Research, Society and Development, v. 11, n. 14, e313111436389 2022
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v11i14.36389

Effects of *Beauveria bassiana* (Hypocreales: Cordycipitaceae) on the midgut of the *Chrysomya megacephala* (Fabricius, 1794) (Diptera: Calliphoridae) maggots

Efeitos de *Beauveria bassiana* (Hypocreales: Cordycipitaceae) sobre o intestino médio em larvas da *Chrysomya megacephala* (Fabricius, 1794) (Diptera: Calliphoridae)

Efectos de *Beauveria bassiana* (Hypocreales: Cordycipitaceae) en el intestino medio de las larvas da *Chrysomya megacephala* (Fabricius, 1794) (Diptera: Calliphoridae)

Received: 10/11/2022 | Revised: 10/21/2022 | Accepted: 10/22/2022 | Published: 10/28/2022

Vagner José Deves
ORCID: https://orcid.org/0000-0003-4890-0422
State University of Maringá, Brazil
E-mail: vagnerdvs@hotmail.com
Bruno Vinicius Daquila
ORCID: https://orcid.org/0000-0003-3540-3187
State University of Maringá, Brazil
E-mail: bv.ds@hotmail.com
Elton Luiz Scudeler
ORCID: https://orcid.org/0000-0002-6005-1139
São Paulo State University, Brazil
E-mail: elton.scudeler@unesp.br
Ronaldo Roberto Tait Caleffe
ORCID: https://orcid.org/0000-0002-5661-105X
State University of Maringá, Brazil
E-mail: ronaldo_caleffe@hotmail.com
Helio Conte
ORCID: https://orcid.org/0000-0002-2090-0554
State University of Maringá, Brazil
E-mail: hconte@uem.br

Abstract
Beauveria bassiana is an entomopathogenic fungus widely used in pest management. After contact with target organisms, fungal conidia germinate and colonize tissues and organs, causing death by starvation and/or septicemia. *Chrysomya megacephala* is an insect pest with worldwide distribution. Its larvae cause secondary myiasis in animals of interest, and adults are pathogen vectors. This study aimed to analyze the effects of the Ballvéria® biopesticide on the midgut of *C. megacephala* third-instar maggots. Four concentrations (1, 1.5, 2, and 4%) of the biopesticide were applied to an artificial diet, followed by conditioning of the maggots. Mortality data and samples for histological and ultrastructural analysis were collected every 24 h, for 144 h. Mortality data were analyzed using SPSS 25.0, and lethal concentrations (LC_{50} and LC_{90}) were calculated using Probit regression. Concentrations of 2 and 4% resulted in mortality rates of 26 and 36%, respectively. LC_{50} and LC_{90} were estimated at 5.3 and 10.9%, respectively. Observational, histological, and ultrastructural analyses revealed the presence of tegumentary melanizations, conidia in the midgut, spacing in the basal labyrinth, degeneration of microvilli, absence of the peritrophic membrane, fungal extrusion on the external surface of the midgut, and dispersion of hyphae, conidiophores, and conidia close to muscle fibers. Internally, hyphae are located on microvilli and cell projections. Our data confirm that the Ballvéria® biopesticide causes cytotoxic effects in the midgut of *C. megacephala* maggots and can be used as a sustainable alternative in its biological control for Integrate Pest Management.
Keywords: Biotechnology; Biological control; Entomopathogen; Fungi; Insect pest; Myiasis.

Resumo
Beauveria bassiana é um fungo entomopatogênico amplamente utilizado no manejo de pragas. Após contato com organismos-alvo, os conídios fúngicos germinam e colonizam tecidos e órgãos, causando sua morte por inanição e/ou septicemia. *Chrysomya megacephala* é um inseto-praga com distribuição mundial, suas larvas causam miíase secundária em animais de interesse, e os adultos são vetores de patógenos. Este estudo objetivou analisar os efeitos do biopesticida Ballvéria® sobre o intestino médio de larvas da *C. megacephala* em terceiro instar. Quatro concentrações (1; 1,5; 2 e 4%) do biopesticida foram aplicadas sobre dieta artificial, seguida pelo acondicionamento das larvas. Dados de mortalidade e amostras para analises histológicas e ultraestruturais foram coletados a cada 24 h, por 144 h.

Os dados de mortalidade foram analisados no software SPSS 25.0, e as concentrações letais (CL_{50} e CL_{90}) calculadas pela regressão de Probit. As concentrações 2 e 4%, resultaram na mortalidade de 26 e 36% das larvas. As CL_{50} e CL_{90} foram estimadas em 5,3 e 10,9%, respectivamente. Analises observacionais, histológicas e ultraestruturais revelaram a presença de melanizações tegumentares, conídios no intestino médio, espaçamentos no labirinto basal, degeneração das microvilosidades, ausência da membrana peritrófica, extrusão fúngica na superfície externa do intestino médio e a dispersão de hifas, conidióforos e conídios próximas as fibras musculares. Internamente, hifas estão sobre as microvilosidades e projeções celulares. Nossos dados confirmam que o bioinseticida Ballvéria® ocasiona efeitos citotóxicos no intestino médio de larvas da *C. megacephala*, podendo ser utilizado como uma alternativa sustentável em seu controle biológico pelo Manejo Integrado de Pragas.
Palavras-chave: Biotecnologia; Controle biológico; Entomopatógeno; Fungos; Insetos-praga; Miíase.

Resumen

Beauveria bassiana es un hongo entomopatógeno ampliamente utilizado en el manejo de plagas. Después del contacto con los organismos plagas, los conidios fúngicos germinan y colonizan tejidos y órganos, provocando su muerte por inanición y/o septicemia. *Chrysomya megacephala* es un insecto plaga de distribución mundial, sus larvas causan miasis secundaria en animales de interés y los adultos son vectores de patógenos. Este estudio tuvo como objetivo analizar los efectos del biopesticida Ballvéria® en el intestino medio de larvas de *C. megacephala* en tercer estadio. Se aplicaron cuatro concentraciones (1; 1,5; 2 y 4%) del biopesticida sobre una dieta artificial, seguido del acondicionamiento de las larvas. Los datos de mortalidad y las muestras para análisis histológico y ultraestructural se recogieron cada 24 h, durante 144 h. Los datos de mortalidad se analizaron con el software SPSS 25.0 y las concentraciones letales (LC_{50} y LC_{90}) se calcularon mediante regresión Probit. Las concentraciones del 2 y 4% resultaron en la mortalidad del 26 y 36% de las larvas. La CL_{50} y CL_{90} se estimaron en 5,3 y 10,9%, respectivamente. Los análisis observacionales, histológicos y ultraestructurales revelaron la presencia de melanizaciones tegumentarias, conidios en el intestino medio, espaciamiento en el laberinto basal, degeneración de microvellosidades, ausencia de la membrana peritrófica, extrusión fúngica en la superficie externa del intestino medio y dispersión de hifas, conidióforos y conidios, cerca de las fibras musculares. Internamente, las hifas se encuentran en las microvellosidades y proyecciones celulares. Nuestros datos confirman que el bioinseticida Ballvéria® provoca efectos citotóxicos en el intestino medio de las larvas de *C. megacephala* y puede utilizarse como una alternativa sostenible en su control biológico a través del Manejo Integrado de Plagas.
Palabras clave: Biotecnología; Control biológico; Entomopatógeno; Hongos; Insectos plaga; Miasis.

1. Introduction

The oriental latrine fly, *Chrysomya megacephala* (Fabricius, 1794) (Diptera: Calliphoridae), is an insect with distribution worldwide (Sontigun, et al., 2018). Its synanthropic behavior, added to your habit to the visitation of decaying organic matter, allows it to mechanically disperse pathogens, posing a risk to the health of animals and humans (Greenberg, 1973).

Studies that demonstrate the efficiency of chemical agents such as permethrin and deltamethrin in the control of *C. megacephala* are available in literature (Sukontason, et al., 2005; Oliveira, et al., 2021). However, the indiscriminate use of chemical controllers causes environmental damage and exposes living organisms to harmful molecules (Nascimento & Melnyk, 2016; Nicolopoulou-Stamati, et al., 2016), besides selecting populations of resistant insect pests (Sparks & Nauen, 2015).

The use of biological controllers can be as alternatives to reduce the use of chemical agents. Microbial entomopathogen-based biopesticides have high specificity for pest control (Caleffe, et al., 2019). *Beauveria bassiana* (Hypocreales: Cordycipitaceae) is a filamentous fungus with entomopathogenic properties (Eley, et al., 2007) indicated for its propriety biocontroller of various insect pests (Zimmermann, 2007), with for example, dipterans (White, et al., 2021a; 2021b).

After contact with target organisms, fungal conidia germinate and colonize insect organs; in this process, mycotoxins and enzymes are released, leading to insect death (Bergamo, et al., 2019; Wang, et al., 2021). The midgut is the main target of biopesticides, and thus, it is a key organ to be investigated to analyze the effects of ingested biopesticides (Scudeler, et al., 2016). In addition, this organ has been useful for evaluating sublethal effects, which can compromise the physiological functions of insects (Scudeler, et al., 2013).

The midgut of *C. megacephala* maggots comprises a single layer of cuboidal epithelial cells, which project for the intestinal lumen from their basement membranes, and each possesses long microvilli covering their apical surfaces, being responsible for absorption of nutrients and secretion of different molecules; within its lumen, it is present the peritrophic membrane, a structure that acts as a mechanical barrier for cell protection (Boonsriwong, et al., 2011). Damage to this organ interferes with the homeostasis of the organism and causes death (Daquila, et al., 2019).

Considering the risks posed by *C. megacephala* to the health of animals and humans, we investigated potential biocontroller and possible morphological alterations in the midgut epithelium of *C. megacephala* maggots treated with the commercial product Ballvéria®, a commercial biopesticide containing *B. bassiana* isolate IBCB-66 conidials. This study provides data that may help better understand entomopathogen action on susceptible insects and serve as a model for future studies involving target and non-target organisms.

2. Methodology

2.1 Insects

C. megacephala maggots were supplied by the Laboratory of Biological Control and Bioprospection of Insects of the State University of Maringá (23° 25′ 30″ S and 51° 56’ 20″ W), Maringá, Paraná, Brazil. The insects were maintained in glass Petri dishes (90 × 15 cm) and fed an artificial diet (Aguirre-Gil 2013) without any antibiotics. Glass Petri dishes were maintained in a climatized room at 25 ± 2 °C, photoperiod 12:12 h and 70 ± 10% RH (Von-Zuben, et al., 2000).

2.2 Commercial product

Bioassays were carried out using Ballvéria® (Ballagro Agro Tecnologia LTDA, Bom Jesus dos Perdões, São Paulo, Brazil; product registration number MAPA: 07312), a commercial biopesticide product containing *B. bassiana* isolate IBCB-66. A product containing 1×10^9 UFC/g viable conidials/g (equivalent to 300 g/kg) was formulated.

The application of the product was based on the manufacturer’s guidelines for dipteran control. Four concentrations, 1% (10 g/L), 1.5% (15 g/L), 2% (20 g/L), and 4% (40 g/L), were prepared by diluting the commercial product in sterile distilled water pH 7.0 at 25 °C. Aliquots (300 µL) of biopesticide solutions were added to the surface of polyethylene Petri dishes (90 × 15 cm) with 10 mL of the artificial diet. Sterile water (pH 7.0) was then added to the control group. Each Petri dish contained 10 maggots. Each bioassay treatment was performed in five replicates (n= 50 per group). The dishes were maintained in a climatized room at 25 ± 2 °C, a 12:12 h photoperiod, and 70 ± 10% RH. After bioassays, observations were made every 24 h using a Zeiss stereomicroscope (Carl Zeiss, Oberkochen, Germany). Mortality was measured every 24 h for 144 h.

2.3 Light microscopy

For light microscopy, control and treated maggots were collected 48 h after beginning the bioassays (n= 4 per group). Samples of the midgut were fixed in alcoholic Bouin solution (7.5 mL picric acid, 2 mL formaldehyde, and 0.5 mL acetic acid) for 24 h at room temperature (25 °C). Samples were dehydrated in increasing concentrations of ethanol (70, 80, 90, and 100%; v/v), diaphanized in xylol, embedded in Paraplast® (Leica Biosystems, Wetzlar, Germany), and sectioned into 6 µm sections using a Leica RM2250 microtome (Leica Biosystems, Wetzlar, Germany). The sections were stained with hematoxylin and eosin (Junqueira & Junqueira, 1983). Images were analyzed using an Omicron medical microscope (Axioskop 40, Carl Zeiss, Gottingen, Germany) and acquired using AxioCam MRc (Carl Zeiss, Oberkochen, Germany).

2.4 Scanning electron microscopy (SEM)

For SEM, maggots were quickly cryoanesthetized at -20 °C for 3 min and then their midguts were dissected in insect physiologic solution (0.1 M NaCl, 0.1 M KH_2PO_4, 0.1 M Na_2HPO_4) using a stereomicroscope (Carl Zeiss, Oberkochen, Germany). Control and treated maggots were collected 48 h after beginning the bioassays (n= 4 per group). Maggots and dissected midguts were fixed in alcoholic Bouin solution (7.5 mL formaldehyde, 2 mL picric acid, and 0.5 mL acetic acid) for 24 h at room temperature (25 °C). The samples were dehydrated in increasing concentrations of ethanol (70, 80, 90, and 100%, v/v). After dehydration, samples were subjected to critical point drying (Leica EM CPD030, Leica Biosystems, Wetzlar, Germany), and the maggots underwent fracture with a stainless-steel blade. All the samples were coated with a gold layer in an IC-50 metalizer (Shimadzu, Kyoto, Japan) and analyzed using a Quanta 250 scanning electron microscope (FEI Company, Eindhoven, Netherlands) at the Microscopy Center of the Complex of Research Support Centers of the State University of Maringá, Paraná, Brazil.

2.5 Transmission electron microscopy (TEM)

For TEM, control and treated maggots from the bioassays (n= 4 per group) were selected and dissected. Samples of the midgut were fixed in 2.5% (v/v) glutaraldehyde and 4% (v/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) for 24 h at room temperature (25 °C), and post-fixed in 1% (w/v) osmium tetroxide diluted in the same buffer for 2 h. The samples were washed in distilled water and a 0.5% (w/v) aqueous solution of uranyl acetate for 2 h, dehydrated in increasing concentrations of acetone (50, 60, 70, 80, 90, and 100% v/v), and embedded in Araldite® resin (Huntsman Advanced Materials, Salt Lake City, UT, USA). Ultrathin sections (0.5 μm) were stained with toluidine blue for preliminary sample selection. Ultrathin sections of selected samples were contrasted with uranyl acetate and lead citrate. The samples were analyzed using a Tecnai™ Spirit transmission electron microscope (FEI Company, Eindhoven, Netherlands) at the Electron Microscopy Center of the Institute of Biosciences of Botucatu, São Paulo State University, Botucatu, São Paulo, Brazil.

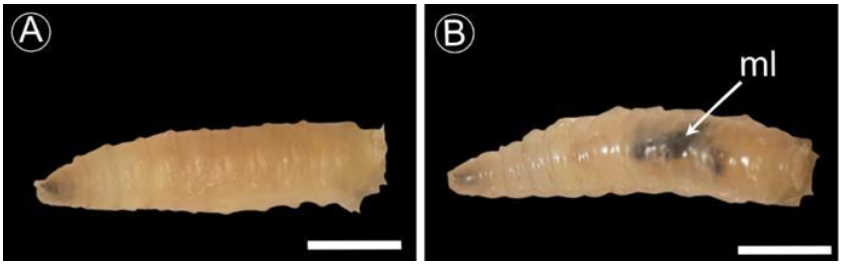
2.6 Statistical analyses

Normality and homogeneity were verified using the Kolmogorov-Smirnov and Bartlett tests, respectively. One-way analyses of variance by Kruskal-Wallis and post hoc Dunn tests were performed (IBM, 2017), with α= 0.05. SPSS (version 25.0; IBM, Armonk, NY, USA) was used for the Probit regression analyses. The results from the bioassays were used to estimate LC_{50} and LC_{90}. GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA) was used to design mortality graphs.

3. Results

The control maggots had a vermiform format and cream color (Figure 1A). Maggots treated with all concentrations of Ballvéria® maintained the body shape seen in the control group but demonstrated a gradual reduction in mobility until they ceased to move; some regions showed color alterations in the tegument, initially brown, and black after 48 h (Figure 1B).

Figure 1 - Whole mounting of *Chrysomya megacephala* maggots in third instar. (A) Control maggots. (B) Maggots treated with Ballvéria® in a concentration of 2%. In B, it is possible to observe the melanized (ml) region in this insect, after 48 h bioassays. Scale bar = 100 µm.

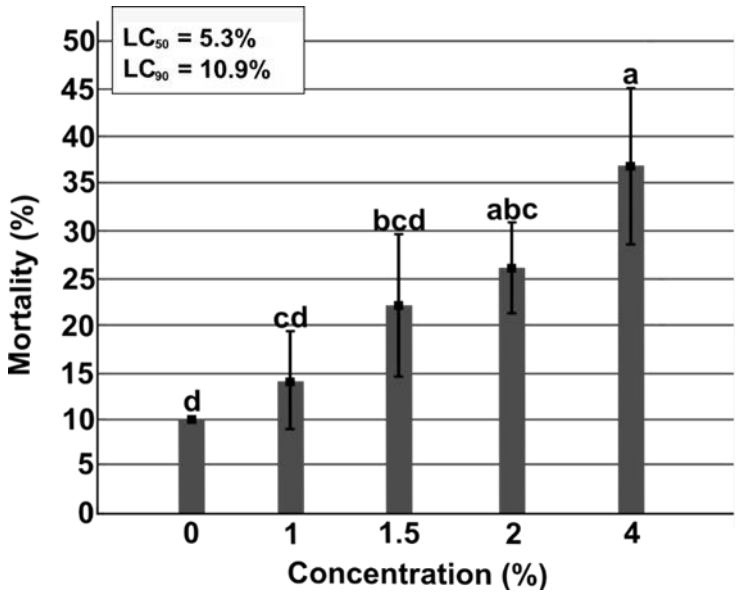


Source: Authors.

3.1 Mortality of *C. megacephala* maggots

The Kruskal-Wallis test indicated significant differences in *C. megacephala* mortality and entomopathogen concentration (X^2= 12.476, p= 0.005). Comparisons between the control and treatment groups indicated significant differences (Dunn's test, $p < 0.05$) (Figure 2).

Figure 2 - Mortality rates induced by different concentrations of Ballvéria® in *Chrysomya megacephala* maggots, after 144 h bioassays. Different letters denote significant differences (Kruskal-Wallis and Dunn test; $p < 0.05$). Lethal concentrations (LC) for death of 50% and 90% maggots. A concentration of 0% represents the control group.



Source: Authors.

In multiple comparison analysis, control, 1, and 1.5% concentrations did not differ significantly according to Dunn's test (1%, p= 0.863; 1.5%, p= 0.057). The other concentrations differed significantly from that of the control (2%, p= 0.007; 4%, p= 0.000). The mortality in the group exposed to a concentration of 1% did not differ between the groups exposed to concentrations of 1.5% (p= 0.324) and 2% (p= 0.057); however, differences were observed in the group exposed to a

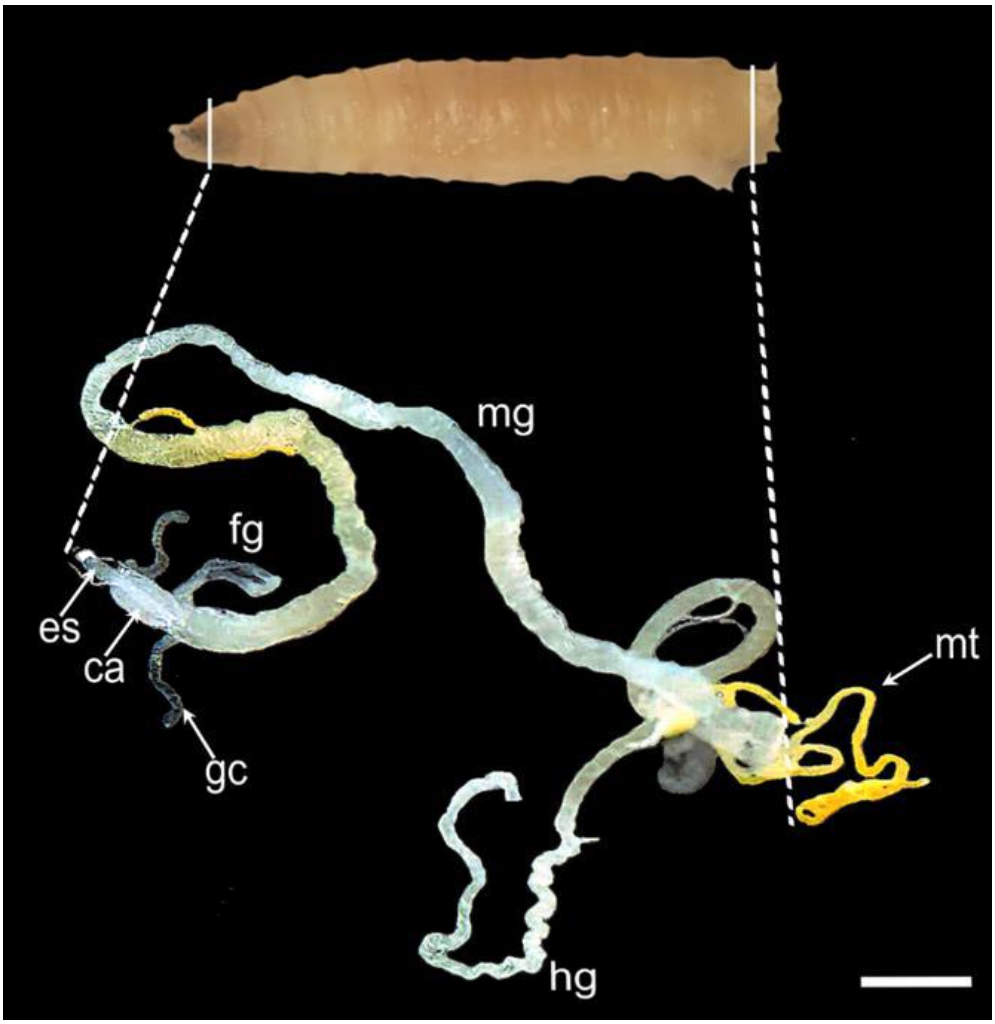
concentration of 4% (p= 0.000). The mortality in the group exposed to a concentration of 1.5% was not different from that in the group exposed to a concentration of 2%; however, there were differences in the group exposed to a concentration of 4% (p= 0.020). The comparison between the 2 and 4% groups did not demonstrate differences (p= 0.145) (Figure. 2).

Our data indicated that Ballvéria® showed toxicity against *C. megacephala* maggots at all concentrations. After 144 h of exposure to the product, insects treated with the concentrations 2 and 4% showed 26 and 36% mortality, respectively. In addition, the LC_{50} calculated with Probit regression was 5.3% (Confidence Interval [CI:3.9-10.1%]), whereas the LC_{90} was 10.9% (CI:7.5-23.5%) (Figure 2).

3.2 Digestive system

C. megacephala maggots presented a tubular digestive system, varying at the anterior, medial, and posterior regions: foregut, midgut, and hindgut, respectively. The esophagus, cardia, and gastric cecae were present in the anterior region. Malpighian tubes were inserted into the hindgut and projected onto the hindgut (Figure 3).

Figure 3 - Digestive system of *Chrysomya megacephala* maggots in third instar. The digestive system demonstrates three regions: foregut (fg), midgut (mg), and hindgut (hg); in the foregut are present the esophagus (es), cardia valvule (ca), and gastric cecae (gc). Malpighian tubules (mt) are projected off the hindgut. Scale bar = 100 µm.



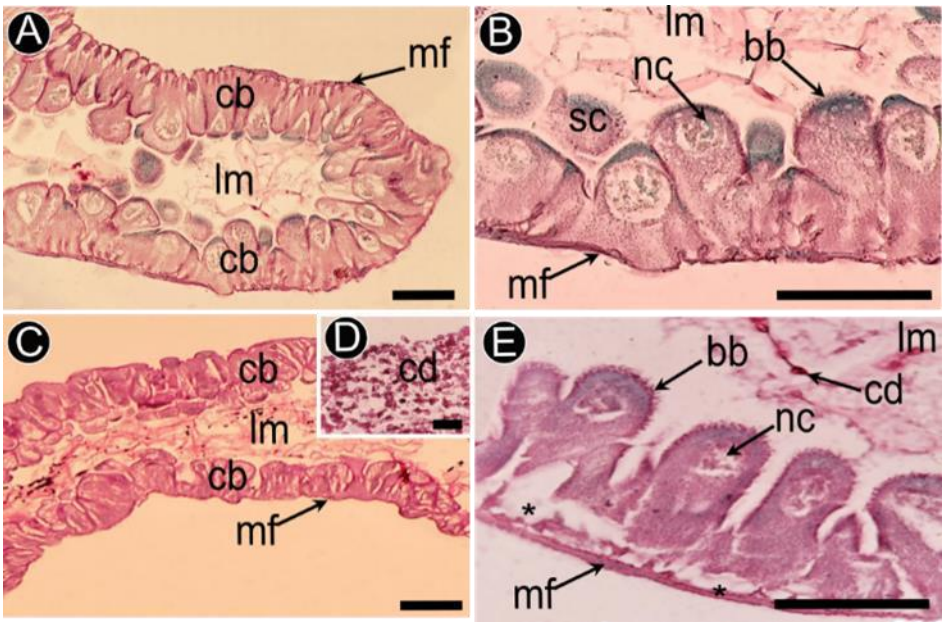
Source: Authors.

3.3 Light microscopy

Histologically, the midgut epithelium displayed a simple epithelium supported by muscle fiber bundles and predominantly cuboid cells (Figures 4A and C). In the control samples, the apical region of the cells presented striated border projections into the lumen, and secretion was observed at the cell apex (Figure 4B). Cuboid cells presented acidophilic cytoplasm and basophilic nuclei, which demonstrated an oval shape and were localized in the apical region of the cells (Figure 4B).

After 48 h of treatment with 2% Ballvéria®, the presence of conidia was also recorded in the midgut lumen (Figures 4C and D). The cells presented alterations in the nuclear region; in addition, cell injuries were observed, with cell lysis and extracellular spaces (Figure 4E).

Figure 4 - Light microscopy image of the midgut of *Chrysomya megacephala* maggots. (A-B) Dissection of third-instar maggot control; displaying a simple epithelium with evident lumen (lm), supported by muscle fiber bundles (mf), and presenting predominantly cuboid cells (cb). The apical region of the cells presented striated border (bb) projections into the lumen; secretion (sc) was observed in the cell apex with hematoxylin and eosin stain. (C, D and E): Maggots in third instar, treated with 2% solution of Ballvéria®; after 48 h, the presence of conidials (cd) was also recorded in the midgut, and the cells presented alterations in the nuclear region (nc); in addition, extracellular spaces (*) were visible in the basal region of the epithelium. Scale bars A-E = 10 µm.

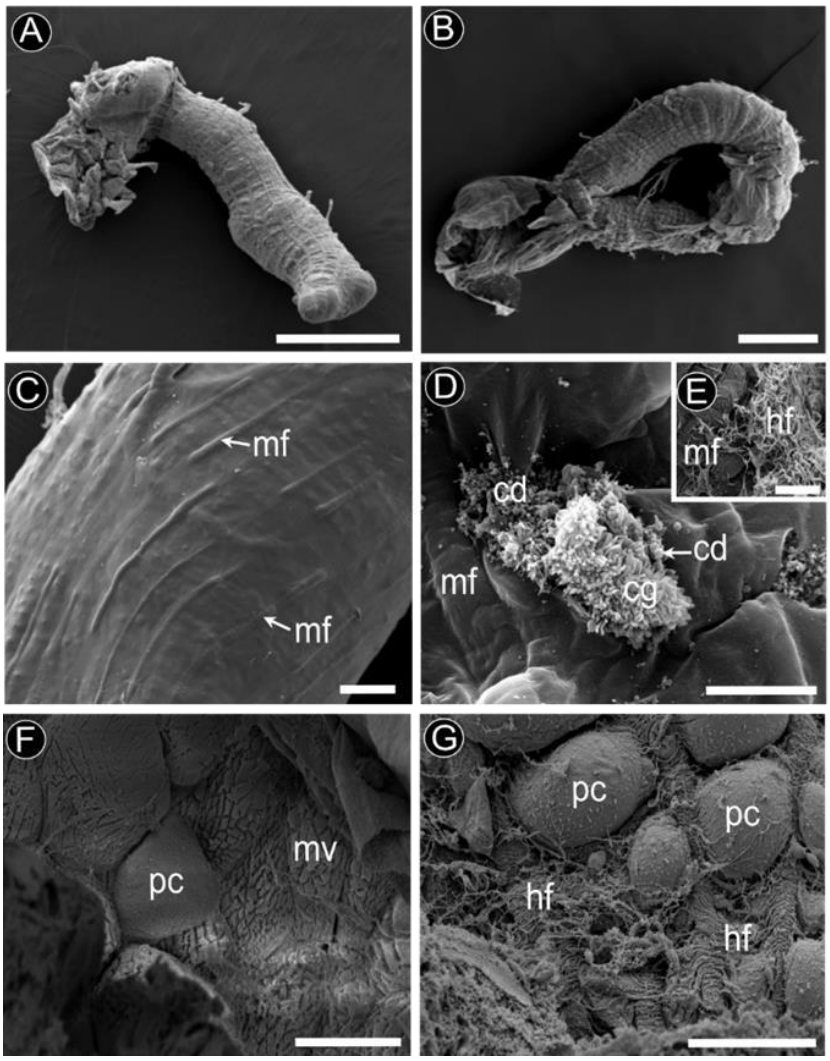


Source: Authors.

3.4 SEM

Ultrastructurally, the midgut of third-instar *C. megacephala* maggots presented a tubular form (Figures 5A and B). In the control group, we externally observed some muscle fiber bundles (Figure 5C). Internally, cell projections and microvilli were observed (Figure 5F). After 48 h, the midgut of maggots treated with a 2% solution of Ballvéria® presented extrusion of fungi, and we observed hyphae, conidiogenous cells, and conidia dispersed on the muscle fiber bundles (Figures 5D and E). Internally, in the midgut lumen, it was possible to observe hyphae present over the microvilli and cell projections (Figure 5G).

Figure 5 - Scanning electron microscopy image of the midgut of *Chrysomya megacephala* maggots. (A, C and F): Dissection of third instar maggot control; externally, we observed some muscle fiber bundles (mf), and internally, cell projections (pc), and microvilli (mv) were observed. (B, D, E and G): Maggots in third instar, treated with a 2% solution of Ballvéria®; after 48 h, extrusion fungi were present, and we observed hyphae (hf), conidiogenous cells (cg), and conidials (cd) dispersed on the muscle fiber bundles. (G) Internally, hyphae were present in the lumen of midgut on the microvilli and cell projections. Scale bars A-G = 100 µm.



Source: Authors.

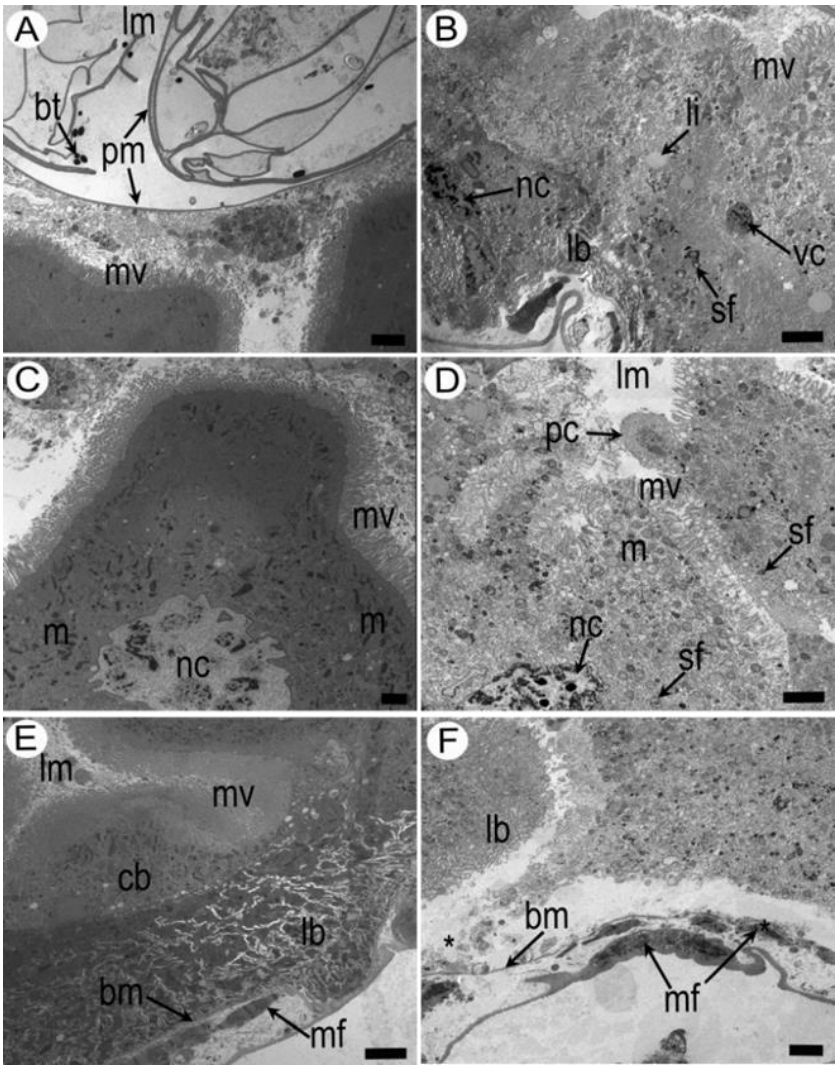
3.5 TEM

In control maggots, the midgut comprised cuboid cells with prolonged microvilli to the lumen (Figures. 6A, C, and E). Certain bundles of peritrophic membranes were present on microvilli, together with certain bacteria that formed the microbiota of this insect (Figure 6A). The basal and infranuclear regions of the cuboid cells presented a well-developed basal labyrinth supported by a basement membrane that was externally surrounded by muscle fiber bundles (Figure. 6E). The apical regions showed electron-dense mitochondria (Figure. 6C). The nuclei were localized in the apical region of the epithelial cells and presented nucleoli with heterochromatic lumps dispersed in the nucleoplasm (Figure. 6C).

After treatment with 2% Ballvéria®, microvilli degenerate, different mitochondrial morphologies accumulate, with an electron-lucent aspect, cell projections occur in the apical region, and bundles of peritrophic membranes are absent (Figures.

6B and D). Spherites and digestive vacuoles were present in the cytoplasm (Figs. 6B and D). Nuclear regions showed nucleolar disintegration in electron-dense small agglomerates (Figures 6B and D). In the basal region of the midgut epithelium, the basal labyrinth demonstrates extracellular space formation between the basement membrane and the basal region of epithelial cells (Figure 6F).

Figure 6 - Transmission electron microscopy of the midgut of *Chrysomya megacephala* maggots. (A, C and E): Control maggots in third instar; the apex of cells presented microvilli (mv) directional by lumen (lm) region. Some bundles of peritrophic membrane (pm) are present on microvilli. Bacteria (bt) are present. Apical regions showed accumulation of mitochondria (m). The nuclei (nc) contained heterochromatic lumps dispersed. The basal and infranuclear regions of cells presented basal labyrinth (lb) well developed supported by basement membrane (bm) which is externally surrounded by muscle fiber bundles (mf). (B, D and F): Maggots in third instar, treated with 2% solution of Ballvéria®; the microvilli were degenerates, occurs accumulation of different mitochondrial morphologies and cell project (pr). In the cell cytoplasm are present spherites (sf), lipid droplets (li) and digestive vacuoles (vc). Nuclear regions presented nucleolus disintegration at electron-dense small agglomerates. In basal region, the basal labyrinth demonstrates extracellular space formation (*) between basement membrane and basal region of epithelial cells. Scale bars A-F = 50 µm.



Source: Authors.

4. Discussion

A review of the biological control of Calliphoridae by Caleffe et al. (2019) showed that the major fungal species used to control this family were isolates of *Metarhizium anisopliae* and *B. bassiana*. In addition, studies utilizing different isolates of *B. bassiana* for the control of dipterans are available in literature (White, et al., 2021a; 2021b; Quintero-Zapata, et al., 2022). However, to the best of our knowledge, there are no data on the effects of the *B. bassiana* isolate IBCB-66 on *C. megacephala* maggots.

In this study, the action of Ballvéria® commercial product on maggots could have occurred in three ways: i) contact with a contaminated diet, ii) ingestion of the contaminated diet, or iii) both (contact and ingestion). However, we did not observe the presence of fungal structures in the tegumentary epithelium of treated maggots during the observation period, indicating that the mortality data for *C. megacephala* maggots are related to ingestion.

The absence of fungal structures in the integumentary epithelium may be associated with the locomotion of insects on the moist substrate, providing mechanical removal of conidia before germination, and the cuticle of dipterans can demonstrate physical characteristics different from those observed in other host insects, interfering with the adhesion of conidia in their body (White, et al., 2021a). Fungal conidia show greater interactions with hydrophobic and non-polar surfaces (Holder & Keyhani, 2005), but dipteran maggots show cuticles with polarized characteristics because of the elasticity of the cuticle during this stage of development (Hillerton & Vincent, 1983).

The pathogenicity effects of *B. bassiana* vary according to the toxins synthetized by fungal isolates and the host in question (Whang, et al., 2021), factors that may interfere with the biocontrol of different insects. The application of the Ballvéria® (*B. bassiana* isolate IBCB-66) at concentrations of 2 and 4% resulted in higher mortality, controlling 26% and 36% of the maggots, respectively. These results were inferior to those described by Quintero-Zapata et al. (2022) in *Aedes aegypti* (Diptera: Culicidae) treated with solutions in 1.5×10^7 conidia/mL of the *B. bassiana* isolate NB3, and similar to isolate GHA, the authors observed that the application of the entomopathogenic fungi resulted in 63 and 30.7% mortality, respectively.

In *Anastrepha ludens* (Diptera: Tephritidae) treated with different solutions (between 1×10^8 and 1.6×10^8 conidia/mL) of eight isolates of *B. bassiana*, La-Rosa et al. (2002) observed that the application of entomopathogenic fungi to an artificial diet resulted in 4% mortality. Mochi et al. (2010) also reported different results using the *B. bassiana* isolates JAB06, JAB07, and AM09 at a concentration of 2×10^9 conidia/mL on *Haematobia irritans* (Diptera: Muscidae) maggots; there were no significant results for maggot mortality.

Hyphae observed in the midgut of maggots indicated that germination of the conidials still occurred in the digestive system of maggots. Hyphae secrete various insecticidal proteins (e.g., beauvericin, bassianin, bassianolide, and beauverolides) and enzymes (e.g., chitinase and lipase) (Lewis, et al., 2009; Wanchoo, et al., 2009). In this manner, the insect immune system activates distinct mechanisms for its protection against molecules, such as phagocytosis, encapsulation, coagulation, and melanization (Jiravanichpaisal, et al., 2006; Lemaitre & Hoffmann, 2007; Strand, 2008; Fauvarque & Williams, 2011).

The melanization observed in this study is a process controlled by proteases (Kanost, et al., 2012), enzymes that trigger a cascade of serine proteases, culminating in the activation of phophenoloxidases, a melanogenesis-controlling enzyme, and phenoloxidase, which oxidizes tyrosine to dihydroxyphenylalanine, resulting in the production of dihydroxyphenylalanine and dopamine, precursors of melanin (Dubovskiy, et al., 2008; Nakhleh, et al., 2016). Melanization is related to the generation of reactive oxygen species (ROS) (Kumar, et al., 2003). Its occurrence in dipterans has been confirmed in some studies, for example, the sand fly *Lutzomyia longipalpis* (Diaz-Albiter, et al., 2012).

ROS originate from the mitochondria (Huang, et al., 2018; Wen, et al., 2016), and their accumulation results in oxidative stress (Felton & Summers, 1995), which in turn can cause cell death via apoptosis, autophagy, or necrosis (Radogna,

et al., 2016; Luckhart, et al., 2013). Characteristics may be associated with the presence of autophagic vacuoles and mitochondria with different morphologies observed in TEM; the absence of electron density in mitochondria, becoming more electron lucent, indicates the process of cristolysis.

However, in vitro studies have shown that different mycotoxins significantly reduce cell viability (Bogus, et al., 2021), inhibiting immune responses. The *B. bassiana* isolate ARSEF 252 secretes oosporein, which is responsible for downregulating the synthesis of antimicrobial peptides and dual oxidase expression in the midgut, reducing immune responses (Wei, et al., 2017). In addition, fungi upregulate the expression and translation of oxidative stress-related genes (e.g., upregulation of superoxide dismutase accelerates the conversion of superoxide ions into molecular oxygen and hydrogen peroxide), elevating the tolerance of *B. bassiana* to oxidative stress (Xie, et al., 2010).

These changes, combined with the observed histopathological symptoms, were similar to those described in studies using plant-derived toxins and insecticides (Almehmadi, 2011; Ling & Zhang, 2011; Qi, et al., 2011; Scudeler & Santos, 2013; Scudeler, et al., 2016). The extracellular spaces in the basal labyrinth of midgut cells, observed by light microscopy and TEM, were also reported by Daquila et al. (2019), who studied the action of *Bacillus thuringiensis* on the midgut of *Diatraea saccharalis* (Lepidoptera: Crambidae) larvae. These alterations may be caused by toxins released by entomopathogenic microorganisms, resulting in their detachment from the basement membrane of epithelial cells.

5. Conclusion

Our data show that the Ballvéria® has negative effects on the midgut of *C. megacephala* maggots. These changes in epithelial cells interfere with nutrient absorption and digestive system homeostasis, causing insect death. In addition, studies using different combinations of *B. bassiana* isolates with other entomopathogenic microorganisms or plant extracts are encouraged. Biopesticides containing the *B. bassiana* isolate IBCB-66 showed biocontrol effects in *C. megacephala* maggots, suggesting its potential for use in the Integrate Pest Management.

Acknowledgments

The authors thank the Coordination for the Improvement of Higher Education Personnel (CAPES) for their scholarship. The authors would also like to thank the Complex of Research Support Centers (COMCAP), State University of Maringá (UEM), and Center of Electron Microscopy of the Institute of Biosciences of Botucatu (IBB), Paulista State University (UNESP).

References

Almehmadi, R. M. (2011). Larvicidal, histopathological and ultra-structure studies of *Matricharia chamomella* extracts against the rift valley fever mosquito *Culex quinquefasciatus* (Culicidae: Diptera). Journal of Entomology, 8(1): 63-72. https://dx.doi.org/10.3923/je.2011.63.72

Bergamo, R. H. S., Daquila, B. V., & Conte, H. (2019). Sustentabilidade agrícola com fungos entomopatogênicos. In: Neto, B. R. S. (ed.). Principais grupos e aplicações biotecnológicas dos fungos. Atena editora: Ponta Grossa, pp. 41-52. https://dx.doi.org/10.22533/at.ed.3071918105

Bogus, M. I., Wronska, A. K., Kaczmarek, A., & Bogus-Sobocinska, M. (2021). In vitro screening of 65 mycotoxins for insecticidal potential. Plos One, 16(3): e0248772. https://doi.org/10.1371/journal.pone.0248772

Boonsriwong, W., Sukontason, K., Vogtsberger, R. C., & Sukontason, K. L. (2011). Alimentary canal of the blow fly *Chrysomya megacephala* (F.) (Diptera: Calliphoridae): An emphasis on dissection and morphometry. Journal of Vector Ecology, 36(1): 1-10. https://doi.org/10.1111/j.1948-7134.2011.00135.x

Caleffe, R. R. T., Oliveira, S. R., Schoffen, R. P., Oliveira-Junior, V. A., & Conte, H. (2019). Biological Control of Diptera Calliphoridae: A Review. Journal of Entomological Research Society, 21(2): 145-155.

Daquila, B. V., Scudeler, E. L., Dossi, F. C. A., Moreira, D. R., Pamphile, J. A., & Conte, H. (2019). Action of *Bacillus thuringiensis* (Bacillales: Bacillaceae) in the midgut of the sugarcane borer *Diatraea saccharalis* (Fabricius, 1794) (Lepidoptera: Crambidae). Ecotoxicology and Environmental Safety, 184: 109642. https://doi.org/10.1016/j.ecoenv.2019.109642

Diaz-Albiter, H., Sant'Anna, M. R. V., Genta, F. A., & Dillon, R. J. (2012). Reactive oxygen species-mediated immunity against *Leishmania mexicana* and *Serratia marcescens* in the phlebotomine sand fly *Lutzomyia longipalpis*. Journal of Biological Chemistry, 287(28): 23995-24003. https://doi.org/10.1074/jbc.M112.376095

Dubovisky, I. M., Krukova, N. A., & Glupov, V. V. (2008). Phagocytic activity and encapsulation rate of *Galleria mellonella* larval hemocytes during bacterial infection by *Bacillus thuringiensis*. Journal of Invertebrate Pathology, 98(3): 360-362. https://doi.org/10.1016/j.jip.2008.03.011

Eley, K. L., Halo, L. M., Song, Z., Powles, H., Cox, R. J, Bailey, A. M., Lazarus, C. M., & Simpson, T. J. (2007). Biosynthesis of the 2-Pyridone Tenellin in the Insect Pathogenic Fungus *Beauveria bassiana*. ChemBioChem, 8(3): 289-297. https://doi.org/10.1002/cbic.200600398

Fauvarque, M-O., & Williams, M. J. (2011). *Drosophila* cellular immunity: a story of migration and adhesion. Journal of Cell Science 124(9): 1373-1382. https://doi.org/10.1242/jcs.064592

Felton, G. W., & Summers, C. B. (1995). Antioxidant systems in insects. Archives of Insect Biochemistry and Physiology, 29(2): 187-197. https://doi.org/10.1002/arch.940290208

Greenberg, B. (1973). Flies and Disease. Biological and Disease Transmission. (Second edition). Princeton University Press, New Jersey, pp. 92-99.

Hillerton, J. E., & Vincent, J. F. V. (1983). Consideration of the importance of hydrophobic interactions in stabilizing the insect cuticle. International Journal of Biological Macromolecules, 5(3): 163-166. https://doi.org/10.1016/0141-8130(83)90032-6

Holder, D. J., & Keyhani, N. O. (2005). Adhesion of the entomopathogenic fungus *Beauveria* (Cordyceps) *bassiana* to substrata. Applied and Environmental Microbiology, 71(9): 5260-5266. https://doi.org/10.1128/AEM.71.9.5260-5266.2005

Huang, H., Long, S., Li, M., Gao, F., Du, J., Fan, J., & Peng, X. (2018). Bromo-pentamethine as mitochondria-targeted photosensitizers for cancer cell apoptosis with high efficiency. Dyes and Pigments, 149: 633-638. https://doi.org/10.1016/j.dyepig.2017.11.010

IBM Corporation. (2017). IBM SPSS Statistics for Windows IBM, Armonk, New York, 25.0.

Jiravanichpaisal, P., Lee, B. L., & Söderhäll, K. (2006). Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. Immunobiology 211(4): 213-236. https://doi.org/10.1016/j.imbio.2005.10.015

Junqueira, L. C. U., & Junqueira, L. M. M. S. (1983). Técnicas Básicas de Citologia e Histologia. Editora Santos, São Paulo, pp. 1-23.

Kanost, M. R., & Clem, R. J. (2012). Insect proteases. In: Lawrence, I. G. (ed). Insect Molecular Biology and Biochemistry. Elsevier: London, pp. 1-16 .

Kumar, S., Christophides, G. K., Cantera, R., Charles, B., Han. Y. S., Meister, S., Dimopoulos, G., Kafatos, F. C., & Barrilas-Mury, C. (2003). The role of reactive oxygen species on *Plasmodium* melanotic encapsulation in *Anopheles gambiae*. PNAS, 100(24): 14139-14144. https://doi.org/10.1073/pnas.2036262100

La-Rosa, W., Lopez, F. L., & Liedo, P. (2002). *Beauveria bassiana* as a pathogen of the Mexican fruit fly (Diptera: Tephritidae) under laboratory conditions. Journal of Economic Entomology, 95(1): 36-43. https://doi.org/10.1603/0022-0493-95.1.36

Lemaitre, B., & Hoffmann, J. (2007). The host defense of *Drosophila melanogaster*. Annual Review of Immunology. 25: 697-743. https://doi.org/10.1146/annurev.immunol.25.022106.141615

Lewis, M. W., Robalino, I. V., & Keyhani, N. O. (2009). Uptake of the fluorescent probe FM4-64 by hyphae and haemolymph-derived in vivo hyphal bodies of the entomopathogenic fungus *Beauveria bassiana*. Microbiology, 155(9): 3110-3120. https://doi.org/10.1099/mic.0.029165-0

Ling, S., & Zhang, R. 2011. Effect of friponil on brain and muscle ultrastructure of *Nilaparvata lugens* (Stal) (Homoptera: Delphacidae). Ecotoxicology and Environmental Safety, 74(5): 1348-1354. https://doi.org/10.1016/j.ecoenv.2011.03.011

Luckhart, S., Giulivi, C., Drexler, A. L., Antonova-Koch, Y., Sakaguchi, D., Napoli, E., Wong, S., Price, M. S., Eigenheer, R., Phinney, B. S., Pakpour, N., Pietri, J. E., Cheung, K., Georgis, M., & Riehle, M. (2013). Sustained activation of akt elicits mitochondrial dysfunction to block *Plasmodium falciparum* infection in the mosquito host. Plos Phatogens, 9(2): e1003180. https://doi.org/10.1371/journal.ppat.1003180

Mochi, D. A., Monteiro, A. C., Machado, A. C. R., & Yoshida, L. (2010). Efficiency of entomopathogenic fungi in the control of eggs and larvae of the horn fly *Haematobia irritans* (Diptera: Muscidae). Veterinary Parasitology, 167(1): 62-66. https://doi.org/10.1016/j.vetpar.2009.09.046

Nakhleh, J., El-Moussawi, L., & Osta, M. A. (2016). The melanization response in insect immunity. Advances in Insect Physiology, 52: 83-109. https://doi.org/10.1016/bs.aiip.2016.11.002

Nascimento, L., & Melnyk, A. (2016). A química dos pesticidas no meio ambiente e na saúde. Revista Mangaio Acadêmico, 1(1): 54-61.

Nicolopoulou-Stamati, P., Maipas, S., Kotampasi, C., Stamatis, P., & Hens, L. (2016). Chemical pesticides and human health: the urgent need for a new concept in agriculture. Frontiers in Public Health, 4: 148. https://doi.org/10.3389/fpubh.2016.00148

Oliveira, S. R., Caleffe, R. R. T., Gigliolli, A. A. S., Moreira, D. R., Conte, H., & Ruvolo-Takasusuki, M. C. C. (2021). Developmental changes in larvae of the oriental latrine fly, *Chrysomya megacephala*, exposed to deltamethrin. Parasitology Research, 120: 1-7. https://doi.org/10.1007/s00436-020-06933-8

Qi, Z. J., Shi, B. J., Hu, Z. N., Zhang, Y., & Wu, W. J. (2011). Ultrastructural effects of Celangulin V on midgut cells of the oriental armyworm, *Mythimna separata* walker (Lepidoptera: Noctuidae). Ecotoxicology and Environmental Safety, 74(3): 439-444. https://doi.org/10.1016/j.ecoenv.2010.10.004

Quintero-Zapata, I., Flores-González, M. S., Luna-Santillana, E. J., Arroyo-González, N., & Gandarilla-Pacheco, F. L. (2022). Late effects of *Beauveria bassiana* on larval stages of *Aedes aegypti* Linneo, 1762 (Diptera: Culicidae). Brazilian Journal of Biology, 82: e237789. https://doi.org/10.1590/1519-6984.237789

Radogna, F., Cerella, C., Gaigneaux, A., Christov, C., Dicato, M., & Diederich, M. (2016). Cell type-dependent ROS and mitophagy response leads to apoptosis or necroptosis in neuroblastoma. Oncogene, 35: 3839-3853. https://doi.org/10.1038/onc.2015.455

Scudeler, E. L., Garcia, A. S. G., Padovani, C. R., Pinheiro, P. F. F., & Santos, D. C. (2016). Cytotoxic effects of neem oil of the predator *Ceraeochrysa claveri*. Micron, 80: 96-111. http://dx.doi.org/10.1603/ICE.2016.113375

Scudeler, E. L., & Santos, D. C. (2013). Effects of neem oil (*Azadirachta indica* A. Juss) on midgut cells of predatory larvae *Ceraeochrysa claveri* (Navas, 1911) (Neuroptera: Chrysopidae). Micron 44: 125-132. https://doi.org/10.1016/j.micron.2012.05.009

Sontigun, N., Sukontason, K. L., Klong-Klaew, T., Sanit, S., Samerjai, C., Somboon, P., Thanapornpoonpong, S-N., Amendt, J., & Sukontason, K. (2018). Bionomics of the oriental latrine fly *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae): temporal fluctuation and reproductive potential. Parasites & Vectors, 11: 415. https://doi.org/10.1186/s13071-018-2986-2

Sparks, T. C., & Nauen, R. (2015). IRAC: mode of action classification and insecticide resistance management. Pesticide Biochemistry and Physiology, 121: 122-128. https://doi.org/10.1016/j.pestbp.2014.11.014

Strand, M. R. (2008). The insect cellular immune response. Insect Science, 15(1): 1-14. https://doi.org/10.1111/j.1744-7917.2008.00183.x

Sukontason, K., Chaiwong, T., Tayutivutikul, J., Somboon, P., Choochote, W., Piangjai, S., & Source, K. L. S. (2005). Susceptibility of *Musca domestica* and *Chrysomya megacephala* to Permethrin and Deltamethrin in Thailand. Journal of Medical Entomology, 42: 812-814. https://doi.org/10.1093/jmedent/42.5.812

Von-Zuben C. J., Stangenhaus, G., & Godoy, W. A. C. (2000). Competição larval em *Chrysomya megacephala* (F.) (Diptera: Calliphoridae): efeitos de diferentes níveis de agregação larval sobre estimativas de peso, fecundidade e investimento reprodutivo. Revista Brasileira de Biologia, 60(2): 195-203. https://doi.org/10.1590/S0034-71082000000200002

Wanchoo, A., Lewis, M. W., & Keyhani, N. O. (2009). Lectin mapping reveals stage-specific display of surface carbohydrates in in vitro and haemolymph-derived cells of the entomopathogenic fungus *Beauveria bassiana*. Microbiology 155(9): 3121-3133. https://doi.org/10.1099/mic.0.029157-0

Wang, H., Peng, H., Li, W., Cheng, P., & Gong, M. (2021). The Toxins of *Beauveria bassiana* and the strategies to improve their virulence to insects. Frontiers in Microbiology, 12: 705343. https://doi.org/10.3389/fmicb.2021.705343

Wei, G., Lai, Y., Wang, G., Chen, H., Li, F., & Wang, S. (2017). Insect pathogenic fungus interacts with the gut microbiota to accelerate mosquito mortality. PNAS, 114(23): 5994-5999. https://doi.org/10.1073/pnas.1703546114

Wen, L. V., Zhang, Z., Zhang, K. Y., Yang, H., Liu, S., Xu, A., Guo, S., Zhao, Q., & Huang, W. (2016). A mitochondria-targeted photosensitizer showing improved photodynamic therapy effects under hypoxia. Angewandte Chemie, 55(34): 9947-9951. https://doi.org/10.1002/anie.201604130

White, R. L., Geden, C. J., & Kaufman, P. E. (2021a) Exposure timing and method affect *Beauveria bassiana* (Hypocreales: Cordycipitaceae) efficacy against house fly (Diptera: Muscidae) larvae. Journal of Medical Entomology, 58(1): 372-378. https://doi.org/10.1093/jme/tjaa156

White, R. L., Geden, C. J., Kaufman, P. E., & Johnson, D. (2021b). Comparative virulence of *Metarhizium anisopliae* and four strains of *Beauveria bassiana* against house fly (Diptera: Muscidae) adults with attempted selection for faster mortality. Journal of Medical Entomology 58(4): 1771-1778. https://doi.org/10.1093/jme/tjab027

Xie, X. Q., Wang, J., Huang, B. F., Ying, S. H., & Feng, M. G. (2010). A new manganese superoxide dismutase identified from *Beauveria bassiana* enhances virulence and stress tolerance when overexpressed in the fungal pathogen. Applied Microbiology and Biotecnology, 86: 1543-1553. https://doi.org/10.1007/s00253-010-2437-2

Zimmermann, G. (2007). Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*. Biocontrol Science and Technology, 17(6): 553-596. https://doi.org/10.1080/09583150701309006