UNIVERSIDADE FEDERAL DO PAMPA

ALLAN P. LEAL

Atividade entomotóxica do veneno do sapo *Rhinella icterica* (Spix, 1824) em baratas da espécie *Nauphoeta cinerea*.

São Gabriel

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Dissertação apresentada ao programa de Pós-graduação *stricto sensu* em Ciências Biológicas da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Mestre em Ciências Biológicas.

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São Gabriel

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Dedico esta dissertação aos meus pais.

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“As grandes ideias surgem da observação O teu trabalho vai preencher uma grande parte da tua vida, e a única maneira de ficares realmente satisfeito é fazeres o que tu acreditas ser um grande trabalho. E a única maneira de fazeres um excelente trabalho é amares o que fazes. Se ainda não encontraste, continua à procura. Não te acomodes. Tal como acontece com todos os assuntos do coração, saberás quando o encontrares.”.

Steve Jobs

RESUMO

Venenos animais são importantes fontes de toxinas estruturalmente diversas e com alto grau de seletividade. O sapo *Rhinella icterica* produz, por meio de glândulas especializadas, uma secreção venenosa rica em compostos de diferentes classes químicas, as quais apresentam uma série de atividades farmacológicas com potencial biotecnológico. Nesse sentido a secreção tóxica de *R. icterica* (RITS) foi ensaiada frente a sua atividade entomotóxica em baratas da espécie *Nauphoeta cinerea*. Primeiramente foram feitos protocolos de atividade locomotora, nos quais verificaram-se que RITS induz alterações no comportamento exploratório dos animais, caracterizadas pela redução significativa da distância total percorrida (38 ± 14%) e pelo aumento de 90 ± 6% no número de episódios de imobilidade ($n=33$, $p<0.05$, respectivamente). RITS (4, 8 e 16µg/200µl) também induziram cardiotoxicidade demonstrada pela redução de até 40% ($n=4$, $p<0.05$) da frequência cardíaca das baratas, de forma irreversível, em 30 min de registros. Em preparação neuromuscular *in vivo*, RITS (20, 50 e100µg/g) produziu inibição tempo-dependente, da resposta contrátil, em todas as doses ensaiadas, atingindo o bloqueio total para a dose de 20µg/g ($n=6$, $p<0.05$, respectivamente). Além disso, RITS 10µg/g, alterou o comportamento de *grooming* de perna dos animais, induzindo aumento de 128 ± 10%, ($n=29$, $p<0.05$). Quando RITS foi ensaiado em animais previamente tratados com fentolamina (0.1 µg/g), houve redução de 90 % do efeito do veneno sobre o *grooming* de perna, indicando que a atividade neurotóxica do composto pode estar relacionada às vias octopaminérgicas. Os registros eletrofisiológicos de potenciais de ação sensoriais compostos espontâneos (SNCAP) em perna de baratas, demonstraram que o tratamento com RITS 20µg/g aumenta significativamente o número de potenciais, bem como o tempo de surgimento e a duração desses eventos ($n=6$, $p<0.05$). Em conclusão os ensaios realizados nesse trabalho demonstraram que RITS induz atividade entomotóxica em *Nauphoeta cinerea*, caracterizada pela modulação do sistema nervoso central e periférico dos animais. O mecanismo de toxicidade parece estar relacionado a uma atividade direta das toxinas presentes na secreção venenosa sobre as vias octopaminérgicas dos insetos. Ensaios futuros de caracterização bioquímica e farmacológica poderão auxiliar na identificação dos

compostos tóxicos presentes na secreção, bem como contribuir para a elucidação das interações biológicas, reforçando o potencial biotecnológico desse composto natural.

Palavras-chave: Venenos animais, anuros, potencial inseticida, sistema nervoso, neurotoxicidade central e periférica, transmissão octopaminérgica.

ABSTRACT

Animal poisons are important sources of structurally diverse toxins with a high degree of selectivity. The *Rhinella icterica* toad produces, through specialized glands, a poisonous secretion rich in compounds of different chemical classes, which present a series of pharmacological activities with biotechnological potential. In this sense the toxic secretion of *R. icterica* (RITS) was tested against its entomotoxic activity in cockroaches of the Nauphoeta cinerea species. Initially, locomotor activity protocols were established, in which RITS induced alterations in the exploratory behavior of the animals, characterized by a significant reduction in the total distance traveled (38 ± 14%) and by a 90 ± 6% increase in the number of episodes of immobility (n = 33, p <0.05, respectively). RITS (4, 8 and 16µg / 200µl) also induced cardiotoxicity demonstrated by the reduction of up to 40% (n = 4, p <0.05) of cockroaches heart rate, irreversibly, in 30 min of records. In neuromuscular preparation in vivo, RITS (20, 50 and 100 µg / g) produced time-dependent inhibition of the contractile response for all doses tested, achieving total blockade at the of 20 µg / g dose (n = 6, p <0.05 , respectively). In addition, RITS 10µg / g, altered the leg grooming behavior of the animals, inducing increase of 128 ± 10%, (n = 29, p <0.05). When RITS was assayed in animals previously treated with phentolamine (0.1 µg / g), there was a 90% reduction in the effect of venom on leg grooming, indicating that the neurotoxic activity of the compound may be related to octopaminergic pathways. The electrophysiological records of spontaneous compound sensory potentials (SNCAP) in the legs of cockroaches demonstrated that treatment with RITS 20µg / g significantly increased the number of potentials as well as the time of onset and duration of these events (n = 6 , p <0.05). In conclusion the tests performed in this work demonstrated that RITS induces entomotoxic activity in Nauphoeta cinerea, characterized by central and peripheral nervous system modulation. The mechanism of toxicity seems to be related to a direct activity of the toxins present in the poisonous secretion on the octopaminergic pathways of the insects. Future trials of biochemical and pharmacological characterization may help identify the toxic compounds present in the secretion, as well as contribute to the elucidation of biological interactions, reinforcing the biotechnological potential of this natural compound.

Keywords: Animal poisons, Anuran, insecticide potential, nervous system, central and peripheral neurotoxicity, octopaminergic transmission.

LISTA DE FIGURAS

Figura 1. Em **A** está representado um exemplar de Rhinella icterica. Em **B** a distribuição da espécie.. 26
Figura 2. Ilustração da barata Nauphoeta cinerea.....27
Figura 3. Ilustração do sistema nervoso central de baratas e suas subdivisões.. 29
Figura 4. Ilustação dos diferentes padrões de grooming. Em (A) representado o grooming de perna. Em (B) está representado o gromming de antena.....31

LISTA DE ABREVIATURAS

AMP_c – Adenosina 3',5'-monofosfato cíclico

AO – Octopamina

DA – Dopamina

DAG – Diacilglicerol

DUM – Dorsal unpaired median neurons

GABA – Gamma-aminobutyric acid

GLU – Glutamate

IP3– Inositol 1,4,5-trisfosfato

PKA – Proteína quinase A

PKC – Proteína quinase C

RITS – *Rhinella icterica* toxic secretion

SISBIO – Brazilian Biodiversity Information and Authorization System

SNC – Sistema nervoso central

SNCAP – Spontaneous neural compound action potentials

SNP – Sistema nervoso periférico

TSE-LF – Lethal factor

VUM – ventral unpaired median neurons.

APRESENTAÇÃO

Na seção **INTRODUÇÂO (1)** é apresentado ao leitor uma breve revisão da literatura sobre o tema abordado na presente dissertação. A metodologia, bem como resultados obtidos neste estudo serão apresentados sob forma de manuscrito, descrito na seção **MANUSCRITO (2)** o qual foi previamente submetido à revista Toxicology (Elsevier). No manuscrito constam as seções: Introdução, materiais e métodos, resultados, discussão e conclusão. O item **CONSIDERAÇÕES FINAIS (3)**, o qual se encontra no final desta dissertação, oferece ao leitor uma breve revisão com comentários sobre a dissertação em geral, assim como as conclusões a respeito desse estudo. A seção de **REFERÊNCIAS (4)** contém somente as citações presentes no corpo da dissertação, com exceção do manuscrito. O **ANEXO (5)** traz as principais publicações do autor, no período de vigência do curso.

SUMÁRIO

1 INTRODUÇÃO 25

1.1 VENENOS ANIMAIS E SEU POTENCIAL BIOTECNOLÓGICO 25

***1.2 RHINELLA ICTERICA (SPIX, 1824)* 25**

1.3 CARACTERÍSTICAS, QUÍMICAS, BIOLÓGICAS E FARMACOLÓGICAS DO VENENO 26

1.4 *NAUPHOETA CINEREA* COMO MODELO EXPERIMENTAL 27

1.5 O SISTEMA NERVOSO DE INSETOS E COMPORTAMENTO 28

1.5.1 Aminas biogênicas e a orquestração do comportamento em insetos 29

1.5.2 Sistema cardiovascular de baratas 31

3 MANUSCRITO SUBMETIDO AO PERIÓDICO PESTICIDE BIOCHEMISTRY AND PHYSIOLOGY (ELSEVIER). EM 12/02/2018 35

4 CONSIDERAÇÕES FINAIS 58

5 REFERÊNCIAS BIBLIOGRÁFICAS DA DISSERTAÇÃO 59

5.1 ARTIGO PUBLICADO DURANTE O PERÍODO DE VIGÊNCIA DO MESTRADO 60

5.2 RESUMOS PUBLICADOS EM ANAIS DE CONGRESSOS DURANTE O PERÍODO DE VIGÊNCIA DO MESTRADO 60

1 INTRODUÇÃO

1.1 Venenos animais e seu potencial biotecnológico

Durante milhões de anos o processo evolutivo vem atuando e modificando os seres vivos para uma melhor adaptação ao meio. Dessa forma a síntese de secreções tóxicas por animais venenosos e peçonhentos têm, ao longo de gerações, contribuído para a evolução dessas espécies, favorecendo a sua seleção em detrimento de outras. Assim, animais venenosos tornaram-se de grande interesse para a ciência, não só pelo óbvio contexto clínico, mas por produzem uma série de toxinas bioativas, estruturalmente diversas, com alto grau de seletividade e consequentemente de grande interesse biotecnológico (Da Silva *et al.*, 2014).

1.2 Rhinella icterica (SPIX, 1824)

A espécie *R. icterica* (Figura 1 A) foi descrita em 1824, inicialmente como *Bufo icterica*, posteriormente foi denominada de *Chaunus ictericae*, atualmente, *Rhinella* icterica. Estes animais são anfíbios de grande porte, o macho adulto possui tamanho médio de 100-166 mm, enquanto a fêmea adulta, de maior porte, mede entre 135-190 mm. Possuem grandes glândulas paratóides localizadas próximas à cabeça, uma característica de outros anfíbios do gênero *Rhinella.* Indivíduos fêmeas e juvenis possuem a região dorsal amarelada com padrão regular de manchas pretas e uma faixa clara da linha média; já nos machos a cor do dorso é bastante peculiar, possuindo padrões amarelo esverdeado brilhante, com apenas algumas manchas negras (Amphibia Web. 2017). Ocorrem na região central, sudeste e sul do Brasil, incluindo o Bioma Pampa, no nordeste da Argentina e no Paraguai oriental (Figura 1 B), em altitudes que variam de 0-1200 m. São encontrados em uma grande diversidade de hábitats, desde florestas abertas a áreas como o Cerrado (Débora Silvano, 2010).

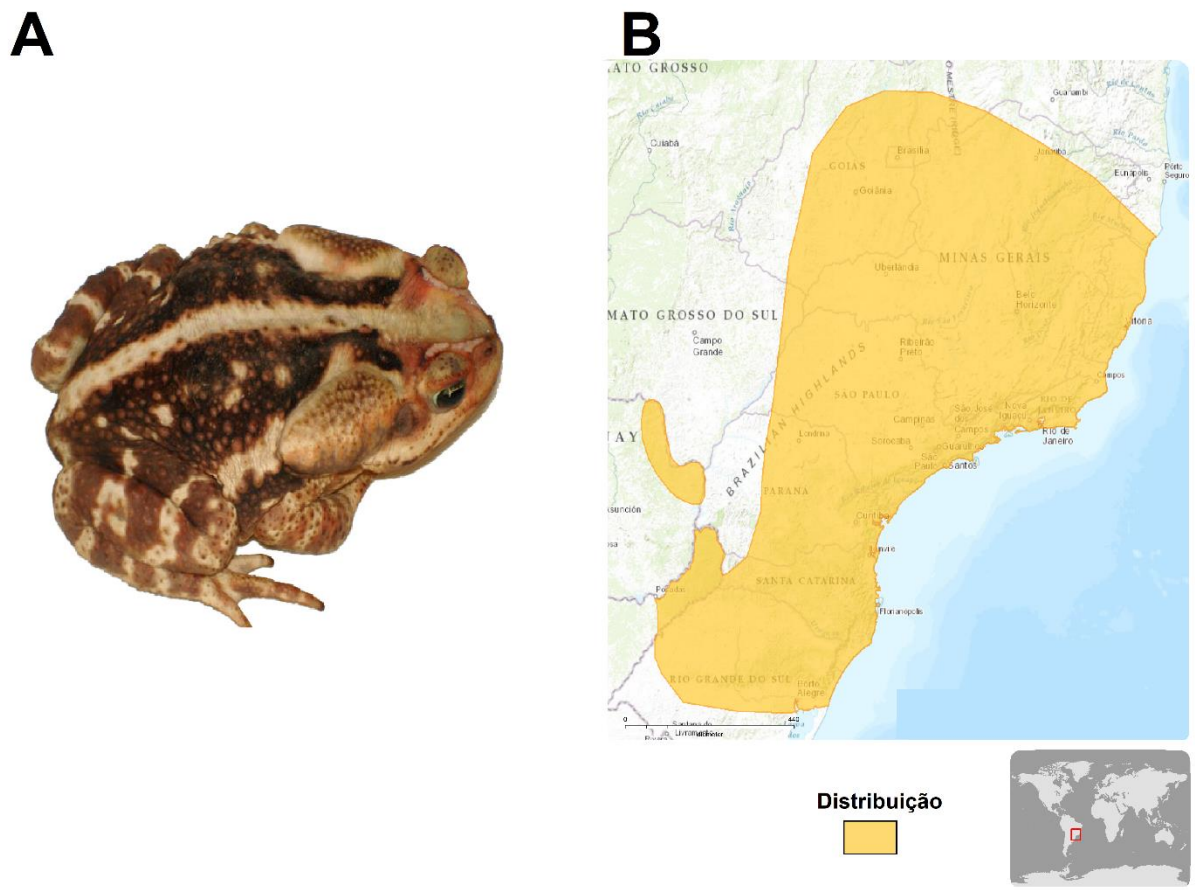

Figura 1. Em **A** está representado um exemplar de *Rhinella icterica*. Registrado por Cháristo André Dal Belo. Em **B** a distribuição da espécie. Fonte: (Débora Silvano, 2010.) alterado pelo autor.

1.3 Características, químicas, biológicas e farmacológicas do veneno

Compostos encontrados em venenos de sapos podem ser divididos basicamente em dois grupos distintos: Aminas biogênicas e derivados esteroides (Sakate e Oliveira, 2000). As aminas biogênicas incluem adrenalina, noradrenalina, bufoteninas, dihidrobufoteninas e bufotioninas. As bufoteninas, dihidrobufoteninas e bufotioninas são responsáveis pelos efeitos alucinógenos no sistema nervoso central e aumentam a liberação de neurotransmissores no sistema nervoso periférico em vertebrados (Sonne *et al.*, 2008; Rostelato-Ferreira *et al.*, 2011). Já os derivados esteroides incluem colesterol, ergosterol, bufotoxinas e bufadienolídeos. Os bufodienolídeos e bufotoxinas são responsáveis dentre outros efeitos, pela inibição da Na+,K+-ATPase (Cunha-Filho *et al.*, 2010; Rang *et al.*, 2015; Oliveira *et al.*, 2017).

1.4 *Nauphoeta cinerea* como modelo experimental

Baratas são conhecidas por serem animais muito resistentes, possuindo a capacidade de habitar uma grande variedade de ambientes nas mais vastas regiões do planeta, sendo bem adaptadas à vida urbana. Existem cerca de 4000 espécies de baratas compreendendo a subordem Dyctioptera, sendo muito diversas entre si (Huber, Masler e Rao, 1990). As baratas estão inseridas em um grupo com cinco famílias, as quais pertencem às duas maiores linhagens filogenéticas (Blattoidea e Blaberoidea), separadas de acordo com as estratégias reprodutivas e morfologia (Huber et al., 1990). Dessa forma, o corpo das baratas possui formato ovular e deprimido, e seu tamanho pode variar de alguns milímetros até quase dez centímetros. A espécie *Nauphoeta cinerea* (Figura 2) descrita por Oliver em 1789 é considerada por muitos como espécie praga. Essa espécie vem ganhando espaço como modelo experimental no estudo toxinológico, devido às analogias em seu sistema nervoso com modelos vertebrados já consagrados (Stankiewicz *et al.*, 2012).

Embora anatomicamente diferente de qualquer outro modelo experimental, princípios biofísicos, bem como a presença de sistemas de neurotransmissão comums entre vertebrados e invertebrados, colocam as baratas e, consequentemente a *N. cinerea*, como um modelo experimental alternativo (Stankiewicz *et al.*, 2012). Fatores como fácil manutenção, baixo custo e ciclo de vida relativamente curto contribuem para a escolha desses insetos como uma opção bastante viável na escolha de um modelo experimental.

Figura 2. Ilustração da barata *Nauphoeta cinerea*. Fonte: Bruna Borges.

1.5 O Sistema Nervoso de Insetos e comportamento

Princípios básicos envolvendo a neurotransmissão em insetos, bem como modelos vertebrados seguem padrões bastante semelhantes. Sinapses químicas geram sinais elétricos, denominados potenciais de ação, esses impulsos são distribuídos pelo sistema nervoso central (SNC) de forma ordenada e localizada permitindo a liberação do neurotransmissor em local específico. O controle dessa complexa função inicia-se no nível dos canais iônicos e é mantido, posteriormente, pelo efeito desses canais sobre a atividade de redes neurais altamente organizadas (Cavalheiro *et al.*, 1991).

Todo comportamento animal é derivado da atividade orquestrada por neurônios no SNC. Dessa forma, cada neurônio pode ser recrutado por compostos químicos denominados neurotransmissores, neuromoduladores e neurormônios. Neurotransmissores são liberados nas sinapses, onde eles agem diretamente em receptor específico e induzem uma resposta rápida, modulada por canais iônicos e durante um curto período de tempo (ms), levando à uma rápida mudança na atividade elétrica dos neurônios. Já os neuromoduladores são liberados em uma área maior, assim como os neurotransmissores, esses também possuem alvos específicos, muitas vezes, se ligam a receptores acoplados a proteínas G. Esses receptores são conhecidos por iniciarem uma cascata de sinalização celular, que por fim poderá levar, de forma mais lenta, a mudanças no potencial elétrico do neurônio. Além de induzirem a modulação sobre canais iônicos, neuromoduladores são capazes de alterar a síntese de proteínas, de atividade enzimática e regulação da expressão gênica (Libersat e Pflueger, 2004). Ao contrário dos neurotransmissores e neuromoduladores, neurormônios encontram-se dispersos por toda a hemolinfa exercendo uma ação completa no organismo (Libersat, 2003). Dentre os vários neurotransmissores presentes no SNC de baratas, os principais moduladores desse sistema são acetilcolina, dopamina, octopamina, 5-hidroxitriptamina e histamina (Taylor e Newburgh, 1979; Osborne, 1996).

O SNC de baratas é composto basicamente de estruturas morfológicas distintas: cérebro, gânglios e seus conectivos (Figura 3). Gânglios são agregados de neurônios dispostos em toda a extensão corporal do inseto, conectando-se ao SNP, o qual inerva os membros e o sistema estomatogástrico (Osborne, 1996). Conectivos possuem a função fundamental de permitir a comunicação entre gânglios e o SNP, permitindo a passagem de impulsos nervosos. Estruturalmente o SNC é composto por onze gânglios: dois presentes na cabeça (supra- e subesofágico), três no tórax (o pró-, meso e metatorácico) e por fim os seis últimos localizados no abdômen (Fourtner e Kaars, 1990).

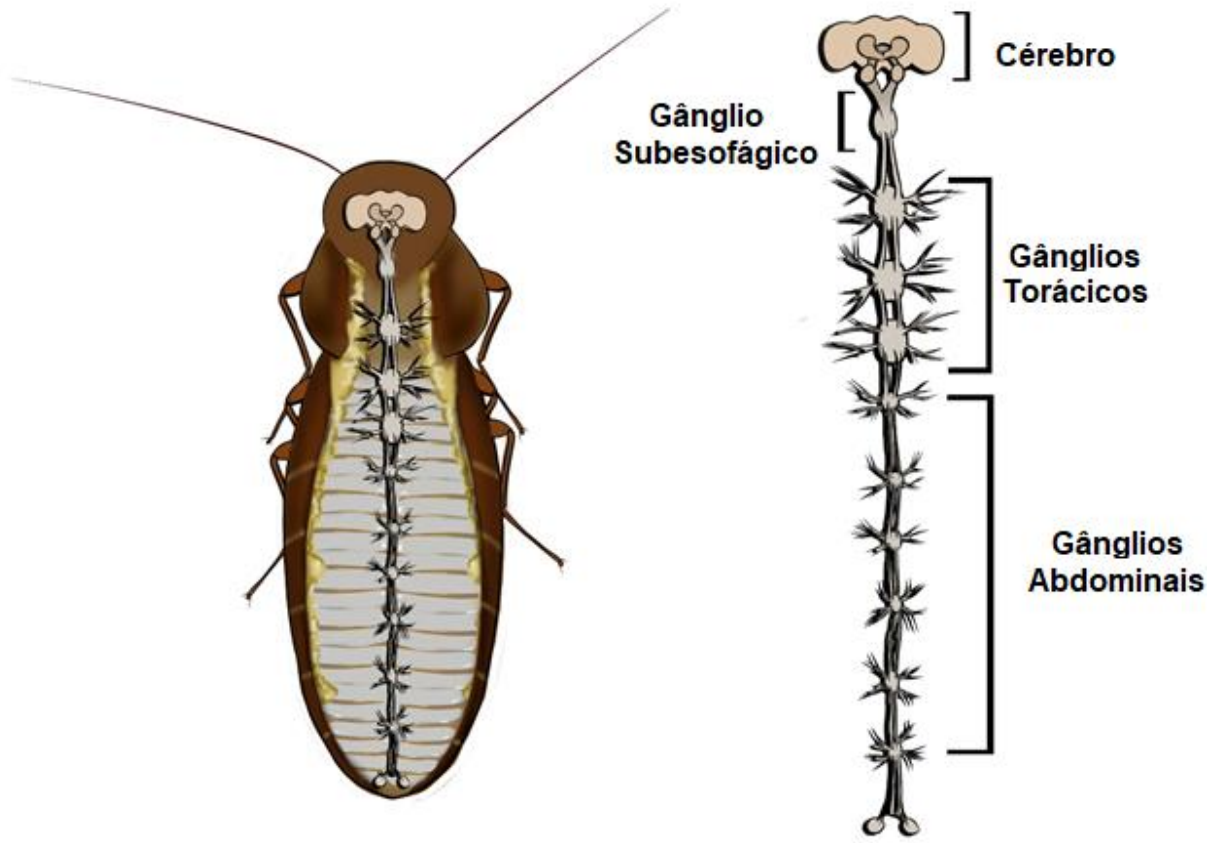

Figura 3. Ilustração do sistema nervoso central de baratas e suas subdivisões. Fonte: Bruna Borges.

1.5.1 Aminas biogênicas e a orquestração do comportamento em insetos

As monoaminas são neuromoduladores conhecidos por interagirem em uma série de comportamentos bem definidos, quer sejam eles em modelos de vertebrados ou invertebrados. Dentre essa classe de compostos, os mais conhecidos por modularem o sistema nervoso são: octopamina, dopamina e a noradrenalina (derivados da tirosina), e a serotonina, obtida a partir do triptofano (Libersat e Pflueger, 2004). Monoaminas e seus receptores sinápticos são considerados uma classe de moléculas com funções altamente conservadas na transmissão sináptica em todos os animais (Walker *et al.*, 1996).

A octopamina (OA) atua como neuromodulador, neurotransmissor e neurormônio no sistema nervoso de insetos, influenciando em uma série de efeitos fisiológicos. Como neuromodulador, regula a dessensibilização de impulsos sensoriais, excitação, iniciação e manutenção de um grande número de comportamentos rítmicos e complexos como aprendizado e memória (Farooqui, 2007). Como neurotransmissor essa monoamina é capaz de regular a atividade endócrina de glândulas. Quando a octopamina age como neurormônio,

induz a mobilização de gorduras e carboidratos, auxiliando na manutenção do metabolismo energético (Farooqui, 2007).

Os receptores octopaminérgicos pertencem à superfamília dos receptores acoplados à proteína G, os quais compartilham o motivo estrutural de sete domínios transmembrana. A ativação via ligante dessas proteínas específicas leva à produção de diferentes segundos mensageiros (Farooqui, 2007). Os segundos mensageiros gerados incluem a adenosina 3',5'-monofosfato cíclico (AMP cíclico), cálcio, diacilglicerol (DAG) e inositol 1,4,5-trisfosfato (IP3). O AMP cíclico ativa a proteína quinase A (PKA), enquanto que cálcio e DAG ativam a proteína quinase C (PKC) e trifosfato inositol (IP3) mobiliza estoques intracelulares de cálcio (Farooqui, 2007). A geração desses segundos mensageiros, mediada por octopamina, está associada às mudanças nas respostas celulares que afetam o comportamento dos insetos (Farooqui, 2007).

Hoyle e Barker (1975) demonstraram que a ativação dos DUM (do inglês, *dorsal unpaired median neurons*), pela octopamina, induzia a contração muscular de *Locusta migratoria*. Esse efeito fisiológico podia ser mimetizado pela aplicação de baixas concentrações de octopamina e altas concentrações de dopamina e noradrenalina diretamente na hemolinfa do inseto.

A dopamina (DA), uma das clássicas catecolaminas utilizadas como neurotransmissor em mamíferos desempenha papel crucial na neurotransmissão central e periférica em insetos. Essa monoamina possui importante função na regulação do comportamento motor em invertebrados (Weisel-Eichler *et al.*, 1999; Libersat e Pflueger, 2004). O comportamento de *grooming* (Figura 4) está relacionado à limpeza da superfície do corpo do inseto, sinalização social, comportamento de corte, podendo interferir até mesmo no deslocamento do inseto (Weisel-Eichler *et al.*, 1999; Libersat e Pflueger, 2004). Estudos demonstram a relação do sistema dopaminérgico com a modulação positiva do comportamento de *grooming* de antena bem como o sistema octopaminérgico com o aumento no tempo do *grooming* de perna (Weisel-Eichler *et al.*, 1999; Libersat e Pflueger, 2004; Carrazoni *et al.*, 2016).

Figura 4. Ilustação dos diferentes padrões de *grooming*. Em (A) representado o *grooming* de perna. Em (B) está representado o *gromming* de antena. Fonte: Bruna Borges.

1.5.2 Sistema cardiovascular de baratas

O coração das baratas possui doze câmaras interligadas onde a hemolinfa é bombeada através pares de aberturas, percorrendo todo o sistema circulatório, o qual caracteriza-se por ser aberto (Cornwell, 1968). As pulsações do coração desses insetos seguem três fazes: sístole (contração), diástole (relaxamento) e diástase (período de repouso) (Collins e Miller, 1977).

Estudos recentes demonstram que o coração das baratas é modulado principalmente pela ação da via octopaminérgica e colinérgica. Para ambos os casos o efeito sobre o ritmo cardíaco demonstra ser do tipo concentração-dependente e também espécie-dependente. (Papaefthimiou e Theophilidis (2011)) demonstraram que a octopamina exerce um efeito bifáscio sobre a frequência cardíaca de insetos já que, em altas concentrações, é capaz de aumentar a frequência cardíaca, enquanto em baixas concentrações diminui a frequência cardíaca.

2 OBJETIVO

Avaliar a atividade do veneno do *sapo Rhinella icterica* quanto ao potencial entomotóxico em baratas da espécie *Nauphoeta cinerea*.

2.1 OBJETIVOS ESPECÍFICOS

- Determinar os mecanismos de entomotoxicidade central e periférica do veneno de *Rhinella icterica (RITS)* por meio de ensaios comportamentais de locomoção em *N. cinerea*;
- Investigar o efeito de RITS sobre a freqüência cardíaca de baratas da espécie *N. cinerea;*
- Determinar o efeito do RITS em preparação neuromuscular *in vivo* de baratas da espécie *N. cinerea*;
- Determinar o mecanismo de neurotoxicidade central induzida pelo veneno de *R. icterica* por meio de registros da atividade de *grooming* em baratas da espécie *N. cinerea*;
- Avaliar o efeito de RITS em preparações *in situ* de pernas de *N. cinerea* para registros eletrofisiológicos dos potenciais de ação compostos neurais espontâneos;

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Entomotoxic activity of *Rhinella icterica* (Spix, 1824) toad skin secretion in *Nauphoeta cinerea* cockroaches: an octopamine-like modulation.

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Highlights

- The entomotoxicity of *Rhinella icterica* toxic secretion was investigated
- Its mechanism relies on the interplay among, octopaminergic and electrophysiological alterations pathways
- *Rhinella icterica* toxic secretion induces profound behavior alterations culminating in irreversible neuromuscular blockade.

ABSTRACT

Rhinella icterica is a poisonous animal whose toxic secretion has never been studied against entomotoxic potential. Sublethal doses of *Rhinella icterica* toxic secretion (RITS) was assayed in *Nauphoeta cinerea* cockroaches, in order to understand the physiological and behavioral parameters, over the insect central and peripheral nervous system. RITS (10 µg/g) injections induced behavioral impairment as evidenced by a significant decrease (38 ± 14%) in the distance traveled (p<0.05), followed by an increase (90 ± 6%) of immobile episodes (p<0.001, n=33, respectively). In cockroaches semi-isolated heart preparations, RITS (16 µg/200 µl) induced a significant irreversible dose-dependent negative chronotropism reaching ~ 40% decrease in heart rate in 20 min incubation. In *in vivo* cockroach neuromuscular

preparations, RITS (20, 50 and 100 µg/g of animal weight) induced a time-dependent inhibition of twitch tension that was complete for 20 µg/g, in 120 min recordings. RITS (10 µg/g) also induced a significant increase in the insect leg grooming activity (128 ± 10%, n=29, $p<0.01$), but not in the antennae counterparts. The RITS increase in leg grooming activity was prevented in 90% by the pretreatment of cockroaches with phentolamine (0.1 µg/g). The electrophysiological recordings of spontaneous neural compound action potentials showed that RITS (20 µg/g) induced a significant increase in the number of events, as well as in the rise time and duration of the potentials. In conclusion, RITS showed to be entomotoxic, being the neuromuscular failure and cardiotoxic activity the main deleterious effects. The disturbance of the cockroaches behavior together with the electrophysiological alterations, may unveil the presence of some toxic components present in the poison with inherent biotechnological potentials.

Key words: Anuran secretion, entomotoxicity, insect behavior, neuromuscular blockade, octopaminergic modulation.

1. Introduction

Poisonous animals are present worldwide and have representatives from many biological taxa. Animal poisons contain substances with unique biological active molecules that have a variety of molecular targets and biological functions (Utkin, 2015). Among others, anuran amphibians are poisonous animals able to inhabit most regions of our planet, especially tropical areas.

The *Rhinella* genus, which includes *Rhinella icterica* species, is characterized by the presence of parotoid glands located on the body surface, which are mostly involved in the synthesis and release of a poisonous secretion, used for defense against predators and pathogens (Toledo and Jared, 1995). *Rhinella icterica* has a fairly wide area of occurrence, being found in central, southeastern and southern Brazil, including the Pampa Biome, that spreads to northeastern Argentina and eastern Paraguay, at altitudes ranging from 0-1200 m. Due to their coverage area, this specie is found in a great diversity of habitats, from open forests to tropical seasonal zones such as the Cerrado biome including areas with considerable anthropic alterations (Débora Silvano, 2010.).

Anurans poison detains a wide variety of biological compounds such as: biological amines, alkaloids, peptides, proteins and steroids (Siano et al., 2014). Several studies suggest

that most of the alkaloids found in amphibians are either acquired or produced through bacteria in the digestive system and later stored in the parotoid glands (Daly, 1995; Daly et al., 2005; Saporito et al., 2004). Among all toxins presented in the *R. icterica* toxic secretion (RITS), Oliveira et al. (2017) demonstrated a substantial presence of bufalin, a potent calcium channel blocker. Effects of this channel blocker have never been described in insects' nervous system.

For a long time, toxinological studies demonstrating the biotechnological potential of animal poisons have been performed only on vertebrate models. In recent decades, however, suitable animal models have also been sought among invertebrates. Basic principles involving neurotransmission in insects as well as vertebrate models follow fairly similar patterns. Among the many neurotransmitters present in the cockroaches' central nervous system (CNS), the main modulators are acetylcholine, dopamine, octopamine, 5-hydroxytryptamine and histamine that exert important roles related to the functioning of the organism as well as the behavior of these insects (Osborne, 1996; Taylor and Newburgh, 1979). Besides simplicity, greater accessibility, and an increasing applicability for experimental procedures are the most relevant factors that make cockroaches a very viable model for toxicological studies (Scharrer, 1987; Stankiewicz et al., 2012).

The aim of this work was to investigate the physiological and behavioral effects induced by the *R. icterica* toxic secretion (RITS), in both central and peripheral nervous system (PNS) of *Nauphoeta cinerea* cockroaches. Hence, the rationale of this study is to determine the sensitivity of insect preparations to *R. icterica* poison as well as the main locus of interaction for entomotoxicity.

2. Materials and Methods

2.1. Experimental Animals

All experiments were performed on adult *N. cinerea* cockroaches of both sexes (3-4 months after adult molt). The animals were reared with water and food *ad libitum* at controlled temperature and lighting (± 25 °C and 12-hour light/dark cycles).

2.2. Rhinella icterica *toxic secretion (RITS)*

Toads were collected at Derrubadas (27° 15' 57" S, 53° 51' 45" W), a region located in the northwest of Rio Grande do Sul, Brazil, with prior approval of the Brazilian Biodiversity Information and Authorization System (SISBIO): collector license n. 24041-2. Poison extraction was performed by manual compression of the parotoid glands and the resulting secretion was partially solved in methanol followed by lyophilization (Liobras, Liotop K105, São Paulo, Brazil). The powdered extract was than dispersed in insect saline solution previous to the biological assays.

2.3. Reagents and solutions

All chemicals and reagents used were of the highest purity available and were obtained from Sigma-Aldrich, Merck, Roche, Life Technologies or BioRad. Test-solutions were prepared daily by dilution in insect saline immediately before use. The insect saline is a buffered solution prepared with the following composition in mM: NaCl, 214; KCl, 3.1; $CaCl_2$, 9.0; sucrose, 50; HEPES buffer, 5.0 and pH 7.2 (Stürmer et al., 2014). Except when stated otherwise, all drugs were injected into the abdominal hemocoel, in 10 µl volumes, by means of a Hamilton syringe.

2.4. Locomotory activity

The influence of *R. icterica* poison on the locomotory activity of *N. cinerea* was assayed in animals randomly selected and placed individually in a white polystyrene box (25 cm in

length × 15 cm in width × 7 cm in height) (Fig. 1A). Their behavior was recorded during 10 minutes by a logitech® HD WEBCAM (Fig. 1B). Behavioral parameters were automatically measured with video-tracking software (IDtracker, Stoelting, CO, USA). Locomotory activity was assessed through computational analysis (Fig. 1C) by using the software ID tracker following an *ad-hoc* script developed at Matlab® software (30 days free-trial license). To ensure standard experimental conditions, all experiments were performed during the same period of the day (from 10:00 a.m. to 4:00 p.m.).

2.5.Semi-isolated cockroach heart preparation

A semi-isolated cockroach' heart bioassay was assembled essentially as described by Rodríguez et al. (2012). Briefly, adult cockroaches were anesthetized by chilling (5-7 min) until immobile and placed ventral side up on a dissection plate. The lateral margins of the abdomen were cut along each side, and the ventral abdominal body wall was pulled out to show the viscera. After moving the viscera carefully aside, the heart was exposed still contracting, while attached to the dorsal body wall. The heart preparations were washed by bathing it in 200 µL of insect saline solution at room temperature (21-24 ^{o}C). After 5 min of heart beat stabilization, the treatments were delivered by exchanging the bathing solution. The beats/min average in the first 5 min was taken as a reference. Heart beat frequency was monitored under a stereoscopic microscope, for 30 min. Four cockroaches were used in each group of treatment. In the control group, only the saline solution was used to bath the heart.

2.6. In vivo cockroach metathoracic coxal-adductor nerve-muscle preparation

To analyze the effect induced by RITS on insect neuromuscular junctions the *in vivo* cockroach metathoracic coxal-adductor muscle preparation was used essentially as described by Martinelli et al. (2014). Briefly, the animals were immobilized by chilling and mounted,

ventral side up, in a plate covered with 1.0 cm soft rubber, where the animal was firmly attached using entomologic needles, then the metathoracic coxal could be firmly tied to the isometric transducer. The left leg was then tied at the medial joint with a dentistry suture line connected to a 1.0 g force isometric transducer (AVS Instruments, São Carlos, SP, Brazil). The transducer was coupled to a manipulator, which allowed muscle length adjustment. A bipolar electrode was inserted in the nerve 5, which includes the motor axon to the muscle, to provide electrical stimulation. The nerve was stimulated at 0.5 Hz/5 ms, during 120 min. Twitch tensions were recorded, digitalized and retrieved using a computer-based software AQCAD (AVS Instruments, São Carlos, SP, Brazil). Data were further analyzed using the software ANCAD (AVS Instruments, São Carlos, SP, Brazil). The preparations went through a period of at least 20 min of stabilization, before the RITS injection into the insect's hemocoel.

2.7.Grooming activity

Grooming behavior was evaluated according to Stürmer et al. (2014). Cockroaches were examined for grooming behavior immediately after injection with RITS in different doses. Pretreatment of the cockroaches with phentolamine (0.1 µg/g) was done by injecting the drug 10 minutes prior to RITS administration. After the treatments, the time of continuous grooming was measured in seconds over a 30 min period. The animals were never been placed in the testing arena previously, so it was a novel environment for all cases. Tests were performed 2–8 h after the beginning of the light cycle and the room was maintained at 22–25 °C. Control cockroaches were injected only with insect saline.

2.8. In vitro extracellular recordings of spontaneous neural compound action potentials (SNCAP)

For the recordings of SNCAP, the leg was carefully fixed on the SpikerBox (Backyard Brains, USA) cork plate with a pair of electrodes. One of the electrodes was connected to the ground connector of the amplifier and the other to its indifferent (-) connector. The third electrode was placed into the femur as the active recording electrode (+). The signals were recorded at a sampling rate of 1.0 kHz and digitalized by the Neuron SpikerBox that converts the sound of each spike in a digitalized deflection on the computer screen. The action potentials were visualized, recorded and retrieved for later analysis in the computer-based software BYB Spike Recorder (Backyard Brains, USA) and the analysis was done by the software WinWCP (John Dempster, University of Strathclyde, Scothland). In this set of experiments, the animals were injected with RITS, as previously described, 30 min before the procedure for the detachment of the metathoracic leg, as close as possible to the body, to ensure that the thigh, femur, tibia and tarsus remained intact. Using the described conditions, the preparation could be used for at least one hour without changes in the characteristics of the potentials.

2.9. Statistical Analysis

The results were expressed as mean ± SEM. Each experiment was repeated at least three times. For comparison between means of two different experimental groups the Student “t” test was employed. When data from more than two experimental groups were analyzed, One-Way ANOVA was performed followed by Tukey (all groups were compared with each other) or Dunnet (the groups were compared with a positive control or saline). When the Two-way ANOVA analyze was required, it was followed by Bonferroni (the groups were compared with a positive control or saline) as post hoc. All statistical analyses were performed using the GraphPad Prism 6.0 (Software Inc., San Diego, CA). The values were considered significantly different when $p \leq 0.05$.

3. Results

3.1. Locomotory activity

The 10 minrecordings of cockroach's locomotory parameters in control saline condition and RITS-treated groups (n = 33) are presented in Fig. 2. The analyses of the data regarding 10 µg/g RITS treatment revealed a 38 ± 14% decrease in the total distance traveled (Fig. 2A), followed by a 90 ± 6% increase in immobile episodes (Fig. 2B) when compared to the control saline (p<0.05). General disturbance induced by different doses of RITS, on the cockroaches' locomotory activity were featured in Fig. 2C. Overall, RITS treatment causedlocomotory damage when compared to control animals.

3.2. Semi-isolated cockroach heart preparation

The addition of RITS in different concentrations to *in vivo* cockroach semi-isolated heart preparations showed that the poison is cardiotoxic. The control values (insect saline) and the negative chronotropic effect induced by RITS (n=4) are presented in Fig. 3. When RITS was added to the preparation, there was a dose and time-dependent decrease in the cockroach chronotropic response. In this set of protocols, the most significant decrease (p < 0.001) in the chronotropic response, about 40%, was observed with the highest concentration (16 µg/200 µl) after 20 min incubation. The negative chronotropism induced by RITS was sustained and it was not reversed by the preparation washout.

3.3. In vivo cockroach metathoracic coxal-adductor nerve-muscle preparation

The effect of RITS on cockroach peripheral nervous system was analyzed. Overall, RITS induced an irreversible dose-independent neuromuscular blockade, in 120 min recordings. The neuromuscular responses and the respective representative timeline traces of neuromuscular recordings are shown in Figures 4A and B. In this protocol, injection of insect

saline in animals did not affect the normal contractile response (n=6) in 120 min (Fig. 4A). When RITS (20, 50 and 100 µg/g of animal weight) were administered, there was a significant time-dependent neuromuscular blockade. In all the doses of RITS assayed, there was a complete inhibition of the muscle twitches in 120 min recordings.

3.4. Modulation of Grooming activity by RITS

The treatment of cockroaches with RITS (2, 10 and 20 µg/g of animal weight) induced a significant alteration of the grooming behavior. Thereby, in control saline-group the mean activity for leg grooming was 153 ± 10 s and 70 ± 6 s for antennae (Fig. 5A). When RITS at 2 µg/g was administered, there was a significant decrease in the antennae grooming activity (40±6 s), while there was a tendency of increase in leg activity (Fig. 5A). The treatment with 10 µg/g produced 128 ± 10% ($p< 0.0001$) increase on leg grooming without substantial and significant variation in antenna grooming (Fig. 5A). On the other hand, when the dose of 20 µg/g was assayed, there was an increase in both antennae and leg grooming behavior, although only the antennal activity was significant (n=29, $p <0.05$).

To verify the octopaminergic pathway involved in RITS entomotoxic activity, animals were treated with the selective blocker of octopamine receptor, phentolamine. The animal treatment with phentolamine (0.1 µg/g) reduced the normal grooming activity to 7 ± 2 s and 20 ± 3 s, respectively, for leg and antenna grooming. In animals pre-treated with phentolamine (0.1 µg/g) 15 min prior to RITS (10 µg/g) injection, there was a reversal of the poison effect of about 89 ± 13% on leg grooming (n=30, $p< 0.05$) (Fig. 5B).

3.5.Effect of RITS on spontaneous neural compound action potentials (SNCAP)kinetic

The effect of RITS (20 µg/g) over *N. cinerea* leg´s SNCAPs was characterized by a significant increase, (60 ± 4.0%), in the numbers of events (n=6) (Fig. 6). The analysis of the

action potential kinetic revealed that RITS treatment also increased the duration (23 ± 5%) and the time of rising (23 ± 4%), when compared to the control saline parameters ($p< 0.05$, respectively) (n=6). Other parameters analyzed in this set of experiments, such as area, peak, T90 and Tau have not been changed.

4. Discussion

In this work, we have characterized the entomotoxic activity induced by RITS in the cockroach *Nauphoeta cinerea*. Aspects involved in the modulation of the insect neurophysiological systems as well as on their behavior will be discussed therein.

In our experimental conditions, the treatment of the animals with RITS induced a series of alterations in the locomotory activity of the cockroaches. The increase in the number of immobile episodes is in accordance to a consequent decrease in the distance traveled. In this regard, cockroaches exploratory and behavior parameters are complex, aiming to prioritize defensive strategies that make it possible to avoid and escape from predators (Adedara et al., 2015). These later behavioral modulations may reflect the activity of the poison, both in central and peripheral insect nervous system. The poison has also increased the time for the insect grooming activity, which is a direct evidence of the toxicity upon the central nervous system (Stürmer et al., 2014). Thus, despite the neural center involved in the grooming behavior in insects be not completely understood, it has been demonstrated that the main neurotransmitter associated with the antennae movements is dopamine (Weisel-Eichler et al., 1999), while octopamine is related to front legs movement (Weisel-Eichler et al., 1999). Hence, the increase in the leg grooming activity by RITS and its prevention by phentolamine, suggests a direct involvement of an octopaminergic pathway in the modulation of this behavior by the poison (Stürmer et al., 2014; Weisel-Eichler et al., 1999).

Despite the central nervous system activity of RITS, the decrease of the distance traveled and the increase of immobile episodes may also reflect a direct inhibition of the insect neuromuscular junctions, by the poison toxic components. In fact, using vertebrate neuromuscular preparations, Rostelato-Ferreira et al. (2011) have shown that the *Rhinella schneideri* poison induce neuromuscular failure, similarly to the RITS activity, in our experimental conditions. Other toad toxins are also claimed for inducing neuromuscular blockage, an effect attributed to the presence of the peptide bufalin (Yoshida and Sakai, 1973, 1974) and the lethal factor, TSE-LF (Das et al., 2001). In this view, the insect neuromuscular junctions are mostly recognized for being orchestrated by two specific neurotransmitters, glutamate (GLU) as the main excitatory one and gamma-aminobutyric acid (GABA), as the inhibitory counterpart, which correspond to no more than 20% of the total innervated muscle fibers (Briley et al., 1982; Huber et al., 1990; Osborne, 1996). The modulation of the insect skeletal muscle also involves the innervation through octopaminergic/tyraminergic (OA/TA) neurons, which receive direct inputs from the dorsal unpaired median neurons (DUM) and the ventral unpaired median neurons (VUM), respectively (Carrazoni et al., 2016; Libersat and Pflueger, 2004; Weisel-Eichler et al., 1999). Laboratory assays have shown that the activation of DUM neurons by very low concentrations of octopamine,inhibit the contraction of the locust metathoracic extensor tibia muscle fiber (Hoyle and Barker, 1975). Thereby, this later information may indicate that the neuromuscular blockade induced by RITS involves the modulation of the octopaminergic pathway by the activation of DUM neurons.

The electrophysiological recordings of the cockroach hair sensilla spikes have also contribute to improve the knowledge about the mechanism of RITS entomotoxicity. The increase in the rise time, duration and the area of the spontaneous neural compound action potentials suggests a depolarizing activity induced by the poison over the insect nerve

membranes. Indeed, recently our group has demonstrated that RITS is able to impair the activity of the electrogenic pump (Oliveira et al., 2017), a pro-depolarizing activity (Sokolove and Cooke, 1971) that would explain both the effects of RITS on the compound action potentials and a consequent activation of DUM neurons. The ultimate result of this pharmacological modulation induced by RITS on insect neurotransmissions will be the animal behavior disturbance, that in nature, would have serious consequences with individual and ecological impacts (Zhukovskaya et al., 2013).

Finally, the entomotoxic activity of RITS also involves the insect carditoxicity, as the poison at any dose assayed, has decreased significantly the heart rate of the animals. The circulation of hemolymph in insects is maintained mainly by a muscular pump, called the dorsal vessel, which is tubular in shape and located dorsally. The control of rhythmic contractions in the insect heart is a complicated process. The insect heart receives innervation from several sources, which includes cholinergic (Miller and Metcalf, 1968), octopaminergic (Papaefthimiou and Theophilidis, 2011) and proctolinergic (Sliwowska et al., 2001), among others. In the case of octopamine, this biogenic amine induce a biphasic activity over the insect heart rate, acting as an agonist in higher concentrations and as an antagonist in lower amounts (Papaefthimiou and Theophilidis, 2011). We cannot rule out about the exact mechanism involved in the negative chronotropic activity induced by RITS in *N. cinerea* heart rate. However, since in the central nervous system phentolamine prevented the poison activity on grooming behavior, the activation of octopamine-related nerves by the poison components cannot be disregarded.

5. Conclusion

The poison of *Rhinella icterica* showed entomotoxicity in *Nauphoeta cinerea* cockroaches. The main activities comprehend neurophysiological alterations involving central and peripheral neurotoxicity and cardiotoxicity. The results suggest that the neurotransmitter octopamine is involved, at least in part, in the poison toxicological profile in insects. The increase in the SNCAP frequency may result from a depolarizing activity over the insect muscle that is reinforced by the RITS blocking activity on the electrogenic pump. The disturbance of the cockroaches behavior together with electrophysiological alterations, may unveil the presence of some toxic components present in the poison with biotechnological potentials.

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Legends

Figure 1: Schematic representation of the setup for locomotory behavior recordings. On (A), the set of boxes where the animals were kept during the video monitoring. (B), webcam mounted above the system. (C), computer-based system for recording, retrieving and posterior analysis of the videos.

Figure 2: Modulation of cockroach locomotory activity by *Rhinella icterica* venom. The figure shows locomotion and motor endpoints evaluated in control and RITS treatment (n=33, respectively). (A), the graph of total distance traveled. On (B), the graph of immobile episodes. On (C), representative images of the animals tracking during the experiments. Data were expressed as mean ± S.E.M and analyzed by One-way Anova followed by the Dunnett's test, *p<0.05, ** p< 0.01, *** p< 0.001 or Tukey, for comparing means between treatments, #p<0.05 and ###p< 0.0001.

Figure 3: Cardiotoxicity induced by *R. icterica* toxic secretion at *in vivoNauphoeta cinerea* semi-isolated heart preparations. The graph represents the mean ± S.E.M of four experiments. Significance was given by applying two-way Anova followed by Bonferroni as a post test. Notice that the poison induces a significant decrease in heart rate in 30min recordings. W: washout; *p < 0.05; ** p < 0.01; *** p < 0.001.

Figure 4: Neuromuscular blockade induced by *R. icterica* toxic secretion (RITS) on *in vivo* cockroach metathoracic coxal-adductor nerve-muscle preparations. On (A), the dose-response graph of RITS activity over cockroach leg-muscle twitch-tension. In the graph, each point represents the mean ± S.E.M. of five experiments. On (B), representative traces of the insect neuromuscular recordings during the onset of RITS (100µg/g) treatment. Data were analyzed by two-way Anova followed by the Bonferroni's post test. *p < 0.05, ** p < 0.01, *** p < 0.001 (n=6).

Figure 5: Modulation of grooming behavior induced by *Rhinella icterica* poison (RITS) on *Nauphoeta cinerea* cockroaches. (A), shows the graph related to the effect of RITS (2, 10 and 20µg/g) on the insect leg and antennae grooming. Notice the significant increase in leg and antenna grooming activity. On (B), protective activity induced by phentolamine (0.1µg/g of animal weight) upon the positive modulation of leg grooming activity by RITS 20µg/g in *N. cinerea* (n=29). The results were expressed as mean ± S.E.M. of the total time spending grooming in (s). Statistics were done using One-way Anova followed by the Dunnett's test: *p < 0.05, ** p < 0.01, ***p < 0.0001 or Tukey to compare significances between treatments: #p<0.05 (n=29).

Figure 6: Electrophysiological recordings of cockroach sensorial compound action potentials (SNCAP) under saline control and *Rhinella icetrica* poison (RITS) (20µg/g) treatment. Note that treatment with RITS induced an increase in the duration (ms) (A), number of events (n) (B) and rise time (ms) (C) of the potentials. Data were expressed as means ± S.E.M. of at least 6 replicates. One-way Anova followed by Dunnett's test accomplished significance: *p < 0.05, ** p < 0.01 (n ≥ 6).

Figure 1

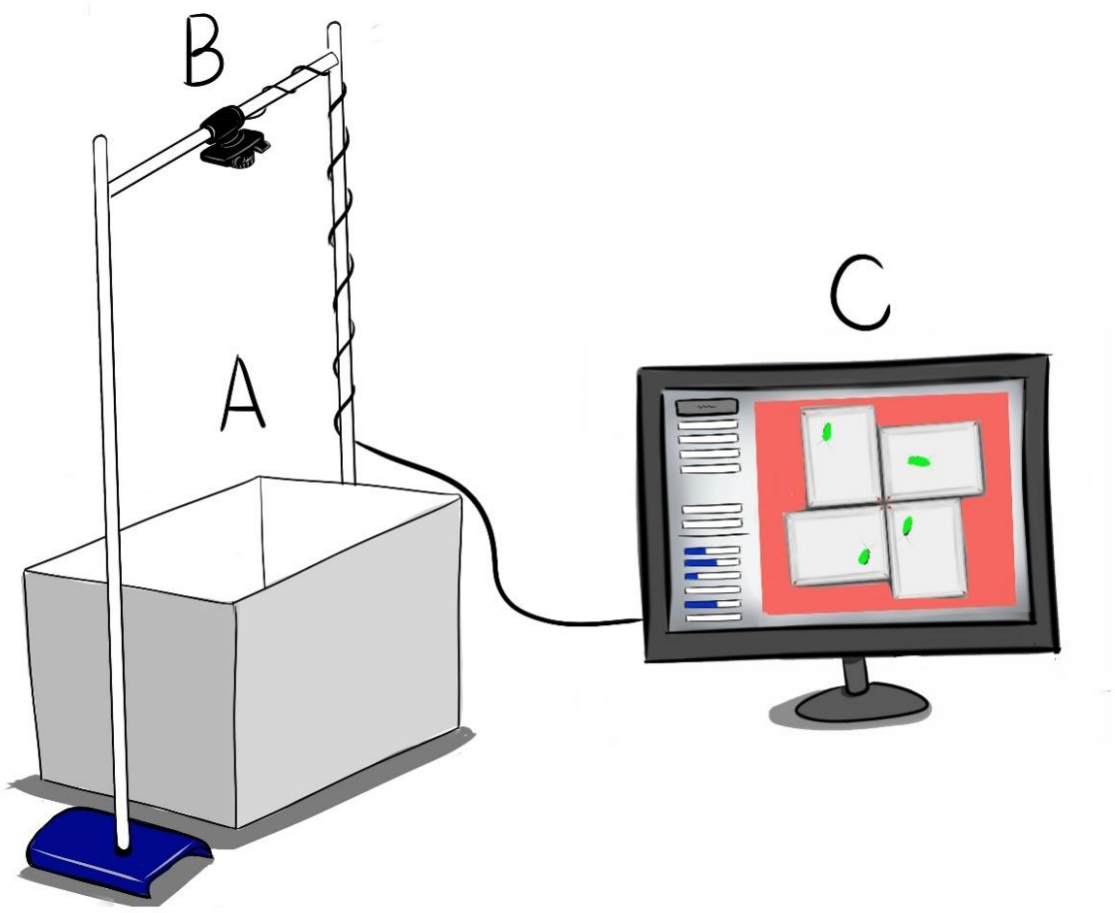


Figure 2

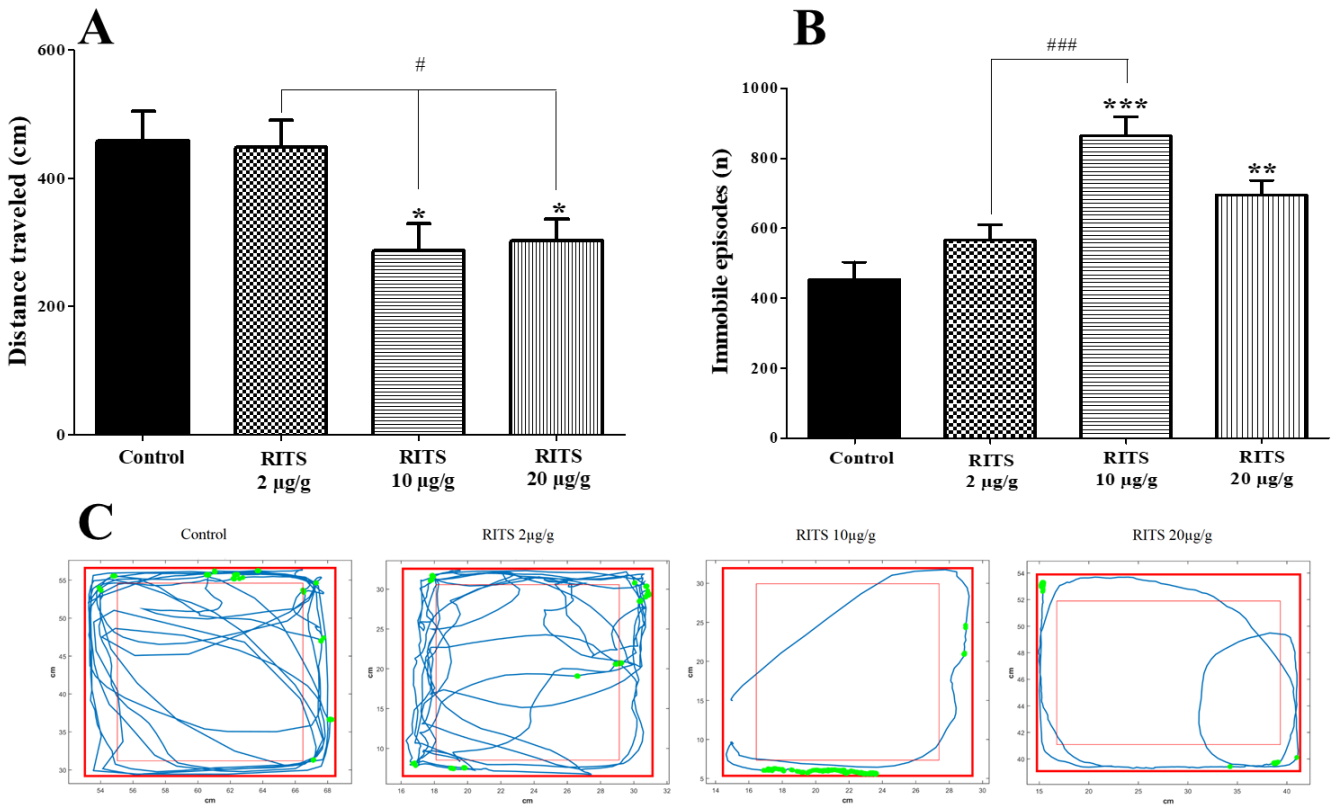

A
Distance traveled (cm)
600
400
200
0
#
*
*
Control
RITS 2 µg/g
RITS 10 µg/g
RITS 20 µg/g
B
Immobile episodes (n)
1000
800
600
400
200
0
###
***
**
Control
RITS 2 µg/g
RITS 10 µg/g
RITS 20 µg/g
C
Control
RITS 2µg/g
RITS 10µg/g
RITS 20µg/g

Figure 3

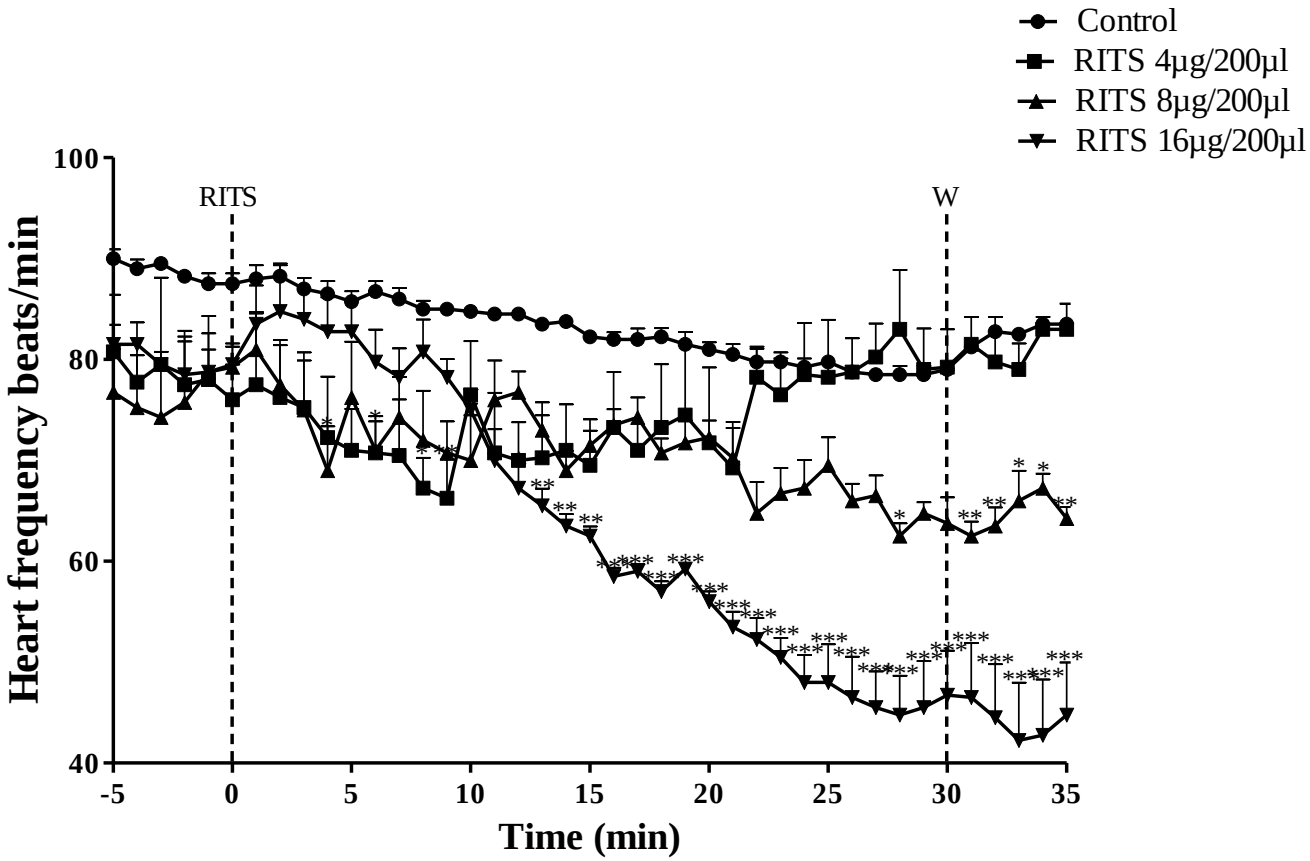

Control
RITS 4µg/200µl
RITS 8µg/200µl
RITS 16µg/200µl
RITS
W
Heart frequency beats/min
Time (min)
100
80
60
40
-5
0
5
10
15
20
25
30
35

Figure 4

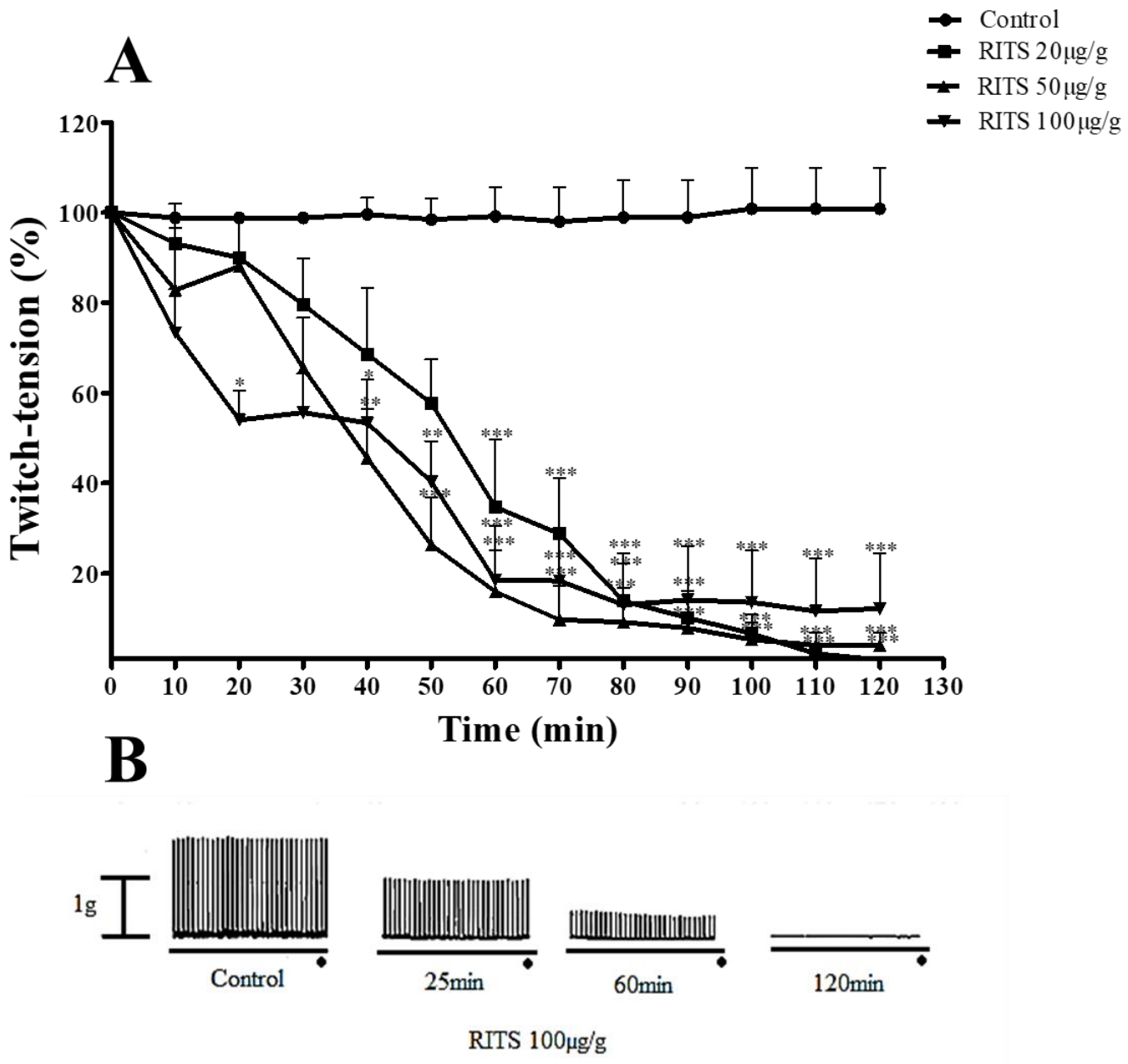

A
Control
RITS 20μg/g
RITS 50μg/g
RITS 100μg/g
Twitch-tension (%)
120
100
80
60
40
20
0
10
20
30
40
50
60
70
80
90
100
110
120
130
Time (min)
B
1g
Control
25min
60min
120min
RITS 100μg/g

Figure 5

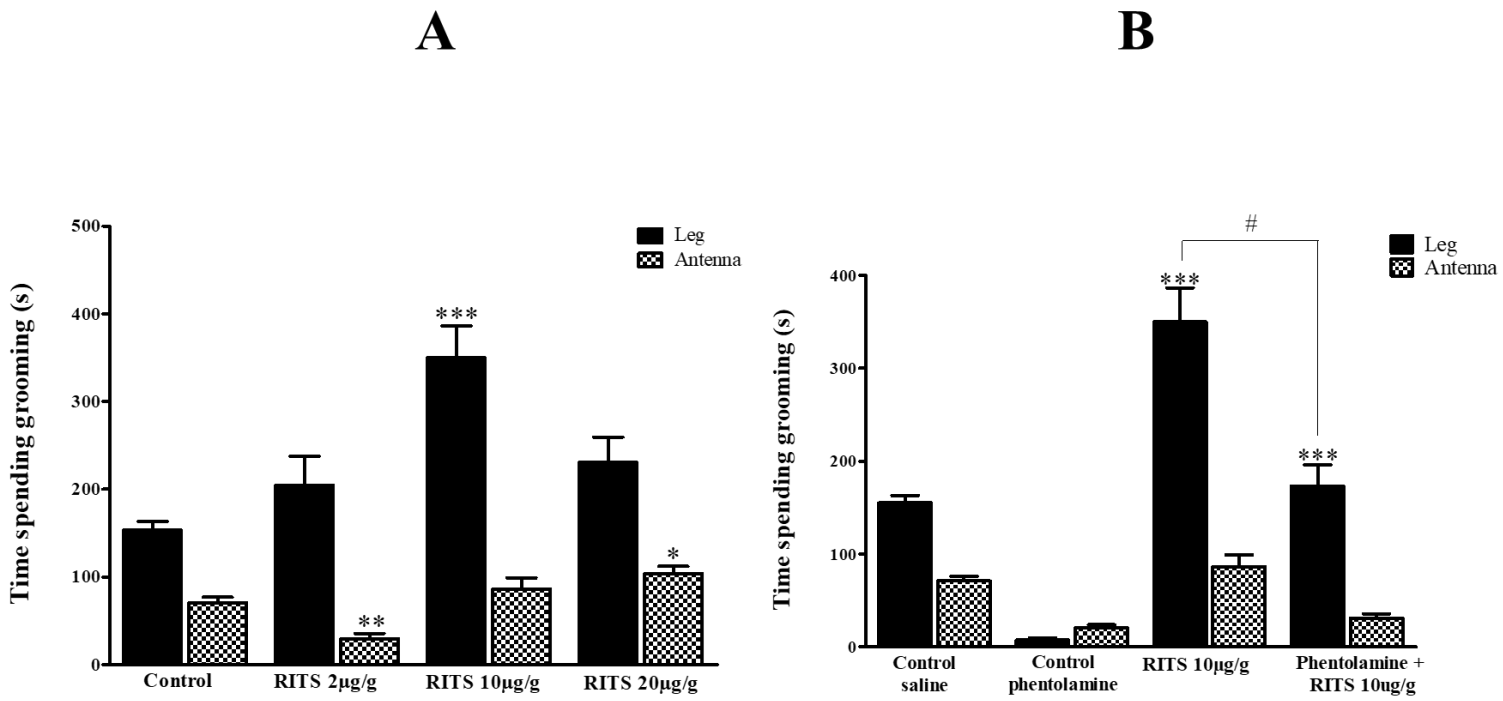
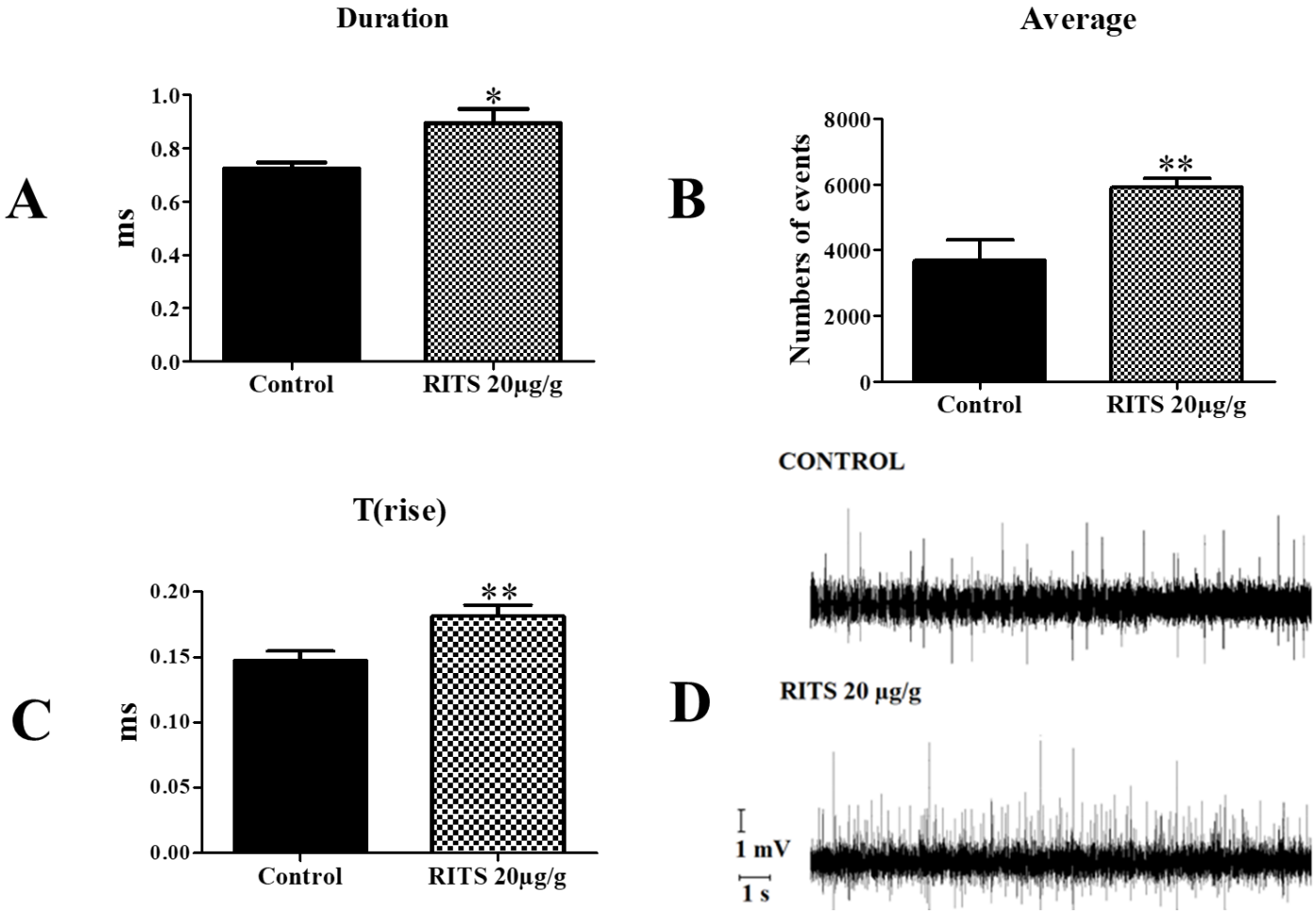

A
B
Time spending grooming (s)
Leg
Antenna
500
400
300
200
100
0
***
**
*
Control
RITS 2µg/g
RITS 10µg/g
RITS 20µg/g
#
Control saline
Control phentolamine
RITS 10µg/g
Phentolamine + RITS 10ug/g

Figure 6


A
Duration
ms
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
RITS 20µg/g
B
Average
Numbers of events
8000
6000
4000
2000
0
**
Control
RITS 20µg/g
C
T(rise)
ms
0.20
0.15
0.10
0.05
0.00
**
Control
RITS 20µg/g
D
CONTROL
RITS 20 µg/g
1 mV
1 s

4 CONSIDERAÇÕES FINAIS

Com base nos resultados observados foi possível delinear as seguintes considerações:

- O efeito cronotrópico negativoinduzido por RITS parece estar ao efeito bifásico induzido pela octopamina na frequência cardíaca de insetos. Outra possível resposta ao efeito observado, é o bloqueio produzido por RITS na bomba de Na+/ K+ ATPase.
- RITS induziu o bloqueio total da junção neuromuscular, em preparações *in* vivo nervo-músculo coxal-abdutor metatorácico de baratas. A modulação dos neurônios do tipo DUM pelo sistema octopaminérgico é uma possível resposta ao efeito observado
- RITS aumentou o *grooming* de perna via rotas octopaminérgicas.
- Dentre as alterações comportamentais observadas,notou-se a diminuição do perfil exploratório do animal juntamente com o aumento do número de paradas.Esse efeito pode estar relacionado a somatória dos danos eletrofisiológicos, eletromigográficos e o aumento no tempo de *grooming* de perna.
- Danos ao sistema nervoso e cardíaco, seguido de transtornos comportamentais induzidos por RITS em *N. cinerea* revelam a presença de toxinas com potencial biotecnológico.

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