
31. Reid S, Cross R, Snowe C (1996) Combined Hoechst 33342 and merosyanine 540 staining to examine murine B cell cycle stage, viability and apoptosis. *J. Immunol. Meth.* **192,** 43-54.
32. Rembold H, Czoppelt CH, Rao PJ (1974) Effect of juvenile hormone treatment on caste differentiation in honeybee *Apis mellifera. J. Insect Physiol.* **127,** 1193-1202.
33. Saunders JW (1996) Death in embryonic system. *Science* **154,** 604-612.
34. Snodgrass RE (1956) *Anatomy of Honeybee.* Comstock Publishers: Ithaca.
35. Steller H (1995) Mechanism and genes of cellular suicide. *Science* **267,**1445-1449.
36. Wyllie AH, Kerr JFR, Currie AR (1980) Cell death: the significance of apoptosis. *Int. Rev. Cytol,* **68,** 251-306.
37. Wyllie AH, Duvall E, Blow JJ (1984) Intracellular mechanism in cell death in normal and pathological tissues. In: *Cell Aging and Cell Death* (Davies I, Singer DC, eds). pp. 269-294. Cambridge University Press: Cambridge.

_____

Received: March 13, 2006
Accepted: August 14, 2006