UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

AVALIAÇÃO DA CAPACIDADE DE RENOVAÇÃO DO EPITÉLIO DO MESÊNTERO E SEUS EFEITOS NO POTENCIAL REPRODUTIVO DE FÊMEAS ADULTAS DE *Ceraeochrysa claveri* (NEUROPTERA: CHRYSOPIDAE) ALIMENTADAS DURANTE A FASE LARVAL COM ÓLEO DE NIM (*Azadirachta indica* A. JUSS)

ELTON LUIZ SCUDELER

Tese apresentada ao Instituto de Biociências, Câmpus de Botucatu, UNESP, para obtenção do título de Doutor no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração *Biologia Celular Estrutural e Funcional.*

Profa. Dra. Daniela Carvalho dos Santos

BOTUCATU – SP
2016

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Palavras-chave: Biopesticida; Crisopídeo; Predador; Toxicidade; Ultraestrutura.

E não somente isso, mas também nos gloriamos nas próprias tribulações,
sabendo que a tribulação produz perseverança;
e a perseverança, experiência; e a experiência, esperança.
Romanos 5:3-4

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SUMÁRIO

RESUMO

Devido ao uso indiscriminado de inseticidas de largo espectro na produção agrícola e a vulnerabilidade dos inimigos naturais a este tipo de exposição, efeitos colaterais podem afetar a função biológica destes inimigos, tornando sua utilização no controle biológico impraticáveis. A busca por pesticidas mais seletivos sobre esses organismos benéficos é necessária, e entre as alternativas, enfatizamos o aumento do uso dos biopesticidas. Um dos biopesticidas amplamente utilizados é o óleo de nim (*Azadirachta indica*), e sua segurança e compatibilidade com os inimigos naturais têm sido mais esclarecidos através de estudos ecotoxicológicos que verificam os efeitos da exposição indireta através da ingestão de presas contaminadas. Assim, este estudo analisou a resposta das células epiteliais do mesêntero de adultos do crisopídeo *Ceraeochrysa claveri* quando expostos por ingestão de presa contaminada com óleo de nim durante a fase larval e a ocorrência de efeitos letais e subletais em seu desenvolvimento. Larvas de *C. claveri* foram alimentadas com ovos de *Diatraea saccharalis* tratados com óleo de nim nas concentrações de 0,5%, 1% e 2% durante todo período larval. Fêmeas adultas obtidas a partir destes tratamentos foram utilizadas em análises morfológicas e ultraestruturais da resposta celular do mesêntero. Além disso, parâmetros biológicos também foram avaliados durante o desenvolvimento pós-embrionário. O óleo de nim provoca efeitos citotóxicos no mesêntero através de pronunciadas alterações, como dilatação celular, perda da integridade do córtex celular, cisternas dilatadas do retículo endoplasmático rugoso, mitocôndrias inchadas, Complexo de Golgi com aparência vesiculada e invaginações dilatadas no labirinto basal. As células epiteliais responderam a essas lesões por diferentes mecanismos citoprotetores e de detoxificação tais como proliferação celular, aumento de grânulos citoplasmáticos contendo cálcio, expressão da HSP 70, processos autofágicos e desenvolvimento de retículo endoplasmático liso, mas eles não foram suficientes para recuperar todos os danos celulares. Da mesma forma, destacamos a baixa sobrevivência das fêmeas no período de pré-oviposição, e significativos efeitos na reprodução os quais ocasionaram redução da fecundidade, fertilidade e viabilidade de ovos para todas as doses avaliadas. Estes efeitos evidenciam a ausência de compatibilidade, impedindo um sucesso no controle biológico ao tentar integrar este predador com o uso do óleo de nim.

Palavras-chave: biopesticida; crisopídeo; predador; toxicidade; ultraestrutura.

ABSTRACT

Due to the indiscriminate use of broad-spectrum insecticides in agricultural production and the vulnerability of natural enemies to this type of pesticide exposure, side effects can affect the biological function of these enemies, making their use for biological control unfeasible. A search for more selective pesticides on these beneficial organisms is necessary, and among the alternatives, we emphasize the increased use of biopesticides. One of the biopesticides widely used is the neem oil (*Azadirachta indica*), and its safety and compatibility with natural enemies have been more clarified through ecotoxicological studies which check the effects of indirect exposure by intake of poisoned preys. Thus, this study analyzed the response of midgut epithelial cells of adult of lacewing *Ceraeochrysa claveri* when exposed by intake of contaminated prey with neem oil during the larval stage and the occurrence of lethal and sublethal effects on its development. Larvae of *C. claveri* were fed on eggs of *Diatraea saccharalis* treated with neem oil at a concentration of 0.5%, 1% and 2% throughout the larval stage. Adults female obtained from these treatments were used in the morphological and ultrastructural analyses of cellular response of midgut. Moreover, biological parameters were also measured during the post embryonic development. Neem oil causes cytotoxic effects on the midgut through pronounced alterations as cell swollen, loss of integrity of cell cortex, dilated cisternae of the rough endoplasmic reticulum, swollen mitochondria, Golgi complex with vesiculated appearance and dilated invagination of basal labyrinth. Epithelial cells responded to these injuries by different cytoprotective and detoxification mechanisms such as cell proliferation, increase of cytoplasmatic granules containing calcium, expression of HSP 70, autophagic processes and development of smooth endoplasmic reticulum, but they were not enough to recover all the cellular damages. Likewise, we highlighted the low survival of females in the preoviposition period and the significant effects on reproduction for which a reduction occurred in fecundity, fertility and egg viability for all evaluated doses. These effects show the absence of compatibility, preventing a success in the biological control when trying to integrate this predator with the use of the neem oil.

Keywords: biopesticide; lacewing; predator; toxicity; ultrastructure.

1.INTRODUÇÃO

A família Chrysopidae engloba atualmente aproximadamente 4910 espécies (OSWALD, 2013), se destacando na ordem Neuroptera por exercer importante papel econômico em diferentes agroecossistemas. Dentre as diversas espécies desta família, algumas se sobressaem por apresentarem potencial para serem utilizados em programas de Controle Biológico, uma vez que são predadores polífagos na fase de larva com ampla plasticidade ecológica, apresentam curto tempo de desenvolvimento, fácil criação massal e alto potencial reprodutivo na fase adulta. Por estas razões, são amplamente pesquisados em estudos entomológicos aplicados a fim de verificar seu potencial de uso como agente de controle biológico (ADAMS; PENNY, 1985; DE FREITAS; PENNY, 2001; FREITAS, 2001, 2002; PAPPAS; BROUFAS; KOVEOS, 2011).

O gênero *Ceraeochrysa* apresenta cerca de 80 espécies descritas no mundo (OSWALD, 2013), distribuídas desde o Canadá até o norte da Argentina, sendo a ampla maioria das espécies encontradas na região Neotropical (ADAMS; PENNY, 1985; DE FREITAS; PENNY, 2001). Em ecossistemas brasileiros De Freitas e Penny (2001) relataram a ocorrência 15 espécies de *Ceraeochrysa*, dentre estas *Ceraeochrysa claveri* (Navás, 1911).

Os crisopídeos, forma popular para descrever os insetos da família Chrysopidae, apresentam metamorfose completa, ou seja, são holometábolos (Fig. 1), diferindo a aparência e hábitos das larvas e dos adultos. A larva predadora de *C. claveri* apresenta o hábito de carregar detritos em seu dorso, detritos que podem incluir desde o exoesqueleto de suas presas, fibras vegetais, exúvias de artrópodes, cascas de árvore e demais partículas que a larva pode encontrar durante sua vida e manter presos e amparados pela ação de longas cerdas existentes na superfície dorsal e nos tubérculos laterais presentes no tórax e abdômen. Este comportamento faz *C. claveri* e demais espécies que apresentam este mesmo hábito serem conhecidas vulgarmente por bicho-lixeiro. O transporte destes detritos na superfície dorsal protege a larva por atuar como camuflagem ou barreira física contra os inimigos naturais e fatores ambientais (GEPP, 1984; FREITAS, 2001; ALBUQUERQUE, 2009).

C. claveri apresenta três ínstares larvais, e após o final do ultimo ínstar a larva tece um casulo de seda que é produzido com precursores de seda secretados pelos túbulos de Malpighi e posteriormente excretados pelo ânus durante a fiação do casulo. O período correspondente ao início da confecção do casulo até a última ecdise que ocorre no interior do casulo originando a pupa corresponde à fase de pré-pupa. A pupa é do tipo exarada, por apresentar seus apêndices livres, e adquire coloração verde com o decorrer do desenvolvimento do adulto. Uma pupa móvel ou adulto farato emerge do casulo, fixa-se no substrato e passa por ecdise, emergindo um adulto apto a se locomover e alimentar-se (GEPP, 1984; CANARD;

PRINCIPI, 1984; LAMUNYON, 1988; FREITAS, 2001; BEZERRA *et al.*, 2009; SCUDELER *et al.*, 2013).

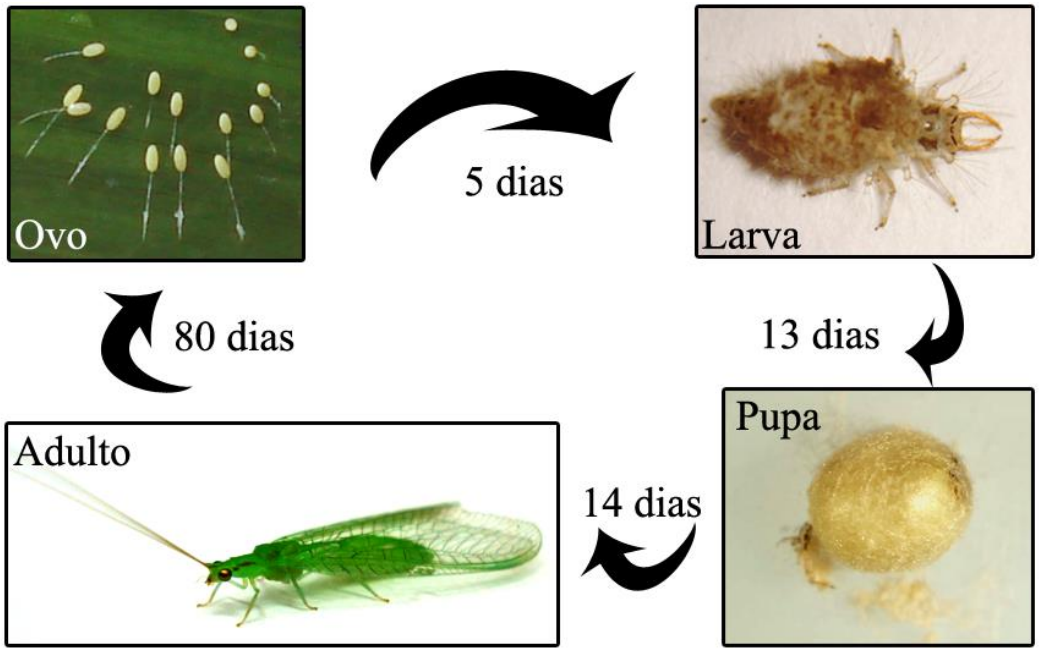

Figura 1. Ciclo de vida de *Ceraeochrysa claveri*, com duração média de cada fase em condições laboratoriais (25 ± 1°C; 70 ± 10% RH; fotoperíodo de 12L:12E).

Na fase adulta, os hábitos alimentares são variáveis, sendo glico-polinívoros, utilizando principalmente nutrientes como açúcares, aminoácidos e lipídios encontrados no pólen, néctar de plantas, e *honeydew* excretados por algumas espécies de insetos sugadores de seiva. Em condições laboratoriais, uma dieta artificial a base de mel e levedura de cerveja (1:1) é rotineiramente utilizada como fonte de alimento para os adultos (FREITAS, 2001, 2002; ALBUQUERQUE, 2009).

A preocupação e conscientização da sociedade com o impacto da agricultura no ambiente e na contaminação da cadeia alimentar com pesticidas estão alterando a produção agrícola, uma vez que a sustentabilidade, segurança alimentar e proteção do ambiente fazem parte da crescente demanda que consumidores esperam obter, consumindo alimentos livres de produtos nocivos a saúde humana e prejudicial à qualidade do ambiente. A necessidade de manutenção da qualidade ambiental, conseqüentemente a manutenção e aumento da biodiversidade de inimigos naturais abre portas para o uso do controle biológico como um dos componentes utilizados em programas de Manejo Integrado de Pragas (MIP) (PARRA *et al.*, 2002; KOUL; CUPERUS, 2007; VENZON; JUNIOR; PALLINI, 2010).

Por serem predadores polífagos encontrados em muitas culturas de interesse econômico, os crisopídeos exercem importante papel no controle biológico, reduzindo a densidade populacional de diversos artrópodes considerados praga. A preservação e manutenção dos crisopídeos nos agroecossistemas devem ser consideradas ao se estabelecer um programa de manejo de pragas que seja efetivo, sustentável e viável. Isso dependerá da compatibilidade com outros métodos de controle, especialmente aqueles relacionados ao uso de inseticidas, por isso a necessidade de buscar e utilizar produtos seletivos a esta população de inimigo natural (CARVALHO *et al.*, 2003; SILVA *et al.*, 2005; PAPPAS; BROUFAS; KOVEOS, 2011; CLOYD, 2012).

Uma alternativa para a substituição do uso de pesticidas poucos seletivos no controle de insetos pragas que vem ganhando cada vez mais adeptos é a utilização de biopesticidas, especialmente os derivados de plantas. Segundo Bailey *et al.* (2010) biopesticida é todo produto produzido em massa, com base biológica, fabricado a partir de microrganismos vivos ou produto natural que é comercializado para o controle de pragas em plantas, incluindo deste modo os derivados de plantas. Por apresentar baixo risco para o ambiente, especialmente sobre a saúde humana, os inseticidas botânicos estão ganhando mercado e tornando-se uma ferramenta promissora no manejo de populações de artrópodes pragas (ISMAN, 2006).

Biopesticidas naturais obtidos a partir da árvore do nim Indiano (*Azadirachta indica* A. Juss) (Meliaceae) são amplamente usados em todo mundo em culturas agrícolas contra artrópodes pragas. O produto mais popular e de fácil acesso é o óleo de nim, obtido a partir da prensagem a frio das sementes do nim (SCHMUTTERER, 1990; MORDUE (LUNTZ); BLACKWELL, 1993; ISMAN, 2006; BAILEY *et al.*, 2010) O componente predominante do óleo de nim é a azadiractina, um triterpenoide da classe dos limonoides, que apresenta diferentes atividades contra os insetos, como repelência, efeito antialimentar, regulação do crescimento, ecdise incompleta com malformação de pupas e adultos, supressão da reprodução com redução da fecundidade e fertilidade, e mortalidade; podendo atuar por contato ou ingestão (SCHMUTTERER, 1990, 1997; MORDUE (LUNTZ) *et al*., 1998; MORDUE (LUNTZ); NISBET, 2000; ISMAN, 2006; MORGAN, 2009; CLOYD, 2012).

Componentes do óleo de nim como azadiractina, salanina e outros limonoides afetam a biosíntese de ecdisteroides, inibindo a atividade da ecdisona 20-monooxigenase, enzima responsável pela conversão do hormônio ecdisônio em sua forma ativa 20-hidroxiecdisona (MITCHELL *et al*., 1997; MORGAN, 2009). A azadiractina de modo geral interfere na função endócrina e neuroendócrina, afetando tanto a biosíntese do hormônio ecdisônio quanto do hormônio juvenil, acarretando alterações no desenvolvimento pós-embrionário e no ciclo

reprodutivo nos insetos (MALCZEWSKA; GELMAN; CYMBOROWSKI, 1988; REMBOLD, 1989; MEURANT; SERNIA; REMBOLD, 1994; SAYAH, 2002).

Na tentativa de integrar agentes de controle biológico com o uso de produtos a base de nim, muitos estudos vem apontando os efeitos diretos e indiretos da exposição dos inimigos naturais ao nim. O nim não tem se mostrado totalmente seguro, não apresentando seletividade para organismos não alvo como os crisopídeos, seja por exposição direta ou indireta por meio da ingestão de presas contaminadas (QI *et al.*, 2001; AHMAD *et al.*, 2003; MEDINA *et al.*, 2003, 2004; AGGARWAL; BRAR, 2006; CORDEIRO *et al.*, 2010; SCUDELER; SANTOS, 2013; SCUDELER; PADOVANI; SANTOS, 2014).

Avaliar os efeitos subletais tem sido o foco de muitos estudos que procuram evidenciar os efeitos prejudiciais que podem atingir uma população de inimigos naturais, e, portanto afetar suas funções fisiológicas e biológicas nos agroecossistemas (DESNEUX *et al.*, 2007; CLOYD, 2012). O mesêntero, por ser considerado um dos primeiros locais a entrar em contato quando analisado os efeitos da ingestão de pesticidas, tem se tornado um importante órgão alvo nos estudos ecotoxicológicos em insetos (MALASPINA; SILVA-ZACARIN, 2006; CATAE *et al.*, 2014), uma vez que alterações morfológicas e ultraestruturais neste órgão podem ser úteis para avaliar os efeitos subletais em organismos não alvo.

O mesêntero, também descrito como intestino médio, é o segundo maior órgão do corpo dos insetos, ocupando a maior parte da cavidade corporal dos crisopídeos e tem como função a produção e secreção de enzimas digestivas, absorção de água, nutrientes, íons e manutenção da homeostase. Possui origem endodérmica, sendo um tubo de diâmetro variável formado por um epitélio pseudoestratificado, apoiado sobre uma membrana basal em torno da qual dispõem uma camada mais interna de fibras musculares estriadas circulares e outra mais externa de fibras longitudinais (SNODGRASS, 1993; LEHANE; BILLINGSLEY, 1996; CHAPMAN, 1998; HAKIM; BALDWIN; SMAGGHE, 2010).

O epitélio do mesêntero encontrado em *C. claveri* é constituído por células colunares, regenerativas e endócrinas (SCUDELER; SANTOS, 2013, 2014). Células colunares, também descritas como enterócitos, digestivas ou principais, é o tipo celular predominante do mesêntero. Devido às suas características morfológicas e ultraestruturais são responsáveis pela síntese e secreção de enzimas digestivas, além de atuarem na absorção dos produtos da digestão, água e íons (SNODGRASS, 1993; BILLINGSLEY; LEHANE, 1996; CHAPMAN, 1998; GIGLIOLLI *et al.*, 2015).

Células regenerativas, também descritas por "*stem cells*" são células indiferenciadas, encontradas isoladas, em pares ou formando grupos de regeneração (nidi), localizados na base do epitélio, tendo função de reposição das células epiteliais envelhecidas ou lesionadas, devido à sua capacidade massiva de proliferação e diferenciação em células epiteliais, tais como colunares e endócrinas (RAES *et al.*, 1994; EVANGELISTA *et al.*, 2003; UWO; UI-TEI; TAKEDA, 2002; ROST, 2006; NARDI; BEE, 2012; GIGLIOLLI *et al.*, 2015).

O terceiro e último tipo celular presente no mesêntero de *C. claveri* são as células endócrinas, localizadas na base do epitélio, sendo caracterizadas principalmente pela sua ultraestrutura e imunoreatividade, por apresentarem grande quantidade de grânulos de secreção em seus citoplasmas. Algumas de suas funções estariam relacionadas com a coordenação de processos digestivos e metabólicos, através do controle hormonal dos movimentos peristálticos, regulação da síntese enzimática e secreção, controle da proliferação e diferenciação das células regenerativas (SEHNAL; ZETNAN, 1996; ROST-ROSZKOWSKA *et al.*, 2008). Dois subtipos de células endócrinas foram descritas no mesêntero de *C. claveri* com base na ultraestrutura de seus grânulos citoplasmáticos nos três estágios do ciclo de vida (SCUDELER; SANTOS, 2014).

A manutenção da integridade do mesêntero é de fundamental importância para a manutenção da homeostase e realização de processos digestivos e absortivos, a fim de sustentar o desenvolvimento do inseto e de sua reprodução através do acúmulo de reservas corporais. No entanto, a exposição indireta por meio da ingestão de presa contaminada com o óleo de nim ocasiona lesões celulares neste órgão, o que pode vir a comprometer as funções do mesmo nas fases de larva e pupa (SCUDELER; SANTOS, 2013; SCUDELER; PADOVANI; SANTOS, 2014). A não recuperação destas lesões e sua permanência na fase adulta podem vir a afetar a reprodução de *C. claveri.*

Para muitas espécies de insetos como *C. claveri* os recursos energéticos armazenados durante a fase larval não são suficientes para total desenvolvimento de seu aparelho reprodutor, e no caso das fêmeas, a maturação dos oócitos e processo de vitelogênese. Segundo Rousset (1984), na emergência dos adultos de crisopídeos, o sistema reprodutor feminino não é funcional, sendo necessário um período para ocorrer sua maturação, período correspondente a pré-oviposição.

Com base no índice de ovigenia, que permite identificar o grau de maturação do aparelho reprodutivo de fêmea adulta recém emergida, fêmeas de crisopídeos seriam extremamente sinovigênicas, ou seja, não possuem oócitos maduros na emergência (JERVIS,

2001). Neste caso, a nutrição durante a fase adulta servirá tanto para manutenção do metabolismo quanto para maturação dos oócitos, que dependem da qualidade e quantidade de nutrientes adquiridos através da alimentação na fase adulta (JERVIS; FERNS, 2004; JERVIS; BOGGS; FERNS, 2005; MILANO *et al.*, 2010). Segundo Rousset (1984) a qualidade da alimentação na fase adulta desempenha papel primordial durante a pré-oviposiçao e oviposição dos crisopídeos.

Considerando a importância ecológica e econômica de *C. claveri* na agricultura face aos benefícios gerados por este predador nos diferentes agroecossistemas; a necessidade de ampliar e aprofundar o conhecimento a respeito da biologia deste inseto; colaborar com estudos científicos direcionados a ampliar o desenvolvimento de novas metodologias de uso do controle biológico; o potencial do uso do óleo de nim no controle de artrópodes praga e a carência de estudos sobre a morfologia e ultraestrutura do mesêntero deste inseto, avaliamos a capacidade de renovação do epitélio do mesêntero de adultos de *C. claveri* e sua ligação com o processo reprodutivo de fêmeas, devido à danos sofridos pela ingestão do óleo de nim durante a fase larval e que podem prejudicar a captura e armazenamento de nutrientes na fase adulta para realização do processo reprodutivo. Através destes dados, será possível analisar a existência de efeitos subletais, que prejudicariam a continuidade de um controle biológico natural em um agroecossistema onde se faz uso do óleo de nim, efeitos que comprometeriam a perpetuação da espécie neste ambiente devido a alterações em seu potencial reprodutivo.

2. OBJETIVOS

Considerando que a exposição indireta por meio da ingestão de óleo nim durante a fase larval de *Ceraeochrysa claveri* ocasiona lesões celulares no mesêntero, neste trabalho propusemo-nos:

- ✓ Avaliar a capacidade de renovação das células epiteliais do mesêntero de fêmeas adultas de *C. claveri*, alimentadas durante toda fase larval com ovos de *Diatraea saccharalis* tratados com óleo de nim;
- ✓ Verificar possíveis alterações no período de pré-oviposição, fecundidade e fertilidade em fêmeas adultas resultantes de larvas alimentadas com óleo de nim.

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4. RESULTADOS

4.1. CAPÍTULO 1

Cytotoxic effects of neem oil in the midgut of the predator *Ceraeochrysa claveri*

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ABSTRACT

Studies of morphological and ultrastructural alterations in target organs have been useful for evaluating the sublethal effects of biopesticides regarded as safe for non-target organisms in ecotoxicological analyses. One of the most widely used biopesticides is neem oil, and its safety and compatibility with natural enemies have been further clarified through bioassays performed to analyze the effects of indirect exposure by the intake of poisoned prey. Thus, this study examined the cellular response of midgut epithelial cells of the adult lacewing, *Ceraeochrysa claveri*, to neem oil exposure via intake of neem oil-contaminated prey during the larval stage. *C. claveri* larvae were fed *Diatraea saccharalis* eggs treated with neem oil at concentrations of 0.5%, 1% and 2% throughout the larval stage. The adult females obtained from these treatments were used at two ages (newly emerged and at the start of oviposition) in morphological and ultrastructural analyses. Neem oil was found to cause pronounced cytotoxic effects in the adult midgut, such as cell dilation, emission of cytoplasmic protrusions, cell lysis, loss of integrity of the cell cortex, dilation of cisternae of the rough endoplasmic reticulum, swollen mitochondria, vesiculated appearance of the Golgi complex and dilated invaginations of the basal labyrinth. Epithelial cells responded to those injuries with various cytoprotective and detoxification mechanisms, including increases in cell proliferation, the number of calcium-containing cytoplasmic granules, and HSP 70 expression, autophagic processes and the development of smooth endoplasmic reticulum, but these mechanisms were insufficient for recovery from all of the cellular damage to the midgut. This study demonstrates that neem oil exposure impairs the midgut by causing sublethal effects that may affect the physiological functions of this organ, indicating the importance of studies of different life stages of this species and similar species to evaluate the safe and compatible integrated use of biopesticides.

Keywords: biopesticide; Chrysopidae; green lacewing; ultrastructure; toxicity.

1. Introduction

Increasing environmental awareness has revealed the importance of natural enemies in agricultural production; however, it has also highlighted limitations associated with traditional studies of the impacts of pesticides on beneficial arthropods. Stop using just the direct mortality measurements and start evaluating the sublethal effects has been the focus of current studies that seek to determine the potential effects of pesticides that may reach a population of natural enemies and thus affect their physiological and biological functions in agroecosystems (Desneux et al., 2007; Cloyd, 2012).

One alternative that has gained an increasing number of adherents is the use of biopesticides, particularly those that are derived from plants. With their low risks to the environment and to human health, botanical insecticides are gaining a share of the market and are becoming a promising tool in the management of arthropod pest populations (Isman, 2006). Despite their bioefficacy, different mechanisms of action on insects, low toxicity and rapid degradation in the environment, their safety and compatibility with biological control agents have been thoroughly assessed and often refuted (Cloyd, 2012; Biondi et al., 2013).

Products derived from neem (*Azadirachta indica* A. Juss) (Meliaceae) are among the most widely used biopesticides worldwide. Their main active component is azadirachtin, which is responsible for the major sublethal effects on insects, including delayed growth and reductions in feeding, fecundity and fertility (Schmutterer, 1990; Mordue (Luntz) et al., 1998; Mordue (Luntz) and Nisbet, 2000; Morgan, 2009). Many studies have shown both the direct and indirect effects of exposure of natural enemies to neem products, questioning the safety and compatibility of neem-based biopesticides in combination with biological control agents. The sublethal effects observed in lacewings, polyphagous predators commonly found in horticultural and agricultural cropping systems, are an example of low selectivity to natural enemies (Medina et al., 2003; Aggarwal and Brar, 2006; Cordeiro et al., 2010; Scudeler and Santos, 2013; Scudeler et al., 2013, 2014).

The midgut is considered one of the primary paths of contact when evaluating the effects of ingested pesticides, and it has become an important target organ for ecotoxicological studies (Malaspina and Silva-Zacarin, 2006; Catae et al., 2014). Whether by direct ingestion of insecticide or by indirect ingestion of contaminated prey, the morphological and ultrastructural alterations of this organ have been useful for evaluating the sublethal effects on non-target organisms.

Previous studies on the neotropical green lacewing *Ceraeochrysa claveri* (Navás, 1911) (Neuroptera: Chrysopidae) have shown that intake of neem oil-contaminated prey during the larval stage affects epithelial cells of the midgut at both the larval and pupal stages (Scudeler and Santos, 2013; Scudeler et al., 2014). This finding indicates that cell damage is present during the pupal stage, which is when the larval midgut epithelium is exchanged for new epithelium during metamorphosis.

The permanence of cell injuries in this organ indicates the presence of cytotoxic effects that can compromise the physiological functions of this predator because digestion and the absorption of nutrients are altered (Scudeler and Santos, 2013, 2014). During the adult stage, the permanence of cell damage in the midgut may compromise female reproduction because the maturation and development of oocytes occurs from adult emergence until the start of oviposition, and these events depend on the quality and quantity of nutrients obtained from the diet during adulthood (Rousset, 1984). We therefore evaluated both the occurrence of cell damage in the midgut of adult females resulting from the intake of neem oil-contaminated prey during the larval stage and the existence of possible cellular response mechanisms against the damage to midgut epithelial cells.

2. Materials and Methods

2.1. Insects

The newly hatched *C. claveri* larvae (0–12 h old) used in the toxicological 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 ± 10% RH; 12L:12D photoperiod).

2.2. Bioassays

Emulsifiable neem oil (Natuneem®, Natural Rural Ind. e Com. de Produtos Orgânicos e Biológicos Ltda, Araraquara-SP, Brazil) (organic product, certified by BCS OKO – Garantie, Doc. Natur - 9009/09.05/7331-BR), a pure cold-pressed neem oil extracted from neem seeds containing 1500 ppm azadirachtin A, was used in bioassays to chronically expose

larvae to neem oil via ingestion. Three concentrations (0.5, 1 and 2%) (v/v) were used based on the recommended rate of usage in Brazilian agricultural fields and on the concentrations previously evaluated in *C. claveri* larvae and pupae (Scudeler and Santos, 2013; Scudeler et al., 2014).

The neem oil solutions (0.5, 1 and 2%) were diluted in distilled water and freshly prepared from the commercial formulation each day. Fresh *D. saccharalis* egg clusters were collected and dipped once in the neem oil solution for 5 s and then air-dried at room temperature for 1 h. For the control group, the egg clusters were dipped in distilled water.

Newly hatched larvae were randomly selected and placed in individual polyethylene boxes (2 cm height x 6 cm diameter). The larvae were divided into four experimental groups (n = 30 per group), and each experimental group included five replicates. The *C. claveri* larvae in the treated groups (0.5, 1 and 2%) were fed with neem oil-treated *D. saccharalis* eggs *ad libitum*. Likewise, the control group was fed with water-treated eggs *ad libitum*. This intake of neem oil-treated or untreated eggs occurred throughout the larval period until pupation (an average of 13 days). Due to the sensitivity of the neem oil components to ultraviolet light, the egg clusters that were not preyed upon were replaced every 3 days.

After the pupal stage, the sex of the freshly emerged adults (0-12 h old) was determined, and one freshly emerged female from each treatment group was paired with two freshly emerged control males. This trio of insects was kept in a polyethylene box (9 cm height x 18 cm diameter) until the start of oviposition (on average 17 days). During this period, the adults were fed an artificial diet (1:1 honey/yeast solution) and provided with a cotton wick soaked in distilled water every 5 days. The entire experiment was conducted under the same environmental conditions as those described for insect rearing.

2.3. Light microscopy

2.3.1. Histology and histochemistry

Adult females of two ages (0-1 day old and first day of oviposition) were quickly cryoanesthetized, and their guts were dissected in saline solution for insects (0.1 M NaCl, 0.1 M Na_2HPO_4 and 0.1 M KH_2PO_4). The insects were cut dorsally, and the midguts were isolated and fixed in a solution of 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) for 24 h for histological analysis. The midguts were removed and fixed in a 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.3) for 12 h for histochemistry and immunohistochemistry. Ten midguts of females from each age/group were

used for histology, and five were used for histochemistry and immunohistochemistry. For the histological and morphometric studies, the midguts were dehydrated in graded ethanol solutions (70–95%) and then embedded in glycol methacrylate (Leica HistoResin Embedding Kit, Leica Biosystems, Wetzlar, Germany) according to the manufacturer's instructions. Sections (3 µm thick) were cut with a Leica RM 2045 microtome and stained with hematoxylin and eosin (HE) (Pearse, 1972).

For histochemical analyses, the specimens were dehydrated in graded ethanol solutions (70–100%), cleared in xylene and embedded in Paraplast® (Leica Biosystems, Richmond, IL, USA). The blocks were cut into 5 µm thick sections, and histochemical analyses were performed on the slides to detect calcium (von Kossa technique) (Junqueira and Junqueira, 1983). The slides were analyzed and documented using a Leica DM500 photomicroscope.

2.3.2. Morphometric analysis

The length and epithelium height of the midgut were measured using one longitudinal HE-stained midgut section each from ten females from each experimental age/group. Ten measurements were collected from random locations in each midgut region, including the anterior and posterior regions, using Axiovision image-processing software (Carl Zeiss, Oberkochen, Germany).

2.3.3. Immunohistochemical analyses

The tissue section slides were deparaffinized, rehydrated and treated for antigen retrieval in 0.01 M citrate buffer (pH 6.0) in a 500 W microwave for 15 min. Thereafter, the slides were left to cool at room temperature for 30 min. After washing in phosphate-buffered saline (PBS, 0.01 M, pH 7.3), the endogenous peroxidase in the sections was blocked by treatment with 0.3% H_2O_2 in PBS for 15 min at room temperature. The sections were then rinsed with PBS and covered with Protein Block (SPD-125, Reveal Polyvalent HRP-DAB Detection System, Spring Bioscience, Pleasanton, CA, USA) for 10 min at room temperature to block non-specific background staining. Then, they were incubated for 2 h at room temperature with the following primary antibodies: mouse anti-PCNA (1:1200 dilution) (clone PC10, ab80576, Abcam® Inc., Cambridge, MA, USA) as a cell proliferation marker and mouse anti-heat shock protein 70 (HSP 70, 1:100 dilution) (clone 5A5, ab2787, Abcam®

Inc., Cambridge, MA, USA) as a marker of cellular stress. After incubation with the primary antibodies, the sections were analyzed with an immunoenzymatic antigen detection system (SPD-125, Reveal Polyvalent HRP-DAB Detection System, Spring Bioscience, Pleasanton, CA, USA) according to the manufacturer's instructions. The chromogen diaminobenzidine (DAB) develops a brown reaction product for positive signals. Finally, the slides were lightly counterstained with Harris hematoxylin. A negative control was obtained by omitting the primary antibody. The slides were examined and photographed with a Leica DM500 photomicroscope.

2.3.4. Cell proliferation index

The cell proliferation index was measured by counting PCNA-positive nuclei. Three longitudinal sections of the midgut from five specimens of each experimental age/group were used, and all positive and negative nuclei in both regions of the midgut were counted (~250 nuclei per region of midgut/specimen). The cell proliferation index was calculated as a percentage by dividing the number of positive nuclei by the total number of nuclei analyzed (positive and negative), and the final value was multiplied by 100.

2.3.5. TUNEL assay

To determine quantitative differences in cell death in the midgut epithelium, the TUNEL assay, a widely used microscopic technique for identifying cell death, was used to label the 3' ends of fragmented DNA. The tissue sections (5 µm) were deparaffinized, rehydrated and washed in Tris-buffered saline (TBS, 20 mM Tris, pH 7.6, 140 mM NaCl). Then, the sections were permeabilized by incubation in Proteinase K (20 µg/ml in 10 mM Tris pH 8) at room temperature for 30 min. Further procedures were performed according to the manufacturer's instructions (TdT-FragEL^{TM} DNA Fragmentation Detection Kit, Calbiochem®, Merck KGaA, Darmstadt, Germany). Diaminobenzidine (DAB) was used as a chromogen, and finally, the slides were counterstained with Harris hematoxylin. All TUNEL-positive nuclei in each midgut region were counted in three longitudinal sections for five samples in each experimental age/group, and the percentage of positive nuclei was calculated.

2.4. Confocal microscopy

Adult females of two ages (0-1 day old and first day of oviposition) (n = 5 per age/group) were dissected in saline solution for insects (0.1 M NaCl, 0.1 M Na_2HPO_4 and 0.1 M KH_2PO_4), and the midgut was fixed in 2% glutaraldehyde and 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.3) for 12 h. Next, the samples were washed in PBS for 5 h and embedded in Paraplast®. The tissue sections (7 µm) were deparaffinized, rehydrated, washed in phosphate-buffered saline (PBS, 0.01 M, pH 7.3) several times and incubated with Phalloidin-TRITC (500 µg/1 ml methanol, diluted 1:900 in PBS) (Sigma-Aldrich®, St. Louis, MO, USA) for 15 min at room temperature. Then, the sections were rinsed several times with PBS, and mounting medium containing DAPI (Fluoroshield with DAPI, Sigma-Aldrich®, St. Louis, MO, USA) was added to the sections. Fluorescence images of the samples were captured with a Leica TCS SP5 confocal microscope at the Electron Microscopy Center of the Institute of Biosciences of Botucatu, UNESP, Brazil and were then examined.

2.5. Electron Microscopy

2.5.1. Scanning electron microscopy (SEM)

The midguts of adult females (n = 5 per age/group) were dissected, rinsed with insect saline solution (0.1 M NaCl, 0.1 M Na_2HPO_4 and 0.1 M KH_2PO_4) and then 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, post-fixed 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 CO_2 and coated with gold (Scudeler and Santos, 2013). The samples were examined and documented using an FEI Quanta 200 scanning electron microscope (FEI Company, Eindhoven, Netherlands) at the Electron Microscopy Center of the Institute of Biosciences of Botucatu, UNESP, Brazil.

2.5.2. Transmission electron microscopy (TEM)

Midguts were isolated from five adult female specimens of each age from each experimental group and were then fragmented, fixed and processed for TEM as indicated below. All samples were analyzed 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.

2.5.2.1. Conventional transmission electron microscopy

The midgut fragments were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.3) for 24 h at room temperature and then post-fixed 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. Ultra-thin sections were contrasted with uranyl acetate and lead citrate and were then analyzed with a Tecnai Spirit transmission electron microscope.

2.5.2.2. Ultrastructural cytochemical analysis

Lanthanum nitrate was used as a tracer of the extracellular space. The tissue fragments were fixed in a solution of 2.5% glutaraldehyde, 2% paraformaldehyde and 1% lanthanum nitrate in 0.1 M sodium cacodylate buffer (pH 7.2) for 12 h and then post-fixed in 1% osmium tetroxide and 1% lanthanum nitrate in cacodylate buffer for 2 h (adapted from Revel and Karnovsky, 1967). Dehydration and embedding were performed according to the routine procedures for conventional analysis.

For the ultrastructural localization of calcium deposits, the midgut fragments were fixed in 2.5% glutaraldehyde with 5 mM $CaCl_2$ in 0.1 M sodium cacodylate (pH 7.2) for 2 h, washed in cacodylate buffer with 5 mM $CaCl_2$, and post-fixed in a solution containing 1% osmium tetroxide, 5 mM $CaCl_2$ and 0.8% potassium-ferrocyanide in cacodylate buffer for 2 h (adapted from Forbes et al., 1977). The subsequent steps were similar to those described for conventional analysis.

The acid phosphatase activity, a lysosomal marker, was analyzed by fixing the tissues in a 2.5% glutaraldehyde and 2% paraformaldehyde solution in 0.1 M sodium cacodylate buffer (pH 7.2) with 5% sucrose for 20 min, washing several times in sodium cacodylate buffer with 5% sucrose, and washing twice in 0.05 M acetate buffer (pH 5.0) with 5% sucrose. Next, the tissues were incubated in a solution of cytidine-5'-monophosphate and 1% lead nitrate in acetate buffer (pH 5.0) for 1 h at 37ºC (Pino et al., 1981). Post-fixation was performed in a solution of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium

cacodylate buffer (pH 7.2) with 5% sucrose for 45 min and with 1% osmium tetroxide in cacodylate buffer for 1 h. Finally, the samples were dehydrated and embedded according to the procedures for conventional analysis.

To study the sub-cellular compartments that react with zinc iodide-osmium tetroxide (ZIO), the midgut fragments were fixed for 12 h with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 6.8) with 8.5% sucrose and incubated for 18 h at 4ºC in ZIO incubation medium (zinc powder, resublimed iodide, osmium tetroxide and Tris-aminomethane buffer) (Reinecke and Walther, 1978). The specimens were then washed in the same buffer, dehydrated and embedded using routine procedures. For all cytochemical analyses, unstained ultra-thin sections were examined using a Tecnai Spirit transmission electron microscope.

2.6. Statistical analysis

The quantitative data were compared using a two-factor analysis of variance complemented with Tukey's multiple comparison test (Zar, 2009). The results are expressed as the mean ± SD. The tests were performed at a significance level of 5%.

3. Results

The midgut of newly emerged adult *C. claveri* in the control group is composed of a pseudostratified epithelium, which is externally surrounded by muscle fiber bundles that formed an internal circular tunic and another external longitudinal tunic. In the anterior region of the midgut, columnar cells possess basophilic cytoplasm, apical cytoplasmic granules, and an acidophilic striated border. Spherical nuclei are centrally located with homogeneously dispersed chromatin. Groups of regenerative cells are present in the basal area of the epithelium and exhibit a strongly basophilic cytoplasm, with the nucleus having a morphology similar to that of the cells, varying from prismatic to oval (Fig. 1A). In the posterior region, the columnar cells showed more elongated, with larger numbers of apical cytoplasmic granules and more oval nuclei, similar to the more elongated morphology of the cells in this region (Fig. 1B).

In *C. claveri* adults from the three neem oil treatment groups, we demonstrated the occurrence of swelling of the apical surface of columnar cells and intense emission cytoplasmic protrusions toward the lumen; more elongated columnar and regenerative cells; the formation of folds in the epithelium; and loss of integrity of the apical membrane with the

occurrence of cell lysis in both midgut regions (Fig. 1C-F). Due to the increased presence of elongated cells, the midgut epithelium height was also altered (Table 1).

At the start of oviposition, the midgut of the control group insects exhibited an increased length and epithelial height in both regions compared with the newly emerged adults. The cytoplasm of the columnar cells was slightly acidophilic and was filled with spherites and apical cytoplasmic granules, and the apex had an acidophilic striated border (Fig. 1G) (Table 1). For the groups exposed to neem oil during the larval stage, the midgut also displayed morphological changes at the start of oviposition, particularly epithelial folds, elongated columnar cells, mainly in the anterior region of the midgut, and the issuance of cytoplasmic protrusions (Fig. 1H-J) (Table 1). There was no change in the length of the midgut following the neem treatments for any of the evaluated ages (Table 1).

Histochemical analysis for calcium detection revealed positivity of the apical cytoplasmic granules in columnar cells, which were more abundant in the posterior region of the midgut in the newly emerged adults in the control group (Fig. 2A and B). When the von Kossa technique was applied to the each of the three neem oil-treated adult midguts, we observed increases in the positive calcium signals for all the evaluated neem dosages in both regions of the midgut (Fig. 2C-E). At the start of oviposition, this positivity for calcium was reduced, and the responses were similar in the control and treated groups and in the anterior and posterior regions of the midgut (Fig. 2F-K).

Evaluation of the integrity of actin via phalloidin staining revealed the presence of positive signals at the striated border and particularly in the cell cortex, with F-actin bundles in the apical regions of columnar cells in the midguts of the control insects of both ages (Fig. 3A-B and E). For the newly emerged females in all of the neem oil-treated groups, we observed that regions of the cell cortex of columnar cells lacked F-actin-positive staining, indicative of changes in organization of the cytoskeleton (Fig. 3C-D). There was no such response at the start of oviposition, and all groups were positive for actin filaments in a region of the cell cortex, indicating recovery of the cytoskeleton in these cells (Fig. 3F).

Immunohistochemical analyses revealed low levels of HSP 70 expression in the cytoplasm and nuclei of columnar and regenerative cells of the midgut from the freshly emerged females (Fig. 4A-B) compared with the cells from the neem oil-treated insects of the same age (Fig. 4C-F). The increased HSP 70 expression in the neem oil-treated groups did not differ between doses or midgut regions. Analysis of the midgut at the start of oviposition revealed that the HSP 70 expression level was low and that it was similar between the controls and neem-treated females in both regions of the midgut (Fig. 4G-J).

All tested concentrations of neem oil induced cell death in the anterior and posterior regions of the midgut, but only at the start of oviposition (Table 2), which is when we also observed lower HSP 70 expression (Fig. 4G-J). However, the proliferation index indicated that all concentrations of neem oil induced cell proliferation only in the anterior region of the midgut at both evaluated ages (Table 3).

Ultrastructural analysis of the epithelial surfaces of freshly emerged *C. claveri* adults revealed differences between the anterior and posterior regions of the midgut. We demonstrated that columnar cells of the controls had abundant long microvilli in the anterior region (Fig. 5A) and more juxtaposed cells, with smaller and regular microvilli covering the apical surface in the posterior region (Fig. 5B). However, the adults that developed from the treated larvae showed swelling of the apical surface, leading to the formation and release of cytoplasmic protrusions, the disorganization and loss of microvilli, and apical membrane rupture in both regions of the midgut (Fig. 5C-E). At oviposition, the control group showed more juxtaposed cells in both midgut regions (Fig. 5F-G), and the epithelium in the neem-treated females no longer exhibited severe cell damage, only showing disorganization of the microvilli and small protrusions (Fig. 5H-J).

The ultrastructural observations of epithelial cells from the freshly emerged control group indicated the presence of regularly distributed microvilli on the apical surfaces of columnar cells, followed by a cytoplasm with a predominance of mitochondria, abundant rough endoplasmic reticulum and spherites in the perinuclear cytoplasm (Fig. 6A-B). Nests of regenerative cells with large nuclei and a granular cytoplasm with a predominance of mitochondria were observed at the base of the epithelium (Fig. 6C). Using lanthanum nitrate percolation, we noted the presence of narrow invaginations in the basal labyrinth of the columnar cells (Fig. 6D). Positive calcium signals were observed in concentric rings of the spherites (Fig. 6E). Similarly, lysosomes, which are mainly located in the apical cytoplasm of columnar cells, were identified by acid phosphatase activity (Fig. 6F). Using the ZIO technique, we observed that the Golgi apparatuses in columnar and regenerative cells displayed numerous polarized cisternae with adjacent vesicular elements (Fig. 6G-I).

Epithelial cells of insects of the same age developed severe ultrastructural changes due to exposure to neem oil during the larval stage. Columnar cells showed sparse, dilated and deformed microvilli; dilated cisternae of the rough endoplasmic reticulum; swollen mitochondria in the cristolysis process; dilated invaginations of the basal labyrinth; and Golgi apparatus with numerous vesicles (Fig. 7A-B; E; H-I). Calcium was identified in thick rings of the spherites (Fig. 7F), and acid phosphatase activity was observed in vacuoles with a

predominance of myelin structures (Fig. 7G). Regenerative and endocrine cells also showed pathological changes, including perinuclear space and mitochondrial dilation (Fig. 7C and D).

The same ultrastructural standard described for the epithelial cells in newly emerged adults was observed at the start of oviposition in the control group, in which we identified columnar cells with increased accumulation of spherites in the cytoplasm and a well-developed endomembrane system, with predominant rough endoplasmic reticulum and Golgi cisternae (Fig. 8A-B; G-I). Using ultrastructural cytochemical techniques, we observed that the invaginations in the basal labyrinth remained narrow (Fig. 8D), lysosomes were concentrated in the apical cytoplasm (Fig. 8E), and calcium was present in some spherites with few concentric rings (Fig. 8F). Regenerative cells were present at the base of the epithelium, with a morphology similar to that described for the freshly emerged females (Fig. 8C).

The ultrastructure of the epithelial cells of the neem-treated groups still showed signs of cellular injury at the start of oviposition. Columnar cells contained protrusions that were mainly filled with vacuoles with membranous contents and cytoplasm with developed smooth endoplasmic reticulum and dilated invaginations of basal labyrinth (Fig. 9A-B and E). Using cytochemical techniques, we noted the presence of calcium in electron-dense spherites, bulky autolysosomes in the apical cytoplasm that were digesting heterogeneous membranous contents, Golgi complex with a vesiculated appearance, and the development of smooth endoplasmic reticulum (Fig. 9F-I). Endocrine cells also exhibited changes, with a predominance of swollen mitochondria and dilated cisternae of the rough endoplasmic reticulum (Fig. 9D). Only regenerative cells showed no signs of cell damage at this age (Fig. 9C).

4. Discussion

The *C. claveri* midgut exhibited the same histological pattern in adulthood as has been described for the species during the immature stage (Scudeler and Santos, 2013), with cellular morphological characteristics appropriate for functions in the synthesis and secretion of digestive enzymes and the subsequent absorption of nutrients; growth and tissue regeneration; and control of metabolic processes, cellular proliferation and differentiation, which are assigned to columnar, regenerative and endocrine cells, respectively (Sousa et al., 2009; Hakim et al., 2010; Teixeira et al., 2013; Scudeler and Santos, 2013, 2014; Gigliolli et al., 2015).

Although we have characterized two morphological regions of the *C. claveri* midgut based on morphometric data on epithelial height, both regions of the midgut contained columnar cells with ultrastructural features indicative of high levels of metabolic activity associated with enzyme secretion and absorption, including apical microvilli and abundant mitochondria, rough endoplasmic reticulum, Golgi apparatus and lysosomes. The absorption, storage and transport of organic and inorganic elements associated with the spherites and basal labyrinth are also involved in some of the functions of these cells (Sousa et al., 2009; Scudeler and Santos, 2013; Teixeira et al., 2013; Gigliolli et al., 2015).

The growth of the midgut between the two ages evaluated in the experiment was identified using morphometry, and it was due to the high rate of cell proliferation identified in the midgut epithelium. Cell proliferation was detected by immunohistochemical staining of proliferating cell nuclear antigen (PCNA), a highly conserved nuclear protein whose synthesis and expression is involved in DNA synthesis, making it a marker of cell proliferation (Ng et al., 1990; Kubben et al., 1994; Zudaire et al., 2004). Zudaire et al. (2004) have reported that PCNA is a useful and simple marker for assessing cell proliferation in insects, particularly in the digestive system. Histological and ultrastructural analyses revealed the basal localization of nests of regenerative cells and demonstrated that these cells had strongly basophilic cytoplasm due to the predominance of free ribosomes and scarce organelles, such as rough endoplasmic reticulum and mitochondria, highlighting the bulky and central nucleus, similar to what has been described by Sousa et al., 2009; Teixeira et al., 2013; Scudeler et al., 2014; and Gigliolli et al., 2015.

Regarding the adults who experienced chronic exposure to neem oil throughout the larval stage, all of the evaluated concentrations induced morphological changes, which were not concentration dependent. A set of histological changes could be grouped because they occurred jointly, including cell swelling, emission of cytoplasmic protrusions and cell lysis. Cellular hypertrophy is one of the first cellular changes observed in cell injury due to the loss of control of the ionic balance and water inflow, causing dilation of cells (Bauer and Prankratz, 1992; Cheville, 1994, 2009), and this alteration has been commonly reported in treatments with plant-derived, toxins and insecticides (Reed and Majumdar, 1998; Rey et al., 1999; Scudeler and Santos, 2013; Almeida et al., 2014; Scudeler et al., 2014).

The loss of cytoskeletal integrity, particularly in the cell cortex, contributes to the formation and release of cytoplasmic protrusions. Anuradha et al. (2007) have reported that azadirachtin induces the depolymerization of actin filaments, which favor the formation of protrusions. By labelling the actin filaments with phalloidin, we noted the occurrence of spots

in the cell cortex that were not positively stained for actin, which corroborates the observations of Anuradha et al. (2007). Nogueira et al. (1997) have reported a similar loss of actin microfilaments in midgut epithelial cells of *Rhodnius prolixus* following azadirachtin administration. Due to the dysfunction of the cellular cytoskeleton at these points, the hydrostatic pressure that is already altered in columnar cells cannot be adequately controlled by apical membrane tension, leading to the formation of protrusions and cell lysis (Barros et al., 2003; Charras and Paluch, 2008; Cheville, 2009).

In light of the histopathological symptoms described here, the columnar cells responded to these injuries by expanding the extracellular spaces of the basal labyrinth, which were percolated with lanthanum nitrate. According to Cruz-Landim et al. (2013), invaginations of the basal labyrinth serve to increase the cell's surface area to promote exchanges with the medium. The dilation observed in the columnar cells at both adult ages may indicate an attempt to increase the transport of excess fluid from the dilated cells to the hemocoel (De Lello et al., 1984; Bauer and Prankratz, 1992; Scudeler and Santos, 2013).

Using histologic and morphometric analyses, it was possible to observe changes in the morphology of the midgut, such as an increased epithelial height and the formation of epithelial folds. These changes may indicate epithelial reorganization due to acceleration of the cellular renovation process (Nogarol and Fontanetti, 2010). Exposure to neem oil resulted in increased cell proliferation for all treatments, particularly in the anterior region of the midgut. The increase in cell proliferation and the failure to induce cell death resulted in the reshaping of the epithelium with the creation of epithelial folds. Although at the start of oviposition there may be an increase in cell death in both regions of the midgut at all of the evaluated concentrations of neem oil, this cell death was not sufficient to eliminate or avoid the presence of epithelial folds at this age because cellular proliferation remained significant in the anterior region of the midgut.

Despite the occurrence of severe cellular changes in the midgut of the freshly emerged adults, neem oil did not induce high levels of cell death. We performed TUNEL assay to identify the rates of epithelial cell death by the microscopic identification of individual cell nuclei that were positive for necrosis or apoptosis, achieved by the labeling of the 3' ends of DNA strand breaks. Moreover, internucleosomal DNA fragmentation is widely used in studies of cell death (Higuchi, 2003; Fink and Cookson 2005; Mizuta et al., 2013).

In addition to the lack of cell death, we observed increased immunoreactivity for HSP 70 in the cytoplasm and nuclei of epithelial cells in all of the groups exposed to neem oil. Exposure to neem oil caused injury and therefore cellular stress, inducing the expression of

HSP 70 and the inhibition of cell death. Significant cell death rates were detected in the treated adults only at the start of oviposition, a period during which we observed low levels of HSP 70 expression in epithelial cells. Based on the data presented, we can confirm the cytoprotective action of HSP 70 in the midgut of *C. claveri* only in freshly emerged adults. Heat shock proteins (HSPs) are a class of proteins used as cellular markers of cell stress or aggression, and their induction is correlated with resistance to stress induced by various factors, such as heat, pesticides, drugs and pollutants (Feder and Hofmann, 1999; Aït-Aïssa et al., 2000; Malaspina and Silva-Zacarin, 2006; Ferreira et al., 2013; Rozza et al., 2014). One of the cytoprotective effects of HSP 70 is an anti-apoptotic response (Beere, 2004; Silva-Zacarin et al., 2006; Rozza et al., 2014), which contributed to the lack of cell death in the treated groups. The reduced HSP 70 expression at the start of oviposition and, consequently, greater incidence of cell death may be related to the age of the adults. Benoit et al. (2011) have observed that HSP 70 expression decreases with age in *Aedes aegypti* females, impairing the repair mechanisms in epithelial cells of the midgut, suggesting that relationships may exist among the ability to synthesize HSP 70, aging and egg production.

The increase in cytoplasmic granules containing calcium, as evidenced by the von Kossa and ultrastructural cytochemical techniques, was another observed cytoprotective response that contributed to the low incidence of cell death. The increase in cytoplasmic granules indicates the activation of the detoxification process in these cells (Nogarol and Fontanetti, 2010; Souza and Fontanetti, 2011); furthermore, the observed increase of calcium prevents damage to the endoplasmic reticulum, including vesiculation and loss of its function, and also stimulates an autophagic response (Hoyer-Hansen et al., 2007; Cerella et al., 2010).

Although the midgut has different cytoprotective responses against neem oil-induced injuries, ultrastructural analysis showed that these responses were not completely effective because the ultrastructural changes were consistently observed in different types of midgut epithelial cells. The main ultrastructural lesions identified here included the microvilli, which were reduced in quantity and became sparse and deformed; swollen mitochondria in the cristolysis process; dilated cisternae of the rough endoplasmic reticulum; a Golgi complex with a vesiculated appearance; and dilation of the perinuclear space. These changes, combined with the observed histopathological symptoms, are similar to those described in studies using azadirachtin, neem oil, and other plant-derived toxins and insecticides (De Lello et al., 1984; Nasiruddin and Mordue (Luntz), 1993; Lü et al., 2010; Almehmadi, 2011; Ling and Zhang, 2011; Qi et al., 2011; Scudeler and Santos, 2013, 2014; Catae et al., 2014; Scudeler et al., 2014).

Nevertheless, possible attempts at detoxification or responses to cellular stress were observed at the ultrastructural level in the cells, including the development of smooth endoplasmic reticulum (Cheville, 1994, 2009; Catae et al, 2014; Scudeler and Santos, 2013, 2014) and digestion of vacuoles containing myelin structures or heterogeneous membranous contents through the activation of autophagic processes (Kroemer et al, 2010; Murrow and Debnath, 2013). Acid phosphatase plays roles in detoxification processes via its lytic activity, and its level has been reported to be increased in response to cytotoxicity (Amirmohammadi et al., 2012; Valizadeh et al., 2013).

Despite the presence of all of these cellular mechanisms in response to cellular injuries, only regenerative cells present at the beginning of oviposition showed no signs of cellular damage. The commitment of other epithelial cells, such as columnar and endocrine cells, during the adult stage may significantly affect the metabolic functions performed by the midgut, such as digestion and nutrient absorption. The loss of these functions may compromise other insect physiological functions; in addition, it may affect female development and reproduction because cellular lesions were detected in the midgut during the ripeness interval and reproductive development.

In conclusion, these results indicate that neem oil intake during the larval stage of *C. claveri* is toxic to insects in the adult stage by inducing cellular injuries in the midgut epithelium, and this damage can greatly affect midgut functions. These effects suggest that any of the tested concentrations of neem is safe for *C. claveri*. The epithelial cells responded to the injuries with various cytoprotective and detoxification mechanisms, but they were not sufficient to reverse all of the cellular damage in the midgut. This study adds relevant information to ecotoxicological studies by providing details of the injuries and cellular responses of a non-target organism to long-term indirect exposure to biopesticides. Sublethal effects must be considered and assessed at multiple stages of an organism's life cycle to be considered safe for the species.

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TABELAS

Table 1. Morphometric analysis (mean ± SD) of midgut of adult *C. claveri* females fed with neem oil during larval development.

Variable	***Treatments***	**Age**	
		newly emerged	*start of oviposition*
Epithelium height (µm) Anterior midgut region	*Control*	53.90 ± 8.19 aA	67.31 ± 10.61 aB
	0.5%	73.00 ± 8.59 bA	88.89 ± 12.93 bB
	1%	92.21 ± 14.18 cA	91.34 ± 10.69 bA
	2%	88.90 ± 16.48 cA	86.76 ± 20.42 bA
Epithelium height (µm) Posterior midgut region	*Control*	58.50 ± 3.17 aA	80.77 ± 5.98 aB
	0.5%	71.58 ± 8.88 abA	85.00 ± 8.95 aB
	1%	77.39 ± 17.43 bA	94.16 ± 16.99 aB
	2%	71.91 ± 13.72 abA	88.54 ± 16.87 aB
Length of midgut (µm)	*Control*	2507.40 ± 222.94 aA	3966.87 ± 354.01 aB
	0.5%	2494.69 ± 269.67 aA	4141.05 ± 352.24 aB
	1%	2285.86 ± 326.33 aA	4129.73 ± 434.18 aB
	2%	2495.52 ± 552.08 aA	3808.72 ± 531.69 aB

The means followed by different letters are significantly different. Lower case: comparison of treatments within the same age. Upper case: comparison of ages fixed treatments ($p < 0.05$, Tukey test). $n = 10$ adults per treatment/age.

Table 2. Percentage of cell death (mean ± SD) in the midgut epithelium of adult *C. claveri* females treated with neem oil during larval development.

		Treatments			
Midgut region	Age	*Control*	*0.5%*	*1%*	*2%*
Anterior	*newly emerged*	16.38 ± 3.33 aB	18.70 ± 10.91 aB	13.18 ± 3.62 aA	20.71 ± 4.09 aB
	start of oviposition	2.21 ± 0.58 aA	11.09 ± 4.74 bA	12.16 ± 4.14 bA	12.16 ± 5.34 bA
Posterior	*newly emerged*	19.04 ± 3.77 aB	11.89 ± 4.19 aA	16.95 ± 3.06 aA	19.59 ± 6.79 aA
	start of oviposition	4.77 ± 1.82 aA	16.96 ± 5.28 bA	18.90 ± 2.92 bA	18.41 ± 7.07 bA

The means followed by different letters are significantly different. Lower case: comparison of treatments within the same age. Upper case: comparison of ages fixed treatments ($p < 0.05$, Tukey test).

Table 3. Proliferation index (%) (mean ± SD) in the regions of midgut of adult *C. claveri* females fed during throughout larval stage with neem oil.

		Treatments			
Midgut region	Age	*Control*	*0.5%*	*1%*	*2%*
Anterior	*newly emerged*	33.19 ± 3.61 aA	42.17 ± 4.75 bB	44.81 ± 4.90 bB	44.25 ± 6.11 bB
	start of oviposition	27.83 ± 2.76 aA	35.76 ± 3.57 bA	37.08 ± 6.48 bA	36.89 ± 3.35 bA
Posterior	*newly emerged*	38.36 ± 9.04 aA	37.51 ± 1.89 aA	44.78 ± 3.18 aA	40.27 ± 4.68 aA
	start of oviposition	39.32 ± 3.40 aA	44.40 ± 10.53 aA	41.87 ± 7.09 aA	44.11 ± 4.49 aA

The means followed by different letters are significantly different. Lower case: comparison of treatments within the same age. Upper case: comparison of ages fixed treatments ($p < 0.05$, Tukey test).

FIGURAS

Fig. 1. Morphological changes in the midgut epithelium of adult *C. claveri* females after treatment with neem oil during the larval stage. (**A-F**) Newly emerged. (**A-B**) Control group, anterior and posterior regions, respectively. The pseudostratified epithelium is composed of basophilic columnar cells with an acidophilic striated border (B) and regenerative cells (R) with a strongly basophilic cytoplasm. (**C**) Neem oil - 0.5%. Anterior region. (**D-E**) Neem oil - 1%. Anterior and posterior regions. (**F**) Neem oil - 2%. Posterior region. In the neem oil-treated groups, elongated columnar and regenerative cells were observed; with dilation of the apical surfaces of columnar cells and intense emission cytoplasmic protrusions (P); the formation of folds in the epithelium (*arrow*); and loss of integrity of the apical membrane along with cell lysis (►). (**G-J**) Oviposition. (**G**) Control group. Anterior region. Note the presence of spherites (S) in the cytoplasm of the columnar cells. (**H-J**) Neem oil - 0.5, 1 and 2%, respectively. (**H** and **J**) Anterior region. (**I**) Posterior region. The epithelial folds formed by elongated columnar cells remain at this age, as well as the issuance of cytoplasmic protrusions. Nucleus of columnar cell (N); cytoplasmic granules (G); circular musculature (Cm); longitudinal musculature (Lm); muscle fiber (Mf); peritrophic membrane (Pm); lumen (L). Hematoxylin and eosin staining.

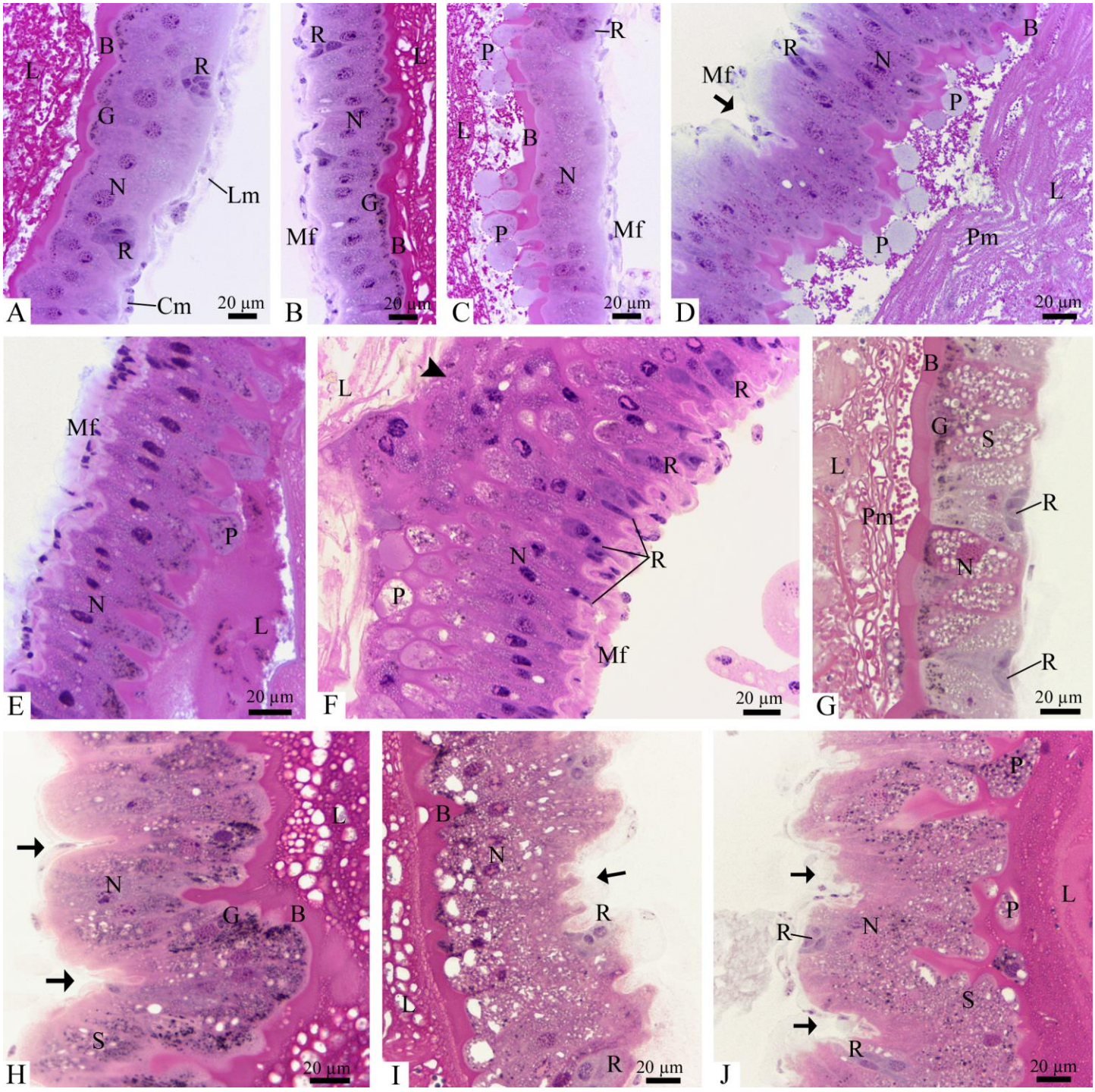
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Fig. 2. Histological sections of the midgut epithelium of adult *C. claveri* females were analyzed for calcium localization by the von Kossa technique. (**A-E)** Newly emerged. (**A-B**) Control group; anterior and posterior regions of the midgut, respectively. Columnar cells showed cytoplasmic calcium granules in the apical region (*arrow*). (**C-E**) Treatment with neem oil at 0.5, 1 and 2%, respectively. Note the increase in calcium granules in the apical region of the columnar cells. (**C** and **E**) Anterior region. (**D**) Posterior region. (**F-K**) Oviposition. Reduction of the cytoplasmic calcium granules in the columnar cells. (**F-G**) Control group, anterior and posterior regions, respectively. (**H-I**) Neem oil - 0.5%, anterior and posterior regions. (**J**) Neem oil - 1%, posterior region. (**K**) Neem oil - 2%, anterior region of the midgut. Lumen (L); striated border (B); regenerative cells (R) and muscle fiber (Mf).

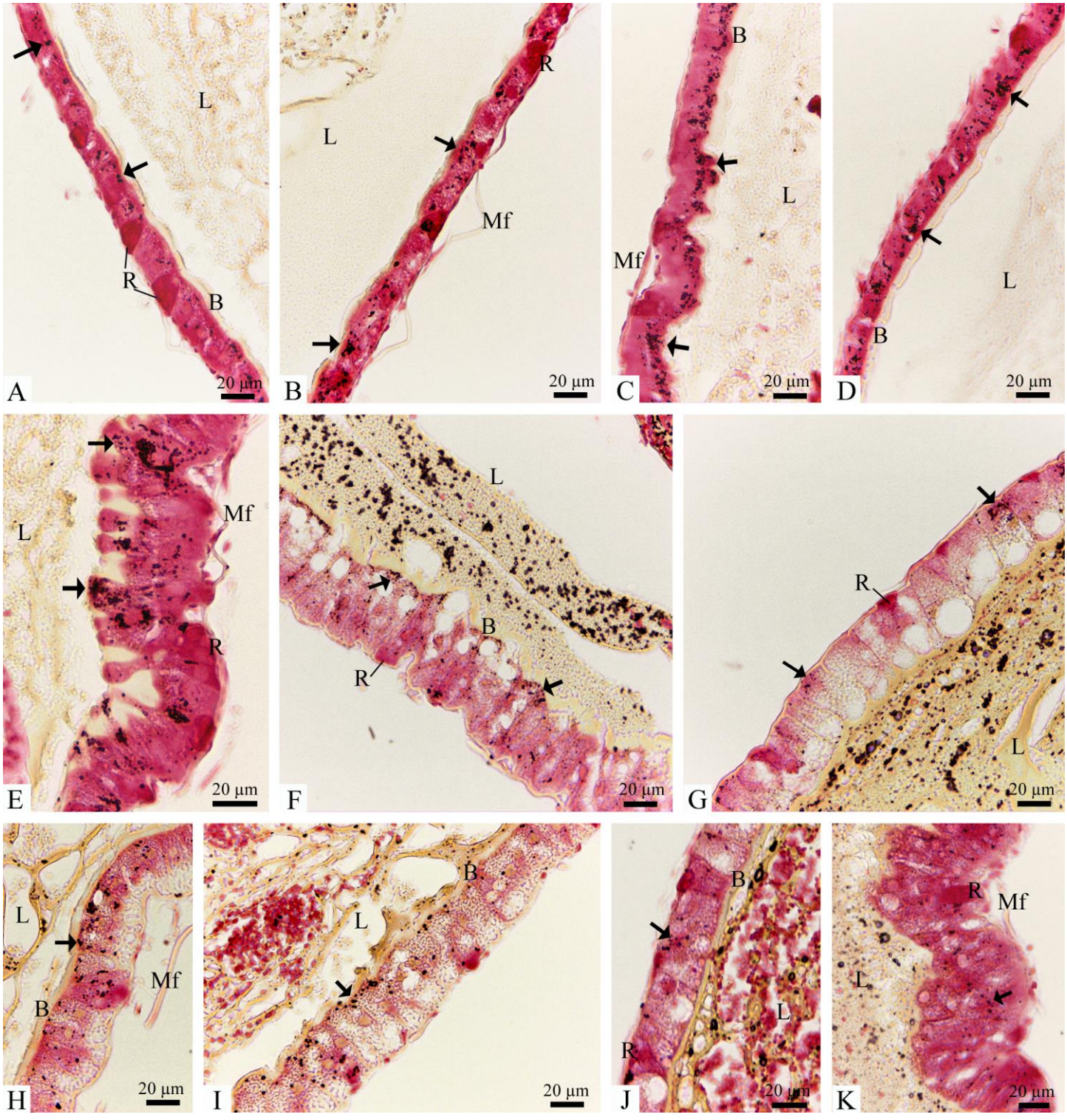
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Fig. 3. Photomicrograph of the midgut of adult *C. claveri* females originating from control and neem oil-treated larvae, shown at the newly emerged stage and at the beginning of oviposition. (**A-D**) Freshly emerged. (**A-B**) Control group, anterior and posterior regions, respectively. The positive reaction to F-actin in the cellular cortex (►) is continuous and is located below the striated border (B). The nuclei of columnar cells (N) and regenerative cells (R) were labeled with DAPI. (**C-D**) Neem oil - 1%, anterior region and neem oil - 2%, posterior region, respectively. Note the regions of the cellular cortex with the absence of F-actin (*). (**E-F**) Oviposition. (**E**) Control group, anterior region. Positive reaction to the cellular cortex (►) and muscle fibers (Mf). (**F**) Neem oil - 2%, posterior region. Note that the presence of the cellular cortex continues below the striated border (B). Lumen (L).

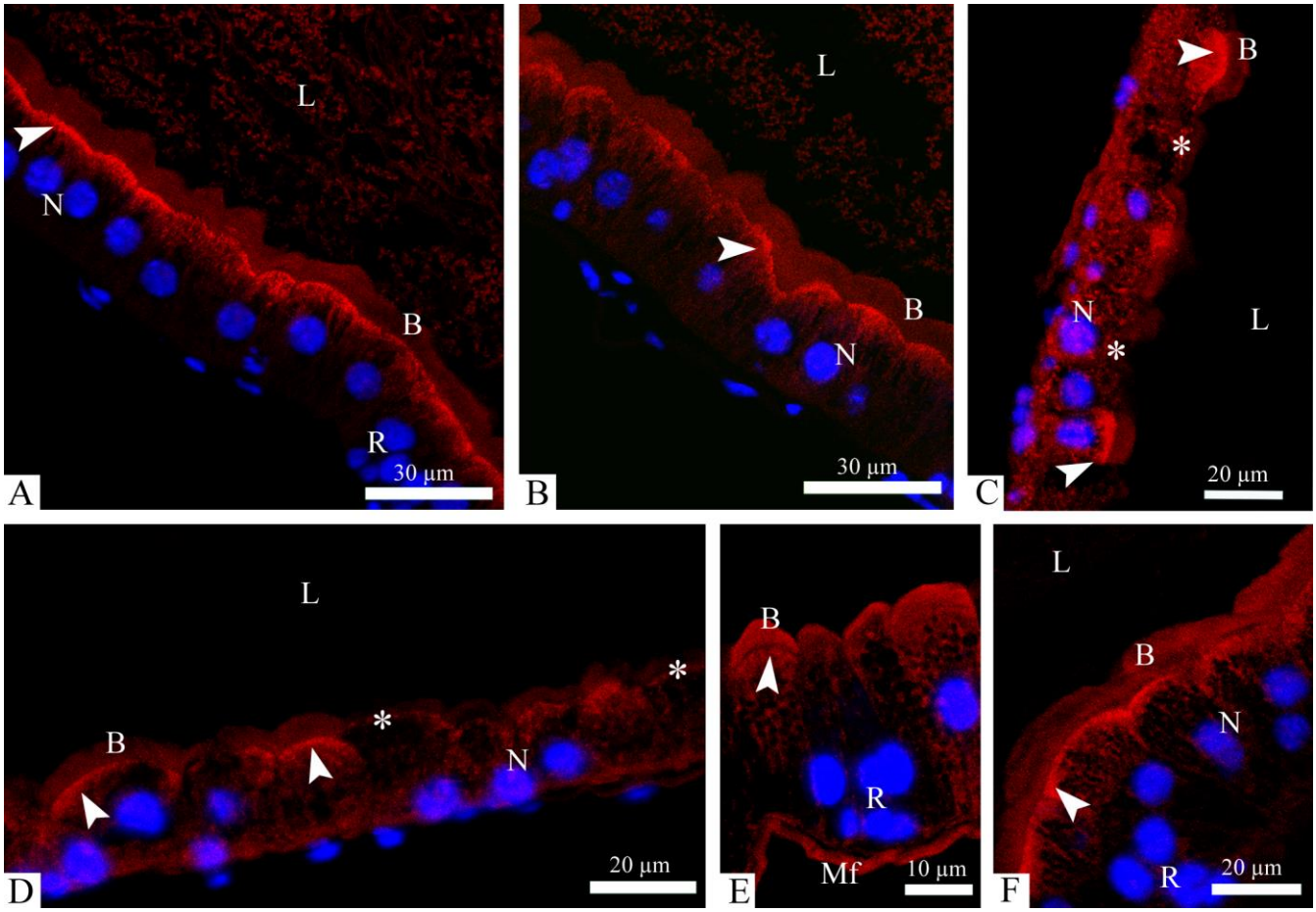
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Fig. 4. Histological sections of the midgut of *C. claveri* were analyzed for cellular stress, represented by HSP 70 immunolocalization. (**A-F**) Freshly emerged. (**A-B**) Control group, anterior and posterior regions, respectively. Note the positive signals in the cytoplasm (*) and nucleus (N). (**C, D** and **F**) Columnar and regenerative cells (R), with more intense staining in the cytoplasm. (**C**) Neem oil - 0.5%, anterior region. (**D**) Neem oil - 1%, posterior region. (**E** and **F**) Neem oil - 2%, anterior region. (**E**) Negative control. (**G-J**) Oviposition in the control, 0.5, 1 and 2% neem oil groups, respectively. Anterior region of midgut. At this age, all groups showed the same immunoreactivity to HSP 70 in the cytoplasm and nuclei of columnar and regenerative cells. Lumen (L); cytoplasmic protrusion (P); muscle fiber (Mf); striated border (B).

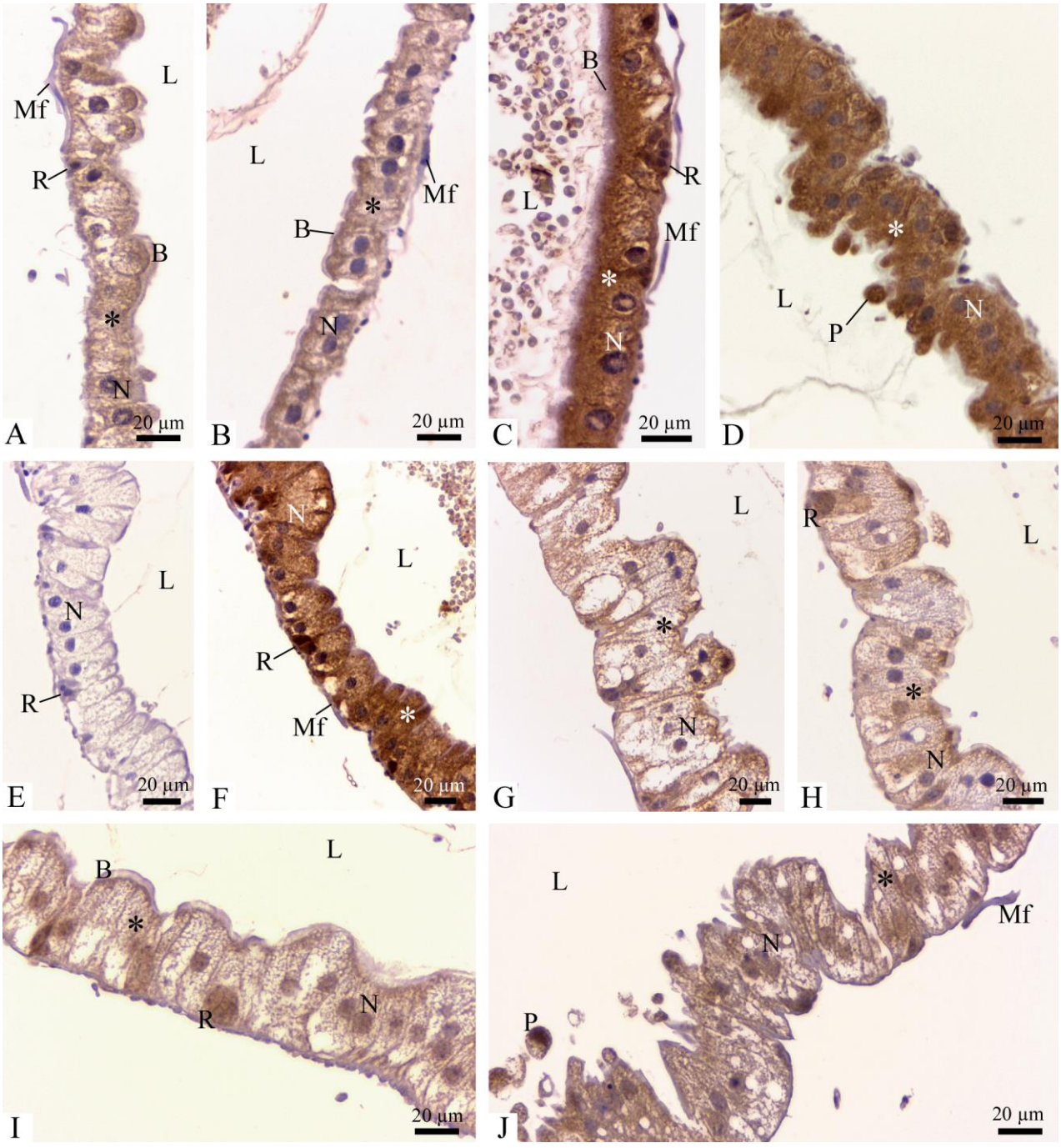
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Fig. 5. SEM images of the midgut epithelial surfaces of adult *C. claveri* females. (**A-E**) Freshly emerged. (**A-B**) Control group, anterior and posterior regions, respectively. (**A**) Note the columnar cells with abundant and long microvilli (Mv). (**B**) The columnar cells were more closely juxtaposed and had smaller, abundant and regular microvilli. (**C-E**) Neem oil - 0.5, 1 and 2%, respectively. (**C**) Anterior region. Details of the formation of cytoplasmic protrusions (*arrow*) and the occurrence of protrusions (P), followed by disorganization and a lack of microvilli (*) (**D**) Posterior region. Occurrence of columnar cells with apical membrane rupture (►) and fewer microvilli (*). (**E**) Posterior region. The cytoplasmic protrusions of columnar cells were present at the dilated apical surface. (**F-J**) Start of oviposition. (**F-G**) Control group, anterior and posterior region. The columnar cells were more closely juxtaposed and had abundant microvilli. (**H-J**) Neem oil - 0.5, 1 and 2%, respectively. (**H-I**) Anterior and posterior region of the midgut, showing cells with disorganized microvilli. (**J**) Posterior region, columnar cells emitting cytoplasmic protrusions (P), as well as fewer and disordered microvilli at specific points of the epithelium.

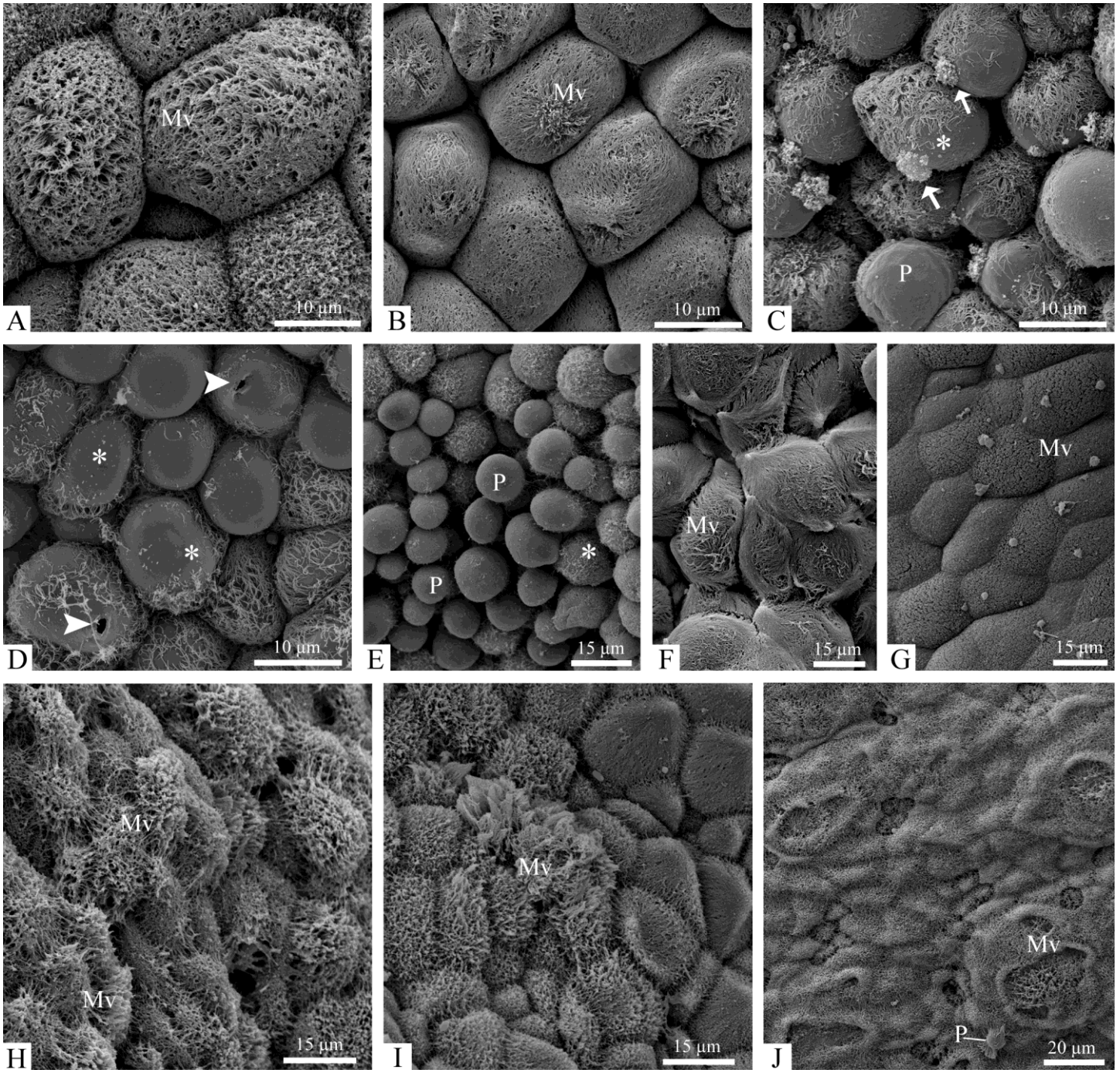
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Fig. 6. TEM micrographs of the midgut epithelium (control) of newly emerged *C. claveri* adults. (**A-E**) Anterior region of the midgut. (**A**) Apical region of a columnar cell, showing long and well-developed microvilli (Mv) and many mitochondria (Mi). (**B**) Perinuclear region of a columnar cell, showing well-developed cisternae of the rough endoplasmic reticulum (Rer), spherites (S), mitochondria (Mi) and endosymbiotic bacteria (Bac). (**C**) Group of regenerative cells with granular cytoplasm, large nuclei (N) and many mitochondria (Mi). (**D**) The basal labyrinth (Bl), with a narrow extracellular space in the basal regions of columnar cells, was visualized by lanthanum nitrate percolation. (**E**) Positive calcium signals were observed using the calcium localization technique in concentric rings of the spherites (*arrow*). (**F**, **G** and **I**) Posterior region of the midgut. (**F**) Apical region of columnar cells, with numerous acid phosphatase-positive lysosomes (Ly) among the mitochondria. (**G**) Basal region of a columnar cell displaying prominent Golgi complexes (Gc) in the cytoplasm among the invaginations of the basal labyrinth (Bl), as detected using the ZIO technique. (**H**) Middle region of the cytoplasm of a columnar cell, with well-structured Golgi complexes among the spherites (S) in the anterior midgut region. (**I**) Regenerative cell with prominent and polarized Golgi complexes around the nucleus (N). Basement membrane (B).

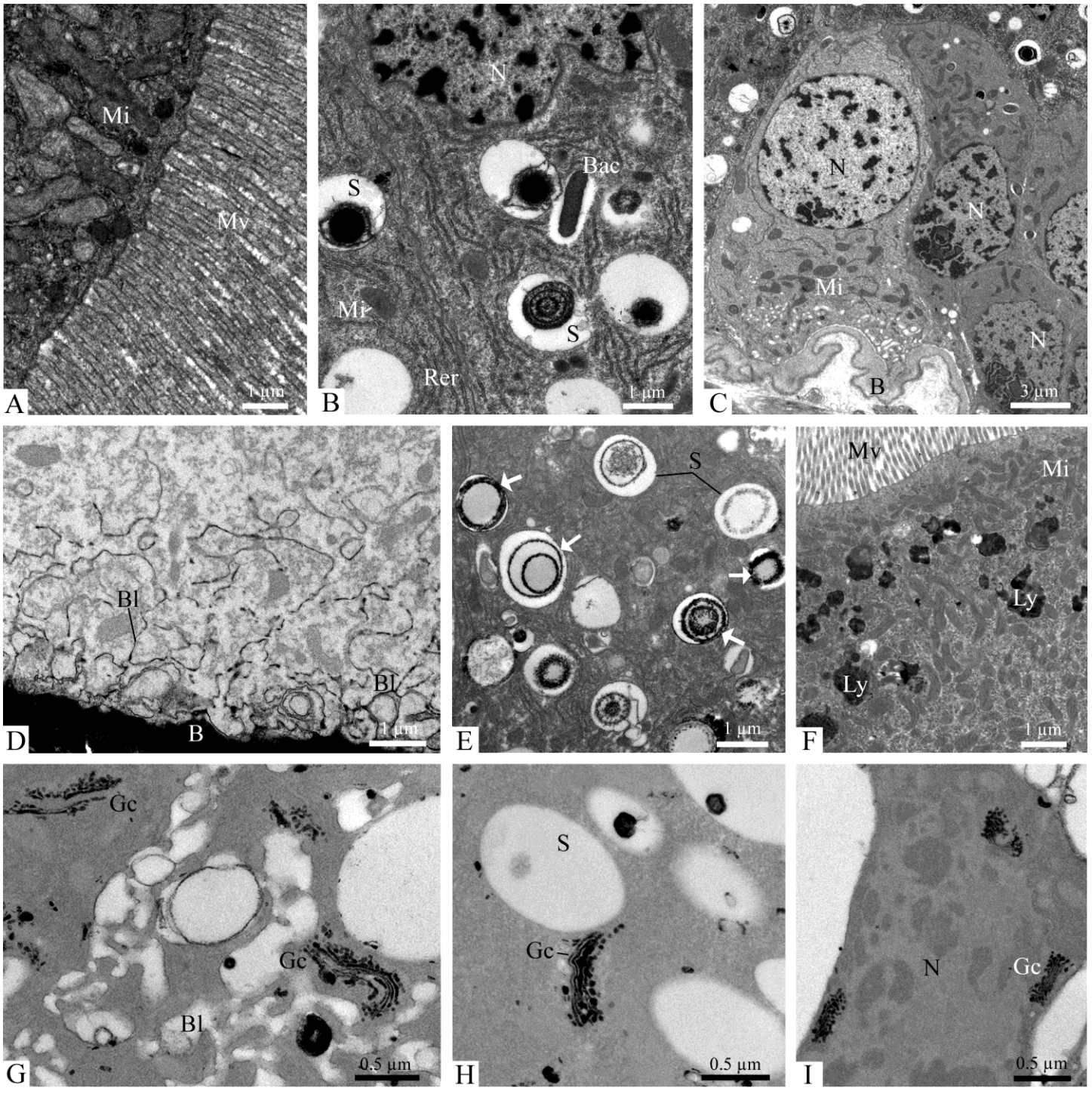
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Fig. 7. Ultrastructural changes in the midgut epithelium of *C. claveri* adults that originated from neem oil-treated larvae. (**A; B** and **D**) Posterior region of the midgut. (**A**) Neem oil - 0.5%. Sparse and deformed microvilli (Mv) in the columnar cells. (**B**) Neem oil - 2%. Supranuclear cytoplasm of a columnar cell containing dilated cisternae of the rough endoplasmic reticulum (Rer), as well as swollen mitochondria (Mi) in the cristolysis process. (**C**) Anterior region. Neem oil - 1%. Regenerative cell (R) with dilated perinuclear space (*arrow*) and mitochondria. (**D**) Neem oil - 2%. Endocrine cells with a vacuolated cytoplasm (V) due to dilation and loss of mitochondrial cristae and dilation of the perinuclear space (*arrow*). (**E; F** and **H**) Posterior region. (**E**) Neem oil - 0.5%. The basal labyrinth (Bl) had more dilated invaginations impregnated by lanthanum nitrate in the columnar cells. (**F**) Neem oil - 2%. Spherites (S) showed thicker concentric rings that were positive for calcium (*arrowhead*) in the columnar cells. (**G**) Neem oil - 1%. Positive reaction for acid phosphatase in myelin structures (Ms). (**H-I**) The ZIO method revealed morphological changes in the Golgi apparatus (Gc), where a few cisternae and abundant adjacent vesicles were observed in the cytoplasm of columnar cells. (**H**) Neem oil - 1%. (**I**) Neem oil - 2%, anterior region of the midgut. Basement membrane (B); lipid droplet (Li), secretory granules (Sg); nucleus (N).

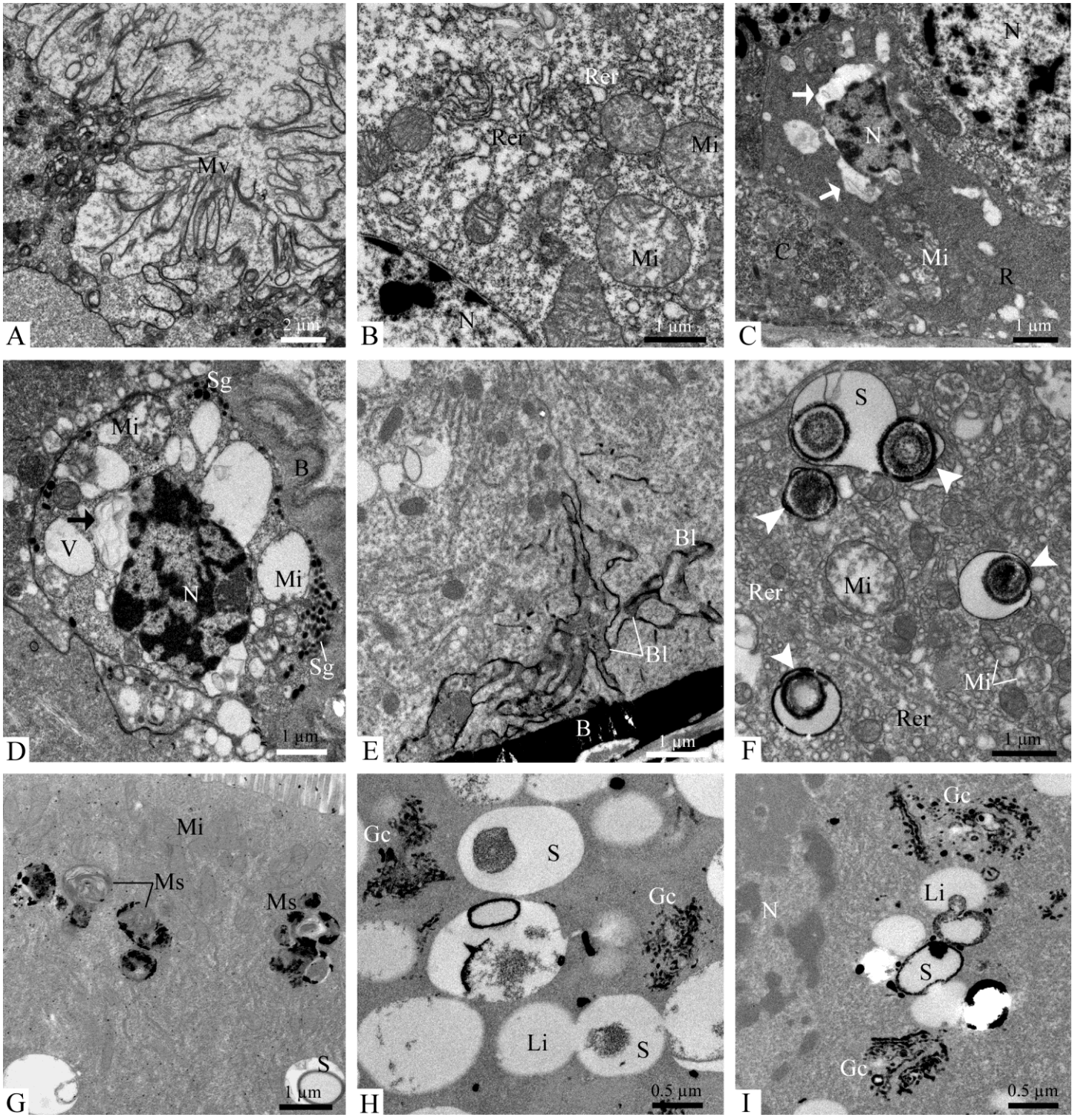
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Fig. 8. Ultrastructure of midgut epithelial cells of adult *C. claveri* females from the control group at the beginning of oviposition. (**A, E, G** and **I**) Posterior region of the midgut. (**B, C, D, F** and **H**) Anterior region. (**A**) Apical region of a columnar cell, showing regular and abundant microvilli (Mv) at the apical surface and a predominance of spherites (S) in the cytoplasm. (**B**) Supranuclear cytoplasm of a columnar cell displaying a predominance of rough endoplasmic reticulum cisternae (Rer) and spherites. (**C**) Group of regenerative cells (R) displaying cytoplasm with abundant mitochondria (Mi) and rough endoplasmic reticulum cisternae, as well as free ribosomes. (**D**) Using the lanthanum technique, we demonstrated slender invaginations of the basal labyrinth (Bl) in columnar cells. (**E**) Lysosomes (Ly) were identified in the apical cytoplasm of the columnar cells by acid phosphatase staining. (**F**) With the calcium localization technique, we identified spherites (*arrowhead*) that were positive for calcium and those that were negative (S), which showed more concentric rings. (**G-I**) ZIO reaction. Note the impregnation of the cisterns of the endoplasmic reticulum, the polarized cisterns of the Golgi complex (Gc) and the perinuclear space of the nuclear envelope in the columnar cell. Lipid droplet (Li); basement membrane (B); nucleus (N); nuclear pores (*arrow*).

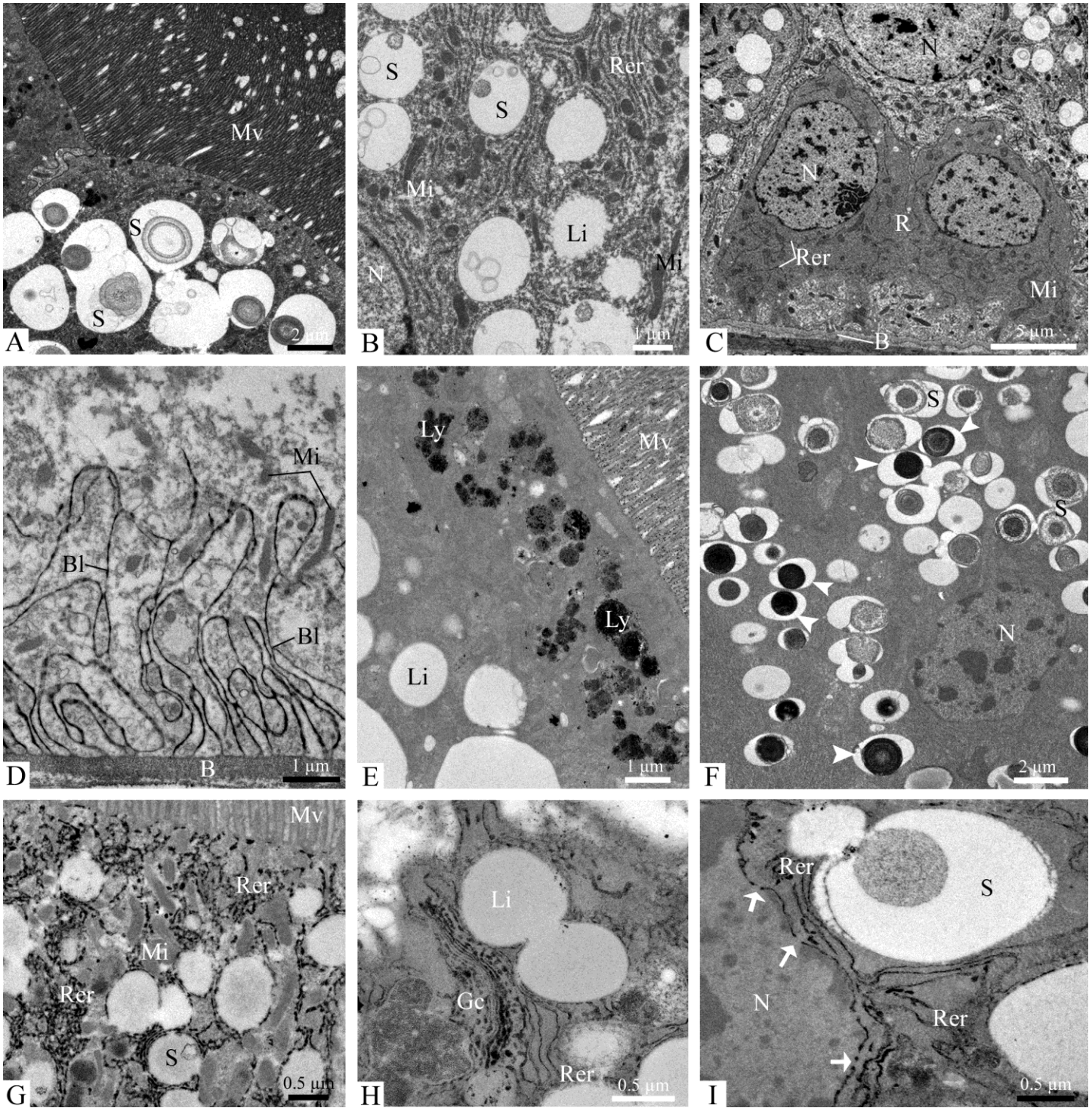
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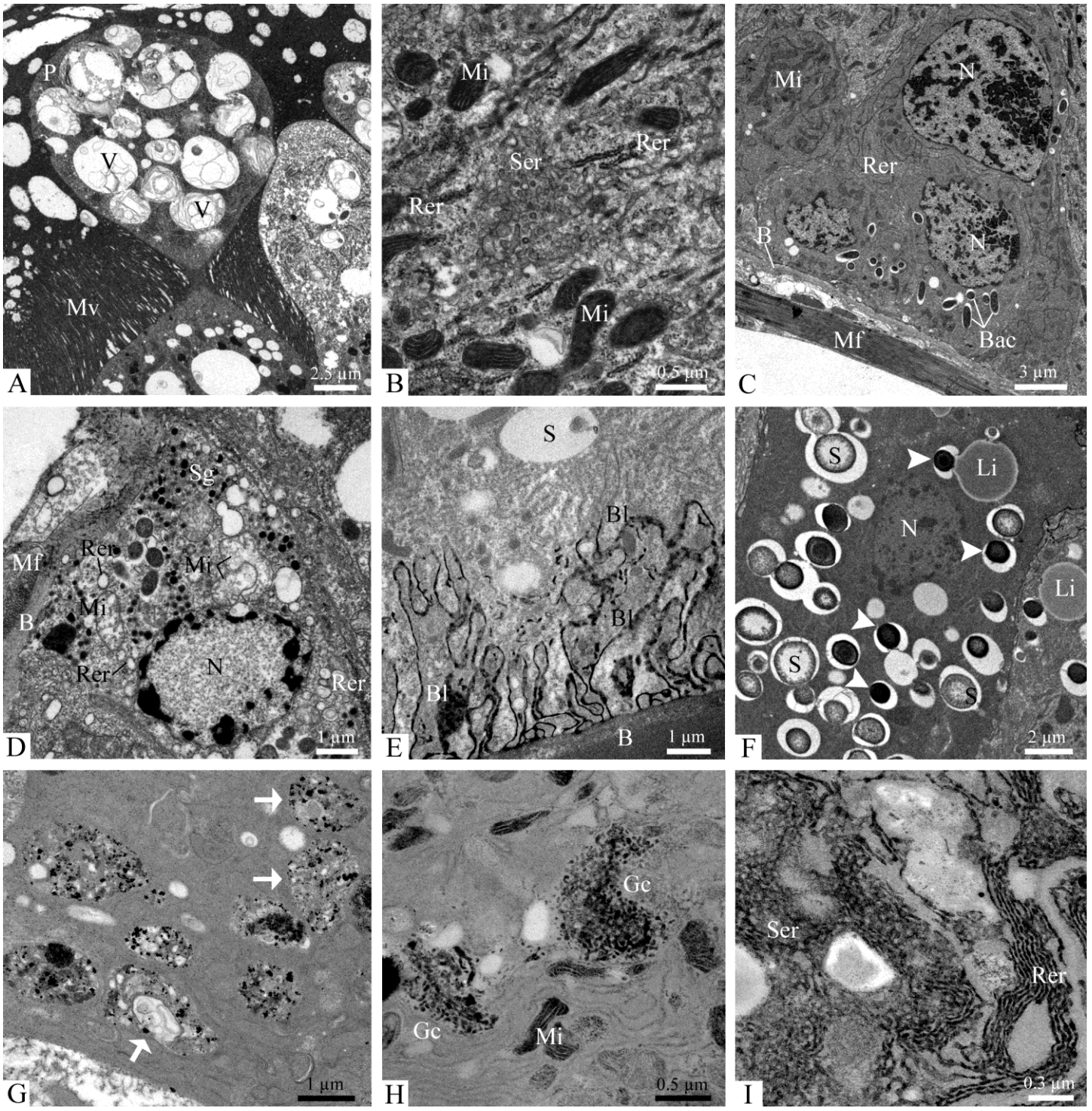
Fig. 9. Transmission electron micrographs of midgut epithelial cells of adult *C. claveri* females that originated from neem oil-treated larvae, sampled at the beginning of oviposition. (**A, C, D** and **G**) Posterior region of the midgut. (**A**) Apical surface of a columnar cell with cytoplasmic protrusions (P) filled with vacuoles (V) containing membranous structures. (**B, E, F, H** and **I**) Anterior region of the midgut. (**B**) Note the presence of tubules of smooth endoplasmic reticulum (Ser) in the cytoplasm of the columnar cell. (**C**) Group of regenerative cells with no signs of cellular injury. (**D**) An endocrine cell that still contains signs of cell injury, including swollen mitochondria (Mi) and the dilation of the rough endoplasmic reticulum cisternae (Rer). (**E**) Using the lanthanum nitrate technique, we found that the invaginations of the basal labyrinth (Bl) still contained large extracellular spaces in the columnar cells. (**F**) Calcium ions were observed in electron-dense spherites (*arrowhead*), among other spherites (S) with small concentric rings. (**G**) The detection of acid phosphatase indicated the presence of larger autolysosomes (*arrow*) in the apical region of the columnar cells and the digestion of membranous structures that originated from autophagic vacuoles. (**H** and **I**) ZIO technique. Note the morphology of the Golgi apparatus (Gc), with a vesiculated appearance, and the presence of branched tubules of the smooth endoplasmic reticulum (Ser) in the cytoplasm of the columnar cell. Muscle fiber (Mf), basement membrane (B); bacterial endosymbionts (Bac); nucleus (N); lipid droplet (Li); microvilli (Mv); secretory granules (Sg).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4.2. CAPÍTULO 2

Are the biopesticide neem oil and the predator *Ceraeochrysa claveri* (Navás, 1911) compatible?

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Running title: Are the neem oil and *Ceraeochrysa claveri* compatible?

Abstract

Due to the indiscriminate use of broad-spectrum insecticides in agricultural production, the vulnerability of natural enemies to this type of pesticide exposure results in side effects that impede the biological function of natural enemies, making their use for biological control in agroecosystems impracticable. The search for more selective pesticides to minimize the effects on these beneficial organisms is necessary, and among the alternatives, we emphasize the increased use of biopesticides, e.g., neem oil (*Azadirachta indica*) (Meliaceae), which is derived from one of the plants among most widely used globally. To determine the compatibility of neem oil with predatory insects in integrated pest management, we evaluated the lethal and sublethal effects on the postembryonic development of the lacewing *Ceraeochrysa claveri* (Navás, 1911) (Neuroptera: Chrysopidae) after neem oil ingestion in the larval instars. Among the measured biological parameters, we highlighted the mortality, the prolongation of the larval instars, the absence of cocoon formation in the prepupa, the low survival of females in the preoviposition period and the significant effects on reproduction for which a reduction occurred in fecundity, fertility and egg viability for all evaluated doses. In laboratory conditions, the predatory lacewing *C. claveri* and the biopesticide neem oil were not compatible. Therefore, the side effects that we observed from the use of neem oil might compromise the long-term population abundance of this natural enemy in agroecosystems.

Keywords: bioinsecticide; Chrysopidae; predator; selectivity; toxicity.

Introduction

Currently, the environmental awareness of the world population is growing, and as a result, the consumption of safe and healthy food is desired. To meet this demand, new forms of food production has been developed and improved; notably, the use of biopesticides and biological pest control in Integrated Pest Management (IPM). For biological control in pest management to be effective, sustainable and viable, the biopesticide used must be compatible with the populations of the biological control agents that occur in agroecosystems (Cloyd 2012, Biondi *et al* 2012, 2013).

Among the biopesticides, 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. The most popular and easily accessible product is neem oil, which is obtained by cold pressing neem seeds (Schmutterer 1990, Mordue (Luntz) & Blackwell 1993, Isman 2006). The predominant active component of neem oil is azadirachtin, which has different activities against insects, such as repellency, antifeedant properties, growth regulation, suppression of reproduction with reductions in fecundity and fertility, and mortality (Schmutterer 1990, Mordue (Luntz) *et al* 1998, Mordue (Luntz) & Nisbet 2000, Isman 2006, Morgan 2009, Cloyd 2012).

Recently, the compatibility of neem products with natural enemies have been evaluated, and the neem products were not completely safe for the biological control agents (Biondi *et al* 2012, 2013, Lima *et al* 2015). The neem products did not show selectivity for nontarget organisms, such as green lacewings, either from direct exposure or from indirect exposure with the ingestion of contaminated prey (Qi *et al* 2001, Ahmad *et al* 2003, Medina *et al* 2003, 2004, Aggarwal & Brar 2006, Cordeiro *et al* 2010, Scudeler & Santos 2013, Scudeler *et al* 2014, 2016). Green lacewings are polyphagous predators of economically important arthropod pests, such as aphids, thrips, mites, whiteflies, and the eggs and larvae of numerous pest insect species (Principi & Canard 1984, Pappas *et al* 2011). Among a number of species of green lacewings, *Ceraeochrysa claveri* (Navás, 1911) (Neuroptera: Chrysopidae) is an important predator of phytophagous pests in many Neotropical agroecosystems (De Freitas & Penny 2001). Because of a broad prey range, high voracity, high search capacity and short developmental time, *C. claveri* can be considered an efficient biological control agent (Principi & Canard 1984, Albuquerque *et al* 2001, Pappas *et al* 2011).

Because of its wide distribution in agroecosystems (De Freitas & Penny 2001), *C. claveri* provided a good biological model to verify the incidence of neem side effects on a predator species. Therefore, our objective was to evaluate the occurrence of lethal and sublethal effects on the postembryonic development of *C. claveri* when exposed to neem oil through oral exposure by feeding on poisoned prey during the larval instars. The focus was to investigate the biological effects of neem oil on the development of the insect to adulthood and to verify that some of these effects compromised the ability of this species to be included in a joint biological control program with the use of neem oil.

Materials and methods

Insects

The *C. claveri* used in the experiments were provided from a continuously reared laboratory colony from the Laboratory of Insects in the Department of Morphology at the Institute of Biosciences, Brazil. In this colony, the *C. claveri* were maintained in an environmental chamber with controlled conditions (25 ± 1°C; 70 ± 10% RH; and photoperiod of 12:12 h L:D). The larvae were reared with the eggs of *Diatraea saccharalis* (Lepidoptera: Crambidae), and the adults were fed an artificial diet (1:1 honey: yeast solution).

Biopesticide and treatments

The formulated product used in all of the experiments was neem oil emulsifiable Natuneem® (Natural Rural Ind. e Com. de Produtos Orgânicos e Biológicos Ltda, Araraquara-SP, Brazil; an organic product, certified by BCS ÖKO – Garantie, Doc. Natur - 9009/09.05/7331-BR), which was a pure cold-pressed neem oil extracted from neem seeds that contained 1500 ppm of azadirachtin A as the active ingredient. Three experimental concentrations of 0.5, 1 and 2% (v/v) were used and were prepared separately with the addition of distilled water. We assessed the recommended label rate for use in Brazilian agricultural fields and the concentrations that were previously evaluated by Scudeler & Santos (2013).

The fresh egg clusters that were recently deposited by the females of *D. saccharalis* were collected, dipped into each neem oil concentration for 5 s and then air-dried at room temperature for 1 h. For the control group, the egg clusters were dipped into distilled water and then were processed identically to the other groups. The bioassays were performed with

the exposure of *C. claveri* larvae to neem oil through the ingestion of the treated eggs. The newly hatched larvae (0-12h) were collected from the stock rearing colony, randomly selected and divided into four experimental groups (n = 30 per group). Each experimental group consisted of 5 replicates per concentration. The larvae were maintained individually in polyethylene boxes (2 cm height x 6 cm diameter) and were tested under identical environmental conditions as described for the rearing of the insects. In the control group, the larvae were fed *ad libitum* the *D. saccharalis* eggs treated with distilled water. For the treated groups (0.5, 1 and 2%), the larvae were fed *ad libitum* the eggs treated with neem oil throughout the larval period until pupation. The egg clusters of *D. saccharalis* were replaced every 3 days because the neem oil compounds were sensitive to ultraviolet light.

Toxicity assessment

The *C. claveri* larvae were exposed chronically to neem oil via ingestion; thus, we measured the lethal and sublethal effects on the larvae, prepupae, pupae and adults emerged from neem treated larvae. For larval instars, the specimens were checked daily to record the developmental time and mortality. A larva was considered dead when it did not move after being touched with a fine brush. The transition of larval instars was confirmed by the exuviae left after each ecdysis and by the color of the larvae. After larval development, when the larva finished a cocoon, the prepupal period began (Fig 1a), which continued until the last larval ecdysis inside the cocoon; the larva remained inside the cocoon and transformed into an exarate pupa during the pupation (Fig 1c). Eventually, a pharate adult emerged from the cocoon. The inability of a larva to spin the cocoon to start the prepupal period (Fig 1b), the pupal mortality inside the cocoon (Fig 1d) and the developmental time of the prepupa and the pupa were monitored daily. The adults that emerged from the larvae that survived the treatments were sexed, and the adult sex ratio was calculated as the proportion of females [females/ (males + females)]. To assess the effects on the biological parameters of the adult stage, one freshly emerged female (0-12h) from neem oil treatment was paired with two freshly emerged males from control. The trio of insects was maintained in a polyethylene box (9 cm height x 18 cm diameter) and was provided an artificial diet (1:1 honey: yeast solution) and a cotton wick with distilled water every 5 days until death. Between seventeen and twenty replicates of the adults were used per concentration of neem oil. From these trios of adults, the biological parameters were measured daily and included the preoviposition and the oviposition periods, survival to oviposition, fecundity, fertility and longevity of females. To

assess the fecundity, the eggs laid per female were collected daily and were counted throughout oviposition period. The eggs were monitored for egg-hatch as the determinate of the fertility. With the fertility and fecundity data, we calculated the percentage of egg viability: [(fertility/fecundity) X 100].

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 abdomen and alimentary canal of female adults emerged from the larvae untreated and treated with neem oil were studied and documented with an Olympus SZX16 stereo microscope with cell^{D} imaging software (Olympus Soft Imaging Solutions GmBH, Münster, Germany) at the Electron Microscopy Center of the Institute of Biosciences, UNESP, Botucatu-SP.

Statistical analyses

The quantitative variables measured following the treatments were examined with analysis of variance, which was complemented with multiple comparison tests. To assess the developmental time of the larvae, prepupae, and pupae, the adult sex ratio and the time of the preoviposition period, the procedure was parametric, which was complemented with the Bonferroni or the Tukey test (Johnson & Wichern 2007, Zar 2009). The non-parametric test Kruskal-Wallis, complemented with the Dunn test (Zar 2009), was performed to detect differences in the oviposition period, fecundity, fertility, viability of the eggs and longevity of females among the treatment concentrations of neem oil and the control group.

The study of association relative to the mortality of larvae, the viability of prepupae and pupae and the survival of newly emerged females until the start of oviposition was conducted using the Goodman test that involved contrasts among and within multinomial populations (Goodman 1964, 1965). All tests were performed at a significance level of 5%.

Results

For the larval period, significant effects of neem oil were observed on the three larval instars. The larval development time was prolonged in the first instar with the exposure to a dose of 0.5% neem oil; the results were similar for all doses in the second instar but only for the highest dose of neem oil in the third instar (Table 1). In addition to the extended periods of larval development, the neem oil preparations significantly reduced the percentage of

survival in the experimental groups because of the accumulated mortality of larvae in the second and third instars. However, a dose-dependent response was not observed, and both the 0.5% and 2% treatments had the highest percent mortalities of the lacewing larvae (20% and 24%, respectively; Table 2).

The effect of neem oil on the ability of the treated larvae to spin a cocoon was measured in the prepupal period with the formation of viable and nonviable cocoons (Fig 1a and b). The quantitative effect of neem oil on the prepupal survivorship is shown in Table 3. The toxic effects on the prepupae were more harmful at 0.5% and 1% than at 2% neem oil treatments. Despite the effect on cocoon formation, no significant difference was found in the viability or the pupal developmental time between the neem treatments and the control (Tables 3 and 4; Fig 1c and d).

The sex ratio of the newly emerged *C. claveri* adults was significantly reduced in the 0.5% neem oil group (Table 5). The females emerged from the neem treated larvae were not severely affected in the preoviposition period, except for a delay in this period in the group treated with 1% neem oil. In the preoviposition period, the low survival percentage of the females that reached oviposition was highlighted, particularly in the 1% and the 2% groups in which just 73.7% and 53% of the females survived, respectively (Table 5). The low survival percentage of the females emerged from the treatments with neem might be related to the difficulty that some females had in the elimination of the meconium from the alimentary canal (Fig 1e - g). Without the elimination of the meconium, the passage of the alimentary bolus was interrupted, which resulted in death.

Similar to the preoviposition period, the group treated with 1% neem oil had a prolonged oviposition period (Table 6). The reproductive parameters of fecundity, fertility and egg viability were all significantly lower than those of the control for all concentrations of neem oil (Table 6). Consequently, these sublethal effects on reproduction significantly affected the production of progeny. The adult longevity was also different among the treatment groups, and the females emerged from larvae in the 0.5% and the 2% neem oil treatments had shorter longevity than the females in the control group (Table 6).

Discussion

In this study, neem oil induced lethal and sublethal effects on the postembryonic lifecycle of the lacewing *C. claveri*. The assessment of the effects caused by the consumption of poisoned prey clearly reflected an important pathway of *C. claveri* exposure. Because the

C. claveri larvae are trash-carriers with collected exuviae, dead prey and several pieces of plant material placed on the dorsum (Pappas *et al* 2011), in the field, the larvae would be protected from the direct sprays of biopesticides.

Increase the knowledge about the use a specific biopesticide and its effects, primarily the indirect consequence on natural enemies is more relevant and gain interest due to expansion of the use of these pesticides held to be safer to natural enemies populations (Desneux *et al* 2007, Cloyd 2012, Biondi *et al* 2012, 2013). The selectivity of neem oil and the active compound azadirachtin on natural enemies was recently questioned in some studies with green lacewings (Qi *et al* 2001, Ahmad *et al* 2003, Medina *et al* 2003, Aggarwal & Brar 2006, Cordeiro *et al* 2010, Scudeler & Santos 2013, Scudeler *et al* 2014, 2016).

In this study, we found that the ingestion of neem oil caused mortality and delay in the development of *C. claveri* larvae, particularly in the second and third instars. Neem oil and the associated compounds affected the larval development of arthropod pests (Rharrabe *et al* 2008, Morgan 2009, Roel *et al* 2010, Ahmad *et al* 2012), but with widespread use in agroecosystems, neem oil might negatively affect the natural enemies, such as predators. Similar to the results described for *C. claveri* in this study, whether for lacewings or coccinellids, the exposure to neem oil via the intake of prey or with contact caused harmful effects on the larval development of these predators (Banken & Stark 1997, Qi *et al* 2001, Ahmad *et al* 2003, Hamd *et al* 2005, Aggarwal & Brar 2006, Kraiss & Cullen 2008). Because of the repellent and antifeedant effects (Mordue (Luntz) & Nisbet 2000, Isman 2006, Morgan 2009), the higher concentrations of neem most likely resulted in larvae of the first instar that did not feed as much as the larvae in the 0.5% treatment group. Thus, it was possible that only the larvae in the 0.5% group had delayed development in the first instar, whereas the other two instars experienced direct negative effects, particularly at the dose of 2%. The antifeedant and repellent effects could also explain the mortality in the larval stage; the low concentration of neem oil (0.5%) repelled fewer larvae from ingestion of the treated eggs, and therefore these larvae fed on large quantities of eggs and became more susceptible to the harmful effects of the neem. By contrast, the high concentration (2%) of neem oil was strongly repellent to the larvae, but even the small amount of eggs consumed by this group resulted in lethal damage. Although larval development was less susceptible to a dose of 1%, all the doses had highly detrimental effects on the predator lifecycle. According to Schmutterer (1997), the neem products were not completely safe for use with all stages of the natural enemy lifecycle, particularly the larval instars.

Because of the high mortality in the larval stages with exposure to 2% neem oil, when we evaluated the prepupa stage, a higher percentage of cocoons not formed were found for the lower doses (0.5% and 1%) than for the high dose. Scudeler *et al* (2013) showed that neem oil intake during the larval stage affected the larval and pupal development of *C. claveri* during the spinning process of the cocoon. These authors demonstrated that the ingestion of neem oil caused morphological and ultrastructural changes in the cocoons and that these specimens were more vulnerable to natural enemies and environmental factors than those that did not ingest neem oil. Although we did not observe significant differences in the viability or the duration of the pupal stage, we believe that this result was because of the manner in which the experiments were conducted, under controlled climatic conditions. Under field conditions, the effects of neem oil on the viability and the development of the pupae might be different from those found in the laboratory evaluations. For many other species that had some form of exposure to neem oil or the components, the harmful effects on the pupal stage were also reported, including for *Coccinella septempunctata* (Banken & Stark 1998), *Harmonia conformis* and *Mallada signatus* (Qi *et al* 2001), *Plodia interpunctella* (Rharrabe *et al* 2008) and *Spodoptera frugiperda* (Roel *et al* 2010).

The adult females were affected by the treatments with neem oil. According to Rousset (1984), in the emergence of adult lacewings, the female reproductive system is not functional and requires some time for the maturation to occur, which is the period that corresponds to preoviposition. Only the group in the 1% treatment showed differences in the preoviposition and the oviposition periods. Ahmad *et al* (2012) reported similar effects on the preoviposition period in *Plutella xylostella*. The physiological effects on reproduction might be one of the most important effects following insect exposure to neem (Schmutterer 1997, Isman 2006, Morgan 2009, Cloyd 2012).

Although the viability of the pupae was high, few females that emerged reached the oviposition period at the larger doses of neem. We observed a dark spot that formed inside the abdomen, a common occurrence in the females treated with neem oil. With an analysis of the internal morphology of these females, we found a dark structure at the end of the hindgut; the females emerged from the cocoon with this structure, which was not be eliminated before reaching adulthood. According to Canard and Principi (1984), this structure is the meconium, which is a small, ovoid and compact mass composed of the wastes that were accumulated by the larva during trophic activities. The meconium must be evacuated through the anus for the adult to become active and to begin to feed. Because the ingestion of neem oil affected the midgut epithelium in the larvae and pupae of *C. claveri* (Scudeler & Santos 2013, Scudeler *et*

al 2014), we believe these changes influenced meconium formation and prevented its elimination, resulting in shorter lives of these insects and the inability to reproduce.

For the reproductive parameters that we examined, neem had harmful effects on the adult females. A decrease in oviposition, fertility and egg viability led to a reduction in the offspring produced by *C. claveri*, which compromised the growth potential of the population. Interference in the oogenesis and vitellogenesis were the most common alteration reported in the females with inhibited ovarian development and a decrease in female reproduction (Sayah *et al* 1996, Medina *et al* 2004, Lucantoni *et al* 2006, Ghazawi *et al* 2007, Tine *et al* 2011, Andrade-Coelho *et al* 2014).

Indeed, the pursuit of new strategies for crop protection is necessary, and we cannot neglect the effects of biopesticides on nontarget arthropods. Our results showed that the predator *C. claveri* was affected indirectly by neem oil through the ingestion of poisoned prey. These indirect effects were reflected in all stages of the lifecycle and would likely inhibit the establishment of this natural enemy and prevent an overall success with biological control when attempting to integrate this predator with the use of the neem oil biopesticide. The exposure of the immature stages to neem oil did not provide safety for this predator from the biopesticide, and all stages of the lifecycle exhibited side effects, which were effects that could endanger the population of this species in an agroecosystem and would prevent synergistic control.

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TABELAS

Table 1 The effects of neem oil on larval development (mean ± SD) of the different larval stages of *C. claveri* when fed the eggs of *D. saccharalis* treated with neem oil.

Treatments	Developmental time (days)		
	First instar	**Second instar**	**Third instar**
Control	3.813±0.915 aA	4.217±0.889 aB	4.738±0.961 aC
0.5%	4.567±1.644 bA	4.621±1.710 bA	4.925±1.291 abA
1%	4.033±0.986 aA	4.752±1.484 bB	5.000±0.828 abB
2%	4.073±1.593 aA	4.805±1.406 bB	5.246±0.992 bC

The means followed by different letters are significantly different: for lower case letters, the comparison of treatments for the same instar; and for upper case letters, the comparison of the instars within a fixed treatment ($p < 0.05$, the Bonferroni test). n = 150 larvae per concentration.

Table 2 Accumulated mortality (%) of *C. claveri* larvae of different instars subjected to bioassays with chronic exposure to neem oil through ingestion during larval development.

Treatments	Accumulated mortality (%)		
	First instar	**Second instar**	**Third instar**
Control	0	4.7 a	6.0 a
0.5%	0	12.0 ab	20.0 b
1%	0	6.0 a	8.0 a
2%	0	18.0 b	24.0 b

The data followed by different letters, within a column, are significantly different. ($p < 0.05$, the Goodman test). n = 150 larvae per concentration.

Table 3 The effects of neem oil on pupation and pupal viability (%) for the formation of adults of *C. claveri*, when larvae were fed the eggs of *D. saccharalis* treated with neem oil during larval development.

Treatments	Prepupa (%)		Pupa (%)	
	Viable	No cocoon formation	Viable	Death inside cocoon
Control	90.0 b	10.0 a	96.3 a	3.7 a
0.5%	78.7 a	21.3 b	94.9 a	5.1 a
1%	78.7 a	21.3 b	94.1 a	5.9 a
2%	82.7 ab	17.3 ab	89.5 a	10.5 a

Percentages followed by different letters, within the same column, are significantly different. ($p < 0.05$, the Goodman test). $n = 150$ prepupae per concentration.

Table 4 Developmental times of the prepupal and pupal stages (mean ± SD) of *C. claveri* treated with neem oil during larval development.

Treatments	Developmental time (days)	
	Prepupa	**Pupa**
Control	4.700±0.618 aA	9.308±0.582 aB
0.5%	5.036±0.614 cA	9.196±0.534 aB
1%	4.829±0.502 abA	9.234±0.485 aB
2%	4.937±0.691 bcA	9.342±0.610 aB

The means followed by different letters are significantly different: for lower case letters, the comparison of treatments for the same stage; and for upper case letters, the comparison of stages within a fixed treatment ($p < 0.05$, the Bonferroni test). $n = 150$ prepupae per concentration.

Table 5 Adult sex ratio, preoviposition period (d) and adult survival to oviposition (%) of the adults that originated from *C. claveri* larvae treated with neem oil during the larval stage.

Treatments	Sex ratio[a]	Preoviposition period (days)	Survival until oviposition (%)
Control	0.532±0.019 b[#]	16.92±3.85 a[#]	97.0 c[Δ]
0.5%	0.399±0.083 a	18.11±6.63 ab	91.6 c
1%	0.515±0.062 b	19.13±5.79 b	73.7 b
2%	0.465±0.075 ab	16.92±4.13 a	53.0 a

[#] The means ± SD followed by different letters, within a column, are significantly different ($p < 0.05$, Tukey test).

[Δ] Percentages followed by different letters, within a column, are significantly different ($p < 0.05$, Goodman test).

[a] Adult sex ratio (adults emerged that originated from treated larvae) calculated as the proportion of females = [females/ (males + females)].

Table 6 Oviposition period (d), fecundity, fertility, egg viability (%), and female longevity (d) [median (minimum; maximum value)] of adult of *C. claveri* fed neem oil during larval development.

Treatments	Oviposition period (days)	Fecundity[a]	Fertility[b]	Egg viability (%)[c]	Female longevity (days)
Control	23.5 (5.0; 67.0) a[#] (18)*	98.5 (13.0; 63.0) b[#] (18)	73.0 (7.0; 130.0) b[#] (18)	73.0 (52.1; 94.9) b[#] (18)	82.5 (60.0; 114.0) b[#] (18)
0.5%	30.0 (1.0; 65.0) ab (20)	32.0 (1.0; 123.0) a (20)	0.0 (0.0; 94.0) a (20)	0.0 (0.0; 85.6) a (20)	70.0 (23.0; 87.0) a (20)
1%	35.0 (14.0; 64.0) b (20)	33.0 (5.0; 107.0) a (20)	0.0 (0.0; 82.0) a (20)	0.0 (0.0; 89.3) a (20)	75.0 (41.0; 101.0) ab (20)
2%	25.0 (5.0; 59.0) a (17)	26.0 (3.0; 135.0) a (17)	0.0 (0.0; 106.0) a (17)	0.0 (0.0; 78.5) a (17)	67.0 (31.0; 90.0) a (17)

[#]Data followed by different letters within columns are significantly different ($p < 0.01$, Dunn test).

[a] Fecundity: total number of eggs laid per female throughout the oviposition period.

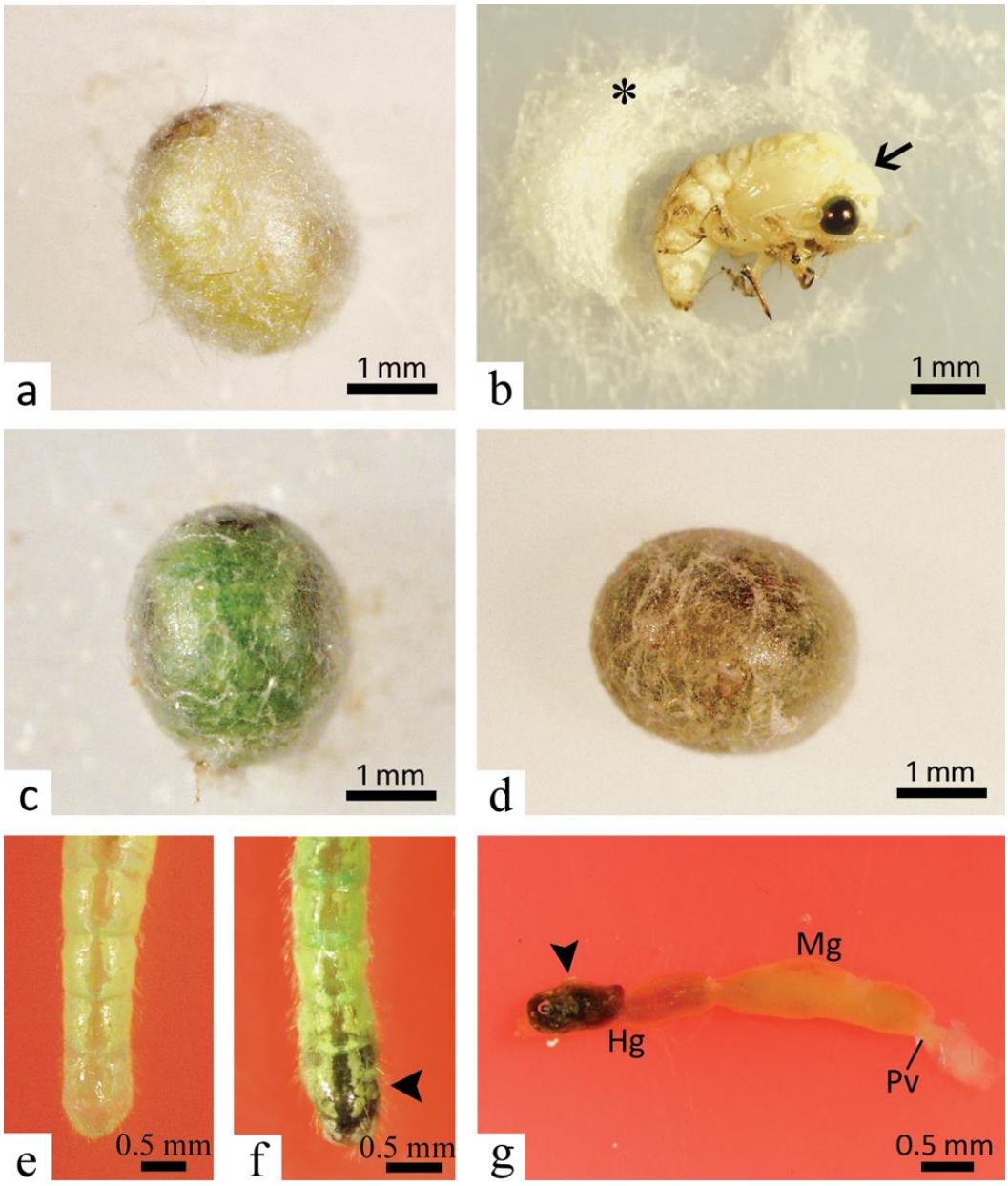
[b] Fertility: total number of eggs hatched.

[c] Egg viability (%): [(fertility/ fecundity) X 100].

* Number of females evaluated per concentration.

FIGURA

Fig 1 The effects of neem oil on the lifecycle of *Ceraeochrysa claveri*. (**a**) A normal cocoon spun by an untreated larva. The prepupa remains inside the cocoon. (**b**) The exarate pupa (*arrow*) outside a malformed cocoon (*). The larva treated with 0.5% of neem oil did not spin the cocoon in the prepupal period. (**c**) A viable pupa inside the cocoon. (**d**) The dark cocoon indicates pupal mortality inside the cocoon. (**e**) The end of the abdomen of a freshly emerged female from control group. It is possible to observe the green color that is common to the adult *C. claveri*. (**f**) A dark structure (*arrowhead*) is visible inside the abdomen of a freshly emerged female after treatment with neem oil at 2%. (**g**) Part of the alimentary canal of a *C. claveri* adult treated with 2% neem oil. Note the dark structure (*arrowhead*) at the end of the hindgut (Hg), which is the meconium that the adult could not eliminate after emerging from the cocoon. (Pv) proventriculus; (Mg) midgut.


a
1 mm
b
*
1 mm
c
1 mm
d
1 mm
e
0.5 mm
f
0.5 mm
g
Mg
Hg
Pv
0.5 mm

5. CONCLUSÕES

- Concluí-se que a ingestão do óleo de nim na fase larval do predador *Ceraeochrysa claveri* induz efeitos citotóxicos no mesêntero dos adultos, apesar das diferentes respostas citoprotetoras como renovação e detoxificação celular, as lesões celulares ainda permaneceram nas células epiteliais do órgão em todas as concentrações avaliadas;

- A exposição de *C. claveri* ao óleo de nim ocasionou significativos efeitos em sua biologia, principalmente na reprodução, onde observamos redução da fecundidade, fertilidade e viabilidade dos ovos para todas as concentrações analisadas, evidenciando a ausência de compatibilidade do uso integrado do predador *C. claveri* e o óleo de nim no manejo integrado de pragas.

6. ANEXO

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Cytotoxic effects of neem oil in the midgut of the predator *Ceraeochrysa claveri*

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ABSTRACT

Studies of morphological and ultrastructural alterations in target organs have been useful for evaluating the sublethal effects of biopesticides regarded as safe for non-target organisms in ecotoxicological analyses. One of the most widely used biopesticides is neem oil, and its safety and compatibility with natural enemies have been further clarified through bioassays performed to analyze the effects of indirect exposure by the intake of poisoned prey. Thus, this study examined the cellular response of midgut epithelial cells of the adult lacewing, *Ceraeochrysa claveri*, to neem oil exposure via intake of neem oil-contaminated prey during the larval stage. *C. claveri* larvae were fed *Diatraea saccharalis* eggs treated with neem oil at concentrations of 0.5%, 1% and 2% throughout the larval stage. The adult females obtained from these treatments were used at two ages (newly emerged and at the start of oviposition) in morphological and ultrastructural analyses. Neem oil was found to cause pronounced cytotoxic effects in the adult midgut, such as cell dilation, emission of cytoplasmic protrusions, cell lysis, loss of integrity of the cell cortex, dilation of cisternae of the rough endoplasmic reticulum, swollen mitochondria, vesiculated appearance of the Golgi complex and dilated invaginations of the basal labyrinth. Epithelial cells responded to those injuries with various cytoprotective and detoxification mechanisms, including increases in cell proliferation, the number of calcium-containing cytoplasmic granules, and HSP 70 expression, autophagic processes and the development of smooth endoplasmic reticulum, but these mechanisms were insufficient for recovery from all of the cellular damage to the midgut. This study demonstrates that neem oil exposure impairs the midgut by causing sublethal effects that may affect the physiological functions of this organ, indicating the importance of studies of different life stages of this species and similar species to evaluate the safe and compatible integrated use of biopesticides.

1. Introduction

Increasing environmental awareness has revealed the importance of natural enemies in agricultural production; however, it has also highlighted limitations associated with traditional studies of the impacts of pesticides on beneficial arthropods. Stop using just the direct mortality measurements and start evaluating the sublethal effects has been the focus of current studies that seek to determine the potential effects of pesticides that may reach a population of natural enemies and thus affect their physiological and biological functions in agroecosystems (Desneux et al., 2007; Cloyd, 2012).

One alternative that has gained an increasing number of adherents is the use of biopesticides, particularly those that are derived from plants. With their low risks to the environment and to human health, botanical insecticides are gaining a share of the market and are becoming a promising tool in the management of arthropod pest populations (Isman, 2006). Despite their bioefficacy, different mechanisms of action on insects, low toxicity and rapid degradation in the environment, their safety and compatibility with biological control agents have been thoroughly assessed and often refuted (Cloyd, 2012; Biondi et al., 2013).

Products derived from neem (*Azadirachta indica* A. Juss) (Meliaceae) are among the most widely used biopesticides worldwide.

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Their main active component is azadirachtin, which is responsible for the major sublethal effects on insects, including delayed growth and reductions in feeding, fecundity and fertility (Schmutterer, 1990; Mordue (Luntz) et al., 1998; Mordue (Luntz) and Nisbet, 2000; Morgan, 2009). Many studies have shown both the direct and indirect effects of exposure of natural enemies to neem products, questioning the safety and compatibility of neem-based biopesticides in combination with biological control agents. The sublethal effects observed in lacewings, polyphagous predators commonly found in horticultural and agricultural cropping systems, are an example of low selectivity to natural enemies (Medina et al., 2003; Aggarwal and Brar, 2006; Cordeiro et al., 2010; Scudeler and Santos, 2013; Scudeler et al., 2013, 2014).

The midgut is considered one of the primary paths of contact when evaluating the effects of ingested pesticides, and it has become an important target organ for ecotoxicological studies (Malaspina and Silva-Zacarin, 2006; Catae et al., 2014). Whether by direct ingestion of insecticide or by indirect ingestion of contaminated prey, the morphological and ultrastructural alterations of this organ have been useful for evaluating the sublethal effects on non-target organisms.

Previous studies on the neotropical green lacewing *Ceraeochrysa claveri* (Navás, 1911) (Neuroptera: Chrysopidae) have shown that intake of neem oil-contaminated prey during the larval stage affects epithelial cells of the midgut at both the larval and pupal stages (Scudeler and Santos, 2013; Scudeler et al., 2014). This finding indicates that cell damage is present during the pupal stage, which is when the larval midgut epithelium is exchanged for new epithelium during metamorphosis.

The permanence of cell injuries in this organ indicates the presence of cytotoxic effects that can compromise the physiological functions of this predator because digestion and the absorption of nutrients are altered (Scudeler and Santos, 2013, 2014). During the adult stage, the permanence of cell damage in the midgut may compromise female reproduction because the maturation and development of oocytes occurs from adult emergence until the start of oviposition, and these events depend on the quality and quantity of nutrients obtained from the diet during adulthood (Rousset, 1984). We therefore evaluated both the occurrence of cell damage in the midgut of adult females resulting from the intake of neem oil-contaminated prey during the larval stage and the existence of possible cellular response mechanisms against the damage to midgut epithelial cells.

2. Materials and methods

2.1. Insects

The newly hatched *C. claveri* larvae (0–12 h old) used in the toxicological 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 ± 10% RH; 12L:12D photoperiod).

2.2. Bioassays

Emulsifiable neem oil (Natuneem®, Natural Rural Ind. e Com. de Produtos Orgânicos e Biológicos Ltda, Araraquara-SP, Brazil) (organic product, certified by BCS OKO – Garantie, Doc. Natur – 9009/09.05/7331-BR), a pure cold-pressed neem oil extracted from neem seeds containing 1500 ppm azadirachtin A, was used in bioassays to chronically expose larvae to neem oil via ingestion. Three concentrations (0.5, 1, and 2%) (v/v) were used based on the recommended rate of usage in Brazilian agricultural fields and on the concentrations previously evaluated in C. claveri larvae and pupae (Scudeler and Santos, 2013; Scudeler et al., 2014).

The neem oil solutions (0.5, 1, and 2%) were diluted in distilled water and freshly prepared from the commercial formulation each day. Fresh *D. saccharalis* egg clusters were collected and dipped once in the neem oil solution for 5 s and then air-dried at room temperature for 1 h. For the control group, the egg clusters were dipped in distilled water.

Newly hatched larvae were randomly selected and placed in individual polyethylene boxes (2 cm height × 6 cm diameter). The larvae were divided into four experimental groups (n = 30 per group), and each experimental group included five replicates. The C. claveri larvae in the treated groups (0.5, 1, and 2%) were fed with neem oil-treated *D. saccharalis* eggs *ad libitum*. Likewise, the control group was fed with water-treated eggs ad libitum. This intake of neem oil-treated or untreated eggs occurred throughout the larval period until pupation (an average of 13 days). Due to the sensitivity of the neem oil components to ultraviolet light, the egg clusters that were not preyed upon were replaced every 3 days.

After the pupal stage, the sex of the freshly emerged adults (0–12 h old) was determined, and one freshly emerged female from each treatment group was paired with two freshly emerged control males. This trio of insects was kept in a polyethylene box (9 cm height × 18 cm diameter) until the start of oviposition (on average 17 days). During this period, the adults were fed an artificial diet (1:1 honey/yeast solution) and provided with a cotton wick soaked in distilled water every 5 days. The entire experiment was conducted under the same environmental conditions as those described for insect rearing.

2.3. Light microscopy

2.3.1. Histology and histochemistry

Adult females of two ages (0–1 day old and first day of oviposition) were quickly cryoanesthetized, and their guts were dissected in saline solution for insects (0.1 M NaCl, 0.1 M Na_2HPO_4 and 0.1 M KH_2PO_4). The insects were cut dorsally, and the midguts were isolated and fixed in a solution of 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) for 24 h for histological analysis. The midguts were removed and fixed in a 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.3) for 12 h for histochemistry and immunohistochemistry. Ten midguts of females from each age/group were used for histology, and five were used for histochemistry and immunohistochemistry. For the histological and morphometric studies, the midguts were dehydrated in graded ethanol solutions (70–95%) and then embedded in glycol methacrylate (Leica HistoResin Embedding Kit, Leica Biosystems, Wetzlar, Germany) according to the manufacturer's instructions. Sections (3 μm thick) were cut with a Leica RM 2045 microtome and stained with hematoxylin and eosin (HE) (Pearse, 1972).

For histochemical analyses, the specimens were dehydrated in graded ethanol solutions (70–100%), cleared in xylene and embedded in Paraplast® (Leica Biosystems, Richmond, IL, USA). The blocks were cut into 5 m thick sections, and histochemical analyses were performed on the slides to detect calcium (von Kossa technique) (Junqueira and Junqueira, 1983). The slides were analyzed and documented using a Leica DM500 photomicroscope.

2.3.2. Morphometric analysis

The length and epithelium height of the midgut were measured using one longitudinal HE-stained midgut section each from ten females from each experimental age/group. Ten measurements were collected from random locations in each midgut region,

including the anterior and posterior regions, using Axiovision image-processing software (Carl Zeiss, Oberkochen, Germany).

2.3.3. Immunohistochemical analyses

The tissue section slides were deparaffinized, rehydrated and treated for antigen retrieval in 0.01 M citrate buffer (pH 6.0) in a 500 W microwave for 15 min. Thereafter, the slides were left to cool at room temperature for 30 min. After washing in phosphate-buffered saline (PBS, 0.01 M, pH 7.3), the endogenous peroxidase in the sections was blocked by treatment with 0.3% H_2O_2 in PBS for 15 min at room temperature. The sections were then rinsed with PBS and covered with Protein Block (SPD-125, Reveal Polyvalent HRP-DAB Detection System, Spring Bioscience, Pleasanton, CA, USA) for 10 min at room temperature to block non-specific background staining. Then, they were incubated for 2 h at room temperature with the following primary antibodies: mouse anti-PCNA (1:1200 dilution) (clone PC10, ab80576, Abcam® Inc., Cambridge, MA, USA) as a cell proliferation marker and mouse anti-heat shock protein 70 (HSP 70, 1:100 dilution) (clone 5A5, ab2787, Abcam® Inc., Cambridge, MA, USA) as a marker of cellular stress. After incubation with the primary antibodies, the sections were analyzed with an immunoenzymatic antigen detection system (SPD-125, Reveal Polyvalent HRP-DAB Detection System, Spring Bioscience, Pleasanton, CA, USA) according to the manufacturer's instructions. The chromogen diaminobenzidine (DAB) develops a brown reaction product for positive signals. Finally, the slides were lightly counterstained with Harris hematoxylin. A negative control was obtained by omitting the primary antibody. The slides were examined and photographed with a Leica DM500 photomicroscope.

2.3.4. Cell proliferation index

The cell proliferation index was measured by counting PCNA-positive nuclei. Three longitudinal sections of the midgut from five specimens of each experimental age/group were used, and all positive and negative nuclei in both regions of the midgut were counted (~250 nuclei per region of midgut/specimen). The cell proliferation index was calculated as a percentage by dividing the number of positive nuclei by the total number of nuclei analyzed (positive and negative), and the final value was multiplied by 100.

2.3.5. TUNEL assay

To determine quantitative differences in cell death in the midgut epithelium, the TUNEL assay, a widely used microscopic technique for identifying cell death, was used to label the 3 ends of fragmented DNA. The tissue sections (5 μm) were deparaffinized, rehydrated and washed in Tris-buffered saline (TBS, 20 mM Tris, pH 7.6, 140 mM NaCl). Then, the sections were permeabilized by incubation in Proteinase K (20 g/ml in 10 mM Tris pH 8) at room temperature for 30 min. Further procedures were performed according to the manufacturer's instructions (TdT-FragEL™ DNA Fragmentation Detection Kit, Calbiochem® , Merck KGaA, Darmstadt, Germany). Diaminobenzidine (DAB) was used as a chromogen, and finally, the slides were counterstained with Harris hematoxylin. All TUNEL-positive nuclei in each midgut region were counted in three longitudinal sections for five samples in each experimental age/group, and the percentage of positive nuclei was calculated.

2.4. Confocal microscopy

Adult females of two ages (0–1 day old and first day of oviposition) (n = 5 per age/group) were dissected in saline solution for insects (0.1 M NaCl, 0.1 M Na_2HPO_4 and 0.1 M KH_2PO_4), and the midgut was fixed in 2% glutaraldehyde and 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.3) for 12 h. Next, the samples were washed in PBS for 5 h and embedded in Paraplast®. The tissue sections (7 μ m) were deparaffinized, rehydrated, washed in phosphate-buffered saline (PBS, 0.01 M, pH 7.3) several times and incubated with Phalloidin-TRITC (500 g/1 ml methanol, diluted 1:900 in PBS) (Sigma–Aldrich® , St. Louis, MO, USA) for 15 min at room temperature. Then, the sections were rinsed several times with PBS, and mounting medium containing DAPI (Fluoroshield with DAPI, Sigma–Aldrich® , St. Louis, MO, USA) was added to the sections. Fluorescence images of the samples were captured with a Leica TCS SP5 confocal microscope at the Electron Microscopy Center of the Institute of Biosciences of Botucatu, UNESP, Brazil and were then examined.

2.5. Electron microscopy

2.5.1. Scanning electron microscopy (SEM)

The midguts of adult females (n = 5 per age/group) were dissected, rinsed with insect saline solution (0.1 M NaCl, 0.1 M Na_2HPO_4 and 0.1 M KH_2PO_4) and then 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, post-fixed 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 CO_2 and coated with gold (Scudeler and Santos, 2013). The samples were examined and documented using an FEI Quanta 200 scanning electron microscope (FEI Company, Eindhoven, Netherlands) at the Electron Microscopy Center of the Institute of Biosciences of Botucatu, UNESP, Brazil.

2.5.2. Transmission electron microscopy (TEM)

Midguts were isolated from five adult female specimens of each age from each experimental group and were then fragmented, fixed and processed for TEM as indicated below. All samples were analyzed 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.

2.5.2.1. Conventional transmission electron microscopy. The midgut fragments were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.3) for 24 h at room temperature and then post-fixed 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. Ultra-thin sections were contrasted with uranyl acetate and lead citrate and were then analyzed with a Tecnai Spirit transmission electron microscope.

2.5.2.2. Ultrastructural cytochemical analysis. Lanthanum nitrate was used as a tracer of the extracellular space. The tissue fragments were fixed in a solution of 2.5% glutaraldehyde, 2% paraformaldehyde and 1% lanthanum nitrate in 0.1 M sodium cacodylate buffer (pH 7.2) for 12 h and then post-fixed in 1% osmium tetroxide and 1% lanthanum nitrate in cacodylate buffer for 2 h (adapted from Revel and Karnovsky, 1967). Dehydration and embedding were performed according to the routine procedures for conventional analysis.

For the ultrastructural localization of calcium deposits, the midgut fragments were fixed in 2.5% glutaraldehyde with 5 mM $CaCl_2$ in 0.1 M sodium cacodylate (pH 7.2) for 2 h, washed in cacodylate buffer with 5 mM $CaCl_2$, and post-fixed in a solution containing 1% osmium tetroxide, 5 mM $CaCl_2$ and 0.8% potassium-ferrocyanide in cacodylate buffer for 2 h (adapted from Forbes et al., 1977). The

subsequent steps were similar to those described for conventional analysis.

The acid phosphatase activity, a lysosomal marker, was analyzed by fixing the tissues in a 2.5% glutaraldehyde and 2% paraformaldehyde solution in 0.1 M sodium cacodylate buffer (pH 7.2) with 5% sucrose for 20 min, washing several times in sodium cacodylate buffer with 5% sucrose, and washing twice in 0.05 M acetate buffer (pH 5.0) with 5% sucrose. Next, the tissues were incubated in a solution of cytidine-5 -monophosphate and 1% lead nitrate in acetate buffer (pH 5.0) for 1 h at 37 °C (Pino et al., 1981). Post-fixation was performed in a solution of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) with 5% sucrose for 45 min and with 1% osmium tetroxide in cacodylate buffer for 1 h. Finally, the samples were dehydrated and embedded according to the procedures for conventional analysis.

To study the sub-cellular compartments that react with zinc iodide-osmium tetroxide (ZIO), the midgut fragments were fixed for 12 h with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 6.8) with 8.5% sucrose and incubated for 18 h at 4 °C in ZIO incubation medium (zinc powder, resublimed iodide, osmium tetroxide and Tris-aminomethane buffer) (Reinecke and Walther, 1978). The specimens were then washed in the same buffer, dehydrated and embedded using routine procedures. For all cytochemical analyses, unstained ultra-thin sections were examined using a Tecnai Spirit transmission electron microscope.

2.6. Statistical analysis

The quantitative data were compared using a two-factor analysis of variance complemented with Tukey's multiple comparison test (Zar, 2009). The results are expressed as the mean ± SD. The tests were performed at a significance level of 5%.

3. Results

The midgut of newly emerged adult *C. claveri* in the control group is composed of a pseudostratified epithelium, which is externally surrounded by muscle fiber bundles that formed an internal circular tunic and another external longitudinal tunic. In the anterior region of the midgut, columnar cells possess basophilic cytoplasm, apical cytoplasmic granules, and an acidophilic striated border. Spherical nuclei are centrally located with homogeneously dispersed chromatin. Groups of regenerative cells are present in the basal area of the epithelium and exhibit a strongly basophilic cytoplasm, with the nucleus having a morphology similar to that of the cells, varying from prismatic to oval (Fig. 1A). In the posterior region, the columnar cells showed more elongated, with larger numbers of apical cytoplasmic granules and more oval nuclei, similar to the more elongated morphology of the cells in this region (Fig. 1B).

In *C. claveri* adults from the three neem oil treatment groups, we demonstrated the occurrence of swelling of the apical surface of columnar cells and intense emission cytoplasmic protrusions toward the lumen; more elongated columnar and regenerative cells; the formation of folds in the epithelium; and loss of integrity of the apical membrane with the occurrence of cell lysis in both midgut regions (Fig. 1C–F). Due to the increased presence of elongated cells, the midgut epithelium height was also altered (Table 1).

At the start of oviposition, the midgut of the control group insects exhibited an increased length and epithelial height in both regions compared with the newly emerged adults. The cytoplasm of the columnar cells was slightly acidophilic and was filled with spherites and apical cytoplasmic granules, and the apex had an acidophilic striated border (Fig. 1G) (Table 1). For the groups exposed to neem oil during the larval stage, the midgut also displayed morphological changes at the start of oviposition, particularly epithelial folds, elongated columnar cells, mainly in the anterior region of the midgut, and the issuance of cytoplasmic protrusions (Fig. 1H–J) (Table 1). There was no change in the length of the midgut following the neem treatments for any of the evaluated ages (Table 1).

Histochemical analysis for calcium detection revealed positivity of the apical cytoplasmic granules in columnar cells, which were more abundant in the posterior region of the midgut in the newly emerged adults in the control group (Fig. 2A and B). When the von Kossa technique was applied to the each of the three neem oil-treated adult midguts, we observed increases in the positive calcium signals for all the evaluated neem dosages in both regions of the midgut (Fig. 2C–E). At the start of oviposition, this positivity for calcium was reduced, and the responses were similar in the control and treated groups and in the anterior and posterior regions of the midgut (Fig. 2F–K).

Evaluation of the integrity of actin via phalloidin staining revealed the presence of positive signals at the striated border and particularly in the cell cortex, with F-actin bundles in the apical regions of columnar cells in the midguts of the control insects of both ages (Fig. 3A, B, and E). For the newly emerged females in all of the neem oil-treated groups, we observed that regions of the cell cortex of columnar cells lacked F-actin-positive staining, indicative of changes in organization of the cytoskeleton (Fig. 3C-D). There was no such response at the start of oviposition, and all groups were positive for actin filaments in a region of the cell cortex, indicating recovery of the cytoskeleton in these cells (Fig. 3F).

Immunohistochemical analyses revealed low levels of HSP 70 expression in the cytoplasm and nuclei of columnar and regenerative cells of the midgut from the freshly emerged females (Fig. 4A and B) compared with the cells from the neem oil-treated insects of the same age (Fig. 4C–F). The increased HSP 70 expression in the neem oil-treated groups did not differ between doses or midgut regions. Analysis of the midgut at the start of oviposition revealed that the HSP 70 expression level was low and that it was similar between the controls and neem-treated females in both regions of the midgut (Fig. 4G–J).

All tested concentrations of neem oil induced cell death in the anterior and posterior regions of the midgut, but only at the start of oviposition (Table 2), which is when we also observed lower HSP 70 expression (Fig. 4G–J). However, the proliferation index indicated that all concentrations of neem oil induced cell proliferation only in the anterior region of the midgut at both evaluated ages (Table 3).

Ultrastructural analysis of the epithelial surfaces of freshly emerged C. claveri adults revealed differences between the anterior and posterior regions of the midgut. We demonstrated that columnar cells of the controls had abundant long microvilli in the anterior region (Fig. 5A) and more juxtaposed cells, with smaller and regular microvilli covering the apical surface in the posterior region (Fig. 5B). However, the adults that developed from the treated larvae showed swelling of the apical surface, leading to the formation and release of cytoplasmic protrusions, the disorganization and loss of microvilli, and apical membrane rupture in both regions of the midgut (Fig. 5C–E). At oviposition, the control group showed more juxtaposed cells in both midgut regions (Fig. 5F and G), and the epithelium in the neem-treated females no longer exhibited severe cell damage, only showing disorganization of the microvilli and small protrusions (Fig. 5H–J).

The ultrastructural observations of epithelial cells from the freshly emerged control group indicated the presence of regularly distributed microvilli on the apical surfaces of columnar cells, followed by a cytoplasm with a predominance of mitochondria, abundant rough endoplasmic reticulum and spherites in the perinuclear cytoplasm (Fig. 6A and B). Nests of regenerative cells with large nuclei and a granular cytoplasm with a predominance of mitochondria were observed at the base of the epithelium (Fig. 6C).

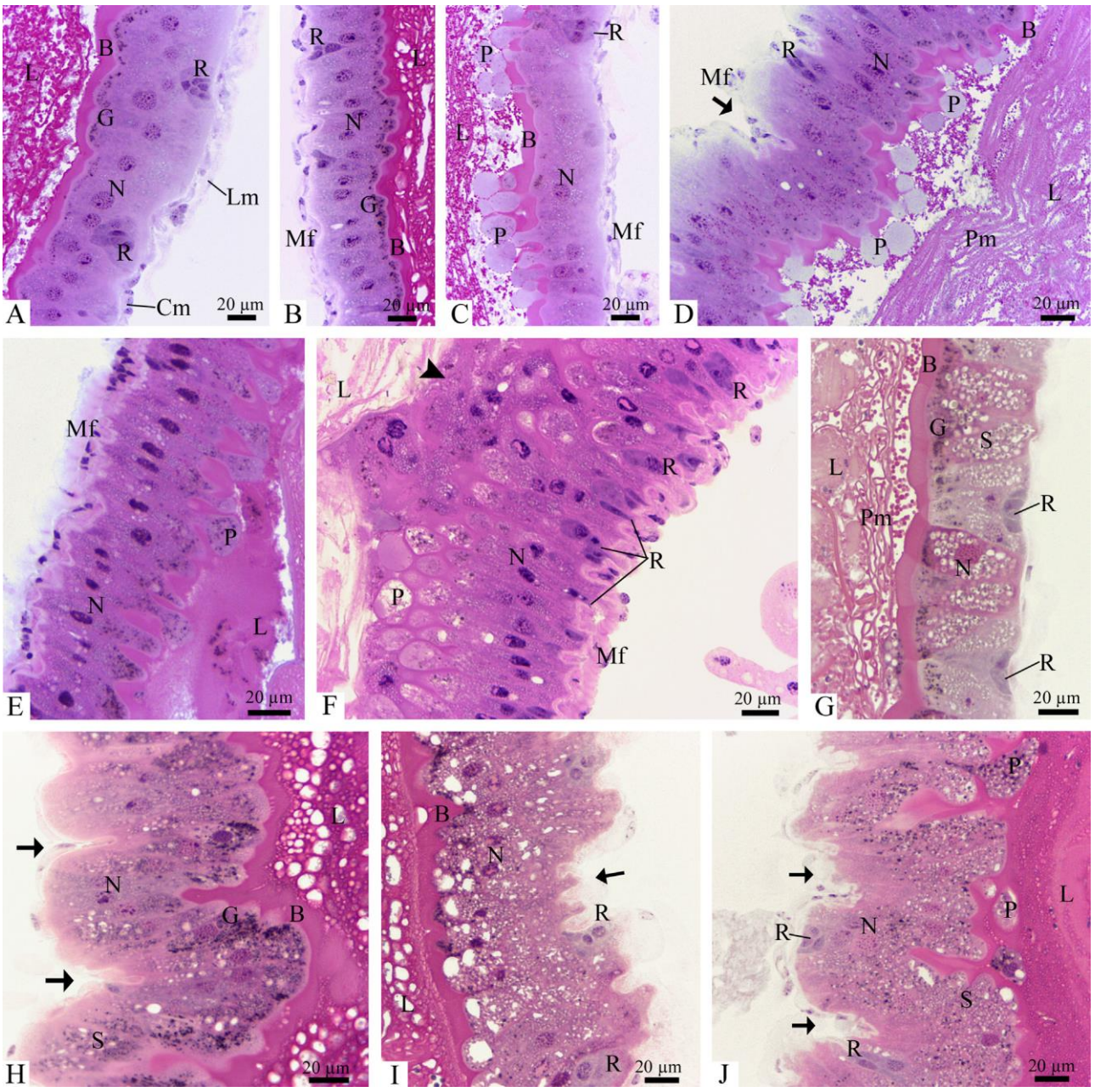

Fig. 1. Morphological changes in the midgut epithelium of adult *C. claveri* females after treatment with neem oil during the larval stage. (A–F) Newly emerged. (A and B) Control group, anterior and posterior regions, respectively. The pseudostratified epithelium is composed of basophilic columnar cells with an acidophilic striated border (B) and regenerative cells (R) with a strongly basophilic cytoplasm. (C) Neem oil—0.5%. Anterior region. (D and E) Neem oil—1%. Anterior and posterior regions. (F) Neem oil—2%. Posterior region. In the neem oil-treated groups, elongated columnar and regenerative cells were observed; with dilation of the apical surfaces of columnar cells and intense emission cytoplasmic protrusions (P); the formation of folds in the epithelium (arrow); and loss of integrity of the apical membrane along with cell lysis (►). (G–J) Oviposition. (G) Control group. Anterior region. Note the presence of spherites (S) in the cytoplasm of the columnar cells. (H–J) Neem oil—0.5, 1 and 2%, respectively. (H and J) Anterior region. (I) Posterior region. The epithelial folds formed by elongated columnar cells remain at this age, as well as the issuance of cytoplasmic protrusions. Nucleus of columnar cell (N); cytoplasmic granules (G); circular musculature (Cm); longitudinal musculature (Lm); muscle fiber (Mf); peritrophic membrane (Pm); lumen (L). Hematoxylin and eosin staining.

Using lanthanum nitrate percolation, we noted the presence of narrow invaginations in the basal labyrinth of the columnar cells (Fig. 6D). Positive calcium signals were observed in concentric rings of the spherites (Fig. 6E). Similarly, lysosomes, which are mainly located in the apical cytoplasm of columnar cells, were identified by acid phosphatase activity (Fig. 6F). Using the ZIO technique, we observed that the Golgi apparatuses in columnar and regenerative cells displayed numerous polarized cisternae with adjacent vesicular elements (Fig. 6G–I).

Epithelial cells of insects of the same age developed severe ultrastructural changes due to exposure to neem oil during the larval stage. Columnar cells showed sparse, dilated and deformed microvilli; dilated cisternae of the rough endoplasmic reticulum; swollen mitochondria in the cristolysis process; dilated invaginations of the basal labyrinth; and Golgi apparatus with numerous vesicles (Fig. 7A, B, E, H, and I). Calcium was identified in thick rings of the spherites (Fig. 7F), and acid phosphatase activity was observed in vacuoles with a predominance of myelin structures (Fig. 7G). Regenerative and endocrine cells also showed pathologi-

Table 1
Morphometric analysis (mean ± SD) of midgut of adult *C. claveri* females fed with neem oil during larval development.

Variable	Treatments	Age	
		Newly emerged	Start of oviposition
Epithelium height (μm)	Control	53.90 ± 8.19 aA	67.31 ± 10.61 aB
Anterior midgut region	0.5%	73.00 ± 8.59 bA	88.89 ± 12.93 bB
	1%	92.21 ± 14.18 cA	91.34 ± 10.69 bA
	2%	88.90 ± 16.48 cA	86.76 ± 20.42 bA
Epithelium height (μm)	Control	58.50 ± 3.17 aA	80.77 ± 5.98 aB
Posterior midgut region	0.5%	71.58 ± 8.88 abA	85.00 ± 8.95 aB
	1%	77.39 ± 17.43 bA	94.16 ± 16.99 aB
	2%	71.91 ± 13.72 abA	88.54 ± 16.87 aB
Length of midgut (μm)	Control	2507.40 ± 222.94 aA	3966.87 ± 354.01 aB
	0.5%	2494.69 ± 269.67 aA	4141.05 ± 352.24 aB
	1%	2285.86 ± 326.33 aA	4129.73 ± 434.18 aB
	2%	2495.52 ± 552.08 aA	3808.72 ± 531.69 aB

The means followed by different letters are significantly different. Lowercase: comparison of treatments within the same age. Upper case: comparison of ages fixed treatments ($p < 0.05$, Tukey test). n = 10 adults per treatment/age.

Table 2
Percentage of cell death (mean ± SD) in the midgut epithelium of adult *C. claveri* females treated with neem oil during larval development.

		Treatments			
Midgut region	*Age*	*Control*	*0.5%*	*1%*	*2%*
Anterior	Newly emerged	16.38 ± 3.33 aB	18.70 ± 10.91 aB	13.18 ± 3.62 aA	20.71 ± 4.09 aB
	Start of oviposition	2.21 ± 0.58 aA	11.09 ± 4.74 bA	12.16 ± 4.14 bA	12.16 ± 5.34 bA
Posterior	Newly emerged	19.04 ± 3.77 aB	11.89 ± 4.19 aA	16.95 ± 3.06 aA	19.59 ± 6.79 aA
	Start of oviposition	4.77 ± 1.82 aA	16.96 ± 5.28 bA	18.90 ± 2.92 bA	18.41 ± 7.07 bA

The means followed by different letters are significantly different. Lowercase: comparison of treatments within the same age. Upper case: comparison of ages fixed treatments ($p < 0.05$, Tukey test).

Table 3
Proliferation index (%) (mean ± SD) in the regions of midgut of adult *C. claveri* females fed during throughout larval stage with neem oil.

		Treatments			
Midgut region	*Age*	*Control*	*0.5%*	*1%*	*2%*
Anterior	Newly emerged	33.19 ± 3.61 aA	42.17 ± 4.75 bB	44.81 ± 4.90 bB	44.25 ± 6.11 bB
	Start of oviposition	27.83 ± 2.76 aA	35.76 ± 3.57 bA	37.08 ± 6.48 bA	36.89 ± 3.35 bA
Posterior	Newly emerged	38.36 ± 9.04 aA	37.51 ± 1.89 aA	44.78 ± 3.18 aA	40.27 ± 4.68 aA
	Start of oviposition	39.32 ± 3.40 aA	44.40 ± 10.53 aA	41.87 ± 7.09 aA	44.11 ± 4.49 aA

The means followed by different letters are significantly different. Lowercase: comparison of treatments within the same age. Upper case: comparison of ages fixed treatments ($p < 0.05$, Tukey test).

cal changes, including perinuclear space and mitochondrial dilation (Fig. 7C and D).

The same ultrastructural standard described for the epithelial cells in newly emerged adults was observed at the start of oviposition in the control group, in which we identified columnar cells with increased accumulation of spherites in the cytoplasm and a well-developed endomembrane system, with predominant rough endoplasmic reticulum and Golgi cisternae (Fig. 8A, B, and G–I). Using ultrastructural cytochemical techniques, we observed that the invaginations in the basal labyrinth remained narrow (Fig. 8D), lysosomes were concentrated in the apical cytoplasm (Fig. 8E), and calcium was present in some spherites with few concentric rings (Fig. 8F). Regenerative cells were present at the base of the epithelium, with a morphology similar to that described for the freshly emerged females (Fig. 8C).

The ultrastructure of the epithelial cells of the neem-treated groups still showed signs of cellular injury at the start of oviposition. Columnar cells contained protrusions that were mainly filled with vacuoles with membranous contents and cytoplasm with developed smooth endoplasmic reticulum and dilated invaginations of basal labyrinth (Fig. 9A, B, and E). Using cytochemical techniques, we noted the presence of calcium in electron-dense spherites, bulky autolysosomes in the apical cytoplasm that were digesting heterogeneous membranous contents, Golgi complex with a vesiculated appearance, and the development of smooth endoplasmic reticulum (Fig. 9F–I). Endocrine cells also exhibited changes, with a predominance of swollen mitochondria and dilated cisternae of the rough endoplasmic reticulum (Fig. 9D). Only regenerative cells showed no signs of cell damage at this age (Fig. 9C).

4. Discussion

The *C. claveri* midgut exhibited the same histological pattern in adulthood as has been described for the species during the immature stage (Scudeler and Santos, 2013), with cellular morphological characteristics appropriate for functions in the synthesis and secretion of digestive enzymes and the subsequent absorption of nutrients; growth and tissue regeneration; and control of metabolic processes, cellular proliferation and differentiation, which are assigned to columnar, regenerative and endocrine cells, respectively (Sousa et al., 2009; Hakim et al., 2010; Teixeira et al., 2013; Scudeler and Santos, 2013, 2014; Gigliolli et al., 2015).

Although we have characterized two morphological regions of the *C. claveri* midgut based on morphometric data on epithelial height, both regions of the midgut contained columnar cells with ultrastructural features indicative of high levels of metabolic activity associated with enzyme secretion and absorption, including apical microvilli and abundant mitochondria, rough endoplasmic reticulum, Golgi apparatus and lysosomes. The absorption, storage and transport of organic and inorganic elements associated with

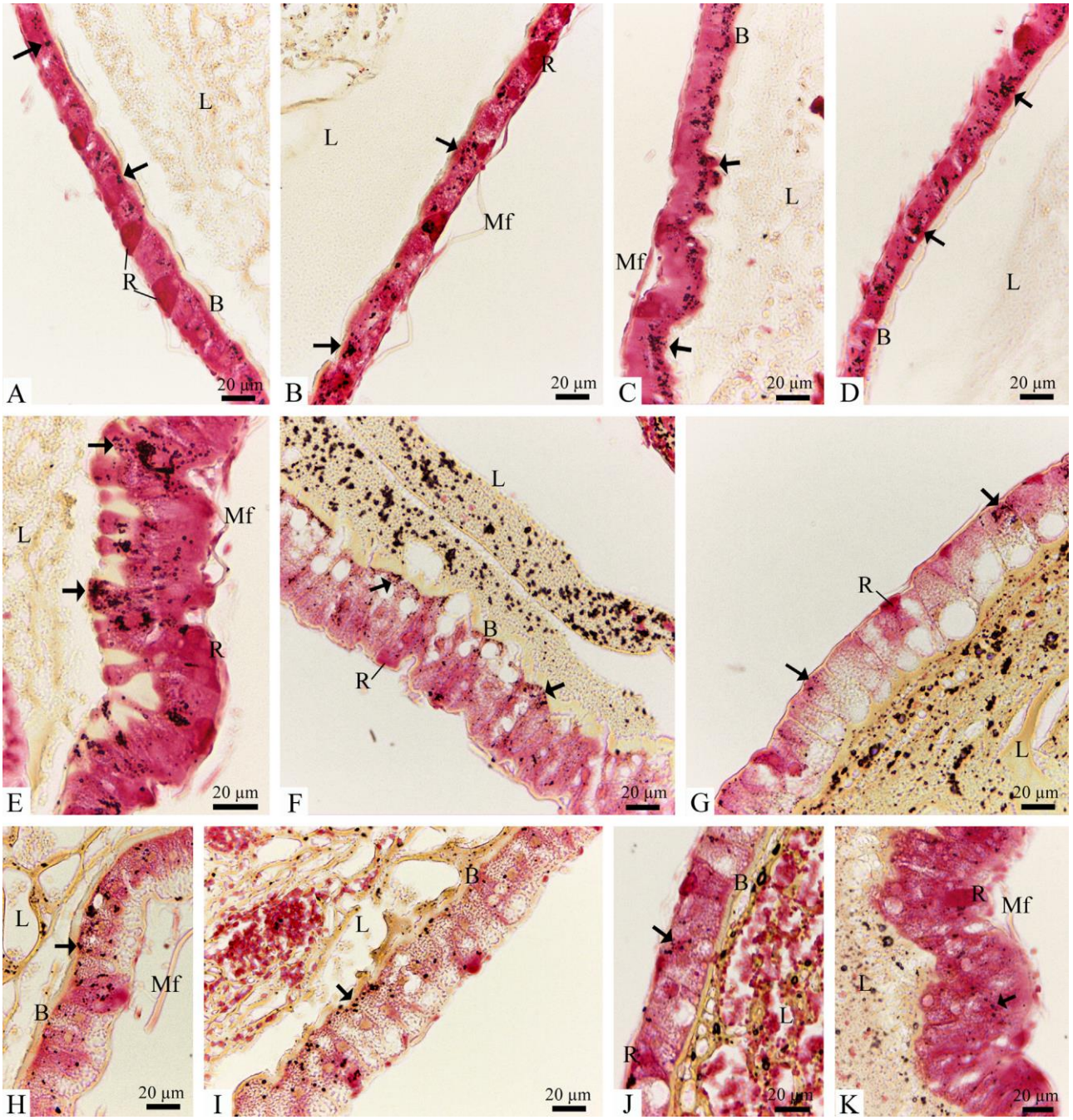

Fig. 2. Histological sections of the midgut epithelium of adult *C. claveri* females were analyzed for calcium localization by the von Kossa technique. (A–E) Newly emerged. (A–B) Control group; anterior and posterior regions of the midgut, respectively. Columnar cells showed cytoplasmic calcium granules in the apical region (arrow). (C–E) Treatment with neem oil at 0.5, 1 and 2%, respectively. Note the increase in calcium granules in the apical region of the columnar cells. (C and E) Anterior region. (D) Posterior region. (F–K) Oviposition. Reduction of the cytoplasmic calcium granules in the columnar cells. (F–G) Control group, anterior and posterior regions, respectively. (H and I) Neem oil—0.5%, anterior and posterior regions. (J) Neem oil—1%, posterior region. (K) Neem oil—2%, anterior region of the midgut. Lumen (L); striated border (B); regenerative cells (R) and muscle fiber (Mf).

the spherites and basal labyrinth are also involved in some of the functions of these cells (Sousa et al., 2009; Scudeler and Santos, 2013; Teixeira et al., 2013; Gigliolli et al., 2015).

The growth of the midgut between the two ages evaluated in the experiment was identified using morphometry, and it was due to the high rate of cell proliferation identified in the midgut epithelium. Cell proliferation was detected by immunohistochemical staining of proliferating cell nuclear antigen (PCNA), a highly conserved nuclear protein whose synthesis and expression is involved in DNA synthesis, making it a marker of cell proliferation (Ng et al., 1990; Kubben et al., 1994; Zudaire et al., 2004). Zudaire et al. (2004) have reported that PCNA is a useful and simple marker for assessing cell proliferation in insects, particularly in the digestive system. Histological and ultrastructural analyses revealed the basal localization of nests of regenerative cells and demonstrated that these cells had strongly basophilic cytoplasm due to the predominance of free ribosomes and scarce organelles, such as rough endoplasmic reticulum and mitochondria, highlighting the bulky and central nucleus, similar to what has been described by Sousa et al. (2009),

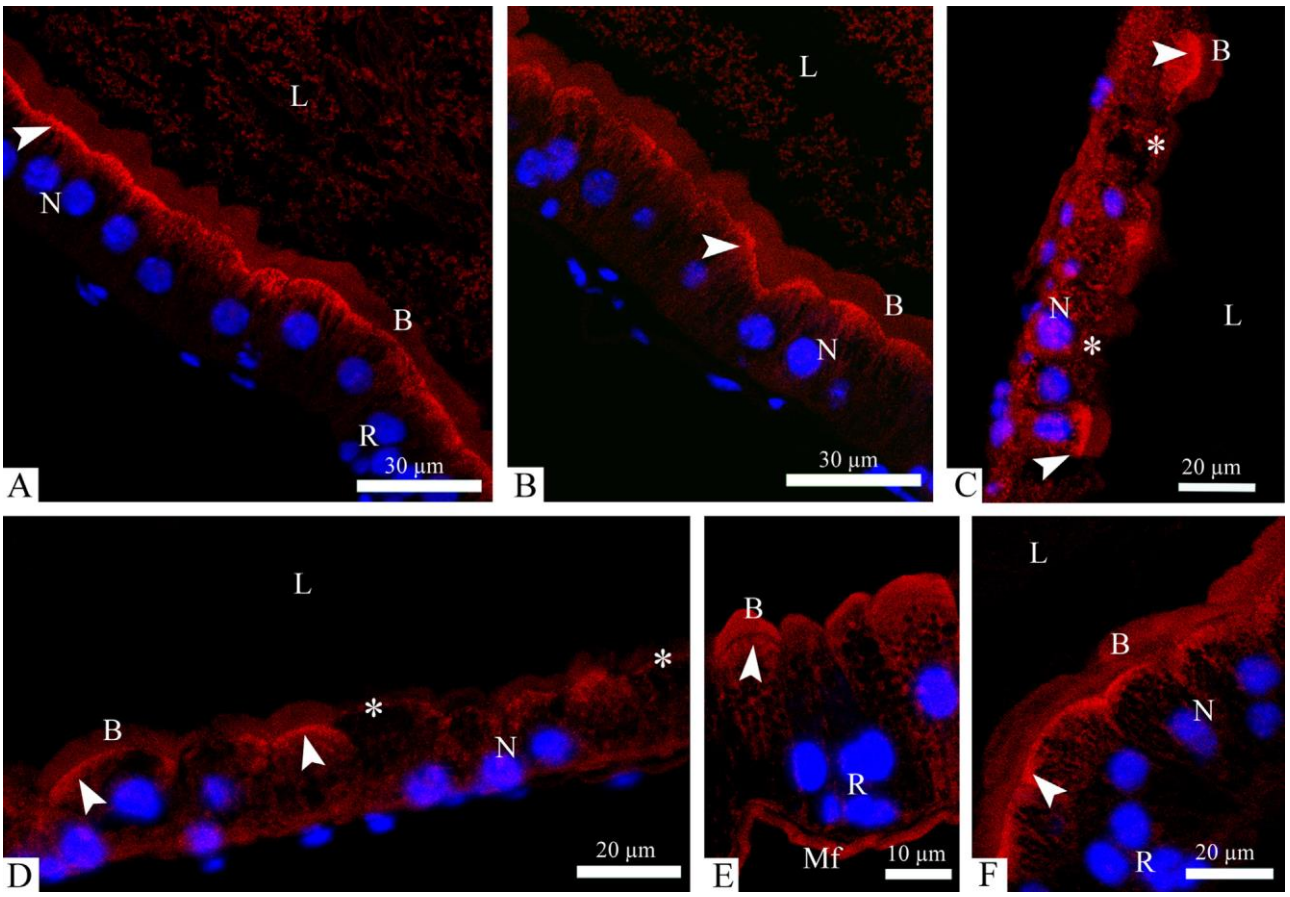

Fig. 3. Photomicrograph of the midgut of adult *C. claveri* females originating from control and neem oil-treated larvae, shown at the newly emerged stage and at the beginning of oviposition. (A–D) Freshly emerged. (A and B) Control group, anterior and posterior regions, respectively. The positive reaction to F-actin in the cellular cortex (►) is continuous and is located below the striated border (B). The nuclei of columnar cells (N) and regenerative cells (R) were labeled with DAPI. (C and D) Neem oil—1%, anterior region and neem oil—2%, posterior region, respectively. Note the regions of the cellular cortex with the absence of F-actin (*). (E and F) Oviposition. (E) Control group, anterior region. Positive reaction to the cellular cortex (►) and muscle fibers (Mf). (F) Neem oil—2%, posterior region. Note that the presence of the cellular cortex continues below the striated border (B). Lumen (L).

Teixeira et al. (2013), Scudeler et al. (2014), and Gigliolli et al. (2015).

Regarding the adults who experienced chronic exposure to neem oil throughout the larval stage, all of the evaluated concentrations induced morphological changes, which were not concentration dependent. A set of histological changes could be grouped because they occurred jointly, including cell swelling, emission of cytoplasmic protrusions and cell lysis. Cellular hypertrophy is one of the first cellular changes observed in cell injury due to the loss of control of the ionic balance and water inflow, causing dilation of cells (Bauer and Prankratz, 1992; Cheville, 1994, 2009), and this alteration has been commonly reported in treatments with plant-derived, toxins and insecticides (Reed and Majumdar, 1998; Rey et al., 1999; Scudeler and Santos, 2013; Almeida et al., 2014; Scudeler et al., 2014).

The loss of cytoskeletal integrity, particularly in the cell cortex, contributes to the formation and release of cytoplasmic protrusions. Anuradha et al. (2007) have reported that azadirachtin induces the depolymerization of actin filaments, which favor the formation of protrusions. By labelling the actin filaments with phalloidin, we noted the occurrence of spots in the cell cortex that were not positively stained for actin, which corroborates the observations of Anuradha et al. (2007). Nogueira et al. (1997) have reported a similar loss of actin microfilaments in midgut epithelial cells of *Rhodnius prolixus* following azadirachtin administration. Due to the dysfunction of the cellular cytoskeleton at these points, the hydrostatic pressure that is already altered in columnar cells cannot be adequately controlled by apical membrane tension, leading to the formation of protrusions and cell lysis (Barros et al., 2003; Charras and Paluch, 2008; Cheville, 2009).

In light of the histopathological symptoms described here, the columnar cells responded to these injuries by expanding the extracellular spaces of the basal labyrinth, which were percolated with lanthanum nitrate. According to Cruz-Landim et al. (2013), invaginations of the basal labyrinth serve to increase the cell's surface area to promote exchanges with the medium. The dilation observed in the columnar cells at both adult ages may indicate an attempt to increase the transport of excess fluid from the dilated cells to the hemocoel (De Lello et al., 1984; Bauer and Pankratz, 1992; Scudeler and Santos, 2013).

Using histologic and morphometric analyses, it was possible to observe changes in the morphology of the midgut, such as an increased epithelial height and the formation of epithelial folds. These changes may indicate epithelial reorganization due to acceleration of the cellular renovation process (Nogarol and Fontanetti, 2010). Exposure to neem oil resulted in increased cell proliferation for all treatments, particularly in the anterior region of the midgut. The increase in cell proliferation and the failure to induce cell death resulted in the reshaping of the epithelium with the creation of epithelial folds. Although at the start of oviposition there may be an increase in cell death in both regions of the midgut at all of the evaluated concentrations of neem oil, this cell death was not

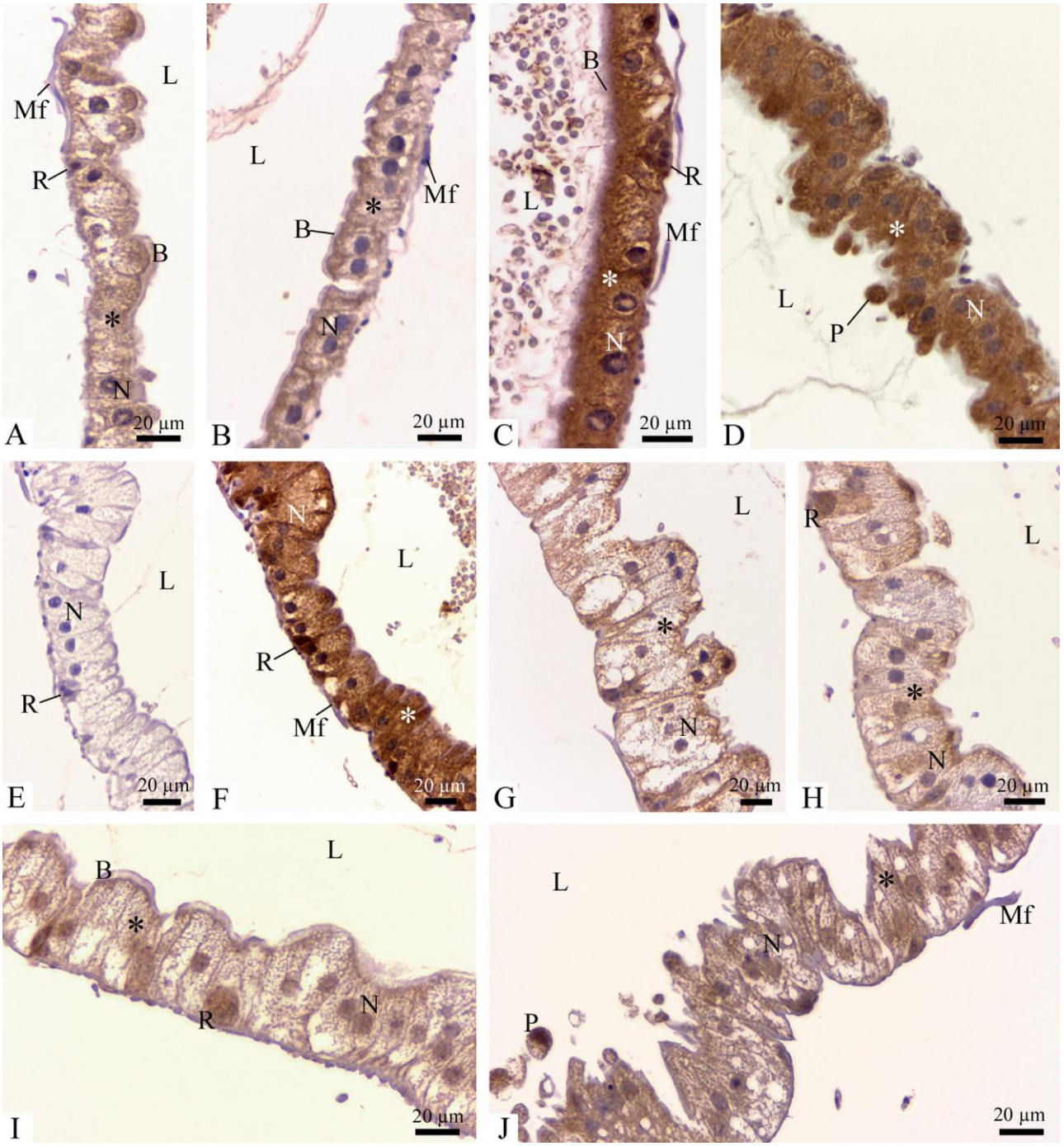

Fig. 4. Histological sections of the midgut of *C. claveri* were analyzed for cellular stress, represented by HSP 70 immunolocalization. (A–F) Freshly emerged. (A and B) Control group, anterior and posterior regions, respectively. Note the positive signals in the cytoplasm (*) and nucleus (N). (C, D, and F) Columnar and regenerative cells (R), with more intense staining in the cytoplasm. (C) Neem oil—0.5%, anterior region. (D) Neem oil—1%, posterior region. (E and F) Neem oil—2%, anterior region. (E) Negative control. (G–J) Oviposition in the control, 0.5, 1 and 2% neem oil groups, respectively. Anterior region of midgut. At this age, all groups showed the same immunoreactivity to HSP 70 in the cytoplasm and nuclei of columnar and regenerative cells. Lumen (L); cytoplasmic protrusion (P); muscle fiber (Mf); striated border (B).

sufficient to eliminate or avoid the presence of epithelial folds at this age because cellular proliferation remained significant in the anterior region of the midgut.

Despite the occurrence of severe cellular changes in the midgut of the freshly emerged adults, neem oil did not induce high levels of cell death. We performed TUNEL assay to identify the rates of epithelial cell death by the microscopic identification of individual cell nuclei that were positive for necrosis or apoptosis, achieved by the labeling of the 3´ ends of DNA strand breaks. Moreover, internucleosomal DNA fragmentation is widely used in studies of cell death (Higuchi, 2003; Fink and Cookson 2005; Mizuta et al., 2013).

In addition to the lack of cell death, we observed increased immunoreactivity for HSP 70 in the cytoplasm and nuclei of epithelial cells in all of the groups exposed to neem oil. Exposure to neem oil caused injury and therefore cellular stress, inducing the expression of HSP 70 and the inhibition of cell death. Significant cell death rates were detected in the treated adults only at the start of oviposition, a period during which we observed low levels

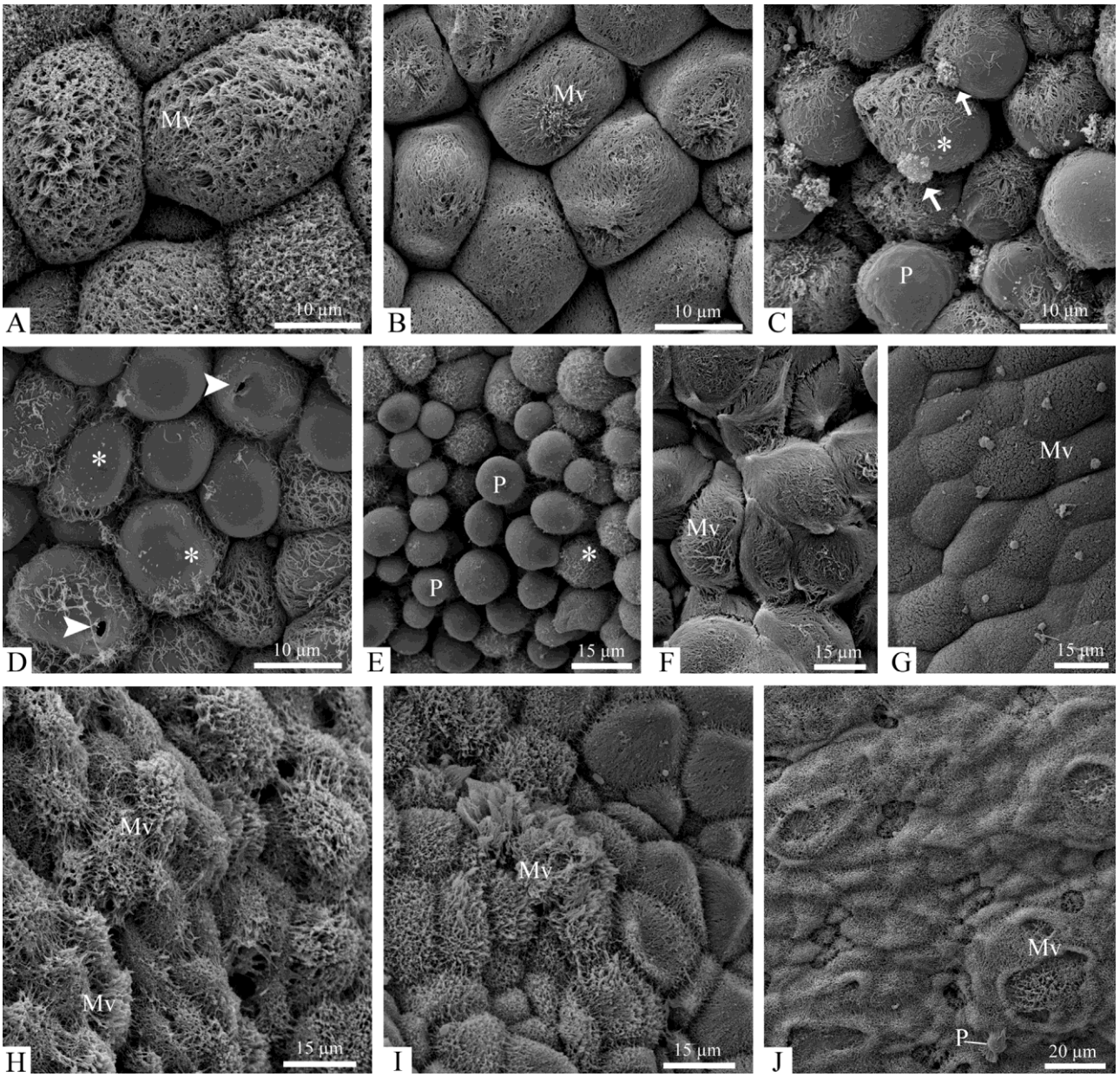

Fig. 5. SEM images of the midgut epithelial surfaces of adult *C. claveri* females. (A–E) Freshly emerged. (A and B) Control group, anterior and posterior regions, respectively. (A) Note the columnar cells with abundant and long microvilli (Mv). (B) The columnar cells were more closely juxtaposed and had smaller, abundant and regular microvilli. (C–E) Neem oil—0.5, 1 and 2%, respectively. (C) Anterior region. Details of the formation of cytoplasmic protrusions (arrow) and the occurrence of protrusions (P), followed by disorganization and a lack of microvilli (*) (D) Posterior region. Occurrence of columnar cells with apical membrane rupture (►) and fewer microvilli (*). (E) Posterior region. The cytoplasmic protrusions of columnar cells were present at the dilated apical surface. (F–J) Start of oviposition. (F and G) Control group, anterior and posterior region. The columnar cells were more closely juxtaposed and had abundant microvilli. (H–J) Neem oil—0.5, 1 and 2%, respectively. (H and I) Anterior and posterior region of the midgut, showing cells with disorganized microvilli. (J) Posterior region, columnar cells emitting cytoplasmic protrusions (P), as well as fewer and disordered microvilli at specific points of the epithelium.

of HSP 70 expression in epithelial cells. Based on the data presented, we can confirm the cytoprotective action of HSP 70 in the midgut of C. claveri only in freshly emerged adults. Heat shock proteins (HSPs) are a class of proteins used as cellular markers of cell stress or aggression, and their induction is correlated with resistance to stress induced by various factors, such as heat, pesticides, drugs and pollutants (Feder and Hofmann, 1999; Aït-Aïssa et al., 2000; Malaspina and Silva-Zacarin, 2006; Ferreira et al., 2013; Rozza et al., 2014). One of the cytoprotective effects of HSP 70 is an anti-apoptotic response (Beere, 2004; Silva-Zacarin et al., 2006; Rozza et al., 2014), which contributed to the lack of cell death in the treated groups. The reduced HSP 70 expression at the start of oviposition and, consequently, greater incidence of cell death may be related to the age of the adults. Benoit et al. (2011) have observed that HSP 70 expression decreases with age in Aedes aegypti females, impairing the repair mechanisms in epithelial cells of the midgut, suggesting that relationships may exist among the ability to synthesize HSP 70, aging and egg production.

The increase in cytoplasmic granules containing calcium, as evidenced by the von Kossa and ultrastructural cytochemical techniques, was another observed cytoprotective response that contributed to the low incidence of cell death. The increase in cytoplasmic granules indicates the activation of the detoxification process in these cells (Nogarol and Fontanetti, 2010; Souza and Fontanetti, 2011); furthermore, the observed increase of calcium prevents damage to the endoplasmic reticulum, including vesicu-

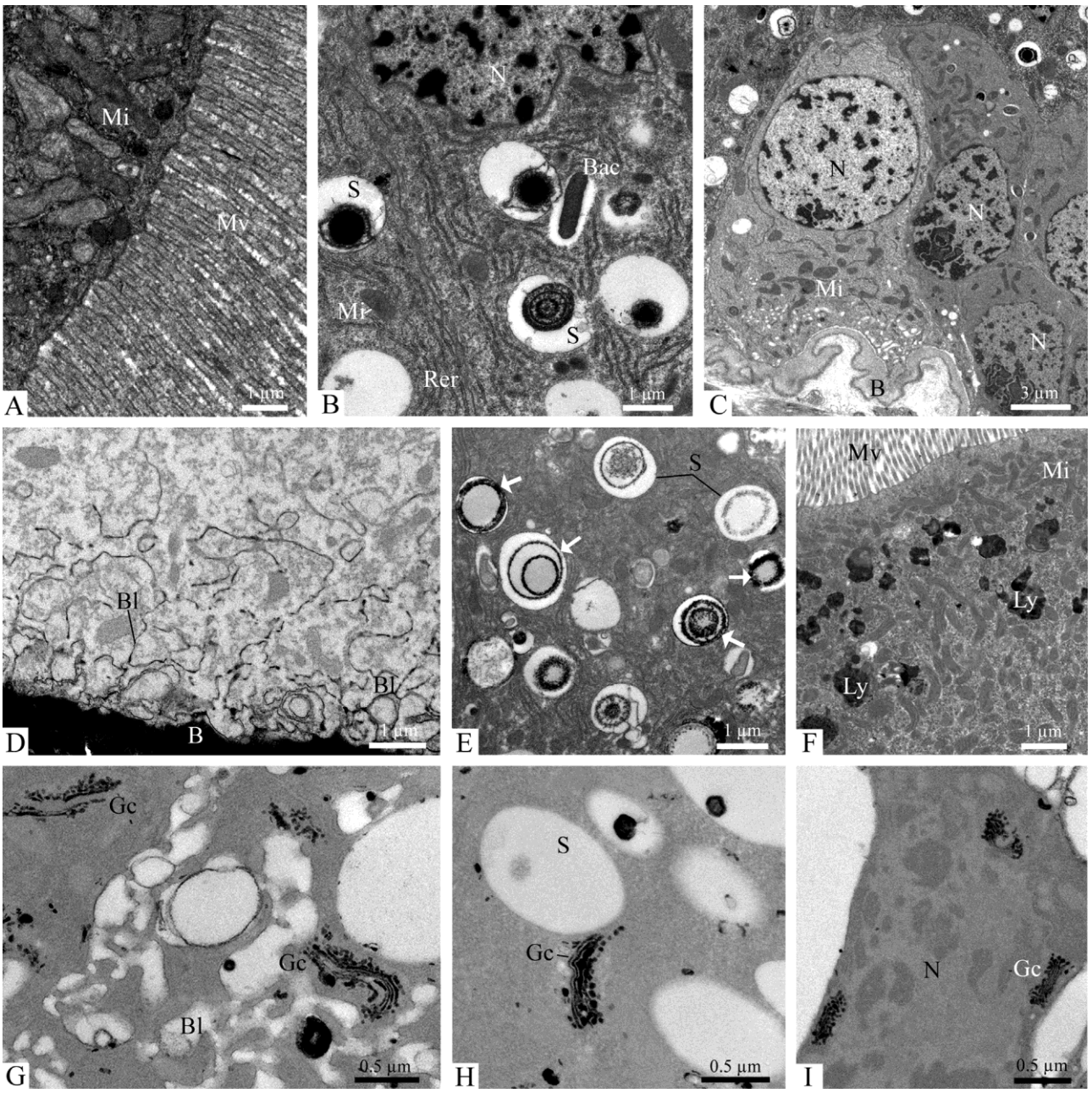

Fig. 6. TEM micrographs of the midgut epithelium (control) of newly emerged *C. claveri* adults. (A–E) Anterior region of the midgut. (A) Apical region of a columnar cell, showing long and well-developed microvilli (Mv) and many mitochondria (Mi). (B) Perinuclear region of a columnar cell, showing well-developed cisternae of the rough endoplasmic reticulum (Rer), spherites (S), mitochondria (Mi) and endosymbiotic bacteria (Bac). (C) Group of regenerative cells with granular cytoplasm, large nuclei (N) and many mitochondria (Mi). (D) The basal labyrinth (Bl), with a narrow extracellular space in the basal regions of columnar cells, was visualized by lanthanum nitrate percolation. (E) Positive calcium signals were observed using the calcium localization technique in concentric rings of the spherites (arrow). (F, G and I) Posterior region of the midgut. (F) Apical region of columnar cells, with numerous acid phosphatase-positive lysosomes (Ly) among the mitochondria. (G) Basal region of a columnar cell displaying prominent Golgi complexes (Gc) in the cytoplasm among the invaginations of the basal labyrinth (Bl), as detected using the ZIO technique. (H) Middle region of the cytoplasm of a columnar cell, with well-structured Golgi complexes among the spherites (S) in the anterior midgut region. (I) Regenerative cell with prominent and polarized Golgi complexes around the nucleus (N). Basement membrane (B).

lation and loss of its function, and also stimulates an autophagic response (Hoyer-Hansenet al., 2007; Cerella et al., 2010).

Although the midgut has different cytoprotective responses against neem oil-induced injuries, ultrastructural analysis showed that these responses were not completely effective because the ultrastructural changes were consistently observed in different types of midgut epithelial cells. The main ultrastructural lesions identified here included the microvilli, which were reduced in quantity and became sparse and deformed; swollen mitochondria in the cristolysis process; dilated cisternae of the rough endoplasmic reticulum; a Golgi complex with a vesiculated appearance; and dilation of the perinuclear space. These changes, combined with the observed histopathological symptoms, are similar to those described in studies using azadirachtin, neem oil, and other plant-derived toxins and insecticides (De Lello et al., 1984; Nasiruddin and Mordue (Luntz), 1993; Lü et al., 2010; Almehmadi, 2011; Ling and Zhang, 2011; Qi et al., 2011; Scudeler and Santos, 2013, 2014; Catae et al., 2014; Scudeler et al., 2014).

Nevertheless, possible attempts at detoxification or responses to cellular stress were observed at the ultrastructural level in the

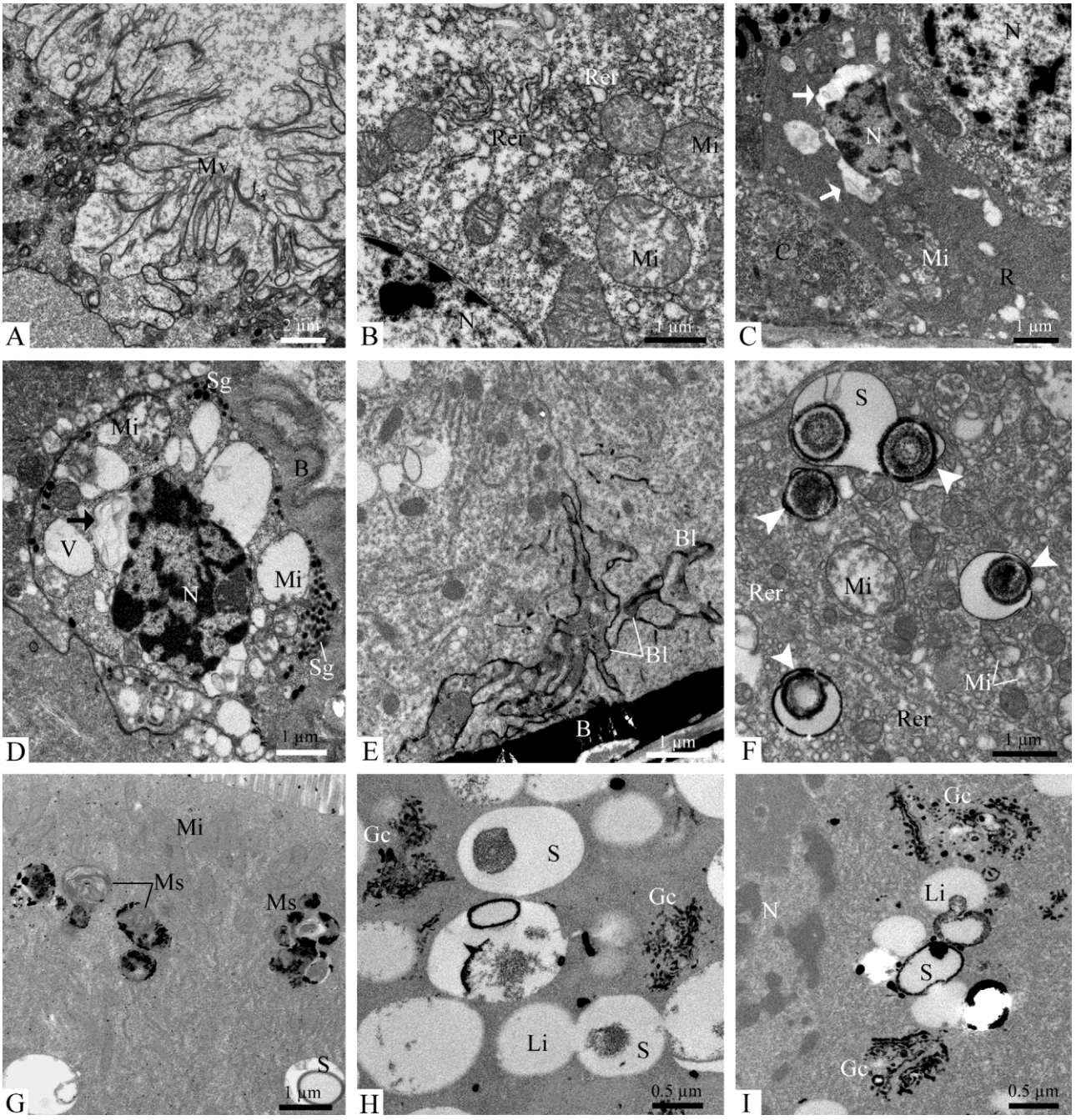

Fig. 7. Ultrastructural changes in the midgut epithelium of *C. claveri* adults that originated from neem oil-treated larvae. (A, B and D) Posterior region of the midgut. (A) Neem oil—0.5%. Sparse and deformed microvilli (Mv) in the columnar cells. (B) Neem oil—2%. Supranuclear cytoplasm of a columnar cell containing dilated cisternae of the rough endoplasmic reticulum (Rer), as well as swollen mitochondria (Mi) in the cristolysis process. (C) Anterior region. Neem oil—1%. Regenerative cell (R) with dilated perinuclear space (arrow) and mitochondria. (D) Neem oil—2%. Endocrine cells with a vacuolated cytoplasm (V) due to dilation and loss of mitochondrial cristae and dilation of the perinuclear space (arrow). (E, F and H) Posterior region. (E) Neem oil—0.5%. The basal labyrinth (Bl) had more dilated invaginations impregnated by lanthanum nitrate in the columnar cells. (F) Neem oil—2%. Spherites (S) showed thicker concentric rings that were positive for calcium (arrowhead) in the columnar cells. (G) Neem oil—1%. Positive reaction for acid phosphatase in myelin structures (Ms). (H and I) The ZIO method revealed morphological changes in the Golgi apparatus (Gc), where a few cisternae and abundant adjacent vesicles were observed in the cytoplasm of columnar cells. (H) Neem oil—1%. (I) Neem oil—2%, anterior region of the midgut. Basement membrane (B); lipid droplet (Li), secretory granules (Sg); nucleus (N).

cells, including the development of smooth endoplasmic reticulum (Cheville, 1994, 2009; Catae et al., 2014; Scudeler and Santos, 2013, 2014) and digestion of vacuoles containing myelin structures or heterogeneous membranous contents through the activation of autophagic processes (Kroemer et al., 2010; Murrow and Debnath, 2013). Acid phosphatase plays roles in detoxification processes via its lytic activity, and its level has been reported to be increased in response to cytotoxicity (Amirmohammadiet al., 2012; Valizadeh et al., 2013).

Despite the presence of all of these cellular mechanisms in response to cellular injuries, only regenerative cells present at the beginning of oviposition showed no signs of cellular damage. The commitment of other epithelial cells, such as columnar and endocrine cells, during the adult stage may significantly affect the

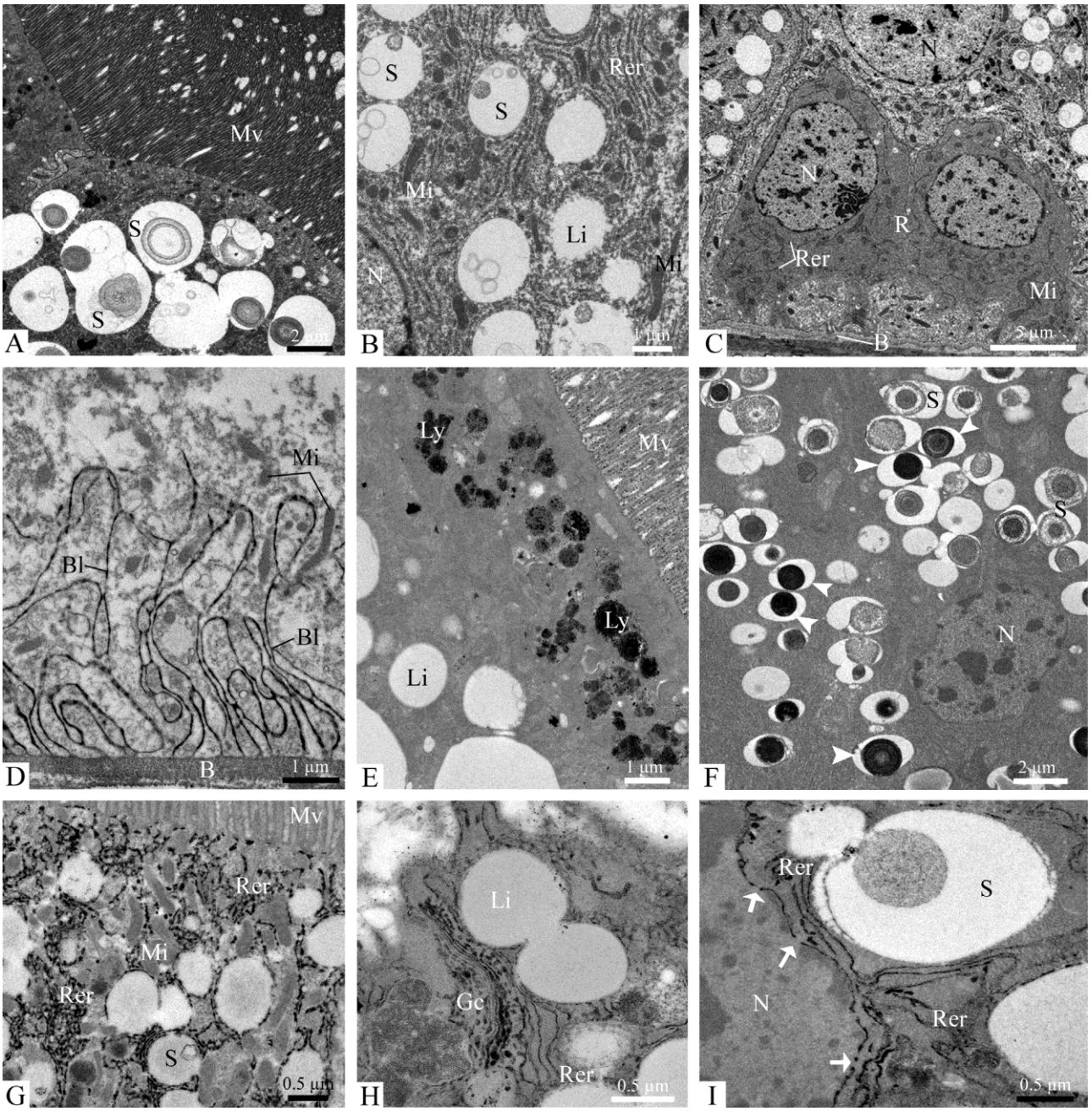

Fig. 8. Ultrastructure of midgut epithelial cells of adult *C. claveri* females from the control group at the beginning of oviposition. (A, E, G and I) Posterior region of the midgut. (B–D, F and H) Anterior region. (A) Apical region of a columnar cell, showing regular and abundant microvilli (Mv) at the apical surface and a predominance of spherites (S) in the cytoplasm. (B) Supranuclear cytoplasm of a columnar cell displaying a predominance of rough endoplasmic reticulum cisternae (Rer) and spherites. (C) Group of regenerative cells (R) displaying cytoplasm with abundant mitochondria (Mi) and rough endoplasmic reticulum cisternae, as well as free ribosomes. (D) Using the lanthanum technique, we demonstrated slender invaginations of the basal labyrinth (Bl) in columnar cells. (E) Lysosomes (Ly) were identified in the apical cytoplasm of the columnar cells by acid phosphatase staining. (F) With the calcium localization technique, we identified spherites (arrowhead) that were positive for calcium and those that were negative (S), which showed more concentric rings. (G–I) ZIO reaction. Note the impregnation of the cisterns of the endoplasmic reticulum, the polarized cisterns of the Golgi complex (Gc) and the perinuclear space of the nuclear envelope in the columnar cell. Lipid droplet (Li); basement membrane (B); nucleus (N); nuclear pores (arrow).

metabolic functions performed by the midgut, such as digestion and nutrient absorption. The loss of these functions may compromise other insect physiological functions; in addition, it may affect female development and reproduction because cellular lesions were detected in the midgut during the ripeness interval and reproductive development.

In conclusion, these results indicate that neem oil intake during the larval stage of *C. claveri* is toxic to insects in the adult stage by inducing cellular injuries in the midgut epithelium, and this damage can greatly affect midgut functions. These effects suggest that any of the tested concentrations of neem is safe for C. claveri. The epithelial cells responded to the injuries with various cytoprotective and detoxification mechanisms, but they were not sufficient to reverse all of the cellular damage in the midgut. This study adds relevant information to ecotoxicological studies by providing details of the injuries and cellular responses of a non-target organism to long-term indirect exposure to biopesticides. Sublethal effects must be considered and assessed at multiple stages of an organism's life cycle to be considered safe for the species.

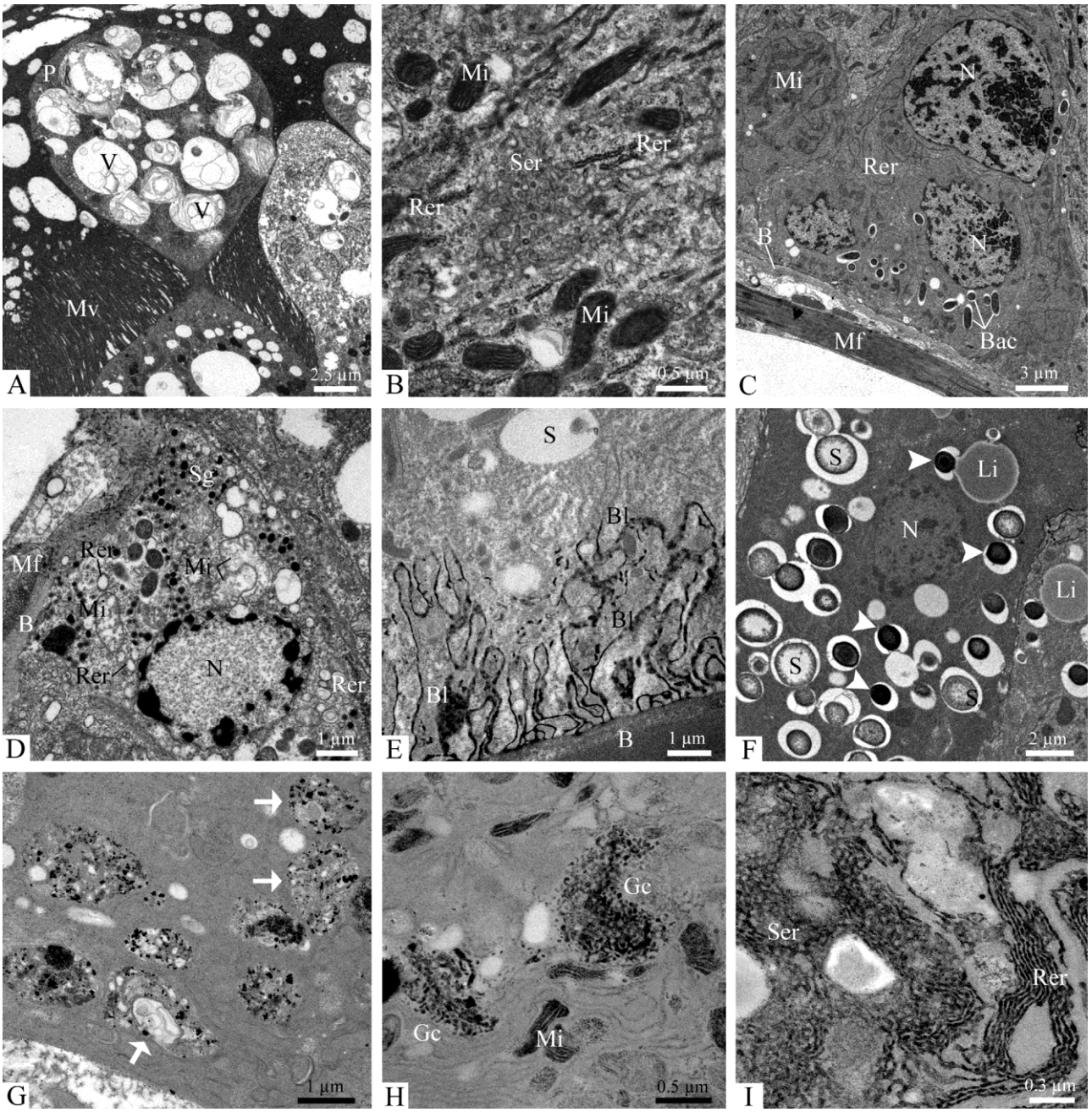

Fig. 9. Transmission electron micrographs of midgut epithelial cells of adult *C. claveri* females that originated from neem oil-treated larvae, sampled at the beginning of oviposition. (A, C, D and G) Posterior region of the midgut. (A) Apical surface of a columnar cell with cytoplasmic protrusions (P) filled with vacuoles (V) containing membranous structures. (B, E, F, H and I) Anterior region of the midgut. (B) Note the presence of tubules of smooth endoplasmic reticulum (Ser) in the cytoplasm of the columnar cell. (C) Group of regenerative cells with no signs of cellular injury. (D) An endocrine cell that still contains signs of cell injury, including swollen mitochondria (Mi) and the dilation of the rough endoplasmic reticulum cisternae (Rer). (E) Using the lanthanum nitrate technique, we found that the invaginations of the basal labyrinth (Bl) still contained large extracellular spaces in the columnar cells. (F) Calcium ions were observed in electron-dense spherites (arrowhead), among other spherites (S) with small concentric rings. (G) The detection of acid phosphatase indicated the presence of larger autolysosomes (arrow) in the apical region of the columnar cells and the digestion of membranous structures that originated from autophagic vacuoles. (H and I) ZIO technique. Note the morphology of the Golgi apparatus (Gc), with a vesiculated appearance, and the presence of branched tubules of the smooth endoplasmic reticulum (Ser) in the cytoplasm of the columnar cell. Muscle fiber (Mf), basement membrane (B); bacterial endosymbionts (Bac); nucleus (N); lipid droplet (Li); microvilli (Mv); secretory granules (Sg).

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