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Exposure to spinosad induces histopathological and cytotoxic effects on the salivary complex of the non-target predator *Podisus nigrispinus*

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HIGHLIGHTS

- The predator *Podisus nigrispinus* is used in biological control programs.
- Toxicity and cytotoxic effects by spinosad were evaluated in the salivary glands.
- Spinosad induces cell death in the cells of the salivary glands.
- Spinosad has side-effect in the salivary glands affecting *Podius nigrispinus* fitness.
- The use spinosad should be evaluated in integrated pest management using predators.

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ABSTRACT

In integrated pest management systems, biological and chemical controls must be compatible. The insecticide spinosad affects some non-target insects and might compromise their fitness. The objective of this study was to evaluate the histopathological and cytotoxic effects of spinosad on the salivary complex of the predatory bug *Podisus nigrispinus* (Heteroptera: Pentatomidae). Spinosad toxicity and insect survival were determined using six concentrations of insecticide. Ultrastructural changes and cell death of salivary glands were analyzed after *P. nigrispinus* exposure to spinosad LC_{50} (3.15 μg L^{-1}). The insecticide caused toxicity to *P. nigrispinus*; survival was 32% after 48 h of exposure to LC_{50}. The main histological changes in the salivary complex were disorganization of the epithelium, cytoplasmic vacuolization, and apocrine secretion into the gland lumen. Cytotoxic effects, such as release of granules and vacuoles into the lumen, presence of autophagosomes, enlargement of basal plasma membrane infoldings, and apoptosis, were observed. Spinosad causes toxicity, decreases survival, and changes the histology and cytology of the *P. nigrispinus* salivary complex. The results suggest that the cellular stress induced by the insecticide affects extra-oral digestion, compromising the potential of *P. nigrispinus* as a biological pest control agent.

1. Introduction

The predatory bug *Podisus nigrispinus* Dallas (Heteroptera: Pentatomidae) is used as a biological control agent in agricultural crops and forest areas because of its easy handling and high predatory capacity (Zanuncio et al., 2008). *Podisus nigrispinus* is a generalist predator found in the Americas that preys on insects of various sizes and stages of development (Torres et al., 2002a; Lemos et al., 2003; Ferreira et al., 2008). Several studies have been conducted on the biology and ecology of *P. nigrispinus*,

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including its development (Medeiros et al., 2000), morphology (Martínez et al., 2014a, 2017), predator–prey interaction (Ferreira et al., 2008), and feeding strategies, such as extra-oral digestion (Fialho et al., 2012; Martínez et al., 2016).

The effective use of *P. nigrispinus* in integrated pest management (IPM) programs depends on the compatibility of the predator with other control methods (De Castro et al., 2015). Chemical control is commonly used to manage insect pests. The impact of insecticides on natural enemies, however, must be taken into account (Desneux et al., 2007). Furthermore, side effects on human health, non-target organisms, and the environment reinforce the need to reduce the use of insecticides (Desneux et al., 2007; Nicholson, 2007; Pedlowski et al., 2012). For predatory bugs, exposure to insecticides can be lethal or can cause physiological damages, affecting behavior, development, longevity, and fecundity (Martínez et al., 2018; 2019).

Podisus nigrispinus has zoophytophagous feeding habits (Torres and Boyd, 2009). Its digestion initiates by injecting saliva into the prey, a process called extra-oral digestion (Cohen, 1995). The saliva is produced in the salivary complex, which consists of a pair of principal salivary glands and a pair of accessory salivary glands (Fialho et al., 2012; Martínez et al., 2014a, 2015). Saliva is composed of water, lipids, carbohydrates, enzymes that play a role in extra-oral digestion and ensure predation efficiency (Martínez et al., 2014a), and protein and non-protein toxic compounds responsible for prey paralysis and death (Martínez et al., 2016). The constitutive production of saliva in large amounts during extra-oral digestion has a high energy cost (Cohen, 1995; Martínez et al., 2016).

Predatory insects generally are tolerant to insecticides, an important characteristic for their potential use in IPM programs (De Castro et al., 2015). The insecticides deltamethrin, imidacloprid, methamidophos, chlorantraniliprole, and spinosad were reported to be toxic to *P. nigrispinus* (De Castro et al., 2013; Malaquias et al., 2014).

Spinosad is effective against some arthropods and has low toxicity to vertebrates (Martínez et al., 2014b; Liu et al., 2016; Kovaríková et al., 2017). The insecticide contains compounds of the spinosyn family produced by the bacterium *Saccharopolyspora spinosa* Mertz & Yao (Actinomycetales: Pseudonocardiaceae). Spinosad is neurotoxic (Salgado, 1998) and has a similar activity to that of neonicotinoids (Orr et al., 2009). It binds to the specific nicotinic acetylcholine and gamma-aminobutyric acid receptors (Watson, 2001). The insecticide is used for the chemical control of caterpillar pests commonly found in Brazilian agricultural crops, such as *Alabama argillacea* Hübner (Silva et al., 2011), *Anticarsia gemmatalis* Hübner (Martins et al., 2009), and *Spodoptera frugiperda* (J.E. Smith) (Noctuidae) (Méndez et al., 2002), which are prey of *P. nigrispinus* (Ramalho et al., 2008). However, there is little information on the histopathological and cytotoxic effects of spinosad on the salivary complex of predators of caterpillar pests.

The objective of this study was to evaluate acute toxicity, histopathology, and cytotoxicity in the salivary complex of *P. nigrispinus* fed on prey exposed to spinosad.

2. Material and methods

2.1. Insects

Podisus nigrispinus and *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) were mass reared in the Insect Biological Control Laboratory of the Federal University of Viçosa (Viçosa, Minas Gerais, Brazil). Insects were maintained at 27 ± 2 °C under relative humidity of 75 ± 5% and a photoperiod of 12:12 h light/dark. *Podisus nigrispinus* adults were fed *T. molitor* pupae and leaves of *Eucalyptus grandis* (W. Hill ex. Maiden) *ad libitum*. *Tenebrio molitor* larvae were fed *ad libitum* with wheat bran (12% protein, 2% lipids, 75% carbohydrates, and 11% minerals/sugar), shoots of *Saccharum officinarum* L. (Poaceae), and fruits of *Sechium edule* (Jacq.) Swartz (Cucurbitaceae) until the pupal stage. *Tenebrio molitor* pupae were kept in plastic trays (60 × 40 × 12 cm). *Podisus nigrispinus* adults and *T. molitor* pupae, without apparent amputations or malformations were used in the bioassays.

2.2. Toxicity bioassay

The insecticide Tracer® SC (480 g L^{-1} spinosad; Dow AgroSciences Industrial Ltd., São Paulo, SP, Brazil) was used from a stock solution of 100 g L^{-1} for acute toxicity tests. Insecticidal efficacy was determined under laboratory conditions by calculating the lethal concentrations (LC_{25}, LC_{50}, LC_{75}, and LC_{90}). Spinosad solutions at six different concentrations were prepared: 0.312, 0.625, 0.125, 0.25, 0.5, and 10 mg L^{-1} (w/v). Distilled water was used as control. *Tenebrio molitor* pupae were immersed for 5 s in 1 mL of each solution and then air dried. Pupae were placed separately in glass vials (2 × 10 cm) as a food source for *P. nigrispinus* adults. Eighty *P. nigrispinus* adults were used per treatment, and the number of dead insects was counted during 48 h.

2.3. Survival bioassay

Newly emerged *P. nigrispinus* adults were exposed by ingestion of *T. molitor* pupae to four lethal concentrations of spinosad (LC_{25}, LC_{50}, LC_{75}, and LC_{90}) following the same procedures of the toxicity bioassay. Distilled water was used as control. The number of dead insects was quantified every 6 h for 48 h.

2.4. Histopathology

Adults of *P. nigrispinus* (n = 10) were fed *T. molitor* pupae immersed in spinosad LC_{50} and were evaluated 0.5, 1, 3, and 6 h after feeding. Subsequently, individuals were cryoanesthetized at −4 °C and dissected in insect saline solution (0.1 M NaCl, 0.1 M KH_2PO_4, and 0.1 M Na_2HPO_4). Principal and accessory salivary glands were fixed in Zamboni's solution (Stefanini et al., 1967) for 12 h at 5 °C. Samples were dehydrated in a graded ethanol series (70%, 80%, 90%, and 95%) and embedded in Historesin (Leica Biosystems Nussloch GmbH, Wetzlar, Germany). Sections (3 μm thick) were stained with hematoxylin and eosin and analyzed under an Olympus BX-60 light microscope (Olympus Corporation, Tokyo, Japan).

2.5. Cytotoxicity

Adults of *P. nigrispinus* (n = 10) were exposed via ingestion of *T. molitor* pupae to spinosad LC_{50} and were cryosanesthetized at −4 °C 6 h after feeding. Insects were dissected in insect saline solution and the principal salivary glands (divided into anterior and posterior lobes) and accessory salivary glands were transferred to 2.5% glutaraldehyde, 0.2 M sucrose, and 0.2 M sodium cacodylate buffer, pH 7.2, solution for 4 h at room temperature. Samples were then post-fixed in 1% osmium tetroxide and 0.2 M sodium cacodylate buffer for 2 h, washed in buffer, dehydrated in a graded ethanol series (70%, 80%, 90%, and 99%), and embedded in LR White resin (London Resin Company Ltd.). Ultra-thin sections (80–90 nm thick) were obtained using a PT-X PowerTome ultramicrotome equipped with glass knives (RMC Boeckeler Instruments Inc., Tucson, AZ, USA), stained with 1% aqueous uranyl acetate and lead citrate (Reynolds, 1963), and examined using a Zeiss EM 109 transmission electron microscope (Carl Zeiss, Jena, Germany).

2.6. Immunofluorescence analysis

Ten principal salivary glands (divided into anterior and posterior lobes) and 10 accessory salivary glands of *P. nigrispinus* fed on *T. molitor* pupae immersed in spinosad LC_{50} were obtained 6, 12, 24, and 48 h after feeding. Samples were fixed in Zamboni's fixative for 2 h. Then, glands were washed with 0.1 M phosphate buffered saline pH 7.2 and 1% Triton X-100 (PBST) and incubated with 1.5% bovine serum albumin in PBST for 2 h. Samples were incubated with rabbit anti-cleaved caspase-3 antibody (1:500; Cell Signaling Technology, Danvers, MA, USA) in PBS for 3 days at −4 °C. After incubation, samples were washed in PBS and incubated for 24 h in the dark at −4 °C with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (1:500; Sigma-Aldrich, St. Louis, MO, USA) diluted in PBS. Glands were washed, and cell nuclei were stained with TO-PRO-3 propidium iodide (Life Technologies, Carlsbad, CA, USA) for 1 h. Samples were mounted on 50% sucrose glass slides and examined using a Zeiss LSM510 META (Carl Zeiss, Jena, Germany) confocal laser scanning microscope.

2.7. Statistical analysis

Lethal concentrations (LC_{25}, LC_{50}, LC_{75}, and LC_{90}) and confidence limits were determined by probit regression analysis (Finney, 1964) using the PROC PROBIT procedure in SAS v. 9.0 (SAS Institute, 2002). Survival bioassay data were submitted to Kaplan–Meier survival analysis and log-rank test using Origin Pro v. 9.1 (Originlab Corporation, 2013). Adults who survived until the end of the experiment were treated as censored data.

3. Results

3.1. Toxicity and survival

Spinosad toxicity to *P. nigrispinus* was assessed using lethal concentrations (LC_{25}, LC_{50}, LC_{90}, and LC_{99}) and estimated by probit analysis ($X^2 = 30.67$; df = 5; $P < 0.001$) (Table 1, Fig. 1A). Spinosad was toxic to *P. nigrispinus*, with LC_{25} of 2.68 μg L^{-1}, LC_{50} of 3.15 μg L^{-1}, LC_{75} of 3.61 μg L^{-1}, and LC_{90} of 4.07 μg L^{-1}. Mortality remained below 1% in the control group.

Survival analysis of *P. nigrispinus* adults exposed to spinosad showed differences among treatments ($X^2 = 79.97$; df = 4; $P < 0.001$) (Fig. 1B). Survival was greater than 93% in the control group after 48 h, decreasing to 82% with exposure to LC_{25}, 32% to LC_{50}, and 0% to LC_{75} and LC_{90}.

3.2. Histopathology

The principal salivary gland of *P. nigrispinus* individuals not exposed to spinosad had an epithelium formed by a single layer of cubic or columnar secretory cells (Fig. 2A) with well-developed spherical nucleus containing predominantly decondensed chromatin and cytoplasm was homogeneous with few vacuoles. The lumen content was homogeneous and acidophilic. The principal salivary gland was externally lined by a thin basement membrane.

Histological changes in the epithelium of the principal salivary gland occurred 30 min after exposure to spinosad. Cells had many large vacuoles (Fig. 2B). One hour after exposure, cytoplasmic granules increased in size and the epithelium became irregular (Fig. 2C). Between 1 and 6 h after exposure, there was an increase in apocrine secretion into the lumen (Fig. 2D). Three hours after exposure, epithelial secretory cells showed irregularly shaped vacuoles and cell disruption with release of cytoplasm content into the lumen (Fig. 2D). The epithelium of the principal salivary gland was disorganized 6 h after exposure to spinosad (Fig. 2E). Cell disruption was observed in some cells and the salivary content of the lumen had been released into the hemocoel.

In control insects, the epithelium of the accessory salivary gland was formed by columnar secretory cells, which lined a narrow lumen (Fig. 2F). The spherical nucleus was well developed with decondensed chromatin and cytoplasm without vacuoles.

The accessory salivary gland showed no histological changes during the first 30 min after spinosad exposure (Fig. 2G). However, after 1 h, there was an increase in the number of vacuoles and granules in the cytoplasm (Fig. 2H). After 3 h, epithelial cells were swollen and exhibited intense vacuolization and ruptured nuclear envelope (Fig. 2I). Secretory cells were completely destroyed after

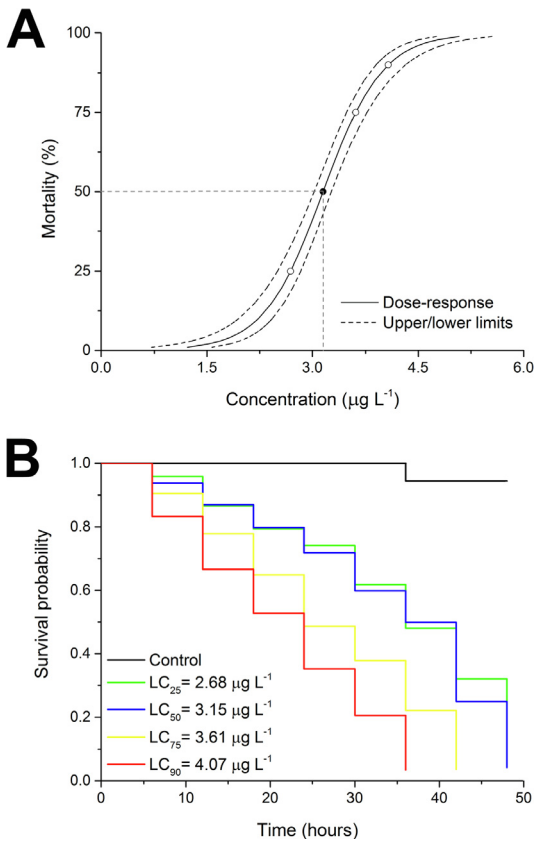

Fig. 1. Toxic effects of spinosad on *Podisus nigrispinus*. A) Adult mortality caused by spinosad at lethal concentrations (LC_{25}, LC_{50}, LC_{75}, and LC_{90}) ($X^2 = 30.67$; df = 5; $P < 0.001$). Dotted lines represent 95% confidence intervals. The black dot represents the concentration (LC_{50}) selected to evaluate histopathological and cytotoxic effects. B) Survival curves of adults during the first 48 h after exposure to different concentrations of spinosad, calculated using the Kaplan–Meier method and compared using the log-rank test ($X^2 = 79.97$; $P < 0.001$).

Table 1
Lethal concentrations of spinosad to *Podisus nigrispinus* determined in 48 h after exposure by ingestion of prey immersed in insecticide. [a]LC, Lethal concentration necessary to kill 25%, 50%, 75%, and 90% of *P. nigrispinus* adults; [b]EV, estimated value (μg L^{-1}); 95% CI, confidence interval (μg L^{-1}); and [d]X^2, chi-square for lethal concentration and fiducial limits based on a logarithmic scale with the significance level set at $P < 0.0001$.

[a]LC	[b]EV	[c]95% CI	[d]X^2
LC_{25}	2.689	2.502–2.833	30.67
LC_{50}	3.151	3.024–3.269	
LC_{75}	3.612	3.483–3.769	
LC_{90}	4.073	3.897–4.315	

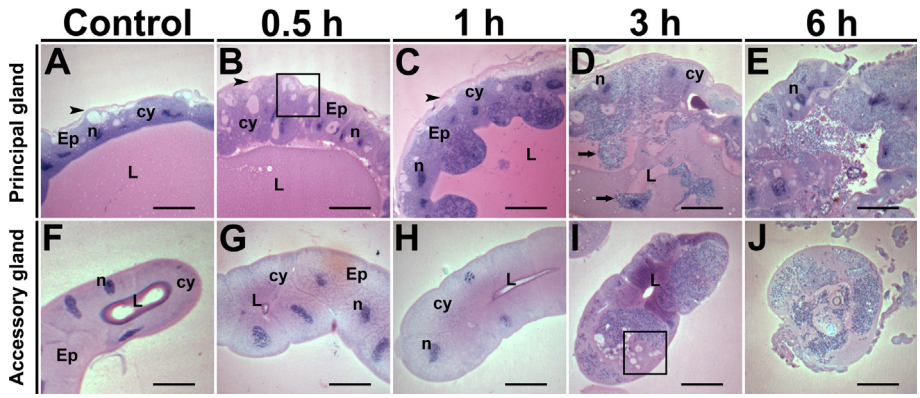

Fig. 2. Histopathology of the salivary complex of *Podisus nigrispinus* 0.5, 1, 3, or 6 h after exposure to spinosad. (A) Principal salivary gland of an individual not exposed to spinosad showing epithelium (*Ep*), nucleus (*n*), cytoplasm (*cy*), and lumen (*L*). (B–E) Principal salivary gland showing the sequential effects of spinosad during the first 6 h after the individual fed on insecticide-treated pupae. Principal salivary gland showing many large vacuoles (*square*) and cytoplasmic protrusions (*arrow*) in the shape of secretory cells. (F) Accessory salivary gland of an individual not exposed to spinosad showing the epithelium (*Ep*), nucleus (*n*), cytoplasm (*v*), and lumen (*L*). (G–J) Accessory salivary gland showing large vacuoles (*square*) and cytoplasmic protrusions (*arrow*) in the shape of secretory cells during the first 6 h after spinosad exposure. Basement membrane (*Head arrow*). (Bars = 40 μm).

6 h of insecticide ingestion, and only cell debris and luminal contents could be observed (Fig. 2J).

3.3. Cytotoxicity

The anterior lobe of the principal salivary gland of *P. nigrispinus* not exposed to spinosad had secretory cells with short apical microvilli and cytoplasm rich in electron-dense vesicles, lysosomes, mitochondria, rough endoplasmic reticulum, and some autophagosomes (Fig. 3A and B). In individuals exposed to spinosad, secretory cells exhibited vacuoles, electron-dense granules, and vacuoles were present in the gland lumen (Fig. 3B). Autophagy was more intensive and observed by high number of autophagosomes.

In control *P. nigrispinus*, the posterior lobe of the principal salivary gland had secretory cells with short apical microvilli and

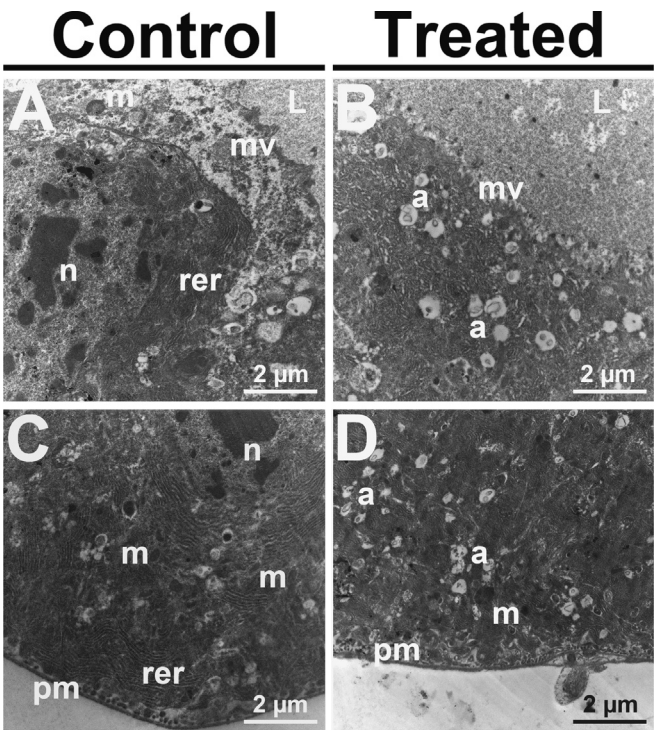

Fig. 3. Spinosad-induced cytotoxicity in the anterior lobe of the principal salivary gland of *Podisus nigrispinus*. (A and C) Epithelial cells not exposed to the insecticide showing short microvilli (*Mv*) in cytoplasm rich in mitochondria (*m*) and reticule endoplasm (*rer*) and nucleus (*n*) with decondensed chromatin in the apical portion. Mitochondria (*m*) and rugose endoplasmic reticulum (*rer*) and plasma membrane (*pm*) are presents with infoldings in the basal portion. (B and D) Epithelial cells exposed to the insecticide showing a large number of autophagosomes (*a*), mitochondria, and granules in the apical portion and dilated plasma membrane (*pm*) infoldings in the basal portion. Vesicles are present in the lumen (*L*).

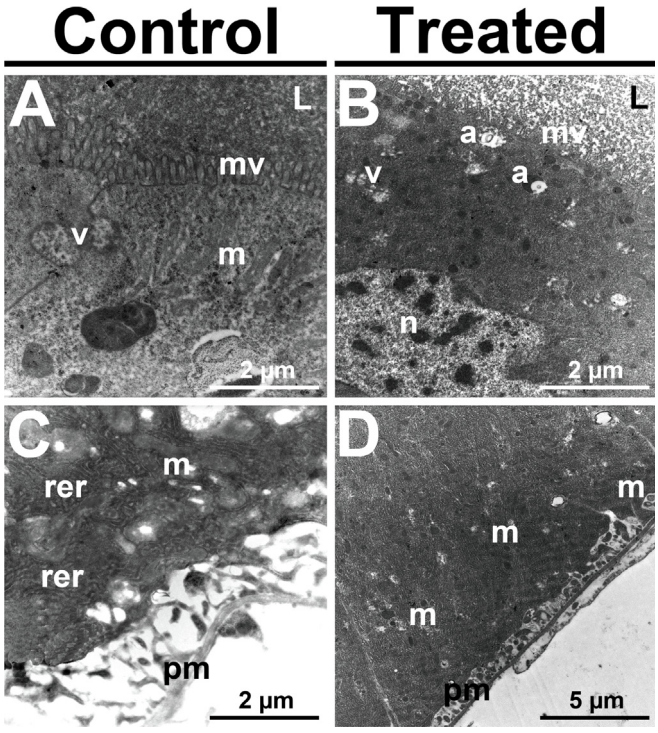

Fig. 4. Spinosad-induced cytotoxicity in the posterior lobe of the principal salivary gland of *Podisus nigrispinus*. (A and C) Control secretory cells showing short microvilli (*Mv*), cytoplasm with many mitochondria (*m*), vacuoles (*v*), and electron-dense vesicles in the apical portion. Cytoplasm rich in mitochondria (*m*) and rugose endoplasmic reticulum (*rer*), and plasma membrane (*pm*) with few and short infoldings. Lumen (*L*). (B and D) Epithelial cells exposed to the insecticide showing large vacuoles (*v*), autophagosomes (*a*) with membrane remnants, and few electron-dense granules. Nucleus (*n*) with decondensed chromatin in the apical portion. Epithelial cells show many mitochondria (*m*) and increased extension of plasma membrane (*pm*) with infoldings in the basal portion.

cytoplasm rich in rough endoplasmic reticulum, vesicles, and some autophagosomes (Fig. 4A and C). In comparison, insects exposed to spinosad had increased numbers of mitochondria, vesicles in the apical portion of the cytoplasm, and autophagosomes as well as greater amounts of apocrine secretion (Fig. 4B and D). Autophagy was more intensive and observed by high number of autophagosomes.

The accessory salivary gland of control *P. nigrispinus* contained secretory cells with short microvilli in the apical surface and cytoplasm with a well-developed rough endoplasmic reticulum (Fig. 5A). In spinosad-exposed *P. nigrispinus*, secretory cells of the accessory salivary gland showed many mitochondria in the apical cytoplasm and release of cytoplasm into the gland lumen (Fig. 5B).

3.4. Immunofluorescence analysis

In insects of the control group, few secretory cells of the salivary complex were positive to cleaved caspase-3 (Fig. 6A, F, and K). However, in *P. nigrispinus* that were fed on *T. molitor* treated with LC_{50} of spinosad, there was an increase in the number of cells cleaved to caspase-3 in the anterior (Fig. 6B–E) and posterior lobes (Fig. 6G–J) of the principal salivary gland and in the accessory salivary gland (Fig. 6KeO) after 6, 12, 24, and 48 h of ingestion.

4. Discussion

The toxicity caused by spinosad in the predator bug *P. nigrispinus* was determined by the occurrence of histological and cytological changes in the salivary complex. *Podisus nigrispinus* was susceptible to spinosad, with an LC_{50} of 2.18 μg L^{-1} (95% CI, 1.89–2.45 μg L^{-1}). Lethality was higher with increasing concentrations. The susceptibility of insects to spinosad varies depending on whether the route of exposure is by contact or ingestion (Mandour, 2009; López et al., 2011; Martínez et al., 2014b). The hemipteran predators *Deraeocoris brevis* Uhler (Miridae), *Geocoris punctipes* Say (Geocoridae), and *Orius laevigatus* Fieber (Anthocoridae) are susceptible to spinosad (Van De Veire et al., 2002; Kim et al., 2006; Myers et al., 2006). Previous studies on *P. nigrispinus* susceptibility to spinosad, however, produced divergent results: Torres et al. (2002b) reported that spinosad is compatible with this predatory bug, but De Castro et al. (2013, 2015) found the insecticide to be toxic. Spinosad has been reported as toxic for other non-target insects, including pollinators, predators, and parasitoids (Cisneros et al., 2002; Williams et al., 2003; Bailey et al., 2005; Penagos et al., 2005).

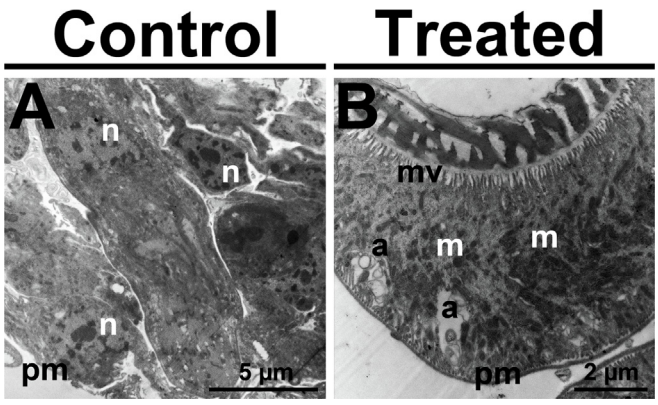

Fig. 5. Spinosad-induced cytotoxicity in the accessory salivary gland of *Podisus nigrispinus*. (A) Epithelial cells not exposed to the insecticide showing nucleus (*n*) with decondensed chromatin cytoplasm with mitochondria, secretory vesicles, electron-dense granules, and plasma membrane (*pm*) with short infoldings. (B) Epithelial cells exposed to the insecticide showing long microvilli (*Mv*), many autophagosomes (*a*), granules, and elongated plasma membrane infoldings associated with mitochondria (*m*) in the basal portion.

Spinosad affected *P. nigrispinus* survival in a short time (48 h). Lethality rate increased with increasing concentrations of insecticide. Spinosad has a rapid effect on *Strategus aloeus* Linnaeus (Coleoptera: Scarabaeidae), acting within 48–72 h after ingestion (Martínez et al., 2014b), whereas in *Sitophilus granarius* Linnaeus and *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) the insecticidal action occurs 12 days after ingestion (Vélez et al., 2017). Low survival of *P. nigrispinus* also occurs when it is exposed via ingestion to azadirachtin (Zanuncio et al., 2016), deltamethrin (De Castro et al., 2013), or permethrin (Martínez et al., 2018). The results suggest that, similarly to these chemicals, spinosad rapidly compromises physiological processes and causes damaging effects on *P. nigrispinus*.

Ingestion of prey contaminated with spinosad produced histopathological effects on the salivary complex of *P. nigrispinus*. Histological changes included features of cellular degeneration, such as cytoplasmic vacuolization, cell disruption, and release of cytoplasmic content into the gland lumen. Similar cell degeneration occurs in the midgut of *Apis mellifera* Linnaeus (Hymenoptera: Apidae) exposed to thiamethoxam (Catae et al., 2014), *Callibaetis radiatus* Navas (Ephemeroptera: Baetidae) exposed to deltamethrin (Gutiérrez et al., 2016), and *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae) exposed to squamocin (Fiaz et al., 2018a). In *P. nigrispinus*, spinosad induced cell degeneration in the salivary complex within 30 min of ingestion, and the effects became more pronounced in the following 3 h, culminating in plasma membrane rupture. Loss of membrane integrity is a typical feature of necrotic cells (Bowen et al., 1996; Benzidane et al., 2011; Gregorc and Ellis, 2011). The effects of spinosad on epithelial cells resulted in irreversible damages to the principal and accessory salivary glands of *P. nigrispinus*, affecting saliva production.

Spinosad caused toxic effects on the salivary complex of *P. nigrispinus* during the first 6 h after ingestion. Increased apocrine secretion was observed, which is suggestive of a cellular detoxification process. The occurrence of detoxification mechanisms is not unexpected, given that the main function of the salivary complex in *P. nigrispinus* is the production of non-proteinaceous compounds (venoms) and proteins (digestive enzymes) that contribute to prey death and extra-oral digestion (Martínez et al., 2014a, 2016; Walker et al., 2016). Enzymes found in the salivary glands of insects, such as cytochrome oxidase (Nicholson et al., 2012), glucose oxidase (Musser et al., 2005), glutathione peroxidase (Carolan et al., 2011), and peroxidase (Vandermoten et al., 2014) play a role in the detoxification of natural and synthetic insecticides. These enzymes are synthesized in secretory cells and released by apocrine secretion (Musser et al., 2005; DeLay et al., 2012; Vogel et al., 2018). Apocrine secretion is intensified when salivary glands are exposed to xenobiotic agents (Aumüller et al., 1999; Cristofoletti et al., 2001). The excess of apocrine secretion observed in the salivary glands of *P. nigrispinus* might be a detoxification response to spinosad, which imposes a high energy cost in cells in an attempt to neutralize the effects of the insecticide during the first hours of exposure.

Cytotoxic effects of spinosad on the salivary complex cells of *P. nigrispinus* resulted in an increase in the number of cytoplasmic granules, mitochondria, vacuoles, and autophagosomes accompanied by excessive apocrine secretion. These findings are in agreement with the degenerative cellular events described in midgut cells of *A. mellifera* exposed to spinosad (Lopes et al., 2018), *P. nigrispinus* exposed to imidacloprid (Martínez et al., 2019), and *A. gemmatalis* exposed to tebufenozide (Fiaz et al., 2018b). The increase in mitochondria in secretory cells might be a response to the oxidative stress and release of pro-apoptotic agents induced by spinosad (Bleicken et al., 2010). The occurrence of autophagosomes

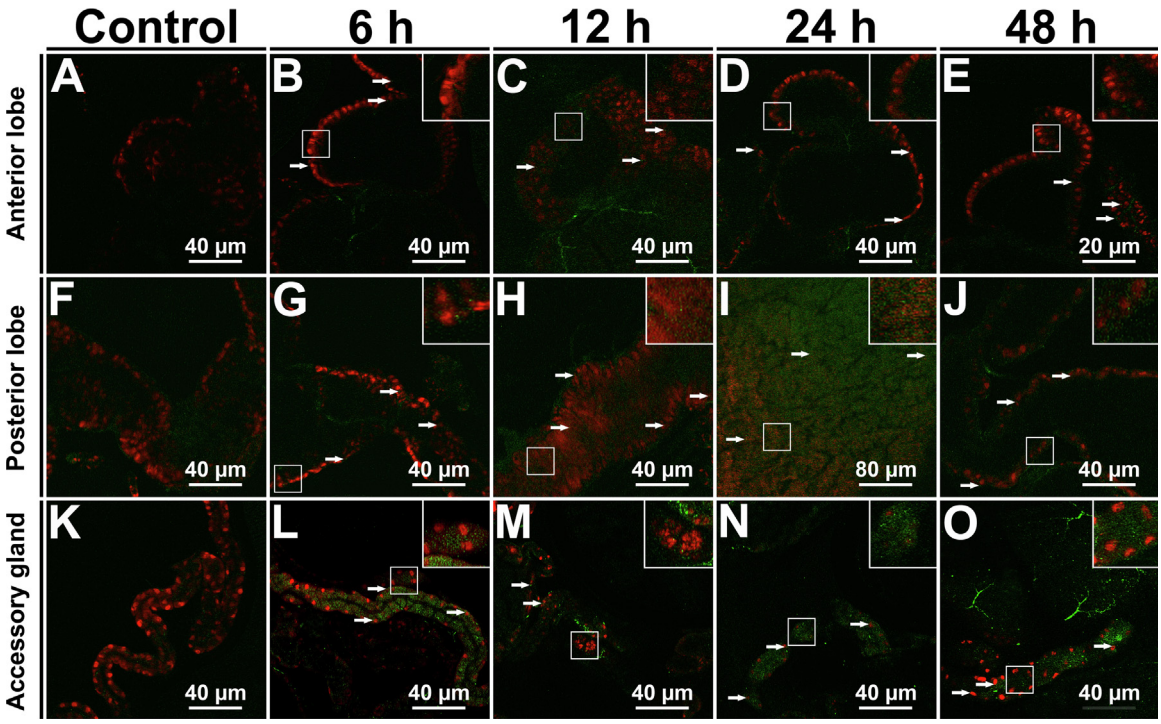

Fig. 6. Immunofluorescence microscopy for apoptosis detection in the salivary complex of *Podisus nigrispinus* 6, 12, 24, and 48 h after spinosad exposure using anti-cleaved caspase-3 antibody (*green*). (A–E) Anterior lobe of the principal salivary gland, (F–J) posterior lobe of the principal salivary gland, and (K–O) accessory salivary gland. Control: individuals not exposed to the insecticide (A, F, and K). Arrows: active caspase-3 (cleaved). Increased image (*white square*) showing specific caspase-3 activity.

indicates autophagy (Clarke, 1990; Lockshin and Zakeri, 2004), probably of cell organelles damaged by chemical/physiological stress. Autophagy does not necessarily result in cell death, as it is a physiological turnover process (Yoshimori, 2004; Ferreira et al., 2013; Rossi et al., 2013). However, if stress is sustained, cells can be completely degenerated by autophagy or can trigger death by apoptosis (Lockshin and Zakeri, 2004; Rossi et al., 2013). In salivary gland cells of *P. nigrispinus*, autophagy precedes to apoptosis induced by spinosad as occur with *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae) exposed to azadirachtin and *S. frugiperda* exposed to pyrethrum, eliminating organs and tissues during the detoxification process (Shao et al., 2016; Xu et al., 2017). The increased apocrine secretion observed in the lumen of the salivary gland of *P. nigrispinus* can be attributed to the continuous production of detoxifying enzymes that participate in the metabolism of pesticides (Yu and Hsu, 1993; Enayati et al., 2005). This mechanism compromises the production of non-protein and protein compounds required for prey immobilization and extra-oral digestion (Martínez et al., 2014a; 2016; Walker et al., 2016). Thus, the cytotoxic effects observed indicate that secretory cells of the salivary gland complex of *P. nigrispinus* cannot maintain saliva production after exposure to spinosad.

Spinosad induces progressive cell death in the salivary complex of *P. nigrispinus*, as shown by the occurrence of cleaved caspase-3-positive cells. The activation of caspases during apoptosis promotes mitochondrial membrane permeabilization and DNA fragmentation, leading to cell destruction (Green, 2005; Nikoletopoulou et al., 2013; Martínez et al., 2019). Chromatin condensation, nuclear fragmentation, and cytoplasmic vacuolization are typical signs of apoptosis (Häcker, 2000; Ziegler and Groscurth, 2004; Rost-Roszkowska et al., 2008; Nikoletopoulou et al., 2013). Neurotoxic insecticides can induce apoptosis in non-target organs, which is exemplified by the action of chlorpyrifos in the salivary glands of *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) (Gupta et al., 2010), thiamethoxam in the optic lobe of *A. mellifera* (Tavares et al., 2015), and permethrin in the midgut of *P. nigrispinus* (Martínez et al., 2018). Our results suggest that spinosad induces cell death in the salivary complex of *P. nigrispinus*, as demonstrated by the occurrence of cleaved caspase-3-positive cells, damaging the metabolism of normal cells and eventually affecting the physiological cell process.

This study showed that spinosad is toxic to the predatory bug *P. nigrispinus* and causes rapid mortality by ingestion. At a low concentration (LC_{50}), spinosad induced irreversible histopathological and cytotoxic effects on the salivary gland complex, affecting the production of saliva, which is necessary for paralyzing and killing prey as well as for extra-oral digestion. The results indicate that the concomitant use of spinosad and *P. nigrispinus* in integrated pest management programs should be avoided.

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