WIANE MELONI SILVA

INSECTICIDE SELECTIVITY TO THE PREDATOR *Podisus nigrispinus* (HETEROPTERA: PENTATOMIDAE) USED IN EUCALYPTUS CULTURE

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência Florestal, para obtenção do título de *Doctor Scientiae*.

Orientador: Jose Cola Zanuncio

Coorientadores: Rosa Angelica Plata-Rueda
Luis Carlos M. Castrillón

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Assentimento:

Wiane Meloni Silva
Autora

Jose Cola Zanuncio
Orientador

Aos meus pais, Aguinaldo e Sidinéia, meu irmão Watson e meu tio Joaquim,

Dedico

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(Albert Einsten)

RESUMO

SILVA, Wiane Meloni, D.Sc., Universidade Federal de Viçosa, setembro de 2021. **Seletividade de inseticidas ao predador *Podisus nigrispinus* (Heteroptera: Pentatomidae) usados na cultura do eucalipto**. Orientador: Jose Cola Zanuncio. Coorientadores: Rosa Angelica Plata-Rueda e Luis Carlos Martínez Castrillón.

O controle de lagartas desfolhadoras na silvicultura inclui o manejo químico e biológico de insetos. O percevejo predador *Podisus nigrispinus* Dallas (Heteroptera: Pentatomidae) é um agente de controle biológico, mas alguns inseticidas podem afetar esse inimigo natural, que necessita de avaliação de risco. Portanto, a seletividade de inseticidas no uso com organismos não alvo em áreas agrícolas e florestais é essencial para o manejo integrado de pragas. O presente trabalho visou preencher lacunas sobre os efeitos dos inseticidas quando utilizados em conjunto com o predador *P. nigrispinus*. No capítulo 1, o objetivo foi avaliar os efeitos colaterais na sobrevivência, respiração, resposta comportamental, preferência e consumo em ninfas de terceiro ínstar de *P. nigrispinus* quando exposto a *Bacillus thuringiensis* (*Bt*), permetrina, tebufenozida e tiametoxam em laboratório. Os inseticidas permetrina e tiametoxam foram os mais tóxicos para ninfas de *P. nigrispinus*. A sobrevivência das ninfas desse predador foi de 93,3%, 66,7%, 56,6%, 0% e 0% no controle, tratamentos com tebufenozida, *Bt*, permetrina e tiametoxam, respectivamente. O *Bt* e os inseticidas tebufenozida, permetrina e tiametoxam reduziram a taxa respiratória de *P. nigrispinus*. Permetrina, tebufenozida e tiametoxam afetaram a locomoção das ninfas desse inseto. Presas tratadas com *Bt*, permetrina e tiametoxam foram menos preferidas por *P. nigrispinus*. Além disso, a redução do consumo de presas, tratadas com inseticidas neurotóxicos, reduziu o potencial predatório desse inimigo natural. *Bt* e tefubenozida apresentaram baixa toxicidade para *P. nigrispinus*, mas os produtos neurotóxicos apresentaram baixa compatibilidade com esse inimigo natural e, portanto, não são recomendados, com esse predador, no manejo de insetos-praga florestais. No capítulo 2 foi investigada a sobrevivência, preferência e consumo de presas e a histopatologia do intestino médio de adultos de *P. nigrispinus* alimentados com presas tratadas com a concentração letal (CL_{50}) de *Bacillus thuringiensis* (*Bt*), permetrina, tebufenozida e tiametoxam. Os inseticidas *Bt*, permetrina e tiametoxam reduziram a sobrevivência e o consumo de presas em *P. nigrispinus* alimentado com presas contaminadas com esses inseticidas. No entanto, os quatro inseticidas testados, incluindo a tebufenozida, causaram alterações histológicas, como arquitetura epitelial irregular, vacuolização do citoplasma e liberação de fragmentos celulares no lúmen do intestino médio de *P. nigrispinus.* Os efeitos subletais de *Bt*, permetrina, tebufenozida e tiametoxam para o inimigo natural sugerem que eles devem ser mais bem avaliados para serem usados junto com *P. nigrispinus* para o manejo integrado de pragas na silvicultura.

Palavras-chave: Compatibilidade. Controle biológico. Organismo não alvo. Pesticidas.

ABSTRACT

SILVA, Wiane Meloni, D.Sc., Universidade Federal de Viçosa, September, 2021. **Insecticide selectivity to the predator *Podisus nigrispinus* (Heteroptera: Pentatomidae) used in eucalyptus culture**. Adviser: Jose Cola Zanuncio. Co-advisers: Rosa Angelica Plata-Rueda and Luis Carlos Martínez Castrillón.

The control of defoliating caterpillars in forestry includes chemical and biological insect management. The predatory stink bug *Podisus nigrispinus* Dallas (Heteroptera: Pentatomidae) is a biological control agent, but some insecticides can affect this natural enemy, which needs risk assessment. Therefore, the selectivity of insecticides for use with non-target organisms in agricultural and forestry areas is essential for integrated pest management. The present work aimed to fill gaps about the effects of insecticides when used together with the predator *P. nigrispinus*. In chapter 1, the objective was to evaluate the side effects on survival, respiration, behavioral response, preference and consumption in third instar nymphs of *P. nigrispinus* when exposed to *Bacillus thuringiensis* (*Bt*), permethrin, tebufenozide and thiamethoxam in the laboratory. The insecticides permethrin and thiamethoxam were the most toxic to *P. nigrispinus* nymphs. The survival of nymphs of this predator was 93.3%, 66.7%, 56.6%, 0% and 0% in the control, treatments with tebufenozide, *Bt*, permethrin and thiamethoxam, respectively. *Bt* and the insecticides tebufenozide, permethrin and thiamethoxam reduced the respiratory rate of *P. nigrispinus*. Permethrin, tebufenozide and thiamethoxam affected the locomotion of this insect's nymphs. Prey treated with *Bt*, permethrin and thiamethoxam were less preferred by *P. nigrispinus*. Furthermore, the reduction in the consumption of prey, treated with neurotoxic insecticides, reduced the predatory potential of this natural enemy. *Bt* and tefubenozide had low toxicity for *P. nigrispinus*, but neurotoxic products had low compatibility with this natural enemy and, therefore, they are not recommended, with this predator, in the management of forest pests. In chapter 2 we investigated the survival, preference and consumption of prey and the histopathology of the midgut of adults of *P. nigrispinus* fed on prey treated with the lethal concentration (LC_{50}) of *Bacillus thuringiensis* (*Bt*), permethrin, tebufenozide and thiamethoxam. The insecticides *Bt*, permethrin and thiamethoxam reduced survival and prey consumption in *P. nigrispinus* fed on prey contaminated with these insecticides.

However, the four insecticides tested, including tebufenozide, caused histological changes, such as irregular epithelial architecture, cytoplasmic vacuolization and release of cell fragments in the lumen of the midgut of *P. nigrispinus*. The sublethal effects of *Bt*, permethrin, tebufenozide and thiamethoxam on the natural enemy suggest that they should be better evaluated to be used in conjunction with *P. nigrispinus* for integrated pest management in forestry.

Keywords: Biological control. Compatibility. Non target organism. Pesticides.

SUMÁRIO

Introdução geral

O aumento da área com plantações de eucalipto tem sido acompanhado pela invasão de insetos e patógenos, com impactos na produtividade dessa cultura (Wingfield et al., 2008). O dano por pragas florestais foi registrado em cerca de 85 milhões de hectares por ano no mundo (Lierop et al., 2015).

O Brasil é o maior produtor mundial de celulose de eucalipto, cultura que representa 72,7% de plantios no país (Indústria Brasileira de Árvores, 2017) e insetos nativos (Zanuncio et al., 2005) como espécies de Lepidoptera (Zanuncio et al., 1993; Santos et al., 2002) que danificam esta planta. As principais pragas Lepidopteras que atacam plantios de eucalipto são *Apatelodes scericea* Schaus (Apatelodidae), *Blera varana* Schaus (Notodontidae), *Eupseudosoma aberrans* Schaus (Arctiidae), *Sarsina violascens* (Henrrich-Schäffer) (Lymantriidae) e *Thyrinteina arnobia* (Stoll) (Geometridae). Além disso, pragas exóticas como *Glycaspis brimblecombei* Moore (Heteroptera: Psyllidae), *Thaumastocoris peregrinus* Carpintero & Dellapé (Heteroptera: Thaumastocoridae) ameaçam esse setor (Wingfield et al., 2008).

O Conselho de Manejo Florestal (FSC) avalia e certifica plantações florestais que fornecem produtos florestais seguindo estratégias de conservação ambiental (Lindenmayer et al. 2000). O FSC estabelece normas para avaliação de inseticidas utilizados no manejo integrado de pragas florestais (Rametsteiner e Simula 2003), incluindo classes de toxicidade de pesticidas, categorizadas como "altamente perigosas" ou proibidas em florestas (Lemes et al. 2017). No Brasil, plantações florestais devem atender os critérios do sistema de certificação para o uso de pesticidas químicos (Lemes et al., 2017).

Inseticidas são os principais métodos usados no manejo integrado de pragas (MIP) (França et al., 2017) e, quando aplicados em ecossistemas agrícolas e florestais, podem causar impactos ambientais, como reduzir a biodiversidade (Mall et al., 2018). Pesquisadores e fabricantes buscam moléculas inseticidas com maior especificidade para pragas alvo, mas a maioria desses compostos afetam inimigos naturais (Fernandes et al., 2010), como percevejos predadores Asopinae (Heteroptera: Pentatomidae). Os ingredientes ativos que vêm sendo mais utilizados para o controle de pragas têm sido do grupo dos piretroides e neonicotinoides, como permetrina e tiametoxam, respectivamente (Santos et al., 2015). Os inseticidas reguladores de crescimento e o bioinseticida *Bt* (*Bacillus thuringiensis*) surgiram como opções de uso no MIP, sendo menos perigosos para os inimigos naturais, mais seletivos e menos tóxicos a esses organismos (Sparks et al., 2019). Esses inseticidas, com diferentes modos de ação, foram escolhidos e utilizados nessa pesquisa pois são certificados pelo FSC e podem ser utilizados em programas de MIP Florestal (Lemes et al. 2017).

Inseticidas com modos de ação específicos podem causar efeitos letais e subletais em predadores (França et al., 2017). A atividade inseticida sobre a biologia, comportamento, fisiologia e a manutenção populacional de inimigos naturais têm sido relatados em estudos toxicológicos e demográficos, podendo reduzir a ação desses organismos no controle biológico (Zanuncio et al., 2013, 2014).

Neonicotinoides atuam como agonistas de receptores nicotínicos da acetilcolina de insetos (nAChRs), ligando-se irreversivelmente e causando atividade muscular incontrolável, paralisia e eventual morte (Moser & Obrycki, 2009). Além de causar esses sintomas neurotóxicos afetam também parâmetros comportamentais como orientação e forrageio de artrópodes predadores (Desneux et al., 2007; Martinou et al., 2014). Esses efeitos subletais sobre inimigos naturais reduzem a capacidade de controlar pragas agrícolas e florestais (Poletti et al., 2007; He et al., 2012), como o tiametoxam que causa mortalidade no predador *Orius insidiosus* Say (Heteroptera: Anthocoridae) em contato com planta e presas tratadas (Camargo et al., 2017).

Os piretroides atuam como moduladores do canal de sódio, causando excitação, tremores e morte no inseto (IRAC, 2021). Destaca-se a deltametrina, o inseticida é tóxico para *Supputius cincticeps* Stal (Heteroptera: Pentatomidae), após exposição por contato causando alterações comportamentais como irritabilidade, diminuição da locomoção e da taxa de predação (Castro et al., 2013). Os piretroides e neonicotinoides são inseticidas neurotóxicos que desencadeiam respostas comportamentais (Desneux et al., 2007, Braga et al., 2011), como exaustão, redução da eficiência na captura da praga e morte (Cordeiro et al., 2010, Biondi et al., 2012, He et al., 2012). A ação desses compostos sobre o sistema nervoso prejudica os neurônios sensoriais e conduções nervosas, reduzindo a locomoção e comportamento de forrageio, consequentemente aumentando o tempo parado e reduzindo a distância percorrida dos insetos (Velki et al., 2014).

Tebufenozida é um inseticida regulador de crescimento (IRC's), de terceira geração de inseticidas com os benefícios de serem menos tóxicos a vertebrados, com modo de ação mais específico, por não persistirem no ambiente por longos períodos e terem menor toxicidade para polinizadores e inimigos naturais (Zibaee et al., 2011; Lee et al., 2018). Tebufenozida é um agonista do ecdisônio que causa a ecdise prematura do inseto por ativar genes que regulam o início do processo, mas permanece alto nos tecidos, impedindo que genes responsáveis por finalizar a muda sejam expressos, levando o inseto a morte (Retnakaran et al., 2001; Choi et al., 2019). Portanto, a toxicidade desse inseticida para predadores naturais deve ser considerada, estes produtos podem ter efeitos subletais sobre o desenvolvimento, a reprodução e sobrevivência dos predadores, quando utilizados no controle de pragas (Evangelista Júnior et al., 2002; Desneux et al., 2007).

Bacillus thuringiensis (*Bt*) é uma bactéria gram-positiva em forma de bastonete que durante a esporulação, produz proteínas tóxicas para insetos (Van Rie et al., 1990). A liberação comercial de plantas geneticamente modificadas expressando genes de *Bt* tem gerado preocupações no desenvolvimento de resistência da praga alvo e seu impacto potencial no meio ambiente e nos grupos de organismos não alvo (Dutton et al., 2002). No entanto, avaliações dos efeitos de bioinseticidas deve ser estudado para definir sua seletividade no uso de manejo integrado de pragas (Dutton et al., 2002).

Os insetos Heteroptera possuem a digestão extra oral (Cohen, 1995), que ocorre no intestino médio, sendo funcionalmente a parte mais importante do trato digestório, participando ativamente da digestão final dos alimentos e absorção de nutrientes (Zhu et al., 2011). E efeitos da redução nutricional na sobrevivência, desenvolvimento e na reprodução dos insetos devido a uso de inseticidas tem sido relatado (Cunha et al., 2015), podendo causar o estresse celular pela exposição e contaminação de inimigos naturais a inseticidas nestas regiões que podem prejudicar processos fisiológicos como a digestão, comprometendo o potencial do predador como agente de controle biológico de pragas (Martinez et al., 2018).

O Brasil é o maior consumidor de agrotóxicos do mundo, e diversos estudos comprovam os malefícios para a saúde humana e ambiental da exposição a esses produtos. Na última década, o Brasil expandiu em 190% o mercado de agrotóxicos, o que colocou o país em primeiro lugar no ranking mundial de consumo desde 2008 (Lopes et al., 2018). E o impacto dos agrotóxicos no meio ambiente evidencia o prejuízo causado sobre os insetos, a água, o solo e os peixes pelo uso dessas substâncias, muitas vezes, por alterarem seu habitat natural (Chelinho et al., 2012). Algumas substâncias já proibidas há décadas no País, como é o caso do Hexaclorociclohexano (HCH), ainda estão sendo detectadas em amostras de águas, poços e mananciais (Kussumi et al., 2011).

Agrotóxicos muito utilizados como a cipermetrina, a lambda-cialotrina e o tiametoxam, podem ser prejudiciais ao desenvolvimento de insetos, como o *Telenomus podisi* (Bastos et al., 2017), e áreas com uso de alguns inseticidas podem alterar a biodiversidade local de insetos (Resende et al., 2016). Estudos também citaram que algumas substâncias, como o spinosad e o imidacloprido, podem estar relacionadas a mortalidade de abelhas e interferindo em suas atividades de voo (Tomé et al., 2015). O número de espécies de abelhas também pode estar prejudicado pelo uso de inseticidas associados a culturas geneticamente modificadas (Pires et al., 2014). Os agrotóxicos interferem diretamente no equilíbrio do ecossistema e, consequentemente, na vida animal e humana, e os impactos afetam desde a alteração da composição do solo, passando pela contaminação da água e do ar, podendo interferir nos organismos vivos terrestres e aquáticos, alterando sua morfologia e função dentro do ecossistema (Lopes et al., 2018).

Neste contexto, a associação de inseticidas com organismos não alvo em programas de MIP deve ser bem avaliada (Castro et al., 2015). A toxicologia, com acaricidas, inseticidas sintéticos, microbiológicos e botânicos vêm sendo estudados no Brasil desde 1992 (Castro et al., 2011; Pires et al., 2015), para identificar compostos tóxicos para insetos pragas e com baixo ou nenhum impacto em predadores (Pires et al., 2015). Assim, o controle biológico surge como um método mais seguro, permanente e econômico em relação a outros métodos de controle, podendo ser usado como alternativa ou complementando o controle químico (França et al., 2017; Janssen & van Rijn, 2021). O controle biológico aplicado é implementado em mais de 30 milhões de hectares no mundo (van Lenteren et al., 2017), e no Brasil seu uso vem aumentando para o controle de algumas pragas (Parra & Coelho Junior, 2019). Asopinae (Heteroptera: Pentatomidae) é composta por predadores com potencial uso em áreas agrícolas e florestais (Ferreira et al., 2008; Araújo et al., 2011). Pentatomidae é uma das maiores famílias de Heteroptera com uma estimativa de 10% das espécies, entre elas *Podisus nigrispinus* (Dallas).

Podisus nigrispinus é um percevejo predador utilizado como regulador populacional de pragas, principalmente Lepidoptera (Oliveira et al., 2002; Zanuncio et al., 2008), e sua ocorrência tem sido registrada em diferentes culturas, como eucalipto (Zanuncio et al., 1994; Zanuncio et al., 2014; Castro et al., 2015). A espécie é zoofitófaga e se alimenta de material vegetal das plantas hospedeiras, de presas e obtendo água e nutrientes como complemento da dieta (Coll & Guershon, 2002; Zanuncio et al., 2002). Esse inimigo natural foi relatado predando mais de 25 espécies de lagartas com eficiência e pode ser criado em laboratórios e liberado em campos agrícolas e florestais para o controle biológico (Saavedra et al., 1997; Torres et al., 2006; Torres et al., 2009).

A seletividade de inseticidas à inimigos naturais é chave no manejo integrado de pragas (MIP) para favorecer a permanência em campo, desses organismos (Santos Junior et al., 2019), ou seja, combinar um eficiente controle da praga com mínima influência negativa sobre a atividade de espécies benéficas, sendo assim compatíveis com as demais estratégias do MIP (Mohaghegh et al., 2000). Nesse sentido, o presente estudo visou a importância de avaliar os efeitos letais e subletais que os inseticidas podem causar em inimigos naturais, impedindo seu uso consorciado com organismos não alvo.

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RESEARCH PAPER 1: Respiration, predatory behavior and prey consumption by *Podisus nigrispinus* (Heteroptera: Pentatomidae) nymphs exposed to some insecticides

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Abstract

Podisus nigrispinus Dallas (Heteroptera: Pentatomidae) preys on insect pests in eucalyptus plantations where it can be exposed to insecticides used in pest control. The effect of insecticides on non-target natural enemies requires further study. The objective of the present study was to evaluate the side effects of *Bacillus thuringiensis* (*Bt*), permethrin, tebufenozide and thiamethoxam on third instar nymphs of the predator *P. nigrispinus* in the laboratory. The toxicity of insecticides for this insect was determined by estimating their lethal concentrations. *Podisus nigrispinus* behavior after exposure to insecticides was analyzed using a video tracking system and the respiratory rate with a respirometer. Prey/nymph consumption was assessed after 24 h of starvation. The preference of *P. nigrispinus* nymphs, for prey treated or not with the insecticides, was evaluated in free choice tests. The insecticides Bt [LC_{50} = 1.10 (0.83 - 1.46) mg mL^{-1}], permethrin [LC_{50} = 0.25 (0.17 - 0.34) mg mL^{-1}], tebufenozide [LC_{50} = 5.71 (4.17 - 7.57) mg mL^{-1}] and thiamethoxam [LC_{50} = 0.04 (0.02 - 0.06) mg mL^{-1}] are toxic to *P. nigrispinus* nymphs. *Bt* and the insecticides tebufenozide, permethrin and thiamethoxam reduced the respiratory rate of *P. nigrispinus*. The insecticides permethrin, tebufenozide and thiamethoxam affect the locomotion of this insect’s nymphs. Prey treated with *Bt*, permethrin and thiamethoxam are less preferred by *P. nigrispinus*. The survival of the nymphs of this predator was 93.3%, 66.7%, 56.6%, 0% and 0% in the control, tebufenozide, *Bt*, permethrin and thiamethoxam treatments, respectively. In addition, the reduction of prey consumption, treated with neurotoxic insecticides, reduces the predatory potential of this natural enemy. *Bt* and tefubenozide present low toxicity for *P. nigrispinus*, but the neurotoxic products have low compatibility with this natural enemy and, therefore, are not recommended, with this predator in the management of forest insect pests.

Keywords: Chemical pesticides, forest culture, natural enemy, non-target organismo, toxicity.

1. Introduction

The predatory stink bug, *Podisus nigrispinus* Dallas (Heteroptera: Pentatomidae) fed on *Apatelodes scericea* Schaus (Apatelodidae), *Blera varana* Schaus (Notodontidae), *Eupseudosoma aberrans* Schaus (Arctiidae), *Sarsina violascens* (Henrrich-Sch€affer) (Lymantriidae) and *Thyrinteina arnobia* (Stoll) (Geometridae) in eucalyptus plantations (Torres et al., 2006). Biology and ecology, including development (De Bortoli et al., 2011), morphology (Martínez et al., 2017), predator-prey interaction (Ferreira et al., 2008) and feeding strategies (Martínez et al., 2014a, 2016) of *P. nigrispinus* have been studied. The use of this predator in pest management can expose it to insecticides in forest plantations (Rosell et al., 2008).

In Brazil, forest plantations must meet certification system criteria for chemical pesticide use (Lemes et al., 2017). *Bacillus thuringiensis* (Bt), permethrin, tebufenozide and thiamethoxam, with different modes of action, are the most commonly used insecticides for forest pest control (EPA, 2011; Lemes et al., 2017). *Bt*, a gram-positive, rod-shaped bacterium produces toxic proteins during their sporulation causing gut cell lysis and insect death (Van Rie et al., 1990). Tebufenozide is an ecdysone receptor agonist inducing premature and lethal ecdises in insect larvae (Fiaz et al., 2018a). Permethrin acts on the axon membrane affecting voltage-gated sodium channels (Martínez et al., 2018). Thiamethoxam binds, irreversibly and specifically, to nicotinic acetylcholine receptors affecting the transmission of nerve impulses in insects (Nauen et al., 2003). These insecticides cause mortality, cell death, movement paralysis and reduced respiration in insect forest pests (Lemes et al., 2017). These insects are preyed upon by P. nigrispinus (Fiaz et al., 2018a; Mbanze et al., 2018; Castro et al., 2019) and, for this reason, the effects of these pesticides on this predator need to be studied.

The objective of this research was to evaluate the survival, respiration, behavior, prey preference and consumption by *P. nigrispinus* nymphs, after exposure to *Bt*, permethrin, tebufenozide and thiamethoxam.

2. Materials and methods

2.1. Insects

P. nigrispinus nymphs and *Tenebrio molitor* Linnaeus (Coleoptera: Tenebrionidae pupae were obtained from mass rearing at the Laboratory of Biological Control of Insects of the Universidade Federal de Viçosa (UFV) in Viçosa, Minas Gerais, Brazil, and maintained at 27 ± 2 ºC, relative humidity of 75 ± 5% and 12:12 h [L: D] photoperiod. *P. nigrispinus* nymphs and adults were fed *T. molitor* pupae na *Eucalyptus grandis* (W. Hill ex. Maiden) leaves ad libitum. *Tenebrio molitor* larvae were fed wheat bran (12% protein, 2% lipids, 75% carbohydrates and 11% minerals), *Saccharum officinarum* Linnaeus (Poaceae) stems and *Sechium edule* (Jacq.) Swartz (Cucurbitaceae) fruits *ad libitum* until the pupal stage. *Tenebrio molitor* pupae were kept in plastic vials (60 x 40 x 12 cm). *Podisus nigrispinus* nymphs and *T. molitor* pupae, without apparent amputations or malformations, were used in the bioassays.

2.2. Concentration-mortality bioassay

The bioinsecticide *Bacillus thuringiensis* (*Bt*) kurstaki variety, HD^{-1} strain (Dipel® SC 33.6 g L^{-1}, Abbot Laboratories Chemical & Agricultural Products Division, North Chicago, IL, USA) and the insecticides tebufenozide (Mimic® SC 240 g L^{-1}, Dow Agro-Sciences, Jacareí, SP, Brazil), permethrin (Permetrina® EC, 384 g L^{-1}, Fersol Indústria e Comércio Ltda., Mainrique, SP, Brazil) and thiamethoxam (Talcord® CE 250gL^{-1}, BASF SA, Guaratinguetá, SP, Brazil) were, each, diluted in 1 L of distilled water to obtain stock solutions, adjusting 100 g L^{-1} for the test concentrations. Six concentrations of each insecticide were used to assess their toxicity and determine the relevant toxicological parameters. Serial dilutions of concentrations of *Bt*, tebufenozide, permethrin, and thiamethoxam (0.78, 1.56, 3.12, 6.25,12.5 and 25 mg mL^{-1}) were performed to determine their concentration-mortality ratio and lethal concentrations (LC_{25}, LC_{50}, LC_{75} and LC_{90}). Distilled water was used as a control. *Tenebrio molitor* pupae were submerged for 5 s in the respective insecticide concentrations, according to the treatments, and air dried. One *T. molitor* pupa was placed per glass tube (2 x 15 cm) as food for a third instar nymph of *P. nigrispinus*. Three replicates, with 30 *P. nigrispinus* nymphs each, were used per concentration and the number of dead insects was counted 96 h after the exposure of *T. molitor* pupae to the insecticides.

2.3. Time-mortality bioassay

Podisus nigrispinus third instar nymphs were individualized in glass tubes (2 x 15 cm), according to the concentration-mortality bioassay, and exposed to the LC50 of each insecticide in addition to the control with distilled water. Exposure procedures, conditions and number of insects were the same as in the concentration mortality bioassay. Three replicates with 30 nymphs each were used per insecticide concentration in a completely randomized design. The live insect data were obtained every 6 h for 10 days.

2.4. Respiration rate

Respiration rate bioassays were conducted for 3 h with *P. nigrispinus* nymphs after exposure to the LC_{50} and LC_{90} insecticide doses according to the procedures in section 3.3.2. Insects treated with distilled water were used in the control. The production of carbon dioxide (CO_2) (µL CO_2 h^{-1}/insect) was measured with a respirator of the type CO_2 Analyzer TR3C (Sable System International, Las Vegas, NE, USA) (Fiaz et al., 2018b; Plata-Rueda et al., 2019a). A *P. nigrispinus* nymph was placed per respirometric chamber (25 mL) connected to a closed system. The CO_2 production was measured for 12 h at 27 ± 2 ºC after acclimatizing the insects. Compressed oxygen gas (99.99% pure) was passed through the chamber at a flow rate of 100 mL min^{-1} for 2 min to quantify the CO_2 produced. The air flow forces the CO_2 molecules produced to pass through an infrared reader attached to the system, continuously measuring the production of this gas by the insects in the chamber. *Podisus nigrispinus* nymphs were weighed, before and after the experiment, on an analytical scale (Sartorius BP 210D, Gottingen, Germany). Fifteen replicates were used per concentration of each insecticide and in the control in a completely randomized design.

2.5. Behavioral response

Podisus nigrispinus nymphs were placed in Petri dishes (90 x 15 mm) with a filter paper disc (9 cm in diameter with 3 mm porosity, 0.5% ash content, and density of 80 g/m^2) (Nalgon Equip. Científicos, Itupeva, SP, Brazil) fixed to their bottom with synthetic glue. Behavioral response bioassays were performed in arenas Half treated with 1 mL of the LC_{50} or LC_{90} of the insecticide dissolved according to the treatment while the other half was treated Only with distilled water as a control. A *P. nigrispinus* nymph was released in the centre of the Petri dish (onto the filter paper) and evaluated for 10 min. Twenty insects were used per lethal concentration in a completely randomized design. The movement of each insect inside the Petri dish, was recorded with a digital camcorder (XL1 3CCD NTSC, Canon, Lake Success, NY, USA) equipped with a 16 x video lens (ZoomXL 5.5-88 mm, Canon). The distance walked and the resting period of each insect, per half of the Petri dish was obtained with a video tracking system (View Point Life Sciences, Montreal, Quebec, Canada). Insects that spent <1 s or <50% of the period in the half of the arena treated with insecticide were considered repelled and irritated, respectively (Plata-Rueda et al., 2018, 2019b).

2.6. Prey preference

The preference of *P. nigrispinus*, between preys treated or not with insecticide, was determined in choice tests. Thirty *P. nigrispinus* nymphs were placed per Petri dish (90 x 15 mm) with a filter paper disc (Whatman No. 1) at the bottom of the plate and used as an arena. The internal walls of the Petri dish were covered with polytetrafluoroethylene (Dupont®, Barueri, SP, Brazil) to prevent insects from escaping. Two *T. molitor* pupae (one treated with the LC_{50} of each insecticide and the other untreated) were placed on opposite sides of the arena and a *P. nigrispinus* nymph was released at the centre and the preferences for the pupae evaluated. The prey was considered preferred when the nymph inserted its stylet into the body and fed for 5 min. Thirty insects were used per concentration of the insecticides in a completely randomized design.

2.7. Prey consumption

Podisus nigrispinus third instar nymphs were individualized in glass tubes (2 x 15 cm) and starved for 24 h. After this period, *T. molitor* pupae, exposed to the lethal concentration (LC_{50}) of the insecticides were supplied to the *P. nigrispinus* nymphs for 48 h. Pupae treated with distilled water were used in the control. Prey consumption was determined by the initial (time 0 before nymphs were supplied) and the final (time 48 h) weight obtained on na analytical scale (Shimadzu Corporation, Kyoto, Japan). Thirty insects were used per insecticide concentration in a completely randomized design.

2.8. Statistics

The concentration-mortality data were submitted to probit analysis, generating a concentration-mortality curve (Finney,1964). Time-mortality data were submitted to survival analysis with the Kaplan-Meier estimator (log-rank test) with Origin Pro software. 9.1. (OriginLab Corporation, 2013). Nymph survival data was withheld until the end of the experiment while data for locomotor behavior, respiration rate and prey consumption were submitted to analysis of variance (ANOVA). The Tukey test (HSD) was used to identify differences between the means at 5% significance. Respiration rate and behavioral response data were arcsine-transformed to fit presuppositions of normality and homoscedasticity and the preference test results were compared by Student's t-test at 5% significance. Toxicity data, respiratory rate, behavioral response, preference and prey consumption were analyzed using SAS for Windows v. 9.0. (SAS Institute, 2002).

3. Results

3.1. Concentration-mortality

The concentration-mortality model was adequate (X^2; $P > 0.05$) proving the toxicity of the insecticides against the predator *P. nigrispinus* (Table 1). The estimated value of the lethal concentration (LC_{50}) showed that the more toxic pesticides were permethrin and thiamethoxam with LC_{50} of 3.75 (3.30-4.21) and 4.34 (3.69-5.20) mg mL^{-1}, respectively, followed by the tebufenozide and *Bt* with LC_{50} of

3.38 (2.98-3.77) and 38.3 (34.2-42.4) mg mL^{-1}, respectively. The mortality of the *P. nigrispinus* nymphs was <1% in the control.

Table 1. Lethal concentrations (LC) and estimated values (EV) of confidential limits (CL) for four insecticides in third instar nymphs of Podisus nigrispinus (Heteroptera: Pentatomidae) by Probit analysis (DF= 5; P > 0. 05)

Insecticides	LC	EV (mg mL^{-1})	CL (95%)	Slope (± SE)	χ^2 (*P*)
Bacillus thuringiensis	25	30.1	25.3-33.7	6.37 (± 0.95)	5.49 (0.36)
	50	38.3	34.2-42.4		
	75	48.9	44.1-56.1		
	90	60.9	53.6-74.6		
Tebufenozide	25	2.62	2.15-2.97	6.03 (± 0.90)	2.75 (0.74)
	50	3.38	2.98-3.77		
	75	4.38	3.92-5.02		
	90	5.52	4.84-6.76		
Permethrin	25	2.81	2.28-3.21	5.37 (± 0.83)	2.91 (0.71)
	50	3.75	3.30-4.21		
	75	5.01	4.46-5.88		
	90	6.50	5.60-8.31		
Thiamethoxam	25	2.75	2.05-3.29	3.41 (± 0.62)	4.89 (0.43)
	50	4.34	3.69-5.20		
	75	6.85	5.63-9.66		
	90	10.3	7.81-17.8		

3.2. Time-mortality

P. nigrispinus nymph survival, after exposure to LC_{50}, differed between insecticides (log-rank test; X^2= 118.43; df= 4; P < 0.001). After 385 h of exposure, survival was 93.33%, 66.67%, 56.67%, 0.00% and 0.00% in the control, tebufenozide, *Bt*, permethrin and thiamethoxam, respectively (Fig. 1).

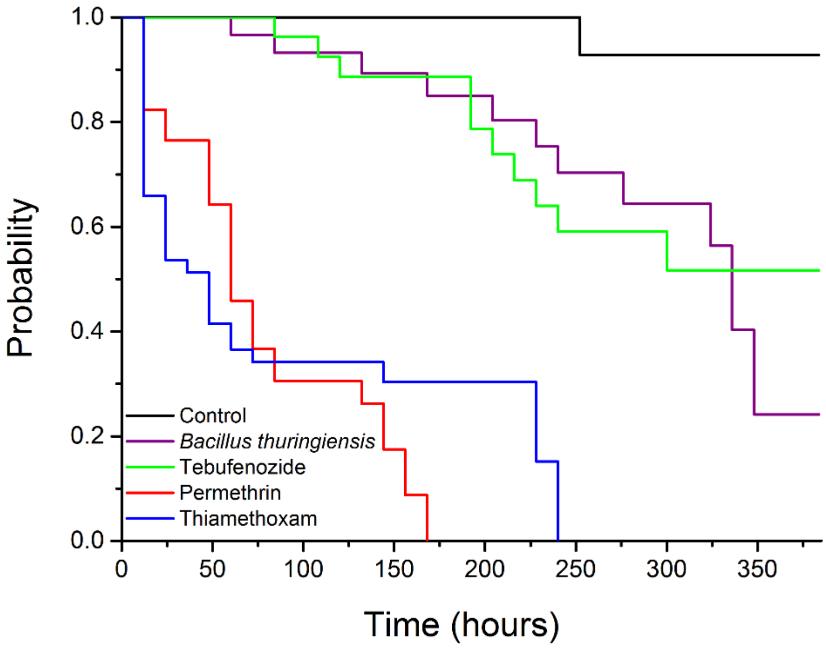

Figure 1: Survival curves of *Podisus nigrispinus* (Heteroptera: Pentatomidae) nymphs exposed to lethal concentration (LC50) of four insecticides using the log-rank test of Kaplan-Meier estimators (X^2 = 118.43; df= 4; P < 0.001).

3.3. Respiration rate

The respiration rate of *P. nigrispinus* nymphs decreased between 1 and 3 h after exposure to the lethal concentrations (LC_{50} and LC_{90}) of the *Bt* ($F_{2,14}$ = 24.45, P < 0.05), tefubenozide ($F_{2,14}$= 25.33, P < 0.05), permethrin ($F_{2,14}$= 361.4, P < 0.05) and thiamethoxam ($F_{2,14}$= 53.04, P < 0.05) (Fig. 2).

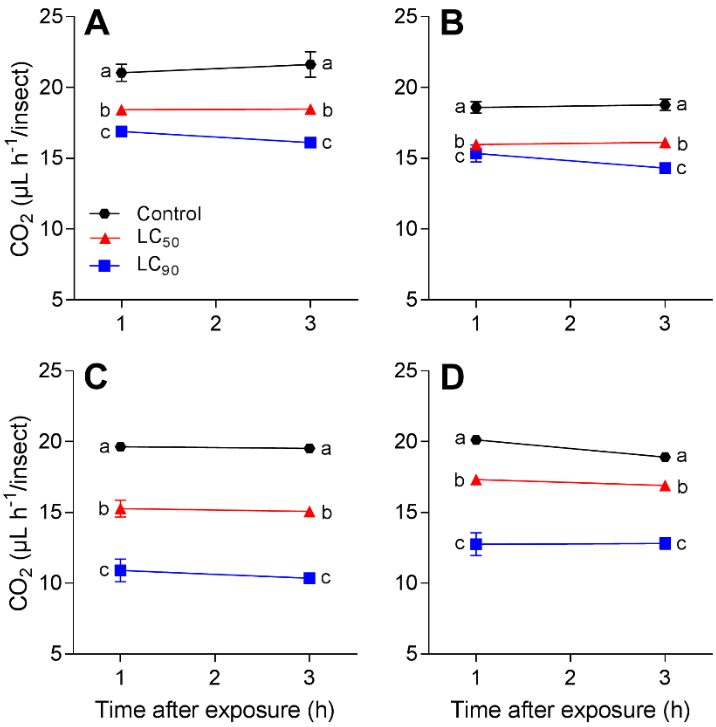

Figure 2: Respiratory rate (mean ± SE) of *Podisus nigrispinus* (Heteroptera: Pentatomidae) nymphs exposed to four insecticides during 3 h. *Bacillus thuringiensis* (A), tebufenozide (B), permethrin (C) and thiamethoxam (D). Means (control and estimated values of LC_{50} and LC_{90}) differ by $P < 0.05$ by Tukey test.

3.4. Behavioral response

The distance walked by *P. nigrispinus* nymphs was similar in the control and with LC_{50} and LC_{90} of *Bt* ($F_{2,14}$= 0.84, P= 0.616). The distance walked with LC_{50} and LC_{90} of tebufenozide was greater ($F_{2,14}$= 4.03, $P < 0.001$) and that with LC_{50} and LC_{90} of permethrin ($F_{2,14}$= 4.52, $P < 0.001$) and thiamethoxam ($F_{2,14}$= 4.54, $P < 0.001$) shorter than in the control (Fig. 3). The resting period was similar in the control and with LC_{50} and LC_{90} of *Bt* ($F_{2,14}$= 1.35, P= 0.258) and longer with tebufenozide ($F_{2,14}$= 5.05, $P < 0.001$), permethrin ($F_{2,14}$= 3.62, $P < 0.021$) and thiamethoxam ($F_{2,14}$= 4.86, $P < 0.001$) (Fig. 3).

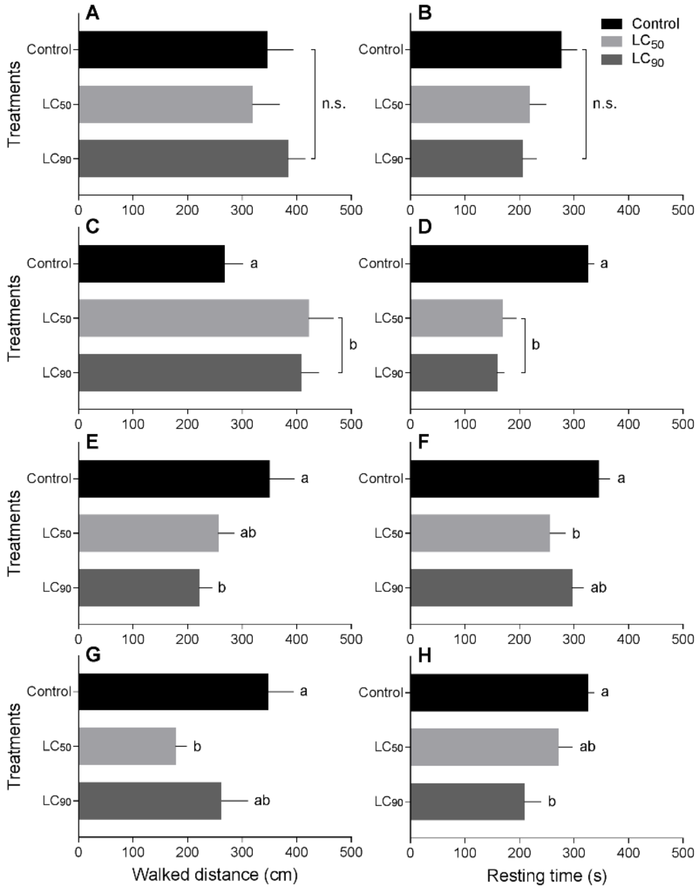

Figure 3: Distance walked and resting time (mean ± SEM) of *Podisus nigrispinus* (Heteroptera: Pentatomidae) exposed to Bt (A and B), tebufenozide (C and D), permethrin (E and F) and thiamethoxam (G and H), (control, estimated LC_{50} and LC_{90} values) for 10 min. Means differ by the HSD Tukey test (P < 0.05).

3.5. Prey preference

The preference of *P. nigrispinus* nymphs for *T. molitor* pupae, treated or not, differed between pesticides, with a higher value for those treated with *Bt* ($t_{1,10}$= 4.26, P < 0.002) and lower with tebufenozide ($t_{1,10}$= 2.49, P < 0.016). The preference for prey exposed to permethrin ($t_{1,10}$= 0.26, P= 0.806) and thiamethoxam ($t_{1,10}$= 0.25, P= 0.804) was similar to that in the control (Fig. 4).

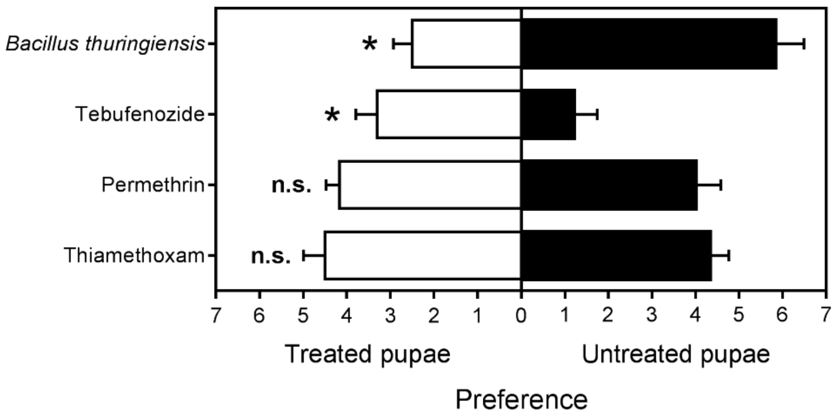

Figure 4: Preference for pupae treated or not with insecticides by *Podisus nigrispinus* (Heteroptera: Pentatomidae) nymphs analyzed by Student's t-test at 5% significance.

3.6. Prey consumption

The prey consumption by *P. nigrispinus* nymphs differed between treatments ($F_{4,29}$= 2.58; P < 0.001) with a higher value in the control (24.5 ± 2.51 mg), followed by tebufenozide (20.9 ± 1.84 mg), *Bt* (16.73 ± 2.49 mg), thiamethoxam (9.33 ± 1.15 mg) and permethrin (6.83 ± 0.69 mg) (Fig. 5).

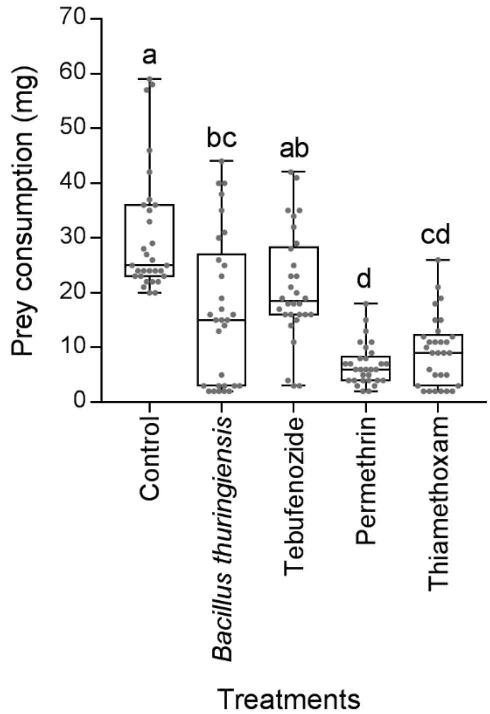

Figure 5: Prey consumption (mean ± SEM) of *Tenebrio molitor* (Coleoptera: Tenebrionidae) pupae exposed to four insecticides by *Podisus nigrispinus*

(Heteroptera: Pentatomidae) nymphs during 48 h. Values in the same column with the same letter are similar at the $P < 0.05$ level by Tukey test.

4. Discussion

Bt, permethrin, tebufenozide and thiamethoxam are toxic for *P. nigrispinus* nymphs feeding on prey exposed to these insecticides. The symptoms in this predator are similar to those reported for permethrin, affecting voltage-gated sodium channels, tebufenozide as an ecdysone receptor agonist, and thiamethoxam biding to nicotinic acetylcholine receptors. Insecticides cause concentration dependent mortality of *P. nigrispinus*, but the effect of *Bt* and tebufenozide on nymphs of this predator, is at doses higher than those recommended for insect pest control. The LC_{50} of *Bt*, to control *Plutella xylostella* Linnaeus (Lepidoptera: Plutellidae) and *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae) is 0.5 mg mL^{-1} and 0.003 mg mL^{-1}, respectively (Magalhães et al., 2015; Fiaz et al., 2018a), without side effects in *P. nigrispinus*. The concentration-mortality bioassay shows that permethrin and thiamethoxam are toxic to *P. nigrispinus* nymphs and, therefore, with reduced compatibility for simultaneous use with this predator in integrated pest management.

The higher toxicity of permethrin and thiamethoxam for *P. nigrispinus* nymphs, from 168 to 240 h, may be due to their neurotoxic action, as observed for the predatory stink bugs *Macrolophus pygmaeus* Rambur (Miridae), *Orius insidiosus* (Say) (Anthocoridae) and *Supputius cincticeps* (Stal) (Pentatomidae) (Zanuncio et al., 1998; Arnó and Gabarra, 2011; Moscardini et al., 2013). The neurotoxic effect of permethrin and thiamethoxam may reduce the compatibility of these insecticides with the predator *P. nigrispinus* in integrated pest management. The high survival of *P. nigrispinus* nymphs after feeding on prey exposed to *Bt*, suggests a reduced effect on this natural enemy that has no Cry receptors in the midgut cells, which are necessary to activate the *Bt* toxin (Magalhães et al., 2015). The slower action and shorter residual period of tebufenozide (Smagghe and Degheele, 1995) may explain the high survival of the predator *P. nigrispinus* after exposure to this insecticide.

The reduction in the respiration rate of *P. nigrispinus* nymphs, between 1 and 3 h, after exposure to lethal concentrations of tefubenozide, permethrin and thiamethoxam indicates physiological stress and an impact on the metabolic defense against toxic compounds in this predator. The decrease in the respiratory rate has also been reported for other insects exposed to different insecticides, such as *Anticarsia gemmatalis* to tebufenozide (Fiaz et al., 2018a), *Hypothenemus hampei* Ferrari (Coleoptera: Curculionidae) to chlorantraniliprole (Plata-Rueda et al., 2019b) and *P. nigrispinus* to the constituents of lemongrass essential oil (Brügger et al., 2019). The reduction in respiratory rates increases the cost of mobility in insects, due to the greater movement for contraction of the abdominal muscles for continuous air renewal in the trachea, affecting physiological processes and leading to high energy consumption (Fiaz et al., 2018b; Rueda et al., 2019a), which may reduce muscle activity causing permanent paralysis (Plata-Rueda et al., 2019a).The reduction in the respiratory rate in insects is due to the absorption of chemical compounds, which, on reaching their place of action (Cotton, 1932; Plata-Rueda et al., 2017) inhibit oxidative phosphorylation and deregulate respiratory activities (Pimentel et al., 2007; Plata-Rueda et al., 2019a), which may be occurring with *P. nigrispinus* nymphs.

The similar distance traveled by *P. nigrispinus* nymphs with both LC_{50} and LC_{90} of *Bt* and the control, the longer distance with tebufenozide, and the shorter distance with thiamethoxam and permethrin than in the control, may be due to the action of these compounds in the nervous system, stimulating or reducing the mobility of insects, as reported for *P. nigrispinus*, *A. gemmatalis*, and *T. molitor* with changes in behavioral responses after exposure to the insecticides tebufenozide, imidacloprid and essential oil of garlic and its constituents (Martínez et al., 2019a; Plata-Rueda et al., 2017; Fiaz et al., 2018b). *Bt* and the insecticides tebufenozide, permethrin and thiamethoxam may affect the olfactory system and the walking behavior of *P. nigrispinus*, and consequently, its substrate recognition, due to their toxic effects, as has been suggested to occur in other insects (Germinara et al., 2015; Plata-Rueda et al., 2018; Fiaz et al., 2019). The lack of repellency by tebufenozide for *P. nigrispinus* nymphs may be due to the irritant side effect of this insecticide, with longer distance walked by the nymphs of this predator when exposed to this insecticide. The shorter distance walked by *P. nigrispinus* nymphs, after exposure to the insecticides permethrin and thiamethoxam, may be due to their neurotoxic activity, blocking potassium voltage-dependent channels in the insect muscles and nerves (Salgado, 1998). The ingestion and exposure of insects to neurotoxic insecticides causes intoxication and alters the contractions of the abdominal intersegmental muscles involved in the hemolymph circulation and respiration (Slama and Miller,1987; Slama,1987). Behavioral reactions of *P. nigrispinus*, after contact with the insecticides permethrin and thiamethoxam include avoidance without previous contact with the insecticide (repellency) as well as irritability by the tebufenozide as was reported for *Tenebrio molitor*, *Hypothenemus hampei* and *Podisus nigrispinus* when exposed to insecticides (Plata-Rueda et al., 2017, 2019b; Martínez et al., 2018). The irritability caused by tebufenozide and repellency by permethrin and thiamethoxam for *P. nigrispinus* nymphs may reduce the efficiency of this natural enemy in capturing prey.

The lower preference of *P. nigrispinus* nymphs for pupae treated with tebufenozide than those with other insecticides may be due to the sensory detection capacity of this predator (olfactory or tactile) for prey contaminated or exposed to toxic compounds. Predatory Hemiptera detect chemical compounds via chemoreceptors in the antennae and mouthparts, identifying specific stimuli before contact with the prey (Sant'ana and Dickens, 1998; van Loon et al., 2000). The pests *Acrosternum hilare* (Say) and *Euschistus servus* (Say) (Hemiptera: Pentatomidae), exposed to plants contaminated or not with the insecticides azadirachtin, pyrethrin and spinosad, preferred those untreated due to the anti-feeding and repelente effect of the insecticides tested (Kamminga et al., 2009). The selection of prey reduces predation, due to increasing searching time for prey not exposed to insecticides (Biondi et al., 2012; Camargo et al., 2017). Prey preference for *P. nigrispinus* and other predators involves sense organs to detect those contaminated with insecticides because feeding on them reduces the predatory capacity of *P. nigrispinus*. The higher prey consumption by *P. nigrispinus* nymphs in the control, followed by tebufenozide, thiamethoxam and permethrin suggests an inhibitory effect on feeding, probably due to the neurotoxic action of these insecticides (Martínez et al., 2019a) that cause paralysis (Martínez et al., 2014b, 2019b). The intoxication of *P. nigrispinus*, after exposure to neurotoxic insecticides, has a side effect on digestion and absorption of nutrientes due to damage to the midgut epithelium after oral exposure to the insecticides imidacloprid (Martínez et al., 2019b), permethrin (Martínez et al., 2018) and spinosad (Santos Júnior et al., 2019a). Neurotoxic insecticides (imidacloprid and permethrin) cause histological changes in the organs involved in digestion such as the *P. nigrispinus* salivary glands and midgut (Martínez et al., 2019b; Santos Júnior et al., 2019b) compromising amino acid absorption, immobilizing digestive enzymes and protecting gut cells Against mechanical damage (Fialho et al., 2013).

The insecticides permethrin and thiamethoxam are toxic for and reduce *P. nigrispinus* survival, with higher mortality between 168 and 240 h and *Bt* and tefubenozide at 384 h, leading to mortality and reducing the survival of nymphs of this predator. Changes in *P. nigrispinus* behavioral response, caused by these insecticides, indicate physiological stress that compromises predation by this natural enemy. The preference for prey treated with *Bt* compared to those treated with tebufenozide, affects predation by *P. nigrispinus* and the lower preference for prey treated with permethrin and thiamethoxam reduces the functional and numerical responses of this predator. The reduction of prey consumption for those exposed to permethrin and thiamethoxam, affects the predatory potential of *P. nigrispinus*, indicating its low compatibility with neurotoxic insecticides. *Bt* and tefubenozide, in commercial field doses, may be compatible with *P. nigrispinus*, but permethrin and thiamethoxam cause lethal and sublethal effects on this natural enemy, suggesting that the combined use of these insecticides with this predator should be further evaluated in integrated pest management.

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RESEARCH PAPER 2: Exposure to insecticides causes effects on survival, prey consumption, and histological changes in the midgut of the predatory bug, *Podisus nigrispinus* (Hemiptera: Pentatomidae)

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Abstract

The control of defoliating caterpillars in forestry includes the use of insecticides and releases of the predatory bug *Podisus nigrispinus*, but some compounds may affect non-target natural enemies, which need evaluation of risk assessment. This research investigates the survival, preference, and prey consumption of *P. nigrispinus* adults fed with prey treated with the lethal concentration (LC_{50}) of *Bacillus thuringiensis* (*Bt*), permethrin, tebufenozide, and thiamethoxam. Moreover, midgut histopathology of *P. nigrispinus* fed with preys treated with LC_{50} of each insecticide was investigated. The insecticides *Bt*, permethrin, and thiamethoxam reduce the survival and the prey consumption in *P. nigrispinus* fed with preys contaminate with these chemicals. However, the four tested insecticides, including tebufenozide, cause histological changes such as irregular epitelial architecture, cytoplasm vacuolization, and release of cell fragments in the midgut lumen of *P. nigrispinus*. The sublethal effects of *Bt*, permethrin, tebufenozide, and thiamethoxam to the natural enemy suggest that they should be better evaluated to be used together with *P. nigrispinus* for integrated pest management in forestry.

Keywords: Chemical hazardous, forestry, histopathology, non-target organismo, predatory bug, toxicity

1. Introduction

The Forest Stewardship Council (FSC) evaluates and certifies forest plantations that supply forest products following environmental conservation strategies (Lindenmayer et al. 2000). The FSC sets standards for assessing insecticides used in forest integrated pest management (Forest IPM) (Rametsteiner and Simula 2003), including pesticide toxicity classes, categorized as “highly hazardous” or prohibited in forestry (Lemes et al. 2017). In Brazil, forestry adopts the FSC criteria reducing the use of pesticides, favoring the sustainability and protection of organism diversity. In this context, the FSC criteria allow the use and establishment of natural enemies such as predators and parasitoids in Forest IPM programs (Zanuncio et al. 2016a).

The predatory bug, *Podisus nigrispinus* Dallas (Hemiptera: Pentatomidae), is an important natural enemy found in forest systems (De Bortoli et al. 2011), but its rational use for biological control depends on the compatibility with other pest control methods (De Castro et al. 2015). The development (Medeiros et al. 2000), predator-prey interactions (Martínez et al. 2014, 2016), and functional/numerical response (Ferreira et al. 2008) of *P. nigrispinus* on preys with different sizes and developmental stages have been investigated. However, some insecticides affect the development, longevity, predation behavior, reproduction, and digestion of predatory bugs (Zanuncio et al. 2016b; Martínez et al. 2018; Silva et al. 2020).

Insecticides with low susceptibility to predatory bugs include *Bacillus thuringiensis* (*Bt*), permethrin, tebufenozide, and thiamethoxam (De Castro et al. 2013; Lemes et al. 2017). *Bt* is a gram-positive, rod-shaped bacterium that produces Cry- or Vip-toxic proteins causing lysis in the midgut cells followed by septicemia (Plata-Rueda et al. 2020a). Permethrin affects voltage-gated sodium channels in the neuron causing paralysis and death (Martínez et al. 2018). Tebufenozide is an ecdysone receptor agonist that induces premature molting with lethality to immature insect (Fiaz et al. 2018). Thiamethoxam interferes in the neuron synapses binding to nicotinic acetylcholine receptors (Nauen et al. 2003). These insecticides, with different modes of action, are certified by the FSC and can be used in Forest IPM programs (Lemes et al. 2017).

Insecticide exposure per os, i.e., through ingestion of treated prey, affects the midgut, the main organ where digestion and absorption take place (Castro et al. 2021). The midgut of *P. nigrispinus* is anatomically divided into the anterior, middle, and posterior regions (Fialho et al. 2013) and, like other hemipterans, the lumen is covered by a lipoprotein perimicrovillar membrane (Teixeira et al. 2013), which play some roles in the compartmentalization of the digestion, amino acid absorption, and conservation of digestive enzymes (Terra and Ferreira 2020). However, insecticides can cross this midgut membrane causing damages on epithelial cells and be transported by the hemolymph reaching their target sites (Denecke et al. 2018; Santos-Junior et al. 2020).

The exposure of *P. nigrispinus* to insecticides such as *Bt*, permethrin, tebufenozide, and thiamethoxam should be studied, because they are toxic to insect pests which are common preys of this predatory bug in Brazilian forest plantations (Zanuncio et al. 2016a; Lemes et al. 2017).

The objective of this study was to investigate the survival, prey preference, prey consumption, and midgut histopathology of *P. nigrispinus*, fed on prey treated with the pesticides *Bt*, permethrin, tebufenozide, and thiamethoxam used for pest control, which are preys of this natural enemy.

2. Materials and Methods

2.1 Insects

Adults of *P. nigrispinus* were obtained from a mass rearing in the Laboratory of Biological Control in the Federal University of Viçosa (Viçosa, Minas Gerais state, Brazil), at 28 ± 2 °C, 78 ± 5% RH, and 12-h photophase. These insects were fed on *Tenebrio molitor* Linnaeus (Coleoptera: Tenebrionidae) pupae (reared in the same *P. nigrispinus* conditions), *Eucalyptus grandis* W. Hill ex. Maiden (Myrtaceae) leaves, and water ad libitum. Newly emerged (less than 48-hour-old) *P. nigrispinus* adults were used in the experiments.

2.2 Concentration-mortality test

The commercial Bt-formulation and chemical insecticides commonly used to control Lepidopteran pests in the Brazilian forestry (Lemes et al. 2017) were used in all experiments. The following Bt, HD-1 strain var kurstaki (Dipel® SC 33.6 g L−1, Abbott Laboratories Chemical & Agricultural Products Division, North Chicago, IL, USA), permethrin (Permetrina® EC, 384 g L−1, Fersol Indústria e Comércio Ltda., Mainrique, São Paulo, Brazil), tebufenozide (Mimic® SC 240 g L−1, Dow Agro-Sciences, Jacareí, São Paulo, Brazil), and thiamethoxam (Talcord® CE 250 g L−1, BASF S.A., Guaratinguetá, São Paulo, Brazil) were prepared in 100 mL of deionized water to obtain a stock suspension, from which dilutions were prepared as needed. Six dilutions (ranging from 2 to 100 g of active ingredient L−1) were used to evaluate the toxicity of each insecticide to *P. nigrispinus* adults, constructo concentration-morality curves, and estimate the lethal concentrations (LC_{50} and LC_{90}). Water alone was used as a control. Subsequently, *T. molitor* pupae were immersed for 5 s in each insecticide dilution and allowed to dry in the environment. In the treatments, one *T. molitor* pupae exposed to the insecticide was placed in a glass vial (20 mL) as a food source for each
P. nigrispinus adult. Three replicates with 30 insects were used per dilution, and the number of dead insects was counted after exposure of up to 96 h to the *T. molitor* pupae with the insecticide.

2.3 Time-mortality test

The time-mortality test for *P. nigrispinus* using lethal concentration (LC_{50}) obtained in the concentration-mortality test of each insecticide was carried per os using the prey *T. molitor pupae*. Then, the pupae were soaked for 5 s in solutions prepared with the calculated lethal concentration (LC_{50}) of each insecticide and air-dried for 30 min. Distilled water was used as control. Adults of *P. nigrispinus* were individualized in glass tubes (2 × 15 cm), and a *T. molitor* pupae treated with the LC_{50} of each insecticide was supplied. Three replicates with 30 insects were used per insecticide in a completely randomized experimental design. The number of live predators was counted each 8 h for 11 days.

2.4 Prey preference

The preference of *P. nigrispinus* for preys treated or untreated with the LC_{50} of each insecticide was determined in free choice tests. Adults of *P. nigrispinus* were individualized in a Petri dish (140-mm diameter) with a Whatman celulose filter paper (Merck KGaA, Darmstadt, Germany) covering the bottom, from here termed arena. The internal wall of the arena was covered with polytetrafluoroethylene (Dupont de Nemours and Company, Willmington, DE, USA) to prevent the insect escape. Two *T. molitor* pupae, one treated with the insecticide as aforementioned and the other untreated, were placed on opposite sides of the arena and one *P. nigrispinus* was released in the center. Preference was confirmed after the predator kept the stylet inserted into the pupa for five min. Thirty repetitions of treated/untreated pupae per insecticide were used in a completely randomized design.

2.5 Prey consumption

Podisus nigrispinus adults were individualized in glass tubes (2 × 15 cm) and starved for 24 h. After this period, *T. molitor* exposed to the lethal concentration (LC_{50}) of insecticides and water as control were supplied to the predatory bugs for 72 h. Prey consumption was calculated by the initial (before predatory bugs were supplied) and the final (72 h) weight of *T. molitor* pupae obtained on a Shimadzu AY220 analytical balance (Shimadzu Corporation, Kyoto, Japan). Twenty *P. nigrispinus* adults were used per treatment in a completely randomized design.

2.6 Histopathology

Adults of *P. nigrispinus* (n = 50; 10 per each insecticide-time exposure and 10 per control) were exposed to the estimated LC_{50} lethal concentration of *Bt*, permethrin, tebufenozide, and thiamethoxam for diferente periods (dissected at 3, 6, 12, and 24 h after insecticide exposure). Water alone was used as a control. Five insects per treatment were cryo-anesthetized at −4 °C for 3 min, the middle region of midgut dissected in saline solution (0.1 M NaCl + 0.2 M KH2PO4 + 0.2 M Na2HPO4), and transferred to Zamboni's fixative solution (Stefanini et al. 1967) for 12 h at 5 °C. Then, the midgut pieces were dehydrated in a graded ethanol series (70%, 80%, 90%, and 95%) and embedded in historesin (Leica Mycrosystems Inc., Buffalo Grove, IL, USA). Slices with 3-μm thickness of the midgut region of *P. nigrispinus* were obtained in a rotative microtome, stained with hematoxylin and eosin, and analyzed with Olympus BX-60 light microscope (Olympus Corporation, Tokyo, Japan).

2.7 Statistic

The concentration-mortality data were submitted to probit analysis to construct a concentration-mortality curve with the PROC PROBIT procedure (SAS Institute, Campus Drive Cary, NC, USA). Time-mortality data of *P. nigrispinus* adults were submitted to Kaplan-Meier survival analysis (log-rank method) with the Origin Pro v. 9.1 software (Origin Lab Corporation, Northampton, MA, USA). Prey preference was compared by Student's t test at a 5% significance level using the SAS User v.9.0. software. Prey consumption data were submitted to analysis of variance (oneway ANOVA) and means of each treatment compared by the Tukey test (HSD) at a significance level of 5% using the SAS User v. 9.0 software.

3. Results

3.1 Concentration-mortality test

The concentration-response model used was suitable (P > 0.05), which confirmed the toxicity of each insecticide to *P. nigrispinus* and provided the estimates of the desired toxicological endpoints for subsequent use (Table 1). For the estimated LC_{50} value, testing indicated that permethrin with LC_{50} = 5.58 (4.62–6.51) g L^{-1} was the most toxic insecticide for *P. nigrispinus*, followed by thiamethoxam with LC_{50} = 7.37 (6.59–8.15) g L^{-1}, tebufenozide with LC_{50} = 8.24 (7.01–9.88) g L^{-1}, and Bt with LC_{50} = 36.1 (29.5–43.5) g L^{-1}. In the control, mortality remained at < 1%.

Table 1. Lethal concentration of four insecticides against *Podisus nigrispinus* adults after 96 h exposure, obtained from probit analysis (df = 5). The chi-square (χ^2) value refers to the goodness of fit test at $P > 0.05$.

Insecticide	Lethal concentration	Estimated concentration (g L^{-1})	95%Confidence interval (g L^{-1})	Slope (±SE)	χ^2 (*P*-value)
Bacillus thuringiensis	LC_{50}	36.1	29.5 – 43.5	2.16 ± 0.21	2.52 (0.64)
	LC_{90}	140.	108. – 201.		
Permethrin	LC_{50}	5.58	4.52 – 6.51	1.61 ± 0.84	2.11(0.83)
	LC_{90}	8.34	7.06 – 11.8		
Tebufenozide	LC_{50}	8.24	7.01 – 9.88	1.37 ± 0.36	3.29 (0.50)
	LC_{90}	13.1	10.6 – 18.3		
Thiamethoxam	LC_{50}	7.37	6.59 – 8.15	1.96 ± 0.64	3.57(0.41)
	LC_{90}	13.5	11.6 – 17.1		

3.2 Time-mortality test

The survival rates were registered when *P. nigrispinus* adults were exposed for 264 h to insecticides and indicated diferences at LC_{50} (log-rank test; $\chi 2 = 57.90$; DF = 4; $P < 0.001$). For the treatments, *P. nigrispinus* survival decreased from 86.67% in the control to 66.67% with tebufenozide, 23.33% with *Bt*, and 0.1% with thiamethoxam and permethrin (Fig. 1).

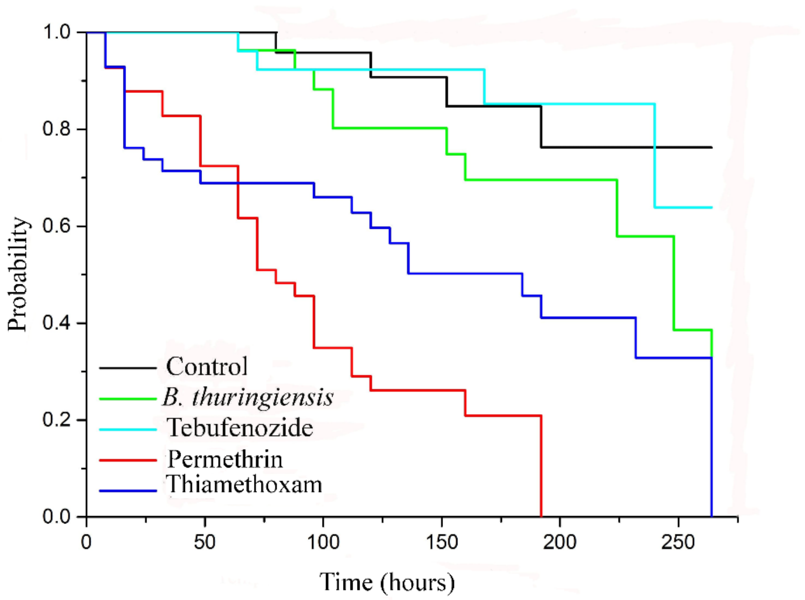

Figure 1: Survival curves of *Podisus nigrispinus* adults exposed to prey treated with four insecticides subjected to survival analysis using the Kaplan-Meier estimators (log-rank test $\chi 2 = 57.90$; DF = 4; P < 0.001).

3.3 Prey preference

The preference of *P. nigrispinus* adults for *T. molitor* pupae treated or not was different according to the insecticides. The lower preference was with prey exposed to *Bt* ($t_{1,29} = 19.79$, $P < 0.001$), where as those treated with tebufenozide ($t_{1,29} = 1.11$, $P = 0.290$), permethrin ($t_{1,29} = 0.988$, $P = 0.347$), and thiamethoxam ($t_{1,29} = 1.534$, $P = 0.156$) had similar preferences in comparison with control (Fig. 2).

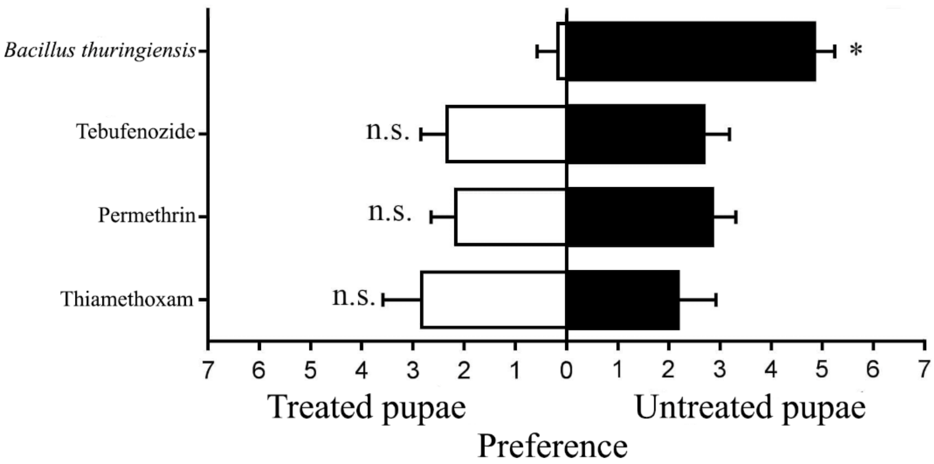

Figure 2: Preference (mean ± SEM) of *Podisus nigrispinus* adults of preys treated or untreated with insecticides subjected by Student's t test. n.s not significant, *significant at 5% significance level.

3.4 Prey consumption

The prey consumption by *P. nigrispinus* adults was affected by prey exposure to different insecticides ($F_{4,19}$ = 15.74; $P < 0.001$) with similar higher prey consumptions in the control (24.5 ± 2.51 mg) and in the prey treated with tebufenozide (20.9 ± 1.84 mg). The prey consumption by the predator decreased significantly in those treated with *Bt* (16.73 ± 2.49 mg), thiamethoxam (9.33 ±1.15 mg), and permethrin (6.83 ± 0.69 mg) (Fig. 3).

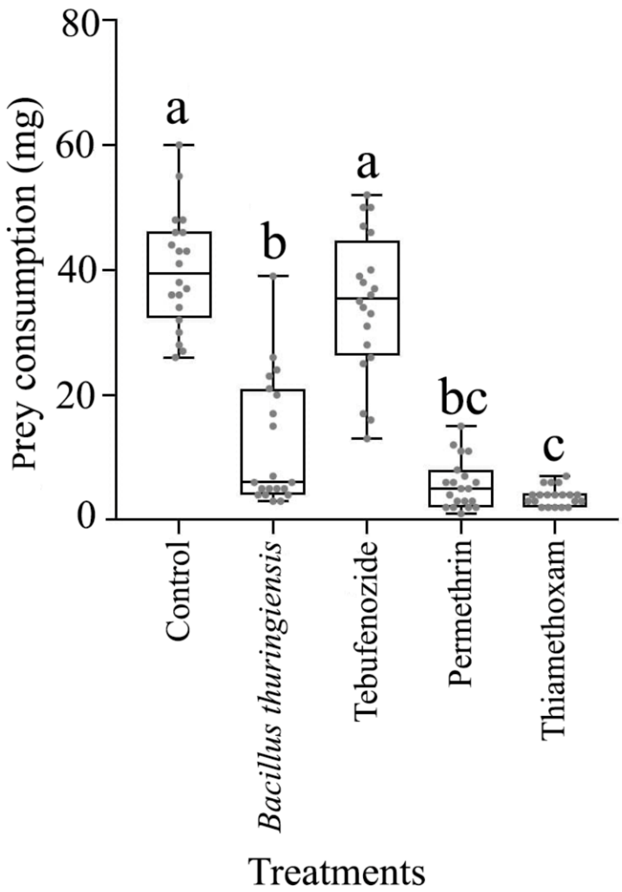

Figure 3: Box plot representing the prey consumption by *Podisus nigrispinus* adults. Insects were fed during 48 h on prey treated with four different insecticides. Different letters indicate statistically significant diferences among treatments when compared by one-way ANOVA follows by Tukey's post-test (P < 0.05)

3.5 Histopathology

The midgut of *P. nigrispinus* had a simple epithelium with columnar digestive cells and nests of regenerative cells scattered in the basal region, whereas the lumen had evidente perimicrovilar membrane (Fig. 4). The digestive cells had a well developed apical brush border, cytoplasm with some vacuoles, and nucleus with predominance of decondensed chromatin (Fig. 4). The adults of *P. nigrispinus*, after 3 h feeding on preys exposed to all insecticides, showed changes in the midgut epithelium, including irregular epithelial architecture, digestive cells with pyknotic nuclei, and apical cell fragments released to the lumen (Fig. 5A, E, I, M). Those insects feeding for 6 and 12 h on pupae treated with the insecticides showed the midgut with additional changes such as increased cytoplasm vacuolization and cell fragments released to the lumen, some with the cell nucleus (Fig. 5B, C, F, G, J, K, N, O). Bacteria were found in the midgut lumen in insects fed on pupae treated with Bt (Fig. 5B). The midgut of *P. nigrispinus* after 24 h feeding on prey exposed to *Bt* had similar features with those reported for 3 h feeding insect, whereas those exposed to tebufenozide, permethrin, and thiamethoxam had additional histological changes, including irregular epithelium and high cytoplasm vacuolization (Fig. 5). The midgut of *P. nigrispinus* feeding on prey exposed to tebufenozide and thiamethoxam for 24 h had higher cytoplasm vacuolization and release of cell fragments to the gut lumen (Fig. 5H, P).

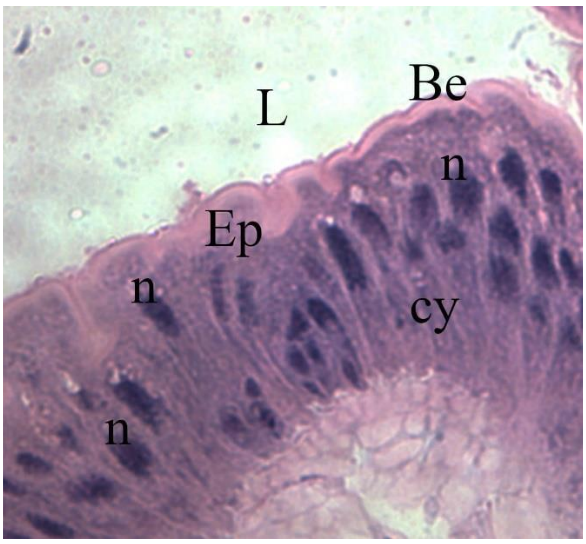

Figure 4: Histological section of the midgut of *Podisus nigrispinus* not exposed to insecticides with epithelium (*Ep*) with digestive cells with nucleus (*n*), cytoplasm (*cy*), lumen (*L*), and striated border (*Be*).

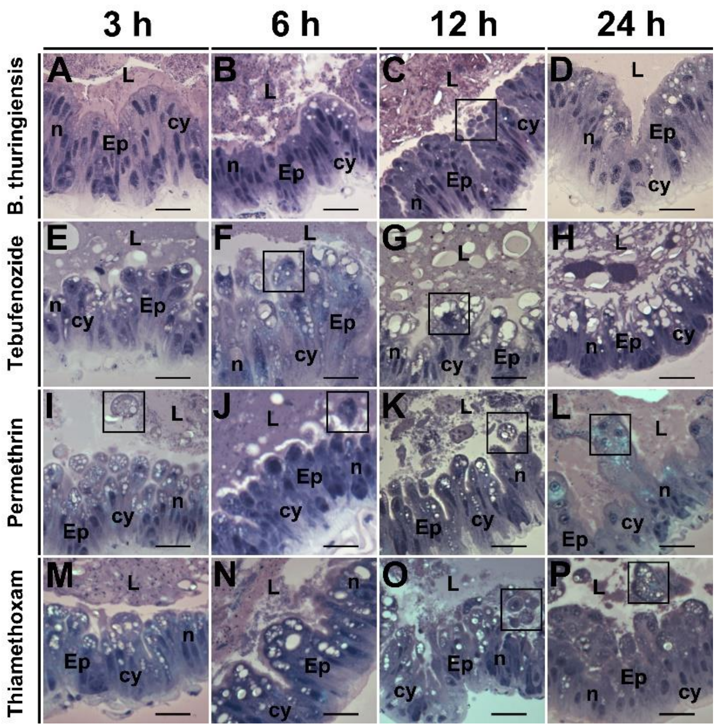

Figure 5: Histological changes of the midgut of *Podisus nigrispinus* exposed to *Bacillus thuringiensis* (**A**, **B**, **C**, and **D**), tebufenozide (**E**, **F**, **G**, and **H**), permethrin (**I**, **J**, **K**, and **L**), and thiamethoxam (**M**, **N**, **O**, and **P**) during intervals of 3, 6, 12, and 24 h. Lumen (*L*); epithelium (*EP*); nucleus (*n*); and cytoplasm (*Cy*). Arrow: bactéria present in the lumen.

4. Discussion

The exposure of prey to various insecticides causes detrimental effects on survival, prey consumption, and histopathological changes in the midgut of the natural enemy *P. nigrispinus* adults. The survival time of *P. nigrispinus* decreases mainly when this predator is fed on prey exposed to permethrin and thiamethoxam confirming toxicity of these insecticides in the nervous system. The neurotoxic effect caused by permethrin is due to its interaction with the voltage-gated sodium channel, blocking nerve impulses and causing the “know-down” effect in insects (Soderlund 2010). Low survival caused by permethrin has been also reported for the predatory wasps *Brachygastra lecheguana* Latreille and *Protonectarina sylveirae* Saussure (Hymenoptera: Vespidae) (Bacci et al. 2009). Thiamethoxam modulates the nicotinic acetylcholine receptors of the postsynaptic membrane (Matsuda et al. 2001) and reduces the survival of predatory bug *Engytatus varians* Distant (Hemiptera: Miridae) (Pérez-Aguilar et al. 2018) and *Orius insidiosus* (Say) (Hemiptera: Anthocoridae) (Camargo et al. 2017). On the other hand, the high survival of *P. nigrispinus* adults fed on prey exposed to tebufenozide may be due to this insecticide is an insect growth regulator that inhibits molt in immature insects (Fiaz et al. 2018) but without effects on the adult stage here evaluated. Indeed, tebufenozide has no effect on survival of other predators, including *Chrysoperla externa* (Hagen) (Neuroptera: Chrysopidae) (Godoy et al. 2004) and *Ceraeochrysa cubana* (Hagen) (Neuroptera: Chrysopidae) (Ono et al. 2017). Similarly, high survival of *P. nigrispinus* adults with *Bt* may occur because the bacterial toxins are activated and bound to the plasma membrane of midgut cells, such as what occurs in Lepidoptera larvae that are the main target of this bioinsecticide (Castro et al. 2019). Survivorship of predatory bugs *Podisus maculiventris* (Say) (Hemiptera: Pentatomidae), *Geocoris punctipes* (Say) (Hemiptera: Geocoridae), and *Nabis roseipennis* Reuter (Hemiptera: Nabidae) have been found after exposure to Bt-toxins (Torres and Ruberson 2008). In our study, low survival of *P. nigrispinus* caused by permethrin and thiamethoxam suggests that these neurotoxic insecticides cause deleterious effects on adults, and can drastically decrease the population level of this predatory bug in forest plantations.

The low preference of *P. nigrispinus* adults, for prey exposed to *Bt*, is similar to found for other insects feeding on insects or plants containing Bt-toxins (Sun et al. 2013; Rolim et al. 2020). Predatory insects may detect prey nutritional quality (Schoonhoven et al. 2005) and avoid those contaminated (Riddick and Barbosa 1998; Meier and Hilbeck 2001). The predator *C. carnea* has preference for the cotton caterpillar *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae) reared on non-transgenic corn in comparison with those fed on Bt-corn (Meier and Hilbeck 2001). Consequently, the energy obtained for prey capture is maximized, reducing the detoxification process if toxins are ingested (Skelhorn and Rowe 2007). In contrast, the predator Harmonia axyridis (Pallas) (Coleoptera: Coccinellidae) has no preference for *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) caterpillar fed on *Bt* and non-Bt transgenic corn (Dutra et al. 2012). Predators avoid prey contaminated with *Bt*, perhaps because toxins stimulate chemical receptors on antennal (Dutton et al. 2003) and labial sensilla (Backus 1988). On the other hand, preys exposed to insecticides permethrin, tebufenozide, and thiamethoxam have no negative impact on the foraging behavior of this natural enemy because it may feed on poisoned preys such as here observed. Similar findings occur in *O. insidiosus* exposed to thiamethoxam (Camargo et al. 2017) and *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae) exposed to spinosad (Nadel et al. 2007). The absence of perception by *P. nigrispinus* to select prey contaminated with the insecticides permethrin and thiamethoxam may result in a severe negative impact because these chemicals reduce the predator survival. Conversely, the absence of a negative effect in the foraging behavior of *P. nigrispinus* for prey exposed to insect growth regulator tebufenozide is an important finding because this insecticide has no impact on the survival and prey capture of this predatory bug. These results indicate that the neurotoxic insecticides permethrin and thiamethoxam with *P. nigrispinus* in IPM should be further evaluated since they cause high mortality and decrease the predation by this natural enemy.

The low consumption of prey exposed to *Bt*, permethrin, and thiamethoxam by *P. nigrispinus* may be due to predator intoxication during feeding, such as reported for predators *C. externa* and *C. cubana* fed on preys exposed to azadirachtin, malathion, and permethrin (Cordeiro et al. 2010). In *P. nigrispinus*, a low prey consumption occurs with *S. frugiperda* caterpillars exposed to imidacloprid (Malaquias et al. 2014). Variations in the prey consumption by predatory insects are a consequence of insecticide toxicity (Moser and Obrycki 2009). Feeding on treated prey can cause disturb on orientation and foraging (Martinou et al. 2014). Insecticides alter the functions of sensory neurons, interrupting the transmission of nerve stimuli and causing paralysis in insects (Martínez et al. 2019a), reducing locomotion and, consequently, effective feeding as here observed with *P. nigrispinus*. An importante finding here observed is that tebufenozide does not affect the prey consumption by *P. nigrispinus* in comparison with control, which together with high survival of the predatory bug exposed to this insecticide indicates that both the insecticide and the natural enemy have potential to be used in Forest IPM. Conversely, the reduction in prey consumption caused by *Bt*, permethrin, and thiamethoxam suggests incompatibility to use these insecticides with *P. nigrispinus* in IPM.

Morphological changes in the midgut of *P. nigrispinus* caused by *Bt*, tebufenozide, permethrin, and thiamethoxam are features of cell degeneration including irregular epithelium architecture, cell fragments released to the lumen, and nuclear pyknosis. Histopathological changes in the insect midgut have been found after pesticides exposure on non-target organisms, like honey bee *Apis mellifera* Linnaeus (Hymenoptera: Apidae) (Lopes et al. 2018; Arthidoro de Castro et al. 2020; Carneiro et al. 2020; Tadei et al. 2020; Serra et al. 2021) and *P. nigrispinus* (Martínez et al. 2018, 2019b; Santos-Junior et al. 2020). In the midgut, cell stress, due to the exposure of *P. nigrispinus* to insecticides, can impair digestibility (Martínez et al. 2018; Santos-Junior et al. 2020) because this is the main organ for digestion and nutriente absorption (Terra and Ferreira 2020), compromising the potential of this predator in biological control of insect pests. The increase in cytoplasm vacuolization in the digestive cells of *P. nigrispinus* exposed to the pesticides according to the time exposure may be due to the detoxification process against substances (Cossolin et al. 2019; Plata-Rueda et al. 2020b). Similar findings were found in this predator fed on prey contaminated with imidacloprid (Martínez et al. 2019b) and spinosad (Santos-Junior et al. 2020).

5. Conclusion

The insecticides *Bt*, tebufenozide, permethrin, and thiamethoxam cause detrimental effects in the non-target predatory bug *P. nigrispinus*. Permethrin and thiamethoxam cause significant effects mainly on the survival, prey consumption, and midgut histopathology of this insect. Overall, this study shows evidence that *Bt*, permethrin, thiamethoxam, and tebufenozide, although recommended for FSC in the pest control, have significant side effects on non-target beneficial insects, such as the natural enemy here evaluated. On the other hand, tebufenozide has no negative effect on survival and prey consumption of *P. nigrispinus*, indicating the potential to be used with this predator in pest management programs, although further studies with chronic exposure or more realistic sublethal doses need to be evaluated.

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Considerações finais

Os inseticidas permetrina e tiametoxam foram mais tóxicos às ninfas do inimigo natural *P. nigrispinus*, levando à mortalidade e reduzindo a sobrevivência desse predador. Esses compostos neurotóxicos causaram maior mortalidade em menor tempo, com relação a tefubenozida e o bioinseticida *Bt*.

As alterações na resposta comportamental de *P. nigrispinus*, causada por esses inseticidas, indicam estresse fisiológico o que pode comprometer a predação por este inimigo natural. A redução do consumo de presas expostas a permetrina e tiametoxam, afetou o potencial predatório de *P. nigrispinus*, indicando sua baixa compatibilidade com inseticidas neurotóxicos.

Bt e tefubenozida, em doses comerciais de campo, podem ser compatíveis com *P. nigrispinus*, mas permetrina e tiametoxam causou efeitos letais e subletais neste inimigo natural, sugerindo que o uso combinado desses inseticidas com este predador deve ser avaliado posteriormente no manejo integrado de pragas.

Este estudo mostrou evidências de que permetrina e tiametoxam, embora recomendado para FSC no controle de pragas, têm efeitos colaterais significativos nesses organismos não alvos, como o inimigo natural aqui avaliado. Por outro lado, a tebufenozida não tem efeito negativo sobre a sobrevivência e o consumo de presas por *P. nigrispinus*, assim como o *Bt* que necessitam altas doses para causar algum efeito subletal nesse predador, dosagens acima das realmente utilizadas em campo, indicando nesse caso o potencial uso desses produtos com este predador em programas de controle de pragas, embora mais estudos com exposição crônica ou mais realista das doses subletais precisam ser avaliadas.

O aumento do uso de agrotóxicos no país tem sido prejudicial ao ecossistema, causando um desequilíbrio no ambiente e na saúde humana, nesse contexto essa pesquisa evidencia a importância de buscar métodos para redução do impacto ambiental, e que há diversas maneiras possíveis de minimizar esses efeitos negativos no meio ambiente, buscando assim a diminuição do uso excessivo de agrotóxicos.