Original Article

# INCREASED CASPASE-3 IMMUNOEXPRESSION AND MORPHOLOGY ALTERATIONS IN OENOCYTES AND TROPHOCYTES OF *APIS MELLIFERA* LARVAE INDUCED BY TOXIC SECRETION OF *EPORMENIS CESTRI*

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**Abstract**

**Toxic honeydew produced by Flatidae *Epormenis cestri* in Uruguay has been shown to cause among honeybees (*Apis mellifera*) colonies a massive larva death called "River disease", but the intrinsic mechanisms are still unknown. Because fat body cells, oenocytes and trophocytes, are known to regulated larvae metabolism, and to be affected by xenobiotics, we tested whether apoptosis of these cells can be an underlying cause of larvae death. Ten colonies were divided into two groups and fed with common honey or toxic honeydew obtained from colonies affected by "River disease". Five-day-old larvae were collected and processed for histology and immunohistochemistry for caspase-3. The area, diameter, and immunostaining area in oenocytes and trophocytes were measured. The oenocyte and trophocyte cellular area decreased in the treated group ($p=0.002$; $p<0.001$ respectively) compared to the control group. The diameter of oenocytes ($p=0.0002$) and trophocytes ($p<0.0001$) decreased in the treated group. Caspase-3 was detected in cytoplasm in the control group but in the cytoplasm and nucleus in the treated group. The caspase-3 immunostaining area increased in oenocytes ($p<0.002$) and trophocytes ($p<0.0001$) of the treated group. The ingestion of toxic honeydew altered the morphology, localization and immunoexpression of caspase-3 in fat body cells, which suggests that the deregulation of the apoptotic mechanism affected the normal development in *A. mellifera* larvae.**

**Keywords: caspase-3, honeybee larvae, oenocytes, trophocytes, River disease**

## INTRODUCTION

Fat body tissue is the main metabolic system during honeybee larvae development (Martins & Ramalho-Ortigão, 2012) and is made up of mainly oenocytes and trophocytes (Cousin et al., 2013). These cells are mainly located below the larval body wall and around the gastrointestinal tract (Makki et al., 2014). Both oenocytes and trophocytes can be isolated or in clusters surrounded by connective tissue in the hemocoel contacting the hemolymph and enhancing the exchange of metabolites (Roma et al., 2010). They are responsible for the production of hemolymph proteins (Ruvolo & da Cruz, 1993), resembling to mammalian hepatocytes, with detoxification and excretion functions, synthesis of proteins, hormones, storage of lipids and carbohydrates (Paes de Oliveira & Da Cruz Landim, 2003; Gutierrez et al., 2007; Landim, 2009). Oenocytes are large, round acidophilic cells derived from the ectoderm and

distributed among the trophocytes (Ruvolo & da Cruz, 1993) and produce cuticulin, the precursor for both the larva and the reproductive adult (Roma et al., 2010). Trophocytes are round oval shaped cells that contain intracellular lipids, proteins, and carbohydrates droplets (Paes de Olivaira & Da Cruz Landim, 2003).

Previous studies determined that oenocytes are morphologically affected by larvae chlorophenol herbicide exposure (Cousin et al., 2013). Furthermore, the starvation of the *Drosophila melanogaster* produced lipid mobilization in larvae and caused a rise in lipid droplets within oenocytes, which maintained a low lipid levels in the hemolymph (Chatterjee et al., 2014). Moreover, exposure to formic and oxalic acids caused apoptosis (Gregorc & Ellis, 2011).

Apoptosis is a normal process in insects and mammals during development, regulated by the activation of cysteinyl aspartate proteases called "caspases" (Gregorc & Bowen, 1997). The starting caspase proteins trigger an enzymatic cascade that activates effectors caspases, including caspase-3. The acaricide amitraz triggers apoptosis in the midgut epithelial cells of the honeybee larvae (Gregorc & Bowen, 1997). Adding to that, recent studies determined that glyphosate increases apoptosis in gut epithelial cells during larvae development (Vázquez, et al., 2018).

A recent, massive death of bee larvae, called "River disease", was caused by the intake of the toxic secretions of the Flatidae *Eporменis cestri* (Hemiptera, Flatidae) living in *Sebastiania schottiana* trees (Invernizzi et al., 2018). Flatidae are widely distributed along the world, including China (Tang et al., 2010), Germany (Nickel & Remane, 2002) and Uruguay. However, the intrinsic mechanisms that lead to this phenomenon remain unknown, hence we hypothesized that this toxic honeydew increases the apoptotic process in bee larvae. Therefore, we tested whether the ingestion of *E. cestri* toxic secretions by honeybee larvae could alter the morphology and apoptosis in fat body cells (oenocytes and trophocytes) during larvae development.

## MATERIAL AND METHODS

### Experimental design

The study was performed on an experimental farm "Campo Experimental N°2" (Faculty of Veterinary, Universidad de la República, Libertad, San José, Uruguay). *A. mellifera* bee colonies (n=10) were divided into the control group, fed with honey (the "healthy group"), and the toxic group fed with toxic honeydew from colonies affected by "River disease" and typified by the QSI Laboratory. The food, a total amount of 4 kg, was given to each colony through internal feeders twice, at hour 0 and hour 48. After a visualization of the eggs and their subsequent hatching, we collected on day 8 the surviving bee larvae at stage 5 (five-day-old larvae) in the toxic group (n=10) and the same number from the healthy group (n=10).

### Tissue processing

Honeybee larvae were fixed and immersed in a fixative solution (ethanol 96% 27 ml, formalin 5% 11 ml, glacial acetic acid 7 ml, and distilled water 55 ml). Samples were then dehydrated in increasing concentrations of ethanol (50%, 70%, 95%, 100%), immersed in chloroform and paraffin embedded. Subsequently, 5 µm sections were obtained using a microtome (Leica Reichert Jung Biocut 2030, Wetzlar, Germany) for staining techniques and immunohistochemistry.

### Hematoxylin - Eosin

The Haematoxylin-Eosin (H&E) technique was used to stain sections with yellow eosin and Mayer's Haematoxylin for histomorphometrical analyses.

### Immunohistochemistry

The immunohistochemistry technique was applied to analyse the caspase-3 in oenocytes and trophocytes. Briefly, the technique included dewaxing of the larvae sections in an oven at 60°C, antigenic recovery and hydration in 0.01 M citrate buffer pH 8.0 and 4 ml of Tween at a high-temperature microwave for three minutes. Subsequently, endogenous peroxidases were blocked with 3% hydrogen peroxide

($H_2O_2$). Afterwards, the slides were incubated for eighteen hours at 4°C with anti-caspase-3 (Polyclonal Rabbit IgG, AF835, 0.2 mg/ml, R & D Systems, USA, dilution 1: 500). Subsequently, biotinylated secondary antibody (anti rabbit secondary IgG- HRP / DAB kit, ab 64261 Abcam) were incubated for thirty minutes. The slides were then incubated for thirty minutes with horseradish-peroxidase-enzymatic-complex (HRP), and finally diaminobenzidine (DAB) chromogen solution (DAB+ hydrogen peroxide) was added for one minute. The slides were then washed with distilled water and counterstained with Harris Hematoxylin. To verify the specificity of the technique, negative controls were performed, replacing the primary antibody with phosphate buffer solution (PBS) pH 7.4. After each step, a rinse in PBS was performed.

**Image capture**

Histological H&E and immunohistochemistry images were retrieved with software (Dino-Capture 2.0 software, AnMo Electronics Corporation, Taiwan) and a digital camera (Dino-Eyepiece, AM-423X, AnMo Electronics Corporation, Taiwan) connected to a binocular microscope (Professional Premiere®, model MRP-5000, Manassas, USA).

**Morphometrical analyses**

The morphometry of oenocytes and trophocytes was determined by image analysis with ImageJ software (ImageJ 1.51 g, Wayne Rasband open source, National Institutes of Health, USA, http://rsb.info.nih.gov/ij/).

**Oenocytes morphometry**

Morphometric analyses were performed in thirty microscopic images of bee larvae oenocytes at high-power field (HPF = 400x). The oenocyte area (µm²) and diameter (µm) were measured with a *Freehand selection* tool, prior to *Set scale and calibration* steps, *Set measurement*, and afterwards measured with *Analysed/Measure* tool including the cellular area and diameter.

**Trophocytes morphometry**

Trophocytes were analysed as described above for oenocytes. The trophocyte area (µm²) and *diameter* (µm) was analysed with thirty microscopic images per healthy and toxic group.

**Immunostaining analysis**

Caspase-3 immunostaining area (%) and intensity (mean gray value of pixels) were measured in oenocytes and trophocytes with ImageJ software. A selection of each cell type was done, afterwards brown areas were selected, using a *Color Segmentation* plugin. The brown colour threshold value was selected for all images, verified, and normalized with controls carried across several runs for calibration. A batch mode was performed to apply the macro to all images contained in the folder. This process provided quantitative values for immunostained and intensity of immunostained area.

**Statistical analysis**

All results for cellular area (µm²), cellular diameter (µm), immunostaining area (%) and intensity of immunostaining (mean gray value of pixels) for caspase-3 in oenocytes and trophocytes were expressed as means ± standard error of the mean (S.E.M.). Differences were compared by Student's t-test (Two-Sample t-test) using PROC TTEST for each dependent variable and SAS statistical analysis (SAS, v. 9.1, SAS Institute Inc., Cary, NC, USA) considering the level of significance to be $p<0.05$.

## RESULTS

**Morphological changes in larvae oenocytes**

Larvae from the healthy group showed defined contours, acidophilus cytoplasm and euchromatic nucleus (Fig. 1a). Oenocytes from the toxic group showed a heterochromatic nucleus and loss of nucleus roundness (Fig. 1b). The morphometric parameters, cellular area and diameter that were normally distributed, decreased in the toxic group compared to the healthy group (area 3274±147 µm² *versus* 3933±147 µm²; p=0.002; diameter 75±1.96 µm *versus* 86±1.96 µm; p=0.0002) (Fig. 1c-d).

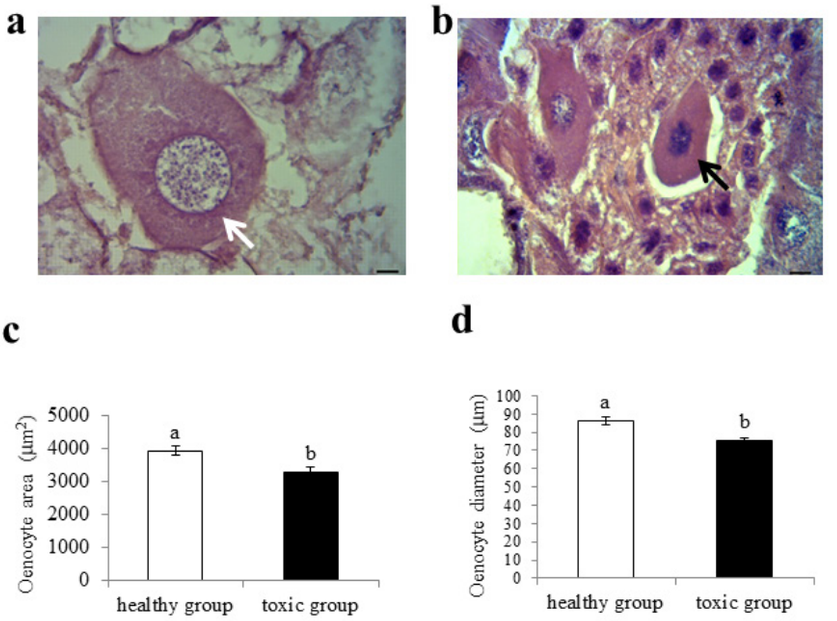


Fig. 1. Morphological effect of toxic honeydew from bee colonies affected by "River Disease" in oenocytes. a) fat body cells from healthy group. Healthy oenocyte (white arrow). b) Fat body cells from toxic group. Oenocyte showing a size decrease and heterochromatic nucleus (black arrow). Scale bar=10 µm. Magnification: 400x. Morphometry results of oenocytes: c) area (µm$^2$), d) diameter (µm). Larvae healthy group (white bars) or with toxic group (black bars). Error bars represent values of mean±SEM. Different literals in columns indicate significant differences (P<0.05). Histological images stained with Hematoxylin-Eosin.

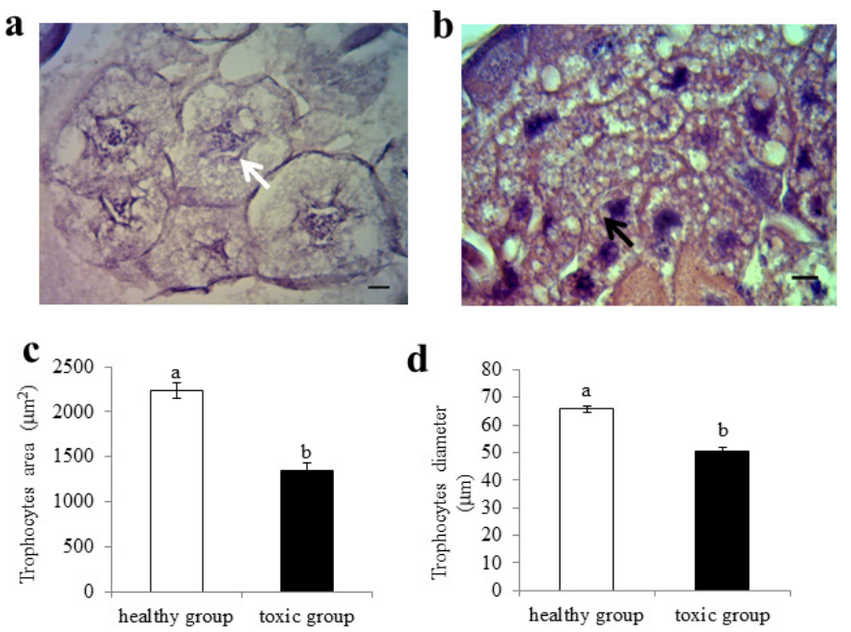


Fig. 2. Effects of toxic honeydew in trophocytes morphology in honeybee larvae at day 5 of development. Histological images with Hematoxylin-Eosin a) trophocytes cytoplasm in healthy larvae fed with honey (white arrow), b) trophocytes in larvae fed with toxic honeydew (black arrow), notice the decrease in cell size and its heterochromatic nucleus compared to healthy larvae. Morphometry results of trophocytes c) trophocyte area (µm$^2$), d) diameter (µm). Larvae fed with honey (white bars) or with toxic honeydew (black bars). Error bars represent values of mean ±SEM. Different literals in columns indicate significant differences (P<0.05).

**Morphological changes in larvae trophocytes**
The trophocytes of the healthy group larvae had a round shape, lipid vacuoles and euchromatic nuclei (Fig. 2a). In contrast, the toxic group trophocytes lacked net cell boundaries and the number of lipid vacuoles was lower (Fig. 2b). The morphometric parameters, cellular area and diameter that were normally distributed, decreased in the toxic group compared to the healthy group (area 1348±85 $\mu m^2$ *versus* 2236 ±85 $\mu m^2$; $p<0.001$; diameter 50±1.41 μm *versus* 65±1.38 μm; $p<0.0001$) (Fig. 2c-d).

**Immunoexpression of caspase-3 in oenocytes**
The immunoexpression of caspase-3 in oenocytes was clearly distributed along the cytoplasm in the toxic group larvae, whereas there was less intense immunoexpression in the healthy group larvae (Fig. 3a-b). The immunohistochemistry variables caspase-3 immunostaining area (%) and intensity (mean gray value of pixels) were normally distributed. The immunostaining area (%) for caspase-3 in oenocytes of the toxic group larvae was higher compared to healthy group larvae (0.85±0.25 *versus* 0.57±0.22 $p<0.03$) (Fig. 3c). Moreover, the intensity of immunostaining (mean gray value of pixels) of caspase-3 in oenocyte toxic group larvae was higher compared to healthy group larvae ($12.3x10^6$±2.9 $x10^6$ *versus* 8.9 $x10^6$±3.4 $x10^6 p<0.0001$) (Fig. 3d).

**Immunoexpression caspase-3 in trophocytes**
The immunoexpression of caspase-3 in trophocytes was intense in both the cytoplasm and nucleus of the toxic group larvae in contrast to the healthy group larvae where the immunoexpression was observed to be light brown at the cytoplasm, more intense in the perinuclear region and clearly negative in the nucleus (Fig. 4a-b). The immunohistochemestry variables caspase-3 immunostaining area (%) and intensity (mean gray value of pixels) were normally distributed. The immunostaining area (%) for caspase-3 of the toxic group larvae was higher compared to that of the healthy larvae (22±0.62 *versus* 20±0.62; $p<0.0001$) (Fig.4c). The immunostaining intensity (mean gray value of pixels) of caspase-3 in trophocytes in the toxic group larvae was higher compared to that in the healthy group larvae ($1.3x10^6$±$3.9x10^5$ *versus* $1.2x10^6$±$3.9x10^5 p<0.0001$) (Fig. 4d).

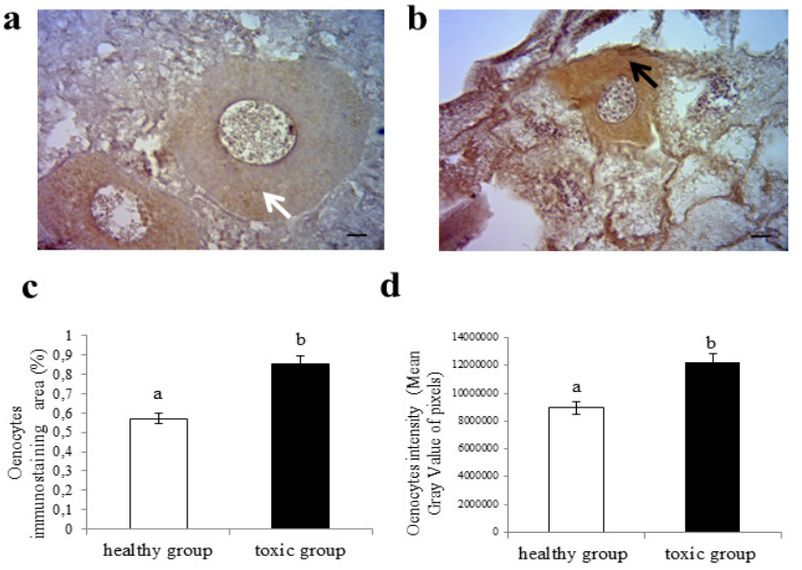


Fig. 3. Effect of toxic honeydew from colonies affected by "River Disease" on honeybee 5 days old larvae. Immunoexpression of caspase-3 in a) oenocytes in healthy larvae showing light intensity of immunostaining in cytoplasm (white arrow), b) oenocytes in larvae with toxic honeydew (black arrow) showing intense brown immunostaining in cytoplasmic region. Scale bar=10 μm. c) Results of caspase-3 immunostaining area (%) and d) immunostaining intensity (mean gray value of pixels) in oenocytes in larvae fed with honey (white bars) or with toxic honeydew (black bars). Error bars represent mean±SEM. Different literals in columns indicate significant differences ($P<0.05$).

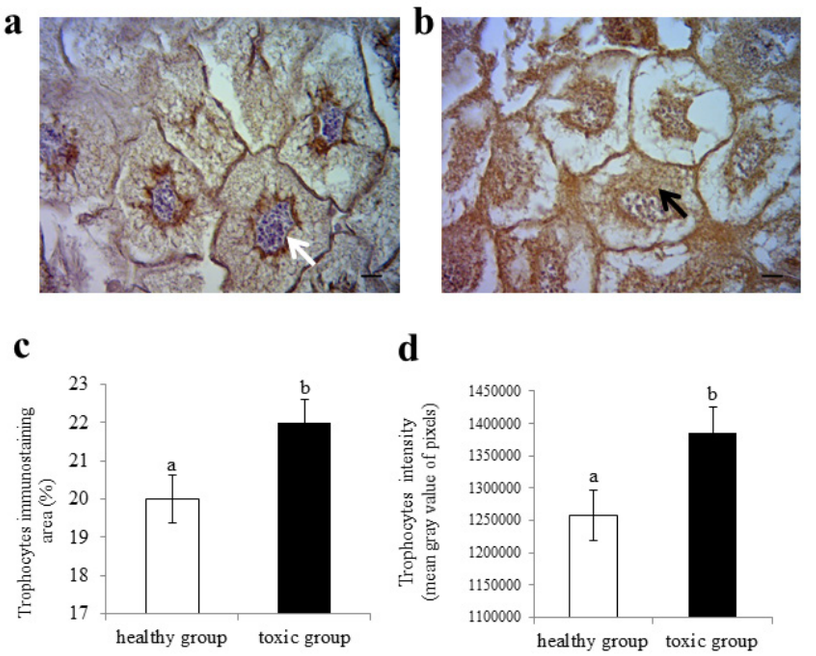


Fig. 4. Effects of toxic honeydew in trophocytes of honeybees at day 5 of development.
Immunoexpression of active caspase-3: a) trophocytes showing perinuclear slight immunostaining in healthy larvae fed with honey (white arrow) and negative basophilic nucleus without immunostaining, b) trophocytes from larvae fed with toxic honeydew showing intense brown immunostaining at nuclear and cytoplasmic region (black arrow). Scale bar=10 µm. Immunostaining area of caspase-3 larvae fed with honey (white bars) or with toxic honeydew (black bars) in trophocytes c) immunostaining area (%), d) immunostaining intensity (mean gray values of pixels). Error bars represent values of mean±SEM. Different literals in columns indicate significant differences ($P<0.05$).

## DISCUSSION

In this study we determined that fat body cell's morphology and apoptotic protein immunoexpression was altered in *A. mellifera* larvae as a consequence of the ingestion of toxic honeydew. The increased immunoexpression of caspase-3 in fat body cells and the changes observed in morphology suggest an increased apoptosis in the fat body cells of the toxic group larvae. It is proposed that this increase in apoptosis could diminish, among others, the function of detoxification of the fat body cells and therefore alter the cellular metabolism that triggers larvae death.

The toxic honeydew ingestion impaired the metabolic action and morphological aspects of the oenocytes and trophocytes. Furthermore, toxic honeydew increased caspase-3 immunoexpression, an executor enzyme involved in cellular apoptosis. These changes match with previous reports on the paraquat intoxication of honeybee larvae, in which oenocytes reduce in size as a consequence of this herbicide (Cousin et al., 2013). Because of morphologically decreased fat body cells in the cross-section area, a possible loss of integrity in the cellular membranes and pyknotic nucleus in the cells affected by the toxic group, we suggest an increase of the apoptosis process.

The increase observed in the caspase-3 immunostaining of oenocytes due to toxic honeydew ingestion is one of the major alterations in larvae as oenocytes secrete ecdysteroid hormones involved in larval development, metamorphosis and remodelling (Cousin et al., 2013). Moreover, the nuclear immunolocalization of active caspase-3 from the cytoplasm compartment to the nucleus suggests that it occurs in the cells of the toxic group larvae; this translocation had been previously detected in culture cells during the progression of apoptosis (Kamada et al., 2005). The observation of caspase-3 at nuclear level could explain the beginning of cell prote-

olysis process as previously described in the apoptosis process (Ramuz et al., 2003). During the larval development of honeybees, apoptosis was observed to occur in the immunoexpression of caspase-3 in oenocytes and trophocytes in the healthy group, while the immunoexpression area occupied by caspase-3 increased in the toxic group.

Moreover, the translocation of caspase-3 to the nuclei in the fat body cells of the toxic group indicates the onset of proteolysis, the disassembly of the nuclear envelope, the intensification of the signal in membranes and proteolysis damage. The immunoexpression of active caspase-3 determined an onset of apoptosis in the nucleus as well as the cytoplasm in the toxic group. The nuclear immunostaining is indicative of apoptosis, which precedes the disruption of cell membranes in this process.

The results contribute to clarifying the cellular mechanisms involved in "*River disease"*, which affected the colonies through massive death of the larvae by the secretions of the Flatidae *E. cestri* (Invernizzi et al., 2018). In summary, we determined that secreted toxic honeydew affected the cellular morphology of oenocytes and trophocytes in *A. mellifera* bee larvae. This includes increased immunoexpression of the pro-apoptotic caspase-3 in fat body tissue.

**List of contributors**

EN, PJ conceived and designed the experiments. HV, ML-P, GP carried out the experiments. HV, ML-P, PL, GP, CI analysed the data. HV, GP, CI wrote the manuscript. JM-V, CI, PJ, PL, GP revised the entire work. All authors gave the final approval of the version to be published.

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