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Short communication

Macro-elements in the hemolymph of adult *Euschistus heros* (Fabr.) (Hemiptera: Pentatomidae) treated with pyriproxyfen

Paulo S.G. Cremonez[a], Janaina F. Matsumoto[a], Avacir C. Andrello[b], Samuel Roggia[c], Daniela O. Pinheiro[d,*], Pedro M.O.J. Neves[a]

[a] *Department of Agronomy, State University of Londrina - UEL, Rod. Celso Garcia Cid, PR-445, Km 380, Mailbox: 10.011, 86057-970 Londrina, Paraná State, Brazil*
[b] *Department of Physics, State University of Londrina - UEL, Rod. Celso Garcia Cid, PR-445, Km 380, Cx. Postal 10.011, CEP 86057-970 Londrina, PR, Brazil*
[c] *Empresa Brasileira de Pesquisa Agropecuária - Embrapa Soja, Rod. Carlos João Strass, PR-545, s/n, Acesso Orlando Amaral, Mailbox: 23, Warta, 86001-970 Londrina, Paraná State, Brazil*
[d] *Department of Histology, State University of Londrina - UEL, Rod. Celso Garcia Cid, PR-445, Km 380, Cx. Postal 10.011, CEP 86057-970 Londrina, PR, Brazil*

A R T I C L E I N F O

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A B S T R A C T

Euschistus heros is an important pest in many crops in Brazil, and different control strategies, mainly involving chemicals, have been evaluated; however, the side effects of these chemicals on the balance of inorganic element levels in the hemolymph are unknown. Thus, the aim of this work was to determine the concentration of inorganic elements (focusing on macro-elements) in the hemolymph of female and male *E. heros* adults, after applying pyriproxyfen at a sublethal concentration ($LC_{30} = 6.68\,mL\,L^{-1}$ diluted in distilled water) to 4th instar nymphs, which were kept in controlled conditions. The hemolymph pool was removed 48 h after adult emergence, centrifuged and placed on an acrylic disk added with Gallium as internal standard for the analysis of total reflection X-ray fluorescence. Most of the elements in the control treatment did not differ between females and males. However, following insecticide application to females and males, respectively, there was a significant increase in sulfur (19 and 51%), chlorine (33 and 137%) and calcium (47 and 82%) in the hemolymph. The significantly higher increase in macro-elements in males' hemolymph indicates that the action of pyriproxyfen may be sex-specific. Phosphorus and potassium concentrations also differed between females and males in the control and treated groups. The observed variation in inorganic elements in the insect's hemolymph may be related to the unknown effects of pyriproxyfen, mainly on immune and reproductive performance.

During the evolution of the Insecta, the chemical composition of the hemolymph developed a balance with the surrounding environment, achieving a status of ionic and mineral homeostasis (Shaw and Stobbart, 1963). The hemolymph is defined as a watery blood consisting of fluid plasma and free nucleated cells, known as hemocytes (Nichols, 1989). Other substances related to the insect's metabolism and physiology such as hormones, lipophorins, phosphatases and trehalose, may also be found in the hemolymph (Wyatt, 1961). Pioneering studies showed that several elements are present in the hemolymph, such as metal ions and macro-elements, mostly in the form of salts, and these play a role in ionic regulation, with variations between different insect species (Snodgrass, 1998).

A series of studies have been published since the last century regarding the chemical composition and biochemistry of hemolymph in arthropods (Beintema et al., 1994; Plantevin, 1967; Shaw and Stobbart, 1963; Sowers et al., 2006; Wyatt, 1961), and some have focused on blood-sucking insects such as mosquitoes and some hemipteran bugs (Clark et al., 2009; Coast, 2009; Fortes et al., 2011; Mantuano et al., 2012).

It is known that some biological control agents can affect the concentrations of inorganic elements in insects such as Lepidoptera. In *Diatraea saccharalis* Fabr. (Lepidoptera: Crambidae) larvae the concentration of macro-elements such as phosphorus (P), sulfur (S), potassium (K) and calcium (Ca) in fat body samples increased after parasitism by *Cotesia flavipes* Cameron (Hymenoptera: Braconidae) (Pinheiro et al., 2010a). In the same insects, a morphological study suggested that ionic imbalance may be associated with alterations in basal labyrinth of columnar midgut cells, as well as in cytoplasm

* Corresponding author at: Biological Sciences Centre, Department of Histology, State University of Londrina, Rod. Celso Garcia Cid, PR-445, Km 380, Mailbox: 10.011, 86057-970 Londrina, PR, Brazil.
E-mail addresses: acandrello@uel.br (A.C. Andrello), samuel.roggia@embrapa.br (S. Roggia), daniela_pinheiro@uel.br (D.O. Pinheiro), pedroneves@uel.br (P.M.O.J. Neves).

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projections that contain mitochondria in the chamber of midgut goblet cells (Pinheiro et al., 2010b); it could also be related to the presence and alteration of spherites in all four types of midgut epithelial cells (Pinheiro et al., 2008). However, studies on the effect of insecticides on macro-element levels in the hemolymph of Pentatomidae have not yet been reported.

Several studies have been carried out to assess the concentration of heavy metals and macro-elements in soils (Adepetu et al., 1988; Jørgensen et al., 2005; Kim et al., 2013), plants (Kim et al., 2012; Ma and Dong, 2014; Pang et al., 2018; Parween et al., 2018), fauna and water (Fianko et al., 2011; Johal and Dua, 1995; Kaur and Dua, 2012) after exposure to synthetic chemical insecticides, which are of great concern to environmentalists as hazardous chemical pollutants (Kabata-Pendias, 2011).

For the control of Neotropical-brown stink bug *Euschistus heros* Fabr. (Hemiptera: Pentatomidae) in Brazilian crops, neurotoxic insecticides such as pyrethroids and neonicotinoids (often in combination) are the main form of control (Tuelher et al., 2018). However, these products are highly toxic to the agroecosystem complex, and are known to cause pest resistance (Castellanos et al., 2018; Santos et al., 2018; Sosa-Goméz and Silva, 2010) and sexual fitness hormesis (Haddi et al., 2016).

The use of biorational control methods, such as Insect Growth Disruptors (IGD), could be a strategy to reduce populations of *E. heros* with lower environmental impact and selection pressure, thus enhancing insecticide resistance management (Ishaaya et al., 2005). Pyriproxyfen, an IGD analog of juvenile hormone (JH), has shown promising results in previous stink bug-pest control bioassays. The effects of JH and its analogous insecticides on the biochemical composition of hemolymph have already been studied (Edwards et al., 1993; Scharf et al., 2005; Yi and Adams, 2000); however, these studies did not focus on macro-element composition in the hemolymph.

The aims of this study were to evaluate macro-element concentrations in the hemolymph of adult *E. heros* treated with a sublethal concentration of pyriproxyfen.

For the bioassays, the insects were reared in polystyrene (PS)-crystal boxes (11 × 11 cm × 3.5 cm). The boxes contained pods of organic common bean, peanuts and soybean as food, and a vial filled with moistened cotton for direct hydration. For this study, only 4th instar nymphs (N4) were used. All procedures were performed under controlled conditions as it follows: 26 ± 1 °C, 65 ± 5% RH and 14 h photoperiod.

A predetermined sub-lethal concentration (LC_{30}) of pyriproxyfen to *E. heros* N4 was used for application using the same methodology as described previously (Cremonez et al., 2017). The commercial product used was Tiger® 100 EC at LC_{30} = 0.668 mL a. i. L^{-1}, diluted in distilled water without adjuvant. For the control treatment, it was used pure distilled water. For each treatment 1 mL was topically applied over a group of ten N4 inside the PS-crystal box using a Potter tower (Burkard Scientific® BS00282, Uxbridge, London) at working pressure of 82.7 kPa. Food and water were added to the insects as described before. Each box was an experimental unit. The survival insects were sexed and individualized in Petri dishes with food, were remained for 48 h until the hemolymph extraction.

The hemolymph of adult's females and males of pyriproxyfen and control treatment was extracted as it follows. Each insect was carefully pined ventral side up in a Petri dish with solid paraffin. Little incisions were made in the lateral abdomen and an aliquot of the flowing hemolymph was carefully drained with a sterile ultrafine needle syringe (8 mm). The collected samples were pooled into a 2.0 mL vial (Eppendorf®) and immediately stored in freezer (−4 °C). After all collection, the vials with the hemolymph pool were centrifuged in room temperature at 6000 rpm with microcentrifuge (HT® CM-610, Hsiangtai Co., Taipei) and the supernatant plasma collected for TXRF analysis.

The sampling order was as it follows: control females; control males; pyriproxyfen-treated females; and pyriproxyfen-treated males. For the procedure, 10 μL of the plasma was placed in an acrylic disk carrier (3 cm diam.) and 10 μL of Ga (10 μg mL^{-1} in deionized water) was added over it as an internal standard (IS). The disks were placed in an oven to dry (60 °C). The samples were analyzed in a TXRF spectrometer (S2 PICOFOX®, Bruker Corp., Billerica, MA), using software Spectra (PICOFOX v 7.2.5.0) following the methodology adapted from Cleto et al. (2016). The samples were analyzed in triplicate at an acquisition time of 300 s per carrier.

The bioassay followed a completely randomized design, with four treatments: pyriproxyfen-treated and untreated (control) females and males. The data were subjected to analysis of variance and the means compared by Tukey test ($p \leq 0.05$). The whole process was formatted and processed with the R® software (R Core Team, 2018).

Initially, it was possible to observe that the absence of background effect indicates no complex matrix effect, with distinct representative peaks related to macro-elements and some minor components (Fig. A.1, Appendix A). The matrix effect is intrinsic from the sample solution and can cause alteration in absorption, leading to systematic errors (Klockenkämper and Von Bohlen, 2015), and the use of certain organic samples may require a preparation technique to attenuate these effects, as seen in high-sugar content beverages (Fernández-Ruiz et al., 2018). However, the data in this study showed that the stink bug's hemolymph present no complex organic matrix that could interfere in the analysis.

The contact with a sublethal concentration of pyriproxyfen increased the macro-element concentration in the hemolymph of adult *E. heros* (Table 1), and a graphic representation is complemented (Fig. 1). Hemolymph levels of S, Cl and Ca in untreated stink bugs did not differ between females and males. However, after insecticide application there was a significant increase in S (19 and 51%), Cl (33 and 137%) and Ca (47 and 82%) in females and males respectively. The concentration of P and K differed between females and males in the control treatment, and P also increased significantly after application of pyriproxyfen in both females and males (22 and 48%, respectively). The levels of K increased by 24% in treated males in relation to the control, however the insecticide did not affect the concentration of this macro-element in the females' hemolymph.

In general, the element concentrations followed two possible

Table 1
Concentration levels (mg L^{-1}) of macro-elements in the hemolymph of females and males of *Euschistus heros* adults treated with pyriproxyfen.

Element	Control treatment Pure distilled water				Pyriproxyfen LC_{30} 0.668 mL a. i. L^{-1}			
	Female		Male		Female		Male	
S	241.00 ± 6.80	a	238.02 ± 2.71	a	285.67 ± 10.37	b	359.34 ± 9.76	c
Cl	237.71 ± 6.41	a	211.71 ± 4.76	a	316.17 ± 22.96	b	502.42 ± 26.90	c
Ca	180.30 ± 4.71	a	185.93 ± 1.34	a	264.99 ± 6.88	b	338.35 ± 8.29	c
P	305.01 ± 15.15	b	268.02 ± 3.47	a	373.39 ± 7.82	c	395.50 ± 19.06	c
K	640.94 ± 12.17	a	751.77 ± 11.77	b	614.33 ± 39.21	a	936.24 ± 44.90	c

Note: Means followed by the same letter in the line did not differ significantly from each other, Tukey test, p ≤ 0,05. a. i. = active ingredient. Internal standard = Ga + deionized H_2O = 10 μg mL^{-1} = 10 ppm.

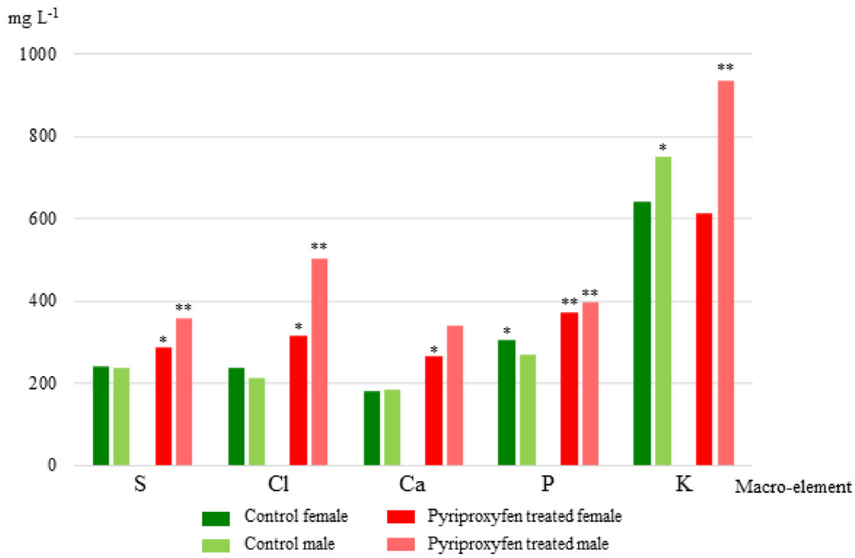

Fig. 1. Concentration and comparison of five macro-elements in the hemolymph of *Euschistus heros* treated with pyriproxyfen. * and ** indicate difference of means within each element by Tukey test (p ≤ 0,05).

patterns: they were similar in untreated females and males, as in the case of S, Cl and Ca; or they differed between sexes before treatment, as in the case of P and K. However, in both situations, concentrations increased after pyriproxyfen treatment (Table 1).

Sulfur is required by all living organisms as basic component of proteins and major metabolites (Roat-Malone, 2002). The sulfur amino acids are important for growth and larval development, and are required for egg-shell formation in females (Shinbo, 1978). The concentration of S may be strongly correlated with major enzymes present in the hemolymph, such as lysozymes and phenoloxidases, which are functionally important in immune humoral responses (Cerenius and Söderhäll, 2004; Nation, 2015), and in hemostasis (blood clotting) in wound injuries (Dushay, 2009). The significant increase in S in pyriproxyfen-treated males and females suggests that degradation of amino acids may have occurred via direct insecticidal action during late juvenile development.

Chlorine is important for ionic homeostasis in insects, being responsible for osmotic adjustment in the ileal region of the hindgut, and its regulation is mediated by a peptide hormone synthesized in the *corpora cardiaca* known as chloride transport-stimulating hormone (CTSH), and supported by ventral ganglia factors (VGFs) (Audsley et al., 1992; Phillips et al., 1996). The increase in Cl in pyriproxyfen-treated males and females suggests that this product may have neurohormonal effects mainly related to homeostasis.

Calcium has many physiological roles, viz. neurotransmitter vesicular transport in synapses, phosphorylase activation, hormonal regulation, gene expression and muscle contraction (Alberts et al., 2014; Chapman et al., 2013). The latter occurs by the regulation of specific Ca^{2+} ion-gated channels known as ryanodine receptors (RyRs) (Ebbinghaus-Kintscher et al., 2006; Nauen, 2006). Specifically, in insects, the cytosolic Ca^{2+} is the main endogenous activator of RyR (Chapman et al., 2013; Sattelle et al., 2008). Furthermore, high concentrations of Ca in the hemolymph can inhibit lipid utilization, ecdysteroid binding and vitellogenesis (Manière et al., 2002; Swevers et al., 2005) and are associated with programmed cell death (Chamberlain, 2004). The hemolymph Ca may be derived from the extracellular contents, and its concentration may reflect the disruption of physiological processes as a side effect of insecticides in *E. heros*.

Phosphorus is a bulk element (Roat-Malone, 2002) and is an important component of many organic molecules, e.g. ADP, ATP and nucleotides, and in the form of the molecular ion PO_4^{3-}, is essential for structural components, energetics and thus general maintenance of life (Filippelli, 2008). The concentration of P is higher in insects with more flight activity (Goldsworthy and Wheeler, 2018; Marden, 2000; Wiesenborn, 2013). Some Pentatomidae such as *E. heros* are considered invasive species and have a high flight capacity and reproductive potential (McPherson et al., 2018), both of which are highly costly metabolic activities (Chapman et al., 2013). On top of that, a previous study on the effect of pyriproxyfen on *Brachynema germari* Kolenati (Hemiptera: Pentatomidae) showed elevated energy allocation in the insect's body, as demonstrated by raised levels of lipids and carbohydrates (Bagheri et al., 2010). The higher amount of P in pyriproxyfen-treated hemolymph of *E. heros* may also be linked to the increased energetic demand of stress-related actions caused by the juvenoid.

Levels of K were higher in the hemolymph of males than in females, and the action of pyriproxyfen seemed to have an additive effect on that disparity. In insects, K plays a role in the ionic regulation of the hemolymph, and in many cases the Na:K ratio is very low, unlike in other invertebrates (Hoyle, 1952). In fact, the K levels vary between species, but in general they are high enough to damage the central nervous system (CNS), a situation that is avoided by insulation of these cells by specialized neurons that create a blood-brain barrier (Banerjee et al., 2006; Desalvo et al., 2011).

Any alterations in K levels can affect normal neuromuscular activity (Hoyle, 1953). Such variations in ionic K^{+} concentration in the hemolymph may be caused by its export from the muscular tissue by the direct flow of hemolymph water and Na^{+} to the gut, which can be triggered by environmental interference, parasitism and associated physiological disorders (Findsen et al., 2014; MacMillan et al., 2012) or even by an alteration in midgut epithelial cells such as goblet and columnar cells, which are responsible for both direct and indirect ionic transport (Anderson and Harvey, 1966; Harvey et al., 1983; Moffett et al., 1995; Pinheiro et al., 2010b; Zeiske et al., 2002). The significant increase in K in females and to a greater extent in males following exposure to pyriproxyfen may be useful for future studies on its side effects on ionic homeostasis and associated neuro-muscular aspects.

Living organisms can be used as indicators of specific environmental parameters of contamination by pollutants due to disturbances in optimal levels of heavy metals and other elements in these organic systems and their components (Al-Hussieny et al., 2015; Andrello et al., 2010; Areington and Varghese, 2017; Kaur and Dua, 2012; Rashed et al., 2009). Such disturbances can arise naturally, but can occur more rapidly and intensely following anthropological interference, such as pesticide application (Kabata-Pendias, 2011). Due to *E. heros* importance and spread throughout the national territory, macro-element alterations in stink bugs' hemolymph treated with pyriproxyfen could be used as a general pesticide contaminant bioindicator in Brazilian agroecosystems. Future studies should be performed to analyze micro- and trace elements in pyriproxyfen-treated pest stink bugs to corroborate our findings.

It is possible to conclude that the insecticide pyriproxyfen significantly increased the concentration of the macro-elements S, Cl, Ca, P and K in the hemolymph of *E. heros*, affecting normal physiological functioning, especially in males. These results indicate that pyriproxyfen may have gender-specific effects. The observed variation in inorganic elements in the insect's hemolymph may also be related to the unknown effects of pyriproxyfen on many physiological pathways.

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Appendix A

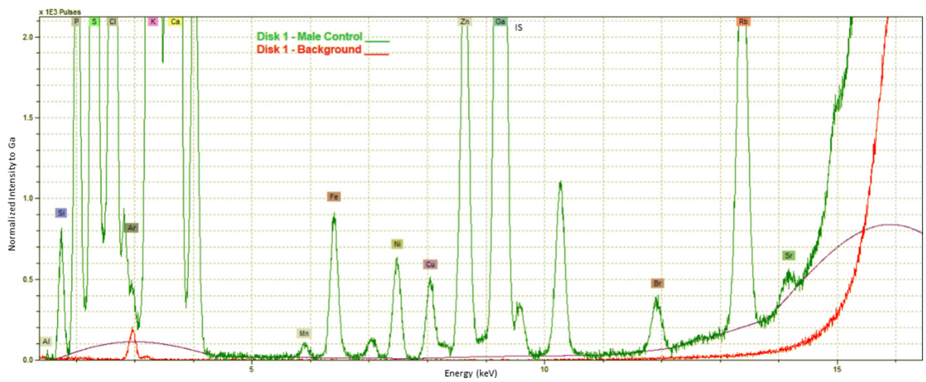

Fig. A.1. Spectra of male *Euschistus heros* hemolymph sample compared with the background reading, presenting the peaks of macro-elements and minor compounds. IS = Internal standard (Ga)

Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2019.02.012.

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