

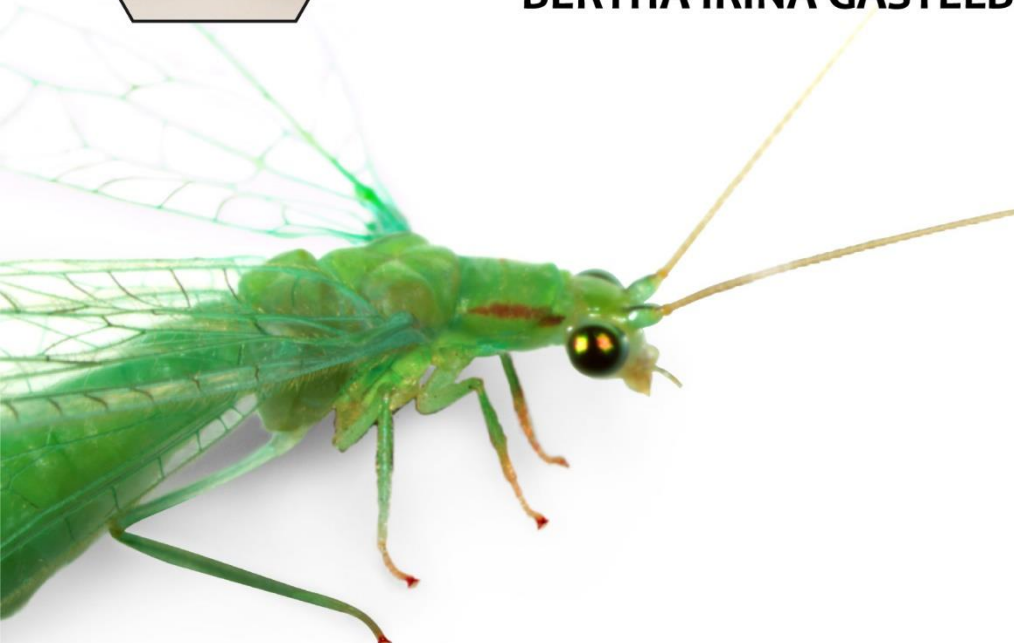


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UNIVERSIDADE ESTADUAL PAULISTA
"Júlio de Mesquita Filho"
INSTITUTO DE BIOCÊNCIAS DE BOTUCATU

**TOXICIDADE E GENOTOXICIDADE DO BIOPESTICIDA
AZAMAX™ EM OVÁRIO E INTESTINO DE *Ceraeochrysa
claveri* (NEUROPTERA:CHRYSOPIDAE)**

BERTHA IRINA GASTELBONDO PASTRANA



**BOTUCATU – SP
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BERTHA IRINA GASTELBONDO PASTRANA

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PROF. DR. FÁBIO HENRIQUE FERNANDES

Tese apresentada ao Instituto de Biociências,
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título de Doutora no Programa de Pós-
Graduação em Biotecnologia, Área de
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*...Muito obrigada Deus, por não ter me dado tudo o que eu quis,
mas, por ter me dado o necessário para eu chegasse até aqui...*

*“E sabemos que todas as coisas contribuem juntamente para o bem
daqueles que amam a Deus, daqueles que são chamados segundo o seu
propósito.”*

Romanos 8:28

Dedico este trabalho...

*Aos meus pais Concepción e Alcibiades,
e aos meus irmãos Alcibiades, Camilo Ernesto e Jorge Arturo.*

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RESUMO

O biopesticida Azamax™ tem sido utilizado como uma alternativa aos inseticidas sintéticos para o controle de pragas em plantações. Além disso, este produto tem obtido destaque por poder se associar ao uso de predadores naturais no controle biológico previsto pelo Manejo Integrado de Pragas (MIP). Dentre os modelos biológicos, destaca-se a espécie *Ceraeochrysa claveri*, sobretudo devido sua característica predadora na fase de larva com ampla variedade de presas e alto potencial reprodutivo na fase adulta. Embora a azadiractina, ingrediente ativo do Azamax™, já tenha sido relacionada com a indução de efeitos citotóxicos em insetos, ainda não há dados na literatura sobre os possíveis efeitos da sua ingestão indireta em células germinativas e intestinais de *C. claveri*. Desta forma, o objetivo deste estudo foi avaliar o efeito tóxico e genotóxico do Azamax™ em células do ovário e do intestino de fêmeas de *C. claveri*. As larvas foram alimentadas *ad libitum*, com ovos de *Diatraea saccharalis* tratados com duas diferentes concentrações do Azamax™ (0,3% e 0,5%) durante todo o seu período larval (n=150 larvas/grupo). As análises morfológicas, ultraestruturais e de genotoxicidade foram realizadas após os indivíduos se desenvolverem até a fase adulta. Os resultados indicaram que a exposição ao Azamax™ somente na fase larval, induziu sinais de toxicidade em todas as fases do desenvolvimento do inseto, como: (a) significativa taxa de mortalidade a partir do 3º instar larval; (b) prepupa e pupas inviáveis; (c) alterações na duração das fases (larva, prepupa e pupa); (d) marcantes malformações nos adultos emergidos; (e) danos primários no DNA de células intestinais e células dos ovários; (f) alterações ultraestruturais significantes nas células ovarianas (desorganização dos cistos/folículos ovarianos, dilatação do envelope nuclear e do retículo endoplasmático das células ovarianas e grande número de células com morfologia sugestiva de morte celular apoptótica. Nas condições experimentais utilizadas, os presentes dados indicam que o Azamax™ afeta o desenvolvimento e o potencial reprodutivo de *Ceraeochrysa claveri*, o que pode afetar a manutenção da espécie no ecossistema e, conseqüentemente, a continuidade do controle biológico natural exercido por esta espécie predadora de pragas.

Palavras-chave: Pesticida natural; reprodução; insetos não alvo; crisopídeo; danos no DNA.

ABSTRACT

AzamaxTM biopesticide has been used as an alternative to synthetic insecticides for pest control in several crops. Besides, this product has been highlighted because this association use with natural predators for biological control in the integrated pest management programs (IPM). Among the biological models, is commonly use the specie *Ceraeochrysa claveri*, above all its predatory characteristic at the larval stage with a wide variety of prey and high reproductive potential in the adult stage. Azadirachtin, active ingredient of AzamaxTM, has been related to the induction of cytotoxic effects in insects, although it has not been published in literature about the possible effects of Azamax and its indirect ingestion in germ and intestinal cells of *C. claveri*. From this, the aim of this study, is to evaluate the toxic and genotoxic effect of AzamaxTM in cells of the ovary and intestine of females of *C. claveri*. Larvae were fed *ad libitum* with treated eggs of *Diatraea saccharalis* at different concentrations of AzamaxTM (0.3% and 0.5%) during all or their larval period (n = 150 larvae/group). Morphological, ultrastructural and genotoxic analyzes carried out when individuals reached adult stage. The results indicated that exposure to AzamaxTM only in the larval phase induced signs of toxicity at all stages of insect development, such as: (a) significant mortality rate from 3rd instar larval; (b) prepupa and pupae inviable; (c) changes in the duration of the phases (larva, prepupa and pupa); (d) marked malformations in the emergent adults; (e) primary damage to the DNA of intestinal cells and ovarian cells; (f) significant ultrastructural alterations in ovarian cells (disorganization of ovarian cysts/follicles, dilatation of nuclear envelope and endoplasmic reticulum of ovarian cells, and large numbers of cells with morphology suggestive of apoptotic cell death. Under experimental conditions used in this study, AzamaxTM affects development and the reproductive potential of *Ceraeochrysa claveri*, and it can affect the maintenance of species in the ecosystem, and consequently, the natural biological control exercised by this predator species of pests.

Keywords: Natural pesticide, reproduction, non-target insects, green lacewings, DNA damage.

1. INTRODUÇÃO



1.1. Considerações iniciais

A poluição ambiental, o aumento do uso de inseticidas sintéticos e os seus riscos à saúde humana são temas atuais que norteiam a discussão sobre o desenvolvimento de uma agricultura sustentável (GARZÓN et al. 2015; SANTOS et al. 2015). A agricultura é hoje um dos setores mais importantes da economia brasileira. No entanto, a incidência de artrópodes pragas nos sistemas de produção agrícola tem sido o principal desafio para a manutenção da produtividade das diferentes culturas (ANSANTE et al., 2015). Neste sentido, tem sido necessário o controle químico das pragas por meio de inseticidas sintéticos, mas o aumento da resistência a estes compostos convencionais, a persistência de resíduos químicos nos ecossistemas, e a crescente demanda para o consumo de alimentos livres de agrotóxicos têm mobilizado a criação de novas metodologias que garantam a sustentabilidade (TIWARI et al., 2011; SANTOS et al., 2015; CARVALHO et al., 2013). Isto tem motivado a implementação de novas e diferentes estratégias para o controle de insetos-pragas, como o uso do sistema de Manejo Integrado de Pragas (MIP), no qual é utilizado o controle biológico por meio de predadores naturais com ou sem associação ao controle químico (ROGERS et al., 2007). No entanto, o MIP recomenda o uso de produtos que preservem, ou ao menos sejam compatíveis com os inimigos naturais das pragas, pertencentes ou inseridos aos agroecossistemas (WIKTELIUS et al., 1999; ROGERS et al., 2007).

1.2. Predadores naturais e *Ceraeochrysa claveri*

Dentre os diversos grupos de insetos considerados predadores naturais, destaca-se a família Chrysopidae (Ordem: Neuroptera), a qual inclui 1.200 espécies de insetos, comumente conhecidos no Brasil como crisopídeos ou bichos-lixeiros, pelo fato de algumas espécies terem o comportamento de carregar detritos no seu dorso como método de camuflagem ou barreira física de proteção (FREITAS; PENNY, 2001; ALBUQUERQUE, 2009). Os crisopídeos, por serem predadores na fase larval com alta voracidade e ampla variedade de presas, além da sua plasticidade ecológica e alto potencial reprodutivo na fase adulta, são atualmente considerados agentes de controle biológico por excelência (PAPPAS et al., 2011). Estes insetos, na sua fase de larva, tem preferência na predação de diversos

tipos de insetos pragas de várias ordens e famílias, como pulgões, cochonilha, mosca-branca, cigarrinhas, tripés, psilideos, ovos e larvas de Lepidoptera, Coleoptera e Diptera; determinando assim, um importante papel no controle biológico natural dos agroecossistemas que englobam as diferentes culturas de grande importância econômica no Brasil e no mundo (CANARD; PRINCIPI, 1984; FREITAS; PENNY, 2001; ALBUQUERQUE, 2009; PAPPAS et al., 2011). No Brasil, de acordo com Freitas e Penny (2001), ocorrem importantes gêneros da família Chrysopidae, com grande potencial para serem usados nos programas de controle biológico de pragas, como são os gêneros *Crysoperla* e *Ceraeochrysa* (ALBUQUERQUE et al., 2001).

O gênero *Ceraeochrysa* possui 56 espécies descritas no mundo inteiro, distribuindo-se no continente americano desde o Canadá até a Argentina e tem principal ocorrência nas regiões neotropicais (FREITAS; PENNY, 2001), sendo 15 espécies identificadas nos ecossistemas agrícolas brasileiros, entre as quais a espécie *Ceraeochrysa claveri* (Navás, 1911). Há na literatura poucas informações sobre *C. claveri*, embora esta espécie já esteja estabelecida como agente de controle biológico de várias pragas agrícolas (FREITAS; PENNY, 2001). *C. claveri* é um inseto considerado holometábolo por apresentar aparência e hábitos alimentares diferentes durante as fases do seu ciclo de vida: ovo, larva, pupa e adulto (Fig. 1) (CANARD et al., 1984; FREITAS; PENNY, 2001).

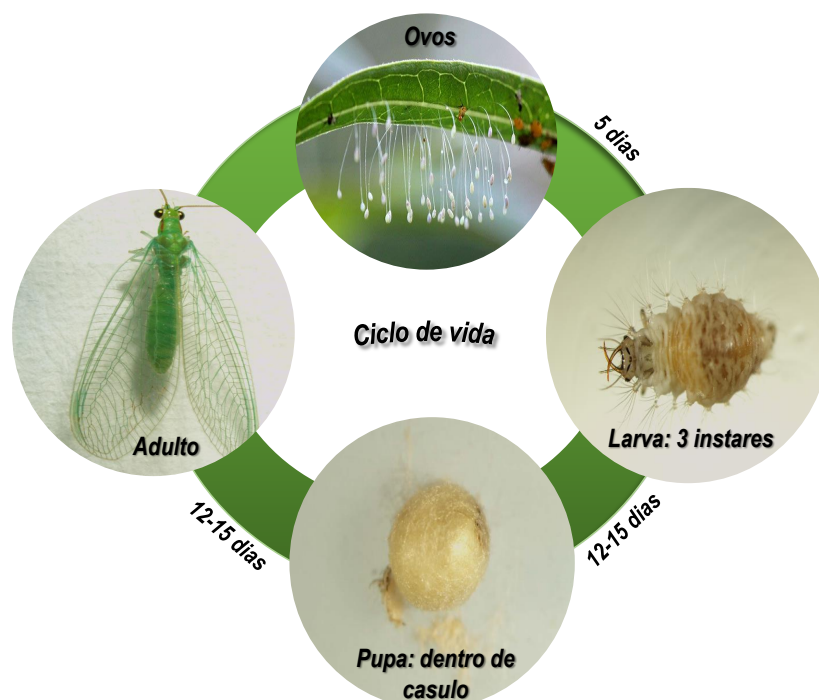


Figura 1. Ciclo de vida de *Ceraeochrysa claveri*. Fonte: Própria.

Os ovos têm forma esférica e são dispostos na extremidade de um pedicelo fino e longo, que tem tamanho variando entre 2 mm e 20 mm, e coloração amarelada ou verde-azulada no período de oviposição, embora tendo a escurecer ao se aproximar o momento de eclosão da larva (GEPP, 1984; ALBUQUERQUE, 2009). Na fase de larva, o inseto passa por três instares, nos quais as larvas apresentam atividade predadora, tendo mandíbulas longas e afiadas. Nessa fase, as larvas também podem preda ovos e larvas recém-eclodidas da mesma espécie, fato que as torna canibais. No entanto, essa característica é comum apenas quando há escassez de alimento (FREITAS; PENNY, 2001; ALBUQUERQUE, 2009).

As larvas de *C. claveri* são terrestres campodeiformes (com pernas torácicas longas e ambulatórias), apresentam corpo oval e um pouco achatado dorso-ventralmente, recoberto por cerdas longas (ALBUQUERQUE, 2009). Após completar o desenvolvimento larval, os indivíduos do 3º instar confeccionam um casulo esférico que contém numerosas camadas de fios finos de seda firmemente aderidos em função de proteger no seu interior a fase de pupa do inseto (GEPP, 1984; CANARD et al., 1984). A pupa é exarada (com apêndices visíveis) e de cor verde, mas tem anteriormente a etapa de prepupa, a qual compreende o período desde a confecção do casulo até a última ecdise larval ocorrida ainda no interior deste, e é caracterizada pela presença da exúvia, vista como um enegrecimento em forma de disco em uma das extremidades do casulo; que evidenciam o acúmulo de resíduos metabólicos produzidos durante o desenvolvimento larval-pupal (ALBUQUERQUE, 2009).

Ao completar a fase de pupa, o adulto farato ou pupa móvel, emerge do casulo por meio de uma abertura circular, e passa pela última ecdise ou estágio imaginal, na qual o adulto atinge sua maturidade, expulsa a exúvia larval em forma de mecônio dentro do casulo pupal e finalmente, adquire asas expandidas e funcionais (ALBUQUERQUE, 2009). Os crisopídeos adultos, medem de 10 a 15 mm de comprimento, são predominantemente verdes, com dois pares de asas membranosas apresentando nervosidades, antenas finas e longas comumente maiores que as asas, três pares de pernas ambulatórias e olhos grandes iridescentes (ALBUQUERQUE, 2009).

A transição dos estágios imaturos até a fase adulta é controlada por dois hormônios morfogenéticos, o hormônio juvenil (HJ) e a 20-hidroxiecdisona (20E), os quais regulam todos os processos de desenvolvimento pós-embrionário, incluindo ecdises, metamorfose e transformações pupa-adulto. Especificamente, o hormônio 20E, do tipo ecdisteróide, induz a ecdise enquanto o HJ determina o tipo de ecdise (larval-larval ou larval-pupal) (TRUMAN; RIDDIFORD, 2002; YAMANAKA et al., 2013).

É importante destacar que o inseto apresenta diferentes hábitos alimentares durante o seu ciclo de vida. No estágio adulto, os Crisopídeos são glicopolínívoros, ou seja, alimentam-se de pólen, néctar e/ou “*honeydew*” (uma excreção dos membros da subordem *Sternorrhyncha: Hemiptera*) (SOUZA et al., 1996; ALBUQUERQUE, 2009). No estágio de larva, o seu hábito alimentar é unicamente polífago, tendo que consumir insetos pragas para completar o desenvolvimento dos seus estágios imaturos, sendo então considerados eficientes predadores (ALBUQUERQUE, 2009).

Pelo fato de *C. claveri* ser um importante predador de pragas fitófagas pertencentes aos agroecossistemas neotropicais, esses insetos contribuem para o controle da densidade populacional dessas pragas no campo e a sua preservação para manter o equilíbrio ecológico se faz muito necessária (ONO et al., 2017). Assim, ao ser incluído dentro dos programas de MIP, juntamente com o controle químico, busca-se o uso de produtos mais seguros para o ambiente e que não causem a eliminação das espécies predadoras (SCHMUTTERER, 1990; LIU; CHEN, 2000; ONO et al., 2017). Neste sentido, inseticidas de origem natural, também chamados de biopesticidas, têm sido muito estudados e têm alcançado boa aceitação devido à contribuição para a redução da dependência aos pesticidas sintéticos convencionais, sobretudo pela rápida degradação no ambiente (REGNAULT-ROGER et al., 2012).

1.3. Bioinseticidas e azadiractina

Entre os inseticidas de origem vegetal atualmente utilizados na agricultura, destacam-se aqueles que contêm a azadiractina (Fig. 2) como princípio ativo, substância obtida das folhas e sementes de *Azadirachta indica*, popularmente conhecida por “Nim”. Desde a sua descoberta em 1966, a azadiractina tem sido estudada por apresentar elevado potencial de inseticida e acaricida, com diferentes mecanismos de ação sobre insetos pragas (MORGAN; THORTON, 1973; MORDUE (LUNTZ); NISBET, 2000; SIDDIQUI et al., 2004; MORGAN, 2009). Devido à mínima ou nula toxicidade induzida em vertebrados de modo geral, e baixa persistência no ambiente, esta substância é considerada “eco-saudável”. Os principais efeitos inseticidas são: atividade anti-alimentar e a ação direta de regulador do crescimento (MITCHELL et al., 1997; MORDUE (LUNTZ); NISBET, 2000; AGGARWAL; BRAR, 2006; MORGAN, 2009).

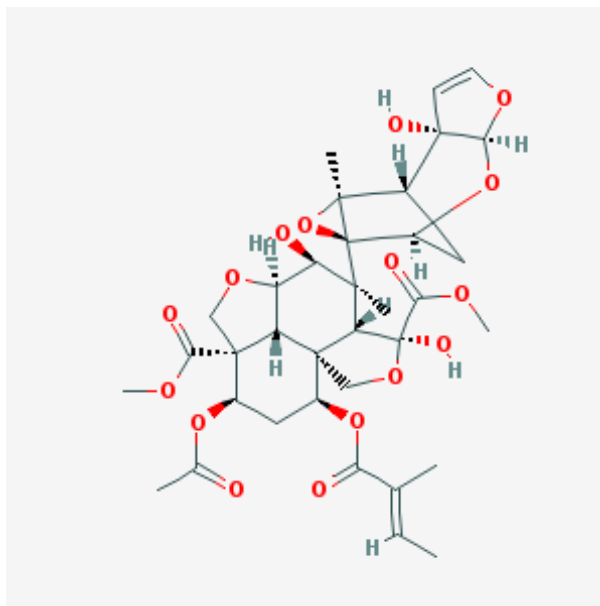


Figura 2. Estrutura química 2D da Azadiractina. Fonte: PUBCHEM/NCBI.

O inseto que ingere azadiractina não morre imediatamente, mas devido ao efeito anti-alimentar, o inseto cessa sua alimentação, o que o leva a morte por inanição (MORDUE (LUNTZ et al., 1996). Desta forma, o composto pode causar diversos efeitos sub-letais, tais quais: atraso no desenvolvimento larval e/ou pupal, ecdise incompleta, presença de larvas permanentes, de pupas e adultos malformados, e diferentes efeitos sobre o ciclo reprodutivo dos insetos (SCHMUTTERER, 1990; NASIRUDDIN; MORDUE (LUNTZ), 1993; MORDUE (LUNTZ); NISBET, 2000; AGGARWAL; BRAR, 2006; MORGAN, 2009; ALMEHMADI, 2011).

De modo geral, a azadiractina pode causar desregulação generalizada dos processos fisiológicos do inseto (MORGAN, 2009). O efeito patológico ocorre principalmente, pela indução de interferência no funcionamento normal dos sistemas endócrino e neuroendócrino (MEURANT et al., 1994; SAYAH, 2002). Tem sido demonstrado, que o mecanismo de ação pode ser considerado multifatorial, por envolver vários outros mecanismos subsequentes (Fig. 3). Um dos efeitos baseia-se no bloqueio da liberação de neuropeptídios precursores de hormônios, que tem a função do controle da síntese e liberação dos hormônios por parte das glândulas endócrinas, especificamente do 20E, que é regulado pelas glândulas pro-torácicas do inseto. Portanto, a azadiractina, bloqueia paralelamente a síntese e a liberação do HJ na hemolinfa, a qual também é dependente da liberação desses neuropeptídios (MORDUE et al., 2005). A azadiractina também interfere na biosíntese de ecdisteroides, pois afeta a conversão do hormônio ecdisônio (20E) em 20-hidroxiecdisona, sua forma mais ativa

fisiologicamente. Além disso, os diferentes compostos do óleo de nim, inibem a atividade da ecdisona 20-monooxigenase, enzima responsável pela conversão da 20E (MITCHELL et al., 1997; MORDUE et al., 2005).

A alteração no sistema hormonal do inseto tem sido associada à ação da azadiractina, a qual atua como antagonista do hormônio ecdisônio, além de interagir com o receptor celular da ecdisona. Portanto, essa interação produz alteração em todos os eventos moleculares subsequentes, os quais ainda não foram totalmente esclarecidos (SOIN et al., 2010; LI et al., 2004). Desta forma, as alterações no sistema endócrino e neuroendócrino do inseto leva a muitas alterações nos órgãos vitais dependentes deste controle hormonal, dentre eles, os órgãos reprodutores (MORGAN, 2009; LAI et al., 2014). Alguns autores classificam o conjunto dessas manifestações/alterações morfológicas à síndrome de toxicidade generalizada, causada pela azadiractina na ecdise do inseto (LAI et al., 2014; BEZZAR-BENDJAZIA et al., 2016).

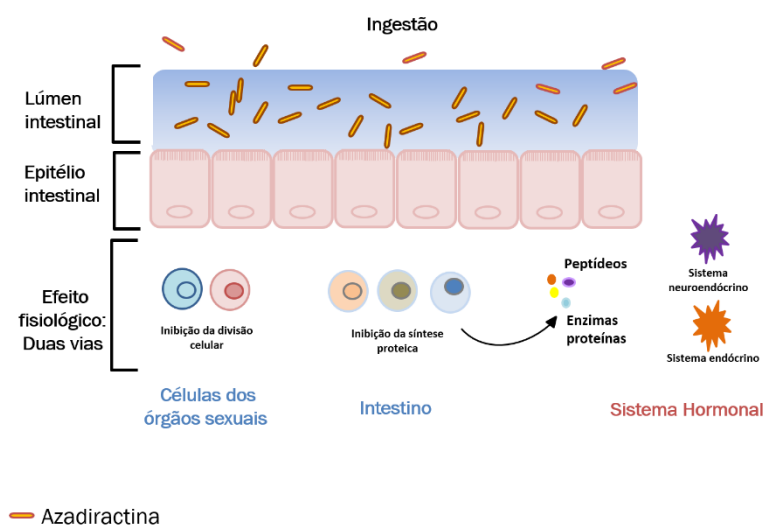


Figura 3. Esquematização do modo de ação da azadiractina após ingestão segundo Mordue et al. (2005). Fonte: Própria.

Os outros efeitos associados à azadiractina, acontecem em dois tipos de células que são principalmente consideradas as células alvo da molécula. A azadiractina inibe a atividade celular em regiões dos órgãos responsáveis pela síntese de enzimas, no caso do intestino atua inibindo a produção de enzimas digestivas e de detoxificação por parte das células colunares (MORDUE et al., 2005). Este composto também atua sobre as células com alta taxa de

divisão celular como é o caso de células ovarianas, inibindo também a atividade desse tipo de células (MORDUE et al., 2005).

A toxicidade da azadiractina tem direcionado diversas pesquisas em insetos não-alvos como os crisopídeos, tendo em vista os diferentes efeitos subletais demonstrados nessas espécies de insetos (MEDINA et al., 2004; GHAZAWI et al., 2007). Existe a recomendação de que o uso desses compostos naturais seja altamente controlado em culturas nas quais se utiliza o biopesticida associado ao controle biológico de pragas no MIP (SANTOS et al., 2015). Alterações morfofisiológicas em órgãos reprodutores e no intestino de várias espécies de insetos têm permitido demonstrar que o efeito generalizado desse biopesticida pode também se apresentar nos inimigos naturais (MEDINA et al., 2003; MEDINA et al., 2004; GAZHAWI et al., 2007; SCUDELER; SANTOS, 2013; SCUDELER; SANTOS, 2014). O óleo de nim mostrou-se tóxico à espécie *Ceraeochrysa claveri* em diferentes abordagens (SCUDELER; SANTOS, 2013; SCUDELER; SANTOS, 2014; SCUDELER et al., 2014; SCUDELER et al., 2016; GARCIA et al., 2019).

Embora estudos demonstrem a eficácia de biopesticidas com o princípio ativo azadiractina (SCHMUTTERER, 1990; NASIRUDDIN; MORDUE (LUNTZ), 1993; MORDUE (LUNTZ); NISBET, 2000; AGGARWAL & BRAR, 2006; MORGAN, 2009; ALMEHMADI, 2011; JASMINE et al., 2012, RIBEIRO et al., 2014; CÉSPEDES et al., 2014), poucos são aqueles voltados para os efeitos deste composto ativo no desenvolvimento de insetos não-alvos. Desta forma, a investigação e o conhecimento dos efeitos de biopesticidas em espécies não-alvos sob diferentes condições (laboratoriais, semi-campo e no campo) são de grande importância para o conhecimento do mecanismo de ação desses compostos nas culturas e nos agroecossistema em que se faz uso do controle biológico de pragas.

A molécula da azadiractina tem sido estudada também pelo seu potencial genotóxico; sugerindo danos no DNA e aberrações cromossômicas após exposição à produtos associados ao neem (AWASTHY et al., 1995; KHAN; AWASTHY, 2003; CHANDRA; KHUDA-BUKHSH, 2004; PACKIAM et al., 2015; DUMAN; ALTUNTAŞ, 2018). Um dos mecanismos moleculares de ação da azadiractina é a inibição ou alteração da transcrição ou tradução de proteínas expressas em estágios específicos do ciclo celular (MORDUE et al., 2005). Ao inibir especificamente a transcrição de proteínas que participam da síntese e montagem do sistema de microtúbulos celular altera-se consequentemente diferentes eventos mitóticos (MORDUE et al., 2005). Neste sentido, considera-se que azadiractina

inibe a proliferação celular; pois ao não se dar a montagem correta dos microtúbulos as células são induzidas à apoptose (MORDUE (LUNTZ) et al., 2005). Essas alterações no ciclo celular induzem a quebras na fita cromossômica ou podem também produzir distúrbios do fuso mitótico levando consequentemente ao desenvolvimento de efeitos genotóxicos tardios (AWASTHY et al., 1995).

A maioria dos métodos utilizados para identificar uma resposta celular induzida pela azadiractina é de avaliação toxicológica clássica utilizando-se biomarcadores histológicos, morfológicos e fisiológicos (LAI et al., 2014). Contudo, a técnica de avaliação de danos no DNA (teste do cometa) vem sendo uma opção para elucidar o mecanismo de ação de compostos ricos em azadiractina, tais como o Azamax™ (JHA, 2008; AUGUSTYNIK et al., 2016).

Atualmente, várias formulações comerciais com base na azadiractina estão disponíveis para utilização na agricultura, entre as quais Azadirex®, Neemix®, Neemix® nos Estados Unidos e NeemAzal T/S e o NeemAzal F disponíveis na Alemanha, Austria, Italia, Espanha, Holanda e India, onde existem mais de 20 formulações comerciais derivadas do óleo de nim (MORGAN, 2009). No Brasil, utiliza-se uma formulação de concentrado emulsionável denominada Azamax™, (UPL e *United Phosphorus* do Brasil Ltda., Indianópolis, SP, Brasil) que é autorizada para o controle de pragas artrópodes em diferentes culturas e sistemas de produção (Agrofit, 2016). A formulação do Azamax™ tem tido crescente aceitação no mercado pelo fato de reduzir as desvantagens do extrato puro da azadiractina, como o curto período residual e a possível resistência para certas insetos pragas. O Azamax™ é um concentrado emulsificável composto por limonóides, tendo a azadiractina como o principal, e o 3-tigloylazadirachtol em menor quantidade (SANTOS et al., 2015).

O direcionamento de novas pesquisas que permitam detalhar as características toxicológicas do Azamax™, hoje considerado “moderadamente tóxico e com mecanismo de ação desconhecido” (MAPA, 2018), é fundamental, segundo o *Guidelines for Reproductive Toxicity Risk Assessment* da EPA (US Environmental Protection Agency) (1996), para avaliar o risco toxicológico e estabelecer o potencial reprotóxico do composto químico, considerando, quando for possível, outras manifestações de toxicidade como a genotoxicidade ou mutagênese e outras formas de toxicidade sistêmica geral (EPA, 1996).

2. OBJETIVOS

Diante do exposto, o atual estudo tem como objetivo geral investigar os efeitos do Azamax™ na biologia reprodutiva e entérica de *C. claveri*.

Objetivos específicos:

- Padronizar a técnica do ensaio cometa nos ovários e intestino de *C. claveri*.
- Avaliar os efeitos do biopesticida Azamax™ quanto aos níveis de danos primários (quebras de cadeia e sítios álcali-lábeis) no DNA de células intestinais e ovarianas de fêmeas adultas de *C. claveri*.
- Avaliar os efeitos do biopesticida Azamax™ no desenvolvimento dos insetos (duração das fases do ciclo de vida), assim como na morfologia e ultraestrutura dos ovários de fêmeas adultas de *C. claveri*.

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4. RESULTADOS

4.1. *CAPÍTULO 1*



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The comet assay in *Ceraeochrysa claveri* (Neuroptera: Chrysopidae): a suitable approach for detecting somatic and germ cell genotoxicity induced by agrochemicals

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Abstract

Some agrochemicals are genotoxic to several organisms. Nevertheless, few protocols are currently available for measuring the toxicogenetic effects of these compounds in target and non-target field-collected species of insects important to agriculture. Herein, we used the species *Ceraeochrysa claveri* (Neuroptera: Chrysopidae), a non-target predator insect, to investigate the ability of an azadirachtin-based biopesticide (Azamax™) to induce DNA damage. The alkaline version of the comet assay was standardized to evaluate genetic instability caused by the toxicant in somatic (gut) and germ (nurse cells and oocytes) cells of *C. claveri*. For this, *C. claveri* larvae were distributed into three groups (10/each) and treated with Azamax™ at 0, 0.3% or 0.5% throughout the larval stage. DNA damage (tail intensity) was measured in adult insects, four days after emerged. The data showed that both doses of Azamax™ (0.3% and 0.5%) were able to significantly ($p < 0.05$) increase DNA damage in somatic and germ cells of *C. claveri*. In conclusion, *C. claveri* (intestinal and ovarian cells) was a sensitive bioindicator for identifying Azamax™ genotoxic potential, whereas the comet assay was a useful tool for detecting the genotoxic hazard of the pesticide in the field-collected insect species. Given that estimation of adverse effects of pollutants on ecosystems is an essential component of environmental risk assessment, the approach used can be recommended to estimate the ecotoxicity of agricultural chemicals.

Keywords: azadirachtin, biopesticide, DNA damage, ecotoxicity, ovary, gut.

4.2. *CAPÍTULO 2*

Artigo sometido

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Azadirachtin-based biopesticide acts as a hazardous product by affecting fitness and ovarian development in the natural enemy *Ceraeochrysa claveri* (Neuroptera: Chrysopidae)

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Abstract

Ceraeochrysa claveri is an important non-target predator insect. Searches for new control methods that minimize the adverse effects of synthetic insecticides have initiated a resurgence of the use of botanical insecticides. Azadirachtin-based biopesticides are the more prominent botanical products commercialized today and represent an alternative to these compounds. Despite extensive studies of the physiological effects on pest insects, little attention has been given to multiple toxic effects of azadirachtin under authorized concentrations in natural enemies of insects important to agriculture. In the present study, we used the *C. claveri*, to assessment the toxic effect of an azadirachtin-based biopesticide (Azamax™) on fitness and ovarian development. *C. claveri* larvae were distributed in 3 groups (150 larvae per group) and treated with Azamax™ at 0 (control), 0.3 and 0.5 % during all the larval stage (15 days) to evaluate the effects of the biopesticide on the survival, behavior, external morphology, development, and the impairment of ovarian follicles of *C. claveri*. The two doses tested corresponding at the minimum and maximum concentrations that is used in the field. Results showed that Azamax™ (i) reduced survival (ii) change larval and pupal developmental time, (iii) induced impairment in the animal body and (iv) in the ultrastructure of adult ovaries – thinner ovariole sheath, complete shrinkage, alterations in cellular organization and cell death –. Together, these effects could potentially compromise the health of *C. claveri*. In conclusion, Azamax™, known as a reduced risk insecticide, induces hazard effects in lifespan and ovarian development of *C. claveri*, an economically important non-target insect.

Keywords: ovary, non-target insects, growth regulation, natural pesticides.

1. Introduction

Ceraeochrysa species are being studied due to their current use in commercial agriculture (Pappas et al., 2011). Many of these are distributed over variety of ecosystems and a restricted number have been reported from agroecosystems (Albuquerque et al., 2001). Among these species, *Ceraeochrysa claveri* is considered a predator insect with great potential for the biological control in neotropical agroecosystems; it has been recognized due to the key effect of natural enemy on a certain pest species (Albuquerque et al., 2001; Freitas and Penny, 2001). At larval stage, *C. claveri* has a broad range of prey, such as aphids, thrips, mites, witheflies, eggs and larvae of economically important insect pests. Due to larval feeding habits of green lacewings such as *C. claveri*, they are recommended to be included in integrated pest management (IPM) programs (Albuquerque et al., 2001; Freitas and Penny, 2001; Pappas et al., 2011).

Considering the use of these natural enemies in IPM schemes, associated with conventional synthetic insecticides for rotation or botanical products as a substitute, is an important alternative for pest control (Garzón et al., 2015; Santos et al., 2015). This strategy is necessary to establish and maintaining a sustainable agriculture, decreasing pesticide dependency, reducing environmental pollution and human contamination. Therefore, preserving these non-target beneficial insects in the agroecosystems could contribute to an effective and natural pest management (Wikteliuss et al., 1999; Rogers et al., 2007).

However, to thoroughly verify the compatibility of the conventional pesticide and biopesticides with beneficial insects is necessary testing the side effects of these preparations for guaranteeing sustainability, safety and credibility of this technology (Santos, 2015). Reduced-risk pesticides are generally compatible with natural enemies (Rimoldi et al., 2012; Guedes et al., 2016), but still previous to their joint use, it is necessary to assessment the side effects in non-target insects. The results of different research could be used by agronomist

and environmental agencies to choose safe products for use in IPM programs and to facilitate the registration of new enable products for use in agriculture (Garzón et al., 2015).

Among the reduced-risk products, the interest is high for natural compounds, primarily derived from plants. The natural pesticides obtained from the Indian neem tree (*Azadirachta indica* A. Juss) (Meliaceae) are used worldwide in agricultural crops against arthropod pests (Charbonneau et al., 2007; Grimalt et al., 2011; Biondi et al., 2012). The most promising and effective insecticide extracted of the neem tree is Azadirachtin, a limonoid with different modes of action, acts mainly as a repellent, antifeedant and insect growth inhibitor, and it interferes with the mating behaviour, fecundity and fertility of female arthropod pests with different eating habits (Weathersbee and McKenzie, 2005; Abedi et al., 2014; Sanchez-Ramos et al., 2014). It combines antifeedant action (Blaney et al., 1990) with growth regulatory and sterilitant effects, caused mainly by alterations of ecdysteroid and juvenile hormone titers (Mordue (Luntz) et al., 1998). Negative effects of extracts from the neem tree on reproduction have also been reported in a number of insects (Schmutterer and Rembold, 1995). Thus, taken together, it is assumed that azadirachtin has direct effects on a variety of tissues and organs and, as a consequence, several modes and sites of action. These multiple activities of azadirachtin involve several pathways (Lai et al., 2014); however, previous studies have tended to focus on single physiological effects of azadirachtin, with little attention on multiple and combined toxicity effects or possible interactions.

The increasing demand of these biopesticides has led to a strong interest in studying their potential to cause deleterious effects on non-target species. Besides activity against arthropod pests, azadirachtin-based products may also cause deleterious effects on beneficial insects, such as *C. claveri* (Scudeler and Santos, 2013; Scudeler and Santos, 2014; Scudeler et al., 2014; Scudeler et al., 2016; Garcia et al., 2018; Scudeler et al., 2019; Gastelbondo-Pastrana et al., 2019). Several azadirachtin-based commercial formulations are

commercially available for use in agriculture. In Brazil, the biopesticide is available in an emulsifiable concentrate formulation termed Azamax™, and it is authorized for use in the control of arthropod pests in different crops and production systems (Agrofit, 2018).

Prior to large-scale application of this novel biopesticide for the control of insect pests, it is important to confirm its multiple sublethal effects in non-target insects, as part of an effective pesticide risk assessment (Bernardes et al., 2018; Zhang et al., 2018). In this sense, we evaluate the effects of this azadirachtin-based product in the biological parameters and on ovarian development in *C. claveri*. It is important to assess the adverse effects of biopesticides on natural enemies, and besides is particularly necessary evaluate defects can be passed onto the next generations. From this, in this study was assessment effects not only the animal directly affected with the biopesticide.

2. Materials and methods

2.1. Chemicals

The biopesticide analyzed was available as an emulsifiable concentrate formulation, Azamax™ (active ingredient 12 g/L; 1.2% m/m) obtained from UPL e United Phosphorus do Brazil Ltd. (Sao Paulo, Brazil) containing azadirachtin (main component) (Santos et al., 2015).

2.2. Insects maintenance

The newly hatched *Ceraeochrysa claveri* larvae (0–12 h old) used in the bioassays were obtained from the continuous laboratory colony of the Laboratory of Insects in the Department of Morphology at the Institute of Biosciences of Botucatu at UNESP, Brazil. The *C. claveri* colony was reared with *Diatraea saccharalis* (Lepidoptera: Crambidae) eggs during the larval stage and with an artificial diet (1:1 honey/yeast solution) for adults. The

insects were maintained in an environmental chamber under controlled conditions (25 ± 1 °C; $70 \pm 10\%$ RH; 12L:12D photoperiod) (Scudeler et al., 2016).

2.4. Bioassays

Fresh egg clusters recently deposited by females of *D. saccharalis* (Lepidoptera: Crambidae) were collected and dipped once in each dose tested of Azamax™, establishing a minimal dose of 36 mg/L (0,3%) and a high dose of 60 mg/L (0,5%) according to the official regulation for the phytosanitary pesticide system of the Ministry of Agriculture, Livestock and Food Supply of Brazil for use in the field in agricultural crops in which the lacewings occur (MAPA, 2018). Eggs of each dose of treatment (diluted in distilled water to obtain the desired concentrations) were immersed for 5 s and air-dried at room temperature for 1 h. For control group, egg clusters were dipped in distilled water (Scudeler and Santos, 2013). Newly hatched larvae were selected randomly and placed in individual polyethylene cups (2 cm height x 6 cm diameter), these were divided into three experimental groups). The groups were tested under the same environmental conditions as described for rearing. In the control group (n = 150), larvae were fed *ad libitum* on *D. saccharalis* eggs treated with distilled water. In the experimental groups of Azamax™ at 0,3 % (n = 150) and 0,5% (n = 150) doses, larvae were fed *ad libitum* on eggs treated with the biopesticide throughout the larval period (15 days) until pupation. After cocoon spinning, specimens remained in the same polyethylene cups under the same controlled conditions for the entire experiment and until the adult emerged. Adults were kept in a polyethylene box (9 cm height x 18 cm diameter) and fed with an artificial diet (1:1 honey/yeast solution). One day after they have emerged, female insects were used to obtain the ovaries to perform the morphological analysis.

2.5. Toxicity analysis

For larval instars, the specimens were checked daily by monitoring larval mortality for fifteen days. Individuals without movement move after being touched with a fine brush, were considered dead as reported by Scudeler et al. (2016). The number of individuals used per treatment (i.e., biopesticide dose, including the control) for this analysis was 150, totaling 450 larvae sampled. At the prepupal period, when the larva finished a cocoon, the eventual inability of a larva to spin the cocoon to start the prepupal period were considered dead prepupa (Fig. 1b). And at the pupal stage, in which larva remained inside the cocoon and transformed into an exarate pupa (Fig. 1c), the pupal mortality was considered when it dead inside the cocoon (nonviable pupa). Larval mortality, percentage of pupae formed and successful adult emergence from those pupae were recorded. Cumulative mortality was the percentage of total individuals who failed to reach the complete adult form.

To assist in the description of the change in the prepupae, pupae and adults, the morphology of the normal and the malformed cocoons spun by larvae in the prepupal period, the viable and the nonviable pupae, the malformations of adults emerged from the larvae untreated and treated with Azamax were studied and documented with an Olympus SZX16 stereo microscope with cellD imaging software (Olympus Soft Imaging Solutions GmbH, Münster, Germany) at the Electron Microscopy Center of the Institute of Biosciences, UNESP, Botucatu-SP according to reported by Scudeler et al. (2016).

2.6. Development time

The developmental time of the larval, prepupa and the pupal stages were monitored daily. The total duration of lifecycle was also recorded. The transition of larval instars was

confirmed by the exuviae left after each ecdysis and by the color of the larvae as reported by Scudeler et al. (2016).

2.7. Morphology of the female reproductive system

2.7.1 Transmission Electron Microscopy (TEM)

For TEM, Adult ovarioles were collected 24 h after emergence and were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.3) (Karnovsky, 1965) for 24 h at room temperature and then postfixed in 1% osmium tetroxide in the same buffer for 2 h. After being washed with distilled water, the samples were contrasted with an aqueous solution of 0.5% uranyl acetate for 2 h at room temperature, dehydrated in a graded acetone series (50%, 70%, 90% and 100%), and embedded in Araldite resin. Ultrathin sections were contrasted with uranyl acetate and lead citrate and were then observed and photographed with a Tecnai Spirit transmission electron microscope (FEI Company, Eindhoven, Netherlands) at the Electron Microscopy Center of the Institute of Biosciences of Botucatu, SP, Brazil.

2.7.2 Scanning Electron Microscopy (SEM)

The ovaries samples were fixed for 48 h at room temperature in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3). Thereafter, the samples were washed in distilled water, postfixed in 1% osmium tetroxide diluted in distilled water for 30 min at room temperature, dehydrated through a graded series of ethanol, critical point-dried with CO₂ and coated with gold. The samples were examined and photographed using an FEI Quanta 200 scanning electron microscope (FEI Company, Eindhoven, Netherlands) at the Electron Microscopy Center of the Institute of Biosciences of Botucatu, SP, Brazil.

The wings of *C. claveri* adults were also analyzed and documented by SEM. Ten samples of wings of three experimental groups, were collected and processed for SEM. The samples were desiccated overnight, mounted on stubs, and critical point-dried with CO₂ and then sputter coated with gold (Bal-Tec SCD 050). They were examined in an FEI Quanta 200 SEM with an accelerating voltage of 12.5 kV at the Electron Microscopy Center of the Bioscience Institute, UNESP, Botucatu-SP. Both wings of insects were analyzed.

2.8. Statistics analysis

The study of association relative to the mortality of larvae, percentage of pupation and emergence and cumulative mortality were conducted using the Chi-square test that involved contrasts among and within multinomial populations. All tests were performed at a significance level of 5%.

To assess the developmental time of the larvae, prepupae, pupae and total duration of life cycle data are presented as mean $\pm$ standard error. ANOVA was used to evaluate mean differences groups, previously checking for normality and variance homogeneity, using Kolmogorov–Smirnov and Bartlett tests, respectively. When normality was not achieved, the non-parametric test Kruskal-Wallis, complemented with the Dunn test was used instead. The criterion of significance was set at $P < 0.05$.

3. Results and discussion

In the present study, we examined the potential effects of Azamax™ on insect fitness and its developmental consequences on whole insect and on ovarian follicles development, after indirect ingestion (prey poisoning) during larval period of *C. claveri*. Results show that the mortality of immature stage increases when tested both doses of this biopesticide and the prepupal and pupal stages represents the most sensitive parts of the life cycle of *C. claveri* leading to an inhibition of adult emergence.

Evaluation of azadirachtin toxicity in immature stages

In larval period, accumulated mortality was significantly more increased at third instar, as demonstrated in Table 1. These larvae died at about the time of the molting to pre-pupa. At this stage, the percentage mortality recorded in controls was only 6.8%. When treated third instar remaining larvae trying to molt to the pre-pupa few individuals were able to ecdyse, at both doses of treatment (0.3% and 0.5) a high percentage of the larvae of last instar were unable to undergo or terminate ecdysis.

These treated larvae remained in this condition. The inability of treated larvae to spin a cocoon was the main effect of Azamax in this period; it was documented by the presence of nonviable prepupae like a resultant deformed pre-pupa without cocoon that finally died or with incomplete cocoons (Fig.1a).

The quantitative effect of Azamax on the prepupal survival is shown in table 2 and it was represented by percentage of pupation (percentage of pupae formed). Significant differences were found among treatments and control in pupation. Both doses of Azamax (0.3% and 0.5%) reduced significantly the percentage of pupation when compared by the control (92%); 54.7% and 40.0% of treated larvae were able to moult into the pupa, respectively. As occurred at the prepupal stage, at undergo on the pupal development treated insects also have shown several malformations and finally dying at this stage (Fig. 1b). At the pupal stage were demonstrated other differences in external morphology between the Azamax treatments and the control, the treated pupae were not able to complete ecdyse and metamorphosis inside de cocoon (malformed pupa) and others died inside the cocoon. Malformed pupa and died pupa are represented in the Figure 1b. The malformed specimens

at the end of the pupal development presented characteristics of both pupa and adult indicating incomplete metamorphosis.

To explain the innocuous effects of this biopesticide at freshly eclodding larvae (first and second instar), it is likely that at this period the larvae had been exposed by lower time than at became third instar larval. According to Garzón et al. (2015) the higher accumulative larval mortality at third instar larvae was probably, higher because these larvae can be more easily damaged due to they walk on treated surfaces and cannot fly, and this overexposure can be by ingestion and inhalation at the same time. Scudeler et al. (2019) also observed the toxic effect of azadirachtin compounds by ingestion exposure in the third larvae of *C. claveri*. These compounds induce antifeedant effect, a feeding deterrent or antifeedant has been defined as a chemical which inhibits feeding but does not kill the insect directly, the insect often remaining near the treated plant and possibly dying through starvation (Morgan, 2009). The antifeedant effect could also explain the mortality at the last larval stage; 0.3% and 0.5% of Azamax seems to be no lethal for *C. claveri* larvae. Certain doses are lower than required for feeding deterrence; some insects ingesting azadirachtin not die immediately but soon stop feeding and will show developmental toxicity (Morgan, 2009). Moreover, azadirachtin deterrent feeding activity has also been reported in other insect species (Mordue (Luntz) and Nisbet, 2000; Morgan, 2009).

Despite its slightly toxic effect on larvae of third instar, this developmental stage showed less susceptibility than the prepupa and pupa. The significant alterations showed during larval-pupal development of *C. claveri* treated insects, demonstrate that Azamax™ mainly affect prepupal and pupal stage. Deleterious metamorphic effects and prevent to pupation seems to be associated with the interference of azadirachtin on the endocrine system (Mordue et al., 2005). These actions of azadirachtin present in Azamax™ probably resulting in growth inhibition and molting defects in the insect (Min-li et al., 1987; Feder et al., 1988).

This compound is known to cause degenerative structural changes of the nuclei in all endocrine glands (prothoracic gland, corpus allatum and corpus cardiacum) responsible for controlling moulting and ecdysis in insect which would contribute to a generalized disruption of neuroendocrine function (Mordue (Luntz) & Nisbet, 2000). Bezzar-Bendjazia et al. (2016) examined the lethal and sublethal effects of azadirachtin on *Drosophila melanogaster* and reported that the mortality of immature stage increases when the doses of azadirachtin are increased and the pupae represent the most affected stage as observed in our results. In addition, Scudeler et al. (2013) and (2016) showed similar results about the no formation of pupae with increase percentage of non-viable prepupa due to no cocoon formation when *C. claveri* larvae were exposed to different treatments of neem oil that contained 1500 ppm of azadirachtin A as the active ingredient. These authors confirmed that azadirachtin interfere with the molting and cocoon spinning, decreasing wall thickness and impairing ability to attach to a substrate. These negative effects may reduce the effectiveness of the mechanical and protective functions of cocoons during pupation, which makes the specimen more vulnerable to environmental factors (Scudeler et al., 2013).

Medina et al. (2003) also reported toxicological effects in the pupal stage in a green lacewing. These authors noted that Azadirachtin was harmful at higher doses during the formation the pupa when the predator *Chrysoperla carnea* was exposed by topical application. Pupae of queens of *Partamona helleri* that ingested azadirachtin-contaminated diet during the post-embryonic development also presented malformations and most of them were unable to walk or feed (Bernardes et al., 2018). In contrast, Medina et al. (2001) demonstrated not deleterious activities in pupae of *C. carnea* after topical azadirachtin treatment of young and old pupae. They explain these results due to the silken ovoid cocoon that covers the decticious pupae might inhibit uptake in the insect body after topical application. In the same study, Azadirachtin, however, significantly reduced the percentage

of pupae when last-instar *C. carnea* larvae were exposed by residual contact (Medina et al., 2001). Indicating that susceptibility to azadirachtin varies with application method and duration of exposure as reported by Zhang et al. (2018).

Regarding cumulative mortality represented by the percentage of individuals on immature stages who failed to reach the complete adult form, the results show significant differences compared with the control as showed in the Table 2. At the dose of 0.5% of Azamax more than half (64.7%) of treated insects no reached to adult stage. In a quantitative sense, because the cumulative prepupal and pupal mortality in the treated groups were increase compared with the control, cumulative mortality total were also increased; from this, at both doses tested of the Azamax, this compound is considered substantially toxic to *C. claveri* on the immature stages. Conversely, at increasing Azamax™ doses, it was decreased emergence rate at adult stage as observed in Table 2.

Indeed, azadirachtin affects various biological processes including larval and pupal development by regulating the genes involved in insect growth (Lai et al., 2014). In holometabolous insects, such as *C. claveri*, pupal stage encountering a drastic remodeling of most tissues and organs and represent a critical phase for adult formation (Ureña et al., 2014). Because, this stage is a vulnerable part of the lifecycle of *C. claveri* which azadirachtin can induce various morphogenetic alterations on the puparium preventing the complete emerging of the adult (Bezzar-Bendjazia et al., 2016).

The molecular mechanisms of sublethal effects of ingested azadirachtin is unclear, but studies in *D. melanogaster* larvae have demonstrated that azadirachtin appears to mainly affect post-transcriptional enzyme regulation, proteins involved in cytoskeleton development, and transcription, translation, and regulation of hormones, and energy metabolism (Lai et al., 2014; Asaduzzaman et al., 2016). In addition, a recent study

demonstrated genotoxicity of Azamax treatment in female adults of *C. claveri* after larval exposure to this product (Gastelbond-Pastrana et al., 2019).

On the other hand, a great percent of adult emerged of treated groups presented malformations as detailed by Gastelbond-Pastrana et al. (2019) in other part of this study. Adults emerged with malformations were considered non-viable adults and consequently died. The malformations including insects with incomplete metamorphosis, insects with parts of the old edcye remained adhering to the adult body, lower size, contorted antennae, abnormal legs and wings as showed in Figure 1c. Morphology of wings are detailed in Figure 2. Other authors support these findings: Ghazawi et al. (2007), Barbosa et al. (2015), Bernardes et al. (2018). In accordance with Zhang et al. (2018), Azadirachtin reduce the lifespan.

Effects on developmental time

The duration of larval development is given in Figure 3. Treatment of freshly larvae altered the duration of larval development at the two tested doses. At the first instar, the results did not show significant differences; in contrast, at second instar Azamax™ at the minimal dose induce delay of development and a high dose it reduces developmental time. Conversely, at the last instar Azamax™ reduces the duration of the period at 0.3% (minimal dose) and increased this time at 0.5% (maximum dose). In the prepupal stage was observed decreased of developmental time at 0.3% of Azamax but the same group of treated insects at pupal stage showed delay in the duration of the stage as observed inf the Figure 4. And the pupal period of the treated insect at 0.5% presented a reduction on the duration when it was compared with the control insects. No statistically significant differences were found for total time of life cycle among treatments.

In the current experiments, azadirachtin treatment not altered the total of life cycle but it affects the development of each stage of the insect . Previously, azadirachtin is reported to impair development and survival of different insect's species (Kraiss and Cullen, 2008; Lai et al., 2014; Asaduzzaman et al., 2016; Scudeler et al., 2016). Furthermore, azadirachtin was found to induce robust developmental delays in the larva-to-pupa and the pupae-to-adult transition. Lai et al. (2014) previously reported a delay in pupariation time in azadirachtin treated third-instar of *D.melanogaster* and showed that azadirachtin down regulated expression of genes related to hormonal regulation which may be related to the development and the deleterious metamorphic effect observed in our results. Similar with our results, Bernardes et al. (2018) reported that azadirachtin treatment resulted in reduced survival, altered development time and caused deformations. Moreover, Qiao et al. (2013) reported that the neurotoxic effect of azadirachtin might interfere with different endocrinological and physiological actions in insects.

Effects on ovarian development

The reproductive system of female adults of *C. claveri* showed morphological differences when compared treated insects with the control insects. The azadirachtin treatment impaired development of the reproductive system as recently reported by Bernardes et al. (2018). Several effects of azadirachtin on reproduction have been reported in several insects (Medina et al., 2004; Ghazawi et al., 2007; Bezzar- Bendjazia et al., 2016; Scudeler et al., 2016; Bernardes et al., 2018; Garcia et al., 2018; Gastelbondo-Pastrana et al., 2019). In the presente study was observed external abnormalities on the reproductive system and significant alterations on the normal ovarian follicle.

Effects of Azamax™ on external superficie of ovarioles

In control group, normal ovarioles are elongated and externally covered by a connective tissue sheath which contains a reticulum of muscle, the ovariole sheath (Garbiec and Kubrakiewicz, 2012). According to Ruickshank (1972) the ovariole sheath in insects, building by a cellular network with small and large meshes seems to be continuous over the ovarian follicles cells but discontinuous over the areas between these structures. This author was observed that large pores were present in the sheath only between the oocyte chambers and externally large pores were observed between strands of sheath. In the current study, we observed a type of structure like a large pores in the external superficie on the ovariole sheath of the control ovarioles (Fig. 5A,C). In *C. claveri* ovaries, each pair of ovarioles are connected by one unique terminal filament (Fig. 5A). The terminal filament is shorter than the proximal region to the oviduct have a larger size as observed in the Figure 5A.

At both doses of treatment, ovarioles of females treated with Azamax™ presented amount of protuberances like as large pores on the ovariole sheath and subsequently the sheath seems to be thinner than the observed and control females ovarioles as detailed in the Figure 5D. The external morphology of treated ovarioles are detailed in the Figure 5D. In accordance to reported by Ruickshank (1972), this increase of ovariole large pores on the sheath likely to be associated with larger intercellular spaces between the cells that compose ovarian follicles. These changes can be comparables to citronela oil induced damages on ovarioles of *Spodoptera frugiperda* reported by Dos santos et al. (2015). Oil-treatment ovarioles showed follicular cell stratification and removal, and a thinner conjunctiva sheath. Giorgi et al. (1990) proposed that the enclosure and closeness exhibited by developing ovarioles through sheaths is designed for guaranteeing protection to the oocyte and to allow a directional flow of hemolymph through the insect ovary. From this, the external disorganisation observed in ovarioles of Azamax™ treated insects can result in diminished reproduction.

Moreover, as observed in the figure 5B, ovarioles of treated insect at both doses presented shrinkage of ovarioles represented by larger terminal filament and the vitellarium and germarium region seems to be smaller when compared to the control ovarioles. The Grasshopper *Heteracris littoralis* treated with azadirachtin suffered shrinkage of the ovary as showed by Ghazawi et al. (2007). These authors associated this fact to azadirachtin interference with the vitellogenesis process at the ovary. Vitellogenesis is a rather complicated process, involving the deposition of yolk in the oocyte, resulting in a very rapid increase in size (Ghazawi et al., 2007). Bernardes et al. (2018), also described this effect of azadirachtin demonstrating reduced size of the queens' reproductive organs after treatment with a azadirachtin-based biopesticide on stingless bee, *Partamona helleri*.

Effects of Azamax on ovarian follicle organization

The pair of ovaries of this insect are formed by polytrophic meroistic ovarioles, which containing ovarian follicles in different stages of development (Garbiec and Kubrakiewicz et al., 2012). Ultrastructure analyzes showed ovarian follicles composed by the oocyte, nurse cells and follicular cells in different stages of development. In control group we observed germ cells (oocyte and nurse cells) surrounded by follicle cells. The nuclei of the germ cells are round with regular contour and decondensed chromatin and the follicle cells exhibit small nuclei with irregular contour as observed in the Figure 6A-B.

At both doses, AzamaxTM treatment seems to be toxic to the adult ovaries, as the ultrastructure results revealed significant alterations in cellular organization into the ovaries. In group treated with 0.3% it is possible to see a large dilatation of the endoplasmic reticulum cisterns and also an important nuclear envelope dilation. Moreover, we also detected condensed nucleus and many autophagosomes with heterogeneous material and part of organelles. The alterations of this group are detailed in the Figure 7A-D.

At 0.5% of Azamax™ the intercellular spaces detected in control group between somatic and germ cells and also between adjacent germ cells, seems to present a discrete enlargement. Finally, we also observed many apoptotic bodies indicating apoptotic cell death process as observed at Figure 7E-F. Azamax™ seems to be more aggressive to the ovaries at maximum dose, since the cellular alterations were more prominent in 0,5% treated group. However, these alterations suggested the absent of oocytes reaching to the egg stage at both doses of treatment. The ovarian follicle failed to complete its development as compared with the normal cells in different stages of development observed in the control ovaries.

The inhibition of ovarian follicle development was also reported by Ghazawi et al. (2007). They reported that when azadirachtin is administered at an appropriate time, it can cause severe damage to the oocytes. Medina et al. (2004) pointed out that a commercial product based on azadirachtin (Align) interfered with vitellogenin synthesis and/or its uptake by developing oocytes; thus, in this study growing follicles in treated females were significantly smaller than those of the controls. Azadirachtin may inhibit vitellogenin synthesis or absorption which eventually leads to: inhibition of both oogenesis and ovarian ecdysteroid synthesis, inhibition of ovarian development, delaying of the vitellogenin synthesis process and interference with vitellogenin synthesis and its absorption by the follicles (Schmutterer, 1990; Mordue et al., 2005). Lower ovarian proteins levels like vitellogenin suggesting a reduced level of vitellogenesis as reported in *Spodoptera exempta* (Tanzubil and McCaffery, 1990) and *D. melanogaster* (Boulahbel et al., 2015) treated with azadirachtin. This compound also alters or prevents the formation of new actin cytoskeleton resulting in disruption of cell division and blocked transport which may affect the process of dumping of the cytoplasmic contents of nurse cells to the oocyte (Mordue et al., 2005). Indeed, Anuradha et al. (2007) reported that azadirachtin induces in *D. melanogaster*, a depolymerisation of actin leading to arrest of cells and subsequently apoptosis.

Bernardes et al. (2018) in accordance with their results suggest that azadirachtin treatment impaired development of the reproductive system, which might be critical for insect maintenance and reproduction. The impact of azadirachtin on female reproduction could be explained by this interference of Juvenile Hormone and ecdysteroids (Mordue et al., 2005), and eventually lead to abnormal ovaries and an associated decrease in fecundity (Zhang et al., 2018).

4. Conclusions

In conclusion, our results suggest that the natural enemy *C. claveri* may be extremely harmed by indirect ingestion of an azadirachtin-based biopesticide, eliciting a range of sub-lethal effects in this beneficial insect, including mortality, disturbance on fitness, development and reproduction. So, the application of these botanical products must be considered carefully for use in IPM programs involving populations of *C. claveri*. Assessment of the side-effects of natural products on different developmental stages of non-target species is particularly important for guaranteeing safety for use together with predator insects such as *C. claveri*.

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Table 1. Effects on cumulative larval mortality (%) of *Ceraeochrysa claveri* during larval exposure to azadirachtin (Azamax™) using the maximum and minimum field recommended concentration for agricultural crops (n = 150 larvae/group)

Treatment	First instar	Second instar	Third instar
Control	2.0 ^a	5.4 ^a	6.8 ^a
Azamax™ (%)			
0.3	6.7 ^a	13.1 ^a	22.3 ^b
0.5	6.0 ^a	12.4 ^a	21.5 ^b

Different letters means significant difference ($p < 0.05$; Chi-Square).

Table 2. Effects on development and mortality of *Ceraeochrysa claveri* after larval exposure to azadirachtin (Azamax™) using the maximum and minimum field recommended concentration for agricultural crops (n = 150 larvae/group)

Treatment	Pupation ^a (%)*	Lifecycle time ^b (days)**	Adult emergence ^c (%)*	Cumulative mortality ^d (%)*
Control	92.0 ^a	27.7±0.2 ^a	92.0 ^a	8.0 ^a
Azamax™ (%)				
0.3	54.7 ^b	28.5±0.3 ^a	51.3 ^b	48.7 ^b
0.5	40.0 ^c	27.1±0.3 ^a	35.3 ^c	64.7 ^c

*Different letters means significant difference ($p < 0.05$; Chi-Square).

**Data (mean $\pm$ SE) followed by the same letter did not significantly differ (Kruskal–Wallis, $P < 0.05$) for adult emergence.

^aPercentage of formed pupae compared with the total number of treated larvae.

^bNumber of total days of life cycle per insect.

^cPercentage of emerged adults compared with the number of formed pupae.

^dPercentage of dead larvae, prepupae, pupa and adult that failed to moult.

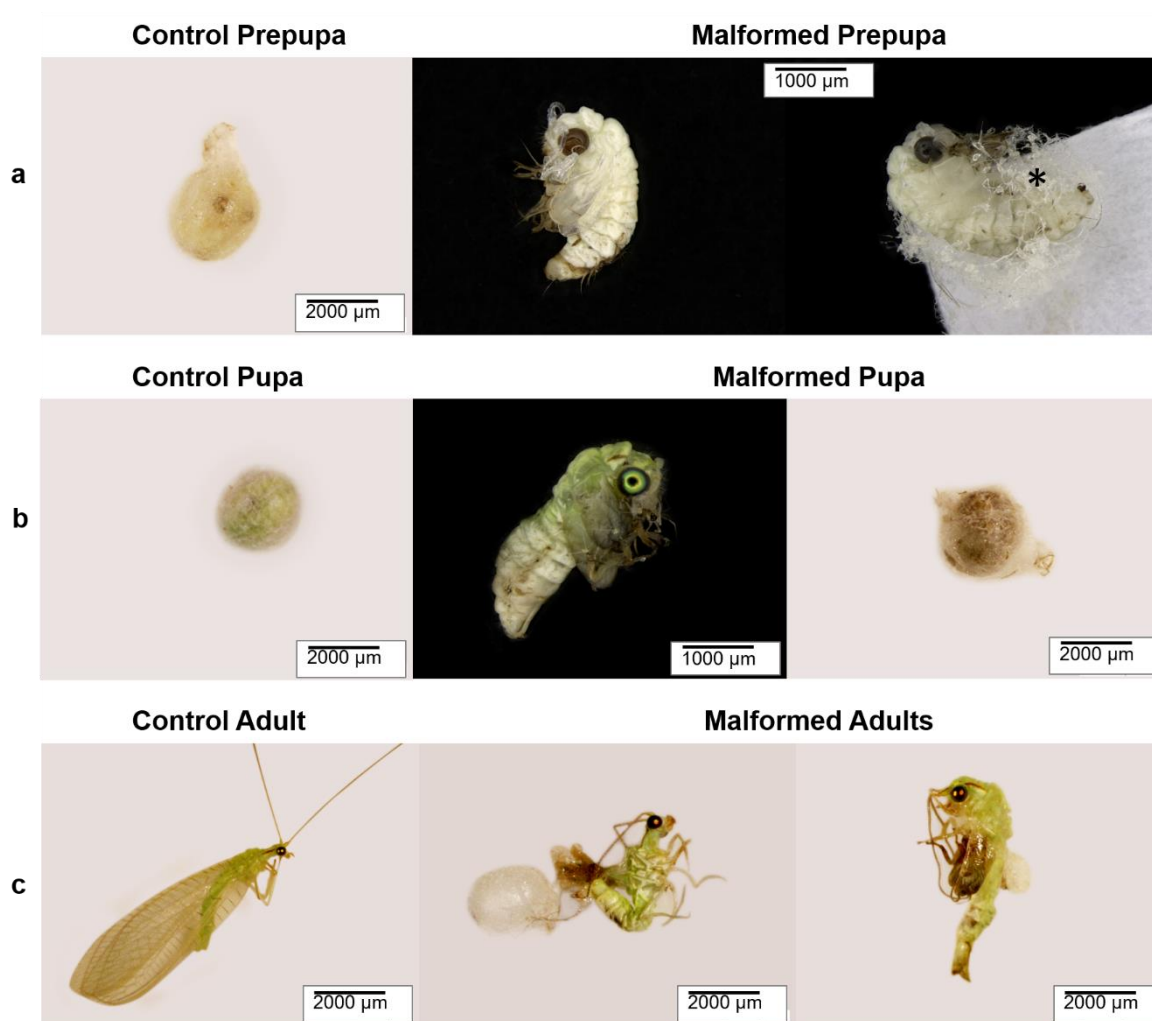


Figure 1. The effect of azadirachtin biopesticide on the lifecycle of *C. claverii*. **Prepupa-(a) Control prepupa:** normal cocoon spun by an untreated larva; **Malformed prepupa:** The exarate prepupa without cocoon; The exarate prepupa outside a malformed cocoon (*). **Pupa-(b) normal cocoon with the viable pupa inside; Malformed pupa:** intermediate stage of development at the pupal emerged after treatment with Azamax™ with incomplete metamorphosis; dark cocoon indicating pupal mortality inside the cocoon. **Adult-(c).** Lateral view of normal emerged adult; **Malformed adults:** Malformed adult trying to emerge of the cocoon with altered tegument, deformed antennae, legs and wings; Malformed adult with intermediate stage of development emerged after exposure during larval period.

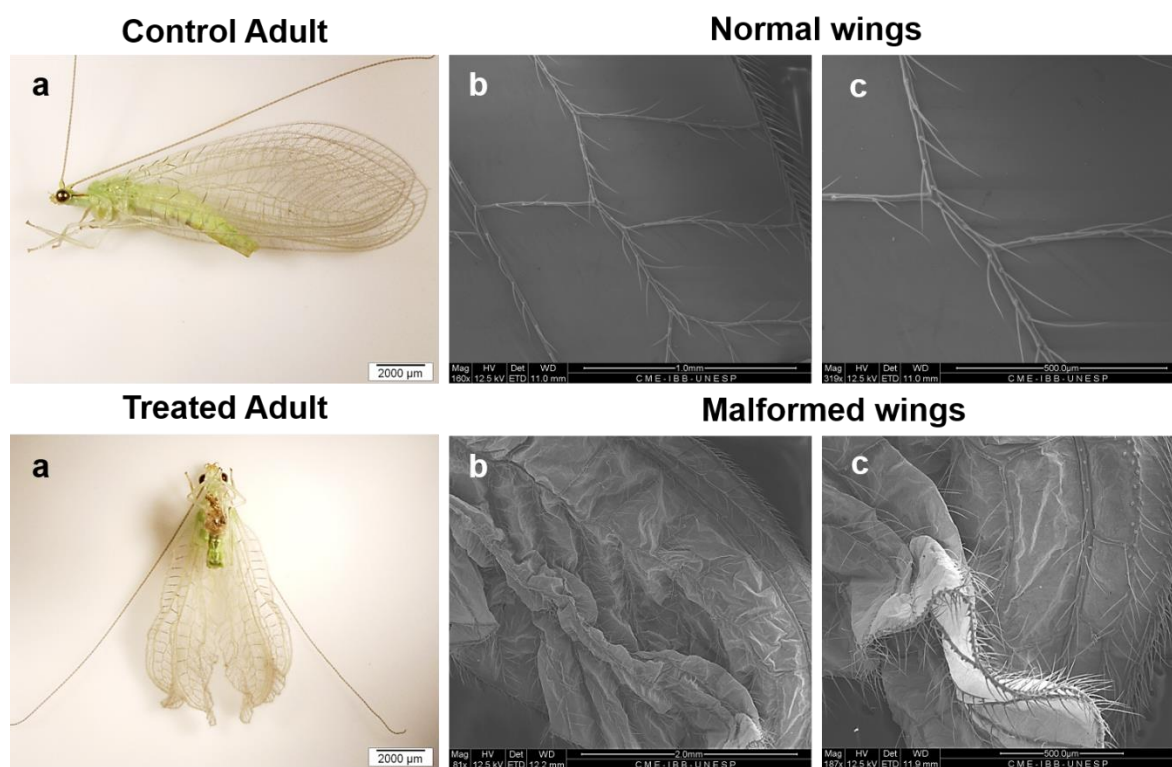


Figure 2. Different malformations of wings of *C. claveri* after exposure of Azamax™ treatment. **Control adult;** (a) adult insect with normal wings; (b-c) SEM micrographs of the normal wings. **Treated adult (0.3 and 0.5% of Azamax;** (a) adult insect with abnormal wings; (b-c) SEM micrographs of the malformed wings.

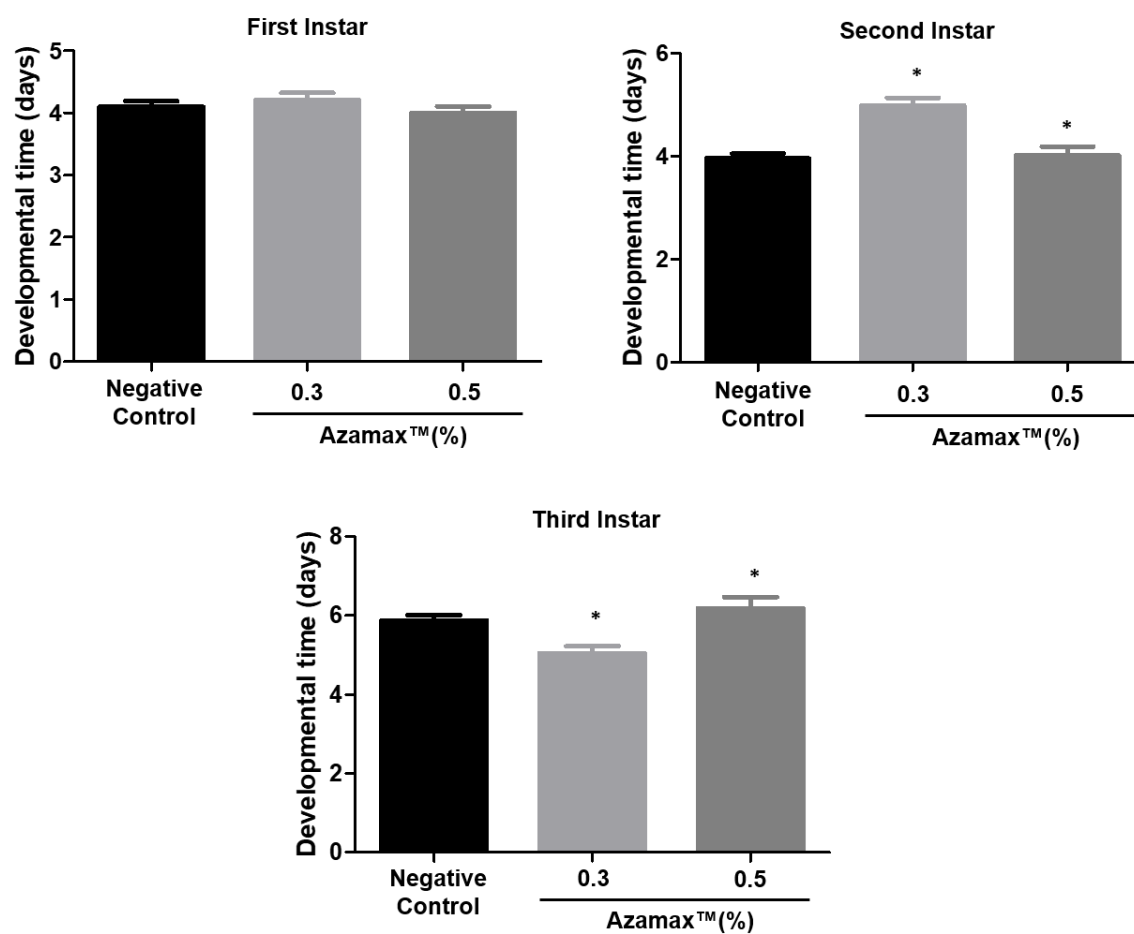


Figure 3. The effects of Azadirachtin biopesticide (AzamaxTM) on larval development (mean $\pm$ SD) of the different larval stages of *C. claveri* when fed the eggs of *Diatraea saccharalis* treated with AzamaxTM.

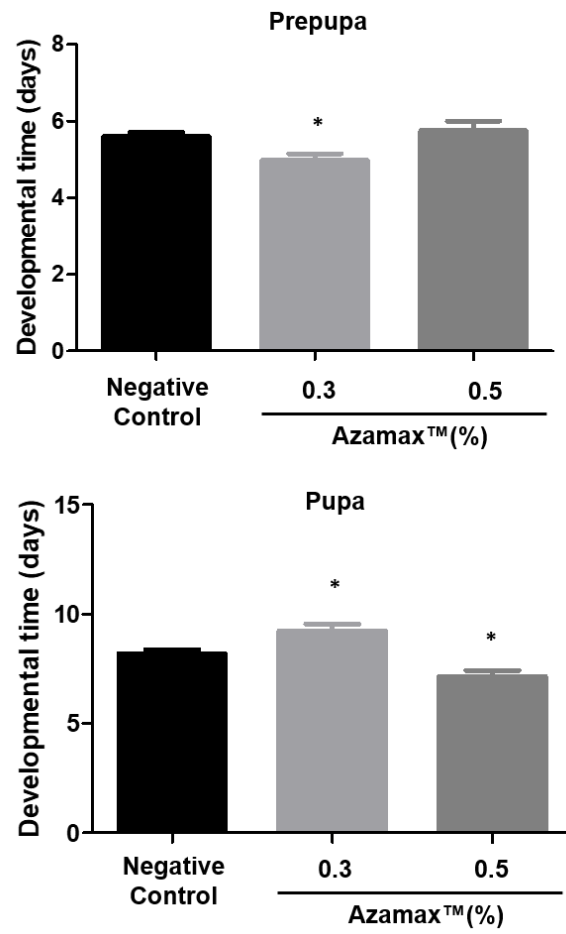


Figure 4. Developmental times of the prepupal and pupal stages (mean $\pm$ SD) of *C. claveri* treated with Azadirachtin biopesticide (Azamax™) during larval development.

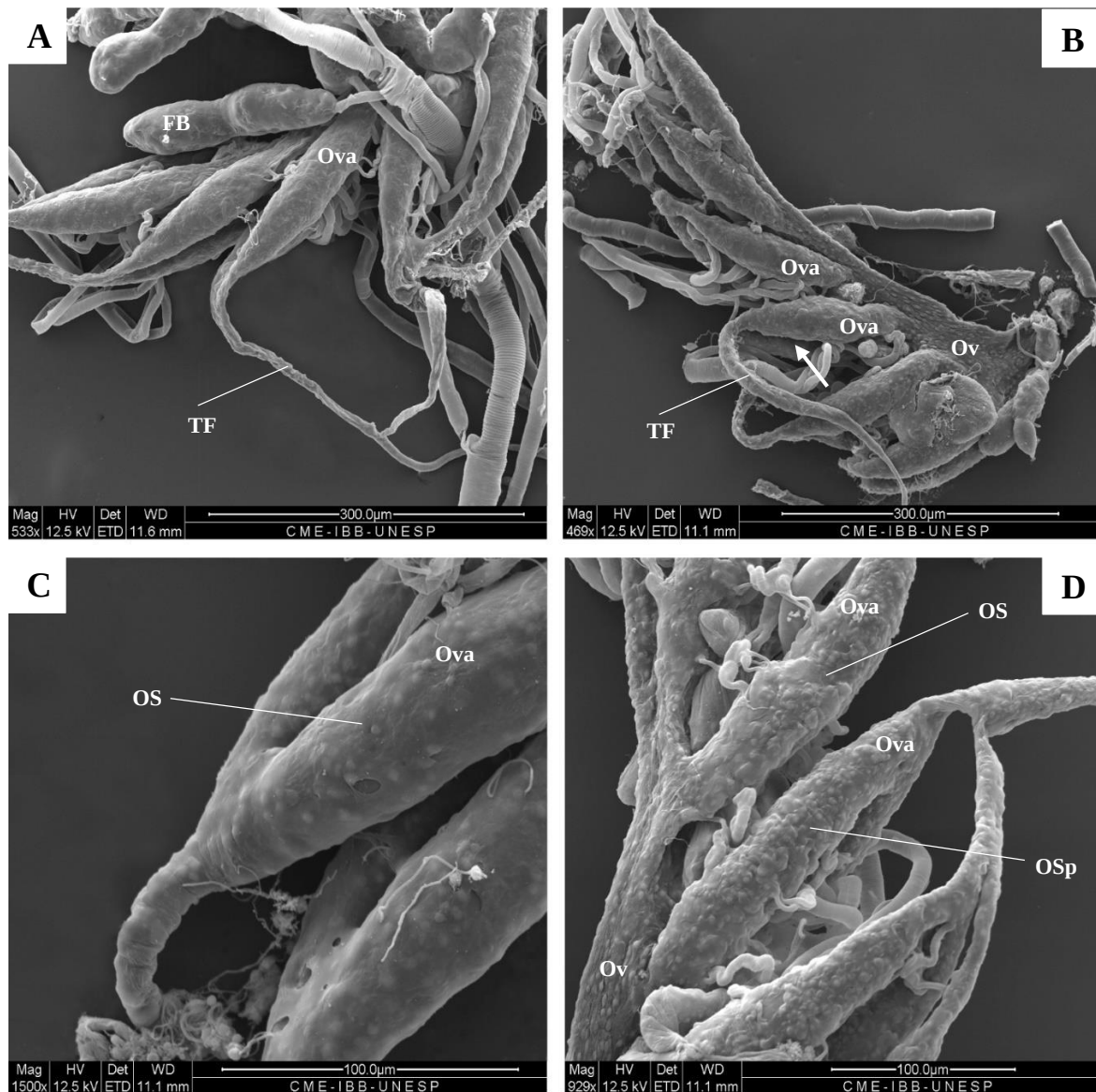


Figure 5. Ultrastructure of ovary (SEM). (A) External morphology of ovaries of a control female, containing ovarioles (Ova) with terminal filaments (TF) and Fat body (FB). (B) External morphology of ovary of treated female showing oviduct (Ov), shorter Ovarioles (Ova) and a longer terminal filament (TF). (C) External superface of ovariole (Ova) of control female showing Ovariole sheath (OS) with normal aspect. (D) External morphology of Ovariole of treated female showing the ovariole sheath with irregular aspect containing protuberances (Osp).

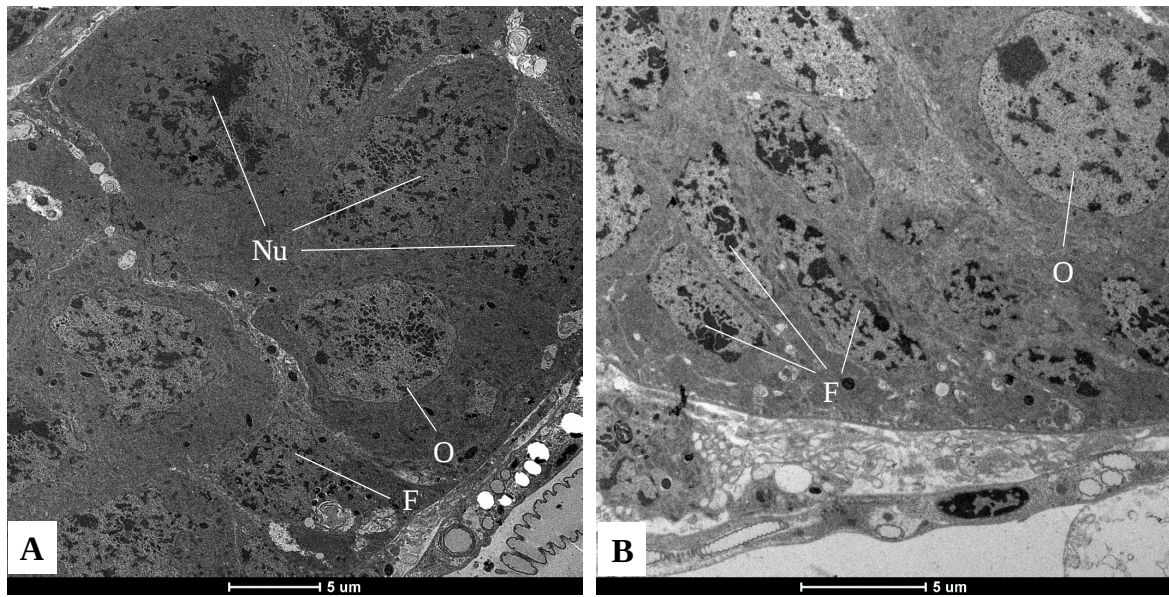


Figure 6. Ultrastructure of ovary (TEM). (A-B) Control Group. Ovaries of female adult at 1 day if age, containing ovarian follicles composed by germ cells such as oocyte (O) and Nurse Cells (Nu) surrounded by Follicular Cells (F).

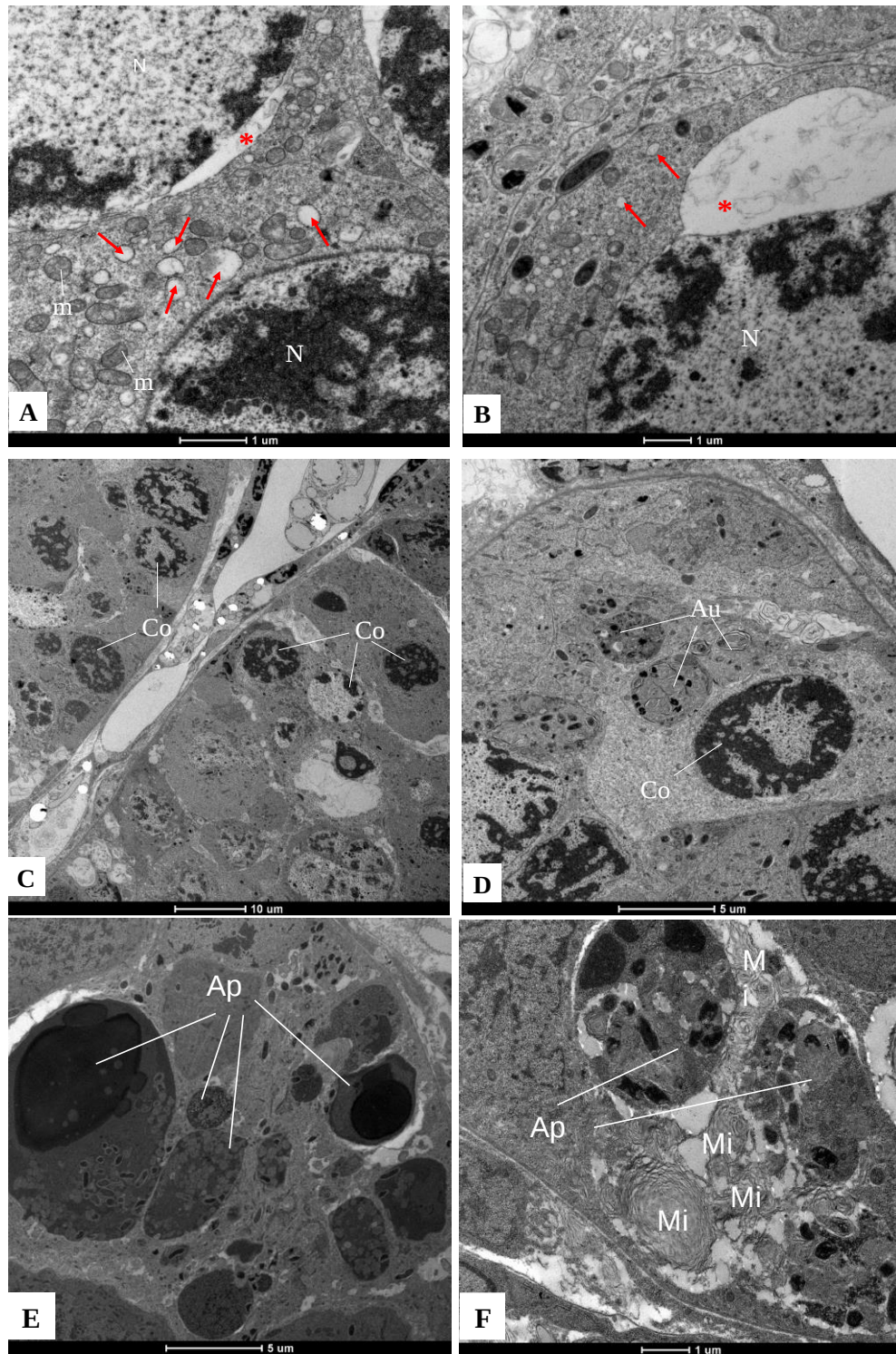


Figure 7. Ultrastructure of ovary (TEM). (A-D) Azamax treatment – 0,3%. Note a large dilatation of the endoplasmic reticulum cisterns (arrows) and an important nuclear envelope dilatation (*) and condensed nucleus (Co) with normal mitochondria (m). Many Autophagosomes (Au) with heterogeneous material and part of organelles. Azamax treatment – 0,5% (E-F). Note a lot of apoptotic bodies (Ap) and Myelin figures (Mi), indicating apoptotic cell death process.

5. CONCLUSÕES

- A ingestão indireta do biopesticida Azamax™ durante a fase larval de *C. claveri* induz efeitos genotóxicos no ovário e no intestino de fêmeas adultas, com lesões celulares severas nas células germinativas e somáticas dos ovários de fêmeas de um dia de idade sobrevivente ao período de exposição;

- A exposição de *C. claveri* ao Azamax™ ocasiona significativos efeitos em sua biologia e desenvolvimento, impactando negativamente na duração das fases do ciclo de vida do inseto, na construção dos casulos, e consequentemente, afetando a sobrevivência do inseto.

6. *ANEXO*





The comet assay in *Ceraeochrysa claveri* (Neuroptera: Chrysopidae): A suitable approach for detecting somatic and germ cell genotoxicity induced by agrochemicals

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HIGHLIGHTS

- *C. claveri* is a sensitive model for environmental genotoxic assessment.
- Azadirachtin has genotoxic effect in a non-target predator insect.
- Somatic and germ cell DNA damage induced by Azadirachtin-based biopesticide.

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ABSTRACT

Some agrochemicals are genotoxic to several organisms. Nevertheless, few protocols are currently available for measuring the toxicogenetic effects of these compounds in target and non-target field-collected species of insects important to agriculture. Herein, we used the species *Ceraeochrysa claveri* (Neuroptera: Chrysopidae), a non-target predator insect, to investigate the ability of an azadirachtin-based biopesticide (Azamax™) to induce DNA damage. The alkaline version of the comet assay was standardized to evaluate genetic instability caused by the toxicant in somatic (gut) and germ (nurse cells and oocytes) cells of *C. claveri*. For this, *C. claveri* larvae were distributed into three groups (10/each) and treated with Azamax™ at 0, 0.3% or 0.5% throughout the larval stage. DNA damage (tail intensity) was measured in adult insects, four days after emerged. The data showed that both doses of Azamax™ (0.3% and 0.5%) were able to significantly ($p < 0.05$) increase DNA damage in somatic and germ cells of *C. claveri*. In conclusion, *C. claveri* (intestinal and ovarian cells) was a sensitive bioindicator for identifying Azamax™ genotoxic potential, whereas the comet assay was a useful tool for detecting the genotoxic hazard of the pesticide in the field-collected insect species. Given that estimation of adverse effects of pollutants on ecosystems is an essential component of environmental risk assessment, the approach used can be recommended to estimate the ecotoxicity of agricultural chemicals.

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