LUIS CARLOS MARTINEZ CASTRILLON

ULTRASTRUCTURE AND FUNCTION OF THE SALIVARY GLANDS OF *Podisus nigrispinus* (Hemiptera: Pentatomidae)

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

VIÇOSA
MINAS GERAIS-BRASIL
2014

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

T
M385u
2014

Martinez Castrillon, Luis Carlos, 1974-
Ultrastructure and function of the salivary glands of Podisus nigrispinus (Hemiptera: Pentatomidae) / Luis Carlos Martinez Castrillon. – Viçosa, MG, 2014.
viii, 37f. : il. (algumas color.) ; 29 cm.

Orientador: José Eduardo Serrão.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. *Podisus nigrispinus* - Controle biológico. 2. Glândulas salivares. 3. Enzimas. 4. N,N-dimetilanilina. 5. 1,2,3-tritiepano. I. Universidade Federal de Viçosa. Departamento de Entomologia. Programa de Pós-graduação em Entomologia. II. Título.

CDD 22. ed. 595.754

LUIS CARLOS MARTINEZ CASTRILLON

ULTRASTRUCTURE AND FUNCTION OF THE SALIVARY GLANDS OF *Podisus nigrispinus* (Hemiptera: Pentatomidae)

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

APROVADA: 23 de maio de 2014.

Stênio Nunes Alves	Walkymário de Paulo Lemos
Leandro Licursi Oliveira	Eugênio Eduardo De Oliveira

José Eduardo Serrão
(Orientador)

To my parents Emiro and Anatilde, they gave me the motivation, encouragement throughout my studies and they well deserve the credit of my work. To my wife Angelica for your unwavering support and encouragement.

ACKNOWLEDGEMENTS

I would like to thank my adviser, Professor Dr. José Eduardo Serrão for his guidance, constant encouragement, valuable suggestion and support throughout my thesis. I wish to thank Dr. José Cola Zanuncio, for his support and invaluable technical help during my PhD thesis. My sincere thanks to Dr. Maria do Carmo Queiroz Fialho, for her excellent support and supervision through constructive criticism. Thanks must also be extended to Dr. Leandro Licursi Oliveira for performing a significant proportion of the venoms extraction, and Dr. Luiz Claudio Almeida Barbosa for performing the chemical analytical assays. To Núcleo de microscopia e microanalise, Núcleo de biomoléculas, Laboratorio de ultraestrutura and Laboratorio de química for their technical support. I am grateful to the laboratory employees and students who were supportive and helpful throughout the years. They have been invaluable in finishing this project. I wish to thanks CAPES, CNPq and FAPEMIG agencies for the financial support. Lastly, I would like to express my gratitude to Departmento de Entomologia, Pró-Reitoria de Pesquisa e Pós-graduação, and Universidade Federal de Viçosa for their support.

CONTENTS

RESUMO

MARTINEZ CASTRILLON, Luis Carlos, D.Sc., Universidade Federal de Viçosa, maio de 2014. **Ultraestrutura e função das glândulas salivares de Podisus nigrispinus (Hemiptera: Pentatomidae).** Orientador: José Eduardo Serrão. Coorientadores: José Cola Zanuncio e Maria do Carmo Queiroz Fialho.

Podisus nigrispinus Dallas (Hemiptera: Pentatomidae) é um inseto zoofitófago com potencial para controle biológico, pois ninfas e adultos predam diversos insetos, inserindo o aparelho bucal e injetando o conteúdo das glândulas salivares no interior das presas, causando a morte das mesmas. Entretanto, os compostos tóxicos da saliva desse inseto, responsáveis pela morte das presas são ainda desconhecidos. Como primeira etapa para a identificação das possíveis substâncias presentes na saliva de P. nigrispinus, este estudo avaliou a ultraestrutura e citoquímica das glândulas salivares de P. nigrispinus. A segunda etapa identificou os compostos da saliva de P. nigrispinus responsáveis pela morte da presa Anticarsia gemmatalis. A avaliação da ultraestrututra das glândulas salivares mostrou que o sistema salivar de P. nigrispinus é composto por um par de glândulas salivares principais bilobadas, formando um lobo anterior curto e um posterior longo e um par de glândulas acessórias tubulares. As células de ambas as glândulas foram bem desenvolvidas com predomínio de mitocôndrias e retículo endoplasmático rugoso, além de invaginações da membrana plasmática basal, indicando que ambas as glândulas tem função de transporte de substâncias a partir da hemolinfa bem como na síntese de proteínas. Os testes citoquímicos mostraram reações positivas no citoplasma para carboidratos, fosfatase ácida e proteínas nas diferentes regiões do sistema glandular. A saliva de P. nigrispinus exposta a inibidores enzimáticos, proteases e na fase de éter causou mortalidade após injeção em lagartas de A. gemmatalis, sendo a CL_{50} de 1,91 e CL_{90} de 4,34 µL do extrato em éter de saliva. Frações do extrato salivar em éter que causaram mortalidade de A. gemmatalis correspondem aos compostos N,N-dimetil anilina e 1,2,3-tritiepano, sendo que a injeção do primeiro composto apresentou CL_{50} de 136,1 nL e CL_{90} de 413,8 nL, sugerindo que este composto não proteico da saliva é um dos responsáveis pela toxicidade da saliva de P. nigrispinus. As características ultraestruturais e citoquímicas sugeriram que as glândulas salivares principais e acessórias participam da secreção de proteínas e substâncias tóxicas não proteicas na saliva desse inseto. Isto foi corroborado pela identificação da N,N-dimetil

anilina e o 1,2,3-tritiepano responsáveis pela morte da A. gemmatalis os quais são reportados pela primeira vez na saliva de insetos.

ABSTRACT

MARTINEZ CASTRILLON, Luis Carlos, D.Sc., Universidade Federal de Viçosa, May, 2014. **Ultrastructure and function of the salivary glands of Podisus nigrispinus (Hemiptera: Pentatomidae).** Adviser: José Eduardo Serrão. Co-Advisers: José Cola Zanuncio and Maria do Carmo Queiroz Fialho.

Podisus nigrispinus Dallas (Hemiptera: Pentatomidae) is a zoophytophagous insect that has a potential in biological control because its nymphs and adults prey on various insects. They insert their mouthparts and inject the contents of their salivary glands in their prey to kill them. Although it is understood that the saliva of this predator is toxic, the toxic compounds responsible for the prey death remains unknown. To identify the components of the saliva of P. nigrispinus, we evaluated the ultrastructure and cytochemistry of the salivary glands of this insect. Cytochemistry evaluation identified the compounds of the saliva of P. nigrispinus responsible for the death of Anticarsia gemmatalis prey. The salivary system of P. nigrispinus has consists of a pair of principal salivary glands, which are bilobed with a short anterior and a long posterior lobe, and a pair of tubular accessory glands. The cells of both the glands are well developed with a predominance of mitochondria and rough endoplasmic reticulum and with basal plasma membrane infoldings, indicating that both the glands play a role in the transport of hemolymph substances and protein synthesis. The cytochemical tests demonstrated positive reactions for carbohydrate, protein, and acid phosphatase in the cells of both the salivary glands. An injection of the saliva of P. nigrispinus exposed to enzyme inhibitors and proteases and extracted at the ether phase caused mortality of A. gemmatalis larvae, with LD_{50} = 1.91 and LD_{90} = 4.34 µL. These saliva fractions were identified as N,N-dimethylaniline and 1,2,3-trithiepane, and the injection of the first compound showed an LC_{50} = 136.1 nL and LC_{90} = 413.8 nL, suggesting that these non-proteineous compound is responsible for the saliva toxicity of P. nigrispinus. The ultrastructural and cytochemical features suggested participation of the principal and accessory salivary glands in the secretion of proteinaceous and non-proteinaceous toxic substances in the saliva of this insect. This was further confirmed by identification of N,N-dimethylaniline and 1,2,3-trithiepane responsible for the death of A. gemmatalis. This is the first study reporting the toxic compounds of the saliva of this insect.

GENERAL INTRODUCTION

Among the most studied methods for the management of agricultural and forest pests is the biological control method because promoting the conservation, inoculation, and inundation of natural enemies. Biological control may be defined as the use of natural enemies such as viruses, bacteria, fungi, nematodes, and insects to reduce the populations of harmful insects damaging plantations with the aim to sustain the agroecosystems with alternatives that reduce the environmental impact (Smith et al. 1997; Letourneau and Altieri 1999; Pearson and Callaway 2003). In this context, biological control is essential for the establishment of sustainable pest management programs by using propagation techniques, handling and release of natural enemies with favorable effects on crop productivity, low cost-benefit ratio, and preservation of native and cultured ecosystems (Bottrell et al. 1998; Thompson 1999; Cortesero et al. 2000). The comprehension of the interactions between different plant-herbivore and natural enemies is important for the implementation of biological control of pests in different ecosystems (Lalonde et al. 1998; Eubanks and Denno 1999; Altieri et al. 2005).

The interaction of predators as natural enemies of insect pests coexisting in natural or artificial habitats has been recognized in biological control (Zanuncio et al. 1994; Eubanks and Denno 1999; De Clercq 2002; Symondson et al. 2002). Predatory insects have an important role in the insect communities, because the variation in food availability or prey has dramatic effects on survival, dispersal, and population dynamics of these organisms (Cohen 1990; Coll and Guershon 2002). The predator representatives of Coleoptera, Diptera, Hemiptera, and Hymenoptera have been reported to feed on one or several preys with specific or generalist preferences (Cohen 1990; Memmott et al. 2000; Richter 2000). Also, these predators can be classified as opportunistic, obligates, or facultatives in function based on the importance of ingested materials such as plant, animal, or other organic sources (Coll and Guershon 2002). The opportunistic predators include phytozoophagous, which are herbivores and which feed on prey, and zoophytophagous, which are carnivores and eventually feed on plants (Memmott et al. 2000; Coll and Guershon 2002).

Podisus nigrispinus Dallas (Hemiptera: Pentatomidae) is a common zoophytophagous insect that is used in the biological control for agricultural and forest crops in America (Cohen 1990; Medeiros et al. 2000; Lemos et al. 2001; Mohaghegh et al. 2001). The potential of P. nigrispinus in biological control has been reported for

defoliating larvae Anticarsia gemmatalis Hübner (Lepidoptera: Erebidae), Alabama argillacea Hübner, Spodoptera exigua Hübner, and Trichoplusia ni Hübner (Lepidoptera: Noctuidae) (Mohaghegh et al. 2001; Medeiros et al. 2003; Ferreira et al. 2008; Neves et al. 2010; De Bortoli et al. 2011) in cotton, tomato, soybean, and eucalyptus plantations (Zanuncio et al. 1994; Matos Neto et al. 2002; Oliveira et al. 2002; Lemos et al. 2003). The importance of P. nigrispinus in biological control has been demonstrated in several studies regarding its development, reproduction, predator–prey interactions, feeding strategies, and extra-oral digestion (Saavedra et al. 1997; Torres et al. 1998; Lemos et al. 2001; Medeiros et al. 2003; Vivian et al. 2003; Oliveira et al. 2006; Ferreira et al. 2008; Fialho et al. 2012).

The extra-oral digestion begins with the injection of saliva into the prey before ingesting it. The saliva contains digestive enzymes that are used to liquefy the body contents of the prey and facilitate the uptake of nutrients from it (Miles 1972; Land et al. 1994; Cohen 1995). The salivary glands of predatory Hemiptera produce saliva containing different compounds such as water, lipids, carbohydrates, and enzymes, favoring the extra-oral digestion (Land et al. 1994; Zeng and Cohen 2000; Oliveira et al. 2006). In this context, P. nigrispinus insert the stylets into the prey bodies and regurgitate saliva, causing rapid paralysis and death of the prey (Cohen 1998; Lemos et al. 2003; Oliveira et al. 2006). However, digestive enzymes, except for collagenase, are not present in the salivary glands of P. nigrispinus, which is used to rupture the internal organs of the prey for facilitating the intake of the tissues (Fialho et al. 2012). The compounds produced in the salivary glands responsible for the death of the prey remain unknown in P. nigrispinus and other predatory Hemiptera.

In insects, prey paralysis and death are caused by the action of toxic compounds or venoms from a predator as a facilitator of predation strategies. Venom is a toxin commonly produced by glands and injected into another organism using specialized apparatuses, and the action of these toxins immobilize or kill prey (Blum 1978; Schmidt 1982). The venoms of insects are chemical compounds containing alkaloids, terpenes, polysaccharides, biogenic amines, organic acids, and amino acids (Blum 1978; Chapman 2013), although the main compounds are macromolecules such as peptides, oligopeptides, and proteins (Schmidt 1982; Calvete et al. 2009). Substances with low molecular weight and short carbon chains has been reported to be responsible for the lethal and sublethal effects on insect, for instance, in Apis cerana Fabricius (Hymenoptera: Apidae) (Schmidt et al. 1997), Galerita lecontei Dejean (Coleoptera:

Carabidae) (Rossini et al. 1997), and Polistes sulcifer Zimmermann (Hymenoptera: Vespidae) (Bruschini and Cervo 2011). However, in these insects, the venom is produced by specialized glands, which are not salivary glands.

The presence of venoms in insects provide a powerful tool for understanding the functioning of the salivary glands and saliva composition of P. nigrispinus and its strategies for predation. We hypothesize that the salivary glands of P. nigrispinus contains non-proteinaceous compounds which cause a toxic response on preys. In order to address this question, the salival compounds from P. nigrispinus may be act as venom and facilitating the extraoral ingestion process. Thus, in this study, we investigated the presence of non-protein substances in the saliva of the predator that imparts toxicity to the venom.

Considering the importance of P. nigrispinus in biological control pests programs and the limited studies on biochemistry and physiology of the salivary glands, this study aimed to evaluate the ultrastructural and cytochemical aspects of the salivary glands and to identify the presence of venom in the saliva of P. nigrispinus so as to facilitate the comprehension of predator–prey interaction and the ecological relationships of predator in biological control.

References

Altieri MA, Ponti L, Nicholls CI (2005) Manipulating biodiversity for improved pest management: case studies from northern California. Int J Bio Sci Manag 1: 191-203.

Blum SM (1978) Biochemical defenses of insects. In: Rockstein, M. (Ed.), Biochemistry of insects. Academic Press, New York, pp. 465-513.

Bottrell DG, Barbosa P, Gould F (1998) Manipulating natural enemies by plant variety selection and modification: a realistic strategy? Annu Rev Entomol 43: 347-67.

Bruschini C, Cervo R (2011) Venom volatiles of the paper wasp social parasite Polistes sulcifer elicit intra-colonial aggression on the nest of the host species Polistes dominulus. Insectes Soc 58: 383-390.

Calvete JJ, Sanz L, Angulo Y, Lomonte B, Gutiérrez JM (2009) Venoms, venomics, antivenomics. FEBS Letters 583: 1736-1743.

Chapman RF (2013) The Insects: Structure and Function. Cambridge University Press, New York, pp. 1-929.

Cohen AC (1990) Feeding adaptations of some predaceous Heteroptera. Ann Entomol Soc Am 83: 1215-1223.

Cohen AC (1995) Extra-oral digestion in predaceous terrestrial Arthropoda. Annu Rev Entomol 40: 85-103.

Cohen AC (1998) Solid-to-liquid feeding: the inside story of extra-oral digestion in predaceous Heteroptera. Am Entomol 44: 103-117.

Coll M, Guershon M (2002) Omnivory in terrestrial arthropods: mixing plant and prey diets. Annu Rev Entomol 47: 267-297.

Cortesero AM, Stapel JO, Lewis WJ (2000) Understanding and manipulating plant attributes to enhance biological control. Biol Control 17: 35-49.

De Bortoli SA, Otuka AK, Vacari AM, Martins MIEG, Volpe HLX (2011) Comparative biology and production costs of Podisus nigrispinus (Hemiptera: Pentatomidae) when fed different types of prey. Biol Control 58: 127-132.

De Clercq P (2002) Dark clouds and their silver linings: exotic generalista predators in augmentative biological control. Neotrop Entomol 31: 169-176.

Eubanks M, Denno RF (1999) The ecological consequences of variation in plants and prey for an omnivorous insect. Ecology 80: 1253-1266.

Ferreira JAM, Zanuncio JC, Torres JB, Molina-Rugama AJ (2008) Predatory behaviour of Podisus nigrispinus (Heteroptera: Pentatomidae) on different densities of Anticarsia gemmatalis (Lepidoptera: Noctuidae) larvae. Biocontrol Sci Techn 18: 711-719.

Fialho MCQ, Zanuncio JC, Neves CA, Ramalho FS, Serrão JE (2012) Prey digestion in the midgut of the predatory bug Podisus nigrispinus (Hemiptera: Pentatomidae). J Insect Physiol 58: 850-856.

Lalonde RG, McGregor RR, Gillespie DR (1999) Plant-feeding by arthropod predators contributes to the stability of predator-prey population dynamics. Oikos 87: 603-608.

Lemos WP, Medeiros RS, Ramalho FS, Zanuncio JC (2001) Effects of plant feeding on the development, survival and reproduction of Podisus nigrispinus (Dallas) (Heteroptera: Pentatomidae). Int J Pest Manage 47: 89-93.

Lemos WP, Ramalho FS, Serrão JE, Zanuncio JC (2003) Effects of diet on development of Podisus nigrispinus (Dallas) (Heteroptera: Pentatomidae), a predator of the cotton leafworm. J Appl Entomol 127: 389-395.

Letourneau DK, Altieri MA (1999) Environmental management to enhance biological control in agroecosystems. In: Handbook of Biological Control. T. Bellows and T. W. Fisher (eds.), pp. 319-345.

Matos Neto FC, Zanuncio JC, Cruz I, Torres JB (2002) Nymphal development of Podisus nigrispinus (Heteroptera: Pentatomidae) preying on larvae of Anticarsia gemmatalis (Lepidoptera: Noctuidae) fed with resistant and susceptible soybeans. Rev Bras Entomol 46: 237-41.

Medeiros RS, Ramalho FS, Lemos WP, Zanuncio JC (2000) Age-dependent fecundity and life-fertility tables for Podisus nigrispinus (Dallas) (Heteroptera: Pentatomidae). J Appl Entomol 124: 319-324.

Medeiros RS, Ramalho FS, Zanuncio JC, Serrão JE (2003) Effect of temperature on life table parameters of Podisus nigrispinus (Het., Pentatomidae) fed with Alabama argillacea (Lep., Noctuidae) larvae. J Appl Entomol 127: 209-213.

Memmott J, Martinez ND, Cohen JE (2000) Predators, parasitoids and pathogens: species richness, trophic generality and body sizes in a natural food web. J Anim Ecol 69: 1-15.

Miles PW (1972) The saliva of Hemiptera. Adv Insect Physiol 9: 183-255.

Mohaghegh J, De Clercq P, Tirry L (2001) Functional response of the predators Podisus maculiventris (Say) and Podisus nigrispinus (Dallas) (Heteroptera: Pentatomidae) to the beet armyworm, Spodoptera exigua (Hübner) (Lepidoptera: Noctuidae): effect of temperature. J Appl Entomol 125: 131-134.

Neves RCS, Torres JB, Zanuncio JC (2010) Production and storage of mealworm beetle as prey for predatory stinkbug. Biocontrol Sci Techn 20: 1013-1025.

Oliveira JEM, Torres JB, Moreira A, Barros R (2002) Efeito das plantas do algodoeiro e do tomateiro, como complemento alimentar, no desenvolvimento e na reprodução do predador Podisus nigrispinus (Dallas) (Heteroptera: Pentatomidae). Neotrop Entomol 31: 101-108.

Oliveira JA, Oliveira MGA, Guedes RNC, Soares MJ (2006) Morphology and preliminary enzyme characterization of the salivary glands from the predatory bug Podisus nigrispinus (Heteroptera: Pentatomidae). Bull Entomol Res 96: 251-258.

Pearson DE, Callaway RM (2003) Indirect effects of host-specific biological control agents. Trends Ecol Evol 18: 456 461.

Richter MR (2000) Social wasp (Hymenoptera: Vespidae) foraging behavior. Annu Rev Entomol 45: 121-150.

Rossini C, Attygalle AB, Gonzalez A, Smedley SR, Eisner M, Meinwald J, Eisner T (1997) Defensive production of formic acid (80-percent) by a carabid beetle (Galerita lecontei). P Natl Acad Sci Usa 94: 6792-6797.

Saavedra JLD, Zanuncio JC, Zanuncio TV, Guedes RN (1997) Prey capture ability of Podisus nigrispinus (Dallas) (Het., Pentatomidae) reared for successive generations on a meridic diet. J Appl Entomol 121: 327-30.

Schmidt JO (1982) Biochemistry of insect venoms. Annu Rev Entomol 27: 339-368.

Schmidt JO, Morgan ED, Oldham NJ, Do Nascimento RR, Dani FR (1997) (Z)-ll-Eicosen-l-ol, a major component of Apis cerana venom. J Chem Ecol 23: 1929-1939.

Smith JW, Wiedenmann RN, Gilstrap FE (1997) Challenges and opportunities for biological control in ephemeral crop habitats: an overview. Biol Control 10: 2-3.

Symondson WOC, Sunderland KD, Greenstone MH (2002) Can generalist predators be effective biocontrol agents? Annu Rev Entomol 47: 561-594.

Terra WR, Ferreira C (1994) Insect digestive enzymes: properties, compartmentalization and function. Comp Biochem Physiol B 109: 1-62.

Thompson SN (1999) Nutrition and culture of entomophagous insects. Annu Rev Entomol 44: 561-592.

Torres JB, Zanuncio JC, Oliveira HN (1998) Nymphal development and adult reproduction of the stinkbug predator Podisus nigrispinus (Het.: Pentatomidae) under fluctuating temperatures. J Appl Entomol 122: 509-14.

Vivan LM, Torres JB, Veiga AFSL (2003) Development and reproduction of a predatory stinkbug, Podisus nigrispinus in relation to two different prey types and environmental conditions. Biocontrol 48: 155-68.

Zanuncio JC, Alves JB, Zanuncio TV, García JL (1994) Hemipterous predators of eucalypt defoliator caterpillars. Forest Ecol Manag 65: 65-73.

Zeng F, Cohen AC (2000) Comparison of a-amylase and protease activities of a zoophytophagous and two phytozoophagous Heteroptera. Comp Biochem Physiol A 126: 101-106.

CHAPTER 1

ULTRASTRUTURE AND CYTOCHEMISTRY OF SALIVARY GLANDS OF THE PREDATOR Podisus nigrispinus (HEMIPTERA: PENTATOMIDAE)

Protoplasma 251(3): 535-543

Luis Carlos Martínez[1], Maria do Carmo Queiroz Fialho[2], José Cola Zanuncio[1]and José Eduardo Serrão[3]

[1]Departamento de Entomologia, Universidade Federal de Viçosa (UFV), 36570-000, Viçosa, Minas Gerais, Brasil.

[2]Departamento de Morfologia, Universidade Federal do Amazonas (UFAM), 69077-000, Manaus, Amazonas, Brasil.

[3]Departamento de Biologia Geral, Universidade Federal de Viçosa (UFV), 36570-000, Viçosa, Minas Gerais, Brasil.

Protoplasma (2014) 251:535–543
DOI 10.1007/s00709-013-0549-0

ORIGINAL ARTICLE

Ultrastructure and cytochemistry of salivary glands of the predator *Podisus nigrispinus* (Hemiptera: Pentatomidae)

Luis Carlos Martínez · Maria do Carmo Queiroz Fialho · José Cola Zanuncio · José Eduardo Serrão

Received: 28 June 2013 / Accepted: 28 August 2013 / Published online: 11 September 2013

Abstract *Podisus nigrispinus* Dallas (Hemiptera: Pentatomidae) is a zoophytophagous insect with a potential for use as a biological control agent in agriculture because nymphs and adults actively prey on various insects by inserting mouthparts and regurgitating the contents of the salivary glands inside the prey, causing rapid paralysis and death. However, the substances found in saliva of *P. nigrispinus* that causes the death of the prey are unknown. As a first step to identify the component of the saliva of *P. nigrispinus*, this study evaluated the ultrastructure and cytochemistry of the salivary glands of *P. nigrispinus*. The salivary system of *P. nigrispinus* has a pair of principal salivary glands, which are bilobed with a short anterior lobe and a long posterior lobe, and a pair of tubular accessory glands. The principal gland epithelium is composed of a single layer of cells enclosing a large lumen. Epithelial cells of the principal salivary gland vary from cubic to columnar shape, with one or two spherical and well-developed nuclei. Cells of the anterior lobe of the principal salivary gland have an apical surface with narrow, short, and irregular plasma membrane foldings; apical and perinuclear cytoplasm rich in rough endoplasmic reticulum; and mitochondria with tubular cristae. The basal portion of the secretory cells has mitochondria associated with many basal plasma membrane infoldings that are short but form large extracellular canals. Secretory granules with electron-dense core and electron-transparent peripheral are dispersed throughout the cytoplasm. Cells of the posterior lobe of the principal salivary gland are similar to those of the anterior lobe, except for the presence of mitochondria with transverse cristae. The accessory salivary gland cells are columnar with apical microvilli, have well-developed nucleus and cytoplasm rich in rough endoplasmic reticulum, and have secretory granules. Cytochemical tests showed positive reactions for carbohydrate, protein, and acid phosphatase in different regions of the glandular system. The principal salivary glands of *P. nigrispinus* do not have muscle cells attached to its wall, suggesting that saliva-releasing mechanism may occurs with the participation of some thorax muscles. The cytochemical and ultrastructural features suggest that the principal and accessory salivary glands play a role in protein synthesis of the saliva.

Keywords Asopinae · Enzymes · Epithelium · Lumen · Secretory cells · Zoophytophagous

Handling Editor: Pavel Dráber

L. C. Martínez · J. C. Zanuncio
Departamento de Entomologia, Universidade Federal de Viçosa, 36570-000 Viçosa, Minas Gerais, Brazil

L. C. Martínez
e-mail: luis.castrillon@ufv.br

J. C. Zanuncio
e-mail: zanuncio@ufv.br

M. do Carmo Queiroz Fialho
Departamento de Morfologia, Universidade Federal do Amazonas, 69077-000 Manaus, Amazonas, Brazil
e-mail: mcqfialho@hotmail.com

J. E. Serrão (✉)
Departamento de Biologia Geral, Universidade Federal de Viçosa, Avenida Peter Henry Rolfs, Campus Universitário Viçosa, 36570-000 Viçosa, Minas Gerais, Brazil
e-mail: jeserrao@ufv.br

Introduction

Insect predators have been studied in different aspects as potential agents for biological control of forest and agricultural pests. Biological control with predators is an alternative to chemical control, which reduces the cost of production and avoids the harmful environmental impact (McPherson et al. 1982; Lacerda et al. 2004; Matos Neto et al. 2004). Within Hemiptera, representatives of Asopinae (Heteroptera: Pentatomidae), *Bronto-*

coris, *Euchistus*, *Podisus*, and *Supputius* have been successfully used to control various insect pests (Zanuncio et al. 1994; Mohaghegh et al. 1999; Medeiros et al. 2000; Panizzi et al. 2000; Tillman and Mullinix 2004; Lemos et al. 2003; Oliveira et al. 2005).

Predation in insects is a complex behavior as it is affected by several factors such as predator and prey density, attack or defense mechanisms, and feeding strategies (Cohen 1995; Mohaghegh et al. 2001; Guedes et al. 2007). Some Heteroptera have been studied in detail for their feeding strategies and extra-oral digestion (Boyd et al. 2002; Swart and Felgenhauer 2003; Bell et al. 2005; Fialho et al. 2009, 2012).

The Asopinae predators insert the stylet into the body of the prey and regurgitate saliva, causing rapid paralysis and death of the prey (Cohen 1990), after which they suck on the prey contents at leisure (Lemos et al. 2005a; Azevedo et al. 2007). The paralysis and death of the prey have been attributed to the action of digestive enzymes, produced by the salivary glands, released inside the prey (Schmidt 1982; Cohen 1990). However, Fialho et al. (2012) did not find any digestive enzymes, except collagenase, in the salivary glands. Thus, saliva compounds responsible for paralysis and death of the prey in Asopinae remain unknown.

Podisus nigrispinus Dallas (Hemiptera: Pentatomidae) has a potential as a biological control agent and is considered as a generalist predator (Cohen 1990; Zanuncio et al. 1998; Lemos et al. 2001). The salivary system of other predatory Hemiptera has a pair of bilobed principal glands, a salivary duct, and a pair of accessory glands (Puri 1924; Barth 1954; Azevedo et al. 2007; Serrão et al. 2008; Castro et al. 2013), but these studies describe gross morphology of the salivary glands without details of the fine structure of their cells.

As a first step to identify the possible substances present in the saliva of *P. nigrispinus*, this study describes the anatomy, ultrastructure, and cytochemistry of its salivary gland system, which may contribute to the comprehension of the saliva compounds that induce paralysis and death of the prey during predation.

Materials and methods

Insects

Adults of *P. nigrispinus* were obtained from mass rearing in the Laboratório de Controle Biológico do Instituto de Biologia Aplicada à Agricultura e Pecuária (Bioagro, Universidade Federal de Viçosa, state of Minas Gerais, Brazil) and maintained at 25±2 °C, 75±5 % relative humidity, and 12:12 h light/day photoperiod. These insects fed on *Tenebrio molitor* (L.) pupae (Coleoptera: Tenebrionidae) and *Eucalyptus grandis* (W. Hill ex. Maiden) leaves ad libitum (Zanuncio et al. 1994; Lemos et al. 2005b).

Scanning electron microscopy

Adults of *P. nigrispinus* were anesthetized at −4 °C, and the salivary glands were dissected in saline solution for insects (0.1 M NaCl, 0.1 M KH_2PO_4, 0.1 M Na_2HPO_4) and transferred to Zamboni's fixative solution (Stefanini et al. 1967) for 12 h at 5 °C. Then, the samples were dehydrated in a graded ethanol series (70°, 80°, 90°, and 99°), transferred to hexamethyldisilazane for 5 min, dried at room temperature, and coated with gold (20 nm thick), and finally observed under the LEO VP1430 scanning electron microscope.

Light microscopy

Adults of *P. nigrispinus* were anesthetized at −4 °C, and the salivary glands were dissected in saline solution for insects and transferred to the Zamboni's fixative solution for 24 h at 5 °C. Then, the samples were dehydrated in a graded ethanol series (70°, 80°, 90°, and 95°), embedded in historesin JB4, sectioned at 3 μm thickness in Leica RM2255, stained with hematoxylin and eosin, and analyzed under Leica DMLS light microscope.

Transmission electron microscopy

Salivary glands of *P. nigrispinus* were dissected and transferred to 2.5 % glutaraldehyde in sodium cacodylate buffer (0.2 M, pH 7.2) containing 0.2 M sucrose for 4 h at room temperature. Then, the principal salivary gland was divided into anterior lobe and posterior lobe, and the accessory gland was isolated and post-fixed in 1 % osmium tetroxide for 2 h in the same buffer at room temperature, followed by washing in buffer and dehydration in 70° ethanol. The samples were embedded in LR White resin, and ultrathin sections (8–90 nm) obtained with glass knife in ultramicrotome Sorvall MT2-BMT2-B were stained with 1 % aqueous uranyl acetate and lead citrate (Reynolds 1963) and examined under the Zeiss EM 109 transmission electron microscope.

Cytochemistry

Carbohydrates

Some ultrathin sections of salivary glands were transferred to copper grids, incubated in 1 % periodic acid for 20 min, and washed in distilled water. Subsequently, the samples were transferred to 0.2 % thiosemicarbazide solution for 30 min, washed in 10 % acetic acid solution (Thiery 1967), and examined under the Zeiss EM 109 transmission electron microscope.

Acid phosphatase

Salivary glands were dissected and transferred to glutaraldehyde–paraformaldehyde 1 % (*v*/*v*) in 0.2 M sodium cacodylate

buffer at pH 7.2 for 4 h at room temperature. Then, the salivary glands were washed in 0.1 M sodium acetate buffer (pH 4.8) and incubated in 0.01 M sodium β-glycerophosphate solution in sodium acetate buffer (0.1 M, pH 5.0) containing 2.5 mM lead nitrate for 45 min at room temperature (Tyder and Bowen 1975). Samples were post-fixed in 1 % osmium tetroxide for 2 h at room temperature, followed by washing in 0.1 M sodium cacodylate buffer, dehydrated in 70° ethanol, and then embedded in LR White resin. Ultrathin sections were stained with 1 % aqueous uranyl acetate and lead citrate (Reynolds 1963) and examined under the Zeiss EM 109 transmission electron microscope.

Lipids

The salivary glands of *P. nigrispinus* were dissected and transferred to 2.5 % glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2) containing 0.2 M sucrose for 4 h at room temperature. Then, salivary glands were washed in sodium cacodylate buffer for 10 min and 0.1 M imidazole buffer (pH 7.5) for 10 min. The glands were post-fixed in 2 % osmium tetroxide in 0.1 M imidazole buffer for 30 min at room temperature in dark (Angermüller and Fahimi 1982). After further washes in imidazole buffer, the samples were dehydrated in 70° ethanol and embedded in LR White resin. Ultrathin sections were stained with 1 % aqueous uranyl acetate and lead citrate (Reynolds 1963) and examined under the Zeiss EM 109 transmission electron microscope.

Proteins

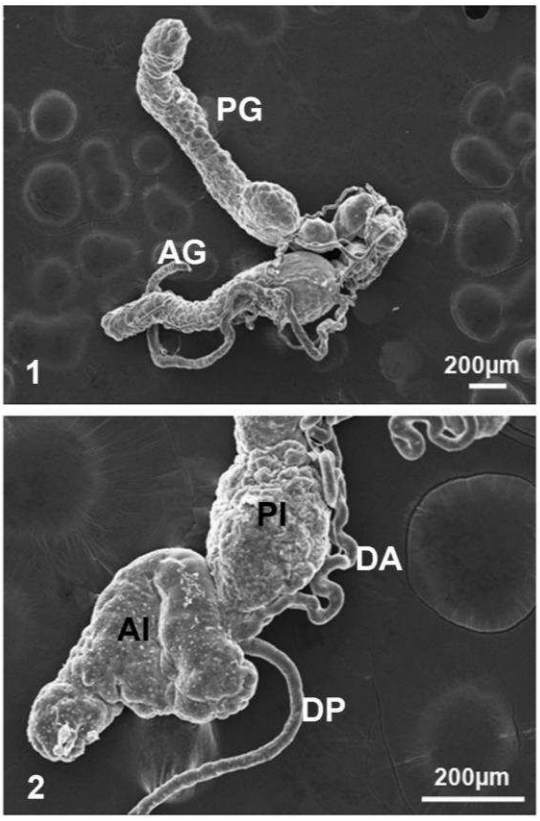
The salivary glands were dissected and transferred to 2.5 % glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2) containing 0.2 M sucrose. The glands were washed in distilled water and incubated in a 0.15 % ammoniacal silver solution for 5 min at room temperature. Next, the glands were transferred to 3 % formaldehyde for 5 min and washed in distilled water. Subsequently, the samples were washed in 0.1 M imidazole buffer (pH 7.5) (McRae and Meetz 1970), followed by dehydration in a graded ethanol series. The glands were embedded in LR White resin and the ultrathin sections were stained with 1 % aqueous uranyl acetate and lead citrate (Reynolds 1963) and then examined under the Zeiss EM 109 transmission electron microscope.

Results

The salivary system of *P. nigrispinus* consisted of a pair of principal salivary glands and a pair of accessory salivary glands extending from the prothorax to the metathorax, which were translucent in saline solution for insects.

The principal salivary glands were bilobed, containing the anterior lobe smaller than the elongated posterior lobe (Fig. 1). The anterior lobe is a semi-oval shaped with a short projection into the insect head and enlarged toward the posterior lobe (Fig. 1). The posterior lobe was located in prothorax, and it was more enlarged at the junction with the anterior lobe and sharper at the posterior end (Fig. 1). In the hilus between the anterior and posterior lobes, a narrow salivary duct was inserted (Fig. 2), which connected inside the head with the salivary duct of the other pair of principal salivary gland to form a single salivary duct that opens in the mouthpart stylet.

Accessory salivary glands were tubular and narrower than the principal salivary glands (Fig. 1), opening by a long and narrow glandular duct in the hilus of the principal salivary gland (Fig. 2). In the portion near the hilus, the duct of accessory salivary gland had a regular U-shaped fold (Fig. 2).



Figs. 1, 2 Scanning electronic micrographs of salivary glands of *P. nigrispinus* (Hemiptera: Pentatomidae): *1* General view showing principal gland (PG) and accessory gland (*AG*). *2* Detail of hylus between anterior (*AI*) and posterior (*PI*) lobes with ducts of accessory gland (*DA*) and principal gland (*DP*)

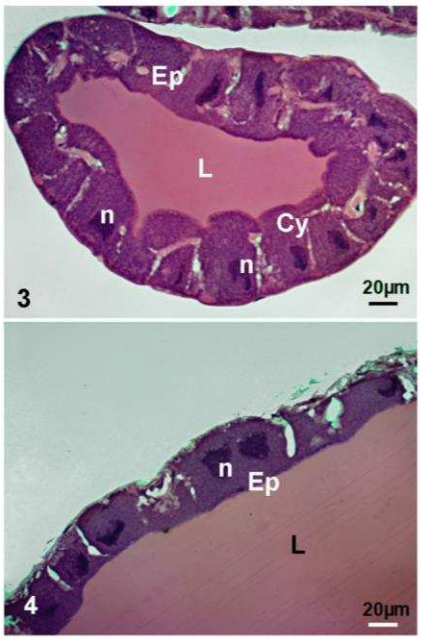
Principal salivary glands

The epithelium of the principal salivary gland had a single layer of cubic or columnar cells (Figs. 3, 4), with one or two well-developed spherical nuclei with a predominance of uncondensed chromatin and evident nucleolus (Fig. 4). The cytoplasm showed homogeneous aspect with few granules. The lumen content was homogeneous and acidophill (Fig. 4). Externally, the principal salivary glands were coated with a thin basement membrane (Fig. 4).

In the anterior lobe of the principal salivary glands, the basal portion of secretory cells had short plasma membrane infoldings that formed large canals (Fig. 5) associated with mitochondria. The cytoplasm had a high amount of rough endoplasmic reticulum (Figs. 6, 7), Golgi apparatus, and vesicles with electron-dense core and an electron-transparent periperical halo (Fig. 8) similar to the luminal content (Fig. 6). Mitochondria were numerous and well developed, with many tubular cristae that resulted in a multivesicular compartment bounded by double membrane (Fig. 7). The nucleus was median and irregular with uncondensed chromatin and well-developed nucleolus (Fig. 9). The apical surfaces of the secretory cells had narrow, short, and irregular plasma membrane foldings (Fig. 5). The luminal content was granular and electron-dense. Cytochemical tests showed positive reactions for carbohydrates near the electron-dense granules (Fig. 9) and for protein in the electron-dense granules (Fig. 10). The cytochemical tests for lipids and acid phosphatase were negative in the anterior lobe of the principal salivary gland tissues.

In the posterior lobe of the principal gland, the basal region of the secretory cell was similar to that found in the anterior lobe, with short plasma membrane infoldings associated with mitochondria that formed large canals (Fig. 11). The cytoplasm was rich in rough endoplasmic reticulum, Golgi complex, mitochondria with many cristae, and vesicles with granular content (Fig. 12). The well-developed nucleus showed a predominance of uncondensed chromatin and multiple nucleoli. The apical surface of the cell was characterized by numerous narrow and long microvilli, reaching the luminal content that showed lower electron density in comparison with the luminal content of the anterior lobe (Fig. 13). Cytochemical tests showed positive reactions for acid phosphatase in vacuoles of different sizes (Fig. 14). Lipids were found in the apical cytoplasm (Fig. 15), and proteins were scattered in the cytoplasm with a high concentration in the basal cell region close to the rough endoplasmic reticulum (Fig. 16). Carbohydrates were weakly identified in these cells.

The secretory cells of both anterior and posterior lobes were onto a thin basal lamina that had an extensive amount of trachea and lacked muscle cells (data not shown).



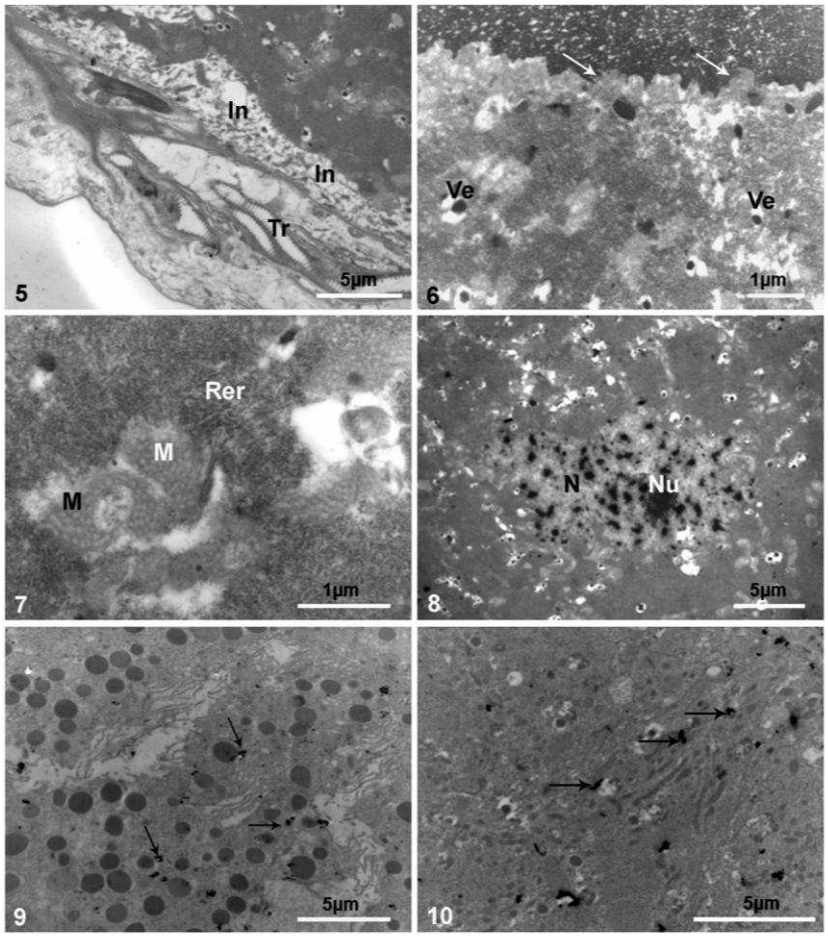
Figs. 3, 4 Histological sections of salivary glands of *P. nigrispinus* (Hemiptera: Pentatomidae). *3* Anterior lobe of principal gland showing columnar epithelium (*Ep*) with well-developed nucleus (*n*) and basophilic cytoplasm (*Cy*). Note acidophill gland content in the lumen (*L*). *4* Posterior lobe of principal gland showing cubical epithelium (*Ep*) with some cells containing two nucleus (*n*) and basophilic cytoplasm (*Cy*)

Accessory salivary glands

The epithelium of accessory salivary glands was composed of columnar cells lining a narrow lumen, whose content remained unstained by hematoxylin and eosin staining (Fig. 17). The cytoplasm of secretory cells of the accessory salivary gland had large vacuoles with electron-transparent or electron-lucent content (Fig. 18), rough endoplasmic reticulum, and mitochondria (Fig. 19). The irregular nucleus showed a predominance of uncondensed chromatin and nucleoli. The basal surface of the cell was characterized by the plasma membrane infoldings (Fig. 20). The apical surface of the glandular cell had narrow and short microvilli (Fig. 21). The secretory epithelium was onto a thin basal lamina rich in trachea. Cytochemical tests showed positive reactions for lipids that were homogeneously distributed in the cytoplasm (Figs. 21, 22) and proteins in the lumen and extracellular spaces (Fig. 23).

Discussion

The salivary system of *P. nigrispinus* is formed by a pair of principal salivary glands and accessory salivary glands, with anatomy similar to that described for *Podisus distinctus* (Castro et al. 2013), *Brontocoris tabidus* (Heteroptera: Pentatomidae) (Azevedo et al. 2007), and other Pentatomidae (Baptist 1941),

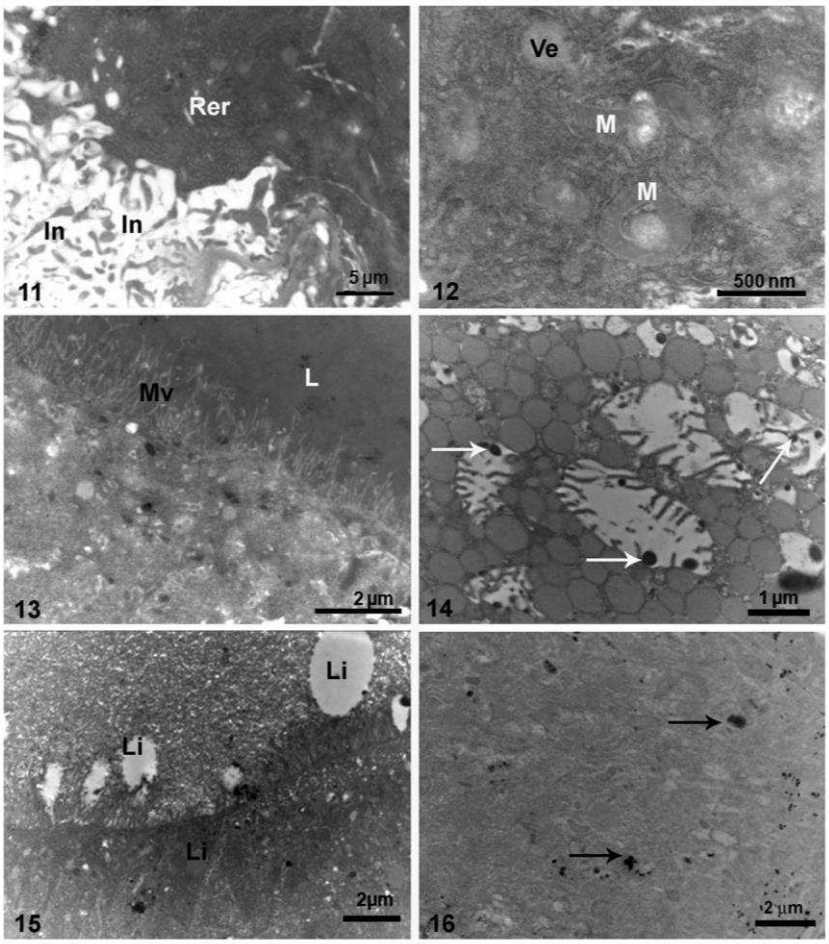


Figs. 5–10 Transmission electronic micrographs of secretory cells of anterior lobe of principal salivary gland *P. nigrispinus* (Hemiptera: Pentatomidae). *5* Basal cell region showing plasma membrane infoldings (*In*). Note peritoneal sheet with trachea (*Tr*). *6* Apical cell region showing irregular cell surface (*arrows*) with electron-dense secretory vesicles (*Ve*). *7* Perinuclear cytoplasm with rough endoplasmic reticulum (*Rer*) and mitochondria (*M*) with tubular cristae. *8* Irregular nucleus (*N*) with decondensed chromatin and well-developed nucleolus (*Nu*). *9* Cytochemical test for carbohydrate showing granules (*arrows*) in the cytoplasm. *10* Cytochemical test for protein showing positive reactions (*arrows*)

suggesting a similar anatomical pattern for Pentatomidae. However, variations have been reported for other Hemiptera such as *Belostoma lutarium* (Belastomatidae) (Swart and Felgenhauer 2003), *Cimex hemipterus* (Cimicidae) (Serrão et al. 2008), *Karenia caelatata* (Cicadidae) (Zhong et al. 2013), *Mahanarva posticata* (Cercopidae) (Roma et al. 2003), and Triatominae species (Lacombe 1999; Weirauch and Schuh 2011). Although the morphological diversity of the salivary glands may be due to different feeding habits of Hemiptera, with zoophagous, phytophagous, zoophytophagous, phytozoophagous, and hematophagous species (Miles 1972; Terra and Ferreira 1994; Cohen 1995; Zeng and Cohen 2000), the anatomy of these glands in Pentatomidae seemed more related to the phylogeny because phytophagous Pentatomidae (Baptist 1941) have salivary glands similar to those of predators *Brontocoris tabidus* (Azevedo et al. 2007), *P. distinctus* (Castro et al. 2013), and *P. nigrispinus*.

The principal salivary glands of *P. nigrispinus* does not have muscle cells attached to its wall, suggesting that saliva-releasing mechanism in *P. nigrispinus* occurs with the participation of some thorax muscles, as reported for secretion release of head glands in termites (Santos et al. 2005).

The accessory salivary glands of *P. nigrispinuns* are tubular, with a narrow lumen and a duct that opens in the hilus between the two lobes of principal salivary gland such as that in *Brontocoris tabidus* (Azevedo et al. 2007) and *P. distinctus* (Castro et al. 2013), suggesting that secretion produced in accessory glands is transported to the lumen of the principal salivary gland. Opening of the accessory salivary gland duct in the principal gland has been reported in other Hemiptera, indicating that the composition of the saliva can be mixed continuously (Terra and Ferreira 1994; Cohen 1995; Zeng and Cohen 2000; Swart and Felgenhauer 2003; Zhong et al. 2013).



Figs. 11–16 Transmission electronic micrographs of secretory cells of posterior lobe of principal salivary gland of *P. nigrispinus* (Hemiptera: Pentatomidae). *11* Basal cell region showing enlarged extracellular canals resulting from basal plasma membrane infoldings (*In*) which extend to perinuclear cytoplasm rich in rough endoplasmic reticulum (*Rer*). *12* Perinuclear cytoplasm with well-developed mitochondria (*M*) and secretory vesicles (*Ve*). *13* Apical cell surface with narrow microvilli (*Mv*) and electron-dense gland content in the lumen (*L*). *14* Cytochemical detection of acid phosphatase (*arrows*) in the digestive vacuoles. *15* Cytochemical detection of lipids (*Li*). *16* Cytochemical detection of proteins in the cytoplasm (*arrows*)

The morphology of salivary glands of *P. nigrispinus* suggests that there are three regions responsible for saliva production: anterior and posterior lobes of principal salivary gland and accessory salivary gland because the occurrence of glandular epithelium with columnar or cubical cells in the three gland regions are indicative of cells with high metabolic rate (Del Bene et al. 1991; Ghanim et al. 2001; Reis et al. 2003; Sais et al. 2003; Oliveira et al. 2006; Azevedo et al. 2007; Serrão et al. 2008).

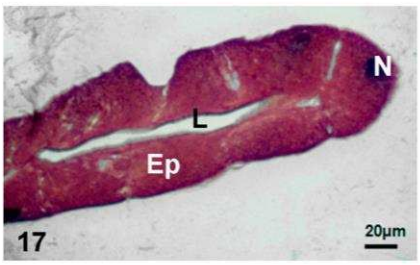
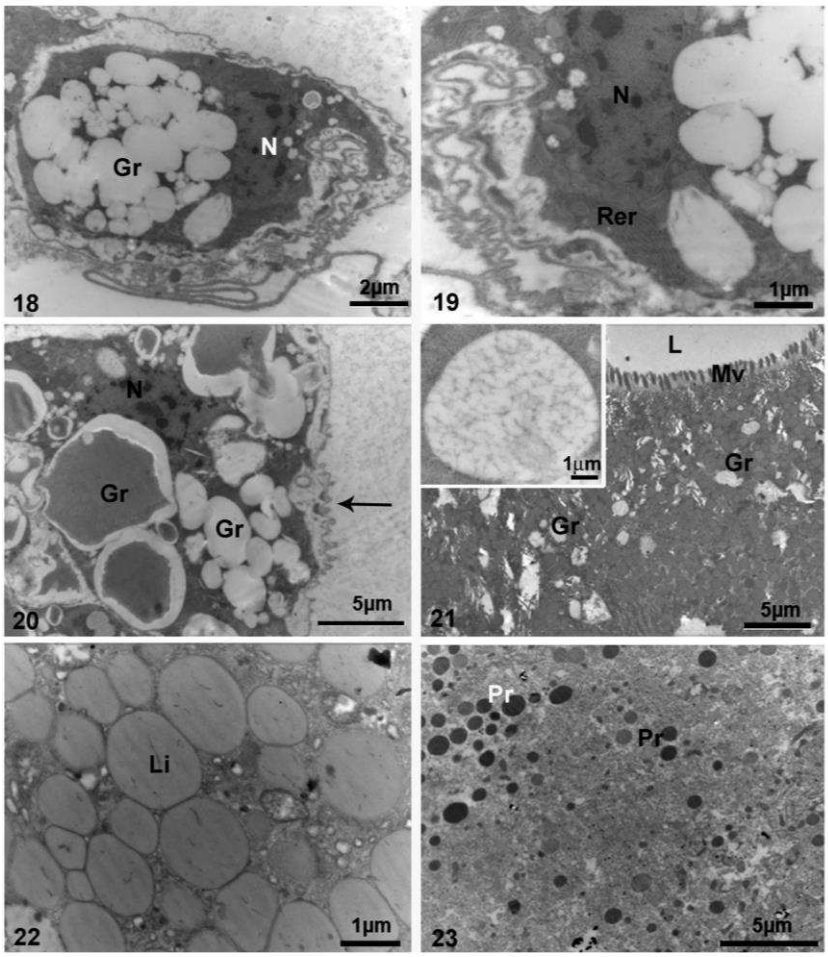


Fig. 17 Histological section of accessory salivary gland of *P. nigrispinus* (Hemiptera: Pentatomidae): Cubic epithelial cells (*Ep*) with well-developed basal nucleus (*N*) and enlarged extracellular space (*arrows*). Note narrow lumen (*L*)

The presence of basal plasma membrane infoldings associated with mitochondria and formation of large canals as well as the presence of carbohydrate reserves demonstrated by cytochemical test in the cells of anterior and posterior lobes of the principal salivary glands of *P. nigrispinus* suggests high transport of substances from the hemolymph. Cells with structural organization are commonly found in the epithelia, which plays an active role in the transport of substances (Alberts et al. 2010), where glycogen is the primary fuel source for ADP to ATP conversion in the mitochondria (Nelson and Cox 2008). Furthermore, cytoplasms rich in rough endoplasmic



Figs. 18–23 Transmission electronic micrographs of secretory cells of accessory salivary gland of *P. nigrispinus* (Hemiptera: Pentatomidae). *18* General view of secretory cell showing nucleus (*N*) and electron-lucent granules (*Gr*). *19* Basal cell region showing nucleus (*N*) with decondensed chromatin and perinuclear cytoplasm rich in rough endoplasmic reticulum (*Rer*). *20* Secretory cell with electron-lucent and electron-dense granules (*Gr*) and irregular plasma membrane foldings (*arrow*). *21* Apical cell surface with short microvilli (*Mv*) and electron-lucent granules (*Gr*). *Inset*, detail of electron-lucent granule. *22* Cytochemical detection of lipids (*Li*). *23* Cytochemical detection of protein (*Pr*)

reticulum and granules containing proteins indicate the occurrence of protein synthesis. Protein secretion by principal salivary glands has been reported for other Hemiptera (Miles 1972; Cohen 1998; Swart and Felgenhauer 2003; Nunes and Camargo-Mathias 2006; Azevedo et al. 2007; Serrão et al. 2008; Fialho et al. 2012; Zhong et al. 2013), showing that the function of the principal salivary glands is conserved in these insects, regardless of their feeding habits.

Epithelial cells of accessory salivary gland of *P. nigrispinus* have ultrastructure suggesting involvement in the transport of substances from hemolymph and proteins secretion. This is an intriguing finding, as the narrow lumen of this gland makes it unlikely to store substances that may be constitutively released in the lumen of the principal salivary glands because the duct of the accessory salivary gland opens in the hilum between the anterior and posterior lobes of the principal salivary gland. Thus, our model suggests that the accessory salivary gland of *P. nigrispinus* has a different function from that suggested by Miles and Slowiak (1976), who pointed out that this gland plays a role in addition of water to saliva.

Cytochemical differences in the quantity and distribution of carbohydrates, acid phosphatase, lipids, and proteins were found in the secretory cells of the anterior and posterior lobes of the principal and accessory salivary glands of *P. nigrispinus*, indicating that different substances in different quantities are produced in these gland compartments. The salivary gland complex of predatory stink-bugs is important in the production of enzymes required for extra-oral digestion (Swart and Felgenhauer 2003; Azevedo et al. 2007). In predatory bugs, the principal salivary glands may play a role in the production of digestive enzymes that cause liquefaction of prey body for its ingestion, which characterizes extra-oral digestion (Cohen 1990, 1995, 1998; Mohaghegh et al. 2001; Eubanks et al. 2003). However, in *P. nigrispinus*, the only

digestive enzyme present in the salivary glands is collagenase (Fialho et al. 2012). Thus, the cellular apparatus for protein synthesis in the principal salivary glands of *P. nigrispinus* synthesizes other proteins/peptides than digestive enzymes.

The diversity of substances produced by the salivary glands of *P. nigrispinus* suggests that this predator can feed on large numbers of prey. Moreover, the complexity of the saliva compounds, functional morphology of ingestion organs, and the ecological implications of insect biology need additional studies to extend our understanding of salivary gland function in the feeding behavior as well as in the production of substances responsible for prey paralysis and death.

Acknowledgments The authors are grateful to the Conselho Nacional de Desenvolvimento Científico e Tecnológico CNPq (Brazil), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior CAPES (Brazil), Fundação de Amparo a Pesquisa do Estado de Minas Gerais FAPEMIG (Brasil), and Núcleo de Microscopia e Microanálise of Universidade Federal de Viçosa.

Conflict of interest We declare that there are no conflicts of interest with the organization that sponsored the research.

References

Alberts B, Johson A, Lewis J, Raff M, Roberts K, Walter P (2010) Molecular Biology of the Cell. Garland Science, New York, pp 1–1601

Angermüller S, Fahimi DH (1982) Imidazole-buffered osmium tetroxide: an excellent stain for visualization of lipids in transmission electron microscopy. Histochem J 14:823–825

Azevedo DO, Zanuncio JC, Zanuncio JJS, Martins GF, Marques-Silva S, Sossai MF, Serrão JE (2007) Biochemical and morphological aspects of salivary glands of the predator *Brontocoris tabidus* (Heteroptera: Pentatomidae). Braz Arch Biol Techn 50:469–477

Baptist BA (1941) The morphology and physiology of the salivary glands of Hemiptera-Heteroptera. Q J Microsc Sci 83:91–139

Barth R (1954) Estudos anatômicos e histológicos sobre a subfamilia Triatominae (Heteroptera-Reduvidae). IV Parte: O complexo das glândulas salivares de *Triatoma infestans*. Mem I Oswaldo Cruz 53:517–585

Bell HA, Down RE, Edwards JP, Gatehouse JA, Gatehouse AMR (2005) Digestive proteolytic activity in the gut and salivary glands of the predatory bug *Podisus maculiventris* (Heteroptera: Pentatomidae), effect of proteinase inhibitors. Eur J Entomol 102:139–145

Boyd DWJ, Cohen AC, Alverson DR (2002) Digestive enzymes and stylet morphology of *Deraeocoris nebulosus* (Hemiptera: Miridae), a predaceous plant bug. Ann Entomol Soc Am 95:395–401

Castro AA, Canevari GC, Pikart TG, Ribeiro RC, Serrão JE, Zanuncio TV, Zanuncio JC (2013) Salivary gland histology of the predator *Supputius cincticeps* (Heteroptera: Pentatomidae). Ann Entomol Soc Am 106:273–277

Cohen AC (1990) Feeding adaptations of some predaceous Heteroptera. Ann Entomol Soc Am 83:1215–1223

Cohen AC (1995) Extra-oral digestion in predaceous terrestrial Arthropoda. Annu Rev Entomol 40:85–103

Cohen AC (1998) Solid-to-liquid feeding: the inside story of extra-oral digestion in predaceous Heteroptera. Am Entomol 44:103–117

Del Bene G, Dallai R, Marchini D (1991) Ultrastructure of the midgut and the adhering tubular salivary gland of *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae). Int J Insect Morphol Embryol 20:15–24

Eubanks MD, Styrsky JD, Denno RF (2003) The evolution of omnivory in heteropteran insects. Ecology 84:2549–2556

Fialho MCQ, Zanuncio JC, Neves CA, Ramalho FS, Serrão JE (2009) Ultrastructure of the digestive cells in the midgut of the predator *Brontocoris tabidus* (Heteroptera: Pentatomidae) after different feeding periods on prey and plants. Ann Entomol Soc Am 102:119–127

Fialho MCQ, Zanuncio JC, Neves CA, Ramalho FS, Serrão JE (2012) Prey digestion in the midgut of the predatory bug *Podisus nigrispinus* (Hemiptera: Pentatomidae). J Insect Physiol 58:850–856

Ghanim M, Rosell RC, Campbell LR, Czosnek H, Brown JK, Ullman DE (2001) Digestive, salivary, and reproductive organs of *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) B type. J Morphol 248:22–40

Guedes BAM, Zanuncio JC, Ramalho FS, Serrão JE (2007) Midgut morphology and enzymes of the obligate zoophytophagous stink-bug *Brontocoris tabidus* (Signoret, 1963) (Heteroptera: Pentatomidae). Pan-Pac Entomol 83:229–235

Lacerda MC, Ferreira AMRM, Zanuncio TV, Zanuncio JC, Bernardio AS, Espindula MC (2004) Development and reproduction of *Podisus distinctus* (Heteroptera: Pentatomidae) fed on larva of *Bombyx mori* (Lepidoptera: Bombycidae). Braz J Biol 64:237–242

Lacombe D (1999) Anatomia e histologia das glândulas salivares nos triatomíneos. Mem I Oswaldo Cruz 94:557–564

Lemos WP, Medeiros RS, Ramalho FS, Zanuncio JC (2001) Effects of plant feeding on the development, survival and reproduction of *Podisus nigrispinus* (Dallas) (Heteroptera: Pentatomidae). Int J Pest Manage 47:89–93

Lemos WP, Ramalho FS, Serrão JE, Zanuncio JC (2003) Effects of diet on development of *Podisus nigrispinus* (Dallas) (Heteroptera: Pentatomidae), a predator of the cotton leafworm. J Appl Entomol 127:389–395

Lemos WP, Ramalho FS, Serrão JE, Zanuncio JC (2005a) Morphology of female reproductive tract of the predator *Podisus nigrispinus* (Dallas) (Heteroptera: Pentatomidae) fed on different diets. Braz Arch Biol Techn 48:129–138

Lemos WP, Zanuncio JC, Serrão JE (2005b) Attack behavior of *Podisus rostralis* (Heteroptera, Pentatomidae) adults on caterpillars of *Bombyx mori* (Lepidoptera, Bombycidae). Braz Arch Biol Techn 48:975–981

Matos Neto FC, Oliveira HN, Zanuncio JC, Holtz AM (2004) Ganância de peso del depredador *Podisus distinctus* (Heteroptera: Pentatomidae) en combinaciones de las presas *Tenebrio molitor* (Coleoptera: Tenebrionidae) e *Musca domestica* (Diptera: Muscidae). Rev Biol Trop 52:1–8

McPherson RM, Smith JC, Allen WA (1982) Incidence of arthropod predators in different soybean cropping systems. Environ Entomol 11:685–689

McRae EK, Meetz GD (1970) Electron microscopy of ammoniacal silver reaction for histones in erythropoietic cells of chick. J Cell Biol 45: 235–245

Medeiros RS, Ramalho FS, Lemos WP, Zanuncio JC (2000) Age-dependent fecundity and life-fertility tables for *Podisus nigrispinus* (Dallas) (Heteroptera: Pentatomidae). J Appl Entomol 124:319–324

Miles PW (1972) The saliva of Hemiptera. Adv Insect Physiol 9:183–255

Miles PW, Slowiak D (1976) The accessory salivary gland as the source of water in the saliva of Hemiptera: Heteroptera. Experientia 15: 1011–1012

Mohaghegh J, De Clercq P, Tirry L (1999) Effects of rearing history and geographical origin on reproduction and body size of the predator *Podisus nigrispinus* (Heteroptera: Pentatomidae). Eur J Entomol 96: 69–72

Mohaghegh J, De Clercq P, Tirry L (2001) Functional response of the predators *Podisus maculiventris* (Say) and *Podisus nigrispinus* (Dallas) (Heteroptera: Pentatomidae) to the beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae): effect of temperature. J Appl Entomol 125:131–134

Nelson DE, Cox MM (2008) Lehninger principles of biochemistry, 5th edn. W.H Freeman, New York

Nunes PH, Camargo-Mathias MI (2006) Ultrastructural study of the salivary glands of the sugarcane spittlebug *Mahanarva fimbriolata* (Stal, 1854) (Euhemiptera: Cercopidae). Micron 37:57–66

Oliveira I, Zanuncio JC, Serrão JE, Zanuncio TV, Pinon TBM, Fialho MCQ (2005) Effect of female weight on reproductive potential of the predator *Brontocoris tabidus* (Signoret 1852) (Heteroptera: Pentatomidae). Braz Arch Biol Techn 48:975–981

Oliveira JA, Oliveira MGA, Guedes RNC, Soares MJ (2006) Morphology and preliminary enzyme characterization of the salivary glands from the predatory bug *Podisus nigrispinus* (Heteroptera: Pentatomidae). Bull Entomol Res 96:251–258

Panizzi AR, McPherson JE, James DG, Javahery M, McPherson RM (2000) Economic importance of stink bugs (Pentatomidae). In: Schaefer CW, Panizzi AR (eds) Heteroptera of economic importance. CRC, Boca Raton, pp 421–474

Puri IM (1924) Studies on the anatomy of *Cimex lectularius* L. Parasitology 16:84–97

Reis MMM, Meirelles RM, Soares MJ (2003) Fine structure of the salivary glands of *Triatoma infestans* (Hemiptera, Reduviidae). Tissue Cell 35:393–400

Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17:208–2012

Roma GC, Camargo-Mathias MI, Arrigoni EB, Marin-Morales MA (2003) Little cicada of sugarcane *Mahanarva posticata* (Homoptera, Cercopidae). A Brazilian agricultural pest. Morpho-histological study of salivary glands. Cytologia 68:101–114

Sais TC, Moraes RM, Ribolla PE, Bianchi AG, Marinotti O, Bijovsky AT (2003) Morphological aspects of *Culex quinquefasciatus* salivary glands. Arthropod Struct Dev 32:219–226

Santos CA, Costa-Leonardo AM, Serrão JE (2005) Morphology of the head and frontal gland in Neotropical Nasutitermitinae (Isoptera, Termitidae). Sociobiology 46:579–593

Schmidt JO (1982) Biochemistry of insect venoms. Annu Rev Entomol 27:339–368

Serrão JE, Castrillon MI, Santos-Mallet JR, Zanuncio JC, Gonçalves TC (2008) Ultrastructure of the salivary gland in *Cimex hemipterus* (Hemiptera: Cimicidae). J Med Entomol 45:991–999

Stefanini M, De Martino C, Zamboni L (1967) Fixation of ejaculated spermatozoa for electron microscopy. Nature 216:173–174

Swart CC, Felgenhauer BE (2003) Structure and function of the mouthparts and salivary gland complex of the giant waterbug, *Belostoma lutarium* (Stall) (Hemiptera: Belostomatidae). Ann Entomol Soc Am 96:870–882

Terra WR, Ferreira C (1994) Insect digestive enzymes: properties, compartmentalization and function. Comp Biochem Physiol B 109:1–62

Thiery JP (1967) Mise em evidence des polysaccharides sur coupes fines en microscopie electronique. J Microsc 6:987–1018

Tillman PG, Mullinix BG (2004) Comparison of susceptibility of pest *Euschistus servus* and predator *Podisus maculiventris* (Heteroptera: Pentatomidae) to selected insecticides. J Econ Entomol 97:800–806

Tyder TA, Bowen ID (1975) A method for the fine structural localization of acid phosphatase activity using rho-nitrophenyl phosphate as substrate. J Histochem Cytochem 23:235–237

Weirauch C, Schuh RT (2011) Systematics and evolution of Heteroptera: 25 years of progress. Annu Rev Entomol 56:487–510

Zanuncio JC, Alves JB, Zanuncio TV, García JL (1994) Hemipterous predators of eucalypt defoliator caterpillars. Forest Ecol Manag 65: 65–73

Zanuncio TV, Torres JB, Zanuncio JC, Santos GP (1998) Ciclo de vida e reprodução de *Podisus distinctus* (Stal) (Heteroptera: Pentatomidae) alimentado com dois tipos de presas. Rev Bras Entomol 41:335–337

Zeng F, Cohen AC (2000) Comparison of a-amylase and protease activities of a zoophytophagous and two phytozoophagous Heteroptera. Comp Biochem Physiol A 126:101–106

Zhong H, Wei C, Zhang Y (2013) Gross morphology and ultrastructure of salivary glands of the mute cicada *Karenia caelatata* distant (Hemiptera: Cicadoidea). Micron 45:83–91

CHAPTER 2

STINK BUG (Podisus nigrispinus) KILLS PREY WITH SALIVARY NON-PROTEINEOUS COMPOUNDS

Luis Carlos Martínez[1], Maria do Carmo Queiroz Fialho[2], Luiz Claudio Almeida Barbosa[3], Leandro Licursi Oliveira[4], José Cola Zanuncio[1] and José Eduardo Serrão[4]

[1]Departamento de Entomologia, Universidade Federal de Viçosa (UFV), 36570-000, Viçosa, Minas Gerais, Brasil.

[2]Departamento de Morfologia, Universidade Federal do Amazonas (UFAM), 69077-000, Manaus, Amazonas, Brasil.

[3]Departamento de Quimica, Universidade Federal de Viçosa (UFV), 36570-000, Viçosa, Minas Gerais, Brasil.

[4]Departamento de Biologia Geral, Universidade Federal de Viçosa (UFV), 36570-000, Viçosa, Minas Gerais, Brasil.

Abstract

Podisus nigrispinus Dallas (Hemiptera: Pentatomidae) is a zoophytophagous insect with potential for biological control because nymphs and adults prey on several insects by inserting their mouthparts and injecting the salivary gland contents in the prey, killing them. However, the toxic compounds from the saliva of this insect that is responsible for the death of the prey remains unknown. This study identified the saliva compounds of P. nigrispinus responsible for the death of the prey A. gemmatalis. The saliva of P. nigrispinus treated with enzyme inhibitors, protease, extracted with ether caused mortality after injection in A. gemmatalis larvae with LC_{50} = 1.91 µL and LC_{90} = 4.34 µL of the saliva extract. The fractions of saliva extract in ether phase responsible for mortality of A. gemmatalis were identified to be N,N-dimethylaniline and 1,2,3-trithiepane, and the injection of the former compound N,N-dimethylaniline showed an LC_{50} = 136.1 nL and LC_{90} = 413.8 nL, suggesting that this non-proteineous compound is responsible for the toxicity of P. nigrispinus saliva.

Keywords: Asopinae, N,N-dimethylaniline, predator, salivary glands, venom, 1,2,3-trithiepane

Introduction

Podisus nigrispinus Dallas (Hemiptera: Pentatomidae) is a zoophytophagous insect used in the biological control in agriculture and forestry in America (Cohen 1990; Medeiros et al. 2000; Lemos et al. 2001; Mohaghegh et al. 2001). P. nigrispinus is used in the biological control for defoliating pests such as Anticarsia gemmatalis Hübner (Lepidoptera: Erebidae), Alabama argillacea Hübner, Spodoptera exigua Hübner, and Trichoplusia ni Hübner (Lepidoptera: Noctuidae) (Mohaghegh et al. 2001; Medeiros et al. 2003; Ferreira et al. 2008; Neves et al. 2010; De Bortoli et al. 2011).

The feeding strategies and extra-oral digestion have been studied for the species of the predatory Hemiptera Belostoma lutarium Stal (Belostomatidae), Deraeocoris nebulosus Uhler (Miridae), and Podisus maculiventris Say (Pentatomidae) (Cohen 1995; Boyd et al. 2002; Swart et al. 2003; Bell et al. 2005). Pentatomidae predators insert their mouthparts into the body of the prey and inject saliva, causing rapid paralysis and death of the prey (Cohen 1990), followed by ingestion of the prey body content (Lemos et al. 2005a; 2005b; Azevedo et al. 2007). The paralysis and death of the prey have been attributed to the action of digestive enzymes produced by the salivary glands and released within the prey (Schmidt 1982; Cohen 1990). The cytochemical and ultrastructural analysis the principal and accessory salivary glands of P. nigrispinus revealed high secretary activity of the proteins and other compounds in the saliva (Martínez et al. 2014). However, Fialho et al (2012) stated that the salivary glands of this insect contain no digestive enzymes, except for collagenase, which could be used to rupture the internal organs of the prey, facilitating its ingestion. The salivary glands compounds responsible for the death of the prey are unknown in P. nigrispinus and other hemipteran predators.

The venoms of arthropods have insecticidal activity against different pests of economic importance (Escoubas et al. 1995; Parkinson et al. 2002; Zhang et al. 2005; Baek et al. 2011). Venom is a toxin produced by glands that is injected into another organism through a specialized apparatus, which can immobilize or kill the prey (Blum 1978; Schmidt 1982). Venoms of some Hemiptera, Hymenoptera, and Lepidoptera have been isolated, identified, and evaluated against other insects and vertebrates (Ramos et al. 2004; Zhang et al. 2005; Sahayaraj and Muthukumar 2011). The transfer of venom into insects can be active like in Hymenoptera through a sting and like in Hemiptera through mouthparts; it may also occur passively like in Lepidoptera larvae through

bristles, spines, or hairs, which transfer the venom via bodily contact with the prey (Schmidt 1982).

The venoms of insects have been described chemically as compounds containing alkaloids, terpenes, polysaccharides, biogenic amines, organic acids, and amino acids (Blum 1978), although the main components are peptides, oligopeptides, and proteins (Schmidt 1982; Calvete et al. 2009).

The identification of the venomous substances in the saliva of P. nigrispinus is important to comprehend the toxic activity responsible for the prey death by the predator. The present study therefore attempted to identify the salivary compounds of this predator responsible for causing death of the prey.

Material and methods

Insects

Adults of P. nigrispinus were obtained from mass rearing in the Laboratório de Controle Biológico do Instituto de Biologia Aplicada à Agricultura e Pecuária (BIOAGRO, Universidade Federal de Viçosa, Minas Gerais, Brazil). They were maintained at 25 ± 2 °C at 75 ± 5% relative humidity and 12-h photophase. The insects were kept in wooden cages (30 × 30 × 30 cm) coated with nylon and glass provided with ad libitum Tenebrio molitor (L.) pupae (Coleoptera: Tenebrionidae), Eucalyptus grandis (W. Hill ex. Maiden) leaves ad libitum and water (Lemos et al. 2001).

Larvae of A. gemmatalis were obtained from a laboratory colony at 26 ± 1 °C at 75 ± 5% relative humidity and 12-h photophase, and placed in polystyrene boxes (15 × 9 cm). The larvae were fed on artificial diet containing 10 g agar, 15.6 g brewer's yeast, 25 g wheat germ, 25 g soy protein, 31.2 g minced beans, 12.5 g casein, and 2.5 mL vitamin solution (1.2% ascorbic acid, 0.03% calcium pantothenate, 0.015% niacin, 0.008% riboflavin, 0.004% thiamin, and 0.004% HCl) (Greene et al. 1976).

Adult males of P. nigrispinus and fifth instar larvae of A. gemmatalis without amputations or malformations were used in the bioassays regardless of size and weight.

Preparation of samples of salivary extract of P. nigrispinus

Males of P. nigrispinus (n = 800) were anesthetized at −4 °C, and the salivary glands were dissected in the saline solution for insects (0.1 M NaCl, 0.1 M KH_2PO_4, 0.1

M Na_2HPO_4) and transferred to four glass vials containing 200 µL of distilled water. The salivary glands were macerated and centrifuged at 10000 ×g at 4 °C for 20 min. The supernatant was removed and stored at −18 °C for bioassays, constituting the aqueous extract of saliva.

Inhibitors

The aqueous extract of saliva of P. nigrispinus (100 µL) was diluted in 100 µL of protease inhibitor cocktail (P2714; Sigma-Aldrich, St. Louis, USA) and other 100 µL of aqueous extract of saliva in 100 µL proteinase K for the degradation of proteins and peptides. The aqueous extract of saliva with protease inhibitor and proteinase K were used as treatments, with 2.7 µL injected into A. gemmatalis larvae using a glass microcapillary with micropipette. As controls, 2.7 µL of protease inhibitor, proteinase K, and distilled water were injected. Fifteen larvae of A. gemmatalis were used for every treatment and, after injection, these insects were individualized in Petri dishes (9 cm diameter) with artificial diet, and the mortality was recorded for 72 h.

Preparation of proteinaceous and non-proteinaceous extract of P. nigrispinus saliva

The aqueous extract of saliva of P. nigrispinus (200 µL) was diluted in 200 µL of petroleum ether. The petroleum ether phase was transferred to a glass vial, air dried, and re-suspended in 200 µL distilled water. A 2.7-µL volume of extracts from the aqueous phase, ether phase, aqueous+ether phases mixture, and distilled water (control) was injected on A. gemmatalis using a micropipette. Fifteen larvae of A. gemmatalis for every treatment were used, individualized in Petri dishes (9 cm diameter) with an artificial diet, and the mortality was evaluated for 72 h.

Mortality bioassay on A. gemmatalis larvae

Non-proteinaceous extract (200 µL) of P. nigrispinus saliva re-suspended from ether phase was obtained as described. Saliva extract at concentrations 1, 2, 3, 4, and 5 µL (adjusted to a 5-µL final volume) and 5 µL distilled water (control) were used to determine the lethal concentration LC_{50} and LC_{90}. Solutions with different concentrations were injected in A. gemmatalis larvae using a micropipette, and the insects were individualized in Petri dishes with an artificial diet. Forty larvae per

concentration were used, and the mortality was recorded for 72 h after injection following correction for natural mortality according to Abbott (1925).

Purification of non-proteinaceous compounds of P. nigrispinus saliva

The salivary extract of P. nigrispinus (200 µL) at the ether phase was air dried, re-suspended in 10 mL of 0.05% (v/v) aqueous formic acid, and fractioned by reverse phase high-performance liquid chromatography (RP/HPLC; Shimadzu, LC20A) using the Vydac® C18-218TP54 column (5 × 250 mm; Deerfield, USA) equilibrated with 0.05 % (v/v) aqueous formic acid. The concentration of acetonitrile in the eluting solvent was raised to 5–50% over 30 min using a linear gradient at a flow rate of 1 mL/min. Absorbance of the column effluent was monitored at $\lambda = 214$ nm and the fractions (1 mL) were collected at 1-min intervals. The fractions were collected at their retention peak and used as test solutions for the toxic activity test; distilled water was used as control. A 3.7-µL aliquot was injected in A. gemmatalis larvae using a micropipette. Fifteen larvae were used per concentration and individualized in glass vial (2.5 × 15 cm) with a 10 g artificial diet. The mortality was recorded every 24 h for 10 days or up the change from larval to pupal stage, and the natural mortality was corrected according to Abbott (1925).

Identification of toxic non-proteinaceous compounds of P. nigrispinus saliva

The toxic fractions for A. gemmatalis larvae were lyophilized and resuspended in 10 µL dichloromethane at 9:1 proportion (v/v). The analyses were performed using a gas chromatograph coupled with a mass detector CG/MS (CGMS-QP 5050A; Shimadzu). For each fraction, 1 µL aliquot was injected in the splitless mode, helium was used as the carrier gas with a flow rate constant of 1.7 mL^{-1} on the Rtx®-5MS capillary column (30 m, 0.25 mm × 0.25 µm; Bellefonte, USA) using the Crossbond® stationary phase (35% diphenyl—65% dimethyl polysiloxane). The initial temperature of injector and detector was 25 °C for 2 min, with a programed temperature of 1 to 230 °C°/min, increasing by 3 °C every 60 min. The mass spectrometer was programmed to detect masses in the range of 29–600 Da with 70 eV ionization energy. The compounds were identified by comparisons of the mass spectra with those available in the Wiley Spectroteca Data Base (7^{th} edition) and by the Retention indices.

Mortality bioassay of commercial substance on A. gemmatalis larvae

The N,N-dimethylaniline compound identified in the saliva of P. nigrispinus was obtained from Sigma-Aldrich. Six different concentrations of N,N-dimethylaniline and the control (552 nL distilled water) were adjusted in volume and used to calculate the lethal concentration LC_{50} and LC_{90}: 18.4, 36.8, 69, 138, 276, and 552 nL. The concentrations were injected into the body of A. gemmatalis larvae using a microinjector (Nanoject II auto nanoliter, Drummond Scientific Co., USA), and the larvae were individualized in Petri dishes with an artificial diet. Ninety larvae were used for each concentration and the mortality was evaluated for 72 h after injection, following correction according to Abbott (1925).

Statistical analyses

The mortality data for inhibitors, phases (aqueous, ether, and aqueous+ether), and saliva fractions were transformed with the formula $\sqrt{x+0.05}$ and analyzed for one-way ANOVA. A Fisher Leans Significant Difference test (LSD) was also used for comparisons of the means in the bioassays at 5% significance level using SAS User software (v. 9.1) for Windows (SAS 2002). The LC_{50} or LC_{90} and their confidence limits were determined by logistic regression in dose–response assays based on the concentration probit–mortality (Finney 1964), with the program XLSTAT-PRO (v. 7.5) for Windows (XLSTAT 2004).

Results

The aqueous extract of P. nigrispinus saliva showed mortality against A. gemmatalis larvae. To determine whether the substances in the saliva of the predator responsible for the mortality of A. gemmatalis were enzymes, the salivary extract containing protease inhibitor cocktail showed 92.29% mortality of larvae. The larvae of A. gemmatalis injected with the salivary extract, whose proteins and peptides were degraded by proteinase K, showed 84.11% mortality, whereas the crude extract of P. nigrispinus saliva without the addition of protease inhibitors or proteinase K showed 96.25% mortality. The larvae mortality was 13.58, 7.03, and 7.03%, respectively, with water, protease inhibitors, and proteinase K (Figure 1). The salivary aqueous extracts of

P. nigrispinus, processed with protease inhibitors and degraded with proteinase K, showed toxic effect on A. gemmatalis larvae ($F_{1,17}$ = 33.67, P < 0.05).

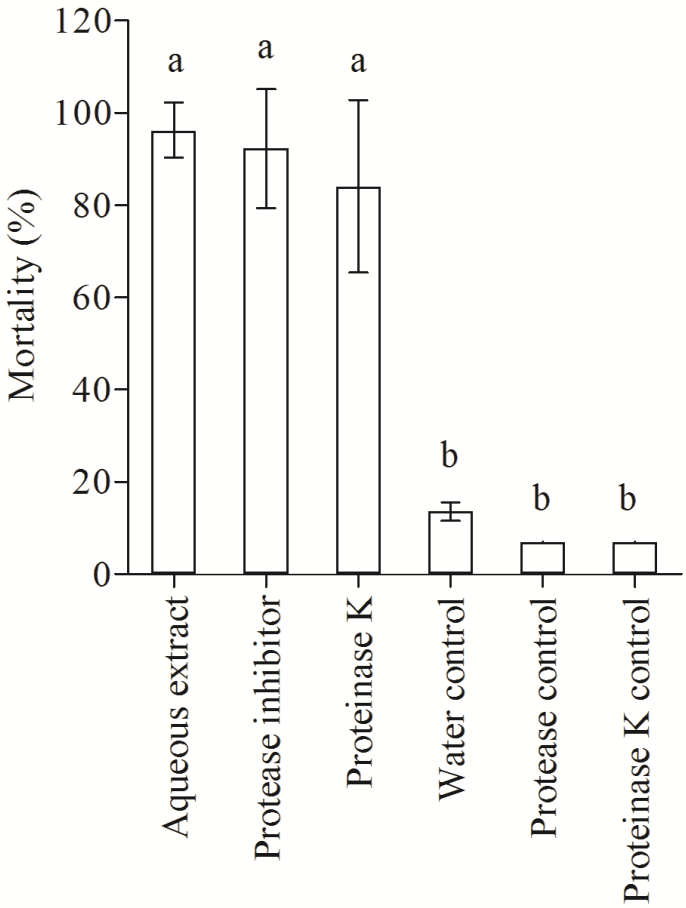


Fig 1. Mortality (Mean ± SD) of Anticarsia gemmatalis larvae caused by the saliva of Podisus nigrispinus (Hemiptera: Pentatomidae) in aqueous extract with protease inhibitor and proteinase K. Letters in columns indicate significant differences by LSD test (P < 0.05).

The salivary gland extract of P. nigrispinus separated in the aqueous, ether, and aqueous+ether mixture phases showed significant mortality of A. gemmatalis larvae ($F_{1,14}$ = 16.02, P < 0.05). The ether and mixture (aqueous+ether) phases showed mortality for A. gemmatalis of 80.02% and 93.32%, respectively; both the values were higher than that for the aqueous extract and control (33.32% and 6.60%, respectively, mortality) (Figure 2).

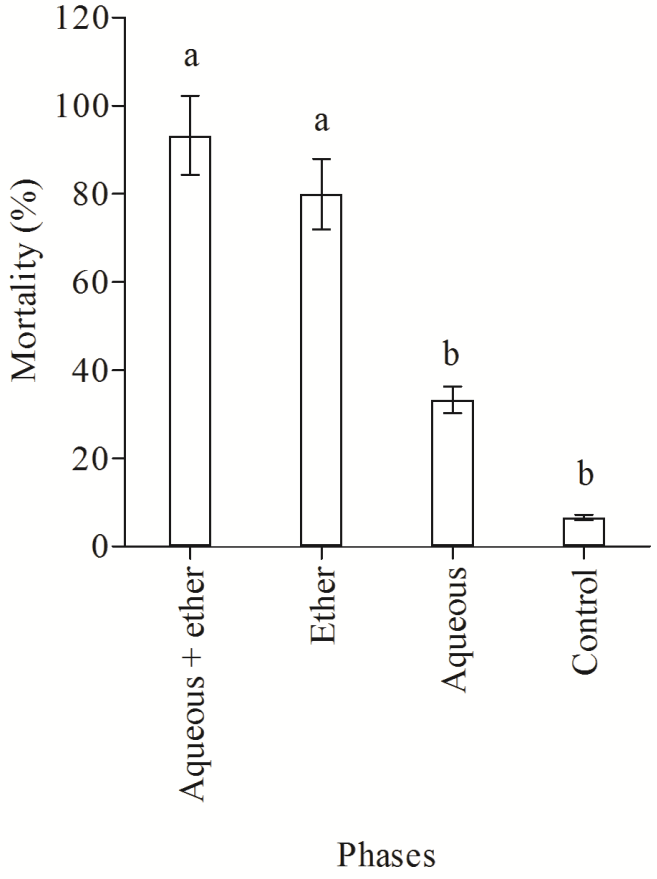


Fig. 2. Mortality (Mean ± SD) of Anticarsia gemmatalis larvae caused by salivary compounds of Podisus nigrispinus (Hemiptera: Pentatomidae) in water, ether phase and aqueous+ether. Letters in the columns indicate significant differences by LSD test ($P < 0.05$).

The dose–response bioassay to different concentrations of the salivary extract of P. nigrispinus in the ether phase on A. gemmatalis showed better result in the concentration of 4 µL of salivary extract ($R^2 = 0.94$, $P < 0.001$), with $LC_{50} = 2.04$ µL for 24 h and $LC_{90} = 3.27$ after 36 h (Table 1). Concentrations below 4 µL induce the symptoms of intoxication in A. gemmatalis larvae as progressive paralysis, low food consumption, regurgitation, and necrosis in the body area injected with the salivary ether extract.

Table 1. Lethal Concentration of the extract in petroleum ether phase of the saliva of Podisus nigrispinus (Hemiptera: Pentatomidae) on Anticarsia gemmatalis larvae at 72 h after injection and estimated in probit values

Concentration (df=3)	Estimated value (µL)	95% Confidence limits		X^2	P>F
		Lower	Upper		
LC_{50}	2.04	0.67	2.59	98.85	<0.0001
LC_{90}	3.27	2.56	4.97		
LC_{95}	3.74	3.02	4.97		
LC_{99}	4.81	3.71	17.3		

The salivary glands extract of P. nigrispinus from ether was separated in five fractions (F1–F5) (Figure 3). These fractions were injected in A. gemmatalis larvae and showed significant mortality response ($F_{1,17}$ = 3.75, P < 0.05), with F3, F4, and F5 fractions showing toxic effect than the F1 and F2 fractions (Figure 4). The mortality for the control was <0.1%.

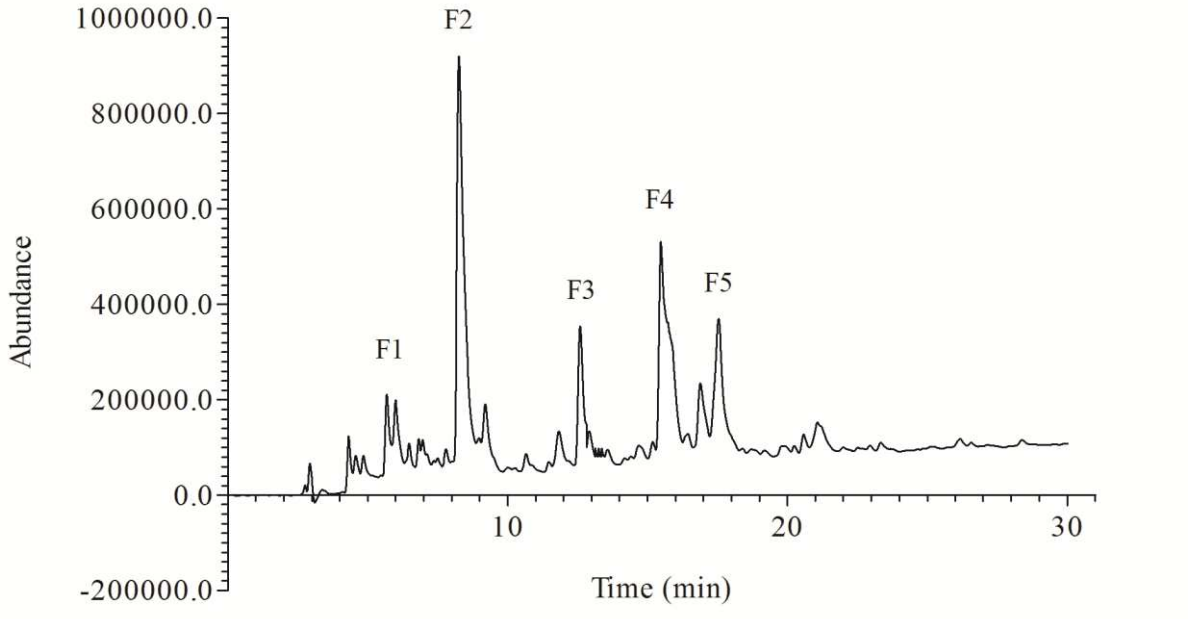


Fig. 3. HPLC chromatogram profile of peak retention of fractions and compounds of the venom of Podisus nigrispinus (Hemiptera: Pentatomidae).

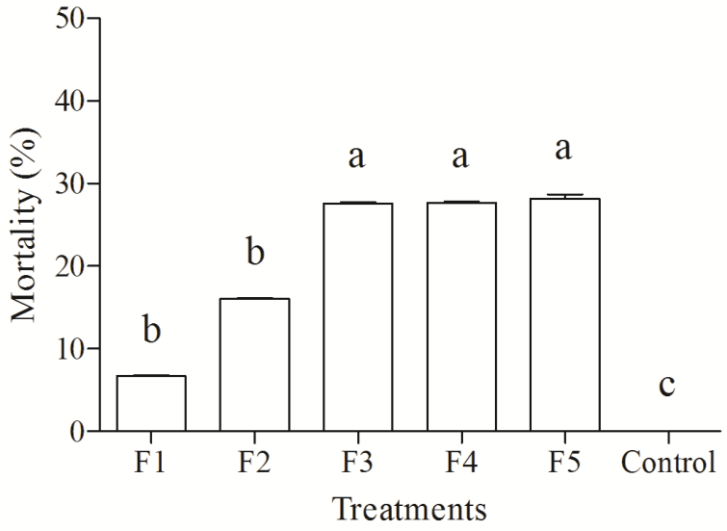


Fig. 4. Mortality (% ± SD) of Anticarsia gemmatalis larvae after 10 days injected with fractions that compose the venom of Podisus nigrispinus (Hemiptera: Pentatomidae). Means followed by different letters in the column differ by LSD test ($P < 0.05$)

The F3, F4, and F5 fractions of the salivary glands extract of P. nigrispinus were identified as hydrocarbons; the F3 and F4 fractions corresponded to N,N-dimethylaniline ($C_8H_{11}N$) and 1,2,3-trithiepane ($C_4H_8S_3$), respectively (Table 2), whereas the F5 fraction, with a retention time of 39.90 min, was not identified.

Table 2. Chemical composition of the fractions of extract of salivary glands Podisus nigrispinus (Hemiptera: Pentatomidae) toxic to Anticarsia gemmatalis

Fraction	Compound	MM	Formula	RI	Ri	Rt	m/z
F3	N,N-dimethylaniline	121	$C_8H_{11}N$	12.5	942	16.61	120.15
F4	1,2,3-trithiepane	152	$C_4H_8S_3$	17.2	1263	29.49	152
F5	n.s	-	-	1.60	641	39.90	31

MM - Molecular mass, RI - Relative intensity, Ri - Retention indices, Rt - Retention time, m/z - Molecular weight

N,N-dimethylaniline was found commercially and its toxic effects in A. gemmatalis were studied at different concentrations (Table 3). Higher mortality was

obtained with the injection of 552 nL of N,N-dimethylaniline (R^2 = 0.84, P < 0.001), with estimated LC_{50} and LC_{90} of 136.1 nL and 413.8 nL, respectively. In this treatment, A. gemmatalis larvae showed symptoms of intoxication and necrosis at concentrations below 552 nL (Figure 5). The lethal time calculated for 36.8, 69, 138, and 276 nL concentrations was 36 h for LC_{50} and 48 h for LC_{90}. The mortality of A. gemmatalis was 99% for all N,N-dimethylaniline concentrations after 72 h.

Table 3. Lethal concentration of N,N-dimethylaniline in Anticarsia gemmatalis larvae at 72 h after injection and estimated in probit values

Concentration (df=3)	Estimated value (nL)	95% Confidence limits		X^2	P>F
		Lower	Upper		
LC_{50}	136.1	3.28	234.4	136.55	<0.0001
LC_{90}	413.89	343.64	520.25		
LC_{95}	531.23	441.06	667.73		
LC_{99}	751.33	623.80	944.38		

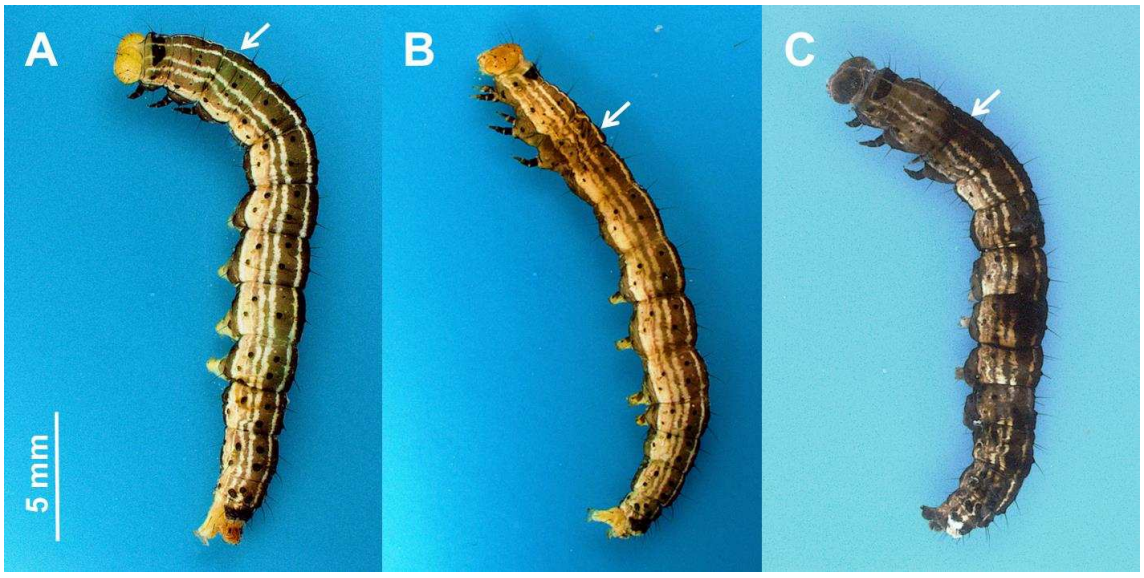


Fig. 5. Time-course of N,N-dimethylaniline on Anticarsia gemmatalis larvae after injection. Sequential necrosis effects at 2 hours (A), 36 hours at LC_{50} (B), and 48 hours at LC_{90} (C). Injection point (arrows).

Discussion

The saliva of P. nigrispinus treated with protease inhibitors and degraded by proteinase K showed toxic activity for A. gemmatalis larvae, indicating that the compounds responsible for the prey death during P. nigrispinus feeding are not proteineous. The saliva of predatory stink bugs has been reported to contain enzymes required for extra-oral digestion (Terra and Ferreira 1994; Swart and Felgenhauer 2003; Azevedo et al. 2007). The action of these digestive enzymes in the saliva of predatory Hemiptera has been assumed to promote internal prey liquefaction for posterior ingestion (Cohen 1990; 1993; 1995; Mohaghegh et al. 2001; Eubanks et al. 2003); however, collagenase was the only digestive enzyme found in the salivary glands of P. nigrispinus (Fialho et al. 2012). In addition, proteins and peptides in the saliva that acts as venoms have been reported in the Heteroptera species to induce paralysis and death in prey. Belostomatidae and stink bugs Reduviidae contain a cocktail of proteins in the saliva, which play a role as a potent venom of immediate lethal action for vertebrates (Silva-Cardoso et al. 2010) and invertebrates (Sahayarajaj and Kanna 2009; Silva-Cardoso et al. 2010; Sahayaraj and Muthukumar 2011). However, these earlier studies reported that the action of proteins in prey death is restricted to the identification of protein profiles present in the saliva and their toxic effect on different prey, whereas our study showed that the toxic compounds in the P. nigrispinus saliva are not proteins or peptides.

The mortality of A. gemmatalis was high with the P. nigrispinus saliva extracted with the petroleum ether phase and with the aqueous+ether phase mixtures, which are low molecular weight substances such as aromatic compounds and hydrocarbons. The mortality of A. gemmatalis larvae injected with the salivary aqueous extract containing only proteins, obtained after extraction of other compounds with ether and enzyme treatment, was lower as compared to that with the compounds obtained after extraction with ether phase and for the mixture of the two extracts, which demonstrates that the venomous effect of P. nigrispinus saliva is not due to proteins and peptides, corroborating our the previous test results. The low molecular weight and short carbon chains venom components of insects have been reported to induce lethal and sublethal effects, such as Apis cerana Fabricius (Hymenoptera: Apidae) (Schmidt et al. 1997), Galerita lecontei Dejean (Coleoptera: Carabidae) (Rossini et al. 1997), and Polistes sulcifer Zimmermann (Hymenoptera: Vespidae) (Bruschini and Cervo 2011). However,

in these insects, the venom is produced by specialized venom glands other than the salivary glands.

Different concentrations of P. nigrispinus saliva extracted with ether showed toxic effect on A. gemmatalis larvae between 12 and 36 h of injection. The dose–response bioassay indicated toxicity for A. gemmatalis with increasing concentrations and 90% mortality. Increasing concentrations of substances isolated and injected into lepidopteran larvae, showed immediate toxic responses 12 h after application (Beard 1963; Boyd et al. 2002; Baek et al. 2011; Sahayaraj and Muthukumar 2011). The LC_{50} (2.04 µL) and LC_{90} (3.27 µL) of P. nigrispinus saliva indicates that low amounts of toxic substances present in the saliva kill A. gemmatalis. The salivary glands of Belostoma lutarium Stal, Lethocerus uhleri Montandon (Belostomatidae), Deraeocoris nebulosus Ulher, Deraeocoris nigritulus Ulher (Miridae), Zelus renardii Kolenati, and Rhynocoris marginatus Fabricius (Reduviidae) produce large volumes of saliva containing toxic substances with the potential to rapidly kill large invertebrates and vertebrates (Cohen 1993; Boyd et al. 2002; Boyd 2003; Swart et al. 2006).

In this study, three fractions of P. nigrispinus saliva demonstrated toxic activity against A. gemmatalis and two compounds were identified as N,N-dimethylaniline and 1,2,3-trithiepane. Injection of N,N-dimethylaniline (DMA) caused mortality of A. gemmatalis larvae (LC_{50} = 136.1 nL). DMA is an organic chemical compound that is a substituted derivative of aniline, consisting of a tertiary amine group and dimethylamine group attached to a phenyl group (Krieger 2010). DMA can modify enzymes via N-demethylation, N-oxidation, and N-hydroxylation as pathways for the formation of toxic compounds in mammalian cells (Gorrod and Goodherman 1981). DMA and xenobiotic compounds derived from N-demethylation, N-oxidation, and N-hydroxylation has been implicated in the degradation of enzymes and with genotoxic, teratogenic and carcinogenic activities (Hlavica and Kiinzel-Mulas 1993; Hover and Kulkarni 2000; Taupp et al. 2006). DMA has been reported to act as a phagostimulant in Anthonomus grandis Boheman (Coleoptera: Curculionidae) and as an insecticide solubilizer in Melophagus ovinus L. (Diptera: Hippoboscidae) by increasing the penetration rate through the body cuticle (Webb 1946; Hedin et al. 1968).

The 1,2,3-trithiepane is a organic chemical compound synthesized from 2-chloretil disulfide by reacting with sodium sulfide and heterocyclic closed chain (Windhager et al. 2007). The synthesis of 1,2,3-trithiepane in living organisms have been reported from the metabolism of proteins rich in sulfide and disulfide (Field and

Foster 1970), and found in species Allium (Alliaceae) and Brassica (Brassicaceae) (Kubec et al. 1997). However, this is the first report of the occurrence of 1,2,3-trithiepane in insects.

Overall, the present study characterized the toxic substances present in the saliva of P. nigrispinus that are responsible for paralysis and death of prey. The toxic substance was identified to be non-proteinaceous compounds—N,N-dimethylaniline and 1,2,3-trithiepane; this is the first report of these compounds in insect saliva. The findings raise the possibility of stink bug to produce a varied cocktail of toxins, so that the resulting venoms are highly efficient, as demonstrated by biochemical and toxicological tests. The presence of venom in the saliva is an evolutionary advantage for a predator, resulting in efficient capture and diversification of the prey. Overall, this research presents insights to the study of the complexity of the venom and its implications in the biology and ecology of the predator due to its importance in the control of forest and agricultural pests.

References

Abbott WS (1925) A method of computing the effectiveness of an insecticide. J Econ Entomol 18: 265–266.

Azevedo DO, Zanuncio JC, Zanuncio JS, Martins GF, Marques-Silva S, Sossai MF, Serrão JE (2007) Biochemical and morphological aspects of salivary glands of the predator Brontocoris tabidus (Heteroptera: Pentatomidae). Braz Arch Biol Techn 50: 469–477.

Bell HA, Down RE, Edwards JP, Gatehouse JA, Gatehouse AMR (2005) Digestive proteolytic activity in the gut and salivary glands of the predatory bug Podisus maculiventris (Heteroptera: Pentatomidae), effect of proteinase inhibitors. Eur J Entomol 102: 139–145.

Baek JH, Ji Y, Shin JS, Lee S, Lee SH (2011) Venom peptides from solitary hunting wasps induce feeding disorder in lepidopteran larvae. Peptides 32: 568–572.

Beard RL (1963) Insect toxins and venoms. Annu Rev Entomol 8: 1–18.

Blum SM (1978) Biochemical defenses of insects. In: Rockstein, M. (Ed.), Biochemistry of insects. Academic Press, New York, pp. 465–513.

Boyd DWJ (2003) Digestive enzymes and stylet morphology of Deraeocoris nigritulus (Uhler) (Hemiptera: Miridae) reflect adaptations for predatory habits. Ann Entomol Soc Am 96: 667–671.

Boyd DWJ, Cohen AC, Alverson DR (2002) Digestive enzymes and stylet morphology of Deraeocoris nebulosus (Hemiptera: Miridae), a predaceous plant bug. Ann Entomol Soc Am 95: 395–401.

Bruschini C, Cervo R (2011) Venom volatiles of the paper wasp social parasite Polistes sulcifer elicit intra-colonial aggression on the nest of the host species Polistes dominulus. Insectes Soc 58: 383–390.

Calvete JJ, Sanz L, Angulo Y, Lomonte B, Gutiérrez JM (2009) Venoms, venomics, antivenomics. FEBS Letters 583: 1736–1743.

Cohen AC (1990) Feeding adaptations of some predaceous Hemiptera. Ann Entomol Soc Am 83: 1215–1223.

Cohen AC (1993) Organization of digestion and preliminary characterization of salivary trypsin-like enzymes in a predaceous heteropteran, Zelus renardii. J Insect Physiol 39: 823–829.

Cohen AC (1995) Extra-oral digestion in predaceous terrestrial Arthropoda. Annu Rev Entomol 40: 85–103.

De Bortoli SA, Otuka AK, Vacari AM, Martins MIEG, Volpe HLX (2011) Comparative biology and production costs of Podisus nigrispinus (Hemiptera: Pentatomidae) when fed different types of prey. Biol Control 58: 127–132.

Escoubas P, Palma MF, Nakajima T (1995) A microinjection technique using Drosophila melanogaster for bioassay-guided isolation of neurotoxins in arthropod venoms. Toxicon 33: 1549–1555.

Eubanks MD, Styrsky JD, Denno RF, 2003. The evolution of omnivory in heteropteran insects. Ecology 84: 2549–2556.

Ferreira JAM, Zanuncio JC, Torres JB, Molina-Rugama AJ (2008) Predatory behaviour of Podisus nigrispinus (Heteroptera: Pentatomidae) on different densities of Anticarsia gemmatalis (Lepidoptera: Noctuidae) larvae. Biocontrol Sci Techn 18: 711–719.

Fialho MDCQ, Zanuncio JC, Neves CA, Ramalho FS, Serrão JE (2012) Prey digestion in the midgut of the predatory bug Podisus nigrispinus (Hemiptera: Pentatomidae). J Insect Physiol 58: 850–856.

Field L, Foster CH (1970) Biologically oriented organic sulfur chemistry. IV. Synthesis and properties of 1,2,5-trithiepane, a model for study of sulfide and disulfide moieties in proximity. J Org Chem 35: 749–752.

Finney DJ (1971) Probit analysis. Cambridge University, London, 333 pp.

Gorrod JW, Gooderham NJ (1981) The in vitro metabolism of N,N-dimethylaniline by guinea pig and rabbit tissue preparations. Eur J Drug Metab Pharmacokinet 6: 195–206.

Greene GL, Leppla NC, Dickerson WA (1976) Velvetbean caterpillar: a rearing procedure and artificial medium. J Econ Entomol 69: 487–488.

Hedin PA, Miles LR, Thompson AC, Minyard JP (1968) Constituents of the cotton bud. Formulation of a boll weevil feeding stimulant mixture. J Agr Food Chem 16: 505–513.

Hlavica P, Kiinzel-Mulas U (1993) Metabolic N-oxide formation by rabbit-liver microsomal cytochrome P-4502B4: involvement of superoxide in the NADPH-dependent N-oxygenation of N,N dimethylaniline. Biochim Biophys Acta 1158: 83–90.

Hover CG, Kulkarni AP (2000) Lipoxygenase-mediated hydrogen peroxide-dependent N-demethylation of N,N-dimethylaniline and related compounds. Chem Biol Interact 124: 191–203.

Krieger R (2010) Hayes' handbook of pesticide toxicology 3th. Academic Press, San Diego, USA.

Kubec R, Velísek J, Dolezal M, Kubelka V (1997) Sulfurcontaining volatiles arising by thermal degradation of alliin and deoxyalliin. J Agric Food Chem 45: 3580–3585.

Lemos WP, Medeiros RS, Ramalho FS, Zanuncio JC (2001) Effects of plant feeding on the development, survival and reproduction of Podisus nigrispinus (Dallas) (Heteroptera: Pentatomidae). Int J Pest Manage 47: 89–93.

Lemos WP, Zanuncio JC, Serrão JE (2005) Attack behavior of Podisus rostralis (Heteroptera, Pentatomidae) adults on Caterpillars of Bombyx mori (Lepidoptera, Bombycidae). Braz Arch Biol Techn 48: 975–981.

Lemos WP, Ramalho FS, Serrão JE, Zanuncio JC (2005) Morphology of female reproductive tract of the predator Podisus nigrispinus (Dallas) (Heteroptera: Pentatomidae) fed on different diets. Braz Arch Biol Techn 48: 129–138.

Martínez LC, Fialho MCQ, Zanuncio JC, Serrão JE (2014) Ultrastructure and cytochemistry of salivar glands of the predator Podisus nigrispinus (Hemiptera: Pentatomidae). Protoplasma 251: 535–543.

Medeiros RS, Ramalho FS, Lemos WP, Zanuncio JC (2000) Age-dependent fecundity and life-fertility tables for Podisus nigrispinus (Dallas) (Heteroptera: Pentatomidae). J Appl Entomol 124: 319–324.

Medeiros RS, Ramalho FS, Zanuncio JC, Serrão JE (2003) Effect of temperature on life table parameters of Podisus nigrispinus (Het., Pentatomidae) fed with Alabama argillacea (Lep., Noctuidae) larvae. J Appl Entomol 127: 209–213.

Mohaghegh J, De Clercq P, Tirry L (2001) Functional response of the predators Podisus maculiventris (Say) and Podisus nigrispinus (Dallas) (Heteroptera: Pentatomidae) to the beet armyworm, Spodoptera exigua (Hübner) (Lepidoptera: Noctuidae): effect of temperature. J Appl Entomol 125: 131–134.

Neves RCS, Torres JB, Zanuncio JC (2010) Production and storage of mealworm beetle as prey for predatory stinkbug. Biocontrol Sci Techn 20: 1013–1025.

Parkinson N, Smith I, Audsley N, Edwards JP (2002) Purification of pimplin, a paralytic heterodimeric polypeptide from venom of the parasitoid wasp Pimpla hypochondriaca, and cloning of the cDNA encoding one of the subunits. Insect Biochem Mol Biol 32: 1769–1773.

Ramos AT, Gonçalves LRC, Ribeiro OG, Rocha Campos AC, Sant'Anna OA (2004) Effects of Lonomia obliqua (Lepidoptera, Saturniidae) toxin on clotting, inflammatory and antibody responsiveness in genetically selected lines of mice. Toxicon 43: 761–768.

Rossini C, Attygalle AB, Gonzalez A, Smedley SR, Eisner M, Meinwald J, Eisner T (1997) Defensive production of formic acid (80-percent) by a carabid beetle (Galerita lecontei). P Natl Acad Sci Usa 94: 6792–6797.

Sahayaraj K, Muthukumar S (2011) Zootoxic effects of reduviid Rhynocoris marginatus (Fab.) (Hemiptera: Reduviidae) venomous saliva on Spodoptera litura (Fab.). Toxicon 58: 415–425.

SAS (2002) The SAS System for Windows, release 9.0. SAS Institute, Cary, N.C.

Schmidt JO (1982) Biochemistry of insect venoms. Annu Rev Entomol 27: 339–368.

Schmidt JO, Morgan ED, Oldham NJ, Do Nascimento RR, Dani FR (1997) (Z)-ll-Eicosen-l-ol, a major component of Apis cerana venom. J Chem Ecol 23: 1929–1939.

Silva-Cardoso L, Caccin P, Magnabosco A, Patrón M, Targino M, Fuly A, Oliveira GA, Pereira MH, Carmo MGT, Souza AS, Silva-Neto MAC, Montecucco C, Atella GC (2010) Paralytic activity of lysophosphatidylcholine from saliva of the waterbug Belostoma anurum. J Exp Biol 213: 3305–3310.

Swart CC, Deaton LE, Felgenhauer BE (2006) The salivary gland and salivary enzymes of the giant waterbugs (Heteroptera: Belostomatidae). Comp Biochem Physiol A 145: 114–122.

Taupp M, Heckel F, Harmsen D, Schreier P (2006) Direct trapping of formaldehyde formed via oxidative N-demethylation of N,N-dialkylarylamines by Bacillus megaterium using cysteamine derivatization. J Microbiol Methods 67: 357–362.

Terra WR, Ferreira C (1994) Insect digestive enzymes: properties, compartmentalization and function. Comp Biochem Physiol B 109: 1–62.

Webb JE (1946) Penetration of Derris through the spiracles and cuticle of Melophagus ovinus L. B Entomol Res 36: 15–22.

Windhager J, Rudolph M, Bräutigam S, Görls H, Weigand W (2007) Reactions of 1,2,4-trithiolane, 1,2,5-trithiepane, 1,2,5-trithiocane and 1,2,6-trithionane with nonacarbonyldiiron: structural determination and electrochemical investigation. Eur J Inorg Chem 2007: 2748–2760.

XLSTAT (2004) XLSTAT for Excel. Addinsoft, U.K

Zhang Z, Ye GY, Cai J, Hu C (2005) Comparative venom toxicity between Pteromalus puparum and Nasonia vitripennis (Hymenoptera: Pteromalidae) toward the hemocytes of their natural hosts, nontarget insects and cultured insect cells. Toxicon 46: 337–349.

FINAL CONSIDERATIONS

The salivary system of P. nigrispinus consists of a pair of principal salivary glands and accessory salivary with similar anatomy to that described for other predatory Hemiptera. The principal salivary glands of P. nigrispinus are bilobed with a short anterior and a long posterior lobe, whereas the accessory glands are tubular.

The cytochemical differences in the quantity and distribution of carbohydrates, acid phosphatase, lipids, and proteins found in the secretory cells of the anterior and posterior lobes of the principal and accessory salivary glands of P. nigrispinus reveal that different substances in different quantities can be produced by these glands. This indicates that the cellular apparatus of salivary glands for this predator is involved in the secretion of substances other than digestive enzymes.

Podisus nigrispinus saliva exposed to protease inhibitors and with its proteins/peptides degraded by proteinase K demonstrated toxic activity against A. gemmatalis larvae, indicating that the compounds responsible for the death of the prey during feeding of the predator are not proteineous. Different concentrations of ether-extracted predator saliva showed toxic effects on A. gemmatalis between 12 and 36 h after injection. This result supports the hypothesis that non-proteineous substances produced in the salivary glands of the predator acts as venom to kill the prey.

In this study, three fractions showed toxic activity against A. gemmatalis, and two compounds were identified as N,N-dimethylaniline and 1,2,3-trithiepane. Injection of commercial N,N-dimethylaniline confirmed the mortality of A. gemmatalis larvae. The two non-proteineous compounds discovered in this study raises the possibility that this stink bug may produce a varied cocktail of toxins, resulting in the production of a highly toxic venom, as demonstrated by biochemical and toxicological analyzes.

In summary, this study evaluated the ultrastructure and function of the salivary glands of P. nigrispinus. The knowledge of ultrastructural and cytochemical features of the salivary glands and the presence of toxic compounds in the saliva of this insect enabled understanding the digestive functions and the mechanism involved in causing death of the prey. This is the most detailed study of prey mortality caused by P. nigrispinus, based on the findings of venom in the saliva. The identification of P. nigrispinus venom has future perspectives to the comprehension of the mechanism of action and direct effects on prey biochemistry, physiology, and immunology. Therefore,

the implications of these findings may have several applications in toxicology, biochemistry, and physiology of insects as well as in understanding the predator–prey interactions better for the development of efficient pharmacological and insecticides agents.