AVALIAÇÃO DA TOXICIDADE DO INSETICIDA NOVALURON EM *Bombyx mori* (LEPIDOPTERA: BOMBYCIDAE)

MARILUCIA SANTORUM

Tese apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para obtenção do título de Doutora no Programa de Pós-Graduação em Biotecnologia, Área de concentração *Biotecnologia aplicada à saúde humana e animal.*

Profa. Dra. Daniela Carvalho dos Santos

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PROFA. DRA. ROSE MEIRE COSTA

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Palavras-chave: Bicho-da-seda; Novaluron; sintomatologia; toxicidade; ultraestrutura.

Meu filho, não se esqueça da minha lei, mas guarde no coração os meus mandamentos,
pois eles prolongarão a sua vida por muitos anos e lhe darão prosperidade e paz.
Que o amor e a fidelidade jamais o abandonem; prenda-os ao redor do seu pescoço, escreva-os na tábua do seu coração.
Então você terá o favor de Deus e dos homens, e boa reputação.
Confie no Senhor de todo o seu coração e não se apoie em seu próprio entendimento;
reconheça o Senhor em todos os seus caminhos, e ele endireitará as suas veredas.
Não seja sábio aos seus próprios olhos; tema o Senhor e evite o mal.
Isso lhe dará saúde ao corpo e vigor aos ossos.
Honre o Senhor com todos os seus recursos e com os primeiros frutos de todas as suas plantações;
os seus celeiros ficarão plenamente cheios, e os seus barris transbordarão de vinho.
Meu filho, não despreze a disciplina do Senhor nem se magoe com a sua repreensão,
pois o Senhor disciplina a quem ama, assim como o pai faz ao filho de quem deseja o bem.
Como é feliz o homem que acha a sabedoria, o homem que obtém entendimento,
pois a sabedoria é mais proveitosa do que a prata e rende mais do que o ouro.
É mais preciosa do que rubis; nada do que você possa desejar se compara a ela.
Na mão direita, a sabedoria lhe garante vida longa; na mão esquerda, riquezas e honra.
Os caminhos da sabedoria são caminhos agradáveis, e todas as suas veredas são paz.

Provérbios 3:1-17

Dedico este trabalho...

Ao meu esposo Junior Cesar Luna,

Aos meus pais Emílio e Lúcia Santorum,

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A vocês, minha amada família que são o meu porto seguro, dedico a conquista deste sonho, que se torna realidade graças ao amor, confiança e apoio que sempre me deram, mesmo na distância, isso me fez e me faz ter força e coragem para continuar sempre seguindo em frente e acreditando que posso vencer!

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SUMÁRIO

RESUMO **6**

ABSTRACT **7**

1. INTRODUÇÃO **8**

2. OBJETIVOS **19**

3. REFERÊNCIAS **21**

4. RESULTADOS **29**

4.1. CAPÍTULO 1: Negative impact of Novaluron on the nontarget insect *Bombyx mori* (Lepidoptera: Bombycidae) **30**

4.2. CAPÍTULO 2: Novaluron impairs the silk gland and productive performance of silkworm *Bombyx mori* (Lepidoptera: Bombycidae) larvae **57**

4.3. CAPÍTULO 3: Reproductive toxicity of Novaluron in *Bombyx mori* (Lepidoptera: Bombycidae) and its impact on egg production **83**

5. CONCLUSÕES **105**

6. ANEXO **107**

RESUMO

O bicho-da-seda, *Bombyx mori* (Lepidoptera: Bombycidae), é o inseto de maior importância econômica na produção de seda. A lagarta se alimenta de folhas de amoreira e é altamente sensível a agrotóxicos, assim o uso destes em culturas agrícolas circunvizinhas às plantações de amoreira pode afetar o desenvolvimento de *B. mori*, acarretando em desequilíbrio nas suas funções metabólicas e, consequentemente, comprometendo a produção de casulos. Entre estes agrotóxicos, destaca-se o Novaluron, inseticida inibidor da síntese de quitina nos insetos e empregado no controle de insetos pragas de culturas agrícolas próximas as plantações de amoreira. Assim, investigamos os efeitos tóxicos de Novaluron no desenvolvimento de *B. mori*. Lagartas de *B. mori*, foram separadas em dois grupos experimentais: grupo controle (GC) e grupo tratamento (GT: tratado com 0, 15 mL/L de Novaluron). Após ecdise do 2° para o 3° instar, lagartas do GT foram alimentadas por 24 horas com folhas de amoreira tratadas com o inseticida. Paralelamente foi realizada uma nova exposição, porém em lagartas que realizavam a ecdise do 4° para o 5° instar. Lagartas, pupas e adultos de *B. mori* foram anestesiadas e segmentos do intestino médio, glândula da seda e órgãos reprodutores retirados e processados convencionalmente para técnicas de microscopias de luz, eletrônica e imunohistoquímica. Além disso, os efeitos no desenvolvimento, reprodução e qualidade do casulo também foram avaliados. O Novaluron provocou efeitos citotóxicos no intestino médio e na glândula da seda de lagartas de *B. mori*, bem como reprotoxicidade nos testículos e ovários de lagartas, pupas e adultos, através de pronunciadas alterações, como extrema rarefação citoplasmática e nuclear, dilatação do retículo endoplasmático, alteração mitocondrial, presença de vacúolos citoplasmáticos e dilatação celular com liberação de protusões citoplasmáticas, além de espaçamento entre células epiteliais e a lâmina basal e o desprendimento de algumas células para o lúmen. Em adição, destacamos vários sintomas de toxicidade de Novaluron em todas as fases do desenvolvimento de *B. mori*, tais como: grande taxa de mortalidade das lagartas, efeito negativo na qualidade do casulo produzido, redução no peso dos casulos construídos, construção de casulos defeituosos, além da significante redução no número de ovos ovipositados pelas fêmeas adultas de *B. mori* tratadas. Estes resultados nos mostram que Novaluron tem grande efeito tóxico em *B. mori*, afetando seu desenvolvimento, bem como a produção dos casulos da seda, o que pode causar sérios prejuízos à sericultura no Brasil.

Palavras-chave: Bicho-da-seda; ultraestrutura; Novaluron; toxicidade; sintomatologia.

ABSTRACT

The silkworm, *Bombyx mori* (Lepidoptera: Bombycidae is the insect of major economic importance in the production of silk. The larvae feeds on mulberry leaves and is highly sensitive to agrochemicals, thus the use of these in agricultural crops surrounding the mulberry plantations can affect the development of *B. mori*, causing an imbalance in its metabolic functions and, consequently, compromising the production of cocoons. Among these agrochemicals, stands out the Novaluron, an insecticide inhibitor of the synthesis of chitin in insects and used in the control of insect pests of crops near mulberry plantations. Thus, we investigated the lethal and sublethal effects of Novaluron on the development of *B. mori*. Larvae were selected into two experimental groups: control group (CG) and treatment group (TG: treated with 0, 15 mL/L Novaluron). After ecdysis from the 2nd to the 3rd instar, the TG larvae were fed for 24 hours with mulberry leaves treated with the insecticide. In parallel, a new exposition was carried out, however in larvae that carry out the ecdysis from the 4th to the 5th instar. *B. mori* larvae, pupae and adults were anesthetized and segments of the midgut, silk gland and reproductive organs were removed and processed conventionally for light microscopy, electron microscopy and immunohistochemistry. In addition, the effects on the development, reproduction and quality of the cocoon were also evaluated. Novaluron caused cytotoxic effects on the midgut and the silk gland of *B. mori* larvae, as well as reprotoxicity on the testes and ovaries of larvae, pupae and adults, through pronounced alterations such as extreme cytoplasmic and nuclear rarefaction, endoplasmic reticulum dilatation, mitochondrial alteration, presence of cytoplasmic vacuoles and cell dilatation with release of cytoplasmic protrusions, as well as spacing between epithelial cells and the basal lamina and the detachment of some cells into the lumen. In addition, we highlight several symptoms of Novaluron toxicity in all stages of the development of *B. mori*, such as: great mortality of larvae, negative effect on the quality of the cocoon produced, reduction in the weight of the cocoons constructed, construction of defective cocoons, besides the significant reduction in the number of eggs deposited by adult *B. mori* females treated. These results show that Novaluron has a great toxic effect on *B. mori*, affecting its development, as well as the production of the silk cocoons, which can cause serious damages to sericulture in Brazil.

Keywords: silkworm; ultrastructure; Novaluron; toxicity; symptomatology.

1. INTRODUÇÃO

A sericicultura é uma atividade agroindustrial, e sua cadeia produtiva abrange o cultivo da amoreira (*Morus* spp.), a produção e o preparo dos ovos do bicho-da-seda, *Bombyx mori* (Lepidoptera: Bombycidae), criação das lagartas, obtenção dos casulos, industrialização dos fios de seda, tecelagem e o comércio da seda (WATANABE; YAMAOKA; BARONI, 2000), sendo a espécie *B. mori*, responsável por 95% da produção comercial total de fios de seda (WATANABE; YAMAOKA; BARONI, 2000; ZANETTI, 2007). Este é um inseto holometábolo, ou seja, com metamorfose completa, o qual apresenta quatro estágios de desenvolvimento durante seu ciclo de vida. O ovo é o primeiro estágio, de onde eclode a lagarta ou larva (segundo estágio), segue-se o estágio de pupa ou crisálida, que por metamorfose transforma-se em mariposa, forma adulta e último estágio do seu ciclo de vida, na qual se dedica exclusivamente à reprodução. O seu ciclo completo finaliza em torno de 35 a 40 dias (Figura 1) (ARUGA, 1994).

A principal fase no ciclo de vida de *B. mori* para a sericicultura, é a fase de lagarta até o início da construção dos casulos de seda, nesta fase *B. mori* alimenta-se exclusivamente de folhas de amoreira e passa por cinco instares ou estádios larvais (1º, 2º, 3º, 4º e 5º). Na passagem de um instar ao outro, o inseto sofre a ecdise ou a muda do exoesqueleto e, assim, passa por quatro ecdises. No final do 5° instar, o inseto cessa a alimentação e começa a tecer o casulo, é no seu interior que realiza a metamorfose transformando-se em pupa. Neste processo, ocorre a morte celular programada ou apoptose em vários tecidos e órgãos, como no intestino, o epitélio larval é reabsorvido, sendo substituído por um epitélio pupal (HAKIM; BALDWIN; SMAGGHE, 2010). Ao término da metamorfose, cerca de 10 a 12 dias, o inseto adulto (mariposa) emerge do casulo (Figura 1) (ARUGA, 1994; FERNANDEZ et al., 2005; HANADA; WATANABE, 1986).

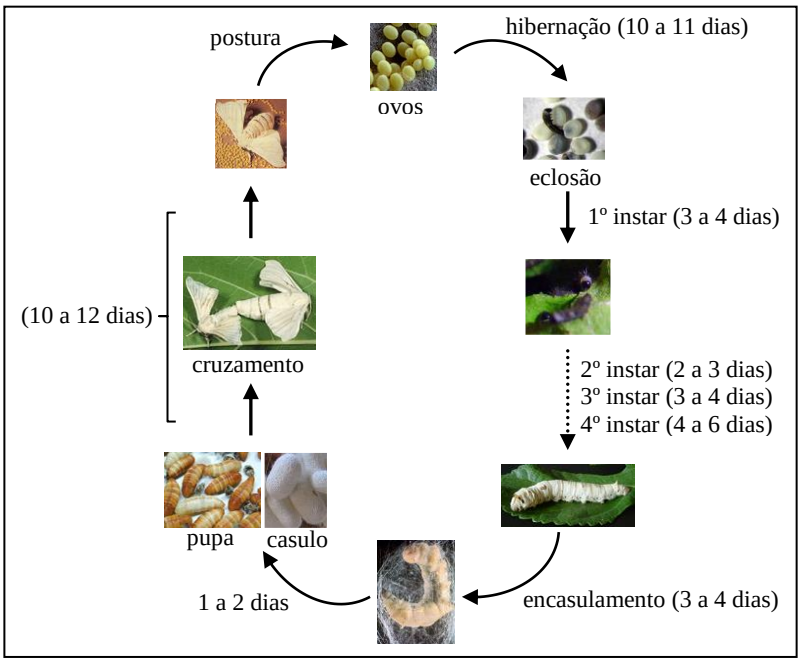

Figura 1. Ciclo de vida de *Bombyx mori* (TAKEDA, 2009).

A sericicultura ou produção de seda é desenvolvida em vários países, movimentando bilhões de dólares por ano no mundo. Neste cenário o Brasil, em 2016, segundo dados do IBGE, produziu 2,8 mil toneladas de casulos de bicho-da-seda, ficando em quinto lugar na produção mundial, atrás da China, Índia, Uzbequistão e Tailândia. A sericicultura nacional é bem desenvolvida no estado do Paraná, o qual tem se destacado nos últimos 10 anos como o maior produtor nacional, responsável por 83%, seguido por São Paulo (13%) e Mato Grosso do Sul (5%) (SEAB, 2017).

No Brasil, esta atividade tem se desenvolvido em pequenas propriedades rurais e se utiliza da mão de obra familiar, proporcionando aos produtores um meio de subsistência. Outro aspecto importante da sericicultura é o baixo impacto ao meio ambiente, uma vez que não é aconselhável o uso de agrotóxicos nas propriedades produtoras, caso contrário as lagartas de *B. mori*, que são muito sensíveis as adversidades do meio, podem morrer intoxicadas (BRANCALHÃO, 2002; DOURADO et al., 2011; PANUCCI-FILHO; CHIAU; PACHECO, 2011; TAKAHASHI, R.; TAKAHASHI, K.; TAKAHASHI, L., 2001).

A atividade sericícola apresenta importância econômica e social e, como relatam Panucci-filho; Chiau; Pacheco (2011) tem influenciado na permanência de famílias nas pequenas propriedades rurais e, com isso, contribuindo diretamente na diminuição da pobreza, minimizando desigualdades sociais, permitindo e assegurando o contato com a

“terra” e a aproximação do ser humano em atividades interdisciplinares de inclusão na sociedade. Neste sentido, a sericicultura é uma atividade sustentável para o país, ao gerar ganhos econômicos, diminuir o êxodo rural e apresentar aspectos ecológicos favoráveis. Entretanto como relata Tsukamoto (2009), esta atividade requer algumas melhorias na produção e nas condições de trabalho, pois exige uma grande jornada diária, principalmente na colheita dos ramos de amoreira, pois é uma das atividades que requer maior esforço, devendo ser feita mesmo em dias chuvosos, dificultando o trabalho no campo, uma vez que as lagartas precisam se alimentar de 3 a 4 vezes ao dia.

Aliado a isso ocorrem outros fatores que podem influenciar a cadeia produtiva da seda, como: baixo valor agregado ao produto; a espera por resultados de pesquisas que envolvam melhoramento genético de *B. mori*; manejo adequado da criação; limpeza e desinfecção do ambiente de criação das lagartas, no objetivo de reduzir a ocorrência de doenças, que podem ser causadas por protozoários, fungos, bactérias e vírus, bem como a deriva de resíduos advindos do uso de agrotóxicos, em culturas circunvizinhas as propriedades produtoras de *B. mori* (BRANCALHÃO, 2002; MARGATHO et al., 2012).

Além da sua grande importância econômica no agronegócio, *B. mori* se destaca também como inseto modelo em pesquisas entre os lepidópteros, sido amplamente utilizado como organismo referência em biologia celular e molecular, fisiologia, biotecnologia, medicina, toxicologia e outros campos (MATSUMOTO et al., 2011; TANSIL et al., 2011; KUNDU et al., 2014; QIN et al., 2012; MAO et al., 2019).

No entanto, o uso de agrotóxicos em culturas agrícolas circunvizinhas as plantações de amoreira, podem afetar a produção de *B. mori*; durante a pulverização aérea, os resíduos destes compostos podem se fixar nas folhas de amoreira, que são a principal fonte de alimento das lagartas, levando assim, à contaminação do inseto. Isso causa desequilíbrio nas funções metabólicas das lagartas, comprometendo seu crescimento e reprodução, incluindo a qualidade do casulo, eclosão e fecundidade (BHOSALE E KALLAPUR, 1985; YIN et al., 2008; KUMUTHA et al., 2013; MUNHOZ et al., 2013; YU et al., 2013; GU et al., 2014; NICODEMO et al., 2014, 2018).

Estudos recentes da Secretaria de Agricultura do Estado do Paraná, Brasil, apontam as grandes percas na sericultura através da deriva de resíduos de inseticidas em culturas agrícolas próximas as propriedades produtoras do bicho-da-seda. No oeste paulista, ocorreu a perca quase total da produção de seda, e a mesma situação foi relatada no Paraná, onde produtores das lagartas de *B. mori* apontaram que a pulverização área de inseticidas

em canaviais contaminou as plantações de amoreira, acarretando em casulos de baixa qualidade, com grande parte da produção perdida, pois suas lagartas não conseguiam completar o ciclo e o casulo ficava transparente, com prejuízo na safra de mais de R$ 200 mil (GLOBO RURAL, 2011; MUNHOZ et al., 2013).

Yin et al. (2008), descreveram o impacto negativo de pesticidas sobre o bicho-da-seda, resultando em drástica redução na produção de seda. Os autores verificaram que a exposição, mesmo com baixas doses ao herbicida Clodinafop-propargyl, houve aumento significativo da porcentagem de dano ao DNA de hemócitos de lagartas de *B. mori*. Além disso, a exposição ao pesticida produziu genotoxicidade em lagartas e a porcentagem de pupação e formação de casulos foi significativamente reduzida nas lagartas tratadas.

Munhoz et al. (2013) relataram a mortalidade de lagartas de *B. mori* localizadas nas proximidades da cultura da cana-de-açúcar e que, segundo registros da Secretaria da Agricultura do Estado do Paraná, houve uma perda de 8.626 kg de casulos no estado. Estas mortes foram atribuídas aos diversos tipos de inseticidas utilizados no controle da Broca-da-cana, *Diatraea saccharalis* (Lepidoptera: Crambidae), como o Chlorantraniliprole. Os autores relataram que o uso desse inseticida acarretou em *B. mori*, ecdise incompleta, casulos defeituosos e comprometimento das funções orgânicas do inseto, observando-se alterações no intestino médio larvário, com células epiteliais colunares liberando corpos apoptóticos, os quais hipotetizaram que a ocorrência de células mortas por apoptose em células do intestino médio comprometeu o desenvolvimento do bicho da seda, bem como a produção de casulos (MUNHOZ et al., 2013).

Alterações no intestino médio requerem muita atenção, uma vez que é o principal órgão responsável pelos principais eventos de digestão e absorção, e seus tipos celulares desempenham funções importantes no organismo. Células colunares, tipo mais abundante, atuam na secreção de enzimas digestivas, bem como, na absorção de nutrientes (CASARTELLI et al., 2001); as caliciformes estão envolvidas no controle da homeostase iônica (WIECZOREK et al., 2000); as regenerativas são indiferenciadas, atuando na renovação do epitélio e substituindo células que danificam ou envelhecem, além de garantir o crescimento do canal alimentar a cada ecdise (CHAPMAN, 1998) e as células endócrinas secretam diferentes hormônios que desempenham um papel na diferenciação de células regenerativas e auxiliam na secreção de enzimas digestivas (WIGGLESWORTH, 1972). Assim, sendo diretamente afetado pelo inseticida durante a alimentação, o intestino médio é um órgão-chave para estudos sobre os efeitos tóxicos dos agrotóxicos nos insetos

(CATAE et al., 2014), uma vez que alterações morfológicas, ultraestruturais e moleculares neste órgão podem comprometer as funções orgânicas do inseto prejudicando seu desenvolvimento com consequências na produção de seda como relatado na literatura.

Outro órgão a ser considerado em estudos toxicológicos de inseticidas e seu impacto na produção de seda, é a glândula da seda (GS), por ser um órgão altamente especializado, responsável por sintetizar e secretar as proteínas da seda, principalmente a fibroína e sericina, que compõem a maior parte da seda e do casulo, sendo deste modo essenciais na sericultura pelo seu alto valor comercial (AKAI, 1984; LI et al., 2014; MONDAL; TRIVEDY; KUMAR, 2007; LIU; ZHANG, 2014).

A GS é uma estrutura par, simétrica e translúcida, com forma tubular, que se estende latero-ventralmente ao canal alimentar, iniciando em um ducto comum no segmento labial alcançando a região caudal. Em *B. mori* este órgão se apresenta ainda muito rudimentar do 1º ao 4º instar larval, produzindo pequena quantidade de seda (DHAWAN; GOPINATHAN, 2003; SEHNAL; AKAI, 1990; TANAKA, 1911), que é secretada no final de cada instar e utilizada para fixar o tegumento a ser descartado após cada muda para o substrato. Porém no início do 5º instar, ocorre a hipertrofia da glândula, com aumento do volume celular, alta biossíntese e secreção de seda, assim o crescimento e o peso da glândula é desencadeado rapidamente, sendo seu peso estimado entre 20% e 40% do peso total do inseto (DHAWAN; GOPINATHAN, 2003; SEHNAL; AKAI, 1990; TASHIRO et al., 1968).

A GS também é comumente conhecida como glândula labial e, devido a diferenças morfológicas e funcionais no decorrer de seu comprimento, é dividida em três regiões: a glândula da seda anterior (GSA), constituída por cerca de 200 células, e tem função de ducto excretor, ou seja, um ducto que realiza a saída da seda para o exterior; a glândula da seda média (GSM), secreta três tipos de sericina ao redor da coluna de fibroína, formada por aproximadamente 300 células secretoras, com cerca de 7 cm de comprimento, podendo ser ainda subdividida em quatro áreas distintas: anterior, anterior-médio, posterior-médio e posterior, devido a diferenças na densidade e morfologia do material contidos em vesículas secretoras, cada área sintetiza um tipo diferente de sericina no lúmen; e a glândula da seda posterior (GSP) que possui cerca de 15 cm de comprimento e aproximadamente 500 células secretoras que realizam a secreção da fibroína e proteínas adicionais (AKAI, 1983; DHAWAN; GOPINATHAN, 2003; MONDAL; TRIVEDY; KUMAR, 2007).

É das folhas de amoreira que *B. mori* obtém os nutrientes para o seu crescimento e para o desenvolvimento de suas funções metabólicas essenciais que permitem a síntese de proteínas, incluindo as proteínas da seda, por isso alterações no seu alimento podem determinar a quantidade e qualidade dos fios de seda produzidos pelas lagartas (TAKANO; ARAI, 1978; PARRA, 1991; KI; PARK, 2013), deste modo a qualidade das folhas de amoreira podem ser comprometidas se estas estiverem contaminadas por inseticidas via deriva de pulverizações (BORA et al., 2012; MUNHOZ et al., 2013). Li et al. (2013, 2014) associam a redução na produção de casulos por *B. mori* ser resultado da exposição a inseticidas. Os autores relataram a redução na produção de casulos de lagartas expostas ao inseticida Phoxin, e acreditam que esta redução pode estar relacionada a toxicidade do inseticida na GS, uma vez que os autores verificaram alterações epiteliais da GS e nos perfis de expressão gênica, redução na síntese de proteínas totais de seda e atribuíram este fato ao comprometimento do tecido da glândula e as expressões de baixos níveis de alguns genes envolvidos na síntese de proteínas da seda.

Em adição, estudos anteriores também tem apontado para os efeitos tóxicos de inseticidas sobre as funções reprodutivas de *B. mori*, prejudicando a oviposição, viabilidade de ovos e a eclosão larval (KURIBAYASHI, 1981; TANG et al., 2018), ponto este importante a ser considerado, uma vez que os órgãos reprodutores e os processos por eles desenvolvidos na reprodução dos insetos são de extrema importância para a manutenção das espécies. No entanto, poucos são os relatos sobre a reprotoxicidade de agrotóxicos em nível celular, assim como o mecanismo de ação do agrotóxico.

Tang et al. (2018), reportam alterações celulares nos espermatócitos de *B. mori* exposto a fluoreto de sódio, utilizado na composição de inseticidas, os autores verificaram ainda características de apoptose e necrose. Em adição, fluoreto de sódio acarretou em fêmeas de *B. mori*, diminuição no número total de ovos, sugerindo alterações nos ovários do inseto. A exposição de lagartas de *B. mori* a doses subletais de pesticidas organofosforados, resultou em danos a reprodução do inseto, com formação de ovos anormais, diminuição no número de ovos totais, grande número de ovos não fertilizados, morte dos embriões em estágio precoce de desenvolvimento, e morte de indivíduos recém emergidos (KURIBAYASHI, 1981). Segundo autores a exposição a químicos induz a esterilidade de insetos, podendo ser relacionado a um dos três fatores, supressão na produção das células envolvidas na espermatogênese ou na oogênese, ocorrência de alterações nestas células após sua

formação ou a inibição de genes na célula sem promover a morte desta (BORKOVEL, 1964; KURIBAYASHI, 1981; THANGARAJ et al., 2018).

Em *B. mori* o sistema reprodutor é formado por um par de gônadas, localizado no lado dorsal do quinto segmento abdominal, sendo após o 3° instar larval fácil a distinção entre testículos e ovários. Os testículos apresentam formato riniforme (SAKAGUCHI, 1978B, SAKAI et al., 2016), e são compostos por células da linhagem germinativa, as células espermáticas, envoltas por células somáticas, as células císticas. Cada testículo encontra-se circundado por uma túnica celular (interna e externa), que emite septos que dividem o testículo em quatro folículos testiculares, estes folículos estão fundidos e fixados em uma estrutura comum, o ducto deferente localizado na extremidade proximal. Na extremidade distal localiza-se o germário, região de proliferação celular, onde ocorre a produção contínua de espermatogônias, e na extremidade proximal estão localizados os cistos de espermatócitos (grupos de células germinativas interconectadas por pontes citoplasmáticas). O crescimento é gradual dos testículos e dos cistos germinativos no decorrer dos estágios de seu ciclo de vida, ocorrendo o processo de maturação do cisto durante a espermatogênese no sentido distal-proximal no interior dos testículos, com cistos germinativos em diferentes estágios de desenvolvimento (SAKAGUCHI, 1978A; SAKAI et al., 2016).

Já os ovários de *B. mori* possuem uma forma triangular e são formados por quatro ovaríolos, que na fase larval ainda são curtos e se tornam mais longos gradativamente, assumindo uma forma tubular nas fases de pupa e adulto (SAKAGUCHI, 1978A; TAKAMI, 1969; SAKAI et al., 2016). Cada ovaríolo é revestido por uma camada acelular contínua, a túnica própria, e mais externamente encontra-se a bainha ovariolar. O ovaríolo é do tipo meroístico politrófico, ocorrendo no seu interior a diferenciação morfofuncional das células germinativas e somáticas, originando os folículos ovarianos. Cada ovaríolo apresenta internamente uma região conhecida por germário, e é nela que as células germinativas se dividem dando origem aos cistos, que juntamente com as células somáticas, diferenciam-se nos folículos ovarianos. Logo abaixo do germário encontra-se a região do vitelário, onde estão presentes células germinativas diferenciadas: um oócito e sete células nutridoras; que são cercadas por uma camada de células somáticas chamadas de células foliculares que formam o epitélio folicular, e então, é nesta região que os folículos ovarianos crescem e suas células sofrem modificações necessárias dando origem ao ovo, ovulado na porção proximal do ovaríolo, seguindo para o oviduto lateral, ducto de

união dos quatro ovaríolos. Em adultos encontra-se aproximadamente 50 a 90 ovos maduros por ovaríolo (HASEGAWA, 1943; TAKAMI, 1969).

Como vimos, lagartas de *B. mori* são sensíveis a agroquímicos e o contato com esses produtos pode comprometer órgãos do inseto e seu desenvolvimento normal, impactando todas as fases do seu ciclo de vida (BORKOVEL, 1964; KURIBAYASHI, 1981; LI et al., 2010, 2011; PENG et al., 2011; YU et al., 2011, 2013; MUNHOZ et al., 2013; GU et al., 2014; KORDY, 2014; NICODEMO et al., 2018; THANGARAJ et al., 2018). E embora os agrotóxicos não sejam utilizados diretamente nas plantações de amoreira, diversos são os inseticidas empregados em culturas agrícolas circunvizinhas as propriedades de produção do bicho-da-seda no intuito de controlar os diversas insetos pragas destas culturas, e assim, através das pulverizações podem atingir as plantações de amoreira e acarretar danos ao inseto não-alvo e de grande importância econômica como *B. mori.*

Entre estes inseticidas destaca-se o Novaluron, (RS)-1-[3-cloro-4-(1,1,2-trifluoro-2-trifluorometoxi-etoxi)fenil]-3-(2,6-difluorobenzoil) uréia, (formulação comercial Rimon Supra - ADAMA Makhteshim Ltd.), registrado no Brasil pelo Ministério da Agricultura, Pecuária e Abastecimento (MAPA) sob nº 14511. Este inseticida é utilizado em culturas agrícolas como milho, soja, feijão, trigo, cana-de-açúcar, batata e tomate, entre diversas outras (MAPA, 2015). Atua como potente supressor de insetos pragas importantes, principalmente das ordens Lepidóptera e Coleóptera, além de algumas espécies Hemípteras e Dípteras.

Novaluron atua como regulador de crescimento de insetos, com modo de ação por contato e ingestão. Pertence ao grupo químico Benzoiluréia, os quais alteram a composição da cutícula dos insetos, especialmente através da inibição de processos bioquímicos que levam à formação da quitina sintetase, causando um depósito anormal da endocutícula que afeta a elasticidade cuticular e firmeza, prejudicando a muda do inseto. Assim, atua principalmente nos estágios larvais, levando a morte (CUTLER; SCOTT-DUPREE, 2007), e de acordo com Retnakaran e Wright (1987) age como ovicida e larvicida, porém têm sido relatados também os seus efeitos sobre a fecundidade, fertilidade e longevidade de insetos adultos (MARCO; PEREZ-FARINOS; CASTANERA, 1998).

Estudos tem sido realizados sobre os efeitos do uso do inseticida Novaluron em importantes insetos da ordem lepidóptera e considerados pragas da agricultura. Como por Storch et al. (2007) com a *Anticarsia gemmatalis* (Lepidoptera:Noctuidae) popular lagarta-da-soja, os quais apontaram os efeitos deletérios do inseticida durante o desenvolvimento

de todas as fases deste inseto. Já em estudo com a lagarta-do-cartucho, *Spodoptera frugiperda* (Lepidoptera:Noctuidae) uma importante praga da cultura do milho, houve redução da fecundidade, afetando o número de espermatóforos encontrados nas fêmeas, sugerindo efeitos no aparelho reprodutor do inseto (ROMANO, 2007).

A literatura aponta os efeitos do Novaluron em outras ordens de insetos pragas. Alyokhin e Choban (2010) verificaram que o inseticida causa mortalidade nos estágios larvais e sugerem que possui propriedades ovicidas, podendo apresentar efeito transovariano nas fêmeas do besouro da batateira (Coleoptera:Chrysomelidae) e reduzir a eclosão de suas larvas. Estudo de Alyokhin, Guillemette e Choban (2009) também ressaltam o efeito de Novaluron na redução da fertilidade dos adultos desta mesma praga da batata. Novaluron afetou negativamente a reprodução da mosca-branca (Diptera: Aleyrodidae), praga do tomateiro, reduzindo a viabilidade de seus ovos (CLOYD; KEITH; GALLE, 2004) .

Novaluron tem sido considerado aceitável para inclusão nos programas de manejo integrado de pragas (MIP), porque a classe Benzoiluréia inicialmente parecia ser mais segura e mais seletiva em seu modo de ação, potencialmente atuando apenas contra as espécies-alvo com riscos reduzidos ao meio ambiente, incluindo mamíferos, aves, animais aquáticos e insetos benéficos e não-alvo (TUNAZ; UYGUN, 2004; DHADIALLA et al., 2005). No entanto, os danos causados por inseticidas ao meio ambiente e para organismos não-alvo ainda precisa ser minimizado (DESNEUX et al., 2007), uma vez que alguns estudos apontaram para efeitos negativos de Novaluron em insetos benéficos não-alvo, incluindo predadores, parasitóides e polinizadores. Nestes organismos não-alvo, Novaluron pode reduzir a eclosão e o desenvolvimento das lagartas, via contato e ingestão. Além disso, Novaluron pode prejudicar a muda, oviposição e viabilidade de ovos de adultos tratados, bem como reduzir sua vida útil (MOMMAERTS et al., 2006; CUTLER; SCOTT-DUPREE, 2007).

Como exposto por Cutler et al. (2006) em estudo com o predador *Podisus maculiventris* (Heteroptera: Pentatomidae), um inimigo natural do besouro da batateira, verificando que insetos provenientes de ovos tratados com o Novaluron foram capazes de emergir, porém os recém-nascidos foram posteriormente incapazes de fazer a muda e as fêmeas adultas tratadas apresentaram redução na longevidade, além de redução significativa na oviposição e viabilidade dos ovos. Mommaerts, Sterk e Smagghe (2006) também observaram efeitos negativos de Novaluron em outro inseto benéfico o *Bombus*

terrestris L. (Hymenoptera), um dos mais importantes polinizadores em cultura como a do tomate. Neste caso, Novaluron reduziu a eclosão das larvas e afetou o desenvolvimento larval via contato e ingestão.

Entretanto, a literatura não traz informações sobre a toxicidade de Novaluron em *B. mori*, e embora este inseticida não ser utilizado diretamente nas plantações de amoreira, há registros de que o uso de inseticidas em culturas vizinhas às amoreiras causem grandes perdas para sericicultura. Em adição, a literatura aponta que a aplicação foliar de Novaluron apresenta persistente atividade biológica em campo e um grande potencial no controle de pragas principalmente as da ordem lepidóptera (CUTLER et al., 2005; ISHAAYA et al., 2002), além de que estudos apontam efeitos negativos de Novaluron em alguns insetos benéficos e não-alvo (CUTLER et al., 2006; CUTLER; SCOTT-DUPREE, 2007; MOMMAERTS; STERK; SMAGGHE, 2006). Tais informações, aliadas à possibilidade de ocorrência de resíduos deste inseticida advindo das pulverizações em culturas circunvizinhas as amoreiras e o importante papel desempenhado pelo bicho-da-seda na sericicultura, reforçam a necessidade e a importância de investigações sobre os efeitos de Novaluron em *B. mori*.

2. OBJETIVOS

Avaliar os efeitos do inseticida Novaluron (formulação comercial Rimon Supra - ADAMA Makhteshim Ltd) em *B. mori*, alimentados na fase larval com folhas de amoreira expostas à concentração de 0, 15 mL/L (0,015g i.a./L).

Assim, propusemo-nos a:

- Verificar os efeitos do inseticida no desenvolvimento do inseto, considerando os seguintes tópicos: sinais e sintomas (sintomatologia) causados pelo Novaluron; taxa de mortalidade; qualidade do casulo produzido; número de ovos ovipositados.

- Analisar os efeitos na morfologia e ultraestrutura do intestino médio e glândula da seda de lagartas de *B. mori*, bem como dos órgãos reprodutores (testículo e ovário) de lagartas, pupas e adultos (mariposas) de *B. mori*.

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4. RESULTADOS

4.1. CAPÍTULO 1

Negative impact of Novaluron on the nontarget insect *Bombyx mori* (Lepidoptera: Bombycidae)

Marilucia Santorum [a, *], Rose Meire Costa Brancalhão [b], Ana Tereza Bittencourt Guimarães [b], Carlos Roberto Padovani [c], Gianluca Tettamanti [d], Daniela Carvalho dos Santos [a, e]

[a] Laboratory of Insects, Department of Morphology, São Paulo State University (UNESP), Institute of Biosciences, Botucatu, Brazil.

[b] Center of Biological Sciences and Health, State University of Western Paraná, - UNIOESTE - Cascavel, PR, Brazil.

[c] Department of Biostatistics, São Paulo State University (UNESP), Institute of Biosciences, Botucatu, Brazil.

[d] Department of Biotechnology and Life Sciences, University of Insubria, 21100 Varese, Italy.

[e] Electron Microscopy Center of the Institute of Biosciences of Botucatu, UNESP, SP, Brazil.

ABSTRACT

Due to increased use of agrochemicals and growing concerns about ecotoxicology, the development of new insecticides, moving away from those with neurotoxic and broad spectrum effects towards insecticides that are safer for the environment and nontarget beneficial species, has been a research priority. Novaluron stands out among these newer insecticides, is an insect growth regulator that is used for the control of insect pests in crops grown close to mulberry plantations. Mulberry serves as food for the silkworm *Bombyx mori*, which is a nontarget insect of great economic importance to silk production. We investigated the lethal and sublethal effects of Novaluron on the development of *B. mori*. Larvae were segregated into experimental groups: the control groups (CGs) and the treatment groups (TGs), which were treated with the Novaluron concentration of 0.15 mL/L. Following exposure, we analyzed: larval mortality, changes in the insect life cicle and cytotoxic effects on the midgut cells. This is the first report about the Novaluron´s effects on *B.mori*. We detected rupture in the integument, complete cessation of feeding, late development, incomplete ecdysis and production of defective cocoons. After 240 h of exposure, there was 100% mortality in TG larvae exposed in the 3rd instar and 20% mortality from larvae exposed in the 5th instar. Cytotoxic effects was observed, such as dilation of cells, emission of cytoplasmic protrusions, extreme rarefaction of the cytoplasm and nuclei, dilation of the endoplasmic reticulum in addition to changes in mitochondria, the presence of large digestive vacuoles and intercellular spaces and the presence of active caspase. Novaluron exposure impairs the midgut and may affect the physiological functions of this organ. Novaluron additionally compromises several phases

of insect development, indicating the importance of toxicology studies that utilize different life stages of nontarget species to evaluate the safe use of insecticides.

Keywords: Silkworm, Novaluron, Toxicity, Ultrastructure, Imunohistochemistry.

Introduction

Due to increased use of agrochemicals and growing concerns about ecotoxicology and mammalian safety, the chemical industry has been developing new insecticides, shifting away from those with neurotoxic and broad spectrum effects towards compounds that are less environmentally harmful to nontarget beneficial species (Tunaz and Uygun, 2004; Dhadialla et al., 2005; Bel, 2014). Novaluron stands out among these newer compounds. Novaluron is an insect growth regulator (IGR) and acts as a chitin synthesis inhibitor. It is used in agricultural crops such as corn, soybeans, beans, and sugarcane, among others (Ishaaya et al., 2002; Mapa, 2018).

Novaluron eliminates Lepidopteran insects as well as other pest orders, such as Coleoptera, Diptera and Hemiptera (Cutler and Scott-Dupree, 2007, Mapa, 2018). Its mode of action is by contact and ingestion of a benzoylphenylurea formulation, which inhibits the biochemical processes leading to the formation of chitin synthetase, causing an abnormal deposition of the endocuticle. This mainly affects larval stages, causing death by abnormal endocuticular deposition and interrupted molting. This class of insecticides has been considered acceptable for inclusion in integrated pest management (IPM) programs because this class initially appeared to be safer and more selective in its mode of action, potentially acting only against the target species with reduced risks to the environment, including mammals, birds, aquatic animals and beneficial and nontarget insects (Tunaz and Uygun, 2004; Dhadialla et al., 2005). However, insecticide damage to the environment and to nontarget organisms still needs to be minimized (Desneux et al., 2007), since studies have pointed out negative effects of Novaluron on beneficial nontarget insects, including predators, parasitoids and pollinators. In these nontarget organisms, Novaluron can reduce larval hatching and affect larval development via contact and ingestion. Furthermore, Novaluron can impair molting, oviposition and the viability of eggs of treated adults, as well as reduce their lifespan (Mommaerts et al., 2006; Cutler and Scott-Dupree, 2007).

The silkworm *Bombyx mori* L. (Lepidoptera: Bombycidae) is a research model insect among Lepidoptera and has been widely used as a model organism in cellular and molecular biology, physiology,

biotechnology, medicine, toxicology and other fields (Matsumoto et al., 2011; Tansil et al., 2011; Kundu et al., 2014; Qin et al., 2012). Its agribusiness applications are of great economic importance, being used in sericulture for the commercial production of silk threads. However, the use of insecticides in crops surrounding mulberry plantations (*Morus spp.*) can affect the production of *B. mori*; during aerial spraying, pesticides can settle on mulberry leaves which are the main food source of *B. mori*, leading to the contamination of the farmed silkworms. This causes an imbalance in the metabolic functions of the larvae, compromising growth and reproduction, including the quality of the cocoon, hatching and fecundity of *B. mori*. Thus studies suggest that exposure of silkworm to insecticides may compromise the production of silk (Bhosale and Kallapur, 1985; Yin et al., 2008; Munhoz et al., 2013; Yu et al., 2013; Gu et al., 2014).

Although sublethal concentrations of insecticides may not cause high population mortality, they can significantly affect the lifespan, fertility, feeding and oviposition of a species (Mommaerts et al., 2006; Cutler and Scott-Dupree, 2007, Munhoz et al., 2013, Scudeler et al., 2016), however, information on the toxicity of Novaluron in *B. mori* is not available. Although it is not used in mulberry plantations, recent studies by the agriculture secretary of Paraná State, Brasil, have pointed to large losses in sericulture, cocoon production and mortality of silkworm larvae, potentially due to the drift of residues from sprays insecticide in agricultural crops near to silkworm farms (Munhoz et al., 2013; Globo Rural, 2011). Such information, coupled with the important role played by silkworms, reinforces the need for investigation of Novaluron's effects on *B. mori*. Thus, the present study analyzed the response of midgut epithelial cells from *B. mori* larvae when exposed by ingestion of Novaluron contaminated mulberry leaves during the larval phase and the occurrence of lethal and sublethal effects in their development.

Materials and methods

Insect and chemicals

Hybrid, second instar *B. mori* larvae were obtained from a silk spinning company producing larvae for commercial purposes in the State of Paraná, Brazil. Larvae were maintained under controlled conditions as described by Santorum et al. (2017).

Novaluron – The commercial formulation, Rimon Supra, was purchased from ADAMA Makhteshim Ltd. This formulation is registered with the Ministry of Agriculture, Livestock and Supply, Brazil (MAPA sob nº 14511).

Bioassays

Each treatment was replicated six times, with ten *B. mori* larvae used per group for statistical analyses of mortality. Parallel groups of larvae received the same experimental treatments for morphological and ultrastructural analyses.

According to the official regulation for the phytosanitary pesticide system of the Ministry of Agriculture, Livestock and Food Supply of Brazil, the minimum approved dose of Novaluron for use in the field is 0.3 ml/L and the maximum dose is 0.5 ml/L for the sugarcane, soybean and corn crops (MAPA, 2018). These crops, often grown near mulberry plantations and silkworm farms. Considering the use of the minimum dose of this insecticide in these cultures and the aerial dispersion of these residues during spraying, we used the concentration 0.15 ml/L that is half the recommended minimum dose.

The treatment groups (TGs) consisted of *B. mori* larvae exposed once to mulberry leaves containing Novaluron at two different instars (1st day of the 3rd instar or 1st day of the 5th instar). Control groups (CG) consisted of *B. mori* larvae fed on mulberry leaves free of Novaluron at two different instars (1st day of the 3rd instar or 1st day of the 5th instar). So in the bioassays, mulberry leaves were immersed in aqueous solutions containing 0.15 ml/L Novaluron, dried at room temperature, and then offered to *B. mori* larvae in TGs for 24 h *ad libitum*. After exposure to Novaluron (24 h), the larvae were again fed mulberry leaves free from the insecticide.

Symptomatology

After exposure to Novaluron, the symptoms manifested by *B. mori* individuals throughout their life cycle were monitored through daily observations and registered in own files and photographed (Fig. 1). The analyzed symptoms were: (1) feeding cessation (daily, the insects of the CG and TG were fed with fresh mulberry leaves, twice a day (8am and 8pm), after the interval between each feeding, it was observed how much of mulberry leaves was consumed by each group, considering the larval instars; (2) late development; (3) irregular ecdysis; (4) production of defective cocoons; (5) defective pupae and (6) defective adults.

Mortality

We observed the mortality of *B. mori* larvae after they were exposed to Novaluron either at the 3rd or 5th instar stage. The mortality rate was recorded every 24 h up to a total of 240 h after exposure. The

palpation method was used to determine mortality as follows: the larva was touched with a soft paintbrush; if it made any movement, it was considered alive; otherwise, it was considered dead.

Morphological procedures

For all the morphological procedures described below, the effects of Novaluron in *B. mori* midgut tissue were evaluated. The larvae were randomly selected from the 3rd and 4th larval instars that were treated on the 1st day of their 3rd instar and the 5th larval instars that were treated on the 1st day of their 5th instar. Selected larvae were anesthetized in a freezer for approximately 5 min, placed in a tube with cotton soaked in ethyl ether, and then dissected, rinsed with insect saline solution (0.1 M NaCl, 0.1 M Na_2HPO4 and 0.1 M KH_2PO_4) and the midgut was removed. After removal, each midgut was submitted to different morphological analyses:

Light microscopy

Each midgut was fixed in DuBosq Brasil (Beçak and Paulete, 1976) for 24 h at 4°C. The midgut samples 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) (Junqueira and Junqueira, 1983). The control slides were subjected to the same preparations. The slides were observed with a Leica DM500 photomicroscope.

Transmission Electron Microscopy (TEM)

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

Scanning Electron Microscopy (SEM)

The midguts were fixed for 48 h at room temperature in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3). Thereafter, the samples were washed in distilled water, postfixed in 1% osmium tetroxide diluted in distilled water for 30 min at room temperature, dehydrated through a graded series of ethanol, critical point-dried with CO_2 and coated with gold. The samples were examined and photographed using an FEI Quanta 200 scanning electron microscope (FEI Company, Eindhoven, Netherlands) at the Electron Microscopy Center of the Institute of Biosciences of Botucatu, SP, Brazil.

TUNEL assay

The midguts were fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.2) for 3 h at room temperature. Specimens were dehydrated in an ethanol series and embedded in paraffin. Sections (8–10 μm thick) were cut with a microtome. For the assessment of cell death, a widely used microscopic technique for identifying DNA fragmentation was used to label the 3' ends of fragmented DNA with the DeadEnd Colorimetric TUNEL System (Promega). Paraffin sections were deparaffinized, rehydrated through graded ethanol washes, and rinsed with PBS. Proteinase K (20 μg/ml) digestion was applied as a pretreatment for 20 min at room temperature. Incubation with the rTdT reaction mix was performed for 1 h at 37 °C, in accordance with the instructions provided by the manufacturer. The reaction was terminated by immersing the slides in 2x standard sodium citrate (15 min). A pretreatment with 3% H_2O_2 (5 min) preceded the incubation with a streptavidin–HRP solution (diluted 1:500 in PBS, 30 min). Diaminobenzidine (DAB) solution was used for signal development. Negative controls were performed by replacing the rTdT enzyme with water in the rTdT reaction mix.

Caspase-3 immunohistochemistry

For caspase-3 immunohistochemistry, paraffin sections of midgut samples were prepared as described in the TUNEL assay. After deparaffinization, the sections were subjected to an antigen unmasking procedure by heating in a 10 mM sodium citrate buffer (pH 6.0) for 5 min and then allowing sections to cool. They were then treated with 3% H_2O_2 for 10 min to inhibit endogenous peroxidases. Sections were incubated for 30 min with a solution of 2% BSA, 0.1% Tween 20 in PBS and then overnight at 4 °C with an anti-cleaved caspase-3 antibody (catalog no. 9661) (Cell Signaling Technology) at a dilution of 1:100. Incubation with an appropriate HRP-conjugated secondary antibody (diluted 1:50) was performed for 1 h. A DAB substrate was used to detect the HRP-conjugated secondary antibody. Antibodies were omitted in control samples.

Statistical Analysis

The delineated experiment was completely randomized with six replicates per treatment. Ten silkworm larvae were used per replicate.

The Goodman test, which involves contrasting binomial proportions, was used to compare the relative rates of mortality of the *B. mori* larvae (Goodman 1964). The results were expressed as the mean ± SD. The test was performed at a significance level of 5%.

Results and discussion

In recent years, Brazilian silkworm producers have reported large losses in cocoon production after spraying of insecticides on crops close to their silkworm rearing properties (Munhoz et al., 2013; Globo Rural, 2011). To eliminate the agricultural insects pests, mainly of the order Lepidoptera, Novaluron can be sprayed on agricultural crops growing near mulberry plantations, and can cause large indirect damage and loss of silkworm larvae and cocoon production. Thus, in this study, we performed bioassays to verify the impact of Novaluron on the development of this important insect by exposing groups of *B. mori* larvae in two different instars to Novaluron.

Symptomatology

Throughout the experimental period, it was possible to observe the symptoms manifested by *B. mori* after exposure to the insecticide. Using the concentration of 0.15 mL/L, corresponding to half of the minimum dose recommended by Mapa (2018) for the main crops grown near mulberry plantations, our results indicated that Novaluron negatively affects the overall growth of *B mori.* The larvae treated with this insecticide exhibited a rapid reduction in feeding leading to complete feeding cessation and, therefore, a notable reduction in body size. Other symptoms observed after treatment included discoloration of the cuticle of the whole body to a dark color (Figs. 1A and B). The treated larvae presented with an extremely fragile integument; ruptures, causing hemolymph extravasation and intestinal exposure (Fig. 1C), as well as irregular ecdysis, where the exuvia remained attached to the body of the insect during molting, promoting incomplete ecdysis (Figs. 1A and B), were observed.

Several studies have evaluated the toxic effects of exposure to agrochemicals on mulberry leaves on the development and growth of *B. mori* (Bhosale and Kallapur, 1985; Kumutha et al., 2013; Munhoz et al.,

2013; Yu et al., 2013; Gu et al., 2014; Tang et al., 2018), however our results are the first description of the Novaluron effects on silkworm development. These data are important since changes in insect feeding compromise the conversion of ingested food to digested food, which can promote subsequent anomalies (Nasr, 2011). We verified symptoms similar to those found by Munhoz et al. (2013) and Bindu (2015) and found that the symptoms appeared very quickly after exposure of the larvae to the insecticide. Novaluron showed similar effects in exposed Lepidopteran insects (Retnakaran and Wright, 1987; Cutler and Scott-Dupree, 2007). The observed symptoms are probably related to the mechanisms and general effects of Novaluron and of the class of benzoylphenylurea insecticides; these insecticides alter the composition of the cuticle, especially through the inhibition of biochemical processes that lead to the formation of chitin synthetase, causing an abnormal deposition of the endocuticle that affects the cuticular elasticity and firmness and interferes with insect molting (Tunaz and Uygun, 2004; Dhadialla et al., 2005).

Formation of the cocoon is an important developmental checkpoint and among the symptoms manifested by the TG larvae exposed in their 5^{th} instar, we observed difficulty in packing silken threads accompanied by a delay in the construction of the cocoon, production of defective and/or thin-shelled cocoons (Fig. 1F), and retention of larval morphological characteristics (Figs. 1E and F) when compared to the CG larvae. The effects of insecticides on cocoon production and quality have been previously reported (Munhoz et al., 2013; Yu et al., 2013; Gu et al., 2014; Tang et al., 2018) and Munhoz et al.,(2013) point out that affected cocoons are not useful for spinning, so sericulture companies do not accept thin-shelled cocoons and silkworm producers must take on the financial losses.

The effects of Novaluron exposure were also observed in adults; moths surviving the TG exposure in the 5^{th} instar presented wing defects, and some, after emerging from cocoons, failed to completely release their exuvia (Fig. 1H and I). Exuvial damage to the terminal region of female moths' abdomens impaired oviposition, and these moths exhibited swollen abdomens and died without being able to lay their eggs (Figs. 1I). Other studies have found that Novaluron compromises the development of adults and that these individuals present morphogenetic abnormalities that reduce their reproductive potential (Tunaz and Uygun, 2004; Mommaerts et al., 2006; Storch et al., 2007), including suppression of embryogenesis (Dhadialla, 2005).

Mortality

We analyzed the mortality of *B. mori* larvae after they were exposed to Novaluron at either of two different instars (1st day of the 3rd instar; 1st day of the 5th instar). *B. mori* larvae exposed to Novaluron in the 3rd instar showed significant mortality (30%) 96 h after initiation of a 24 h exposure to treated mulberry leaves. That is, mortality was observed soon after the larvae molted from the 3rd to the 4th larval instar. 240 h after initiation of a 24 h exposure to treated mulberry leaves, 100% mortality occurred in this group, this time when larvae were molting from the 4th to the 5th instar (Table 1). These two larval mortality peaks coincided with instar molting, proving that the greatest toxic effect of Novaluron is on insect ecdysis, resulting in abortive molting (Retnakaran et al., 1985; Mapa, 2018). Due to the complete mortality of TG - larvae exposed to Novaluron (Table 1) on the 1st day of their 3rd instar, larvae failed to complete their life cycle; that is, they were unable to continue their development, to construct their silk cocoons and to reach adulthood.

Instar	**Hours**	Accumulated Mortality (%)	
		Control	**Treatment**
3rd	24	0 a	0 a
	48	0 a	0 a
	72	0 a	1.7 a
4th	96	0 a	30.0 b
	120	0 a	30.0 b
	144	0 a	35.0 b
	168	0 a	35.0 b
	192	0 a	68.3 b
5th	240	1.7 a	100.0 b

Table 1. Cumulative mortality (%) of *B. mori* larvae during larval development (h) exposed to Novaluron. Values followed by different letters within the same line differ significantly ($p < 0.05$, Goodman's test). n = 60 larvae per group.

Larvae exposed to Novaluron insecticide on the 1st day of their 5th instar showed significant mortality after 96 h of exposure, with cumulative mortality 20% after 240 h of exposure (Table 2). By comparing cumulative mortality rates after the two exposure times (Table 1 and 2), we verified that there was a lower percentage of mortality in the TG larvae exposed in the 5th instar. We believe that this is because larvae exposed at a later instar were more developed, possessing a greater body size and a faster metabolism aimed at the accumulation of energy reserves necessary for metamorphosis and cocoon construction (Santorum et al., 2017) and potentially conferring greater resistance to adverse conditions, such as exposure to Novaluron insecticide.

Instar	Hours	Accumulated Mortality (%)	
		Control	**Treatment**
	24	0 a	3.3 a
	48	0 a	3.3 a
	72	0 a	5.0 a
	96	0 a	6.7 b
5th	120	0 a	6.7 b
	144	3.3 a	13.3 b
	168	3.3 a	16.7 b
	192	3.3 a	18.3 b
	240	3.3 a	20.0 b

Table 2. Cumulative mortality (%) of *B. mori* larvae during 5th instar (h) exposed to Novaluron. Values followed by different letters within the same line differ significantly ($p < 0.05$, Goodman's test). n = 60 larvae per group.

Novaluron's mode of action is another important factor to consider; Novaluron is a growth regulator and chitin synthesis inhibitor. As indicated in the product description, its application to crops is carried out during the early stages of the development of insect pests; that is, Novaluron is applied when first larval instars are present, preventing them from reaching more advanced instars (Mapa, 2018) and completing their development.

Although Novaluron is cited as an insecticide to be used in integrated pest management programs because of its low toxicity to mammals, fish and nontarget beneficial insects like honeybees, parasitic wasps, ants, predaceous mites and others pollinators, parasites and predators (Tunaz and Uygun, 2004; Dhadialla et al., 2005; Alyokhin, 2009; Jiang et al., 2010), our results point to its high toxicity potential for the silkworm, which is a nontarget species of great economic importance. Exposure results in significant larval mortality and compromises all phases of insect development. These results are similar to findings from previous studies that point to the toxic effects of Novaluron in beneficial insect species (Cutler et al., 2006; Mommaerts et al., 2006; Cutler and Scott-Dupree, 2007).

Morphological alterations in the midgut of B. mori larvae

The midguts of *B. mori* larvae from the CG at the 3rd, 4th and 5th larval instars presented as a typical epithelium externally surrounded by muscle fiber bundles that formed an internal circular tunic and another external longitudinal tunic (Fig. 2) (Pinheiro et al. 2008; Correia et al. 2009; Franzetti et al., 2016). We verified the presence of four typical cell types: columnar cells, the most abundant with apical microvilli, and oval and central nuclei; goblet cells, each a with basal nucleus and invaginated cytoplasm forming globular chambers, intercalated between the columnar cells; regenerative cells, found in isolation or in groups in the basal area of the epithelium, with the nucleus having a shape similar to that of the cells, varying from

elongated to oval (Figs. 2A, D and F); and endocrine cells, which were observed more rarely along the epithelium, located in the basal regions close to nests of regenerative cells and possessing a large nucleus and clear cytoplasm.

The morphology of these cells is related to their functions performed in the midgut, such as in the synthesis and secretion of digestive enzymes and subsequent absorption of the nutrients, performed by columnar and goblet cells; in the tissue growth and regeneration; in the control of metabolic processes; and in the control of cell proliferation and differentiation, mediated by regenerative and endocrine cells, respectively (Sousa et al., 2009; Hakim et al., 2010; Teixeira et al., 2013; Gigliolli et al., 2015).

Novaluron caused cytotoxic effects in the midguts of the TG *B. mori* larvae for the three larval instars analyzed, and exposure resulted in pronounced alterations throughout the entire intestinal epithelium, with extreme disorganization observed in the arrangement of cell types typical of this epithelium (Fig. 2). The columnar cells were extremely elongated, with a dilated apical region and cytoplasmic protrusions similar to vesicles visualized along the epithelium and in the intestinal lumen. This cell type also had sparse and/or absent microvilli in some regions. Thus, the apical surface of the TG epithelium demonstrated more irregularities compared to that of the CG (Figs. 2 and 3). The TG goblet cells were also more elongated, following the same trend in columnar cells (Fig. 2). Similar results on the toxic effects of insecticides on insect midgut epithelial cells have been previously reported (Ndione et al., 2007; Correia et al., 2009; Roel et al., 2010; Scudeler and Santos, 2013; Munhoz et al., 2013; Scudeler et al., 2016). As there is no literature demonstrating Novaluron's effects on the midgut morphology and development of *B. mori*, our findings will contribute to understand the great toxic potential presented by this insecticide, which has been considered safer for nontarget species. Munhoz et al. (2013), analyzing the effects of another class of insecticide, the Chlorantraniliprole, usually used for sugarcane borer control next mulberry plantations, reported negative effects in columnar cells of *B. mori* midgut, which presented apoptotic bodies. They hypothesized that the midgut cell death by apoptosis compromised silkworm development as well as the production of cocoons.

Various stimuli may induce apoptosis of midgut epithelial cells in insects, such as inhibitors of ARN and protein synthesis (Palli et al., 1996), bacterial (Gregorc and Bowen, 1999) or viral infection (Garcia et al., 2001), and exposure to biopesticides (Nasiruddin and Mordue (Luntz), 1993; Ndione et al., 2007) and insecticides (Gregorc and Ellis, 2011; Munhoz et al., 2013). When exposed to azadirachtin, a compound that is also an insect growth regulator (Thangaraj et al., 2018), histopathological alterations in the midgut epithelium, such as elongation of the columnar cells, smaller nuclei, loss of the apical cytoplasm and necrosis

were observed in *Spodoptera frugiperda* (Lepidoptera: Noctuidae) (Correia et al., 2009). The midgut is the main location for digestion and absorption of food and therefore is the region most vulnerable to the action of foreign elements (Roel et al., 2010; Yu et al., 2013).

We observed hypertrophy in the regenerative cells and a reduction in their numbers. The clusters of regenerative cells, characteristic of this cell type in Lepidoptera and observed for CG, were no longer visible in TG larvae (Fig. 2). Mordue (Luntz) and Nisbet (2000) found similar changes in these cells induced by azadirachtin; absorption of this compound causes inhibition of cell division and protein synthesis, with effects reflected in the loss of groups of regenerative cells and resulting in deficiencies in epithelial renewal. We believe that the same effects on the regenerative cells of *B. mori* exposed to Novaluron may have occurred.

In some regions of the epithelium, there is a spacing between the epithelial cells and the basal lamina where they are supported, in addition to the separation also occurring between the epithelium and the muscular layer (Fig. 2I and J). We identified the detachment of some cells (Fig. 2J and K) from the epithelium towards the lumen; this detachment is better visualized through SEM, as shown in Fig. 3I, where the presence of empty spaces previously occupied by the epithelial cells is clear. The visualization of these spaces in the epithelium may be related to the lack of epithelial renewal since we observed a decrease in the regenerative cells in the TGs.

We compared the ultrastructural observations of epithelial cells from the midguts of *B. mori* larvae in TGs with those of the CGs via SEM (Fig. 3) and TEM (Fig. 4), and in the 3 instars analyzed, we verified several changes in the 4 typical cell types, such as extreme cytoplasmic and nuclear rarefaction and dilation of the endoplasmic reticulum; mitochondrial changes; the presence of large digestive vacuoles and myelin figures; and intercellular and intracellular spacing (Fig. 4), similar to what has been described by Yu et al. (2013) and Gu et al. (2014) in *B. mori* after exposure to phoxin insecticides. These authors point out that deteriorated mitochondria produce more reactive oxygen species and thus accelerate the process of apoptosis, and Cheville (2009) associates the occurrence of myelin figures with irregular fragments of the reticular endoplasmic membrane that aggregate and reorient in a laminar arrangement as a consequence of degeneration of the endoplasmic reticulum. The formation of intercellular spaces has also been reported in several treatments with biopesticides (Correia et al., 2009; Scudeler and Santos, 2013; Scudeler et al., 2016) and insecticides (Cruz et al., 2009). We believe that the complexes in cell junctions can be broken down, allowing the formation of these spaces. Cheville (2009) mentions that during acute cellular swelling, the

junctions may disintegrate, causing cells to lose their normal cohesion with neighboring cells. All of these changes seem to be associated with possible attempts of detoxification or response to cellular stress against cytotoxicity. (Cheville, 2009; Scudeler and Santos, 2013, 2014; Catae et al, 2014; Scudeler et al. 2016).

In addition to these characteristics, we observed in the goblet cells of the TGs an absence of mitochondria within their microvilli or cytoplasmic projections as they are also known, a common characteristic for this cellular type (Fig. 4H and I). Bong and Sikorowski (1991) found that infection with the cytoplasmic polyhedrosis virus resulted in decreased cytoplasmic projections and absence of mitochondria inside in *Helicoverpa zea* (Lepidoptera: Noctuidae) larvae. Due to the high concentration of potassium in plant tissues, it is known that this cell type plays an important role in insects that feed on plants by executing the transport of the hemolymph ions to the intestinal lumen (Anderson and Harvey, 1966). Thus, changes in these cells due to exposure to the insecticide may lead to an imbalance of the normal function of the intestinal epithelium, compromising the growth and development of the insect.

In some regions, we visualized ruptures in the basolateral membrane of the columnar cells, in the nuclear envelope and in the apical membrane/microvilli. The microvilli were deformed (Fig. 4), and the absence of actin microtubules inside them suggests that the exposure to insecticides and toxins seems to be related to the decrease and depolymerization of the actin microfilaments in the cell that favor the formation of cytoplasmic protrusions (Nogueira et al., 1997; Anuradha et al. 2007; Scudeler et al., 2016). This microfilament deficit also resulted in cell hypertrophy with the formation and release of cytoplasmic protrusions containing cytoplasmic and nuclear residues, which were observed along the epithelium and in the intestinal lumen (Fig. 4F). These changes have been reported frequently in expositions of insecticides and toxins in insects (Scudeler and Santos, 2013; Almeida et al., 2014) and in *B. mori* (Chiang et al., 1986, Munhoz et al., 2013). Cellular hypertrophy is the first change to be observed in cell lesions due to the loss of ionic control and water entry, causing cellular dilation and cellular lysis (Cheville, 2009).

Cell death analysis

To analyse the occurrence of cell death in the midgut epithelium following to the Novaluron exposure, we used several apoptotic markers (Franzetti et al., 2012). Despite the occurrence of severe cellular damage in the midgut of TGs larvae in the three larval instars analyzed, Novaluron did not induce cell death. In fact, only a few positive nuclei were evidenced by the TUNEL assay in the midgut of treated insects (Figs. 5B, C, E, F, H and I), probably due to the normal physiology of the epithelium, rather than the effects of the

insecticide, as also demonstrated in the midgut of *Ceraeochrysa claveri* (Neuroptera: Chrysopidae) in our previous studies with a biopesticide neem oil (Scudeler and Santos, 2013; Scudeler et al., 2014).

Despite the absence of DNA fragmentation, the midgut epithelium of *B. mori* had a reasonable activation of caspase-3 during larval development in response to exposure to the insecticide Novaluron. A positive reaction was not found in the CGs (Figs. 6A, C and E), but a clearly positive reaction was visible in the cytoplasm of the midgut epithelial cells of the TGs in the three larval instars larvae (Figs. 6B, D and F), indicating a toxic response to the insecticide. According to Nicholson (1999), inactive caspases are present in the cytoplasm before initiation of the cell death process, and the activation of these caspases is closely related to programmed cell death. The activation of the starter caspases leads to cleavage and/or activation of effector caspases (or executors). After activation, the effector caspases attack and cleave major intracellular components, promoting several morphological and biochemical characteristics associated with apoptosis, including plasma membrane blebbing and DNA fragmentation. However, according to Accorsi et al. (2015), an even wider role has been hypothesized for caspases of the ‘‘undead’’ cells of the imaginal disc in *Drosophila melanogaster*. ‘‘Undead’’ cells are imaginal disc cells that do not undergo the expected apoptosis when a proapoptotic stress is applied to the *Drosophila* wing imaginal disc. The resistance of ‘‘undead’’ cells towards apoptosis mainly depends on the overexpression of an effector caspase inhibitor, such as p35. We believe that the positive reaction for caspase in treated insects is not directly involved in the process of apoptotic cell death but rather in another type of cell death that is evidenced by ultrastructural features such as those observed in this study.

Although Novaluron is not used directly in mulberry plantations, when applied to surrounding agricultural crops, mainly by aerial spraying, it can adhere to mulberry leaves and damage silkworms that feed on the leaves, it could damage the silk production chain. Several studies have noted disturbances in the life cycle of the silkworm that directly impact the production of quality cocoons (Kuribayashi, 1988; Nath, 1997; Yin et., 2008; Nasr, 2011; Munhoz et al., 2013; Yu et al., 2013; Gu et al., 2014). Although these studies have used different insecticides from Novaluron, the reported toxic effects are very similar to those observed in our study. These effects of Novaluron exposition are also reported on other beneficial and nontarget insects (Cutler et al., 2006; Mommaerts et al., 2006; Cutler and Scott-Dupree, 2007); these show that Novaluron is not always a safe insecticide as is presumed and included in integrated pest management (IPM) programs expressly because it is thought to be of selective toxicity to target insects and not to negatively affect or at least minimally impact nontarget species (Tunaz; Uygun, 2004; Dhadialla et al., 2005).

Conclusions

Our results indicate that with only 24 h of exposure of *B. mori* larvae to Novaluron, it is possible to demonstrate a negative impact on insect development and induces a set of cellular injuries in the midgut epithelium, affecting midgut functions. This study adds relevant information to the toxicology literature by providing details of the cellular injuries suffered by a nontarget organism, as well as, illustrating the consequences of possible long-term exposure, in environmental, of a nontarget organism. Thus, the use of crop protection chemicals requires extreme caution to avoid deleterious damage to the environment and to nontarget species, and sublethal effects must be considered and assessed at all stages of an organism's life cycle in order for crop protection chemicals to be considered safe.

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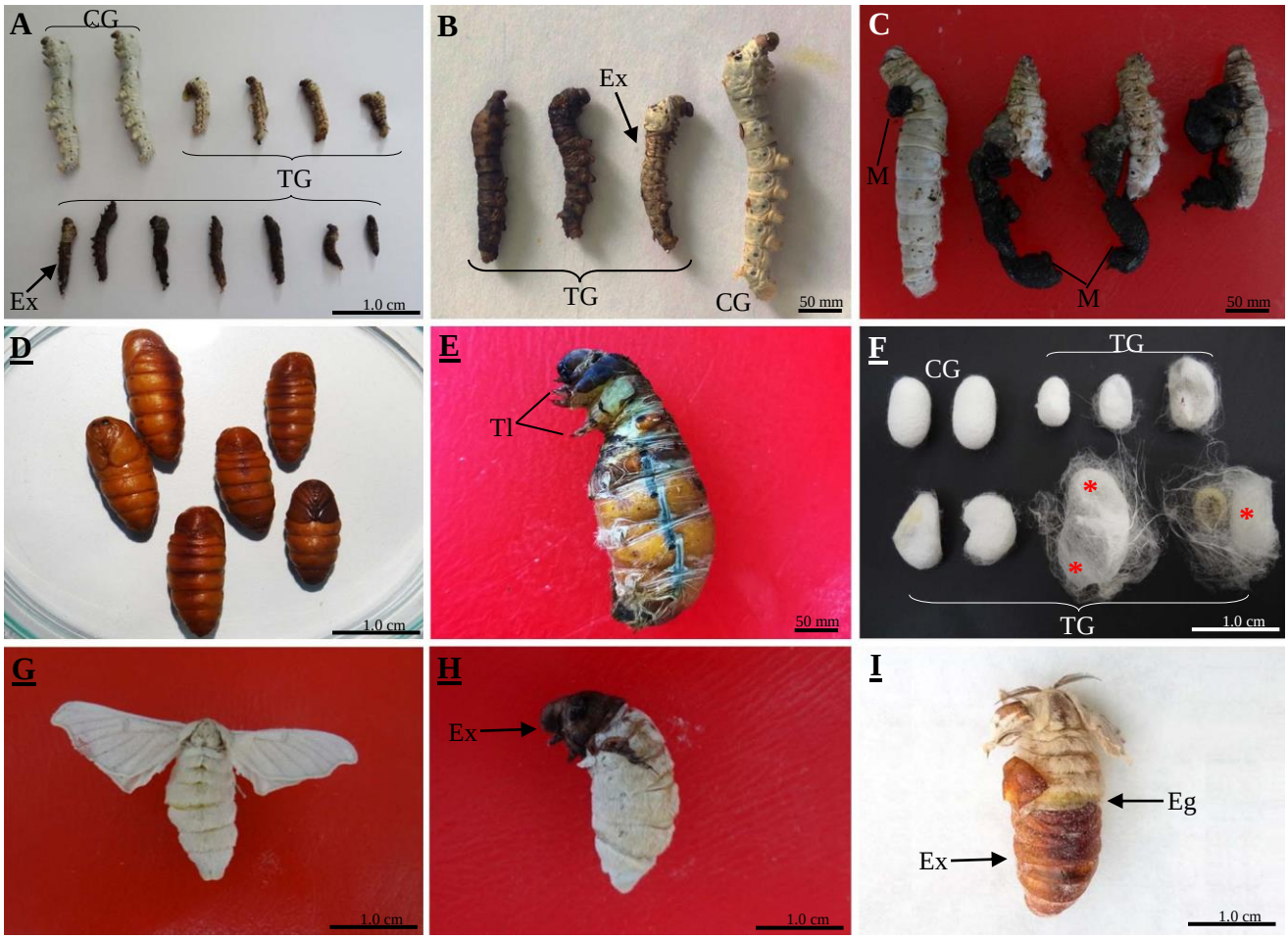

Fig. 1. Symptomatology observed in *B. mori* larvae exposed to Novaluron, treatment group (TG) and control group (CG) larvae for comparison. In **A** and **B**, incomplete ecdysis with exuvia attached to the body of the insect (Ex). Note the changes in the development of TG larvae, which have a smaller size and discoloration of the cuticle of the whole body to a dark color compared to the that of the CG. In **C**, TG larvae with ruptures in the tegument with exposed midgut (M); In **D**, pupae from larvae of the CG for comparison. In **E**, TG larvae that could not complete transformation to pupa, with retention of larval morphological characteristics. Note the presence of the thoracic legs (Tl). In **F**, defective cocoons that are deformed and thin-shelled produced by insects of the TG. Red asterisk (*) showing two cocoons attached. Cocoons constructed by insects of the CG for comparison. In **G**, CG emerged moth. In **H**, a moth emerged from TG with exuvia attached to head and in **I**, a TG moth that failed to release its exuvia (Ex), which is adhered to the final region of the abdomen. Eggs (Eg) trapped inside the abdomen are discernable with the yellow color.

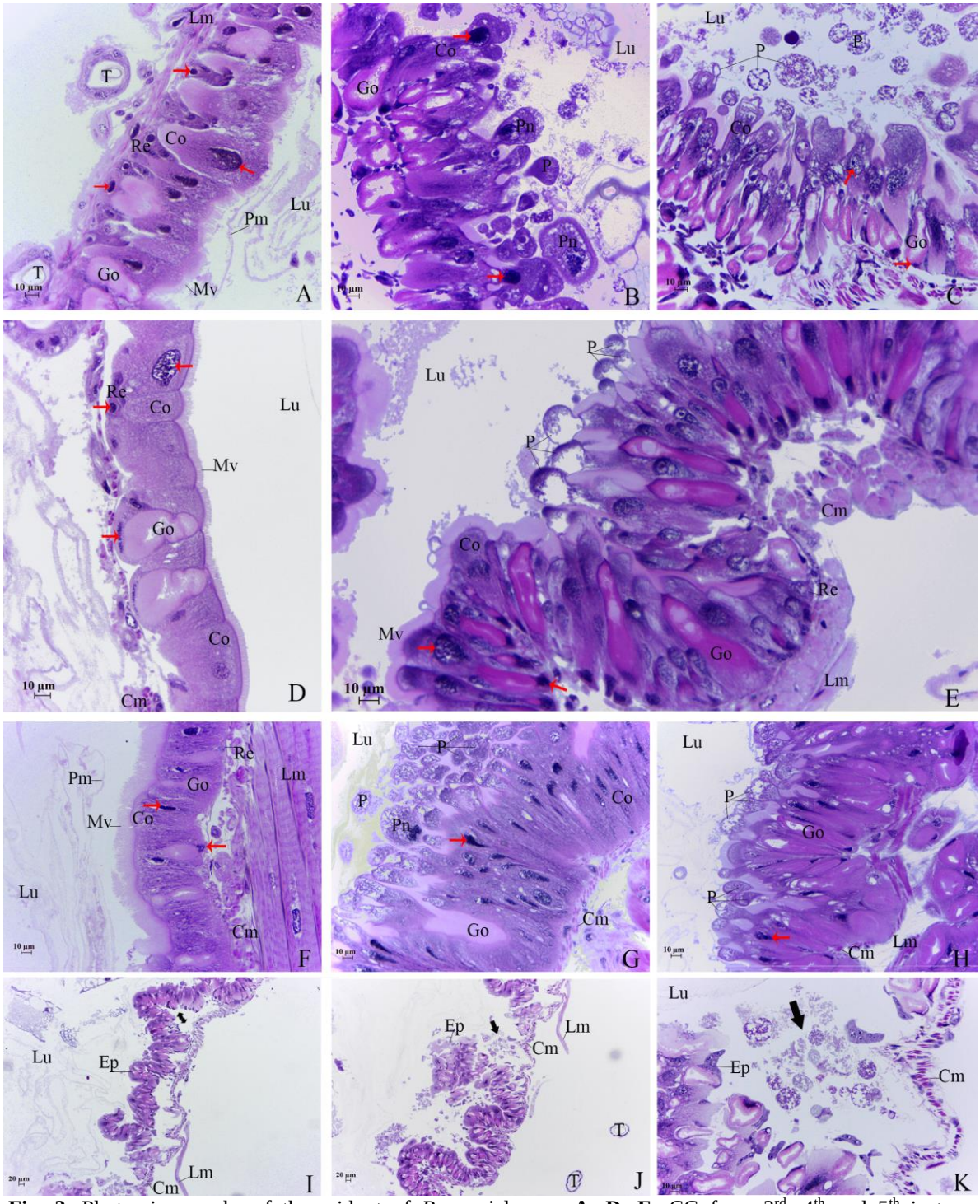

Fig. 2. Photomicrographs of the midgut of *B. mori* larvae. **A, D, F:** CG from 3rd, 4th and 5th instars, respectively, presented columnar cells (Co) with microvilli (Mv) in the apical region facing the lumen of intestine (Lu), goblet cells (Go) intercalated between columnar cells and nests of regenerative cells (Re) at the base of the epithelium. The nucleus (red arrow), peritrophic membrane (Pm), trachea (T), circular (Cm) and longitudinal (Lm) muscle are shown. **B, C, E, G, H:** TG from 3rd, 3rd, 4th, 5th and 5th instars, respectively, where we note the tissue disorganization, with elongated columnar cells (Co), with the most enlarged apical region and with a large number of cytoplasmic protrusions (P) angled towards the lumen of the intestine (Lu) and some protrusions containing cytoplasmic and nuclear material in its interior (Pn). Note the presence of more elongated goblet cells (Go) and a reduction in the presence of regenerative cells (Re). **I, J, K:** TG with the region showing separation between the epithelium (Ep) and the muscular layer (double arrow). In J, the region showing the detachment of the epithelium towards the lumen (black arrow). In K, increased epithelial detachment of the region towards the lumen.

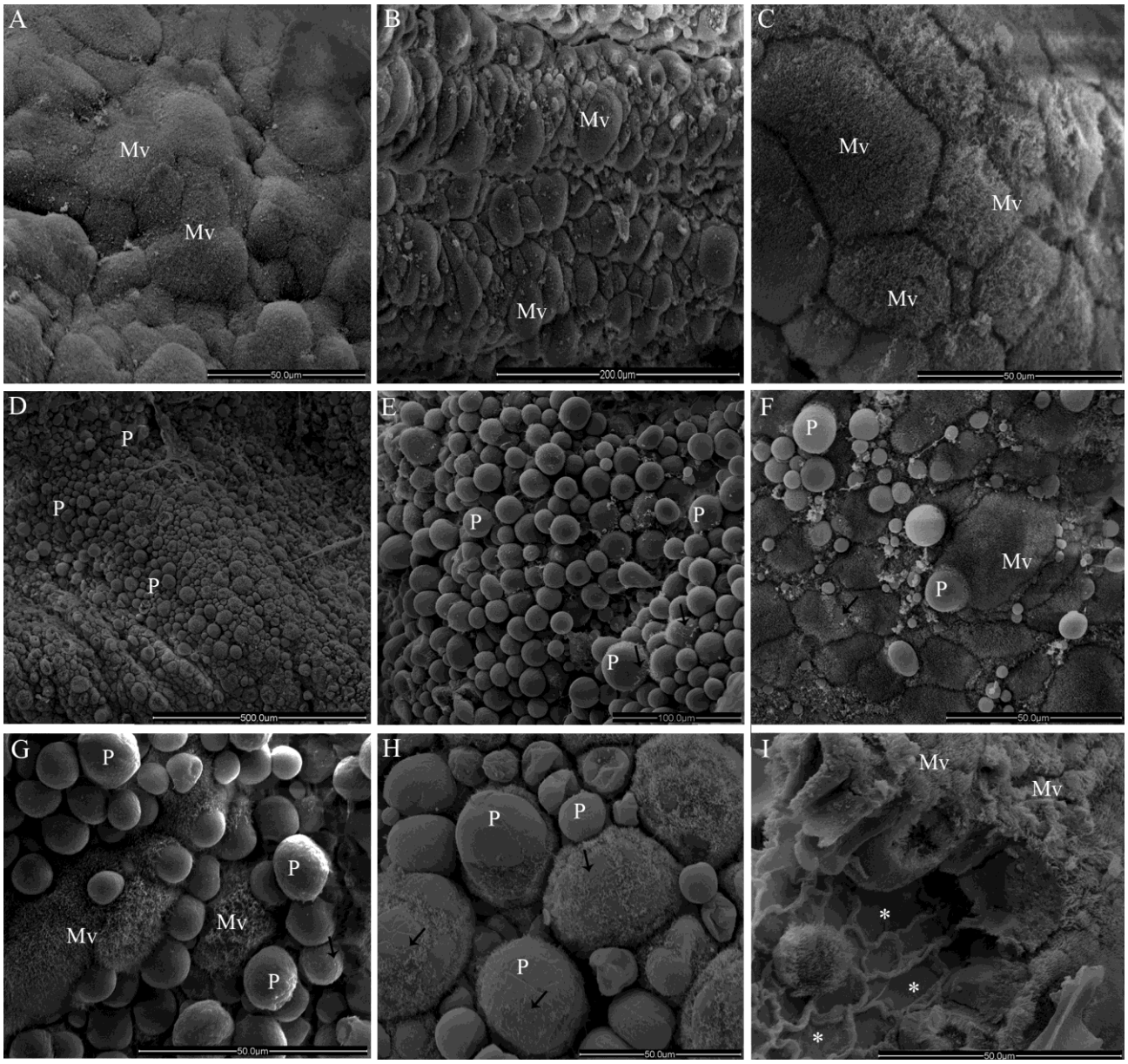

Fig. 3. Electron micrographs of the midgut of *B. mori* larvae. **A, B, C:** CG 3rd, 4th and 5th instars, respectively, presented columnar cells (Co) with a regular pattern of microvilli (Mv). **D, E, F, G, H, I:** TG from 3rd, 3rd, 4th, 4th, 5th, and 5th instars. Note the epithelial surface of the midgut of the TG, with changes in the morphology of the columnar cells showing enlargement of apical regions and with a large number of cytoplasmic protrusions (P) and sparse and/or absent microvilli in some regions (black arrow). In I, notice the empty spaces (asterisks) previously occupied by columnar cells.

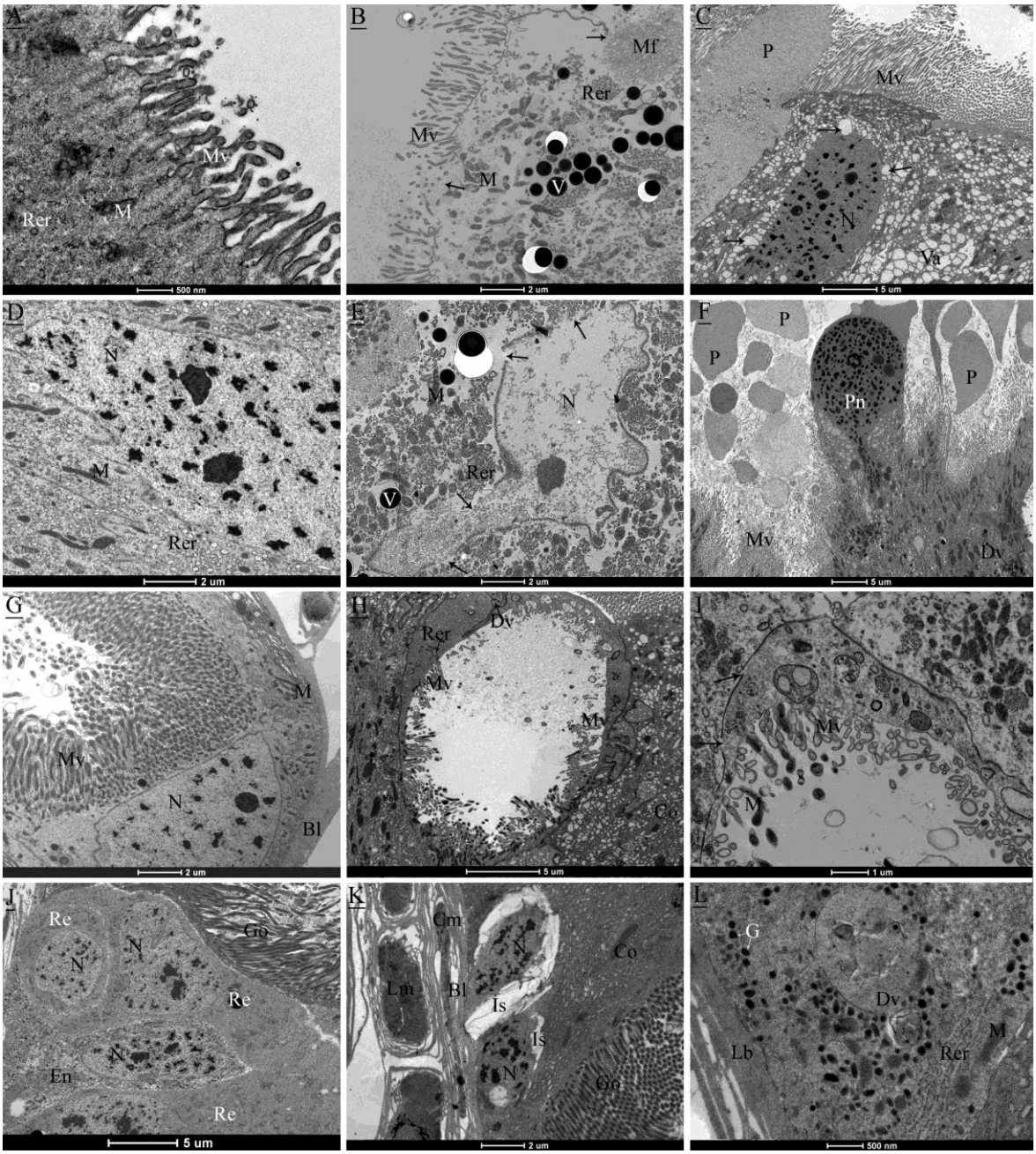

Fig. 4. Electron micrographs of the midgut *B. mori* larvae. . **A, D, G, J:** CG. In A, apical region of the columnar cells with microvilli (Mv), mitochondria (M) and rough endoplasmic reticulum (RER). In D, column cell nucleus (N). In G, basal region of the goblet cell seated on the basal lamina (Bl) and note mitochondria inside microvilli. In J, groups of regenerative cells (Re) interspersed with endocrine cell (En) and goblet cell (Go). **B, C, E, F, H, I, K, L:** TG. In B, apical region of the columnar cells with alterations in microvilli (Mv) with ruptures in the membrane (arrow). Note a large dilation and fragmentation of the rough endoplasmic reticulum, myelin figures (Mf), vesicles with electron-dense content (V).In C, the apical region of the columnar cell emitting cytoplasmic protrusions (P), with extremely vacuolated cytoplasm and dilation of the perinuclear space (arrow).In E, note the rarefaction of the nucleus of the columnar cell with ruptures in the nuclear envelope (arrow) and general appearance of the perinuclear cytoplasm. In F, note the presence of digestive vacuoles (Dv) and large number of cytoplasmic protrusions containing cytoplasmic and nuclear material in their interior and some protrusions released towards the lumen (Pn). In H, goblet cells with changes in microvilli (Mv) and many of them devoid of mitochondria in their interior, and columnar cells (Co). In I, apical region of the goblet cell with membrane ruptures (arrow). In K, regenerative cells with intercellular and intracellular spaces (In), circular muscle (Cm) and longitudinal (Lm). In L, the basal region of the endocrine cell; note the presence of digestive vacuoles, electron-dense granules (G).

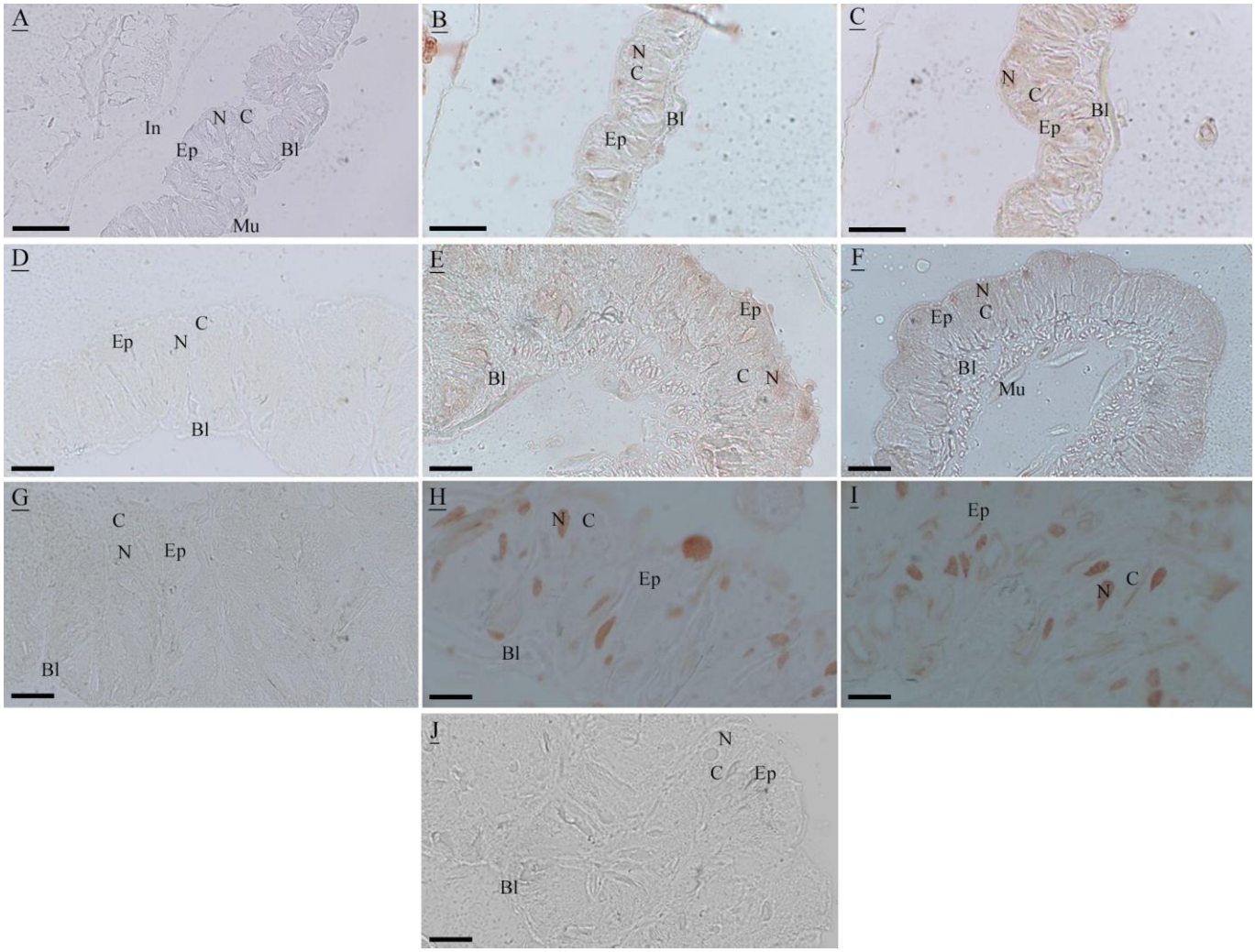

Fig. 5. TUNEL assay in the midguts of *B. mori* larvae. **A, D, G:** CG from 3rd, 4th and 5th instars, respectively. No signal is visible in the midgut epithelium (Ep). Intima (In); nucleus (N); cytoplasm (C); basal lamina (Bl); muscle (Mu). **B, C, E, F, H, I:** TG from 3rd, 3rd, 4th, 4th and 5th instars, respectively, with the nucleus are stained. No staining can be observed in TUNEL-negative control **(J)**. Bars: A - C = 24 μm; D – J = 12 μm.

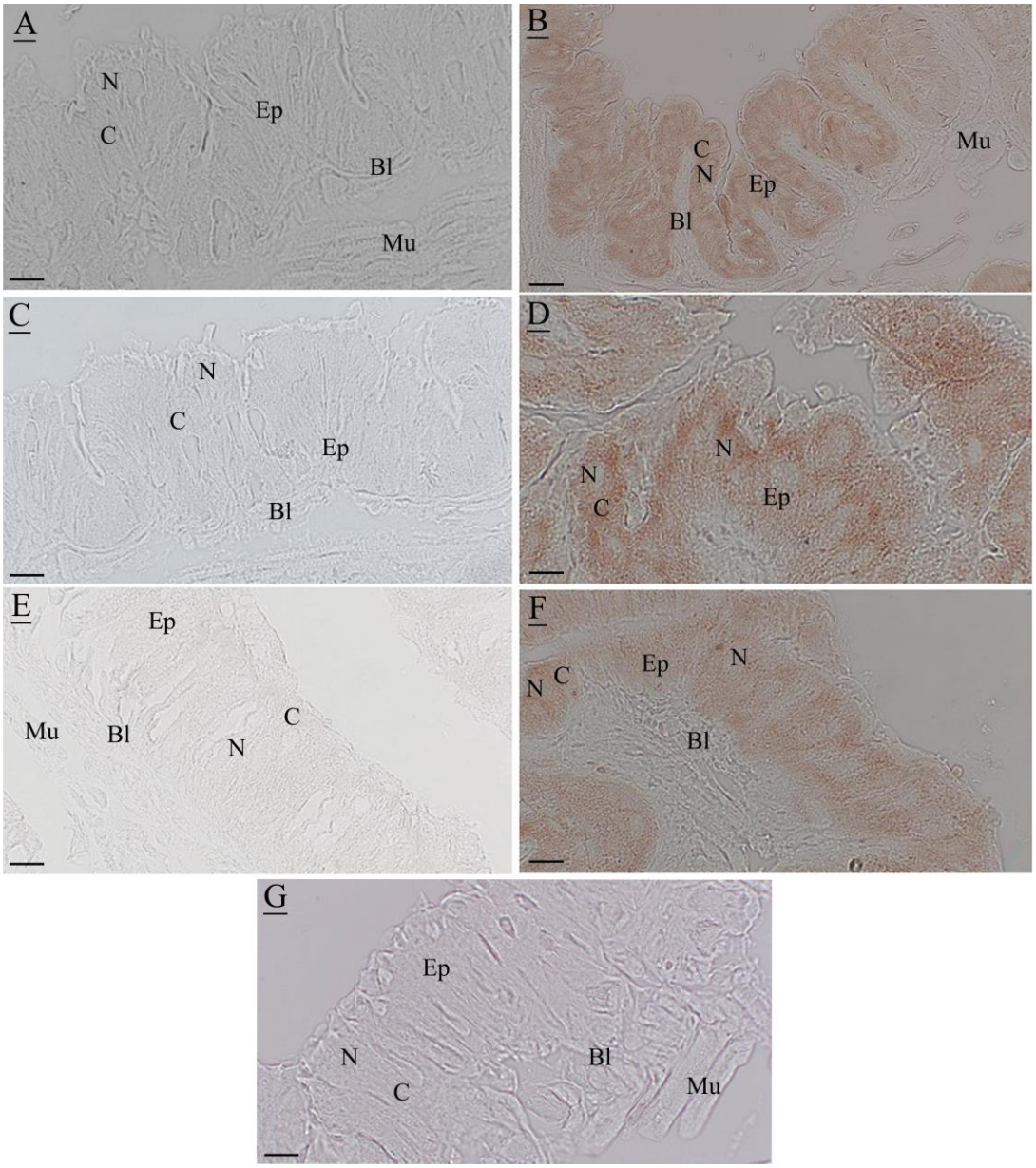

Fig. 6.Immunolocalization of activated Caspase-3 in the midguts of *B. mori* larvae. **A, C, E:** CG from 3rd, 4th and 5th instars, respectively. The antibody does not reveal any positivity for caspase-3 in midgut epithelium (Ep). Nucleus (N); cytoplasm (C); basal lamina (Bl); muscle (Mu). **B, D, F:** TG from 3rd, 4th and 5th instars, respectively, where a signal is visible in cytoplasm of the cells midgut. No staining can be observed in negative control **(G)**. Bars: 12 μm.

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4.2. CAPÍTULO 2

Novaluron impairs the silk gland and productive performance of silkworm *Bombyx mori* (Lepidoptera: Bombycidae) larvae

Marilucia Santorum [a], Rose Meire Costa Brancalhão [b], Gustavo Henrique Dos Reis [c], Daniela Carvalho dos Santos [a, d, *]

[a] Laboratory of Insects, Department of Morphology, São Paulo State University (UNESP), Institute of Biosciences, Botucatu, Brazil.

[b] Center of Biological Sciences and Health, State University of Western Paraná (UNIOESTE), Cascavel, PR, Brazil.

[c] Postgraduate in Pathology, Federal University of Rio de Janeiro (UFRJ), Rio de Janeiro, RJ, Brazil.

[d] Electron Microscopy Center of the Institute of Biosciences of Botucatu, UNESP, SP, Brazil.

* daniela.santos@unesp.br

ABSTRACT

This study investigates the effects of the insect growth regulator Novaluron on the silk gland (SG) and silk cocoon production in a nontarget insect, the silkworm *Bombyx mori*, which is a model research insect among Lepidoptera and of great economic importance for the commercial production of silk threads. Larvae were segregated into experimental groups: the control group (CG) and the treatment group (TG), which was exposed to a Novaluron concentration of 0.15 mL/L. Following exposure, we analyzed the cytotoxic effects on the epithelial cells of the anterior, middle and posterior regions of the SG of *B. mori* larvae in the 3rd, 4th, and 5th instars, as well as the quality of the cocoons from larvae in the 5th instar. Cytotoxic effects were observed in the TG, such as the dilation of cells, emission of cytoplasmic protrusions, extreme rarefaction of the cytoplasm and nuclei, dilation of the endoplasmic reticulum, intracellular and intercellular spaces, spacing between the epithelial cells and the basal lamina and detachment of some cells towards the lumen of the SG, and decreased protein in the lumen, with faults in its composition. In addition, we verified ultrastructural changes in the production of fibers and silk cocoons, including a reduction in the weight of the cocoons constructed by both males and females in the TG and the construction of defective cocoons. Novaluron exposure impairs the SG and may affect the physiological functions of this organ; additionally, it compromises the quality of silk cocoons, potentially causing serious damage to sericulture.

Keywords: Insecticide; Toxicity; Silk cocoons; Ultrastructure; Morphology.

1. Introduction

The silkworm *Bombyx mori* L. (Lepidoptera: Bombycidae) is a model research insect among Lepidoptera and of extreme economic importance in the production of silk worldwide (Tansil et al., 2011; Qin et al., 2012; Kundu et al., 2014). The silk is made by a highly specialized organ, the silk gland (SG), which synthesizes silk proteins and expels them at the end of the 5th larval instar in the form of a long and unique silk thread with a high commercial value (Li et al., 2014; Liu and Zhang, 2014).

This insect feeds on mulberry leaves (*Morus* spp. L.), from which it obtains all of the necessary nutrients for its growth and the development of essential metabolic functions that allow the synthesis of proteins, including silk protein fiber; therefore, changes in their food determine the quantity and quality of the silk thread produced by the larvae (Takano and Arai, 1978; Parra, 1991; Ki and Park, 2013). The quality of this food can be compromised if the mulberry plants are contaminated by insecticides, which should not be used on *B. mori* producing properties (Bora et al., 2012; Munhoz et al., 2013). However, contamination of mulberry plants can occur indirectly via drift when insecticides are used on other crops near the mulberry plantations and properties that produce *B. mori*, especially when the insecticides are applied by aerial spraying, which can reach the mulberry trees used to feed the silkworm larvae and impair sericulture activity (Bora et al., 2012; Munhoz et al., 2013).

A greater emphasis has been placed on studies regarding silkworms poisoned by broad-spectrum insecticides, especially organophosphorus commonly used on crops near *B. mori* production farms (Nath, 1993; Li et al., 2010, 2011; Peng et al., 2011; Yu et al., 2011, 2013; Gu et al., 2014); however, insecticides from other classes can also compromise the metabolic functions of these insects and the production of silk (Munhoz et al., 2013; Kordy, 2014). New insecticides have been described as safer for the environment and for nontarget beneficial species because they are selective in target species (Tunaz and Uygun, 2004; Dhadialla et al., 2005; Bel, 2014; Thangaraj et al., 2018). Among these new compounds, Novaluron, which is of the benzoylphenylurea class, acts by contact and ingestion and stands out as an insect growth regulator (IGR) that inhibits biochemical processes leading to the formation of chitin synthetase, which causes an abnormal deposition of the endocuticle. These changes mainly affect the larval stages and can cause death by abnormal endocuticular deposition and interrupted molting. Novaluron is used on agricultural crops, such as corn, soybeans, beans and sugar cane, to eliminate insect pests, mainly of the order Lepidoptera (Ishaaya et al., 2002; Tunaz and Uygun, 2004; Dhadialla et al., 2005; Cutler and Scott-Dupree, 2007; MAPA, 2018).

Novaluron has been considered acceptable for inclusion in Integrated Pest Management (IPM) programs because it initially appeared to be safer and selective in its mode of action, and it potentially acts against target species and presents reduced risks to the environment, mammals, birds, aquatic animals and to beneficial and nontarget insects, such as honeybees, ants, and others pollinators, parasites and predators (Tunaz and Uygun, 2004; Dhadialla et al., 2005; Alyokhin, 2009). Nevertheless, previous studies have demonstrated the negative effects of Novaluron on beneficial and economic nontarget insects, including *B. mori*, and have shown that it has a toxic capacity at all stages of the life cycle, may affect larval development by impairing insect molting and reducing larval hatching, oviposition and adult egg viability, thereby causing a reduction in their life time (Mommaerts et al., 2006; Cutler and Scott-Dupree, 2007; Santorum et al., 2019).

However, information on the impacts of Novaluron on silk production by *B. mori*, a nontarget insect of extreme economic importance, is not available. Recent studies have shown that great losses have occurred to sericulture by the mortality of *B. mori* larvae and cocoon production has been impacted, which is potentially due to the drift of insecticide residues in agricultural crops near the mulberry plantations and properties producing *B. mori*. These changes indicate the need to investigate the effects of Novaluron in the production of silk cocoons by silkworm larvae. Thus, in this study, we used cellular biomarkers to analyze the response of the SG epithelial cells from *B. mori* larvae exposed to mulberry leaves contaminated by Novaluron during the larval phase and their impact on cocoon production.

2. Materials and methods

2.1. Chemical

Novaluron (commercial formulation: Rimon Supra) was purchased from ADAMA Makhteshim Ltd. This formulation is registered with the Ministry of Agriculture, Livestock and Supply, Brazil (MAPA sob nº 14511).

2.2. Silkworm and Treatment

The larvae of hybrid *B. mori* in the second instar were obtained from a silk spinning company producing larvae for commercial purposes in the state of Paraná, Brazil and then maintained in our laboratory as described by Santorum et al. (2017) under controlled temperature, luminosity and humidity conditions.

Considering the official regulations for the phytosanitary pesticide system of the Ministry of Agriculture, Livestock and Food Supply of Brazil, the minimum approved dose of Novaluron for use in the field is 0.3 ml/L (0.03 g ia/L) and the maximum dose is 0.5 ml/L (0.05 g ia/L) for sugarcane, soybean and corn crops (MAPA, 2018). The aerial dispersion of this insecticide in the target crops generates residues during spraying, and because the crops are often grown near mulberry plantations and silkworm farms, theses residues can reach the mulberry plantations used to feed the silkworms. Therefore, we used the concentration 0.15 ml/L (0.015 g ia/L) (half the recommended minimum dose) in the experiments according to our previous studies (Santorum et al., 2019).

The treatment groups (TGs) consisted of *B. mori* larvae exposed once (24 h) to mulberry leaves containing Novaluron at two different instars:

- 1st day of the 3rd instar for morphological and ultrastructural analyses of the SG.

- 1st day of the 5th instar for morphological and ultrastructural analyses of the SG and for statistical analyses of productivity by analyzing the weight of the cocoons (full and empty/pupae), percentage of defective cocoons and ultrastructural analysis.

Fresh leaves treated with water were provided to larvae of the control groups (CGs). Each treatment was replicated six times, with ten *B. mori* larvae used per group for statistical analyses of productivity and in parallel groups of larvae receiving the same experimental treatments for morphological and ultrastructural analyses of the SG.

2.3. Morphological Evaluation of the Silk Gland

For all morphological procedures, the samples were collected as follows:

1- larvae from the 3rd and 4th larval instars that were treated on the 1st day of the 3rd instar;

2- larvae from the 5th larval instars that were treated on the 1st day of the 5th instar.

Selected larvae were cryoanesthetized for approximately 5 min and placed in a tube with cotton soaked in ethyl ether. The larvae were then dissected and rinsed with insect saline solution (0.1 M NaCl, 0.1 M Na_2HPO4 and 0.1 M KH_2PO_4), and the SG was removed. In the 4th and 5th instar larvae, it was possible to segment the SG into the different regions: the anterior (ASG), middle (MSG) and posterior regions (PSG). In 3rd instar larvae, the SG is still very rudimentary, and it was not possible to segment the SG by region; thus, it was kept whole (Tashiro et al., 1968; Sehnal and Akai, 1990; Dhawan and Gopinathan, 2003). After removal, the SG samples were subjected to different morphological analyses as described below.

2.3.1. Light Microscopy

The SG samples were fixed in DuBosq Brasil (Beçak and Paulete, 1976) for 24 h at 4°C. The SG samples 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) (Junqueira and Junqueira, 1983). The slides were observed and photographed with a Leica DM500 photomicroscope.

2.3.2. Transmission Electron Microscopy (TEM)

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

2.3.3. Scanning Electron Microscopy (SEM)

The SG samples were fixed for 48 h at room temperature in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3). Thereafter, the samples were washed in distilled water, postfixed in 1% osmium tetroxide diluted in distilled water for 30 min at room temperature, dehydrated through a graded series of ethanol, critical point-dried with CO_2 and coated with gold. The samples were examined and photographed using an FEI Quanta 200 scanning electron microscope (FEI Company, Eindhoven, Netherlands) at the Electron Microscopy Center of the Institute of Biosciences of Botucatu, SP, Brazil.

The cocoons were also analyzed and documented by SEM. Ten samples of cocoons of the CG and TG exposed to Novaluron on the 1st day of the 5th instar were collected and processed for SEM. The samples were desiccated in an oven at 37°C overnight, mounted on stubs, and then sputter coated with gold (Bal-Tec SCD 050). They were examined in a FEI Quanta 200 SEM with an accelerating voltage of 12.5 kV at the Electron Microscopy Center of the Bioscience Institute, UNESP, Botucatu-SP. Both the inner and outer sides of the cocoons were analyzed.

2.4. Productivity Analysis

The production variables were evaluated from male larvae and female larvae of the CG and TG that managed to survive exposure to Novaluron performed on the 1st day of the 5th instar and thus reached the spinning phase as described below.

Eight days after the start of the construction of the first cocoon, the cocoons were harvested and classified according to commercial criteria: normal cocoons, defective cocoons and thin-shelled cocoons (Hanada and Watanabe, 1986; Meneguim et al., 2007; Munhoz et al., 2013). From these cocoons, we performed the following analyses for the experimental group: weight of the full cocoons (with pupa and spoil inside) and cocoon shells (without pupa and spoil inside) and percentage of defective cocoons.

2.5. Statistical Analysis

The delineated experiment was composed of six replicates per treatment (CGs and TGs), with ten silkworm larvae per replicate.

Student's t-test was used for independent samples to evaluate the weight of cocoons constructed by both groups (Zar, 2009), and Goodman test, which involves contrasting binomial proportions, was used to compare the percentage in the production of normal and defective cocoons (Goodman, 1964). The results were expressed as the mean ± SD. The test was performed at a significance level of 5%.

3. Results and discussion

Novaluron can be applied to agricultural crops grown in fields close to mulberry plantations and silkworm production facilities to eliminate insect pests, mainly of the order Lepidoptera (MAPA, 2018); however, the application of this and other insecticides has caused great damage to and production losses of cocoons (Munhoz et al., 2013; Santorum et al., 2019). Sericulture in the south and southeast regions of Brazil has experienced great losses in the production of cocoons because of contamination of mulberry trees available to silkworm larvae. According to *B. mori* producers, this loss coincided with the application of insecticides in sugarcane plantations near silk production areas. Such insecticides have caused great losses in production because of the high mortality rate of silkworm larvae and generation of larvae that could not complete their cycle and thus produced defective, translucent and unproductive cocoons, and these losses have exceeded R$ 200 thousand (Globo Rural, 2011; Munhoz et al., 2013).

The negative impacts of Novaluron on the silk production of *B. mori* observed in this pioneering study reinforces the problems associated with insecticides on silk production reported by silkworm farms.

3.1. Morphological Alterations in the Silk Gland of B. mori Larvae

Novaluron caused cytotoxic effects to the three regions of the SG in the three analyzed larval instars of the *B. mori* larvae in the TGs. Only 24 hours of exposure of the larvae to Novaluron resulted in pronounced alterations throughout the entire SG, with extreme disorganization in the arrangement of epithelial cells and organ wall cells (Fig. 1-2). We observed vacuolization of the cytoplasm with the presence of intercellular and intracellular spaces and extreme nuclear and cytoplasmic rarefaction (Fig. 1, 3, 4); thus, fewer Golgi complexes and mitochondria were visualized, and dilation of the nuclear envelope and endoplasmic reticulum was observed (Fig. 3, 4). The apical region of the cells was more elongated, with cytoplasmic protrusions visualized along the SG epithelium, and microvilli were decreased and ruptures were observed in the intima in some regions of the epithelium. Less secretory vesicles containing protein material were observed in the SG of the TGs, and a smaller amount of proteins, fibroin and sericin was seen in the lumen; moreover, we observed significant faults in the deposition of protein layers in the SG lumen, and this factor was more pronounced in the 5th instar larvae. All these changes were visible from the 3rd instar larval after exposure to Novaluron and in the three SG regions, ASG, MSG and PSG, although they became more pronounced in the MSG and PSG and later instars (Fig. 1, 3, 4).

In addition, in some regions of the epithelium, we verified the spacing between the epithelial cells and the basal lamina where they are supported and observed a more severe detachment in the 5th instar larvae with the presence of empty spaces previously occupied by the epithelial cells (Figs. 1-4). These effects were confirmed by SEM analysis as indicated in figure 2, in which the external surface of the SG presented greater fragility, with ruptures in the basal lamina and spacing between the epithelial cells, and external surface protuberances were visualized. All of these alterations caused irregularities in the external surface of the three SG regions in the TG larvae, and the surfaces no longer had the same uniformity observed in the SG regions of the CGs larvae.

The SG of *B. mori* is a highly specialized organ, and its function is to synthesize silk proteins, mainly fibroin (produced in PSG) and sericin (produced in MSG), which are later expelled through the anterior duct (ASG) and spinneret at the end of the 5th instar, and these proteins make up the bulk of the silk

cocoon and are essential for sericulture (Akai, 1984; Li et al., 2014). However, few studies have focused on the cellular alterations undergone by the SG of *B. mori* after exposure to agrochemicals (Li et al., 2013, 2014, 2019) and the changes that occur during the course of the development its three regions. In addition, the effects of Novaluron on the morphology of the *B. mori* SG have not been previously studied; thus, our findings provide insights into the great toxic potential presented by this insecticide, which has been considered safer for nontarget organisms (Tunaz and Uygun, 2004; Dhadialla et al., 2005; Alyokhin, 2009). Additionally, our results suggest that even 24 h of exposure of *B. mori* larvae to Novaluron caused a reduction in the quality of cocoons, and significant pathological changes were observed in the SG and protein deposition decreased inside the SG lumen.

The separation of epithelial cells from the basal lamina and intercellular spaces may be related to the detachment of SG epithelial cells. Beaulaton and Lockshin (1982) indicated that the release of epithelial cells from insect tissues is preceded by the dilation of the intercellular space, and Kerr et al. (1972) showed that cells that undergo the process of apoptosis lose their normal cohesion with neighboring cells. The formation of intercellular spaces has also been reported in several treatments with biopesticides (Correia et al., 2009, Scudeler and Santos, 2013, Scudeler et al., 2016) and insecticides in insect tissues (Cruz et al., 2009; Santorum et al., 2019). We believe that the complexes in cell junctions can be broken down, thus allowing for the formation of these spaces. Cheville (2009) mentioned that during acute cellular swelling, the junctions may disintegrate, which can cause cells to lose their normal cohesion with neighboring cells. All of these changes seem to be associated with possible detoxification attempts or cellular stress responses against cytotoxicity (Cheville, 2009; Scudeler and Santos, 2013, Scudeler et al., 2014; Catae et al., 2014; Scudeler et al. 2016; Santorum et al., 2019).

Cytoplasmic vacuolation in secretory cells of insects (Armbruster et al., 1986; Jones and Bowen, 1993; Silva-Moraes and Bowen, 2000; Silva-Zacarin et al., 2007) and other observed effects, such as intracellular and intercellular spaces, detachment of the basal lamina, dilation and fragmentation of the endoplasmic reticulum, cytoplasmic fragmentation, and irregularity in the apical surface with the formation of protrusions and changes in microvilli, are described in the literature during the process of the degeneration of insect tissues (Matsuura et al., 1968; Bautz, 1979; Catae et al., 2014). In addition to the loss of microvilli, apical dilation of cells with the formation of cytoplasmic protrusions in tissues of insects exposed to insecticides has been associated with insect cell death (Rey et al., 1999; Hacker, 2000; Rello et al., 2005; Munhoz et al., 2013; Scudeler and Santos, 2013).

Li et al. (2013, 2014) reported severe damage to the SG of 5th instar *B. mori* exposed to Phoxim, an organophosphate applied in crops next to mulberry plantations. This insecticide caused vacuolization and scarcity of epithelial cells, the emergence of intercellular spaces and alterations in proteins in the lumen of the SG since large spaces appeared between epithelial cells and protein layers. The authors also verified a 46.05% decrease in total protein concentrations in the SG in larvae exposed to Phoxim and a drastic suppression of the genes involved in the synthesis of sericin in the MSG, and they believed that this set of changes resulted in a lack of cocoon construction by the larvae exposed to the insecticide. Recently similar results are described by Li et al. (2019) occurring with SG of *B. mori* after exposure to the fungicide tebuconazole. We believe that the same changes may have occurred in the SG cells of *B. mori* larvae in response to exposure to Novaluron, with cellular degeneration and alterations in important organelles, such as the endoplasmic reticulum, Golgi complex and mitochondria, which are important in the process of protein synthesis, thus resulting in a decrease in the production of silk proteins, since we observed the change in the deposition of protein layers along the lumen of the three regions of the SG as indicated in the figures 1, 3 and 4.

In relation to the occurrence of more severe changes in the 5th instar larvae of the TG observed in this study, we believe that these changes are related to the high biosynthesis and secretion of silk that should be performed by the secretory cells of the MSG and PSG at this stage of the insect's life cycle, i.e., the end of the 5th instar larval, when *B. mori* begins the construction of its silk cocoon (Tashiro et al., 1968; Montali et al., 2017). These changes were also more evident in the MSG and PSG precisely because they are the secretory regions of the SG that are responsible for the synthesis of the silk proteins; thus, these changes resulted in a decrease in the production and quality of these proteins, which consequently caused the production of defective cocoons.

Although Novaluron is not applied directly in mulberry plantations, when aerial spraying occurs in near agricultural crops, it can adhere to mulberry leaves and damage the silkworms that feed on the leaves and their silk production. Several studies on the effects of insecticides have indicated that disturbances in the life cycle of silkworms directly affect the production of quality cocoons (Kuribayashi, 1988; Nath et al., 1997; Yin et., 2008; Nasr, 2011; Munhoz et al., 2013; Yu et al., 2013; Gu et al., 2014; Li et al., 2013, 2014). Previously, we have shown that disturbances occur in the life cycle of exposed *B. mori* to Novaluron (Santorum et al., 2019), and negative effects have also been reported in other beneficial and nontarget insects (Cutler et al., 2006; Mommaerts et al., 2006; Cutler and Scott-Dupree, 2007). These previous results and

those observed in this study with *B. mori*, a nontarget species of great importance, indicate that Novaluron is not a safe insecticide, although it has been presumed and described to have selective toxicity to target insects without adversely affecting, or at least minimally affecting, nontarget species, which supported its inclusion in IPM programs.

3.2. Productivity Analysis

Novaluron negatively affected the silk productivity of silkworm larvae exposed on the 1st day of the 5th instar at a concentration of 0.15 mL/L. We verified the effects of the insecticide both on the weight of the cocoons and on the defective formation of cocoons of *B. mori* (Tables 1, 2).

When we analyzed the weight of the cocoons from TG larvae, we observed a significant reduction in the weight of all analyzed variables as indicated in Table 1, with a clear reduction in the weight of cocoons constructed by both male and female insects. The difference was significant both in the full cocoon and those with cocoon shells, indicating that in addition to the decrease in the weight of the cocoons constructed by *B. mori* larvae, *B. mori* pupa weight also decreased because Novaluron affects insect development and ecdysis by inhibiting chitin synthesis by decreasing pupation rates (Xu et al., 2017), which likely impaired cocoon construction.

Table 1. Weight of full cocoons (with pupa and spoil inside) and cocoon shells (without pupa and spoil inside) in grams [mean (standard deviation)] from 5th instar *B. mori* larvae exposed to Novaluron.

Cocoon	**Sex**	Groups		
		Control (g)	**Treatment (g)**	**p value**
Full cocoon	Males	1.910 (0.502)	1.480 (0.441)	$p < 0.05$
Cocoon shells	Males	0.473 (0.125)	0.333 (0.146)	$p < 0.01$
Full cocoon	Females	2.283 (0.464)	2.011 (0.313)	$p < 0.05$
Cocoon shells	Females	0.485 (0.128)	0.389 (0.079)	$p < 0.05$

Mean and standard deviation within the same line differ significantly. (Student's t-test).

The effects of Novaluron were also harmful to the quality of the cocoon constructed by the exposed insects. Table 2 shows that both male and female insects of the CG did not produce defective cocoons (0%); however, we observed significant results in the construction of the male and female cocoons in the TG (53.8% and 64.3%, respectively), which produced a large percentage of defective cocoons.

Table 2. Percentage of defective cocoons from 5th instar *B. mori* larvae exposed to Novaluron.

Sex	Groups		
	Control (%)	**Treatment (%)**	**p value**
Males	0	53.8	$p < 0.01$
Females	0	64.3	$p < 0.001$

Percentage, within the same line differ significantly. (Goodman's test).

The effects of Novaluron on cocoon formation was an important checkpoint in silk productivity since TG larvae exposed at the 5th instar presented difficulties in producing and packing silk threads. These effects resulted in the production of defective cocoons and/or thin-shelled cocoons, thus reducing the weight of the cocoons and consequently their quality as indicated in Tables 1 and 2 and Fig. 5. These negative effects observed in *B. mori* are related to the contamination of their food with insecticide, and studies have discussed the direct effects of exposing mulberry leaves to agrochemicals on the development, growth and production of the cocoons of *B. mori* (Bhosale and Kallapur, 1985; Sehnal and Akai, 1990; Yin et al., 2008; Kumutha et al., 2013; Munhoz et al., 2013; Bindu et al., 2015; Gu et al., 2014; Li et al., 2013, 2014). However, our results are the first description of the effects of Novaluron on the silk productivity of silkworms.

Nasr (2011) noted that changes in insect feeding lead to a compromise in the conversion of ingested and digested food, which can promote anomalies in later development. According to Anantharaman et al. (1993, 1994), the quality and quantity of cocoons produced by the silkworm depend on the quality of the mulberry leaves provided and the care taken during the process of producing *B. mori*. The literature reports the importance of the quality of the food consumed by *B. mori* during the larval phase, which is a highly specialized phytophagous insect that consumes and digests the leaves of different varieties of mulberry; however, the degree of nutrient utilization depends on the digestibility and efficiency of the food supplied to the larvae, and factors related to the consumption and the use of food are basic conditions for its development (Fukuda et al., 1960; Parra, 1991; Chowdhary, 1996). The quality of the food ingested in the larval stage affects the rate of growth, development, body weight, survival, and the production of cocoons (Nath, 1993; Li et al., 2010, 2011; Peng et al., 2011; Yu et al., 2011, 2013; Munhoz et al., 2013; Gu et al., 2014; Kordy, 2014; Nicodemo et al., 2014, 2018). In view of all these important factors associated with *B. mori* feeding, when these insects ingest poor quality food that may be contaminated by the dispersion of insecticides from surrounding crops, their development may be compromised, which adversely affects their life cycle and cocoon production and results in disabled adults (Chowdhary, 1996; Fukuda et al., 1960; Parra, 1991). Munhoz et al. (2013) analyzed the effects of another class of insecticide, Chlorantraniliprole, on *B. mori*

larvae; this chemical usually used for sugarcane borer control next to mulberry plantations, and negative effects on *B. mori* and the production of defective and thin-shelled cocoons have been observed. These authors note that affected cocoons are not useful for spinning; therefore, silk spinning companies do not accept thin-shelled cocoons and silkworm producers experience financial losses.

3.3. Ultrastructural Alterations of Silkworm Cocoons

Silkworm cocoons form a compact structure that is usually oval and completely covers the pupa inside (Fig. 5A). The TG showed severe morphological changes in their cocoons after exposure to Novaluron. We observe deformed, smaller, acrylated and thin-shelled cocoons with few silk threads wrapping the pupa (Fig. 5B, C). The cocoon spun by *B. mori* provides mechanical protection during the metamorphosis period (Hakimi et al., 2006; Zhao et al., 2007), protecting the pupa in the interior from possible danger in the natural environment, such as parasitism, predators and bacteria (Zhao et al., 2005; Chen et al., 2012). The production of silk via sericulture for commercial purposes and its increasing use in biotechnology as a sorbent material, gas filter, and biosensor have increasingly called for the modification of the microstructure and improvement of the properties of cocoons silkworm (Chen et al., 2013); thus, changes in the quality of the cocoon become important.

The cocoon is heterogeneous in structure and consists of two distinct types of silk fibers. The outer layer is composed of large-diameter “outer” fibers laid down in a crisscrossing pattern. The inner layer with fibers of smaller diameter incorporated with a solid matrix forms a more tightly woven pelade lining of the cocoon wall (Fig. 5E, I, M). The SEM analysis clearly showed ultrastructural changes in the cocoons of *B. mori* larvae exposed to Novaluron in the 5^{th} instar, and many of the fibers on the outer surface of the cocoon were loose and chaotically arranged with faults in the union of the silk fibers and with a high degree of defective formation of fibers with a nonwoven structure, thus causing a lack of uniformity and leaving the cocoon surfaces with a rough texture (Fig. 5F, G, H). Changes in the external fibers of the cocoon may reflect deleterious effects on the fixation of the cocoon to a substrate, which leads to less protection in a natural environment (Danks, 2002). On the inner surface of the TG cocoon, we also observed faults in fibers, a nonwoven structure and large amounts of fibers that did not mix in the matrix, thus giving a fibrous appearance to the inner surface of the cocoon (Fig. 5J, K, L, N, O, P). The integrity of the innermost layer of the cocoon, the pelade, is of utmost importance because it helps prevent the abrasion of the waterproof

external cuticle of the pupa during its frequent movements within the cocoon. Cocoon fibers also have both antifungal and antibacterial properties (Danks, 2004).

B. mori larvae are sensitive to agrochemicals, and contact with these products may compromise the normal development of the SG and cocoon production (Maribashetty et al., 1999). We believe that the same effect occurred in our study, where the contact of *B. mori* larvae with Novaluron resulted in several alterations in the SG of the insect, thus impairing its ability to synthesize the proteins of the silk to form the silk threads and leading to a decrease in weight and quality of the cocoons produced as indicated in Tables 1 and 2. An important factor to consider in Novaluron's mode of action is the growth regulator and chitin synthesis inhibitor, which compromise insect molting (Ishaaya et al., 2002; Tunaz and Uygun, 2004; Dhadialla et al., 2005; MAPA, 2018). Thus, the exposure of *B. mori* larvae to Novaluron also results in difficulties carrying out the molt from the larval phase to the pupa, which consequently may influence the efficacy of cocoon functions during pupation, make the species more vulnerable to natural enemies and environmental factors, and impair silk production by *B. mori* farmers.

4. Conclusions

Our results indicate that exposure of *B. mori* larvae to Novaluron-contaminated mulberry leaves, even at half the minimum concentration used in agricultural plantations, has toxic effects on the ASG, MSG and PSG epithelium and impacts the production of silk cocoons. All of these factors can cause large losses in silkworm production on sericulture farms.

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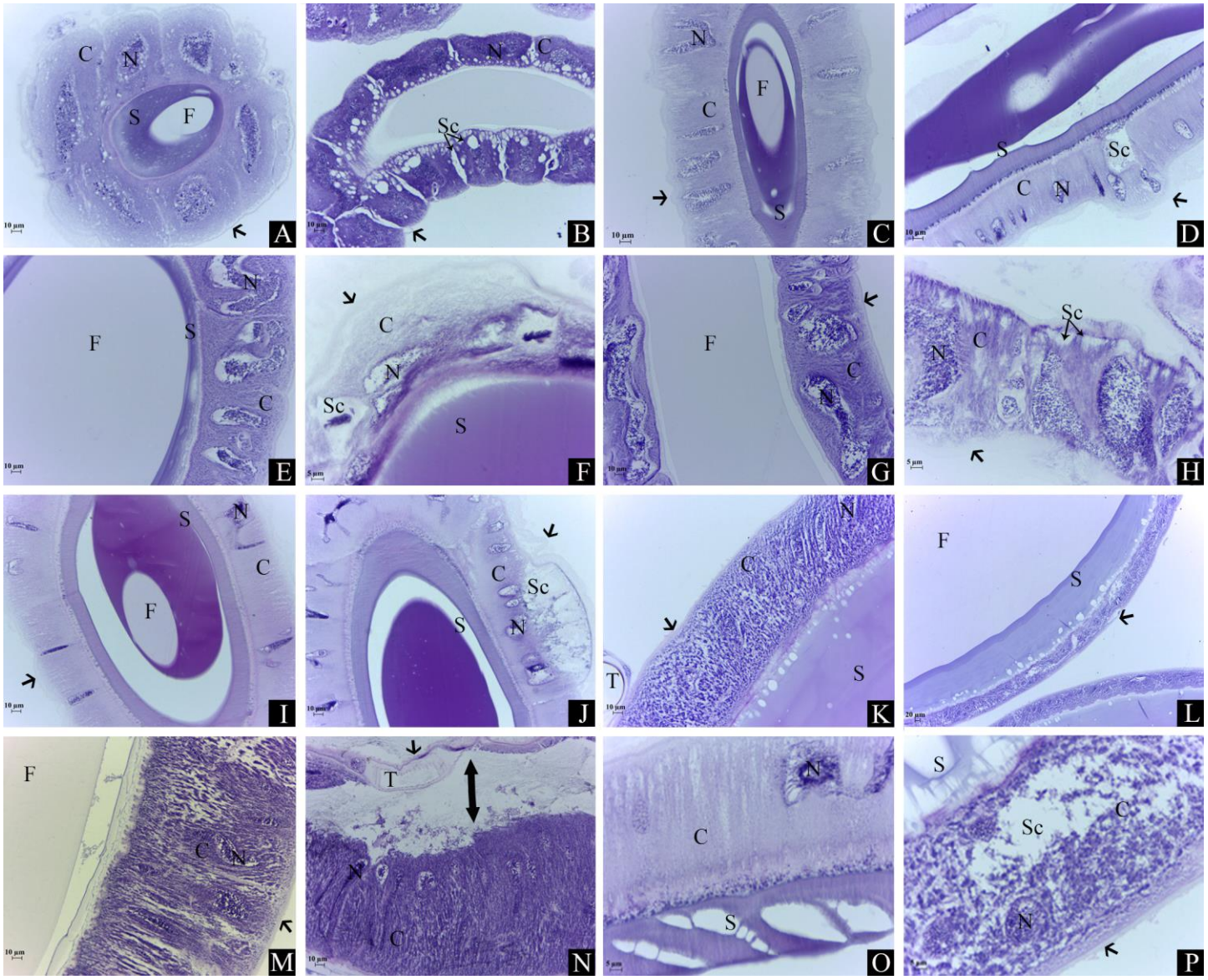

Fig. 1. Photomicrographs of *B. mori* silk gland larvae. **A, B**: SG of CG and TG from 3rd instar, respectively, presented cells seated on the basal lamina (arrow), note in A, the presence of the sericin (S) and fibroin (F) layers in the lumen. Cytoplasm (C), nucleus (N). Note in B the cellular rarefaction with the presence of intracellular and intercellular spaces (Sc). **C, D:** ASG of CG and TG from 4th instar, respectively. **E, F:** MSG of CG and TG from 4th instar, respectively. **G, H:** PSG of CG and TG from 4th instar, respectively. **I, J:** ASG of CG and TG from 5th instar, respectively. **K, L:** MSG of CG and TG from 5th instar, respectively. Trachea (T). **M, N:** PSG of CG and TG from 5th instar, respectively. Note in N the separation of epithelial cells from the basal lamina (double arrow). **O, P:** PSG of TG from 5th instar, in greater increase showing the cellular rarefaction and faults in the deposition of sericin in the lumen.

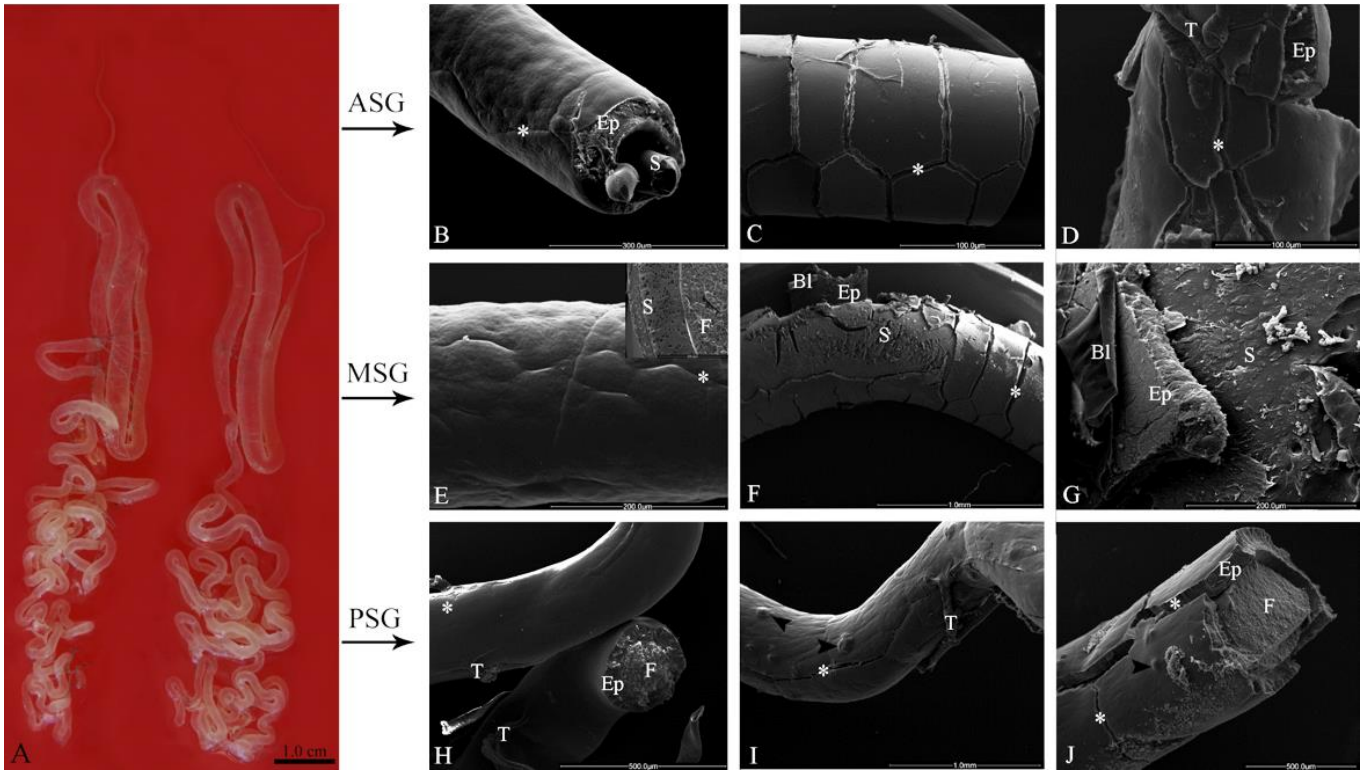

Fig. 2. A: Morphology of the *B. mori* silk gland from CG 5th instar, showing anterior (ASG), middle (MSG), and posterior silk gland (PSG). **B – J:** SEM micrograph of the *B. mori* silk gland from 5th instar. **B, E, H:** ASG, MSG and PSG of CG, respectively. Note the presence of a regular epithelium (Ep), formed by juxtaposed cells (*) and the presence of the sericin (S) and fibroin (F) layers in the lumen of the SG, pointed in the inset in E. Trachea (T). **C, D:** ASG of TG, to note greater fragility of the epithelium of the SG and the separation between epithelial cells (*). **F, G:** MSG of TG with detachment of the epithelial cells from basal lamina (Bl). **I, J:** PSG of TG presenting irregularities in the external surface with protuberances (arrow head).

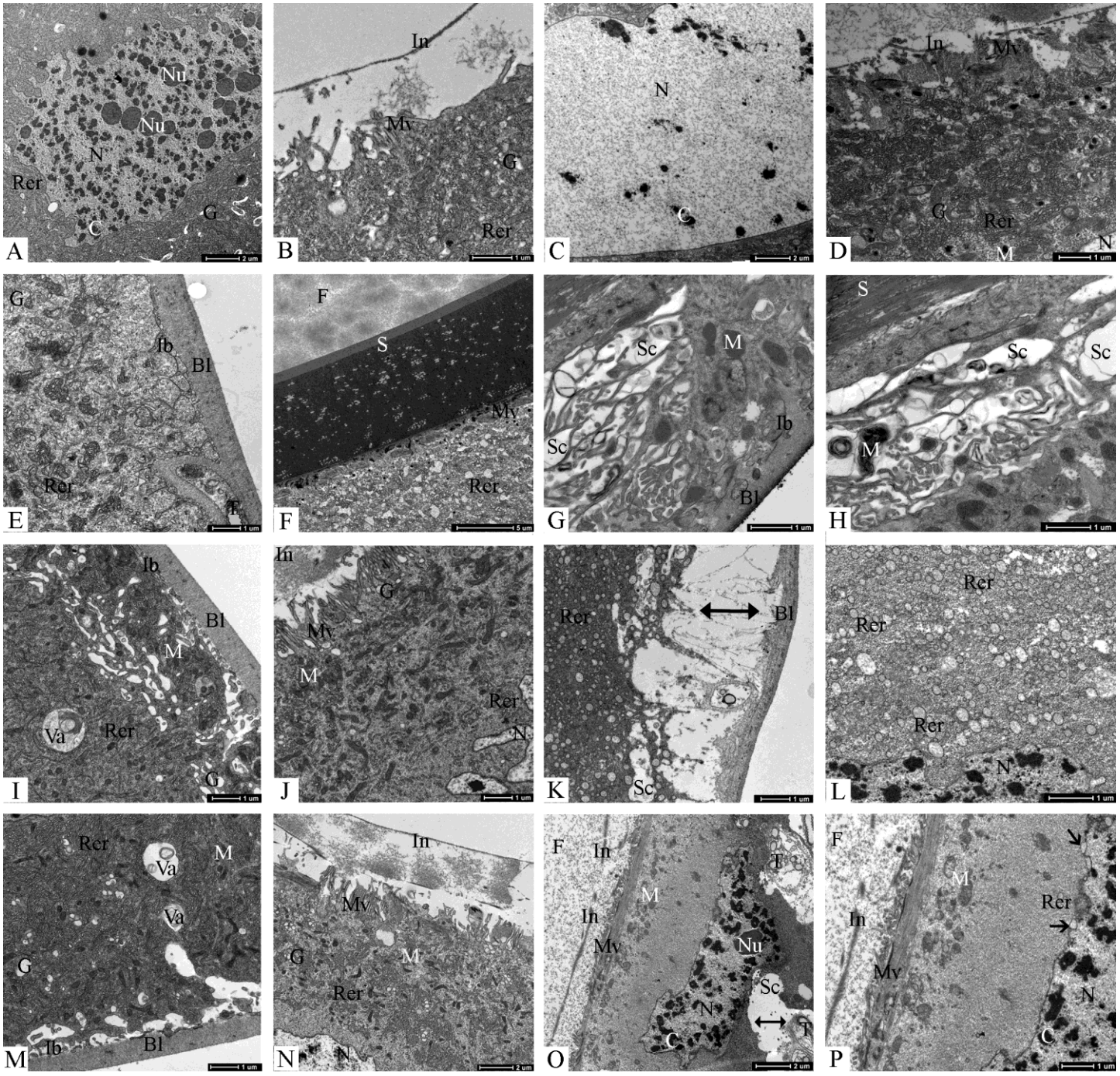

Fig. 3. Electron micrographs of *B. mori* silk gland larvae. **A, B:** CG from 3rd instar. In A, general aspect of the nucleus (N) with nucleoli (Nc), chromatin (C) and perinuclear cytoplasm with golgi complex (G), rough endoplasmic reticulum (Rer). In B, apical region of epithelial cells formed by microvilli (Mv) covered by the intima (In). **C, D:** TG from 3rd instar. Note in C, the rarefaction of the nuclei and in D ruptures in the intima. Mitochondria (M). **E, F:** ASG from CG 4rd instar. In E, basal region of epithelial cells with basal lamina (Bl) and discrete basal invaginations (Ib), trachea (T). In F, apical region and sericin (S) and fibroin (F) layers in the lumen of the SG. **G, H:** ASG from TG 4th instar. To note rarefaction of the cytoplasm with cellular spaces (Sc) and mitochondria (M). **I, J:** MSG from CG 4th instar. Vacuoles with filamentous material (Va). **K, L:** MSG from TG 4th instar. Note in K, the separation of epithelial cells from the basal lamina (double arrow) and in L, large dilation and fragmentation of the rough endoplasmic reticulum (Rer). **M, N:** PSG from CG 4th instar. **O, P:** PSG from TG 4th instar, note the dilation of the nuclear envelope (arrow), ruptures in the intima and alterations in fibroin seen in the lumen.

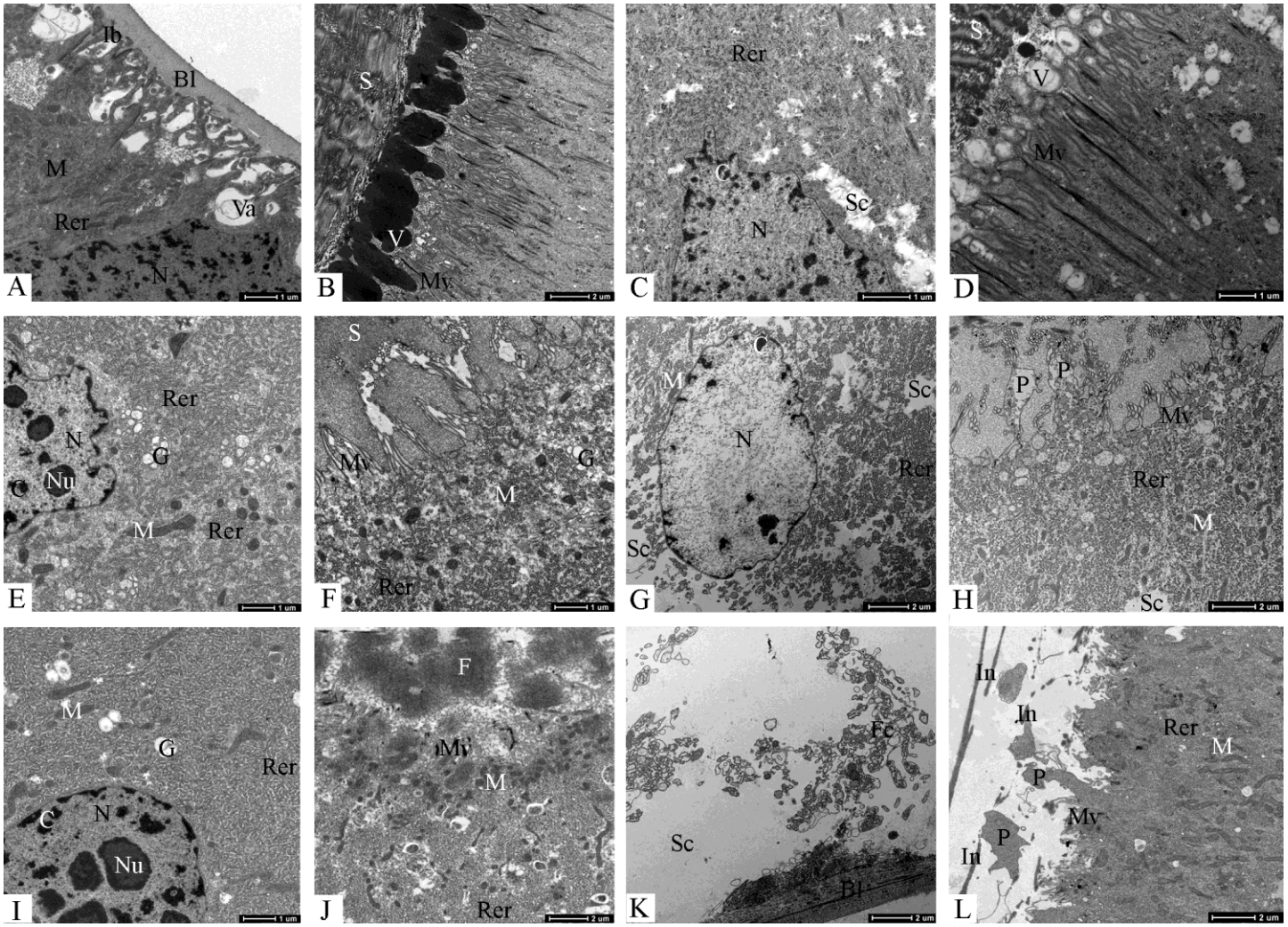

Fig. 4. Electron micrographs of *B. mori* silk gland larvae from 5th instar**. A, B:** ASG from CG. In A, basal region of epithelial cells with basal lamina (Bl), discrete basal invaginations (Ib), vacuoles with filamentous material (Va), rough endoplasmic reticulum (Rer), mitochondria (M) and nucleus (N). In B, apical region of epithelial cells formed by microvilli (Mv) and the presence of secretory vesicles with protein material (V). Sericin (S) in the lumen. **C, D:** ASG from TG, note the presence cellular spaces (Sc) and secretory vesicles without protein material (V). Chromatin (C). **E, F:** MSG from CG. Nucleoli (Nc), golgi complex (G). **G, H:** MSG from TG. Note in G, rarefaction of the nuclei and dilation and fragmentation of the rough endoplasmic reticulum (Rer). In H, note apical region with alterations in microvilli (Mv) with the formation of cytoplasmic protrusions (P) towards the lumen. **I, J:** PSG from CG. Fibroin (F) in the lumen. **K, L:** PSG from TG. Is shown in K the cellular spaces (Sc) with the absence of the epithelial cells seated on the basal lamina (Bl) with the presence of only cellular fragments (Fc). In L, is shown details of the apical region with the cytoplasmic protrusions and ruptures in the intima (In).

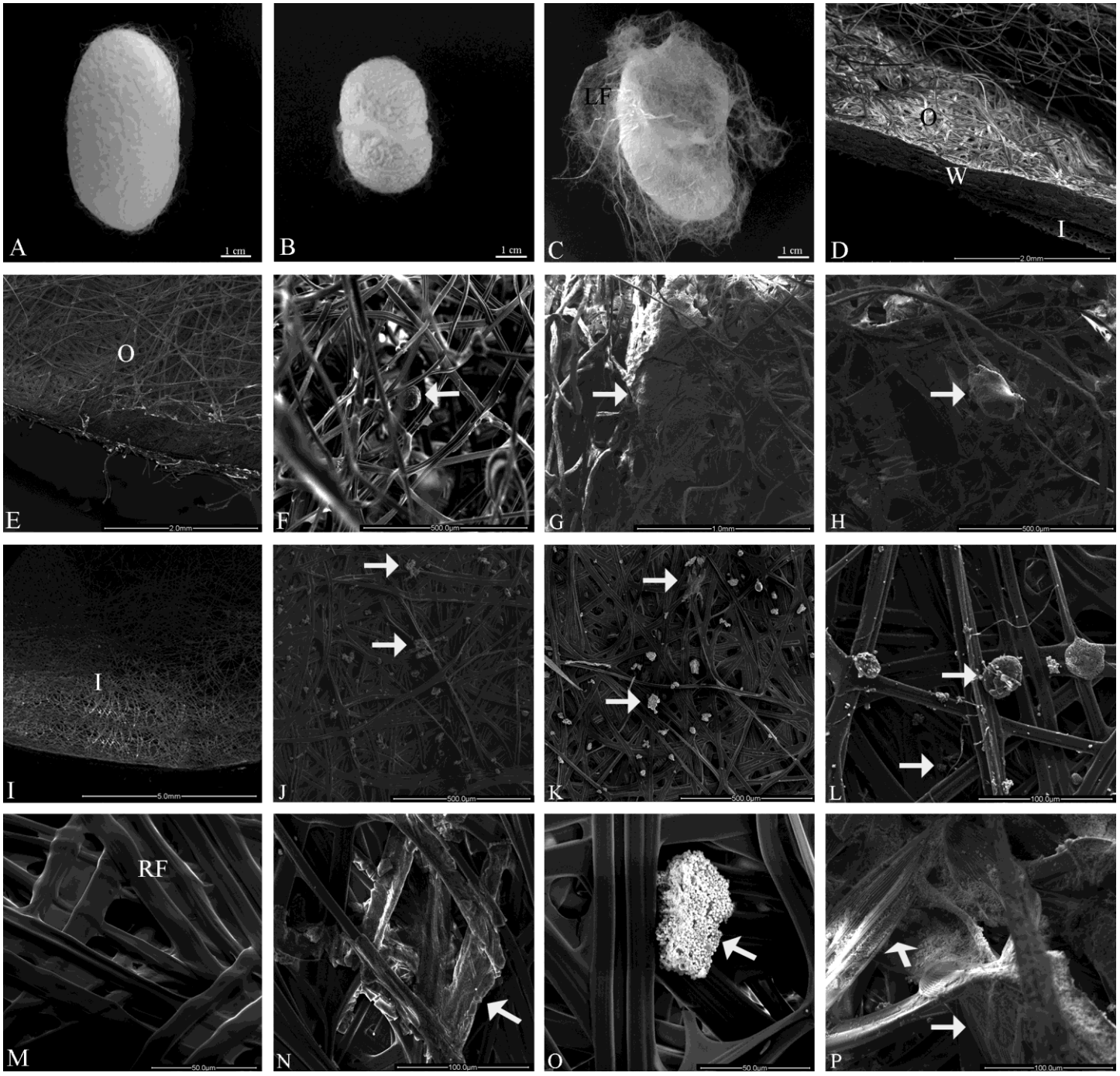

Fig. 5. Morphology of the *B. mori* cocoon. **A:** CG, the cocoon of untreated larvae was regular with a rounded shape and compact. **B, C:** TG, the treated larvae produced defective cocoons with less amount of woven fibers, exhibiting smaller size in B and cocoon of thin-shelled in C with many loose fibers (LF) in the outer surface of the cocoon. **D – P:** SEM micrograph of cocoons of *B. mori*. **D, E, I, M:** CG, section of the entire cocoon wall (W) composed of the fibers of the outer layer (O) and the inner layer (I). Note the outer and inner layers of the cocoon with fibers that are close to each other and in M showing in greater increase the inner layer of the cocoon with regularity in the woven fibers (RF). **F, G, H:** TG outer surface of the cocoon, with many of the loose fibers and faults in the silk fibers and a high degree of nonwoven structure (arrow), causing a lack of uniformity and leaving the cocoon surfaces with a rough texture. **J, K, L:** TG inner surface of the cocoon and in **N, O, P:** greater increase, showing the details of this inner surface of TG.

4.3. CAPÍTULO 3

Reproductive toxicity of Novaluron in *Bombyx mori* (Lepidoptera: Bombycidae) and its impact on egg production

Marilucia Santorum [a], Bertha Gastelbondo Pastrana [a], Márcia Santorum [b], Rose Meire Costa [c], Daniela Carvalho dos Santos [a, d, *]

[a] Laboratory of Insects, Department of Morphology, São Paulo State University (UNESP), Institute of Biosciences, Botucatu, Brazil.

[b] Postgraduate in Agricultural Engineering, State University of Western Paraná, (UNIOESTE), Cascavel, PR, Brazil.

[c] Center of Biological Sciences and Health, State University of Western Paraná (UNIOESTE), Cascavel, PR, Brazil.

[d] Electron Microscopy Center of the Institute of Biosciences of Botucatu, UNESP, SP, Brazil.

* daniela.santos@unesp.br

ABSTRACT

Bombyx mori was used as a model to evaluate the reprotoxicity of the Novaluron in insects. The morphological analyses of the testes and ovaries of *B. mori* throughout their life cycle revealed important alterations in the germinative and somatic cells involved in the spermatogenesis and oogenesis process. We observed that Novaluron affected the organization, distribution and development of the cysts containing male germ cells and it is possible to see morphological features of cell death in all phases of the testicular development. Similar characteristics were also seen in the cells present in the treated *B. mori* ovaries, suggesting the occurrence of cell death in both organs, in addition to significant reduction in oviposition of eggs by female moths. We demonstrated reproductive toxicity of Novaluron to the nontarget beneficial insect silkworm, and the results provide a theoretical basis for revealing the reprotoxicity of this insecticide in other nontarget beneficial insects.

Keywords: Silkworm; Testes; Ovaries; Ultrastructure; Insecticide; Reprotoxicity, Oviposition.

Introduction

Due to the major problems faced by agriculture with insect pests, the biological control of these has been generally carried out with the use of synthetic insecticides which, despite their effectiveness, promote several problems to the ecosystem and nontarget species [1,2], in this scenario insecticides belonging to the benzoylphenylurea class have been considered safer, and thus used in order to present less harmful effects on the environment and on nontarget beneficial species [3–5]. Among the benzophenylureas stands out Novaluron, an insect growth regulator, with a mode of action via contact and ingestion, which inhibits the biochemical processes responsible for the formation of chitin synthetase, causing an abnormal deposition of the endocuticle and interrupting molting [3,4,6,7].

Novaluron has been used in the control of insect pests of the order Lepidoptera, Coleoptera, Diptera and Hemiptera [6,7], but previous studies have shown that Novaluron may have toxic effects on the reproductive functions of nontarget beneficial insects such as predators, parasitoids and pollinators, impairing egg oviposition and viability, and larval hatching [6,8], important point to be considered since the reproductive organs and the processes developed by them in the reproduction of the insects is of extreme importance for the maintenance of the species. However, there are few reports on its reproductive toxicity at the cellular level, and the underlying mechanisms still little elucidated.

The silkworm *Bombyx mori* L. (Lepidoptera: Bombycidae) is a reference insect among Lepidoptera, which is widely used as a model organism in several areas such as cellular and molecular biology, physiology, biotechnology, medicine and toxicology, besides its great importance in the economy by the production of silk [9–11]. In the present study, *B. mori* was used to the study the reproductive toxicity of Novaluron toward insects. Our previous study showed that Novaluron has great toxic potential in the development of *B. mori*, compromising all phases of its life cycle [12]. To better understand the impact of Novaluron at the reproductive level throughout the life cycle of the silkworm, we evaluated its toxic effects on the morphology and ultrastructure of the testes and ovaries throughout their life cycle, as well as egg oviposition moth exposed to the insecticide. The findings provide a theoretical basis for revealing the reprotoxicity of Novaluron toward insects.

Materials and methods

Silkworm and Chemical

Hybrid, second instar *B. mori* larvae were obtained from a silk spinning company producing larvae for commercial purposes in the State of Paraná, Brazil. Larvae were maintained in our laboratory under controlled conditions as described by Santorum et al. [13].

The insecticide Novaluron (commercial formulation: Rimon Supra) was purchased from ADAMA Makhteshim Ltd, it is registered in Brazil by the Ministry of Agriculture, Livestock, and Supply (No. 14511) and is certified internationally and in Brazil for use in agricultural crops.

Bioassays

Our bioassays were based on the official regulation of the phytosanitary pesticide system of the Ministry of Agriculture, Livestock and Food Supply of Brazil, where the minimum approved dose of Novaluron for use in the field is 0.3 ml/L and the maximum dose is 0.5 ml/L for sugarcane, soybean and corn crop [7]. These crops are often grown near the production farms of silkworm and mulberry plantations (*Morus spp.)* insect food, and thus considering the minimum dose of this insecticide used in these cultures and the aerial dispersion of these residues during spraying, we used the concentration 0.15 ml/L that is half the recommended minimum dose. Thus, fresh mulberry leaves were immersed in aqueous solutions containing 0.15 ml/L Novaluron (Rimon Supra), and after air-drying, the mulberry leaves were provided to *B. mori* larvae of the treatment groups (TGs) for 24 h *ad libitum*. After exposure for 24 h, the larvae were fed mulberry leaves free of Novaluron.

The TGs consisted of *B. mori* larvae exposed once to mulberry leaves containing Novaluron at two different instars:

- 1st day of the 3rd instar for morphological analyses of testes and ovaries.

- 1st day of the 5th instar for morphological analyses of testes and ovaries and for statistical analyses of effects of Novaluron on oviposition (counting the number of unfertilized eggs oviposited by adult females - moths).

Fresh leaves treated with water were provided to larvae of the control groups (CGs). Each treatment was replicated six times, with ten *B. mori* larvae used per group for statistical analyses of oviposition and in parallel groups of larvae receiving the same experimental treatments for morphological analyses.

Morphological Analyses

For all morphological procedures described below, the effects of Novaluron on *B. mori* testes and ovaries were evaluated. The samples were collected as follows:

1- larvae from the 3rd and 4th larval instars that were treated on the 1st day of the 3rd instar.

2- larvae from the 5th larval instars that were treated on the 1st day of the 5th instar.

The larvae were selected and cryoanesthetized for approximately 5 min and placed in a tube with cotton soaked in ethyl ether. The larvae were then dissected and rinsed with insect saline solution (0.1 M NaCl, 0.1 M Na_2HPO4 and 0.1 M KH_2PO_4), and the testes and ovaries were removed and submitted to different morphological analyses, as described below.

Light Microscopy

The samples were fixed in DuBosq Brasil [14] for 24 h at 4°C. The samples 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) [15]. The control slides were subjected to the same preparations. The slides were observed with a Leica DM500 photomicroscope.

Transmission Electron Microscopy (TEM)

For TEM, the samples were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.3) [16] for 24 h at room temperature and then postfixed in 1% osmium tetroxide in the same buffer for 2 h. After being washed with distilled water, the samples were contrasted with an aqueous solution of 0.5% uranyl acetate for 2 h at room temperature, dehydrated in a graded acetone series (50%, 70%, 90% and 100%), and embedded in Araldite resin. Ultrathin sections were contrasted with uranyl acetate and lead citrate and were then observed and photographed with a Tecnai Spirit transmission electron microscope (FEI Company, Eindhoven, Netherlands) at the Electron Microscopy Center of the Institute of Biosciences of Botucatu, SP, Brazil.

Scanning Electron Microscopy (SEM)

The samples were fixed for 48 h at room temperature in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3). Thereafter, the samples were washed in distilled water, postfixed in 1% osmium tetroxide diluted in distilled water for 30 min at room temperature, dehydrated through a graded series of ethanol,

critical point-dried with CO_2 and coated with gold. The samples were examined and photographed using an FEI Quanta 200 scanning electron microscope (FEI Company, Eindhoven, Netherlands) at the Electron Microscopy Center of the Institute of Biosciences of Botucatu, SP, Brazil.

Effects of Novaluron on Oviposition (unfertilized eggs)

The oviposition were evaluated from female larvae of the CG and TG that managed to survive exposure to Novaluron performed on the 1st day of the 5th instar and thus reach adulthood, as described below:

Twelve moths that managed to survive the exposure to Novaluron could have their oviposition rate evaluated, while for CG we used twenty moths, thus after the females moths emerged were then individualized in polyethylene boxes (11 cm height x 13,5 cm diameter), maintained in our laboratory under controlled conditions until death and the number of eggs was counted soon after the death of each moths.

Statistical Analysis

The delineated experiment was composed of six replicates per treatment (CGs and TGs), with ten silkworm larvae per replicate.

To evaluate the difference in oviposition rate between the moths of the CG and the TG, we used the nonparametric test of Mann-Whitney U-test [17]. The results were expressed as median (minimum value and maximum value) and the test was performed at a significance level of 5%.

Results and discussion

Effects of Novaluron on Testicular Morphology of B. mori

In the CG, the testes of *B. mori* in the 3rd, 4th, 5th larval instars, pupae and adults (moths) have a kidney shape, with gradual development of their germline cysts (Fig. 1). Each testes is surrounded by a cellular tunica (internal and external), creating a smooth outer surface that is surrounded by a layer of adipose tissue and many trachea. This cell emits septa dividing the testes into four testicular follicles, and these follicles are fused with one another and fixed in a common structure, the duct deferens located at the proximal end (Fig. 1). Each testicular follicle is composed by germ line cells, the sperm cells, surrounded by

somatic ones, the cystic cells. The process of cyst maturation during spermatogenesis occurs from the distal-proximal direction within the testes, where it is possible to observe germline cysts at different stages of development. At the distal end, where the germ line cells are located, a region of cell proliferation, it is possible to see spermatogonia and at the proximal end, it is easy to detected many spermatocyte cysts (groups of germ cells interconnected by cytoplasmic bridges). In the testes of larvae in the 3rd instar, sperm cysts were observed at different stages of development, and the cysts containing spermatogonia and spermatocytes were observed in the three larval instars analyzed (3rd, 4th and 5th). In addition, from larvae of 5th instar to adult stage (moth) it was also possible to visualize the occurrence of cysts of spermatids at different stages of maturation and spermatozoa (Figs, 1 and 2A-D).

Novaluron caused cytotoxic effects in the testes of the TGs in the three instars analyzed (3rd, 4th and 5th), in the pupa and in the adult of *B. mori* exposed to Novaluron, where the exposure resulted in pronounced alterations in the testicular cyst cells, with extreme disorganization in the arrangement of these cells. We observed that the anatomical form of the testes in the TGs does not change with the exposure to Novaluron, but histopathological alterations were observed, such as ruptures in the cellular tunica and septa surrounding the follicles in regions of the cystic cells that surround the spermatic cysts, as well as in some spermatids. It was also possible to see a significant tissue reduction inside the testes, with many spaces between the spermatic cysts, exhibiting a large amount of free cellular residue and making the distribution of cysts in the testicular follicles irregular compared to CG (Fig. 2).

In the observations in TEM, we confirmed that the exposure resulted in extreme disorganization in the arrangement of the cellular tunica and in the cysts of the germ cells, showing the occurrence of the separation between the cells and the presence of many cytoplasmic vacuoles and apoptotic bodies in all phases of the testicular development, thus suggesting the occurrence of cell death in this epithelium (Fig. 3).

Effects of Novaluron on Ovarian Morphology of B. mori

The same analyzes described above for testes were also performed for the *B. mori* ovaries of both experimental groups (CGs and TGs), where the ovaries of the CGs are triangular in shape and are formed by four ovarioles, which in the larval phase are still short and gradually become longer, assuming a tubular shape in the pupa and adult phases (Fig. 4).

In *B. mori* there is a polytrophic meroistic ovarioles, occurring internally the morpho-functional differentiation of germ and somatic cells, originating the ovarian follicles. Each ovariole is externally coated

with a continuous acellular layer, the tunica propria, and more externally to this is the epithelial sheath. Inside each ovariole is the germarium, region where the germ cells are divided originating the cysts, which together with somatic cells, differentiate into ovarian follicles. Just below the germarium, is the vitelarium region, where the growth of the ovarian follicles occurs and the differentiation of germ cells in one oocyte and seven nurse cells per ovarian follicle; which are surrounded by a layer of somatic cells called follicular cells that form the ovarian follicle (Fig. 5A-G).

During the 3rd, 4th and 5th larval instars, pupae and adult, it is possible to see that the development of the ovarian follicles is progressive. In 3rd and 4th larvae instars we observed the presence of many undifferentiated somatic and germ cells in the germarium region and in the vitelarium region, few ovarian follicles formed by the oocyte, nurse cells and surrounding follicular cells, however from the 5th larval instar we found ovarian follicles at a later stage of development, already in the pupae phase, it was possible to visualize the occurrence of degeneration of some nurse cells, leaving the oocyte involved by the follicular cells that decrease in height, and in the adult phase (moth) since they had already performed the egg laying, only the follicular epithelium can be observed (Fig. 5A-H).

Cytotoxic effects of the Novaluron were observed in the ovaries of the TGs in the three instars analyzed (3rd, 4th and 5th), in the pupa and in the adult of *B. mori*, where the exposure resulted in pronounced alterations in the ovarian follicle cells, with extreme disorganization in the arrangement of these cells. We observed alteration in the outer surface of the ovarioles of the pupa and moth of the TG, which was irregular, with a granular aspect exhibiting small protuberances (Fig. 4C and D).

Morphological alterations were observed by light microscopy and TEM, such as extreme disorganization in the epithelial sheath and tunica propria of the ovarioles, in germ cells and somatic cells of the germarium already in the larvae of 3rd and 4th instars, as well as in the ovarian follicles with their nurse cells and oocytes in the 5th larval instar and in the pupae. We verified ruptures in the epithelial sheath and detachment between these cells and tunica propria. There are an increase cell spacing between cells (from epithelial sheath, germ cells and follicular cells) (Figs. 5 and 6). Dilatation and fragmentation of rough endoplasmic reticulum, cytoplasmic vacuoles and apoptotic bodies were also observed in the ovarian follicle cells of the TGs in all stages analyzed (Figs 5 and 6), suggesting that as well as in the *B. mori* testes exposed to Novaluron, the cell death could be induced in the ovarian cells during the ovarian development in treated insects.

Effects of Novaluron on Oviposition (unfertilized eggs)

Novaluron negatively affected the oviposition of females moths *B. mori* from the TG exposed at 1st day of the 5th instar, because we observed a significant reduction in the number of eggs oviposited by the TG compared to CG as shown in table 1. We infer that this reduction in the number of the moths eggs of the TG is consequently associated with the changes in the ovarian of the insect from exposure to the insecticide, and that these effects may thus negatively affect the reproduction of *B. mori* by reducing the amount of eggs.

Table 1. Eggs oviposition (unfertilized) [median (minimum value; maximum value)] of *B. mori* moths exposed to Novaluron.

Groups		
Control	**Treatment**	**p value**
796 (185; 928) (20)*	597 (0; 872) (12)*	$p < 0.005$

Date within the same line differ significantly (Mann-Whitney U-test).
* Number of females evaluated per group.

Discussion

The morphology of the reproductive organs as well as the process of spermatogenesis in the testes and oogenesis in the ovary is similarly pointed out in the literature in *B. mori* [18–20].

However, there are few studies that have focused on morphological and ultrastructural alterations suffered by these important organs of *B. mori* after exposure to agrochemicals [21–23], so little that they indicate the changes that occur during the development of these organs throughout the life cycle of the insect, this being an important point to be analyzed since it can give us a theoretical basis to reveal the reprotoxicity of Novaluron in beneficial species and nontarget. Moreover, our previous studies have found that Novaluron has great toxic potential in the different stages of the life cycle of *B. mori* [15].

Novaluron effects associated with reproductive aspects of insects were previously reported in target insects (crop pests) [24,25] as in some beneficial and nontarget insects [18,19]. Novaluron acts as an insect growth regulator through the inhibition of chitin synthesis, affecting insect molting and mainly affecting larval development [3,4,6,7,12,26], but some reports indicate its effects on fecundity, fertility and longevity of adult insects [6,8].

In this study, we verified that Novaluron negatively affects the testes and the spermatogenesis process in the nontarget *B. mori* insect, due to severe alterations in the testes cells, we clearly demonstrate that with only 24 h of *B. mori* larvae to the insecticide affected the organization, distribution, and

development of sperm cysts. Authors point to similar results in the testes of insects exposed to biopesticide Azadirachtin, that is also insect growth regulator, and they discuss that exposure to this compound inhibits the formation of cysts around spermatogonia, and thus the development of spermatocytes is impaired, Azadirachtin also caused disintegration of the testes epithelium, degeneration of spermatids and spermatozoa, as seen in this study with Novaluron [27,28]. Szöllösi [29] mentions that in the insect testis, the relationship between germ-somatic cell is regulated by the somatic cells, which surrounding the developing gametes, they isolate them from the rest of the body, which promotes permeability restricted to the hemolymph molecules creating the "blood-testis barrier", but allows important communication between germ and somatic cells through gap junctions. We believe that the morphological alterations, such as ruptures in the cellular tunica and septa surrounding the follicles, in regions of the cystic cells that surround the spermatic cysts, as well as in some spermatids visualized in the testis of *B. mori* exposed to Novaluron may be a consequence of the penetration of the insecticide via this "blood-testis barrier" that undergoes degeneration process.

In relation to the presence of many cytoplasmic vacuoles and apoptotic bodies in the testicular follicles and in all phases of the testicular development verified in our study, agrees with results Tang et al. [22] which reported on the exposure of *B. mori* to sodium fluoride, leading to a more dilated and degranulated endoplasmic reticulum in the spermatocytes and presence of many necrotic vacuoles, associating these characteristics with the occurrence of apoptosis and necrosis in the epithelium [22].

In addition to the changes observed in the testes of *B. mori*, alterations were also found in the ovaries of the insect as described in the section results and the changes observed as the alteration in the external surface of the ovarioles, rupture in the epithelial sheath and tunica propria and detachment between them, as well as, occurrence of spacing between the cells of the sheath, between the germ cells and between the follicular ones, are comparable to the damages caused by insecticides and biopesticides in insects, as seen by Silva et al. [2] in females of *Spodoptera frugiperda,* treated with citronella oil, presented several morphological changes in the ovaries, among them stratification of follicular cells, gaps surrounded by the presence of epithelium inside the vitelarium, undeveloped nurse cells and thinner connective sheath. Azadirachtin also caused degeneration of oocytes in *Heteracris littoralis* (Ramb.) (Orthoptera: Acrididae), where the follicular cells were partially destroyed, and with the presence of many vacuoles in the epithelium [30]. The insecticide Lufenuron similar to Novaluron, of the class of benzoylphenylurea and thus, insect growth regulator, was studied previously in *Schistocerca gregraria,* leading to decline and disintegration of follicular epithelial cell layers in ovarioles and organelles disintegrated [31]. Cell damage such as dilatation

and fragmentation of rough endoplasmic reticulum, presence of cytoplasmic vacuoles and apoptotic bodies in the germ and follicular cells from the ovaries were observed and we believe that Novaluron could induce cell death in ovarian follicle of *B. mori* TGs [22] .

We believe that the various alterations in the ovary resulting from the exposure of *B. mori* larvae to Novaluron compromised the cells involved in the oogenesis process and consequently also resulted in decreased egg production and oviposition, since we observed a large reduction in the number of eggs laid by the moths, since according to Takami [32] in the adult phase approximately 50 to 90 mature eggs are present by ovariole. Kuribayashi [33] also observed reproductive damage following exposure of *B. mori* larvae to sublethal doses of organophosphorus pesticides, such as formation of abnormal eggs, decrease in total number of eggs, large numbers of unfertilized eggs, death of early embryos development, and death of newly emerged individuals.

Previous data have indicated damage to reproduction of nontarget beneficial insects by Novaluron, as in females of *Podisus maculiventris* (Heteroptera: Pentatomidae) emerged from treated eggs, resulting in a reduction in their longevity, oviposition and viability of their eggs [34]. In *Bombus terrestris* L., one of the most important pollinators in culture such as tomato, Novaluron resulted in reduction in larval hatching and impaired larval development [8]. It is known that *B. mori* larvae are sensitive to agrochemicals and the contact with these products can compromise the normal development of the insect and impact all stages of its life cycle [12,33,35–38], and we present here the first reports of the great toxicity of Novaluron in the reproductive organs of this important nontarget insect.

Conclusion

Our results indicate that with only 24 h of exposure of *B. mori* larvae to Novaluron, it is possible to demonstrate reprotoxicity in both testes and ovarian of the insect, and negatively affect the reproduction of *B. mori* by reducing the amount of eggs, and thus could compromise the maintenance of the species. Thus, the use of agrochemicals to protect agricultural crops requires extreme caution to avoid harm to beneficial and nontarget species, and the sublethal effects must be considered and evaluated at all stages of the organism's life cycle so that these products can be considered safe.

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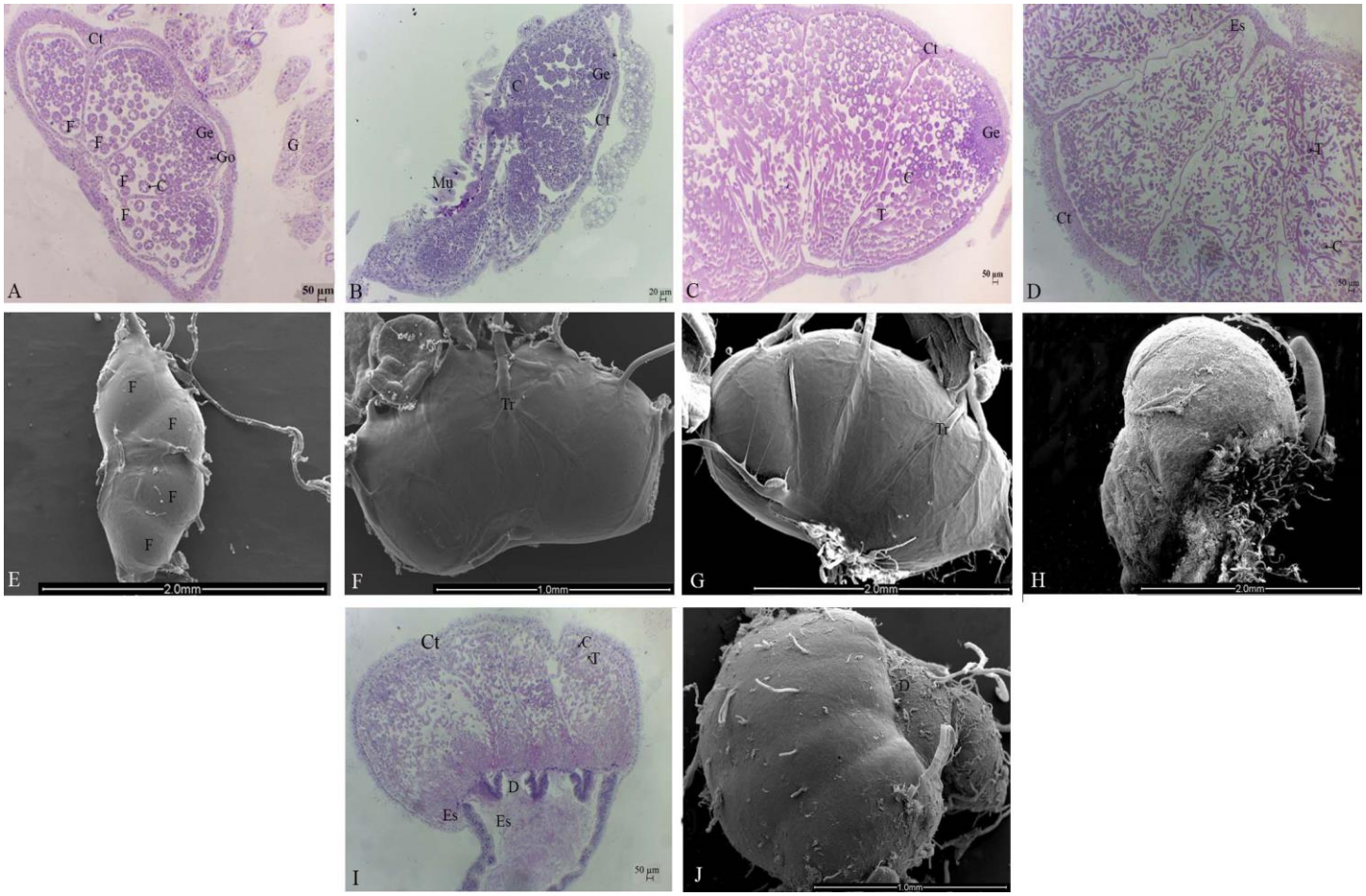

Fig. 1. Morphology of the testis of *B. mori* CG. **A, B, C, D, I:** Photomicrographs from 3rd, 4th and 5th larval instars, pupae and moths**,** respectively. Note the kidney shape of the testis composed of the cellular tunica (Ct); testicular follicles (F); region of the germarium (Ge) with spermatogonia (Go); vitelarium region with spermatocytes (C); spermatids (T) and spermatozoa (S). Externally to the testis we can notice the presence of muscular layers (Mu); dipose tissue (G) and trachea (Tr). In I: the presence of the duct deferens (D) containing spermatozoa inside. **E, F, G, H, J:** Electron micrographs from 3rd, 4th and 5th larval instars, pupae and moths, respectively.

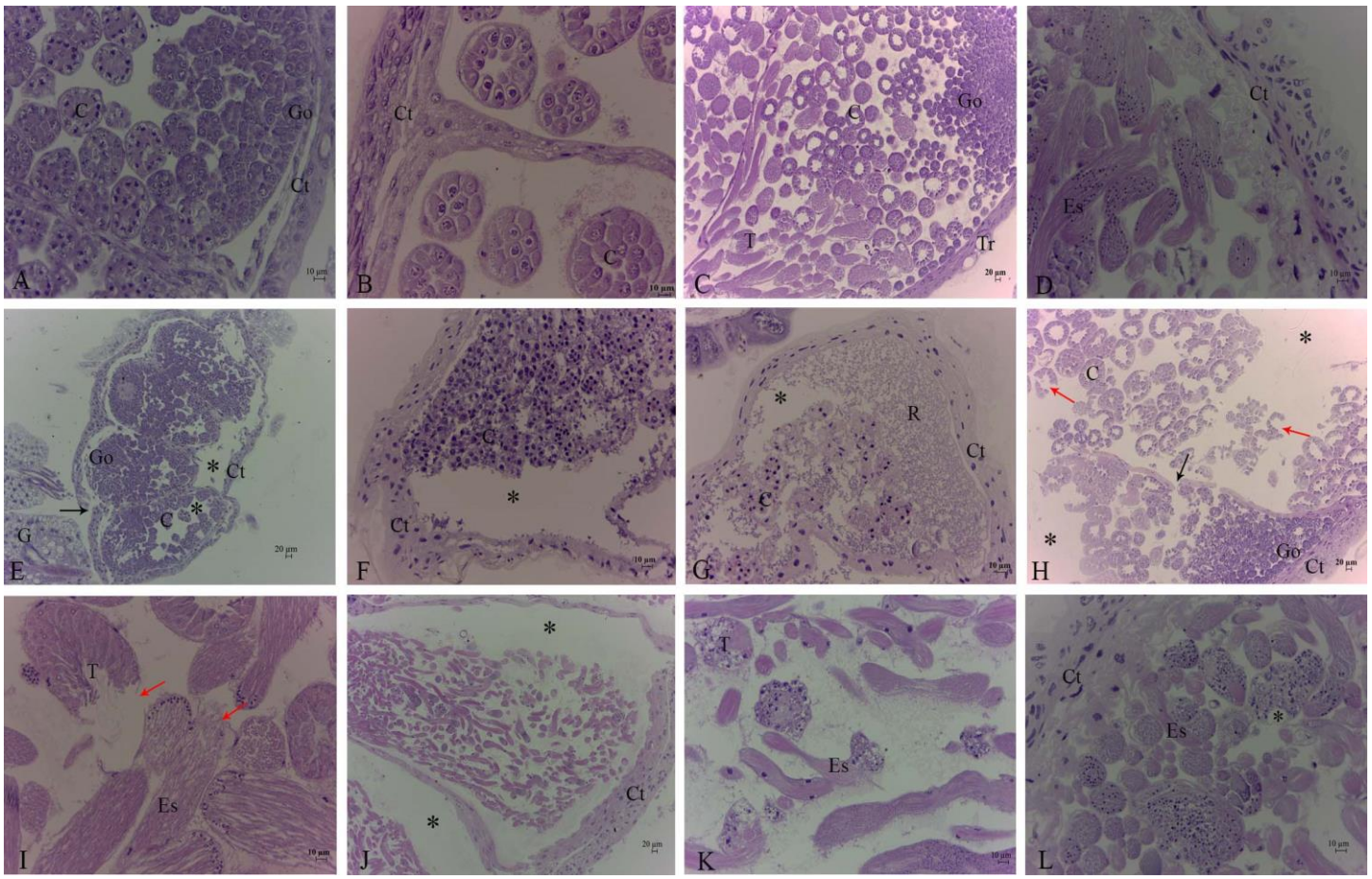

Fig. 2. Photomicrographs of the testis *B. mori*. **A, B, C, D:** CG from 3rd, 4th and 5th larval instars and moths, respectively. Note the presence of the cellular tunic (Ct) covering the testicular follicles formed by spermatogonia (Go); spermatocytes (C); spermatids (T) and spermatozoa (S). Externally there are trachea (Tr). **E, F, G, H, I, J, K, L:** TG from 3rd, 4th, 4th, 5th and 5th larval instars, pupae, pupae and moths, respectively. Note the irregularity in the testis with ruptures in the tunica cell and septa (black arrow); ruptures in the cysts of spermatocytes, spermatids and spermatozoa (red arrow); distance between the cellular tunica and the germline cysts (*) with many empty spaces within the follicles and the presence of cellular residue (R). Note in K and L, degeneration characteristics in spermatids and spermatozoa. Adipose tissue (G).

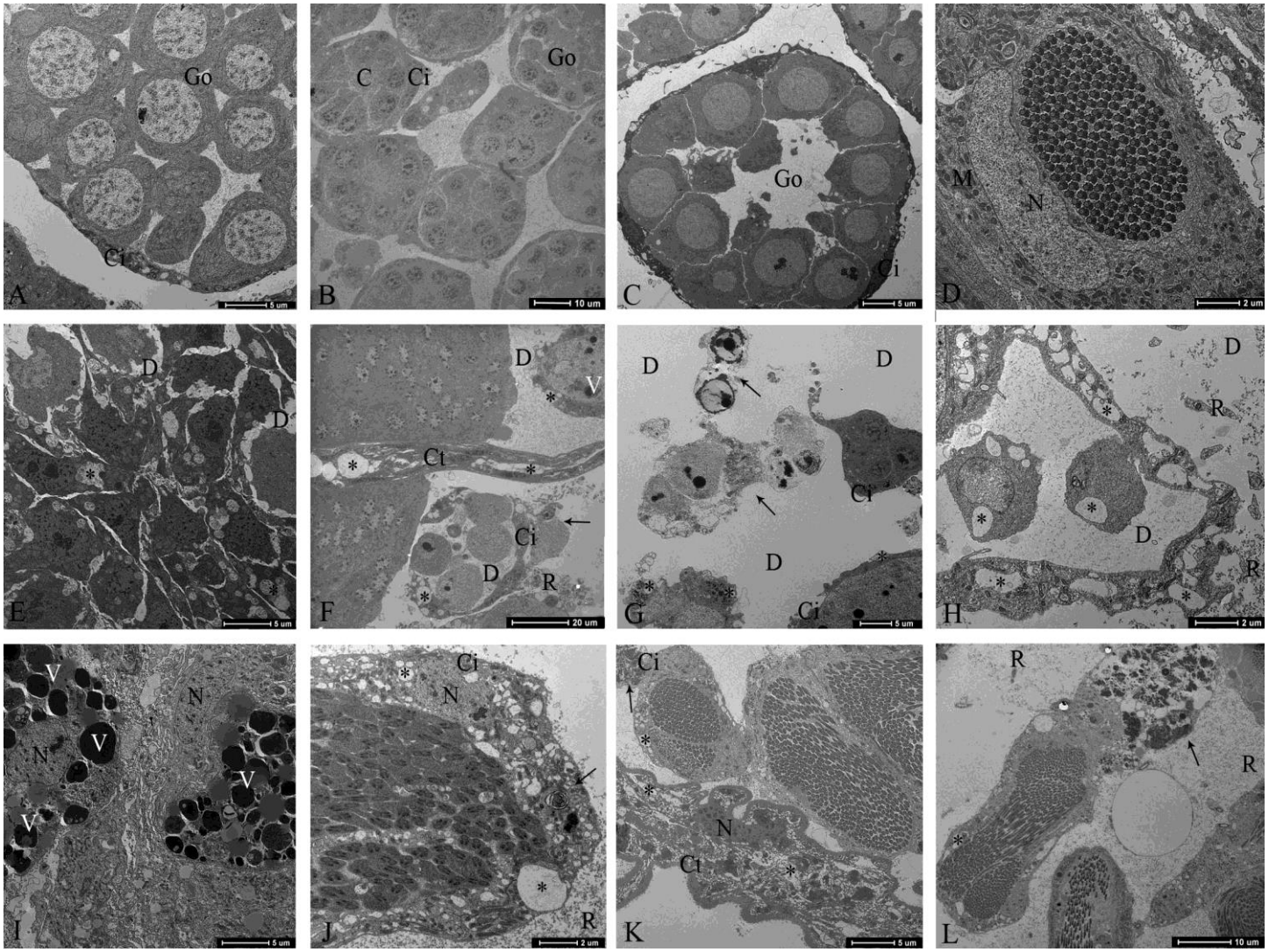

Fig. 3. Electron micrographs of the testis *B. mori*. **A, B, C, D:** CG from 3rd, 4th and 5th larval instars and moths, respectively. Note the presence of the spermatogonia cysts (Go) and spermatocytes (C) surrounded by cystic cells (Ci). In D, germ cell line with nucleus (N) and mitochondria (M). **E, F, G, H, I, J, K, L:** TG from 3rd, 4th, 4th and 5th larval instars, pupae, pupae, moths and moths, respectively. Note the irregularity in the germline cysts and their cystic cells, showing distancing (D) between the cysts and between the cells, presence of spaces (*) in the cells of the tunica cellular and spermatic cysts, cellular residues (R), and characteristics of cell degeneration in the germline cysts, with presence of many vacuoles (V) and apoptotic bodies (arrow).

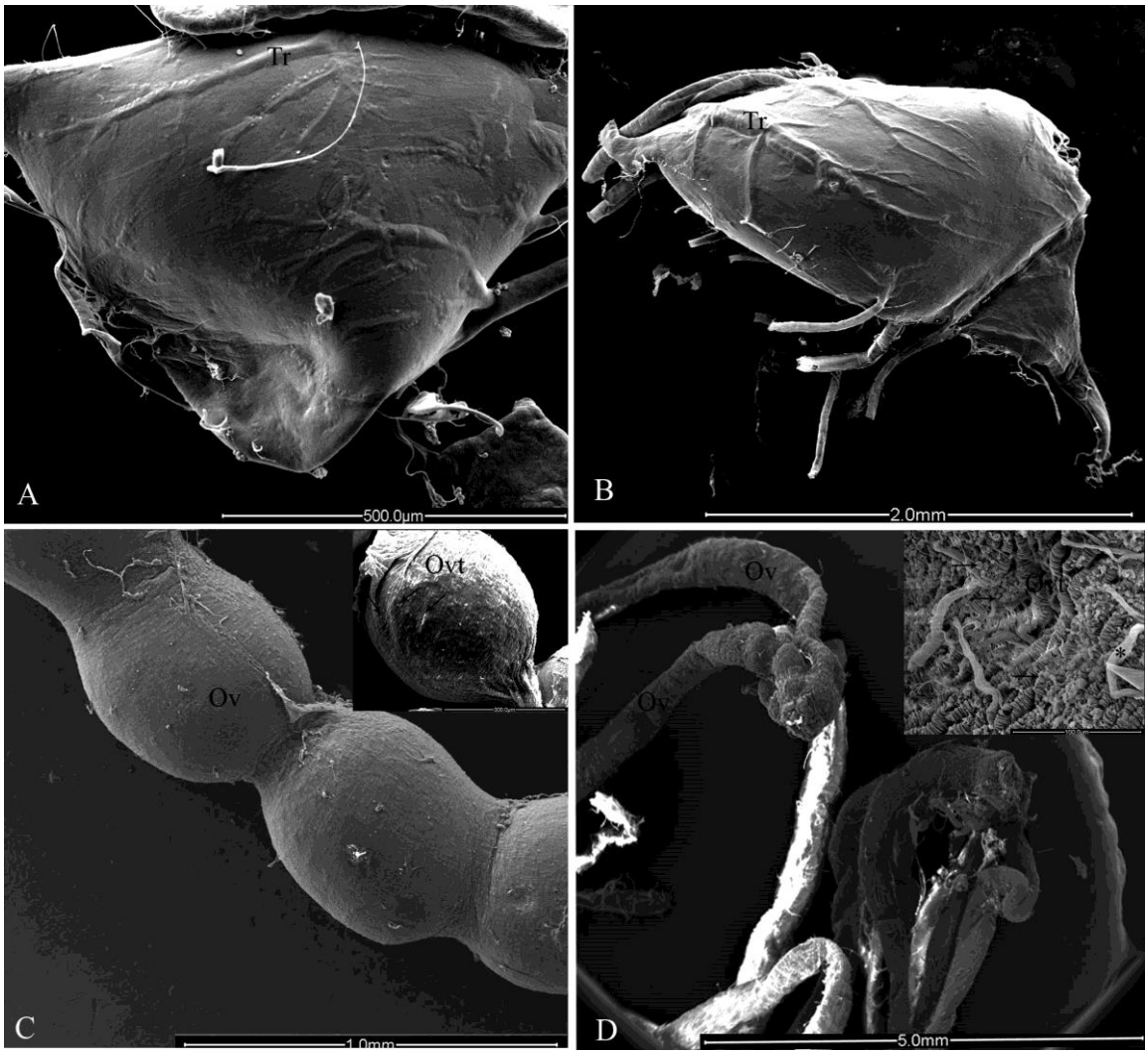

Fig. 4. Electron micrographs showing the morphology of the ovarian *B. mori*. **A, B, C, D:** CG from 4th, 5th larval instars, pupae and moths, respectively. A and B, note the triangular shape of the ovaries in the larval phase and in C and D become longer, assuming a tubular shape in the pupae and adult phases. Note in C and D the regular outer surface of the ovarioles (Ov) of the CG and in detail the outer surface of the ovarioles of the TG (Ovt) with granular appearance exhibiting small protrusions (arrow) and faults (*). Trachea (Tr).

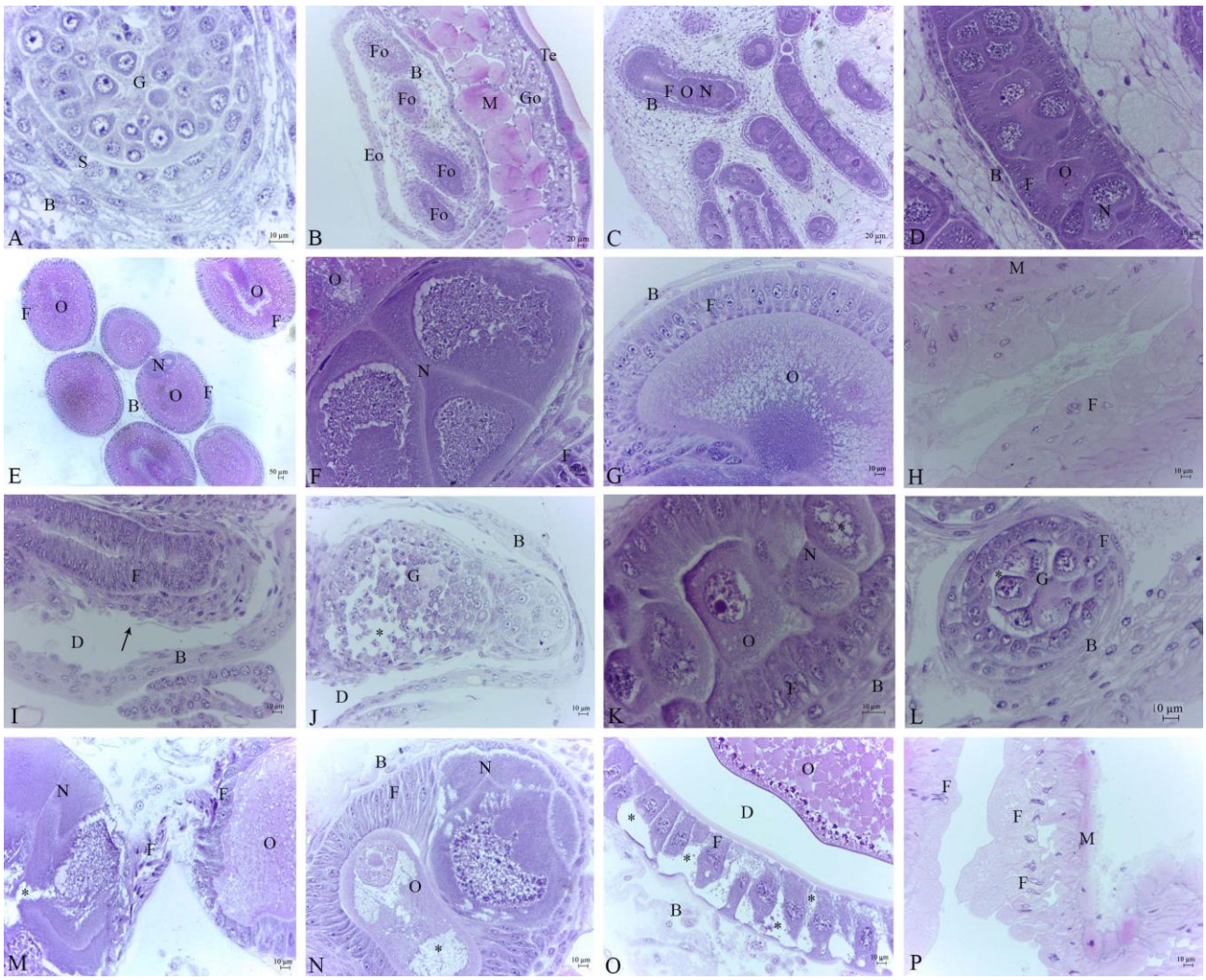

Fig. 5. Photomicrographs of the ovaries *B. mori*. **A, B, C, D, E, F, G, H:** CG from 3rd, 4th, 5th, 5th larval instars, pupae, pupae, pupae and moths, respectively. Note externally to the ovarian the presence of the muscular layers (M); adipose tissue (Go); tegument (Te) and epithelium that covers the ovarian (Eo), formed by ovarian follicles (Fo) that are covered by the ovariolar sheath (B). In A, germ cells (G) and somatic undifferentiated (S); already in C-G, note the presence of the nurse cells (N) and oocyte (O) enveloped by follicular cells (F). Note in E, some advanced ovarian follicles, formed only of the oocyte involved by the follicular cells; and in H, the adult phase (moths) formed only of follicular cells. **I, J, K, L, M, N, O, P:** TG from 3rd, 4th, 5th, 5th larval instars, pupae, pupae, pupae and moths, respectively. Note the irregularity in the ovarian with ruptures in the ovariolar sheath (arrow); distancing between the ovariolar sheath and the ovarian follicles (D); many spaces within the follicles and within the cells (*). Note in P, the follicular cells of the ovarioles moths, more elongated with degeneration characteristics.

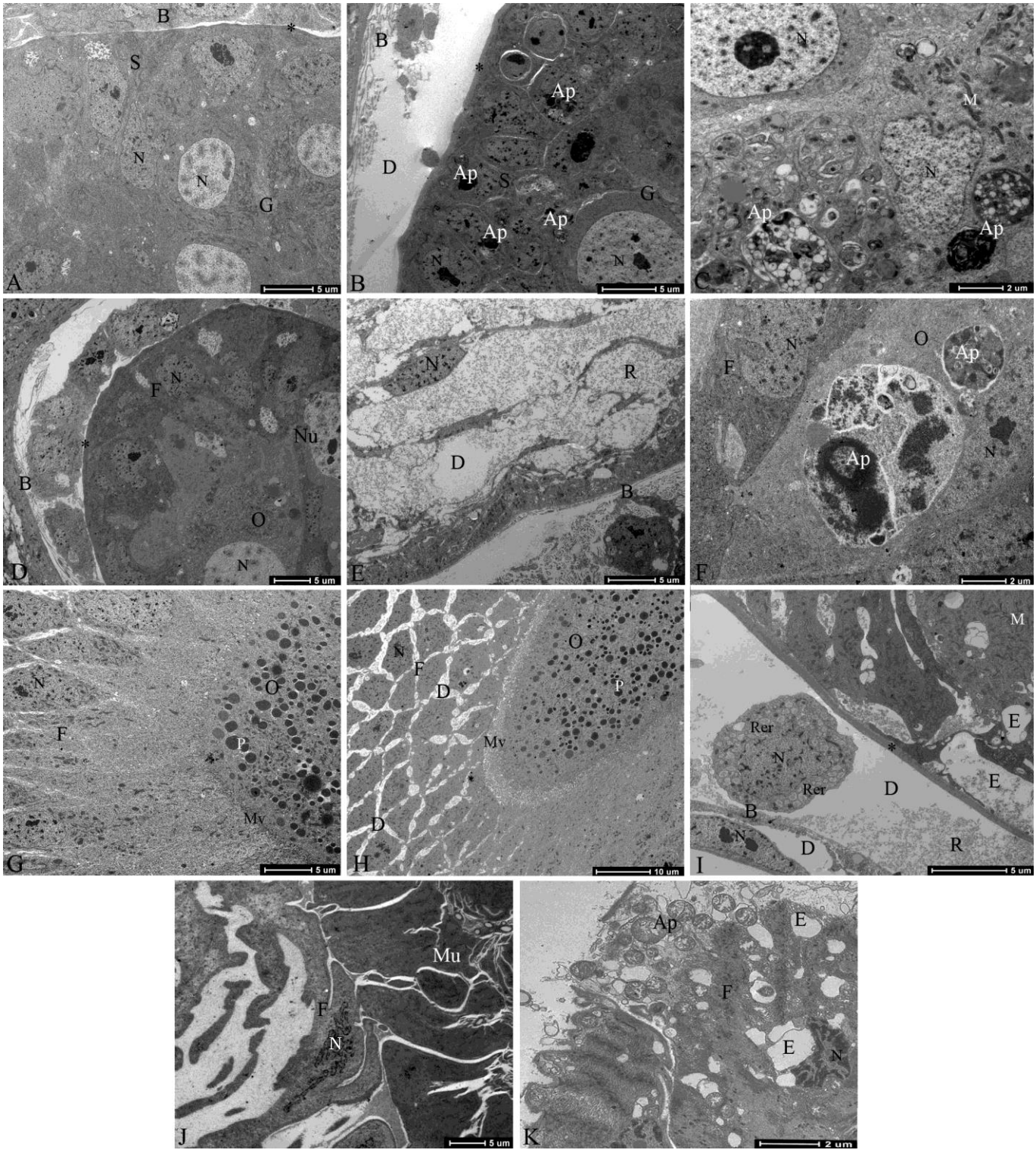

Fig. 6. Electron micrographs of the ovaries *B. mori*. **A, D, G, J:** CG from 4th, 5th larval instars, pupae and moths, respectively. In A, ovariolar sheath (B) and tunica propria (*) involving germinative (G) and somatic cells undifferentiated (S), nuclei (N); in B, presence of the nurse cells (Nu) and oocyte (O) enveloped by follicular cells (F). In G, oocyte with proteins (P) and microvilli (Mv). In J, adult phase (moths) formed only of follicular cells and externally layer muscular (Mu). **B, C, E, F, H, I, K:** TG from 4th, 4th, 5th, 5th larval instars, pupae, pupae and moths, respectively. Note the irregularity in the ovarian with ruptures in the ovariolar sheath; distancing between the ovariolar sheath and the ovarian follicles and between the cells (D); many spaces within the follicles and within the cells (E); presence of apoptotic bodies (Ap), cellular residues (R) and dilatation of rough endoplasmic reticulum (Rer). Mitochondria (M).

5. CONCLUSÕES

- A ingestão do inseticida Novaluron na fase larval de *B. mori* induz efeitos citotóxicos no intestino médio e na glândula da seda de lagartas de *B. mori*, bem como dos órgãos reprodutores (testículo e ovário) de lagartas, pupas e adultos (mariposas);

- A exposição de *B. mori* ao Novaluron ocasiona significativos efeitos em sua biologia e desenvolvimento, comprometendo todas as fases do seu ciclo de vida, principalmente na construção dos casulos de seda, e redução da fecundidade de fêmeas expostas, afetando assim, consequentemente a sobrevivência do inseto;

- Todos esses efeitos comprometem a produção e a manutenção da espécie, podendo trazer sérios prejuízos a toda a cadeia produtiva da seda.

6. ANEXO

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Negative impact of Novaluron on the nontarget insect *Bombyx mori* (Lepidoptera: Bombycidae)☆

Marilucia Santorum [a], Rose Meire Costa Brancalhão [b], Ana Tereza Bittencourt Guimarães [b], Carlos Roberto Padovani [c], Gianluca Tettamanti [d], Daniela Carvalho dos Santos [a, e, *]

[a] Laboratory of Insects, Department of Morphology, São Paulo State University (UNESP), Institute of Biosciences, Botucatu, Brazil
[b] Center of Biological Sciences and Health, State University of Western Paraná, UNIOESTE, Cascavel, PR, Brazil
[c] Department of Biostatistics, São Paulo State University (UNESP), Institute of Biosciences, Botucatu, Brazil
[d] Department of Biotechnology and Life Sciences, University of Insubria, 21100, Varese, Italy
[e] Electron Microscopy Center of the Institute of Biosciences of Botucatu, UNESP, SP, Brazil

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ABSTRACT

Due to increased use of agrochemicals and growing concerns about ecotoxicology, the development of new insecticides, moving away from those with neurotoxic and broad spectrum effects towards insecticides that are safer for the environment and nontarget beneficial species, has been a research priority. Novaluron stands out among these newer insecticides, is an insect growth regulator that is used for the control of insect pests in crops grown close to mulberry plantations. Mulberry serves as food for the silkworm *Bombyx mori*, which is a nontarget insect of great economic importance to silk production. We investigated the lethal and sublethal effects of Novaluron on the development of *B. mori*. Larvae were segregated into experimental groups: the control groups (CGs) and the treatment groups (TGs), which were treated with the Novaluron concentration of 0.15 mL/L. Following exposure, we analyzed: larval mortality, changes in the insect life cicle and cytotoxic effects on the midgut cells. This is the first report about the Novaluron's effects on *B.mori*. We detected rupture in the integument, complete cessation of feeding, late development, incomplete ecdysis and production of defective cocoons. After 240 h of exposure, there was 100% mortality in TG larvae exposed in the 3rd instar and 20% mortality from larvae exposed in the 5th instar. Cytotoxic effects was observed, such as dilation of cells, emission of cytoplasmic protrusions, extreme rarefaction of the cytoplasm and nuclei, dilation of the endoplasmic reticulum in addition to changes in mitochondria, the presence of large digestive vacuoles and intercellular spaces and the presence of active caspase. Novaluron exposure impairs the midgut and may affect the physiological functions of this organ. Novaluron additionally compromises several phases of insect development, indicating the importance of toxicology studies that utilize different life stages of nontarget species to evaluate the safe use of insecticides.

1. Introduction

Due to increased use of agrochemicals and growing concerns about ecotoxicology and mammalian safety, the chemical industry has been developing new insecticides, shifting away from those with neurotoxic and broad spectrum effects towards compounds that are less environmentally harmful to nontarget beneficial species (Tunaz and Uygun, 2004; Dhadialla et al., 2005; Bell, 2014). Novaluron stands out among these newer compounds. Novaluron is an insect growth regulator (IGR) and acts as a chitin synthesis inhibitor. It is used in agricultural crops such as corn, soybeans, beans, and sugarcane, among others (Ishaaya et al., 2002; MAPA, 2018).

Novaluron eliminates Lepidopteran insects as well as other pest orders, such as Coleoptera, Diptera and Hemiptera (Cutler and Scott-Dupree, 2007, MAPA, 2018). Its mode of action is by contact and ingestion of a benzoylphenylurea formulation, which inhibits the biochemical processes leading to the formation of chitin synthetase, causing an abnormal deposition of the endocuticle. This mainly affects larval stages, causing death by abnormal endocuticular deposition and interrupted molting. This class of insecticides

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* Corresponding author.
E-mail address: daniela@ibb.unesp.br (D.C. dos Santos).

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has been considered acceptable for inclusion in integrated pest management (IPM) programs because this class initially appeared to be safer and more selective in its mode of action, potentially acting only against the target species with reduced risks to the environment, including mammals, birds, aquatic animals and beneficial and nontarget insects (Tunaz and Uygun, 2004; Dhadialla et al., 2005). However, insecticide damage to the environment and to nontarget organisms still needs to be minimized (Desneux et al., 2007), since studies have pointed out negative effects of Novaluron on beneficial nontarget insects, including predators, parasitoids and pollinators. In these nontarget organisms, Novaluron can reduce larval hatching and affect larval development via contact and ingestion. Furthermore, Novaluron can impair molting, oviposition and the viability of eggs of treated adults, as well as reduce their lifespan (Mommaerts et al., 2006; Cutler and Scott-Dupree, 2007).

The silkworm Bombyx mori L. (Lepidoptera: Bombycidae) is a research model insect among Lepidoptera and has been widely used as a model organism in cellular and molecular biology, physiology, biotechnology, medicine, toxicology and other fields (Matsumoto et al., 2011; Tansil et al., 2011; Kundu et al., 2014; Qin et al., 2012). Its agribusiness applications are of great economic importance, being used in sericulture for the commercial production of silk threads. However, the use of insecticides in crops surrounding mulberry plantations (*Morus spp.*) can affect the production of *B. mori*; during aerial spraying, pesticides can settle on mulberry leaves which are the main food source of *B. mori*, leading to the contamination of the farmed silkworms. This causes an imbalance in the metabolic functions of the larvae, compromising growth and reproduction, including the quality of the cocoon, hatching and fecundity of *B. mori*. Thus studies suggest that exposure of silkworm to insecticides may compromise the production of silk (Bhosale and Kallapur, 1985; Yin et al., 2008; Munhoz et al., 2013; Yu et al., 2013; Gu et al., 2014).

Although sublethal concentrations of insecticides may not cause high population mortality, they can significantly affect the lifespan, fertility, feeding and oviposition of a species (Mommaerts et al., 2006; Cutler and Scott-Dupree, 2007; Munhoz et al., 2013; Scudeler et al., 2016), however, information on the toxicity of Novaluron in *B. mori* is not available. Although it is not used in mulberry plantations, recent studies by the agriculture secretary of Paraná State, Brasil, have pointed to large losses in sericulture, cocoon production and mortality of silkworm larvae, potentially due to the drift of residues from sprays insecticide in agricultural crops near to silkworm farms (Munhoz et al., 2013; Globo Rural, 2011). Such information, coupled with the important role played by silkworms, reinforces the need for investigation of Novaluron's effects on *B. mori*. Thus, the present study analyzed the response of midgut epithelial cells from *B. mori* larvae when exposed by ingestion of Novaluron contaminated mulberry leaves during the larval phase and the occurrence of lethal and sublethal effects in their development.

2. Materials and methods

2.1. Insect and chemicals

Hybrid, second instar *B. mori* larvae were obtained from a silk spinning company producing larvae for commercial purposes in the State of Paraná, Brazil. Larvae were maintained under controlled conditions as described by Santorum et al. (2017).

Novaluron – The commercial formulation, Rimon Supra, was purchased from ADAMA Makhteshim Ltd. This formulation is registered with the Ministry of Agriculture, Livestock and Supply, Brazil (MAPA sob n^{o} 14511).

2.2. Bioassays

Each treatment was replicated six times, with ten *B. mori* larvae used per group for statistical analyses of mortality. Parallel groups of larvae received the same experimental treatments for morphological and ultrastructural analyses.

According to the official regulation for the phytosanitary pesticide system of the Ministry of Agriculture, Livestock and Food Supply of Brazil, the minimum approved dose of Novaluron for use in the field is 0.3 ml/L and the maximum dose is 0.5 ml/L for the sugarcane, soybean and corn crops (MAPA, 2018). These crops, often grown near mulberry plantations and silkworm farms. Considering the use of the minimum dose of this insecticide in these cultures and the aerial dispersion of these residues during spraying, we used the concentration 0.15 ml/L that is half the recommended minimum dose.

The treatment groups (TGs) consisted of *B. mori* larvae exposed once to mulberry leaves containing Novaluron at two different instars (1st day of the 3rd instar or 1st day of the 5th instar). Control groups (CG) consisted of *B. mori* larvae fed on mulberry leaves free of Novaluron at two different instars (1st day of the 3rd instar or 1st day of the 5th instar). So in the bioassays, mulberry leaves were immersed in aqueous solutions containing 0.15 ml/L Novaluron, dried at room temperature, and then offered to *B. mori* larvae in TGs for 24 h *ad libitum*. After exposure to Novaluron (24 h), the larvae were again fed mulberry leaves free from the insecticide.

2.3. Symptomatology

After exposure to Novaluron, the symptoms manifested by *B. mori* individuals throughout their life cycle were monitored through daily observations and registered in own files and photographed (Fig. 1). The analyzed symptoms were: (1) feeding cessation (daily, the insects of the CG and TG were fed with fresh mulberry leaves, twice a day (8am and 8pm), after the interval between each feeding, it was observed how much of mulberry leaves was consumed by each group, considering the larval instars; (2) late development; (3) irregular ecdysis; (4) production of defective cocoons; (5) defective pupae and (6) defective adults.

2.4. Mortality

We observed the mortality of *B. mori* larvae after they were exposed to Novaluron either at the 3rd or 5th instar stage. The mortality rate was recorded every 24 h up to a total of 240 h after exposure. The palpation method was used to determine mortality as follows: the larva was touched with a soft paintbrush; if it made any movement, it was considered alive; otherwise, it was considered dead.

2.5. Morphological procedures

For all the morphological procedures described below, the effects of Novaluron in *B. mori* midgut tissue were evaluated. The larvae were randomly selected from the 3rd and 4th larval instars that were treated on the 1st day of their 3rd instar and the 5th larval instars that were treated on the 1st day of their 5th instar. Selected larvae were anesthetized in a freezer for approximately 5 min, placed in a tube with cotton soaked in ethyl ether, and then dissected, rinsed with insect saline solution (0.1 M NaCl, 0.1 M Na_2HPO4 and 0.1 M KH_2PO_4) and the midgut was removed. After removal, each midgut was submitted to different morphological analyses:

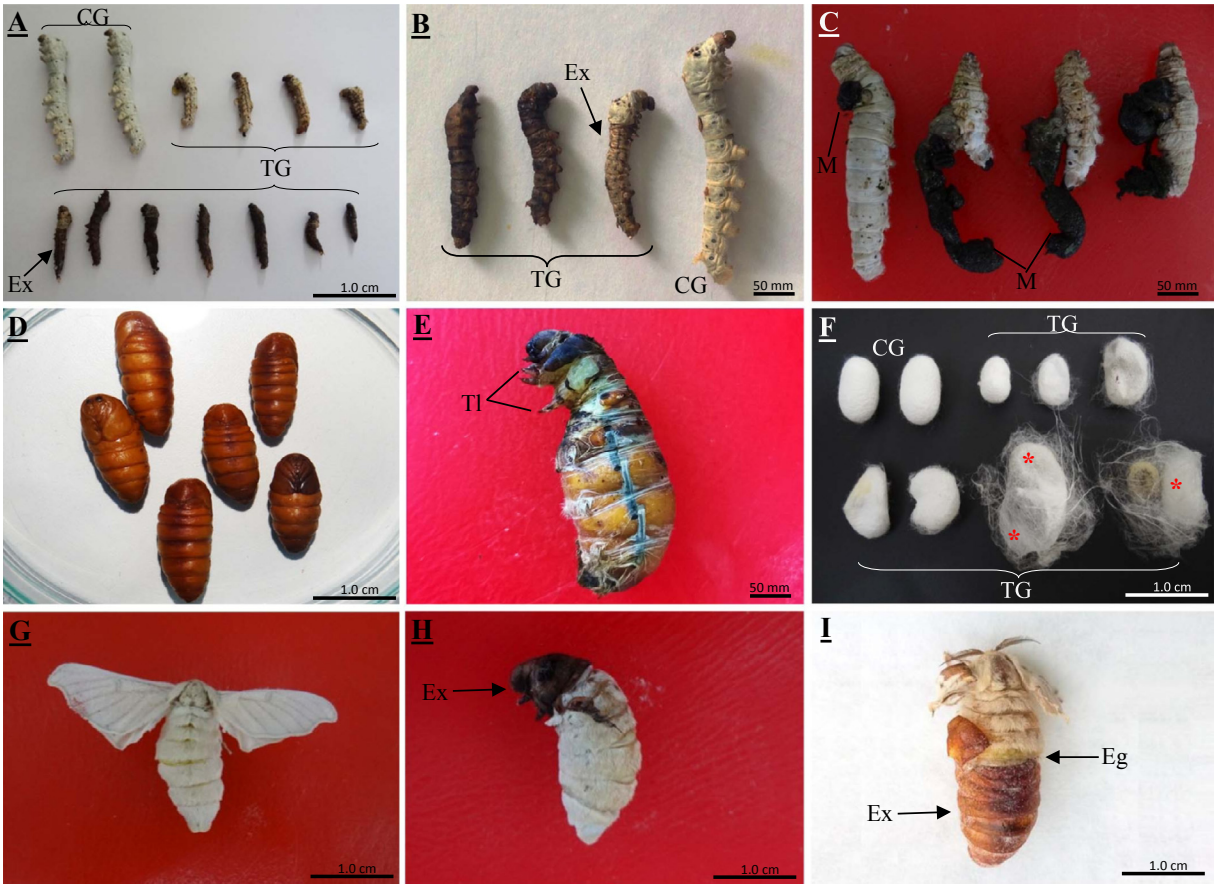

Fig. 1. Symptomatology observed in *B. mori* larvae exposed to Novaluron, treatment group (TG) and control group (CG) larvae for comparison. In **A** and **B**, incomplete ecdysis with exuvia attached to the body of the insect (Ex). Note the changes in the development of TG larvae, which have a smaller size and discoloration of the cuticle of the whole body to a dark color compared to the that of the CG. In **C**, TG larvae with ruptures in the tegument with exposed midgut (M); In **D**, pupae from larvae of the CG for comparison. In **E**, TG larvae that could not complete transformation to pupa, with retention of larval morphological characteristics. Note the presence of the thoracic legs (Tl). In **F**, defective cocoons that are deformed and thin-shelled produced by insects of the TG. Red asterisk (*) showing two cocoons attached. Cocoons constructed by insects of the CG for comparison. In **G**, CG emerged moth. In **H**, a moth emerged from TG with exuvia attached to head and in **I**, a TG moth that failed to release its exuvia (Ex), which is adhered to the final region of the abdomen. Eggs (Eg) trapped inside the abdomen are discernible with the yellow color.

2.6. Light microscopy

Each midgut was fixed in DuBosq Brasil (Beçak and Paulete, 1976) for 24 h at 4 °C. The midgut samples 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) (Junqueira and Junqueira, 1983). The control slides were subjected to the same preparations. The slides were observed with a Leica DM500 photomicroscope.

2.7. Transmission electron microscopy (TEM)

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

2.8. Scanning electron microscopy (SEM)

The midguts were fixed for 48 h at room temperature in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3). Thereafter, the samples were washed in distilled water, postfixed in 1% osmium tetroxide diluted in distilled water for 30 min at room temperature, dehydrated through a graded series of ethanol, critical point-dried with CO_2 and coated with gold. The samples were examined and photographed using an FEI Quanta 200 scanning electron microscope (FEI Company, Eindhoven, Netherlands) at the Electron Microscopy Center of the Institute of Biosciences of Botucatu, SP, Brazil.

2.9. TUNEL assay

The midguts were fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.2) for 3 h at room temperature. Specimens were dehydrated in an ethanol series and embedded in paraffin. Sections (8–10 μm thick) were cut with a

microtome. For the assessment of cell death, a widely used microscopic technique for identifying DNA fragmentation was used to label the 3' ends of fragmented DNA with the DeadEnd Colorimetric TUNEL System (Promega). Paraffin sections were deparaffinized, rehydrated through graded ethanol washes, and rinsed with PBS. Proteinase K (20 μg/ml) digestion was applied as a pretreatment for 20 min at room temperature. Incubation with the rTdT reaction mix was performed for 1 h at 37 °C, in accordance with the instructions provided by the manufacturer. The reaction was terminated by immersing the slides in 2x standard sodium citrate (15 min). A pretreatment with 3% H_2O_2 (5 min) preceded the incubation with a streptavidin–HRP solution (diluted 1:500 in PBS, 30 min). Diaminobenzidine (DAB) solution was used for signal development. Negative controls were performed by replacing the rTdT enzyme with water in the rTdT reaction mix.

2.10. Caspase-3 immunohistochemistry

For caspase-3 immunohistochemistry, paraffin sections of midgut samples were prepared as described in the TUNEL assay. After deparaffinization, the sections were subjected to an antigen unmasking procedure by heating in a 10 mM sodium citrate buffer (pH 6.0) for 5 min and then allowing sections to cool. They were then treated with 3% H_2O_2 for 10 min to inhibit endogenous peroxidases. Sections were incubated for 30 min with a solution of 2% BSA, 0.1% Tween 20 in PBS and then overnight at 4 °C with an anti-cleaved caspase-3 antibody (catalog no. 9661) (Cell Signaling Technology) at a dilution of 1:100. Incubation with an appropriate HRP-conjugated secondary antibody (diluted 1:50) was performed for 1 h. A DAB substrate was used to detect the HRP-conjugated secondary antibody. Antibodies were omitted in control samples.

2.11. Statistical analysis

The delineated experiment was completely randomized with six replicates per treatment. Ten silkworm larvae were used per replicate.

The Goodman test, which involves contrasting binomial proportions, was used to compare the relative rates of mortality of the *B. mori* larvae (Goodman, 1964). The results were expressed as the mean ± SD. The test was performed at a significance level of 5%.

3. Results and discussion

In recent years, Brazilian silkworm producers have reported large losses in cocoon production after spraying of insecticides on crops close to their silkworm rearing properties (Munhoz et al., 2013; Globo Rural, 2011). To eliminate the agricultural insects pests, mainly of the order Lepidoptera, Novaluron can be sprayed on agricultural crops growing near mulberry plantations, and can cause large indirect damage and loss of silkworm larvae and cocoon production. Thus, in this study, we performed bioassays to verify the impact of Novaluron on the development of this important insect by exposing groups of *B. mori* larvae in two different instars to Novaluron.

3.1. Symptomatology

Throughout the experimental period, it was possible to observe the symptoms manifested by *B. mori* after exposure to the insecticide. Using the concentration of 0.15 mL/L, corresponding to half of the minimum dose recommended by MAPA (2018) for the main crops grown near mulberry plantations, our results indicated that Novaluron negatively affects the overall growth of *B mori.* The larvae treated with this insecticide exhibited a rapid reduction in feeding leading to complete feeding cessation and, therefore, a notable reduction in body size. Other symptoms observed after treatment included discoloration of the cuticle of the whole body to a dark color (Fig. 1A and B). The treated larvae presented with an extremely fragile integument; ruptures, causing hemolymph extravasation and intestinal exposure (Fig. 1C), as well as irregular ecdysis, where the exuvia remained attached to the body of the insect during molting, promoting incomplete ecdysis (Fig. 1A and B), were observed.

Several studies have evaluated the toxic effects of exposure to agrochemicals on mulberry leaves on the development and growth of *B. mori* (Bhosale and Kallapur, 1985; Kumutha et al., 2013; Munhoz et al., 2013; Yu et al., 2013; Gu et al., 2014; Tang et al., 2018), however our results are the first description of the Novaluron effects on silkworm development. These data are important since changes in insect feeding compromise the conversion of ingested food to digested food, which can promote subsequent anomalies (Nasr, 2011). We verified symptoms similar to those found by Munhoz et al. (2013) and Bindu et al. (2015) and found that the symptoms appeared very quickly after exposure of the larvae to the insecticide. Novaluron showed similar effects in exposed Lepidopteran insects (Retnakaran and Wright, 1987; Cutler and Scott-Dupree, 2007). The observed symptoms are probably related to the mechanisms and general effects of Novaluron and of the class of benzoylphenylurea insecticides; these insecticides alter the composition of the cuticle, especially through the inhibition of biochemical processes that lead to the formation of chitin synthetase, causing an abnormal deposition of the endocuticle that affects the cuticular elasticity and firmness and interferes with insect molting (Tunaz and Uygun, 2004; Dhadialla et al., 2005).

Formation of the cocoon is an important developmental checkpoint and among the symptoms manifested by the TG larvae exposed in their 5th instar, we observed difficulty in packing silken threads accompanied by a delay in the construction of the cocoon, production of defective and/or thin-shelled cocoons (Fig. 1F), and retention of larval morphological characteristics (Fig. 1E and F) when compared to the CG larvae. The effects of insecticides on cocoon production and quality have been previously reported (Munhoz et al., 2013; Yu et al., 2013; Gu et al., 2014; Tang et al., 2018) and Munhoz et al. (2013) point out that affected cocoons are not useful for spinning, so sericulture companies do not accept thin-shelled cocoons and silkworm producers must take on the financial losses.

The effects of Novaluron exposure were also observed in adults; moths surviving the TG exposure in the 5th instar presented wing defects, and some, after emerging from cocoons, failed to completely release their exuvia (Fig. 1H and I). Exuvial damage to the terminal region of female moths' abdomens impaired oviposition, and these moths exhibited swollen abdomens and died without being able to lay their eggs (Fig. 1I). Other studies have found that Novaluron compromises the development of adults and that these individuals present morphogenetic abnormalities that reduce their reproductive potential (Tunaz and Uygun, 2004; Mommaerts et al., 2006; Storch et al., 2007), including suppression of embryogenesis (Dhadialla et al., 2005).

3.2. Mortality

We analyzed the mortality of *B. mori* larvae after they were exposed to Novaluron at either of two different instars (1st day of the 3rd instar; 1st day of the 5th instar). *B. mori* larvae exposed to Novaluron in the 3rd instar showed significant mortality (30%) 96 h after initiation of a 24 h exposure to treated mulberry leaves. That is, mortality was observed soon after the larvae molted from the 3rd to the 4th larval instar. 240 h after initiation of a 24 h exposure

to treated mulberry leaves, 100% mortality occurred in this group, this time when larvae were molting from the 4th to the 5th instar (Table 1). These two larval mortality peaks coincided with instar molting, proving that the greatest toxic effect of Novaluron is on insect ecdysis, resulting in abortive molting (Retnakaran et al., 1985; MAPA, 2018). Due to the complete mortality of TG - larvae exposed to Novaluron (Table 1) on the 1st day of their 3rd instar, larvae failed to complete their life cycle; that is, they were unable to continue their development, to construct their silk cocoons and to reach adulthood.

Larvae exposed to Novaluron insecticide on the 1st day of their 5th instar showed significant mortality after 96 h of exposure, with cumulative mortality 20% after 240 h of exposure (Table 2). By comparing cumulative mortality rates after the two exposure times (Tables 1 and 2), we verified that there was a lower percentage of mortality in the TG larvae exposed in the 5th instar. We believe that this is because larvae exposed at a later instar were more developed, possessing a greater body size and a faster metabolism aimed at the accumulation of energy reserves necessary for metamorphosis and cocoon construction (Santorum et al., 2017) and potentially conferring greater resistance to adverse conditions, such as exposure to Novaluron insecticide.

Novaluron's mode of action is another important factor to consider; Novaluron is a growth regulator and chitin synthesis inhibitor. As indicated in the product description, its application to crops is carried out during the early stages of the development of insect pests; that is, Novaluron is applied when first larval instars are present, preventing them from reaching more advanced instars (MAPA, 2018) and completing their development.

Although Novaluron is cited as an insecticide to be used in integrated pest management programs because of its low toxicity to mammals, fish and nontarget beneficial insects like honeybees, parasitic wasps, ants, predaceous mites and others pollinators, parasites and predators (Tunaz and Uygun, 2004; Dhadialla et al., 2005; Alyokhin, 2009; Jiang et al., 2010), our results point to its high toxicity potential for the silkworm, which is a nontarget species of great economic importance. Exposure results in significant larval mortality and compromises all phases of insect development. These results are similar to findings from previous studies that point to the toxic effects of Novaluron in beneficial insect species (Cutler et al., 2006; Mommaerts et al., 2006; Cutler and Scott-Dupree, 2007).

3.3. Morphological alterations in the midgut of B. mori larvae

The midguts of *B. mori* larvae from the CG at the 3rd, 4th and 5th larval instars presented as a typical epithelium externally surrounded by muscle fiber bundles that formed an internal circular tunic and another external longitudinal tunic (Fig. 2) (Pinheiro et al., 2008; Correia et al., 2009; Franzetti et al., 2016). We verified the presence of four typical cell types: columnar cells, the most abundant with apical microvilli, and oval and central nuclei; goblet cells, each a with basal nucleus and invaginated cytoplasm forming

Table 1
Cumulative mortality (%) of *B. mori* larvae during larval development (h) exposed to Novaluron.

Instar	Hours	Accumulated Mortality (%)	
		Control	Treatment
3rd	24	0 a	0 a
	48	0 a	0 a
	72	0 a	1.7 a
4th	96	0 a	30.0 b
	120	0 a	30.0 b
	144	0 a	35.0 b
	168	0 a	35.0 b
	192	0 a	68.3 b
5th	240	1.7 a	100.0 b

Values followed by different letters within the same line differ significantly ($p < 0.05$, Goodman's test). n = 60 larvae per group.

Table 2
Cumulative mortality (%) of *B. mori* larvae during 5th instar (h) exposed to Novaluron.

Instar	Hours	Accumulated Mortality (%)	
		Control	Treatment
5th	24	0 a	3.3 a
	48	0 a	3.3 a
	72	0 a	5.0 a
	96	0 a	6.7 b
	120	0 a	6.7 b
	144	3.3 a	13.3 b
	168	3.3 a	16.7 b
	192	3.3 a	18.3 b
	240	3.3 a	20.0 b

Values followed by different letters within the same line differ significantly ($p < 0.05$, Goodman's test). n = 60 larvae per group.

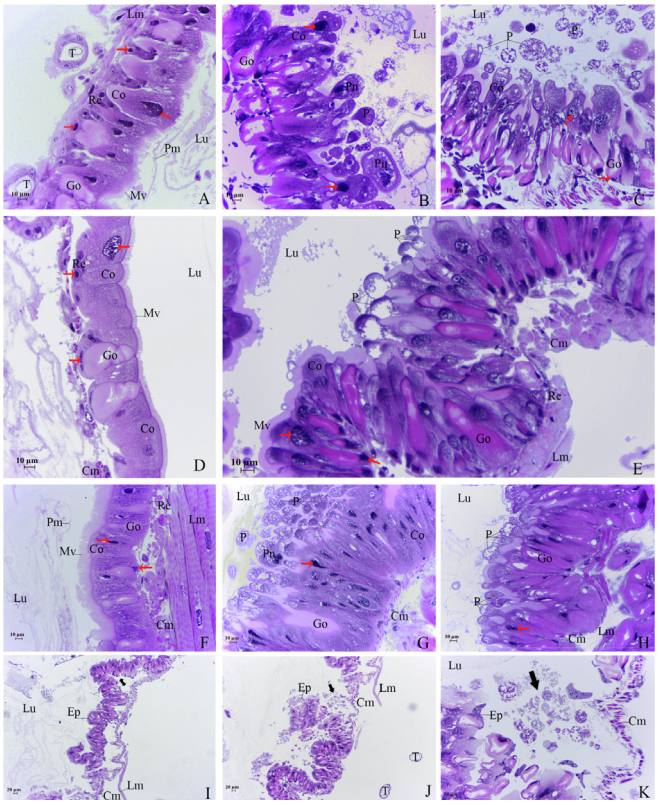

Fig. 2. Photomicrographs of the midgut of *B. mori* larvae. **A, D, F:** CG from 3rd, 4th and 5th instars, respectively, presented columnar cells (Co) with microvilli (Mv) in the apical region facing the lumen of intestine (Lu), goblet cells (Go) intercalated between columnar cells and nests of regenerative cells (Re) at the base of the epithelium. The nucleus (red arrow), peritrophic membrane (Pm), trachea (T), circular (Cm) and longitudinal (Lm) muscle are shown. **B, C, E, G, H:** TG from 3rd, 3rd, 4th, 5th and 5th instars, respectively, where we note the tissue disorganization, with elongated columnar cells (Co), with the most enlarged apical region and with a large number of cytoplasmic protrusions (P) angled towards the lumen of the intestine (Lu) and some protrusions containing cytoplasmic and nuclear material in its interior (Pn). Note the presence of more elongated goblet cells (Go) and a reduction in the presence of regenerative cells (Re). **I, J, K:** TG with the region showing separation between the epithelium (Ep) and the muscular layer (double arrow). In J, the region showing the detachment of the epithelium towards the lumen (black arrow). In K, increased epithelial detachment of the region towards the lumen.

globular chambers, intercalated between the columnar cells; regenerative cells, found in isolation or in groups in the basal area of the epithelium, with the nucleus having a shape similar to that of the cells, varying from elongated to oval (Fig. 2A, D and F); and endocrine cells, which were observed more rarely along the epithelium, located in the basal regions close to nests of regenerative cells and possessing a large nucleus and clear cytoplasm.

The morphology of these cells is related to their functions performed in the midgut, such as in the synthesis and secretion of digestive enzymes and subsequent absorption of the nutrients, performed by columnar and goblet cells; in the tissue growth and regeneration; in the control of metabolic processes; and in the control of cell proliferation and differentiation, mediated by regenerative and endocrine cells, respectively (Sousa et al., 2009; Hakim et al., 2010; Teixeira et al., 2013; Gigliolli et al., 2015).

Novaluron caused cytotoxic effects in the midguts of the TG *B. mori* larvae for the three larval instars analyzed, and exposure resulted in pronounced alterations throughout the entire intestinal epithelium, with extreme disorganization observed in the arrangement of cell types typical of this epithelium (Fig. 2). The columnar cells were extremely elongated, with a dilated apical region and cytoplasmic protrusions similar to vesicles visualized along the epithelium and in the intestinal lumen. This cell type also had sparse and/or absent microvilli in some regions. Thus, the apical surface of the TG epithelium demonstrated more irregularities compared to that of the CG (Figs. 2 and 3). The TG goblet cells were also more elongated, following the same trend in columnar cells (Fig. 2). Similar results on the toxic effects of insecticides on insect midgut epithelial cells have been previously reported (Ndione et al., 2007; Correia et al., 2009; Roel et al., 2010; Scudeler and Santos, 2013; Munhoz et al., 2013; Scudeler et al., 2016). As there is no literature demonstrating Novaluron's effects on the midgut morphology and development of *B. mori*, our findings will contribute to understand the great toxic potential presented by this insecticide, which has been considered safer for nontarget species. Munhoz et al. (2013), analyzing the effects of another class of insecticide, the Chlorantraniliprole, usually used for sugarcane borer control next mulberry plantations, reported negative effects in columnar cells of *B. mori* midgut, which presented apoptotic bodies. They hypothesized that the midgut cell death by apoptosis compromised silkworm development as well as the production of cocoons.

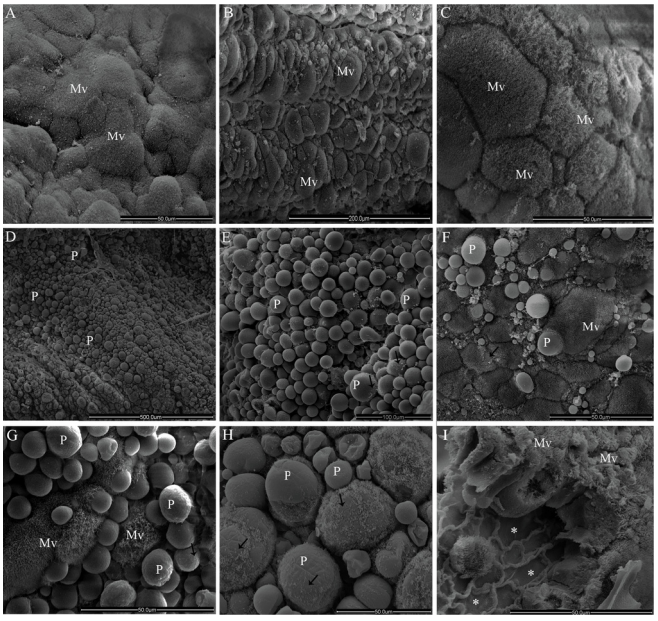

Fig. 3. Electron micrographs of the midgut of *B. mori* larvae. **A, B, C:** CG 3rd, 4th and 5th instars, respectively, presented columnar cells (Co) with a regular pattern of microvilli (Mv). **D, E, F, G, H, I:** TG from 3rd, 3rd, 4th, 4th, 5th, and 5th instars. Note the epithelial surface of the midgut of the TG, with changes in the morphology of the columnar cells showing enlargement of apical regions and with a large number of cytoplasmic protrusions (P) and sparse and/or absent microvilli in some regions (black arrow). In I, notice the empty spaces (asterisks) previously occupied by columnar cells.

Various stimuli may induce apoptosis of midgut epithelial cells in insects, such as inhibitors of ARN and protein synthesis (Palli et al., 1996), bacterial (Gregorc and Bowen, 1999) or viral infection (Garcia et al., 2001), and exposure to biopesticides (Nasiruddin and Mordue (Luntz), 1993; Ndione et al., 2007) and insecticides (Gregorc and Ellis, 2011; Munhoz et al., 2013). When exposed to azadirachtin, a compound that is also an insect growth regulator (Thangaraj et al., 2018), histopathological alterations in the midgut epithelium, such as elongation of the columnar cells, smaller nuclei, loss of the apical cytoplasm and necrosis were observed in *Spodoptera frugiperda* (Lepidoptera: Noctuidae) (Correia et al., 2009). The midgut is the main location for digestion and absorption of food and therefore is the region most vulnerable to the action of foreign elements (Roel et al., 2010; Yu et al., 2013).

We observed hypertrophy in the regenerative cells and a reduction in their numbers. The clusters of regenerative cells, characteristic of this cell type in Lepidoptera and observed for CG, were no longer visible in TG larvae (Fig. 2). Mordue (Luntz) and Nisbet (2000) found similar changes in these cells induced by azadirachtin; absorption of this compound causes inhibition of cell division and protein synthesis, with effects reflected in the loss of groups of regenerative cells and resulting in deficiencies in epithelial renewal. We believe that the same effects on the regenerative cells of *B. mori* exposed to Novaluron may have occurred.

In some regions of the epithelium, there is a spacing between the epithelial cells and the basal lamina where they are supported, in addition to the separation also occurring between the epithelium and the muscular layer (Fig. 2I and J). We identified the detachment of some cells (Fig. 2J and K) from the epithelium towards the lumen; this detachment is better visualized through SEM, as shown in Fig. 3I, where the presence of empty spaces previously occupied by the epithelial cells is clear. The visualization of these spaces in the epithelium may be related to the lack of epithelial renewal since we observed a decrease in the regenerative cells in the TGs.

We compared the ultrastructural observations of epithelial cells from the midguts of *B. mori* larvae in TGs with those of the CGs via SEM (Fig. 3) and TEM (Fig. 4), and in the 3 instars analyzed, we verified several changes in the 4 typical cell types, such as extreme cytoplasmic and nuclear rarefaction and dilation of the endoplasmic reticulum; mitochondrial changes; the presence of large digestive vacuoles and myelin figures; and intercellular and intracellular spacing (Fig. 4), similar to what has been described by Yu et al. (2013) and Gu et al. (2014) in *B. mori* after exposure to phoxin insecticides. These authors point out that deteriorated mitochondria produce more reactive oxygen species and thus accelerate the process of apoptosis, and Cheville (2009) associates the occurrence of myelin figures with irregular fragments of the reticular endoplasmic membrane that aggregate and reorient in a laminar arrangement as a consequence of degeneration of the endoplasmic reticulum. The formation of intercellular spaces has also been reported in several treatments with biopesticides (Correia et al., 2009; Scudeler and Santos, 2013; Scudeler et al., 2016) and insecticides (Cruz et al., 2009). We believe that the complexes in cell junctions can be broken down, allowing the formation of these spaces. Cheville (2009) mentions that during acute cellular swelling, the junctions may disintegrate, causing cells to lose their normal cohesion with neighboring cells. All of these

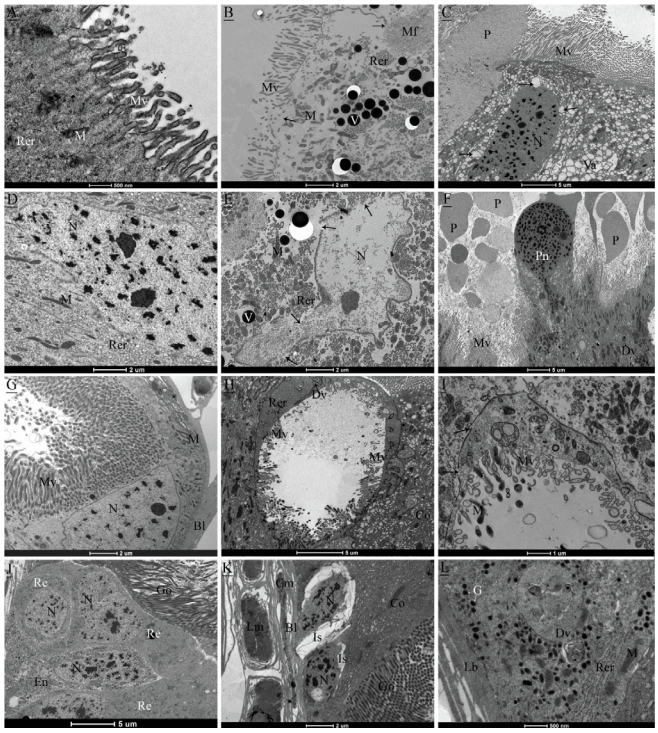

Fig. 4. Electron micrographs of the midgut *B. mori* larvae. . **A, D, G, J:** CG. In A, apical region of the columnar cells with microvilli (Mv), mitochondria (M) and rough endoplasmic reticulum (RER). In D, column cell nucleus (N). In G, basal region of the goblet cell seated on the basal lamina (Bl) and note mitochondria inside microvilli. In J, groups of regenerative cells (Re) interspersed with endocrine cell (En) and goblet cell (Go). **B, C, E, F, H, I, K, L:** TG. In B, apical region of the columnar cells with alterations in microvilli (Mv) with ruptures in the membrane (arrow). Note a large dilation and fragmentation of the rough endoplasmic reticulum, myelin figures (Mf), vesicles with electron-dense content (V).In C, the apical region of the columnar cell emitting cytoplasmic protrusions (P), with extremely vacuolated cytoplasm and dilation of the perinuclear space (arrow).In E, note the rarefaction of the nucleus of the columnar cell with ruptures in the nuclear envelope (arrow) and general appearance of the perinuclear cytoplasm. In F, note the presence of digestive vacuoles (Dv) and large number of cytoplasmic protrusions containing cytoplasmic and nuclear material in their interior and some protrusions released towards the lumen (Pn). In H, goblet cells with changes in microvilli (Mv) and many of them devoid of mitochondria in their interior, and columnar cells (Co). In I, apical region of the goblet cell with membrane ruptures (arrow). In K, regenerative cells with intercellular and intracellular spaces (In), circular muscle (Cm) and longitudinal (Lm). In L, the basal region of the endocrine cell; note the presence of digestive vacuoles, electron-dense granules (G).

changes seem to be associated with possible attempts of detoxification or response to cellular stress against cytotoxicity. (Cheville, 2009; Scudeler and Santos, 2013, 2014; Catae et al., 2014; Scudeler et al., 2016).

In addition to these characteristics, we observed in the goblet cells of the TGs an absence of mitochondria within their microvilli or cytoplasmic projections as they are also known, a common characteristic for this cellular type (Fig. 4H and I). Bong and Sikorowski (1991) found that infection with the cytoplasmic polyhedrosis virus resulted in decreased cytoplasmic projections and absence of mitochondria inside in *Helicoverpa zea* (Lepidoptera: Noctuidae) larvae. Due to the high concentration of potassium in plant tissues, it is known that this cell type plays an important role in insects that feed on plants by executing the transport of the hemolymph ions to the intestinal lumen (Anderson and Harvey, 1966). Thus, changes in these cells due to exposure to the insecticide may lead to an imbalance of the normal function of the intestinal epithelium, compromising the growth and development of the insect.

In some regions, we visualized ruptures in the basolateral membrane of the columnar cells, in the nuclear envelope and in the apical membrane/microvilli. The microvilli were deformed (Fig. 4), and the absence of actin microtubules inside them suggests that the exposure to insecticides and toxins seems to be related to the decrease and depolymerization of the actin microfilaments in the cell that favor the formation of cytoplasmic protrusions (Nogueira et al., 1997; Anuradha et al., 2007; Scudeler et al., 2016). This microfilament deficit also resulted in cell hypertrophy with the formation and release of cytoplasmic protrusions containing cytoplasmic and nuclear residues, which were observed along the epithelium and in the intestinal lumen (Fig. 4F). These changes have been reported frequently in expositions of insecticides and toxins in insects (Scudeler and Santos, 2013; Almeida et al., 2014) and in *B. mori* (Chiang et al., 1986; Munhoz et al., 2013). Cellular hypertrophy is the first change to be observed in cell lesions due to the loss of ionic control and water entry, causing cellular dilation and cellular lysis (Cheville, 2009).

3.4. Cell death analysis

To analyse the occurrence of cell death in the midgut epithelium following to the Novaluron exposure, we used several apoptotic markers (Franzetti et al., 2012). Despite the occurrence of severe cellular damage in the midgut of TGs larvae in the three larval instars analyzed, Novaluron did not induce cell death. In fact, only a few positive nuclei were evidenced by the TUNEL assay in the midgut of treated insects (Fig. 5B, C, E, F, H and I), probably due to the normal physiology of the epithelium, rather than the effects of the insecticide, as also demonstrated in the midgut of *Ceraeochrysa claveri* (Neuroptera: Chrysopidae) in our previous studies with a biopesticide neem oil (Scudeler and Santos, 2013; Scudeler et al., 2014).

Despite the absence of DNA fragmentation, the midgut epithelium of *B. mori* had a reasonable activation of caspase-3 during larval development in response to exposure to the insecticide Novaluron. A positive reaction was not found in the CGs (Fig. 6A, C and E), but a clearly positive reaction was visible in the cytoplasm of the midgut epithelial cells of the TGs in the three larval instars larvae (Fig. 6B, D and F), indicating a toxic response to the

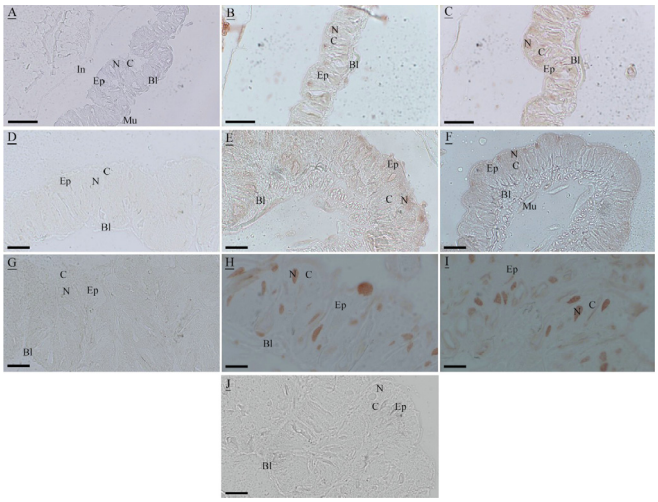

Fig. 5. TUNEL assay in the midguts of *B. mori* larvae. **A, D, G:** CG from 3rd, 4th and 5th instars, respectively. No signal is visible in the midgut epithelium (Ep). Intima (In); nucleus (N); cytoplasm (C); basal lamina (Bl); muscle (Mu). **B, C, E, F, H, I:** TG from 3rd, 3rd, 4th, 4th and 5th instars, respectively, with the nucleus are stained. No staining can be observed in TUNEL-negative control **(J)**. Bars: A - C = 24 μm; D – J = 12 μm.

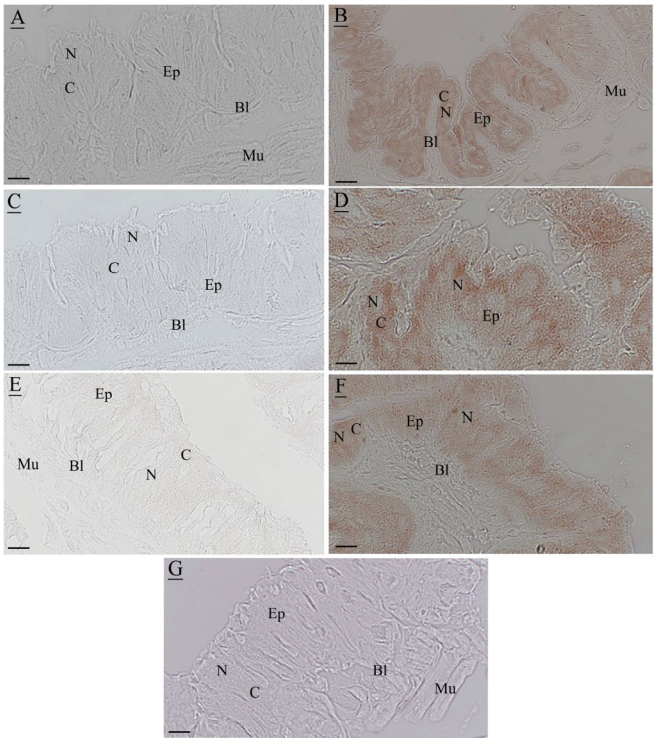

Fig. 6. Immunolocalization of activated Caspase-3 in the midguts of *B. mori* larvae. **A, C, E:** CG from 3rd, 4th and 5th instars, respectively. The antibody does not reveal any positivity for caspase-3 in midgut epithelium (Ep). Nucleus (N); cytoplasm (C); basal lamina (Bl); muscle (Mu). **B, D, F:** TG from 3rd, 4th and 5th instars, respectively, where a signal is visible in cytoplasm of the cells midgut. No staining can be observed in negative control **(G)**. Bars: 12 μm.

insecticide. According to Nicholson (1999), inactive caspases are present in the cytoplasm before initiation of the cell death process, and the activation of these caspases is closely related to programmed cell death. The activation of the starter caspases leads to cleavage and/or activation of effector caspases (or executors). After activation, the effector caspases attack and cleave major intracellular components, promoting several morphological and biochemical characteristics associated with apoptosis, including plasma membrane blebbing and DNA fragmentation. However, according to Accorsi et al. (2015), an even wider role has been hypothesized for caspases of the "undead" cells of the imaginal disc in *Drosophila melanogaster*. "Undead" cells are imaginal disc cells that do not undergo the expected apoptosis when a proapoptotic stress is applied to the *Drosophila* wing imaginal disc. The resistance of "undead" cells towards apoptosis mainly depends on the overexpression of an effector caspase inhibitor, such as p35. We believe that the positive reaction for caspase in treated insects is not directly involved in the process of apoptotic cell death but rather in another type of cell death that is evidenced by ultrastructural features such as those observed in this study.

Although Novaluron is not used directly in mulberry plantations, when applied to surrounding agricultural crops, mainly by aerial spraying, it can adhere to mulberry leaves and damage silkworms that feed on the leaves, it could damage the silk production chain. Several studies have noted disturbances in the life cycle of the silkworm that directly impact the production of quality cocoons (Kuribayashi, 1988; Nath et al., 1997; Yin et al., 2008; Nasr, 2011; Munhoz et al., 2013; Yu et al., 2013; Gu et al., 2014). Although these studies have used different insecticides from Novaluron, the reported toxic effects are very similar to those observed in our study. These effects of Novaluron exposition are also reported on other beneficial and nontarget insects (Cutler et al., 2006; Mommaerts et al., 2006; Cutler and Scott-Dupree, 2007); these show that Novaluron is not always a safe insecticide as is presumed and included in integrated pest management (IPM) programs expressly because it is thought to be of selective toxicity to target insects and not to negatively affect or at least minimally impact nontarget species (Tunaz and Uygun, 2004; Dhadialla et al., 2005).

4. Conclusions

Our results indicate that with only 24 h of exposure of *B. mori* larvae to Novaluron, it is possible to demonstrate a negative impact on insect development and induces a set of cellular injuries in the midgut epithelium, affecting midgut functions. This study adds relevant information to the toxicology literature by providing details of the cellular injuries suffered by a nontarget organism, as well as, illustrating the consequences of possible long-term exposure, in environmental, of a nontarget organism. Thus, the use of crop protection chemicals requires extreme caution to avoid deleterious damage to the environment and to nontarget species, and sublethal effects must be considered and assessed at all stages of an organism's life cycle in order for crop protection chemicals to be considered safe.

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