YEISSON GUTIÉRREZ LÓPEZ

ALTERAÇÕES MORFOFISIOLÓGICAS E COMPORTAMENTAIS MEDIADAS POR DELTAMETRINA EM INSETOS AQUÁTICOS

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

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Aprovada: 20 de outubro de 2014

Raul Narciso C. Guedes

Gerson Adriano Silva

Eugênio E. de Oliveira
(Orientador)

"I've concluded, through careful empirical analysis, and much thought, that someone is looking out for me. Keeping track of what I think about things, forgiving me when I do less than I ought, giving me strength to shoot for more than I might feel capable of. I believe he knows everything that I do (and think) and still love me. I've concluded after careful consideration, that this person keeping score... Is me"

(Adam Savage, Reason Rally 2012)

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Resumo

GUTIERREZ, Yeisson M.Sc., Universidade Federal de Viçosa, Outubro de 2014. **Alterações morfofisiológicas e comportamentais mediadas por deltametrina em insetos aquáticos**. Orientador: Eugênio E. de Oliveira.

Apesar de já existir um considerável arcabouço científico sobre os efeitos letais de inseticidas sobre os organismos alvos, ainda são escassos os registros de efeitos subletais destes compostos e, em se tratando da entomofauna aquática, estas informações são praticamente inexistentes. Os piretroides são potentes inseticidas neurotóxicos que tem os canais de sódio como seus sítio principal de ação. Estes compostos são amplamente utilizados na agricultura e no controle de insetos vetores de doenças, o que tem possibilitado que parte das moléculas aplicadas a campo acabem alcançando ecossistemas aquáticos. Insetos aquáticos são costumeiramente usados como bioindicadores de qualidade de água, o que lhes tornam como excelentes modelos da contaminação de corpos d'água por deltametrina. Dentro deste contexto, este projeto foi conduzido com o objetivo de avaliar a morfofisiologia de ação bem como alterações comportamentais em insetos aquáticos desencadeados pela exposição subletal ao inseticida piretroide deltametrina. Para tanto, os efemeropteros *Callibaetis radiatus* foram utilizados em experimentações que visavam averiguar tanto a toxicidade como os possíveis distúrbios a nível celular ou tecidual no sistema nervoso e intestino médio decorrentes da exposição ao inseticida deltametrina, e os heteropteros predadores da família Notonectidae, *Buenoa tarsalis* e *Martarega bentoi* foram utilizados em experimentações que visavam averiguar a toxicidade de deltametrina e eventuais mudanças na atividade natatória destes percevejos quando expostos a concentrações subletais deste inseticida. Reduções significativas na sobrevivência de *C. radiatus* foram observadas em concentrações de deltametrina $\geq$ 0.5 µg/L. Mudanças citomorfológicas leves no cérebro (presença de núcleos picnóticos)e severas nos gânglios torácicos (vacuolização e deformação dos corpos neuronais, surgimento de espaços vazios entre o perineurium e a região cortical, e presença de núcleos picnóticos); e no intestino médio (retração da camada muscular, vacuolização do citoplasma, desorganização do núcleo e do citoplasma) de *C. radiatus* foram observado quando estes insetos foram expostos à concentração subletal de 0.25 µg/L. Entretanto, análises imunocitoquímicas e de integridade do DNA demonstraram que estas alterações não ocasionaram morte celular. Nas experimentações com os

Heteroptera aquáticos, os resultados obtidos demonstraram que *B. tarsalis* é uma espécie mais sensível do que *M. bentoi* à presencia de deltametrina. A sobrevivência destes organismos foi reduzida em todas as concentrações testadas, e curtas exposições a deltametrina provocaram uma mudança no padrão de ocupação da coluna d'água. Tais distúrbios comportamentais foram mais intensos nas fêmeas do que em machos de *B. tarsalis*, uma vez que a atividade locomotora de fêmeas de *B. tarsalis* foi significativamente reduzida quando expostas a deltametrina (0.05 µg/L). Já para a espécie *M. bentoi*, a exposição a deltametrina não incorreu em diferenças significativas na locomoção destes insetos. Desta forma, os resultados aqui obtidos sugerem que a exposição a concentrações deltametrina, mesmo em baixas concentrações, afeta controle de processos vitais (metabolismo) ou comportamento (e.g. locomoção) de macroinvertebrados aquáticos, o que pode incorrer em sérios desequilíbrios ambientais.

Abstract

GUTIERREZ, Yeisson M.Sc., Universidad Federal de Viçosa, October of 2015. **Morphophysiological and behavioral changes mediated by deltamethrin in aquatic insects**. Advisor: Eugênio E. de Oliveira.

Although there is already a considerable scientific framework on the lethal effects of insecticides on target organisms, there are few records of sublethal effects of these compounds and, in the case of aquatic insect fauna, this information is virtually nonexistent. Pyrethroids are potent neurotoxic insecticides that have sodium channels as their primary sites of action. These compounds are widely used in agriculture and in the control of insect vectors of disease, which has allowed that part of the molecules applied in the field end up reaching aquatic ecosystems. Aquatic insects are customarily used as bio-indicators of water quality, which make them excellent study models of the contamination of water bodies by deltamethrin. Within this context, this project was conducted to evaluate the morphophysiology of action as well as behavioral changes in aquatic insects triggered by sublethal exposure to deltamethrin. To this end, the mayflies *Callibaetis radiatus* were used in experiments aimed at ascertaining both the toxicity and the possible disturbances at cellular and tissue level in the nervous system and midgut resulting from exposure to deltamethrin, and heteropteran predators (Notonectidae), *Buenoa tarsalis* and *Martarega bentoi,* were used in experiments aimed to determine the toxicity of deltamethrin and possible changes in swimming activity of these bugs when exposed to sublethal concentrations of this insecticide. Significant reductions in the survival of *C. radiatus* was observed at concentrations of deltamethrin $\geq$ 0.5 µg/L. Cytomorphological changes were light in the brain (presence of pyknotic nuclei) and severe in thoracic ganglia (neuronal vacuolization and deformation of bodies, appearance of voids between the perineurium and the cortical region, and the presence of pyknotic nuclei) and in the midgut (retraction of the muscular layer, cytoplasm vacuolization, disorganization of the nucleus and cytoplasm) of *C. radiatus* when these insects were exposed to the sublethal concentrations 0.25µg/L. However, immunofluorescence and DNA integrity analysis showed that these changes did not cause cell death. In experiments with aquatic heteropterans, the results showed that *B. tarsalis* is a more sensitive species than *M. bentoi* to the presence of deltamethrin. The survival of these organisms was reduced at all concentrations tested, and short-term exposure to deltamethrin caused a change in the pattern of occupation of the water column. Such behavioral disturbances were more severe in females than in males *of B. tarsalis*, since the locomotory activity of the females was significantly reduced when exposed to deltamethrin (0.05 µg/L). For the species *M. bentoi*, the exposure to deltamethrin did not incur in significant differences in the locomotion of these insects. Thus, our findings suggest that exposure to deltamethrin concentrations, even at low concentrations, affects the control of vital processes (metabolism) or behavior (eg, locomotion) of aquatic macroinvertebrates, which can incur in serious environmental imbalances.

Conteúdo

Introdução Geral

Atualmente existe uma preocupação sobre fontes diretas e indiretas de contaminação por inseticidas, já que são considerados os estressores mais importantes dos ecossistemas aquáticos (Neumann *et al.* 2002, Relyea & Hoverman 2006, Schwarzenbach *et al.* 2006, Butchart *et al.* 2010).

Segundo a World Health Organization (WHO, 1990), estudos toxicológicos comparando 243 pesticidas mostraram que os piretroides estão entre os pesticidas mais tóxicos para organismos aquáticos tais como peixes e crustáceos. Estes compostos possuem um grupo α-ciano que induz inibição de longa duração do fechamento dos canais de sódio, resultando numa permeabilidade prolongada da membrana do axônio ao sódio produzindo choreoathetosis (movimentos involuntários das pernas e convulsões) (Demoute 1989, WHO 1990, Joy 1994, Soderlund *et al.* 2002). Além disto, este inseticida possui como mecanismo auxiliar (ou secundário) de ação, a fosforilação de outros canais iônicos no sistema nervoso de insetos ou vertebrados (Burr & Ray 2004, Ray & Fry 2006).

Nos últimos anos têm sido amplamente documentado os mecanismos físico-químicos responsáveis pelo transporte de inseticidas aplicados em áreas agrícolas até os corpos de água. Estes compostos podem ser transportados à superfície da água por efeito do vento, escoamento superficial, drenagem, águas subterrâneas e vazamento de fontes pontuais (Schulz 2001). Em se tratando de deltametrina, não existem registros sobre as concentrações deste composto em corpos de água parada, mas estudos conduzido por Muir *et al.* (1985) demonstraram que os níveis máximos de concentração de deltametrina nos sedimentos destes ambientes foram alcançados 48h após aplicação no meio aquoso e ainda eram detectáveis 306 dias após uma única exposição. Grande parte deste fenômeno pode estar relacionada com o fato de que devido às propriedades lipofílicas destas moléculas, uma porção deste composto é transportada à superfície e somente eventualmente se depositam no fundo, além de que parte das moléculas dissolvidas na água são adsorvidas a superfícies orgânicas, como macrofitas ou sedimento (Wauchope 1978, Moore *et al.* 2001, Gan *et al.* 2005, Domagalski *et al.* 2010, Fojut & Young 2011).

Grande parte dos estudos sobre os efeitos de pesticidas em macroinvertebrados aquáticos têm utilizado parâmetros de avaliação como mortalidade ou paralisação (Kreutzweiser & Kingsbury 1987, Sibley *et al.* 1991, Beketov & Liess 2008, Heckmann & Friberg 2005, Rasmussen *et al.* 2008). Entretanto, estudos mais recentes têm noticiado outras alterações comportamentais em várias espécies de macroinvertebrados aquáticos que foram decorrentes da exposição aguda a inseticidas piretroides. Dentre estas alterações, encontram-se a redução na taxa de alimentação de alguns trituradores e herbívoros (Lauridsen *et al.* 2006, Rasmussen *et al.* 2008), alterações do comportamento locomotor (Werner & Moran 2008, Nørum *et al.* 2010, Tomé *et al.* 2014, Huynth *et al.* 2014), e também efeitos a longo prazo, que incluem redução da alimentação, fecundidade e sucesso de emergência (Liess *et al.* 1996; Schulz & Liess 2001a,b).

Dentre os inseticidas piretróides, a deltametrina possui um amplo espectro de ação (DHHS 2003, Ray & Fry 2006), podendo agir tanto por ingestão ou contato direto (Tomlin 2006). O sistema nervoso tem sido proposto como o alvo principal de ação dos inseticidas piretroides (Soderlund *et al.* 2002) e estudos têm demonstrado alterações a nível tecidual no sistema nervoso em artrópodes (Roma *et al.* 2013, Roma *et al.* 2014). Porém, quando ingeridos, os piretroides podem ser facilmente absorvidos pelo trato intestinal (Soderlund *et al.* 2002), principalmente no intestino médio, que normalmente é a maior região do trato digestivo e no qual as células secretam enzimas digestivas, absorvem nutrientes e transportam íons (Cioffi 1979, Martoja & Ballan-Dufrancais 1984). Nos organismos aquáticos heterotróficos, o intestino médio é considerado uma das principais rotas de absorção e acumulação de compostos tóxicos (Cunningham & Tripp 1975, Ribeyre & Boudou 1984, Boudou & Ribeyre 1985, King & Davies 1987, Hakim *et al.* 2010), características que explicitam a importância ecotoxicológica deste órgão (Sauter *et al.* 1991).

Desta forma, esta dissertação foi desenvolvida com o objetivo de avaliar a susceptibilidade de insetos aquáticos (*Buenoa tarsalis*, *Martarega bentoi* e *Callibaetis radiatus*) ao inseticida deltametrina e, principalmente, se a exposição subletal a este composto resulta em distúrbios comportamentais ou morfofisiológicos nestes organismos. A sobrevivência destas três espécies foi avaliada quando estas foram expostas a diferentes concentrações de deltametrina. Além disto, possíveis alterações morfológicas no sistema nervoso (gânglios torácicos e cérebro) e intestino médio decorrente da exposição subletal ao inseticida foram investigadas na espécie *C. radiatus*, enquanto que o comportamento natatório das espécies *B. tarsalis* e *M. bentoi* foi analisado em exposições de curta duração a deltametrina.

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Mudanças citomorfológicas causadas por deltametrina em ninfas de *Callibaetis radiatus*

Mudanças citomorfológicas causadas por deltametrina em ninfas de Callibaetis radiatus

Toxicity and cytomorphological changes in the midgut and central nervous system of the mayfly *Callibaetis radiatus* mediated by deltamethrin exposure

Abstract

Deltamethrin is a pyrethroid insecticide commonly used in the agriculture and in the control of insects that are human disease vectors. These compounds have been recognized as important stressors to freshwater ecosystems, especially in benthic organisms, but their actions at cellular levels in aquatic insects still are poorly investigated. Here, we assessed the susceptibility to deltamethrin as well as the cytomorphological changes and cell death (immunofluorescence for apoptosis and analyses for DNA fragmentation) in the central nervous system (proposed target of the pyrethroids) and midgut (tissue of deltamethrin secondary actions) of the mayfly *Callibaetis radiatus.* Insects were sublethally exposed to deltamethrin to 1, 12 and 24h hours. Sub-lethal deltamethrin exposure (up to 24h at the concentration of 0.25 µg a.i/L) induced slight cytomorphological changes in the brain (presence of pyknotic nuclei) and despite the severe effects observed in histological analysis of the thoracic ganglia (vacuolization and deformation of neuronal cell bodies, emergence of empty spaces between the perineurium and cortical region and presence of pyknotic nuclei) and midgut (muscle layer retraction, cytoplasm vacuolization, nucleus and microvilli disorganization), no further support were obtained in the immunofluorescence and DNA fragmentation analysis. However, the time-mortality response of *C. radiatus* was significantly reduced in deltamethrin concentrations $\geq$ 0.25 µg a.i/L (log-rank test, $\chi 2 = 202.9$, df = 4, $P < 0.001$). Thus, the results obtained here indicated that the cellular stress caused by short-term exposure (< 24h) to deltamethrin showed slight evidence of apoptosis. However, longer exposure led to insect death, which might be related to the disruption of physiological processes (e.g. metabolism or electrical signal transmission) rather than to cell death.

Key-words: cytotoxicity, ecotoxicology, sublethal effects, pesticides, pyrethroids

1. Introduction

Deltamethrin is a typical type II pyrethroid insecticide that has been used as adulticides for controlling mosquitoes (Rozilawati *et al.* 2005, Bengoa *et al.* 2014) and immature and adult of many agricultural pests (Hirano 1989, EPA 2013). However, the extensive use of this insecticide has raised concerns about their environmental safety as several studies have reported serious harm in aquatic invertebrates (WHO 1990, Liess *et al.* 1996, Schulz & Liess 2000, 2001a, b, Beketov & Liess 2005, Beketov & Liess 2008, Toumi *et al.* 2013).

The entry of this insecticide on aquatic ecosystems can be via either direct applications over water surfaces for residual control of vector mosquitoes (Rozilawati *et al.* 2005, Bengoa *et al.* 2014) or as a result of normal agricultural usages, including spray drift, runoff and drainage (Schulz 2001, Reichenberger *et al.* 2007, Bereswill *et al.* 2013). Comparatively to organochlorine and other long-lasting insecticides, deltamethrin is rapidly broken down in sunlight, which might reduce the insecticide exposure in some aquatic invertebrates (Day 1989, Wheelock *et al.* 2005, Lawler *et al.* 2008). However, it has not prevented the deltamethrin occurrence not only in the water bodies but also in sediments of aquatic ecosystems (Pawlisz *et al.* 1998, Amweg *et al.* 2006, Weston *et al.* 2006, Feo *et al.* 2010), increasing the chances of deltamethrin intoxication in benthic organisms (Wheelock *et al.* 2005). Indeed, previous investigations of pyrethroids on aquatic arthropods have shown that immature phases of benthic mayflies are among the most sensitive taxa to these compounds (Siegfried 1993, Beketov 2004, Nørum *et al.* 2010).

The main deltamethrin effects on insects are related to the physiological impairment of the voltage-gated sodium channels that are responsible for the initiation and propagation of action potentials in excitable cells. This insecticide prolongs the opening of sodium channels, resulting in membrane depolarization leading to conductance block of the nervous system (Narahashi 1988, Narahashi 2000). Other auxiliary targets, especially voltage-gated calcium and chloride channels, have been implicated in the actions of a subset of pyrethroids (Burr and Ray 2004, Ray and Fry 2006, Soderlund 2012). Furthermore, the functional disruption of ion transporter at epithelial tissues has been related to reductions of food digestion and nutrient absorption in some terrestrial and aquatic insect species (Alves *et al.* 2010, Konus *et al.* 2013).

Although mayfly species has recently become important test organisms in insecticide ecotoxicology (Schulz & Dabrowski 2001, Beketov 2004, Licht *et al.* 2004, Beketov & Liess 2005, Roessink *et al.* 2013), the cytomorphological changes in their central nervous system (proposed target of the pyrethroids) and midgut (tissue of deltamethrin secondary actions) mediated by insecticide exposures have been completely neglected. To address this knowledge gap, we assessed the acute and chronic toxicity for deltamethrin in nymphs of the mayfly *Callibaetis radiatus* (Ephemeroptera: Baetidae), which is well distributed in lentic environments of Argentina, Paraguay and Brazil (Cruz *et al.* 2014). Nothing is known about their susceptibility to pesticides. Thus, considering that *Callibaetis* nymphs are important part of the food web in aquatic ecosystems (Domínguez *et al.* 2006) and Baetidae mayflies have been used as biological indicator of environmental degradation (Buss & Salles 2007), the findings of this study will assist on biomonitoring the influence of pesticides on benthic macroinvertebrate assemblages nearby agricultural and urban areas.

2. Material e Methods

2.1. Test organism

By using a D-net we collected nymphs of *C. radiatus* of 7-8 mm size (body length excluding the terminal filaments) in fish-farming tanks at the Federal University of Viçosa (Viçosa, Minas Gerais State, Brazil). These nymphs were brought to laboratory and were kept under controlled conditions (25± 2°C, 70 ± 5% relative humidity, 12 h of scotophase) for 24 h before were subjected to experiments.

2.2 Concentration-mortality bioassays

Groups of *C. radiatus* nymphs were exposed to commercial formulation of deltamethrin (Decis 25EC®, Bayer CropScience Ltda., São Paulo, Brazil) at concentrations that ranged from 0.25 to 5 µg of a.i/L. These deltamethrin concentrations were selected after preliminary tests with broad concentration range allowing selection of lower (the highest deltamethrin concentration unable to kill *C. radiatus*) and upper (the smallest deltamethrin concentration able to kill 100% of *C. radiatus*) mortality responses. In the control treatments, only dechlorinated tap water was used, since it was the solvent used to dilute the insecticide. The exposure time was 24 h and individuals were considered dead when had no movement of appendages (legs, antennae, and terminal filaments) or the gills after repeated mechanical stimuli with a pipette tip. Thus, the experimental unit consisted of groups of 10 nymphs that were submitted to 0.3L of solution confined in 0.5L beakers (Laborquimi Vidrolabor, São Paulo, Brazil). Five replicates were used for each insecticide concentration.

2.3 Cytomorphology of the midgut and central nervous system under deltamethrin sub-lethal exposure

In order to detect sublethal effects of deltamethrin exposure, groups of 10 *C. radiatus* nymphs were exposed to the smaller deltamethrin concentration (0.25 µg of a.i/L) and individuals that survived the exposure time for 1, 12 and 24h were randomly selected to cytomorphological analysis. A control treatment (i.e. without insecticide application) was performed and consisted of the exposure to dechlorinated tap water. The nymph size and insecticide exposure procedures were similar to the procedures described above. After exposure, nymphs were dissected on Petri dishes containing phosphate buffered saline solution (PBS; NaCl 0.13 M, Na_2HPO_4 0.01 M, KH_2PO_4 0.02 M, pH 7.2). The midgut, brain and thoracic ganglia of five nymphs for each exposure time were removed and fixed for 1-2h in Zamboni solution (Stefanini *et al.* 1967). After fixation, these tissues were washed for one hour in the same buffer and dehydrated in an alcoholic series (70, 80, 90 and 95%) at 15 min intervals. Infiltration was made with historesin JB-4. Sections with 3 µm thickness were mounted on glass slides, stained with hematoxylin–eosin (for pink-orange stained cytoplasm and darkly stained nuclei), and photographed in a photomicroscope (Olympus BX53, Olympus Deutschland, Hamburg, Germany).

2.4 Cell death detection in the tissues studied

As for the previous explained cytomorphological analysis, individuals that survived the exposure to 0.25 µg of a.i/L for 1, 12 and 24h were assessed for the occurrence of cell death. DNA fragmentation test and whole-mount preparation using anti-cleaved-caspase-3 antibody (#2305-PC-100, Trevigen, USA) were performed for the midgut, brain, and thoracic ganglia of five randomly selected insects from each exposure time, including the control treatment (dechlorinated tap water). The methods for immunofluorescence and DNA fragmentation analysis were similar to the previously described by Santos *et al.*, (2014). The tissues were analyzed under a laser scanning confocal microscope (LSM510 META, Zeiss, Thornwood, NY, USA), and the DNA samples were subjected to electrophoresis on 1.2 % agarose gel and stained with ethidium bromide.

2.5 Survival bioassays

For these experiments, groups of *C. radiatus* nymphs were exposed to deltamethrin at four concentrations determined by the concentration-mortality results (0.25, 0.5, 2.5 and 5 µg of a.i/L) or to dechlorinated tap water as control treatment. All the exposure procedures and insect sizes followed were the same as above described for concentration-mortality bioassays. Here, the beakers were covered with organza to prevent losses of emerged adults. The number of dead nymphs and emerged adults were recorded every six hour during 4 days (96 h). Dead individuals were removed from beakers as soon as they were registered in order to prevent cannibalism

2.6 Statistical analysis

Concentration–mortality curves were estimated by probit analyses using the PROC PROBIT procedure (SAS Institute, 2002). The results of the survival bioassays were subjected to survival analysis using the Kaplan–Meier stimator (Log-rank method) with the SigmaPlot 12.0 software (Systat Software, San Jose, California, USA). The nymphs surviving until the end of the experiment as well as the emerged adults were treated as censored data.

3. Results

3.1 Concentration-mortality bioassays

The probit model was suitable for the concentration-mortality results since the goodness-of-fit test provided low $\chi 2$-value (< 3.0) and high P-values (> 0.05). Lethal concentrations (LC_{20}, LC_{50}, LC_{80}, and LC_{99}) of deltamethrin, which were estimated based on dose-mortality bioassays, are shown in the Table 1.

Table 1. Toxicity of deltamethrin to the mayflies *Callibaetis radiatus* (n =170, $\chi^2 = 2.10$, $P = 0.35$). Lethal concentration (LC) values were estimated based on concentration-mortality bioassays using probit analyses. CI denotes confidence interval. Concentrations are expressed in µg of a.i/L.

Slope ± SE	**LC_{20} (95% CI)**	**LC_{50} (95% CI)**	**LC_{80} (95% CI)**	**LC_{99} (95% CI)**
2.03 ± 0.28	0.23 (0.15-0.32)	0.60 (0.46-0.78)	1.56 (1.13-2.25)	3.89 (2.43-8.47)

3.2 Midgut cytomorphology

The midgut of *C. radiatus* nymphs not exposed to deltamethrin presented a single-layered epithelium composed mainly of columnar digestive cells, which oval nuclei were located near the central region and contained similar proportions of hetero and euchromatin. In the apical region of columnar cells is a well-developed striated border. The midgut lumen revealed a peritrophic membrane enclosing the food. Externally, a layer of circular muscle cells were present (Fig. 1.a and b). Cytomorphological changes in this tissue were dependent of the deltamethrin exposure time. Nymphs that survived one hour to deltamethrin exposure showed columnar digestive cells with cytoplasmic vacuolization, especially at basal region. Furthermore, the peritrophic membrane become less thick and, consequently, less eosinophilic (Fig. 1.c and d). Survivors of 12 h exposure to deltamethrin did also present the above described cytomorphological changes and showed a disintegration of muscle layer with thinner and spaced cells (Fig. 1.e and f). After 24 h of deltamethrin exposure, the cytoplasmic vacuolization and the presence of more eosinophilic regions were more intense in the apical region of columnar digestive cells. There was also a separation between the midgut lumen and muscle layer and peritrophic membrane (Fig 1.g and h).

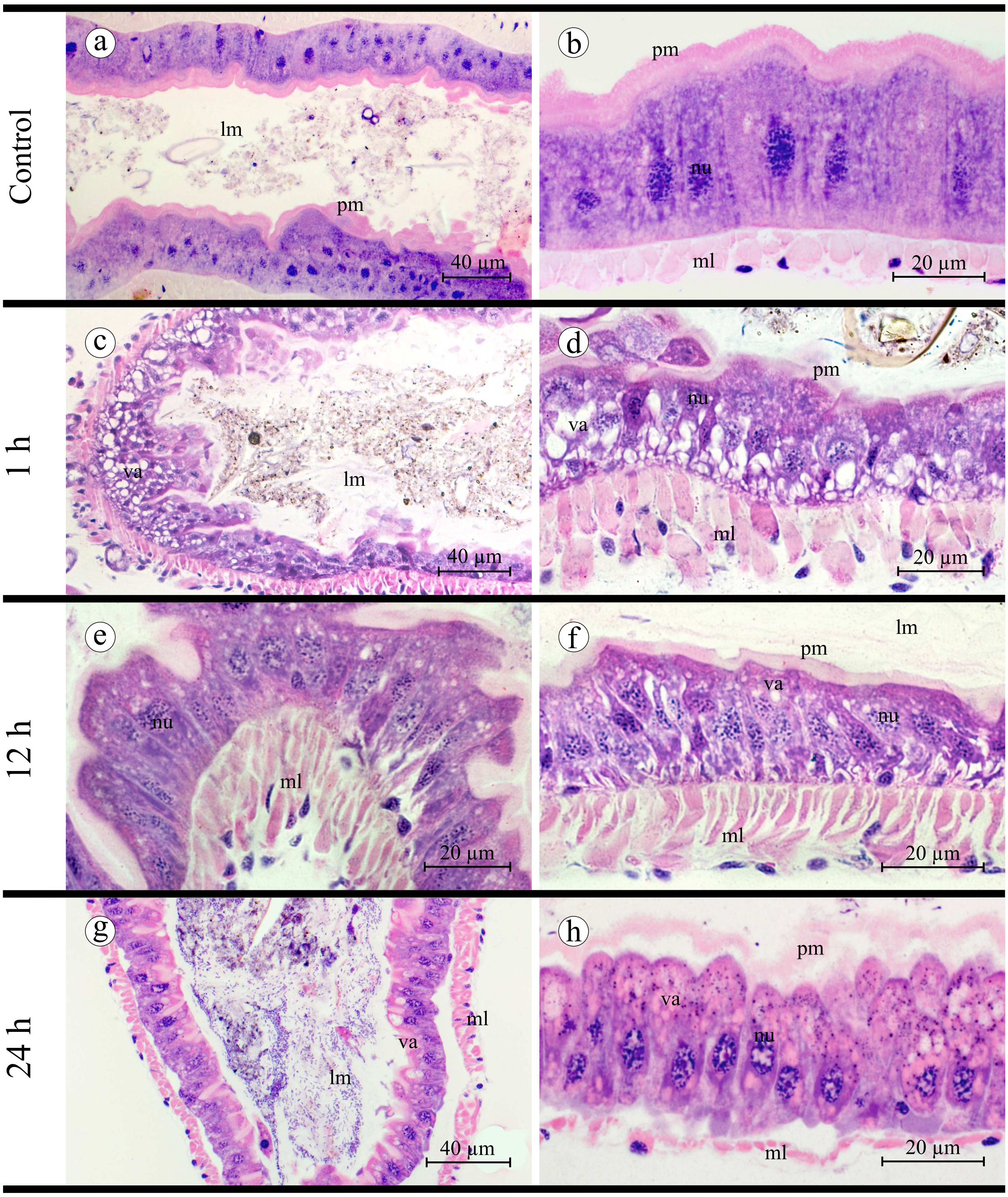

Figure 1. Histological sections of *C. radiatus* midgut stained with hematoxylin and eosin. (**a**) and (**b**), Control treatment with no exposure to insecticide. (**c**) and (**d**), 1h exposure to deltamethrin. (**e**) and (**f**), 12 h exposure to deltamethrin. (**g**) and (**h**), 24 h exposure to deltamethrin. All exposure time were performed for the lowest deltamethrin concentration (0.25 µg of a.i/L) used in this study. **lm:** midgut lumen, **pm**: peritrophic membrane, **nu**: nuclei, **ml**: muscle layer, **va**: vacuolization.

3.3 Thoracic ganglia and brain histomorphology

Each thoracic segment of *C. radiatus* nymphs had a thoracic ganglion with all the nerve cell comprised in the ganglion peripheral region. The diameters of their cell bodies varied between 5 and 20 µm. The central region of the thoracic ganglia was composed by neurite bundles (neuropiles) that were moderately dense organized but without a homogenous distribution, i.e. empty space could be detected among the neurite fibers (Fig. 2.a). Despite the fact that no drastic alterations were observed in the *C. radiatus* thoracic ganglia after one hour exposure to deltamethrin (Fig. 2.d, e and f), vacuolization and deformation of neuronal cell bodies, emergence of empty spaces between the perineurium and cortical region, separation of the lamellae as well as the presence of pyknotic nuclei were consistently observed in these tissues of *C. radiatus* nymphs that survived 12 or 24h of deltamethrin exposure (Fig. 2.g to l). Except for the presence of a few pyknotic nuclei in the brain of *C. radiatus* nymphs that were deltamethrin-exposed (12 or 24h), the exposure to this insecticide did not alter the brain of organization and structuration in these insects (Fig. 3).

3.4 Cell death detection in midgut, thoracic ganglia and brain

The results of the immunofluorescence analysis of tissues from insects exposed to deltamethrin were dependant of the exposure time. Thoracic ganglia and brains cells of untreated and 1 h exposed insects showed no activity of caspase-3, the midgut of the untreated insects showed caspase-3 activity (green areas) with a definitive pattern possibly due to natural auto-fluorescence or artifacts in the preparation of the tissue. For insects that were exposed for 12 and 24 h, few regions in the midgut and thoracic ganglia showed increased caspase-3 activity (Fig 4.) No caspase-3 activity in the brain cells was spotted for any the exposure times assessed. Moreover, DNA fragmentation was completely absent in the analysis of DNA integrity from the midgut, thoracic ganglia and brain of all of the exposure time treatments (Fig. 5).

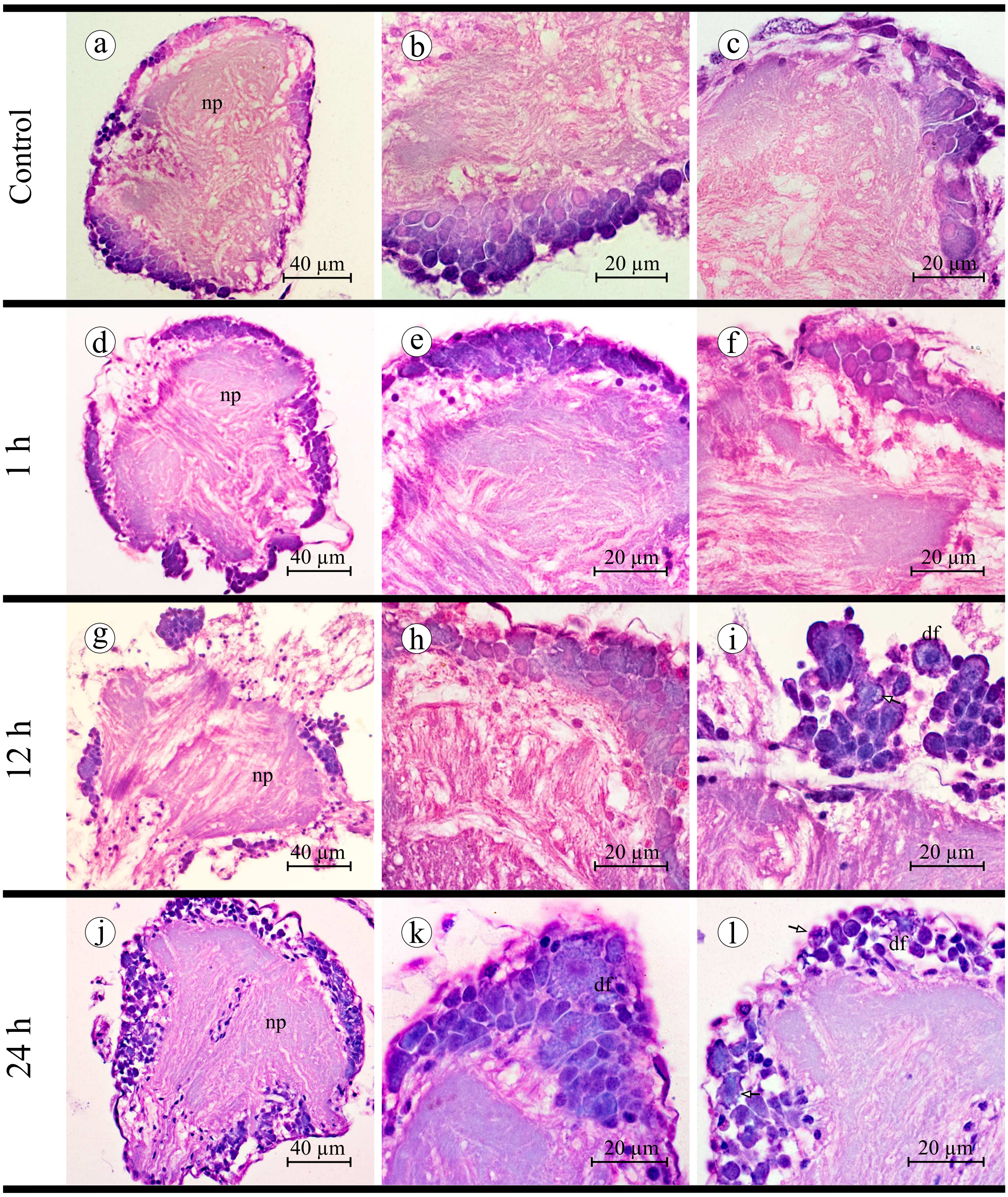

Figure 2. Histological sections of *C. radiatus* thoracic ganglia stained with hematoxylin and eosin. (**a**), (**b**) and (**c**), Control treatment with no exposure to insecticide. (**d**), (**e**) and (**f**), 1h exposure to deltamethrin. (**g**), (**h**) and (**i**), 12 h exposure to deltamethrin. (**j**), (**k**) and (**l**), 24 h exposure to deltamethrin. All exposure time were performed for the lowest deltamethrin concentration (0.25 µg of a.i/L) used in this study. **np**: neuropile, **arrow**: vacuolization, **df**: deformation of neuron,

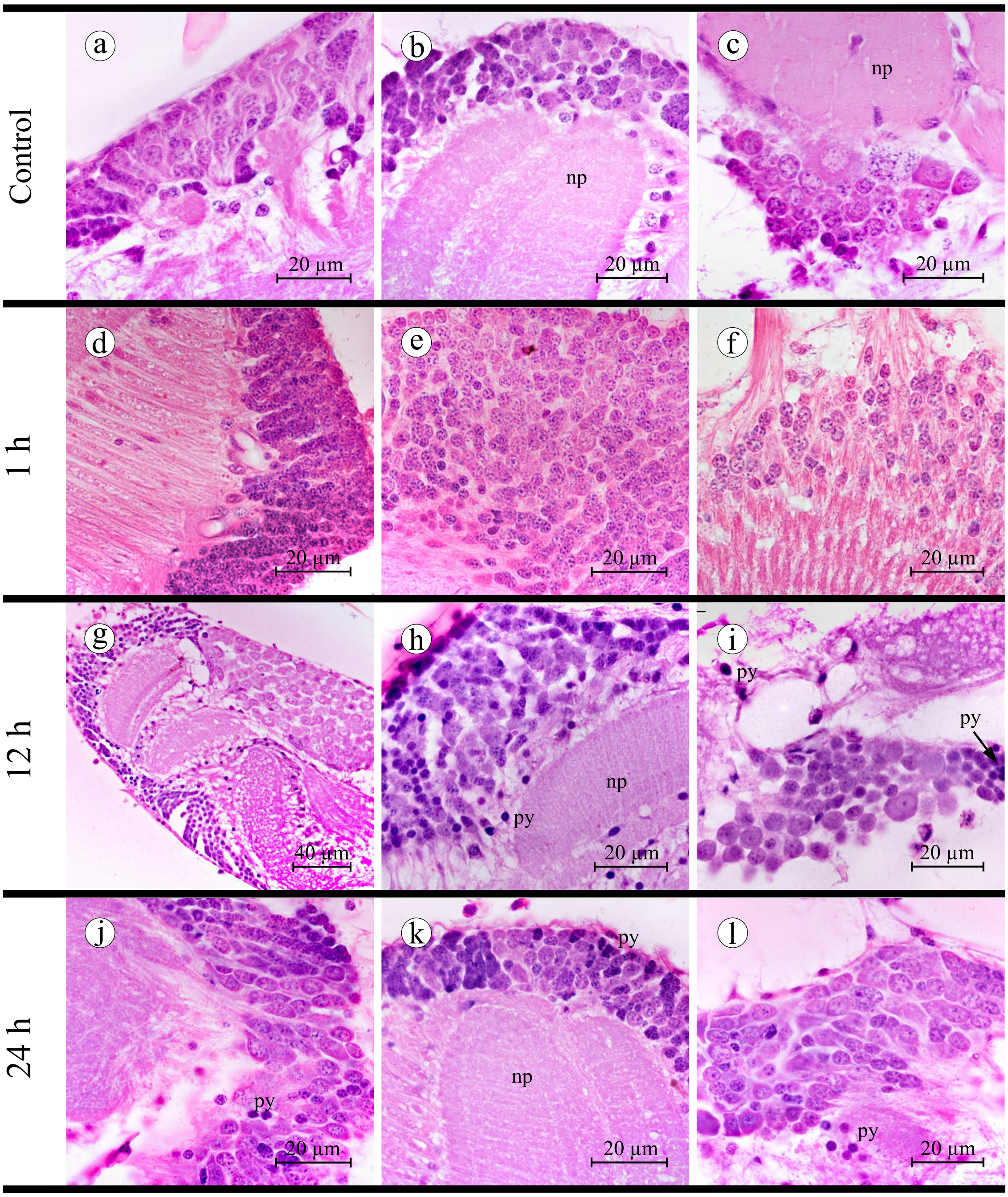

Figure 3. Histological sections of *C. radiatus* bain stained with hematoxylin and eosin. (**a**), (**b**) and (**c**), Control treatment with no exposure to insecticide. (**d**), (**e**) and (**f**), 1h exposure to deltamethrin. (**g**), (**h**) and (**i**), 12 h exposure to deltamethrin. (**j**), (**k**) and (**l**), 24 h exposure to deltamethrin. All exposure time were performed for the lowest deltamethrin concentration (0.25 µg of a.i/L) used in this study. **py**: pyknotic nuclei, **np**: neuropile.

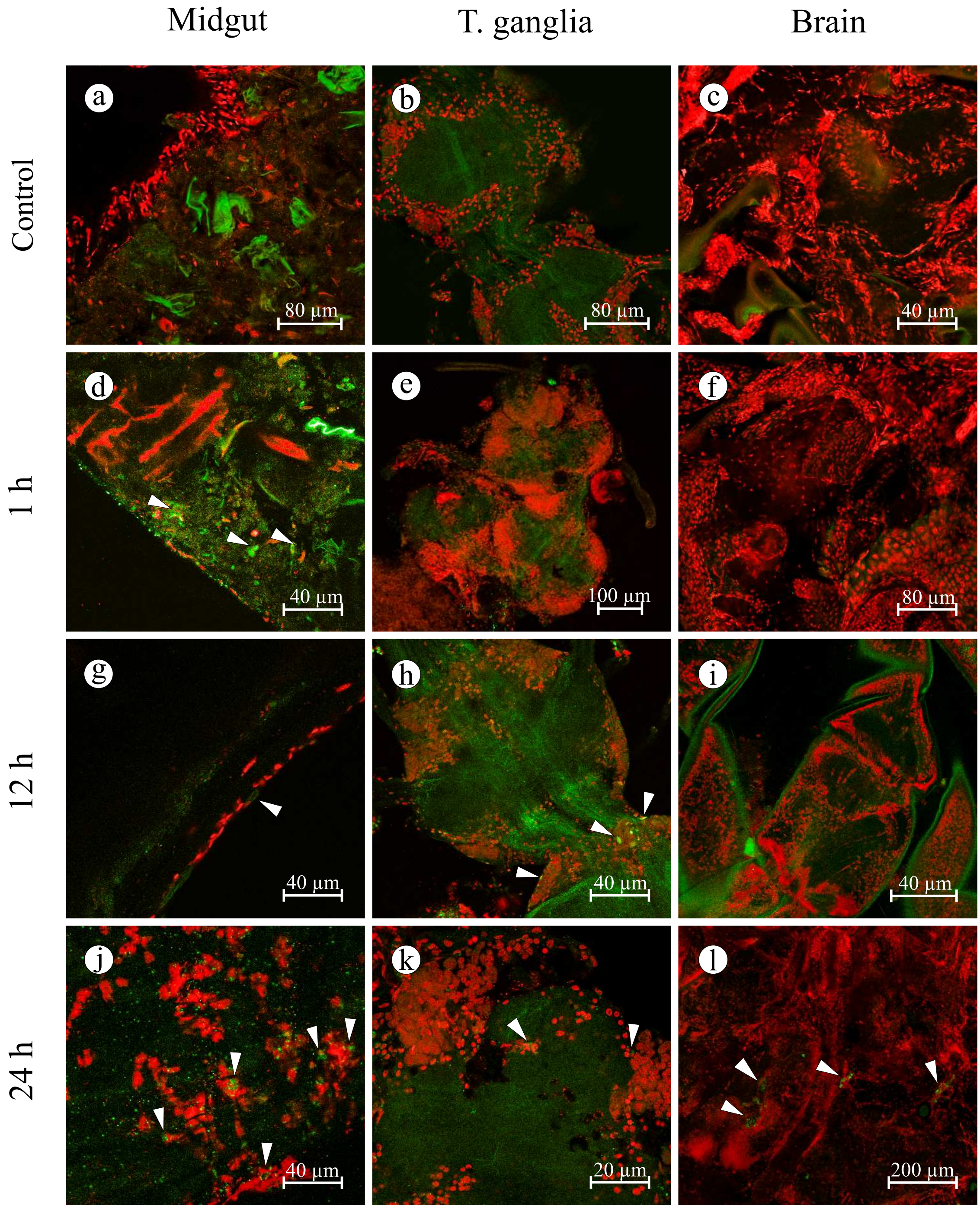

Figure 4. Immunofluorescence of *C. radiatus* tissues using anti-cleaved-caspase-3 antibody (green). (**a**), (**d**), (**g**) and (**j**), midgut control, 1, 12, 24 h treatments correspondingly. (**b**), (**e**), (**h**) and (**k**), thoracic ganglia control, 1, 12, 24 h treatments correspondingly. (**c**), (**f**), (**i**) and (**l**), brain control, 1, 12, 24 h treatments correspondingly. Arrowheads indicate caspase-3 activity.

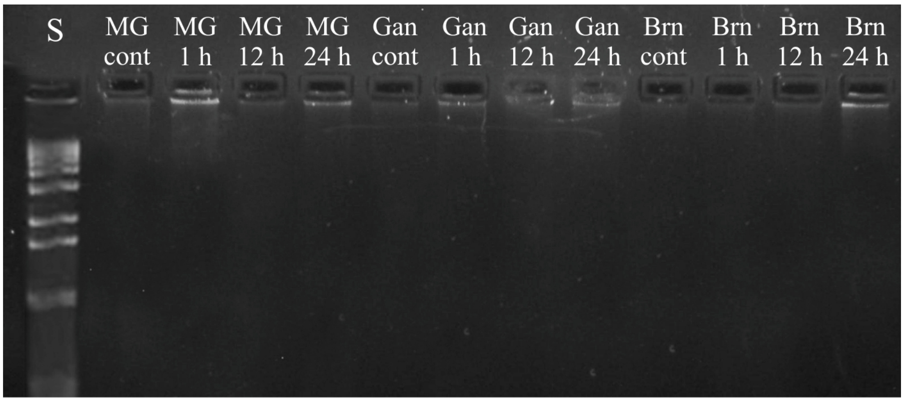

Figure 5. DNA integrity of the Midgut (MG), thoracic ganglia (Gan) and brain (Brn) of *C. radiatus* for the unexposed (cont) and the 1, 12, and 24 h exposure treatments. The first column correspond to the standard (S).

3.5. Survival analysis

The survival analysis of the data from *C. radiatus* nymphs exposed to deltamethrin residues indicated significant differences among the insecticide concentrations (Log-rank test, $\chi 2$ = 204.7, df = 4, $P < 0.001$). After four days of exposure, survival was above 60% for nymphs that had not been exposed to deltamethrin decreasing to around 30% at the lowest deltamethrin concentration (0.25 µg of a.i/L) and dropping to less than 6% at the deltamethrin concentration of 0.5 µg of a.i/L (Fig. 1). Deltamethrin concentration higher than 0.5 µg of a.i/L killed all the nymphs in exposure times less than 48 h (Fig. 6).

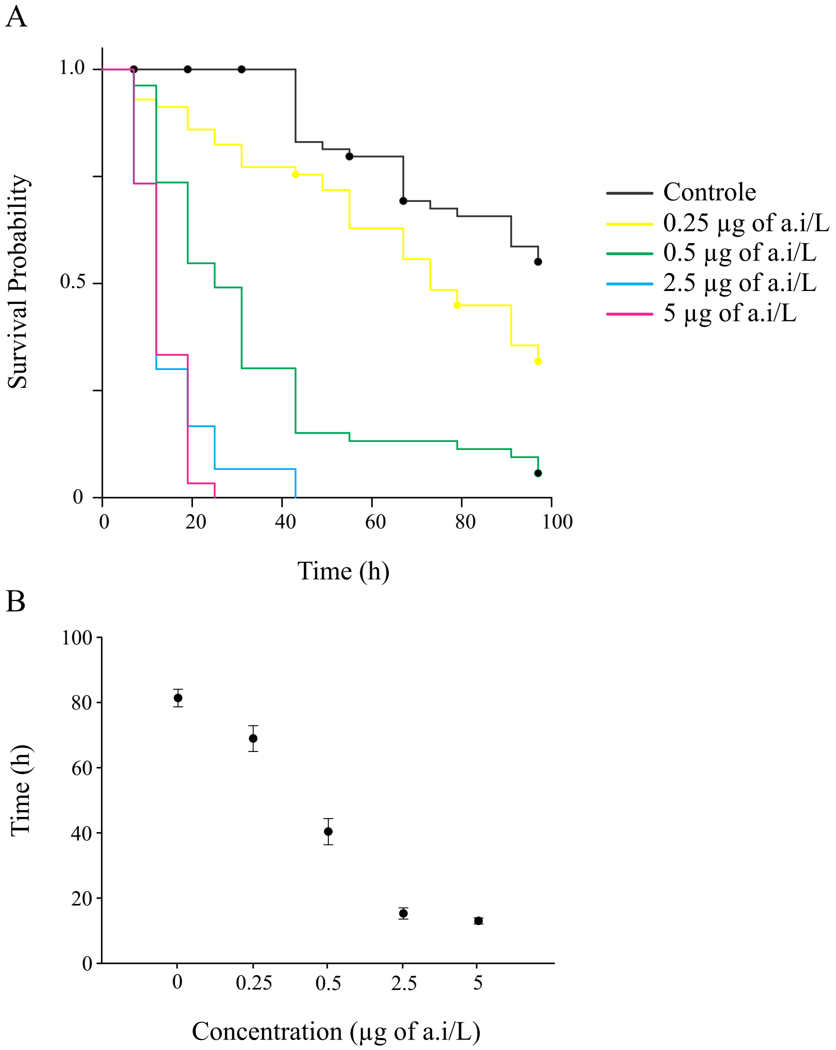

Figure 6. (**A**) Survival curves of *C. radiatus* nymphs up to 96 h deltamethrin exposure. Only the survival curves of the highest concentrations (2.5 and 5 µg of a.i/L) were not significantly different by Holm-Sidak's test ($P > 0.05$). Points represent the censored data (nymphs surviving until the end of the experiment as well as the emerged adults). (**B**) Mean lifetime of *C. radiatus* nymphs under 96 h deltamethrin exposure. Dispersion expressed as SE.

4. Discussion

Pyrethroid insecticides are highly toxic to a number of non-target organisms such as bees, freshwater fishes and other aquatic organisms even at very low concentrations (often <1 µg/L) (Siegfried 1993, Oudou *et al.* 2004). Here, it was evaluated the deltamethrin toxicity to *C. radiatus* nymphs, which is a macroinvertebrate well distributed in lentic environments of Argentina, Paraguay and Brazil (Cruz *et al.* 2014) being important part of the food web in aquatic ecosystems (Domínguez *et al.* 2006) and potential bioindicator of of environmental degradation (Buss & Salles 2007). It was also assessed if the deltamethrin exposure would mediate ciytomorphological changes in important physiological systems (digestive and central nervous system) or in the survival abilities of *C. radiatus* nymphs. Different alteration intensities were observed between tissue types and in a time dependent manner. Despite short-term (up to 24h) exposure do deltamethrin at low concentrations (0.25 ng/L) did not induced cell death, but longer exposure (up to 96h) significantly reduced the survival abilities of *C. radiatus* nymphs.

These cytomorphological differences and the effects severity among the tissues may results from the deltamethrin toxicological characteristics. Despite the deltamethrin mainly actions on the axonal nerve impulse transmission, it may also cause secondary effects that contribute to its toxicity (Soderlund *et al.* 2002, 2011). Osmotic imbalances on the digestive tract can contribute to such deltamethrin secondary actions, since the system represents one of the main routes of toxicant absorption and accumulation in heterotrophic aquatic organisms (Cunningham & Tripp 1975, Ribeyre & Boudou 1984, Boudou & Ribeyre 1985, King & Davies 1987).

Similar to the cytomorphological changes in the thoracic ganglia of *C. radiatus* nymphs, exposure to the pyrethroid cypermethrin mediated cytomorphological changes (cellular swelling, pyknotic nuclei and structural disorganization of the neuropile) in thoracic ganglia of the tick *Rhipicephalus sanguineus* (Roma *et al.* 2013, 2014). Cypermethrin also affected the brain of the anuran tadpoles *Physalaemus biligonigerus* and *Bufo arenarum*, causing massive cell death (apoptosis and DNA fragmentation), and spacing of nerve cells. (Izaguirre *et al.* 2000, Casco *et al.* 2006).

Cytomorphological alteration in the insect midguts caused by insecticide exposures have been explored in different insect taxa (Endo & Nishiitsutsuji-Uwo 1980, Nishiitsutsuji-Uwo & Endo 1981, Percy & Fast 1983, Singh *et al.* 1986, Roel *et al.* 2010, Correia *et al.* 2013). Similar alterations to that here described for *C. radiatus* midgut were reported to *Spodoptera frugiperda* when subjected to neem exposures (Roel *et al.* 2010, Correia *et al.* 2013). Neem exposure caused separation between the midgut lumen and muscle layer, and columnar cells became thin and long in *S. frugiperda*, (Roel et al. 2010, Correia et al. 2013), Other studies have demonstrated changes in the shape of epithelial cells and gradual loss of microvilli after insecticide exposure in several terrestrial insect species (Endo & Nishiitsutsuji-Uwo 1980, Nishiitsutsuji-Uwo & Endo 1981, Percy & Fast 1983, Singh *et al.* 1986).

In addition to the aforementioned changes, cytoplasm vacuolization observed in midgut and thoracic ganglia could represent initial processes of deltamethrin mediated changes, which ultimately can lead to autophagy (de Almeida Rossi *et al.* 2013), apocrine secretion and / or apoptosis (Caetano *et al.* 1994, Serrão & Da Cruz-Landim 1995, Cristofoletti *et al.* 2001, Costa *et al.* 2012). However, loss of apical cytoplasm into the lumen, a common indicator of apoptosis (Tettamanti *et al.* 2007, Rost-Roszkowska 2008, Azevedo *et al.* 2009), was not observed in *C. radiatus* midgut. Furthermore, the changes observed in the nuclei of columnar cells of *C. radiatus* exposed to deltamethrin was disorganized and less dense, different to the apoptotic nuclei reported in previous studies (da Silva Cruz *et al.* 2010, de Almeida Rossi *et al.* 2013).

Although there are several preliminary morphological evidences of cell death in the midgut and thoracic ganglia of *C. radiatus*, measurements of caspase-3 activity, which has been extensively used as an indicator of indicator of apoptosis in animal cells (Zagariya 2012, Kaneko *et al.* 2011, Santos *et al.* 2014), indicated only slightly increases in the midgut. One could argue that these caspase-3 activity increases would indicate an early stage of the apoptosis process in the assessed tissues, but it can not be ruled out that this may be part of the normal cell renewal in tissues remodeling processes (Franzetti *et al.* 2012, Santos *et al.* 2014). Furthermore, this slight increase in caspase-3 activity might result of immunolabeling of food residues inside the midgut, as well as autofluorescence of other insect tissues (Fukatsu *et al.* 1998, Thimm & Tebbe 2003, Koga *et al.* 2009). Lending more evidence to the absence of deltamethrin-mediated cell death, DNA fragmentation was not observed for any of *C. radiatus* tissues in the exposure times assessed.

Survival analysis results suggest that short-term exposure (up to 24 h) to low deltamethrin concentrations did not caused severe reduction of the survival probability of the nymphs of *C. radiatus*. Previous studies pointed out that exposure to deltamethrin sublethal concentrations of can cause morphological changes, which may reduce the animal ability to convert ingested food into nutrients needed for their development and/or reproduction (Terra & Ferreira 1994, Cristofoletti *et al.* 2001, Levy *et al.* 2004).

When exposed to sublethal concentrations of a pesticide, the basal metabolism of insects could increase in response to physiological stress imposed by the pesticide (Rasmussen 2012), which might not affect the survival ability to a single insecticide pulse, but certainly the insect will become less healthy (Mohr *et al.*, 2012). However, repeated exposure to the pesticide could have further adverse effects, especially if the toxicokinetic processes and the elimination of the pesticide from the insect body are more time consuming than the interval between exposures (McCarty & Mackay 1993, Escher & Hermens 2002, Ashauer *et al.* 2010).

The finding obtained in this study lends more support to this hypothesis, since the survival abilities of *C. radiatus* nymphs were significantly reduced under longer exposure (up to 96h) to a deltamethrin concentration that did not induced cell death up 24h of exposure. In such exposure situations, these cytomorphological changes may lead to cell death (Lockshin & Zakeri 2004). The findings of this study will assist on biomonitoring the influence of pesticides on lentic macroinvertebrate assemblages nearby agricultural and urban areas, providing not only toxicological data but also indicating that slight cytomorphological changes observed after short-term exposure to low deltamethrin concentrations might result in reductions of these animals survival abilities .

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Distúrbios locomotores mediados por deltametrina em Notonectidae

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Differential toxicity and swimming behavior impairment mediated by deltamethrin in two notonectid species

Abstract

Assessment of toxicant effects on aquatic insects requires not only the evaluation of the lethality but also the potential impairments of important behaviors mediated by these compounds. Pyrethroid insecticides can be present in these environments as a result of human activities such agriculture (plant protection) and the control of insect vectors of human diseases. Despite the fact that backswimmers (Heteroptera: Notonectidae) are predacious insects found in a wide variety of freshwater habitats, with considerable potential as biological control agents of disease-transmitting mosquitoes and also target of chemical control, because of their capacity of feeding on eggs and immature stages of fishes and tadpoles of economic interest. However, little is known in terms of the deltamethrin effects on these animals. In this study the lethal and sublethal effects of deltamethrin were assessed in the backswimmer species *Buenoa tarsalis* and *Martarega bentoi*. Concentration-mortality and survival of the two species were assessed in concentrations ranging from 0.5 to 5x10^3 ng of a.i/L. In addition, measurements of different swimming behavioral traits in the presence and absence of deltamethrin were separately performed in male and female of both species. In general, *B. tarsalis* was more susceptible to deltamethrin than *M. bentoi* and only *B. tarsalis* females had their swimming behavior affected by deltamethrin. Deltamethrin also disturbed the *B. tarsalis* water column occupation pattern, forcing these insect to stay for longer in more superficial areas. Swimming traits of *M. bentoi* showed significant differences between sexes, where *M. bentoi* males were significantly more active and swam longer distances than *M. bentoi* females. Thus, the findings obtained here showed that *M. bentoi* might be more resilient than *B. tarsalis* to deltamethrin disturbances, since changes in the swimming behavior traits may negatively affect populations of these insects in aquatic ecosystems, as long-term hypoactivity could directly contribute to decreased efficiency in mating, pray catching, and other activities.

Key-words: Backswimmers, ecotoxicology, sublethal effects, pesticides, pyrethroids

1. Introduction

Most aquatic insects are generally considered highly susceptible organisms to a wide variety of chemical compounds, including pyrethroid insecticides (Anderson 1989, Coats *et al.* 1989, Siegfried 1993). Deltamethrin is a typical type II pyrethroid that has been used as adulticide for controlling mosquitoes (Rozilawati *et al.* 2005, Bengoa *et al.* 2014) and to control immature and adult instars of many agricultural pests (Hirano 1989, EPA 2013). The entry routes of this insecticide on aquatic ecosystems can be via either direct applications over water surfaces for residual control of vector mosquitoes (Rozilawati *et al.* 2005, Bengoa *et al.* 2014), draining from residential areas (Weston *et al.* 2005), or as a result of normal agricultural usages, including spray drift, runoff and drainage (Schulz 2001; Reichenberger *et al.* 2007; Bereswill *et al.* 2013). Some of the sublethal effects of deltamethrin reported in aquatic arthropods are as follows: contact irritability in adults (Chareonviriyaphap *et al.* 2006, Kongmeee *et al.* 2004) and disturbances in the swimming activity in juvenile of *Aedes aedypti* (Tomé *et al.* 2014), and feeding inhibition in *Chironomus xanthus* (Moreira-Santos *et al.*, 2005).

In the aquatic toxicology scenario, mayflies and damselflies have normally been recognized as the most susceptible aquatic insects to pesticides (Stephenson 1982, Siegfried 1993, Wogram & Liess 2001, Beketov 2004), which has not reduced the utilization of other aquatic macroinvertebrates, such as backswimmers, as test organisms in ecotoxicological studies (Perschbachur 1989, Lahr 1998, Lahr *et al.* 2001). However, these studies with backswimmers have focused only on the lethal effects and the impact of theses compouds on backswimmer behaviors have not received much attention (Barry *et al.* 2004, Berticat *et al.* 2004, Reynaldi *et al.* 2010).

Knowing the potential impacts of pesticides in backswimmers is important as they are predacious insects found in a wide variety of freshwater habitats and have been recognized as ecologically important, since they are often among the first successive insects to colonize aquatic habitats (Papácek 2000). Furthermore, previous studies have confirmed their potential as biological control agent of disease-transmitting mosquitoes (Rodríguez-Castro *et al.* 2006, Quiroz-Martínez & Rodriguez-Castro 2007, Saha *et al.* 2007, Shaalan & Canyon 2009, Kweka *et al.* 2011). Moreover, in certain cases, backswimmers have been themselves the targets of pesticide application, since they have the capacity of feeding on eggs and immature stages of fishes and tadpoles of economic interest (Bare 1928, Kumar *et al.* 1993, Papácek 2000, Triplehorn & Johnson 2005, Mazzuconi *et al.* 2009). Thus, this studied was carried out aiming to assess the toxicity of the pyrethroid deltamethrin in the backswimmer species *B. tarsalis* and *M. bentoi*, as well as attempting to evaluate the possible disturbances in swimming behavior traits of these species.

2. Material and methods

2.1. Test organisms

Both backswimmers *B. tarsalis* and *M. bentoi* are predatory insects that float upside down and inhabit stagnant water environments (Gittelman 1974, 1976). *Buenoa tarsalis* presents cells with hemoglobin in the abdominal segments 3-7, which gives them neutral buoyancy and allows to remain at a constant level in the water column (Bare 1928, Miller, 1966, Wawrowski *et al.* 2012). Such trait allows *B. tarsalis* to explore the middle region of the water column that is unavailable for other insects (Mazzuconi *et al.* 2009). *Martarega bentoi* constantly swims on the surface of the water or near it (Gittelman, 1974). Adults of *B. tarsalis* and *M. bentoi* were collected using a D-net from pesticides free artificial ponds at the fish-farm station of the Federal University of Viçosa (UFV, Viçosa, MG, Brazil, 20°45´ S, 42°52´ W). The insects were kept under controlled conditions (25 ± 2°C, 70 ± 5% relative humidity, 12h of scotophase) for 24 h before subjected to toxicological assessment or video tracking experiments.

2.2. Exposure medium

All the toxicological and behavioral experiments were conducted using mineral water (Hélios, Dona Eusébia – MG, Brazil) as medium test to minimize between-experiment variability in water chemistry keeing constant deltamethrin bioavailability. The values for physical chemical properties were as follows: HCO_3^- 8.32 mg/L, Na^+ 2.028 mg/L, Ca^{2+} 1.381 mg/L, K^+ 1.381 mg/L, NO_3^- 1.82 mg/L, Mg^{2+} 0.631 mg/L, Cl^- 1.69 mg/L, SO_4^{2-}

0.55 mg/L, F^- 0.02 mg/L, pH 5.61, and conductivity 25.5 µS/cm.

2.3. Concentration-mortality bioassays

For this experiment, adults of the species *B. tarsalis* and *M. bentoi* were exposed to concentrations ranging from 0.5 to $5x10^3$ ng of a.i/L of a deltamethrin commercial formulation (Decis 25EC®, Bayer CropScience Ltda., São Paulo, Brazil). Every group of ten insect was placed in a 0.5 L beaker containing 0.3 L of mineral water and covered with organza to prevent insect escape. The insects were exposed only to mineral water in the control treatment. Four replicates of ten insects for every concentration and species combination were used. Mortality was assessed after 24 h exposure and the individuals were considered dead if they remained motionless after repeated mechanical stimuli with a pipette tip.

2.4. Survival bioassays

Groups of ten individuals were exposed to deltamethrin concentrations determined by the concentration-mortality results. These concentrations ranged from 0.5 to 250 ng of a.i/L for *B. tarsalis* and from 5 to $2.5x10^3$ ng of a.i/L for *M. bentoi.* In the control treatment the insects were exposed only to mineral water. Mortality was assessed every 6 h for three days (72 h); the individuals that remained motionless after repeated mechanical stimuli with a pipette were considered dead and removed from containers to prevent cannibalism.

2.5. Swimming behavior experiments

Activity of the backswimmers was recorded beginning immediately after the insect was transferred to a beaker containing 0.5 L of the exposure medium (9 cm diameter and 8 cm height). The concentrations tested for the swimming experiments were 5 and 50 ng of a.i/L for *B. tarsalis* and, 50 and 500 ng of a.i/L for *M. bentoi.* These concentrations were selected based on the calculated lethal concentrations and the results of the survival bioassays, choosing a low (5 ng of a.i/L for *B. tarsalis* and 50 ng of a.i/L for *M. bentoi*) and a high concentration (50 ng of a.i/L for *B. tarsalis* and 500 ng of a.i/L for *M. bentoi*). Additionally, non-exposed insects (untreated) were also included as control treatment.

Twenty exposed and unexposed adult individuals of each sex of the species *B. tarsalis* and *M. bentoi* were recorded over a 20 minutes period under incandescent light and at a temperature of 25 ± 1°C by using two charge-coupled device cameras (CCD), (one fixed above and one at the lateral of the beaker). The video was digitally transferred to a computer equipped with video-tracking software (VideoTrack System, Viewpoint LifeSciences, Montreal, Canada). The time spent (even in motion or resting) in four depth ranges from 0 to 8 cm was assessed using the lateral view recordings. Additionally, the parameters resting time (s), distance (cm) and velocity (cm/s) were simultaneously measured in both the lateral and top view recordings. The swimming behavioral experiment was separately performed for males and females of both species.

2.6. Statistical analysis

Concentration–mortality curves were estimated by probit analyses using the PROC PROBIT procedure (SAS Institute, 2002). The results of the survival bioassays were subjected to survival analysis performed using the Kaplan–Meier stimator (Log-rank method) with the SigmaPlot 12.0 software (Systat Software, San Jose, California, USA). The swimming behavioral data was analyzed with Generalized Linear Model using the PROC GLM procedure in SAS for both the lateral and top view recording results (SAS Institute, 2002), with behavioral traits (time spent in different depth ranges – only in lateral view -, resting time, distance and velocity) as dependent variables; Fisher's Least Significant Difference (LSD) test was used for multiple comparisons of the means in the same statistical software.

3. Results

3.1. Concentration-mortality bioassays

The probit model was suitable for the concentration-mortality results for both species *B. tarsalis* and *M. bentoi.* Lethal concentrations (LC_{20}, LC_{50}, and LC_{95}) of deltamethrin, which were estimated based on concentration-mortality bioassays, are shown in Table 1. The LC_{50} for *M. bentoi* was almost 26 times higher than that for *B. tarsalis.*

Table 1. Toxicity of deltamethrin to *B. tarsalis* ($n = 290$, $\chi^2 = 1.73$, $P = 0.94$) and *M. bentoi* ($n = 160$, $\chi^2 = 0.68$, $P = 0.71$). Lethal concentrations (LC) values were estimated based on concentration-mortality bioassays using probit analyses. CI denotes confidence interval. Tolerance ratio (TR) was estimated using the CL_{50}. All concentrations are expressed in ng of a.i/L.

Slope ± SE	**LC_{20}** **(95% CI)**	**LC_{50}** **(95% CI)**	**LC_{95}** **(95% CI)**	**TR** **(95% CI)**
Buenoa tarsalis				
1.54 ± 0.16	1.13 (0.66 - 1.70)	4.00 (2,80 - 5,63)	46.83 (28.55 - 93.47)	
Martarega bentoi				
0.65 ± 0.12	6.29 (0.71 - 19.41)	102.5 (39.27 - 228.34)	$2.39*10^4$ ($5,31*10^3$ - $5,27*10^5$)	25.63 (2.47 - 292.02)

3.2. Survival bioassays

The insecticide concentrations tested in this experiment affected significantly the survival (time-mortality response) of both species, *B. tarsalis* (Log-Rank Test, $\chi2 = 246.4$, df = 6, P <0,001) and *M. bentoi* (Log-Rank Test, $\chi2 = 104.0$, df = 5, P <0,001). While the life span of *M. bentoi* in laboratory conditions was shorter than that of *B. tarsalis* , the former species was comparatively less susceptible to the insecticide deltamethrin (Fig. 1). Around 80% of the adults of *B. tarsalis* unexposed to deltamethrin survived after the three days bioassays, whereas survival was about 70% for adults exposed to the lowest deltamethrin concentration (0.5 ng of a.i/L).For the treatments of 2.5 and 5 ng of a.i/L, the survival rates were about 50% and 40% respectively. Deltamethrin concentrations higher than 50 ng of a.i/L killed all the *B. tarsalis* adults in exposure times less than 48h (Fig. 1.A). In the species *M. bentoi* only around 40% of the individuals survived at the end of the bioassays, the lowest concentration tested for this species (5 ng of a.i/L) did not show lower survivorship; when exposed to 50 ng of a.i/L the survival decreasing to around 30%, and the concentrations 500 ng/L of a.i/L and above, caused the mortality of almost 100% of the insects at the end of the bioassays (Fig. 1.B).

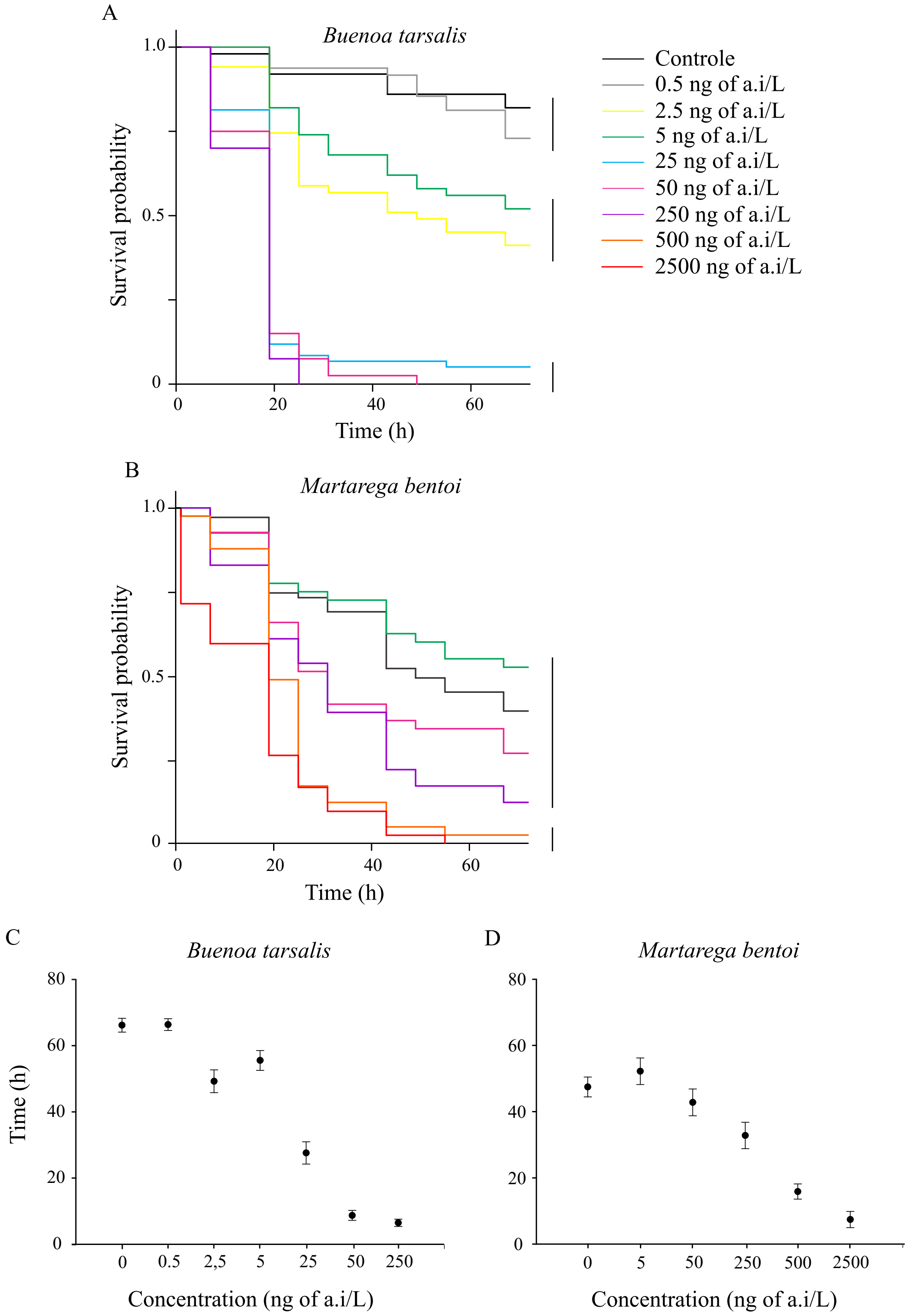

Figure 1. Survival curves for a 72 h exposure to deltamethrin. (**A**) *Buenoa tarsalis*. (**B**) *Martarega bentoi*. Survival curves grouped by the same line are not significantly different by Holm-Sidak's test ($P > 0.05$). (**C**) Mean lifetime of *B. tarsalis* nymphs under 96 h deltamethrin exposure. (**D**) Mean lifetime of *M. bentoi* nymphs under 96 h deltamethrin exposure. Dispersion expressed as SE.

3.3. Swimming behavior experiments

Time spent in the depth ranges

Significant differences on the water column occupancy was observed between species (Table 2). While the *B. tarsalis* preferred to occupy deeper water column parts, *M. bentoi* occupied more superficial parts of it (Fig. 2). Deltamethrin disturbed the *B. tarsalis* water column occupation pattern, forcing these insect to stay longer periods in more superficial areas (Fig. 2) and females of *B. tarsalis* reduced significantly their swimming activity (in lateral view recordings) with increases on the deltamethrin concentration.

Table 2. Results of the GLM analyses for the depth ranges occupancy for males and females of the species *B. tarsalis* and *M. bentoi* . The time spent (s) comprises locomotory and resting activity of the insects.

Sources of variation	**DF**	**Time Spent**	
		F	***P***
Buenoa tarsalis			
Sex (S)	1	0.05	0.82
Concentration (C)	3	0.06	0.94
Depth range (D)	3	167.60	<0.001 *
S x C	3	0.00	0.99
S x D	3	1.54	0.20
C x D	9	3.42	0.0026 *
S x C x D	9	1.21	0.29
Martarega bentoi			
Sex (S)	1	4.51	0.0401 *
Concentration (C)	2	0.29	0.76
Depth range (D)	3	590.07	<0.0001 *
S x C	2	0.43	0.64
S x D	3	23.63	<0.001 *
C x D	6	0.39	0.86
S x C x D	6	0.37	0.87

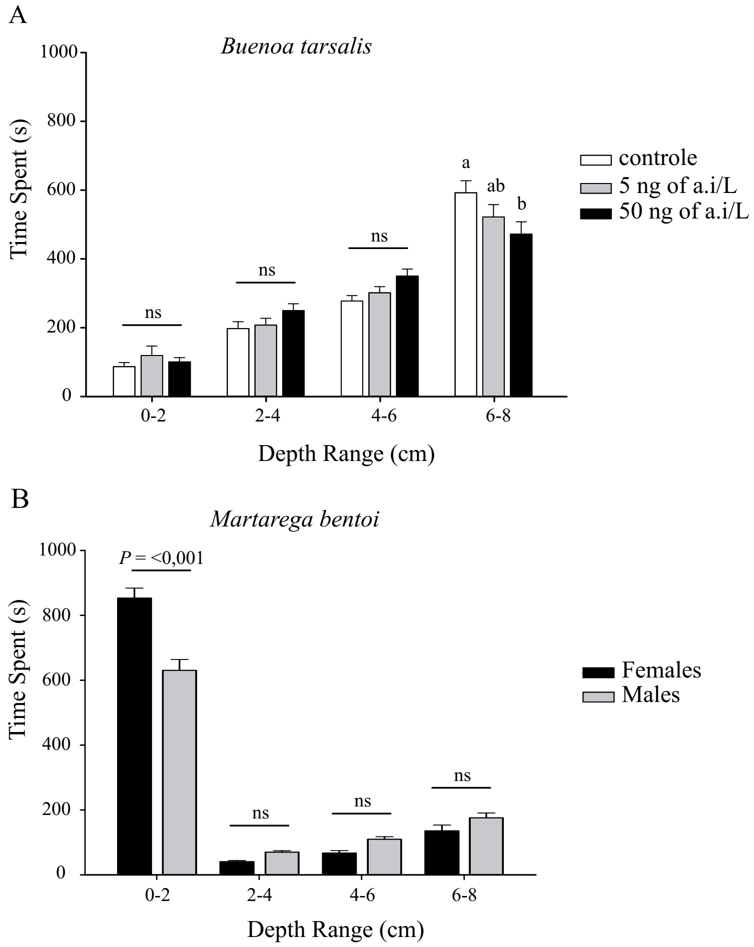

Figure 2. Time spent in different depth ranges in a lateral view of the water column. (**A**) time spent by *B. tarsalis* in every range for exposure to different deltamethrin concentrations. (**B**) time spent in every range by males and females of *M. bentoi.* The time spent comprises locomotory and resting activity of the insects. Each bar represent the mean of 40 (in A) and 60 (in B) replicates. Dispersion expressed as SE.

Locomotory traits from the top and lateral view

Resting time of *B. tarsalis* was the swimming behavioral trait that presented the most striking differences during the exposure to deltamethrin (Table 3). In the lateral view analyses, short-term exposure to deltamethrin concentrations caused and increased in the resting time of the *B. tarsalis* females (Fig. 3.A), but males were unaffected (Fig. 3.B). In both viewpoints, no significant differences were observed for the velocity and distance traveled by *B. tarsalis* in the presence and absence of deltamethrin. For *M. bentoi*, the traits resting time, velocity and distance traveled only showed significant differences between sex (Table 3), whith *M. bentoi* males significantly more active and swam longer distances than *M. bentoi* females. Similar patterns were also observed in the velocity of *M. bentoi* male and females. In this case, males traveled faster than females (Fig. 4).

Table 3. Results of the GLM analyses for the swimming behavior of males and females of the species *B. tarsalis* and *M. bentoi* for top and lateral view.

Viewpoint	Sources of variation	DF	Resting Time (s)		Distance (cm)		Velocity (cm/s)	
			F	*P*	F	*P*	F	*P*
Buenoa tarsalis								
Top	Sex (S)	1	2.34	0.13	0.41	0.52	0.07	0.80
	Concentration (C)	3	3.07	0.05	1.35	0.26	1.24	0.29
	S x C	3	0.65	0.52	0.40	0.67	0.26	0.77
Lateral	Sex (S)	1	1.38	0.24	0.91	0.34	1.14	0.29
	Concentration (C)	3	5.29	0.0063 *	1.22	0.30	1.03	0.36
	S x C	3	3.83	0.0245 *	1.97	0.14	1.83	0.16
Martarega bentoi								
Top	Sex (S)	1	0.45	0.50	0.98	0.32	5.11	0.0257 *
	Concentration (C)	2	0.43	0.65	1.02	0.36	1.25	0.29
	S x C	2	1.29	0.28	2.56	0.08	2.82	0.06
Lateral	Sex (S)	1	36.84	<0.001 *	7.29	0.008 *	12.98	0.0005 *
	Concentration (C)	2	0.77	0.46	1.87	0.16	1.54	0.22
	S x C	2	0.05	0.95	0.27	0.76	0.06	0.94

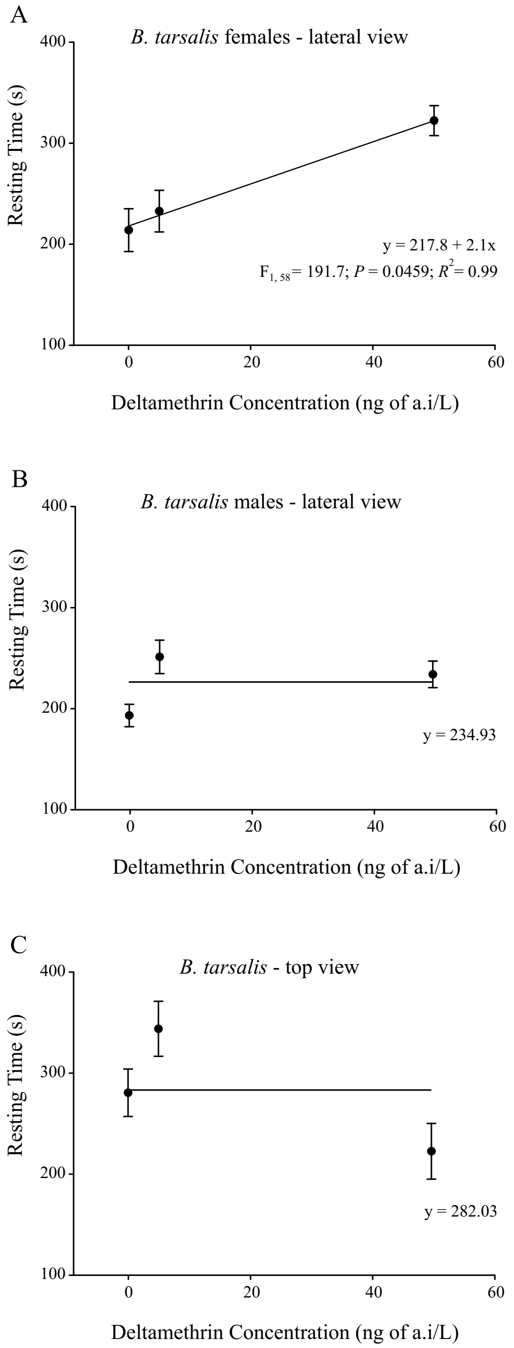

Figure 3. Resting time of *B. tarsalis* males and females . (**A**) linear regression for *B. tarsalis* females in different deltamethrin concentrations, lateral view. (**B**) linear regression for *B. tarsalis* males in different deltamethrin concentrations, lateral view. (**C**) linear regression for *B. tarsalis* for different deltamethrin concentration, top view (males and females combined). Dispersion expressed as SE.

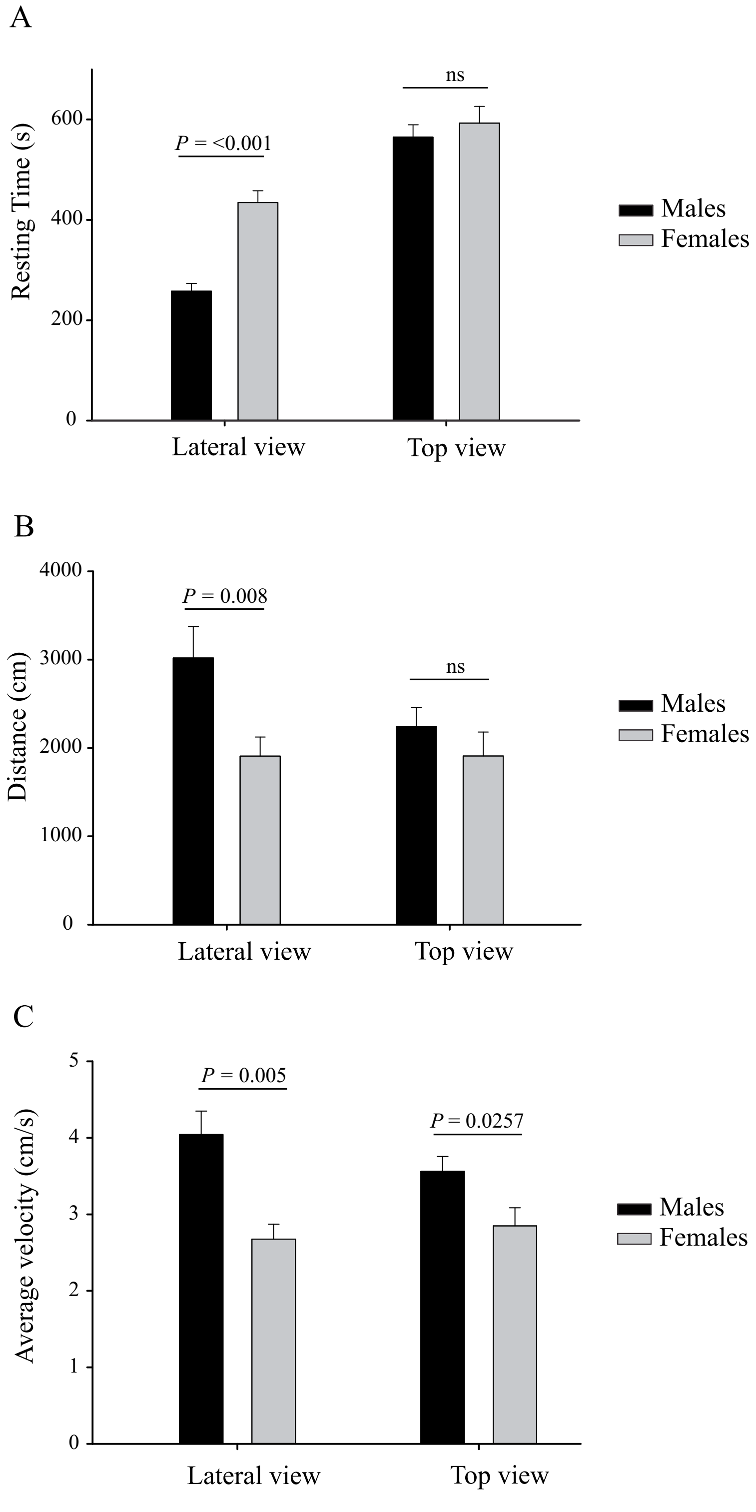

Figure 4. Top and lateral view analysis of the swimming behavior traits of males and females of *M. bentoi.* (**A**) resting time (s). (**B**) distance (cm). (**C**) average velocity (cm/s). Dispersion expressed as SE.

4. Discussion

In this study, the toxicity of the insecticide deltamethrin to the backswimmer species *B. tarsalis* and *M. bentoi*, and the sublethal effects with short-term exposure in several swimming behavior traits were assessed. The results revealed a differential susceptibility to insecticide deltamethrin in two phylogenetically related species that co-habitat the same aquatic environments. While *B. tarsalis* was highly susceptible to deltamethrin in the concentration-mortality and survival bioassays (Fig. 1), as the LC_{50} results suggest (Table 1), *M. bentoi* was more tolerant and their swimming behavior traits were not impaired in short-term exposure to the insecticide.

Backswimmers were previously reported among the most important and sensitive taxa to adverse effects caused by deltamethrin in the field (Lahr 1998), and the results for deltamethrin toxicity to the species *B. tarsalis* assessed in this study is similar to that reported for *Anisops sardeu*, a backswimmer species of the same subfamily of *B. tarsalis* (Anisopinae), for which the estimated LC_{50} was 11 ng of a.i/L (Lahr *et al.* 2001). However, the LC_{50} for the species *M. bentoi* was several times higher than that of *B. tarsalis* (Table 1). This is in accordance with previous studies that have demonstrated the differential susceptibility to insecticides in aquatic species within the same orders or families (Beketov 2004, del Sarto *et al.* 2014). These differences in the susceptibility to chemical compounds may be related to differences in toxicodynamics of insecticide poisoning, or to physiological and biochemical peculiarities associated with the aquatic life of each species (Siegfried 1993).

Besides the toxicological differences between the two species, short-term exposures to different deltamethrin concentrations affected distinctly some of the swimming behavior traits of the backswimmer species included in this study. Differences in the pattern of the depth range occupancy in *B. tarsalis* and *M. bentoi* were expected as several studies report that sympatric and often closely related species are vertically and horizontally stratified (and even different instars of the same species may be similarly stratified) (Gittelman 1976, 1977, Cook & Streams 1984). This stratification can also be influenced by both abiotic and biotic conditions (Streams & Shubeck 1982, Sih 1982, Cockrell 1984a,b, Streams 1987, 1992a,b, Bailey 1987).

B. tarsalis exhibited a definite preference to remain in the deepest ranges (4-6 and 6-8 cm) as they achieve prolonged periods of neutral buoyancy and maintain their position in the water column without continually swimming by using oxygen stored in their hemoglobin to stabilize the volume of the bubble as they breathe from it (Matthews & Seymour 2006). But both males and females exposed to deltamethrin showed a different trend in the occupancy of the water column, spending less time in the deepest range (in comparison with the untreated insects). *M. bentoi*, wich has to periodically renew the air carried in the plastron (Heckman 2011), spent most of the time in the water surface.

Besides of the difference in the pattern of water column occupancy, females of *B. tarsalis* showed a trend to increase the resting time under short-term exposure to deltamethrin, while treated and untreated males exhibited similar swimming activity; and unless the distance swum was not different among the concentrations assessed for both the sexes, the insects unexpectedly achieved to maintain the same average velocity. This could only have happened by either increasing the high speed movements and decreasing the low speed movements, or compensating the lack of movements in some axes with movemente increase in a different one, as discussed below. The deltamethrin concentrations assessed in this study did not impair the swimming behavior of *M. bentoi.* This species was more tolerant to deltamethrin effects in all the aspects, and for this study concentrations higher than 500 ng of a.i/L were not included as this would have been unrealistic. The behavioral traits only showed differences when comparing males and femaleswith males generally more active than females. The females spent more time resting (Fig. 4.A) and remained for longer periods in the water surface (Fig. 2.B), consequently swimming smaller distances (Fig. 4.B) and exhibiting a lower average velocity than males (Fig. 4.C).

Recordings from the lateral view were enough to highlight the most striking differences for all of the behavioral traits assessed in this study (mainly in the case of time spent in the depth ranges and resting time), but the recordings from the top were important as complement of the results since allowed a better understanding of the patterns in the swimming behavior of both species. From this point of view, video tracking experiments has proven to be a useful tool when quantify animal movements in several ecotoxicological studies (Baatrup & Bayley 1993, Nørum *et al.* 2010, Tooming *et al.* 2014). Impairments in the behavior may be markers for fitness-related parameters such as food seeking and predator avoidance (Jensen *et al.* 1997), therefore, assessing the changes in the behavior following contaminant exposure provides a better understanding of the likely environmental consequences of toxic contamination than solely lethal effects (Amiard-Triquet 2009).

In the case of the pyrethroid insecticide deltamethrin, decreasing of the activity seems to be a common impairment in the behavior of exposed arthropods (Everts *et al.* 1991, Tomé *et al.* 2014), which consequently can led to a reduction of the feeding activity (Decoourtye *et al* 2004, Moreira-Santos *et al.* 2005), and alterations of survival (avoiding predators) or reproductive success (Amiard-Triquet 2009). Thereby, presence of deltamethrin in the aquatic environment could cause lethal effects and, when in very low concentrations or under short-term exposures, could induce changes in the swimming behavior traits that may negatively affect the insect populations, as long-term hypoactivity could directly compromise mating, pray catching, and other activities. This can lead to effects in the community structure if inter-specific ecological relations are altered.

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Conclusões gerais

- O inseticida pretróide deltametrina foi altamente tóxico para as três espécies utilizadas na execução deste projeto, sendo o percevejo *B. tarsalis* aquele de maior sensibilidade ao inseticida.

- A avaliação da morfologia do Ephemeroptera *C. radiatus* demonstrou que o inseticida deltametrina pode agir tanto em tecidos considerados alvo da ação (sistema nervoso central), como também pode desencadear efeitos secundários a nível do intestino médio.

- Quando em exposição curta a doses subletais, vários tecidos internos do ephemeroptero *C. radiatus* sofreram alterações significativas. Curtas exposições (até 24h) resultaram em sintomatologia indicativa de apoptose, porém as análises de imunofluorescência e de integridade do DNA não confirmaram tais suspeitas.

- Os resultados da avaliação toxicológica e de atividade natatória dos percevejos aquáticos *M. bentoi* e *B. tarsalis,* permitiram identificar um efeito diferencial do inseticida deltametrina em espécies relacionadas filogeneticamente tanto na mortalidade como na resposta comportamental.

- Na espécie *B. tarsalis*, machos e fêmeas apresentaram alterações no padrão de ocupação da coluna d'água, em quanto que apenas as fêmeas aumentaram o tempo dispendido em repouso quando expostas ao inseticida.