MINI-REVIEW

CELL MARKERS FOR ECOTOXICOLOGICAL STUDIES IN TARGET ORGANS OF BEES

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ABSTRACT

Bees are important pollinators that, because of extensive deforestation of their natural habitats, now forage widely in agricultural areas. This interaction with human agricultural activity has led to a reduction in the number of bee species because of contact with widely used pesticides. However, little is known about the adverse effects that exposure to such agents has on bee tissues and organs. In this review, we discuss the morphological alterations induced by environmental contaminants in the midgut and Malpighian tubules of bees; these two organs are involved in the absorption and excretion of toxic compounds, respectively. We also discuss the role of heat shock proteins, also known as stress proteins, in the cellular response to chemical compounds, and the importance of cell death as an indicator of the toxicity of these compounds. The analysis of these two cellular markers may be useful for monitoring bees that forage in agricultural areas.

Key words: Cell death, heat shock protein, larval salivary glands, Malpighian tubules, midgut

INTRODUCTION

Bees are pollinators that contribute to the maintenance of native plants in the wild. However, there has been a gradual decrease in the number of bee species, probably because of habitat destruction caused by deforestation and the widespread use of pesticides to protect a variety of commercial crops. Balestieri *et al.* [2] compared the diversity of orchid bees in protected areas and in areas damaged by human activity and concluded that *Eulaema nigrita* could be a useful bio-indicator for altered areas because of its abundance in such areas compared to natural habitats.

The use of insects as bio-indicators in terrestrial and aquatic ecosystems is of great interest in ecotoxicological studies [8,9] since a great variety of insects can accumulate toxic environmental compounds in their tissues and organs [35]. Morphological studies of insect gut [42,57,71] and Malpighian tubule cells [68] have been used to detect ultrastructural alterations caused by sub-lethal doses of environmental contaminants.

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Although the toxicity of chemical compounds has been studied in many species of stingless bees [10] and in the honey bee *Apis mellifera* [11,12], there have been no studies of the cytoplasmic and nuclear alterations in the midgut cells and Malpighian tubules of bees exposed to pesticides. These organs are involved in the absorption and excretion of chemical compounds, respectively, and an assessment of their morphology can reveal ultrastructural alterations induced by environmental stressors [8,9,68]. Other organs, such as larval salivary glands (silk glands), could also be useful tools for assessing the toxicological effect of chemical compounds in bees. Larval salivary glands provide a good model for studying the mechanisms of programmed cell death [5], particularly because the morphological changes during the degeneration that precedes metamorphosis are well defined [62]. Since these glands remove substances from the hemolymph [1,43,51], they can absorb toxic compounds that are not eliminated by the Malpighian tubules. These compounds can accelerate cell death in the salivary glands and/or alter their gene expression.

Currently, little is known about the adverse effects resulting from exposure to environmental stressor agents. This lack of information makes it difficult to assess the ecological danger of most

environmental contaminants [32]. For this reason, studies of the morphological alterations in bee organs could improve our understanding of the effects of chemicals released into the environment since bees are wide-ranging foragers.

The use of cellular markers provides a means of assessing the toxicity of a given compound in insect organs. One of the most widely used cellular markers is the family of heat shock proteins (HSP), also known as stress proteins. Heat shock proteins have been widely used in ecosystem biomonitoring because high levels of their expression in organisms under stress serve to protect cells and prevent the induction of cell death [4]. Another cellular marker is the fragmentation of DNA in cells undergoing necrosis or programmed cellular death after exposure to an environmental stressor. This fragmentation is mediated by endonucleases [72] and can be assessed immunocytochemically and biochemically.

Few studies have examined the relationship between stress proteins and cell death in bees. The only studies of HSPs and cell death in bee tissues have been with the midgut of *A. mellifera* larvae infected with *Paenibacillus larvae* or *Bacillus larvae* [30,31] and the larval salivary glands of *A. mellifera* treated with acaricides and antibiotics [65,66]. Comparison of the level of HSP expression and cell death in the organs of contaminated adult bees with those of uncontaminated bees could be useful in establishing the relationship between HSPs and cell death. Similar studies in other invertebrate groups have shown an accumulation of stress proteins in animals from environmentally polluted areas [24,25,41].

The enhanced expression of HSPs seen in cells from stressed bees suggests that this phenomenon could be a defense mechanism to prevent against cell death. In this context, the immunocytochemical detection of HSP coupled with an assessment of DNA fragmentation can provide an indication of the level of cellular stress such that cells with a significant increase in HSP expression probably will show little or no DNA fragmentation. In such an analysis, the presence of either a few or a large number of cells with DNA fragmentation may indicate that the stressor substance was active at sub-acute or acute concentrations, respectively, and that the activation of HSPs was insufficient to inhibit cell death (whether by programmed cell death or by necrosis).

In the following discussion, we describe the morphological and molecular alterations generally seen in the organs of bees exposed to environmental stressor agents.

Literature review

Ecotoxicological bio-monitoring has revealed high levels of HSPs in the tissues of invertebrates collected in contaminated areas compared to tissues from animals collected in non-contaminated areas. The persistently high levels of HSPs in the cells of organisms exposed to stress account for the induced or acquired tolerance of these organisms and their adaptation to environmental fluctuations [4].

HSPs are classified into four families based on their molecular mass: HSP90 (90 kDa), HSP70 (70 kDa), HSP60 (60 kDa) and the small HSPs [28]. Although this classification may vary slightly with the presence of additional proteins, depending on the organism [44], each HSP family consists of constitutively expressed proteins as well as proteins that are induced by intra- or extracellular factors [28]. HSPs act as molecular chaperones that allow cells to adapt to gradual environmental changes and to survive under adverse conditions. Thus, HSPs occur in large quantities in the cells of *Drosophila melanogaster* exposed to increasing temperatures [27,44,58]. HSPs may be involved in the transportation of proteins into cellular compartments, in protein folding in the cytosol, endoplasmic reticulum and mitochondria, in the degradation of unstable proteins, in the dissolution of protein complexes, in the prevention of undesirable protein aggregation, in the correction of inadequate protein folding and in the control of regulatory proteins [28].

Chronic exposure to sub-lethal doses of chemicals can activate the expression of HSPs. The activation of HSP70 in diplopods, for instance, is a useful tool for monitoring the presence of toxic agents in soil [41]. Similarly, the immunocytochemical detection of HSP70 and HSP90 is useful in the pathological analysis of larval tissues in *A. mellifera* infected with *Paenibacillus larvae* [41]. The enhanced expression of HSP family members increases the probability of cell survival by preventing the activation of programmed cell death [6].

HSPs can act at multiple points in the intracellular signalling pathway in apoptosis [28]. In mammals, HSP27 and HSP70 are anti-apoptotic, whereas HSP60 and HSP10 are pro-apoptotic. The role of HSP90 in apoptosis is still unclear since some members of

the HSP90 family are anti-apoptotic while others are pro-apoptotic, depending on the stimuli for cell death [28]. HSP90 is associated with various signalling proteins, including transcription factors that are ligand-dependent, and its main function is to promote the conformational maturation of these signalling proteins, including the signal transducer protein kinase [44].

Bierkens [4] pointed out that the detection of members of more than one HSP family indicated the involvement of various cellular responses to stress. This author also stressed the importance of using HSPs in the biomonitoring of animals in contaminated areas where there may be chronic exposure to low levels of many environmental contaminants. This situation is very difficult to replicate in the laboratory, where generally only lethal and sub-lethal doses of a given chemical compound are evaluated. In addition, many physical, chemical and biological factors present in the field cannot be reproduced in artificial conditions [55].

More studies have been done on the effects of pesticides on *A. mellifera* than for other bee species, mainly because of this species´ economic importance and extensive geographical distribution. Knowlton [39,40] described an extensive bibliographic survey of poisoning in bees, and Hocking [34] provided a list of chemical products used in agriculture with toxic effects on bees. This toxicity varied according to biotic and non-biotic factors related to the pesticides used and the type of vegetation.

Silva-Zacarin *et al.* [65] examined the effect of acaricides on *A. mellifera* larvae in apiaries, particularly with regard to the activation of HSPs in silk glands. Under stress, the HSP90 chaperones were indirectly anti-apoptotic since salivary gland cell death induced by acaricides was limited by the enhanced expression of HSP90 and by the sustained level of HSP70. In larvae treated with rotenone, there was no change in the normal level of cellular death nor were there significant morphological alterations in the secretory gland cells [66]. In larvae treated with oxalic acid, the secretory cells showed varying degrees of morphological alteration and an increase in cell death. The immunocytochemical detection of HSP suggested that the increase in HSP70 expression had an anti-apoptotic effect in the salivary glands of larvae treated with rotenone, while the enhanced expression of HSP90 suggested a role for this protein as a chaperone [66].

Under high levels of stress, the protective effect of HSPs in cells is insufficient to prevent programmed cell death, which includes necrosis in the majority of tissues and organs and apoptosis in a few cells. Apoptosis and necrosis are easily distinguished morphologically and the induction of one or the other of these two types of cell death indicates the level of cellular stress involved. Many studies have examined the mechanisms of programmed cell death in insect tissues [6,7,16,19,20,22,23,29-31,33,38,45-50,59,63] and have raised doubts concerning the current classification of the phenomena involved, particularly since the typical features of apoptosis established in vertebrates are not always seen in insect cells.

Most studies of programmed cell death in insects have focused on the tissue reorganization that occurs during metamorphosis or on the natural regression of certain organs in adult life. Little is known about cell death in insect tissues and organs in response to environmental stressor agents. Some studies have highlighted the ultrastructural alterations in cultured insect cells exposed to organic and inorganic mercury [9] and cadmium [8]. Sub-lethal concentrations of these metals caused ultrastructural alterations in mitochondria, an increase in the number of lysosomes and free ribosomes, chromatin condensation, irregularities in the nuclear envelope, nucleolar proliferation, dilation of the rough endoplasmic reticulum cisternae and the formation of cytoplasmic blebs. Although these ultrastructural alterations are not characteristic of necrosis, they are typical diagnostic markers of programmed cell death. Under these circumstances, the protein synthetic machinery is directed towards the synthesis of HSPs in response to the agents being tested.

Few studies have examined the effect of chemicals in insect organs. The Malpighian tubules are the excretory and osmoregulatory organs of insects [3,26,56]. Their simple tubular organization, convenient size and excretory function make these tubules one of the most studied organs in ecotoxicological investigations in insects. Sorour [68] evaluated the ultrastructural alterations in the Malpighian tubules of *Lethocerus niloticum* (Hemiptera: Belostomatidae) caused by lake pollution. The most prominent characteristics were mitochondrial alterations, an increase in the number of lysosomes, chromatin condensation, irregularity of the nuclear envelope and the lysis of some cells.

These findings suggested that alterations in the Malpighian tubules of *L. niloticum* could be used as a bioindicator of lake pollution [68].

Another organ of extreme importance in ecotoxicological studies of insects is the gut. The digestive tract of insects generally consists of a foregut (stomodeum), midgut (ventriculus) and hindgut (proctodeum). Most of the digestion and absorption of nutrients and chemicals occurs in the midgut. In this region in most insects, the food is enveloped by a semipermeable acellular layer known as the peritrophic membrane that has several functions [13], including protection of the midgut epithelium against the mechanical action of food, the formation of a physical barrier against infestation by microorganisms, and the prevention of enzyme excretion by allowing the endo-ectoperitrophic circulation of digestive enzymes [69,70]. The principal cells of the midgut epithelium in adult insects are the columnar or digestive cells and the regenerative cells. The columnar cells are responsible for the production and secretion of digestive enzymes and for the absorption of digested substances and water, while the regenerative cells are found in the base of the epithelium and replace the columnar cells that are lost through abrasion and cell ageing; this replacement involves the division and differentiation of regenerative cells [13].

Gregorc and Bowen [30] showed that the number of columnar cells undergoing degeneration in the midgut of *A. mellifera* larvae increased during infestation by *Bacillus larvae*, which suggested an increase in the frequency of cell death in pathological situations. Thiboldeaux *et al.* [71] used bioassays to assess the effect of 5-hydroxy-1,4-naphthoquinone on the midgut morphology of *Actias luna* (Lepidoptera, Saturniidae) and *Callosamia promethea* (Lepidoptera, Saturniidae) larvae. The histological alterations observed included a partial loss of the microvilli and fragmentation of the epithelium.

The morphological organization of the midgut of adult *A. mellifera* and various species of stingless bees has been described [18,37,60,61,67], although there are histological, ultrastructural and functional variations among the species examined. More recent work suggests that these regions probably differ in their sensitivity to boric acid [36] since the morphological alterations induced by this substance were more accentuated in the posterior region of the midgut in *A. mellifera* workers. In this region, the production of digestive enzymes was interrupted, as indicated by the lack of apocrine secretion at the apex of the digestive cells. The disappearance of the peritrophic membrane in the midgut of larvae treated with boric acid probably affected the digestive and absorptive functions of the midgut and the protection of the epithelium. Consequently, there was greater shedding of digestive cells into the lumen in the posterior midgut and there was also partial fragmentation of the epithelium, with the digestive cells showing high levels of cytoplasmic vacuolization and nuclear pycnosis [36].

In the midgut of *A. mellifera* larvae treated with boric acid [21], the epithelial cells also showed morphological alterations that included intense basal vacuolization, a dilated intercellular space and various degrees of nuclear pycnosis. There was an intense shedding of cells and cytoplasmic material into the gut lumen. The Malpighian tubules of *A. mellifera* larvae exposed to boric acid showed morphological alterations that included flattening of the epithelium and distension of the lumen. This distension probably resulted from the increased intraluminal pressure caused by the accumulation of fluid that had not been excreted by the damaged cells [21]. In adult bees, the region where the Malpighian tubules were connected to the gut were the most affected by boric acid. In this region, the Malpighian tubule cells showed vacuolization of the apical cytoplasm, nuclear pycnosis and the accumulation of material in the lumen, indicating a loss of function in this region of the tubule [36].

Although the morphological structure of the midgut of bees is well established, there have been few studies of the morphology of the Malpighian tubules in these insects. Mello and Bozzo [53] resported a histochemical and ultrastructural study of the Malpighian tubules in *Melipona quadrifasciata*, and Cruz-Landim [14] analyzed the cellular specializations in the tubules of this same species, including during the metamorphosis or larvae into adults [15]. Silva de Moraes and Cruz-Landim [64] conducted a morphological, histochemical and cytophotometric study of the Malpighian tubules of larvae, prepupae and adult workers of *Melipona quadrifasciata anthidioides*. Mello [52] described a histochemical analysis of the mucous secretion produced by Malpighian tubules of the bumble bee *Bombus atratus*; a similar study was also

reported for the stingless bees *Plebeia droryana* and *Scaptotrigona postica* [54]. As in other insects, the number of Malpighian tubules in bees varies among species [17]. Hence, additional morphological studies of the Malpighian tubules need to be done in various species of bees in order to allow a more complete assessment of the usefulness of these structures for ecotoxicological studies.

PERSPECTIVES

The study of cellular markers, such as the expression of HSPs and the extent of cell death in Malpighian tubules and the midgut, will be of great importance in ecotoxicological studies in bees, particularly in agricultural areas. A comparison of these cellular markers in the organs of contaminated and non-contaminated adult bees should allow better planning for the use of agricultural areas visited by pollinator bees.

REFERENCES

1. Armbruster L, Levy M, Mathieu MN, Bautz AM (1986) Acid phosphatase activity in the haemolymph, hemocytes, fat body and salivary glands during larval and prepupal development in *Calliphora erythrocephala* (Diptera: Calliphoridae). *J. Biochem. Physiol.* **84B**, 349-354.
2. Balestiari JBP, Alves-Júnior VV, Moraes-Alves MMB, Silva ACG (2002) Levantamento de abelhas Euglossinae ao longo do transecto Dourados-Sidrolândia-Campo Grande/MS, associado à linha de instalação do gasoduto. In: *Anais do XIV Congresso Brasileiro de Apicultura*. Campo Grande (MS), Brazil, 16-20 July. p. 45.
3. Berridge MJ, Oschmann JL (1972) *Transporting Epithelia.* Academic Press: New York.
4. Bierkens JGEA (2000) Applications and pitfalls of stress-proteins in biomonitoring. *Toxicology* **153**, 61-72.
5. Bowen ID, Lockshin RA (1981) *Cell Death in Biology and Pathology.* Chapman and Hall: New York.
6. Bowen ID, Morgan SM, Mullarkey K (1993) Cell death in the salivary glands of metamorphosing *Calliphora vomitoria. Cell Biol. Int.* **17**, 13-33.
7. Bowen ID, Mullarkey K, Morgan SM (1996) Programmed cell death in the salivary gland of the blow fly *Calliphora vomitoria. Microsc. Res. Tech.* **34**, 202-217.
8. Braeckman B, Brys K, Rzeznik U, Raes H (1999) Cadmium pathology in an insect cell line: ultrastructural and biochemical effects. *Tissue Cell* **31**, 45-53.
9. Braeckman B, Raes H (1999) The ultrastructural effect and subcellular localization of mercuric chloride and methylmercuric chloride in insect cells (*Aedes albopictus* C6/36). *Tissue Cell* **31**, 223-232.
10. Brito AS, Batista ARPL, Castro MS (2002) Toxicidade de praguicidas usados no controle da mosca branca *Bemisia argentifolli* (Hemiptera, Aleyrodidae) para as abelhas sem ferrão do gênero *Scaptotrigona* (Hymenoptera: Apoidea). In: *Anais do XIV Congresso Brasileiro de Apicultura*. Campo Grande (MS), Brazil, 16-20 July, p. 36.
11. Carvalho EM, Carvalho SM, Carvalho CF, Carvalho GA, Souza B (2002) Efeito da pulverização de alguns inseticidas usados em cucurbitáceas sobre adultos de *Apis mellifera* Linnaeus, 1758 (Hymenoptera: Apidae). In: *Anais do XIV Congresso Brasileiro de Apicultura.* Campo Grande (MS), Brazil, 16-20 July, p. 112.
12. Carvalho EM, Carvalho SM, Carvalho CF, Carvalho GA, Souza B (2002) Impacto de inseticidas fornecidos a adultos de *Apis mellifera* Linnaeus, 1758 (Hymenoptera: Apidae) por meio de pasta cândi contaminada. In: *Anais do XIV Congresso Brasileiro de Apicultura.* Campo Grande (MS), Brazil, 16-20 July, p. 114.
13. Cavalcante VM, Cruz-Landim C (1999) Types of cells present in the midgut of the insects: a review. *Naturalia* **24**, 19-40.
14. Cruz-Landim C (1998) Specializations of the Malpighian tubules cells in a stingless bee, *Melipona quadrifasciata anthidioides* Lep. (Hymenoptera, Apidae). *Acta Microsc.* **7**, 26-33.
15. Cruz-Landim C (2000) Replacement of larvae by adult Malpighian tubules during metamorphosis of *Melipona quadrifasciata anthidioides* Lep. (Hym: Apidae, Meliponinae): morphological studies. *Ciênc. Cult.* **52**, 59-63.
16. Cruz-Landim C, Giometti SEE (1990) Histólise dos músculos torácicos larvais durante a metamorfose e miogênese dos músculos do vôo, em *Melipona quadrifasciata anthidioides* Lep. (Hymenoptera, Apidae, Meliponinae). *Rev. Brasil. Biol.* **50**, 983-1002.
17. Cruz-Landim C, Mello ML, Rodrigues L (1969) Nota sobre o número de tubos de Malpighi em abelhas. *Ciênc. Cult.* **21,** 734-735.
18. Cruz-Landim C, Melo RA (1981) *Desenvolvimento e Envelhecimento de Larvas e Adultos de Scaptotrigona postica Latreille (Hymenoptera, Apidae): Aspectos Histológicos e Histoquímicos.* ACIESP: São Paulo.
19. Cruz-Landim C, Silva de Moraes RLM (1977) Estruturas degenerativas nas glândulas hipofaríngeas de operárias de *Apis mellifera* (Apidae). *Rev. Brasil. Biol.* **37**, 681-692.
20. Cruz-Landim C, Silva de Moraes RLM (2000) *Morte Celular Programada em Abelhas como uma Forma de Redirecionar a Morfologia e a Fisiologia Adaptativa.* Ed. Tipografia Costa: Rio Claro.
21. Cruz AS, Silva-Zacarin ECM, Malaspina O (2005) Morphological and histochemical studies of the midgut and Malpighian tubules of *Apis mellifera* larvae treated with boric acid. *Braz. J. morphol. Sci.* **22 (Suppl.),** 171-172.
22. Dai JD, Gilbert LI (1997) Programmed cell death of the prothoracic glands of *Manduca sexta* during pupal-adult metamorphosis. *Insect Biochem. Molec. Biol.* **27**, 69-78.

23. Dai JD, Gilbert LI (1999) An *in vitro* analysis of ecdysteroid-elicited cell death in the prothoracic gland of *Manduca sexta*. *Cell Tissue Res.* **297**, 319-327.
24. Eckwert E, Alberti G, Kohler HR (1997) The induction of stress proteins (hsp) in *Oniscus asellus* (Isopoda) as a molecular marker of multiple heavy-metal exposure: I. Principles and toxicological assessment. *Ecotoxicology* **6,** 249-262.
25. Eckwert E, Kohler HR (1997) The indicative value of the Hsp70 stress response as a marker for metal effects in *Oniscus asellus* (Isopoda) field populations: variability between populations from metal-polluted and uncontaminated sites. *Appl. Soil Ecol.* **6**, 275-282.
26. Eichelberg D, Wessing A (1975) Morphology of the Malpighian tubules of insects. *Fortsh. Zool.* **23**, 124-147.
27. Feder ME, Cartano NV, Milos L, Krebs RA, Lindquist SL (1996) Effect of engineering Hsp 70 copy number on Hsp 70 expression and tolerance of ecologically relevant heat shock in larvae and pupae of *Drosophila melanogaster*. *J. Exp. Biol.* **199**, 1837-1844.
28. Garrido C, Gurbuxani S, Ravagnan L, Kroemer G (2001) Heat shock proteins: endogenous modulators of apoptotic cell death. *Biochem. Biophys. Res. Commun.* **286,** 433-442.
29. Gregorc S, Bowen ID (1996) Programmed cell death in honeybee (*Apis mellifera*) larvae midgut. *Cell Biol. Int.* **21,** 151-158.
30. Gregorc S, Bowen ID (1998) Histopathological and histochemical changes in honeybee larvae (*Apis mellifera* L.) after infection with *Bacillus larvae*, the causative agent of American foulbrood disease. *Cell Biol. Int.* **22**, 137-144.
31. Gregorc A, Bowen ID (1999) In situ localization of heat-shock and histone proteins in honeybee (*Apis mellifera* L.) larvae infected with *Paenibacillus larvae*. *Cell Biol. Int.* **23**, 211-218.
32. Hacon SS (2003) Avaliação e gestão do risco ecotoxicológico à saúde humana. In: *As Bases Toxicológicas da Ecotoxicologia* (Azevedo FA, Chasin AAM, eds). pp. 245-322. RiMa: São Carlos.
33. Hartfelder K, Steimbruck G (1997) Germ cell cluster formation and cell death are alternatives in caste-specific differentiation of the larval honey bee ovary. *Invert. Reprod. Dev.* **31**, 237-250.
34. Hocking B (1950) The honeybee and agricultural chemicals. *Bee World* **31**, 49-53.
35. Introna F, Campobasso CP, Goff ML (2001) Entomotoxicology. *Forensic Sci. Internat.* **120**, 42-47.
36. Jesus D, Silva-Zacarin ECM, Malaspina O (2005) Histological studies in the midgut and Malpighian tubules of *Apis mellifera* workers treated with boric acid. *Braz. J. morphol. Sci.* **22 (Suppl),** 168.
37. Jimenez DR, Gilliam M (1989) Age-related changes in midgut ultrastructure and trypisin in the honey bee, *Apis Mellifera*. *Apidologie* **20**, 287-303.
38. Jones HE, Bowen ID (1993) Acid phosphatase activity in the larval salivary glands of developing *Drosophila melanogaster*. *Cell Biol. Int.* **17**, 305-315.
39. Knowlton GF (1944) Poisoning of honey bee. *Utah Agric. Exp. Stn. Mimeo* **310**, 1-11.
40. Knowlton GF (1948) Some relationships of newer insecticides to honeybees. *Utah State Agr. Coll. Extn. MS.* 725.
41. Kohler HR, Triebskorn R, Stocker W, Kloetzel PM, Alberti G (1992) The 70 kD heat shock protein (Hsp 70) in soil invertebrates: a possible tool for monitoring environmental toxicants. *Arch. Environ. Contam. Toxicol.* **22**, 334-338.
42. Lauverjat S, Ballan-Dufrançais C, Wegnez M (1989) Detoxification of cadmium. Ultrastructural study and electron-probe microanalysis of the midgut in a cadmium-resistant strain of *Drosophila melanogaster*. *Biol. Metals* **2**, 97-107.
43. Levy M, Bautz AM (1985) Degeneration of larval salivary glands during metamorphosis of the blow-fly *Calliphora erythrocephala* Meigen (Diptera: Calliphoridae). *Int. J. Insect Morphol. Embryol.* **14**, 281-290.
44. Lindquist S, Craig EA (1988) The heat-shock proteins. *Annu. Rev. Genet.* **22**, 631-677.
45. Lockshin RA (1981) Cell death in metamorphosis. In: *Cell Death in Biology and Pathology.* (Bowen ID, Lockshin RA, eds). pp. 79-121. Chapmam & Hall: London.
46. Lockshin RA (1985) Programmed cell death. In: *Comprehensive Insect Physiology, Biochemistry and Pharmacology. Vol. 2.* (Kerkut GA, Gilbert LI, eds). pp. 301-317. Pergamon Press: Oxford.
47. Lockshin RA, Beaulaton J (1974) Programmed cell death. Cytochemical evidence for lysosomes during the normal breakdown of the intersegmental muscles. *J. Ultrastruct. Res.* **46**, 43-62.
48. Lockshin RA, Beaulaton J (1974) Cytochemical appearance of lysosomes when the death of the intersegmental muscles is prevented. *J. Ultrastruct. Res.* **46**, 63-78.
49. Lockshin RA, Williams CM (1964) Programmed cell death. II - Endocrine potentiation of breakdown of the intersegmental muscles of silkmoths. *J. Insect Physiol.* **10**, 643-649.
50. Martau T, Romer F (1998) Degeneration of molting glands in male crickets. *J. Insect Physiol.* **44**, 981-989.
51. Meirelles RMS, Silva ECM, Silva de Moraes RLM (2001) Lipid distribution in salivary glands of larvae and adult bees (Hymenoptera: Apidae). *Cytobios* **106,** 57-66.
52. Mello ML (1979) A mucous secretion in the Malpighian tubes of a neotropical bumblebee, *Bombus atratus* Franklin. *Protoplasma* **99**, 147-158.
53. Mello ML, Bozzo L (1969) Histochemistry, refractometry and fine structure of secretory globules in larval

Malpighian tubes of *Melipona quadrifasciata* (Hym, Apoidea). *Protoplasma* **68**, 241-251.
54. Mello ML, Kerr WE (1984) Histochemistry of salivary gland and Malpighian tubule secretions contribuiting to the cocoon in *Plebeia droryana* and *Scaptotrigona postica* (Hym., Apoidea). *Zool. Anz. Jena* **213**, 177-189.
55. Morse RA (1961) The effect of sevin on honeybee. *J. Econ. Entomol.* **54**, 566-568.
56. Pannabecker T (1995) Physiology of the Malpighian tubule. *Annu. Rev. Entomol.* **40**, 493-510.
57. Pawert M, Triebskorn R, Graff S, Berkus M, Schutz J, Kohler HR (1996) Cellular alterations in collembolan midgut cells as a marker of heavy metal exposure: ultrastructure and intracellular metal distribution. *Sci. Total Environ.* **181**, 187-200.
58. Petersen NS, Mitchell HK (1985) Heat shock proteins. In: *Comprehensive Insect Physiology, Biochemistry and Pharmacology. Vol. 10.* (Kerkut GA, Gilbert LJ, eds). pp. 348-365. Pergamon Press: New York.
59. Reginato RD, Cruz-Landim C (2002) Morphological characterization of cell death during the ovary differentiation in worker honeybee. *Cell Biol. Int.* **26**, 243-251.
60. Serrão JE, Cruz-Landim C (1995) Comparative size and histology of the proventriculus and midgut among the female castes and the males of *Scaptotrigona postica* Latreille (Hymenoptera: Apidae, Meliponinae). *Biociências* **3**, 85-94.
61. Serrão JE, Cruz-Landim C (1998) O intestino médio de abelhas – comparação morfológica entre operárias em diferentes fases do desenvolvimento e idades. In: *Anais do 3º Encontro sobre Abelhas*. Ribeirão Preto (SP), Brazil, 11-14 June. pp. 128-131.
62. Silva ECM (2002) Glândulas salivares larvais das abelhas. In: *Glândulas Exócrinas das Abelhas*. (Cruz-Landim C, Abdalla FC, eds). pp. 21-49. FUNPEC: Ribeirão Preto.
63. Silva de Moraes RLM, Bowen ID (2000) Modes of cell death in the hypopharyngeal gland of the honey bee (*Apis mellifera* L.). *Cell Biol. Int.* **24**, 737-743.
64. Silva de Moraes RLM, Cruz-Landim C (1976) Estudos comparativos dos tubos de Malpighi de larva, pré-pupa e adulto de operárias de *Melipona quadrifasciata anthidioides* Lep. (Apidae, Meliponinae). *Pap. Avulsos Zool.* **29,** 249-257.
65. Silva-Zacarin ECM, Gregorc A, Silva de Moraes RLM (2002) Análise imunocitoquímica de proteínas de choque térmico (HSPs) e a morte celular programada em glândulas salivares larvais de *Apis mellifera carnica*, tratadas com acaricidas e antibióticos. In: *Anais do 5º Encontro sobre Abelhas*. Ribeirão Preto (SP), Brazil, 4-7 September. p. 242.
66. Silva-Zacarin ECM, Gregorc A, Silva de Moraes RLM (2006) *In situ* localization of heat-shock proteins and cell death labeling in the salivary gland of acaricide-treated honeybee larvae. *Apidologie* (in press).
67. Snodgrass RE (1956) *Anatomy and Physiology of the Honeybees.* Comstock Publishing Associates: New York.
68. Sorour J (2001) Ultrastructural variations in *Lethocerus niloticum* (Insecta: Hemiptera) caused by pollution in Lake Mariut, Alexandria, Egypt. *Ecotox. Environ. Safe.* **48**, 268-274.
69. Terra WR (1996) Evolution and function of insect peritrophic membrane. *Ciênc. Cult*. **48,** 317-324.
70. Terra WR, Espinozafuentes FP, Ribeiro AF, Ferreira C (1988) The larval midgut of the housefly (*Musca domestica*): ultrastructure, fluid fluxes and secretion in relation to the organization of the digestion. *J. Insect Physiol.* **34,** 463-472.
71. Thiboldeaux RL, Lindroth RL, Tracy JW (1998) Effects of juglone (5-hydroxy-1,4-naphthoquinone) on midgut morphology and glutathione status in Saturniid moth larvae. *Comp. Biochem. Physiol.* **120,** 481-487.
72. Wyllie AH (1980) Cell death: a new classification separating apoptosis from necrosis. In: *Cell Death in Biology and Pathology.* (Bowen ID, Lockshin RA, eds). Chapman & Hall: New York.

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