MILTON LEONCIO MONTAÑO CAMPAZ

EVALUATION OF THE EFFECT OF PESTICIDES ON THE BIOLOGICAL TRAITS OF *Chironomus columbiensis*

Thesis submitted to the Ecology Graduate Program of the Universidade Federal de Vicosa in partial fulfillment of the requirements for the degree of Doctor Scientiae, with co-tutelagem with the Doctor (PhD) in Agricultural Sciences at the University of Caldas of Manizales, Colombia.

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I dedicated to my family, especially to my daughter Saray Montaño Quiñones

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“Who is afraid but still dares their dreams will come true”
(Donald Kendall)

GENERAL ABSTRACT

MONTAÑO-CAMPAZ, Milton L., D.Sc., Universidade Federal de Vicosa, July 2022. **Evaluation of the effect of pesticides on the biological traits of *Chironomus columbiensis***. Advisers: Eugenio Eduardo de Oliveira and Lucimar Gomes Dias.

The use of pesticides such as pyrethroids, neonicotinoids and diamides in agriculture has generated a substantial change in agricultural production, but they have also generated great environmental damage, with direct and indirect effects on a wide variety of non-target organisms. These chemical compounds trigger a series of response mechanisms in organisms, which can occur at the physiological, reproductive, morphological, behavioral and genomic levels. For this reason, the objective of this thesis was to evaluate the effects of pesticides on biological traits of *Chironomus columbiensis* under controlled laboratory conditions, and also to evaluate biorational tools that cause less toxicological risks in the environment. To develop these objectives, four assays were carried out to evaluate the individual response mechanisms and processes that occur mainly at the physiological, reproductive and morphological levels. The first experiment (**Chapter II**) presents results on how sublethal exposure of immature *C. columbiensis* to deltamethrin intra- and transgenerationally altered reproduction and wing shape. In addition, it was evaluated whether the populations after exposure to the insecticide could show a recovery of reproduction (increase in the number of eggs per spawning) and wing shape after the elimination of deltamethrin in one or two generations. The second experiment (**Chapter III**) demonstrates sex-dependent changes (emergence, weight, reproduction and shape) in the adaptive responses of *Chironomus columbiensis* to acute and chronic sublethal exposures to imidacloprid. The third experiment (**Chapter IV**) we demonstrate sex-dependent changes (emergence, time to reach the adult stage, body mass, reproduction) and variations in egg and wing shape in responses of *Chironomus columbiensis* to sublethal exposures to chlorantraniliprole and its combination with imidacliprid. The fourth study **(Chapter V)** presents the results of the Chemical composition of essential oils of *Siparuna guianensis* and *Siparuna gesnerioides* and its effect on the *Aedes aegypti* and its predator. This work contains exclusively laboratory data, which evidences the responses of the non-target insect *C. columbiensis* to pesticide stressors such as deltamethrin, imidacloprid and chlorantraniliprole. The understanding the response mechanisms that occur at the individual and population level (e.g., physiology, reproduction, and morphology) is key to the population dynamics of these non-target aquatic insects. Our evidence highlights the need to adopt appropriate management strategies to mitigate the unwanted effects of synthetic insecticides on non-target insects.

Keywords: Synthetic insecticides. Biorational insecticides. Essential oils. Non-target insects

RESUMEN GENERAL

MONTAÑO-CAMPAZ, Milton L., D.Sc., Universidade Federal de Vicosa, Julio 2022. **Evaluación del efecto de plaguicidas sobre rasgos biológicos de *Chironomus columbiensis*.** Orientadores: Eugenio Eduardo de Oliveira y Lucimar Gomes Dias.

El uso de plaguicidas como los piretroides, neonicotinoides y diamidas en la agricultura ha generado un cambio sustancial en la producción agrícola, pero también han generado grandes daños ambientales, afectando directa e indirectamente a una gran variedad de organismos no objetivo. Estos compuestos químicos desencadenan una serie de mecanismos de respuesta en los organismos que pueden darse a nivel fisiológico, reproductivo, morfológico, conductual y genómico. Por ello, el objetivo de esta tesis fue evaluar los efectos de los plaguicidas sobre los rasgos biológicos de *Chironomus columbiensis* en condiciones controladas de laboratorio, así como evaluar herramientas biorracionales que provoquen menores riesgos toxicológicos en el medio ambiente. Para desarrollar estos objetivos se realizaron cuatro ensayos, en los cuales se evaluaron los mecanismos y procesos de respuesta individuales que se producen principalmente a nivel fisiológico, reproductivo y morfológico. El primer experimento (**Capítulo II**) presenta los resultados sobre cómo la exposición subletal de *C. columbiensis* inmaduros a deltametrina alteró intra y transgeneracionalmente la reproducción y la forma del ala. Además, se evaluó la recuperación de estos rasgos biológicos en las poblaciones tras la eliminación de la deltametrina. El segundo experimento (**Capítulo III**) demuestra cambios dependientes del sexo (emergencia, peso, reproducción y forma) como respuestas adaptativas de *Chironomus columbiensis* a exposiciones subletales agudas y crónicas a imidacloprid. El tercer experimento (**Capítulo IV**) demostramos cambios dependientes del sexo (emergencia, tiempo para alcanzar el estado adulto, masa corporal, reproducción), además de variaciones en la forma de los huevos y las alas como respuesta de *Chironomus columbiensis* a las exposiciones subletales al clorantraniliprol y su combinacion con el imidacloprid. En el cuarto estudio **(Capítulo V)** presenta los resultados de la composición química de los aceites esenciales de *Siparuna guianensis* y *Siparuna gesnerioides* y su efecto sobre el vector *Aedes aegypti* y su depredador. Este trabajo contiene exclusivamente datos de laboratorio, que evidencian las respuestas del insecto no objetivo *C. columbiensis* a estresores plaguicidas como la deltametrina, el imidacloprid y el clorantraniliprol; además de evaluar las respuestas de *Aedes aegypti* y su depredador frente a la exposición a aceites esenciales de dos especies del género *Siparuna*. La comprensión los mecanismos de respuesta que se producen a nivel individual y poblacional (por ejemplo, fisiología, reproducción y morfología) es clave para la dinámica poblacional de estos insectos acuáticos. Nuestras pruebas ponen de manifiesto la necesidad de adoptar estrategias de gestión adecuadas para mitigar los efectos no deseados de los insecticidas sintéticos sobre los insectos no objetivo.

Palabras clave: Insecticidas sintéticos. Insecticidas biorracionales. Aceites esenciales. Insectos no objetivo

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SUMMARY

CHAPTER I

General introduction and overview

General introduction and overview

1. Impact of agricultural activities and use of pesticides on aquatic ecosystems aquatic ecosystems

Freshwater ecosystems are considered one of the most important natural resources for life. In terms of their biological value, these ecosystems stand out for containing a rich and varied biota, including a high diversity of fish, plants, algae, bryophytes, bacteria and mainly invertebrates (Jonsson et al. 2001; Barón et al. 2003). Despite their importance, the transformation of the landscape by human activities has generated negative consequences for aquatic ecosystems. Changes in land use and productive activities developed near the banks of rivers and streams have progressively affected the water quality of many watersheds globally (Murgueitio & Ibrahim, 2009; Dudgeon, 2013; García-Moreno et al., 2014).

In the Colombian Andean ecosystems, alterations due to agricultural activities are among the most frequent (García & Beck, 2006; Mena & Hofstede, 2006; Sánchez-Vega & Dillon, 2006; Defensoría del Pueblo Colombia, 2010). It is considered that 70% of the forest cover in the region has been transformed, mainly due to increased water demand for agricultural and industrial activities (Murgueitio & Ibrahim, 2001; IDEAM, 2008). These activities affect soil structure and, consequently, water resources and their associated fauna and flora (Wardle, 1995; Filser et al., 1995; Lal, 1998; Marín & Feijoo, 2005). In addition, it is known that intensive agriculture, monocultures and the application of agrochemicals promote the reduction of biodiversity and an inevitable environmental imbalance (Brévault et al., 2007).

Studies have shown that pesticides used in agricultural activities can have toxic properties in aquatic fauna, both in sublethal and chronic doses, acting as endocrine and immune system disruptors (Taxvig et al., 2008), modulating and acting exogenously on certain cell receptors. In addition, other authors have shown that some pesticides can affect the reproduction of aquatic fauna and cause male sterility in certain fish species (Stahlschmidetallner et al., 1997; Babín et al., 2005; Laier et al., 2006; Trösken et al., 2006; Kinnenberg et al., 2007).

In natural aquatic systems near areas with agricultural activities, certain pesticides are likely to be present in low concentrations that are persistent compounds. These pesticides usually resist biotic and abiotic degradation and cause sublethal effects (reproduction, development, morphological deformities, etc.) in a large number of species in the ecosystem.

There is enormous variability in bioavailability and toxic characteristics between and within different groups of contaminants (Rand, 1995).

2. General aspects of pesticides

Pesticides are substances or mixtures of substances used to prevent, control or destroy a pest (FAO). Their use dates back to the 19th century, starting with highly toxic compounds such as calcium arsenate, lead arsenate, hydrogen cyanide and sulfur, used to control fungi, insects and bacteria (Zacharia, 2011). Due to their high toxicity, they were no longer applied and were replaced by second-generation pesticides, which were known as synthetic organic compounds, such as DDT, among others (Zacharia, 2011).

Pesticides include:

- Products that can be administered to combat insects, arachnids or other pests.
- Substances used as plant growth regulators, defoliants, desiccants, agents to prevent premature fruit drop.
- Substances applied to crops before or after harvest to prevent deterioration during storage and transport (FAO, 1990).

Today there are more than 1500 active ingredients that, in different mixtures and concentrations, generate more than 50000 products registered worldwide as pesticides (Villaamil et al., 2013). Due to the large number of synthetic pesticides that vary in their identity, chemical and physical properties, mechanisms of action and toxicity, these are classified into different groups according to need, target pest, chemical structure, mode of action and origin (CASAFE, 2015).

Depending on the target pest, pesticides are classified as herbicides, algaecides, insecticides, acaricides, molluscicides, rodenticides, avicides, fungicides, bactericides and virucides (Zacharia, 2011).

According to the chemical structure, it has the advantage of grouping substances with similar effects on pests, in the environment and similar intoxications in humans. One of the most commonly used classifications combines the chemical group with the mechanism of action on pests, i.e. the specific physiological process that is affected by the pesticide (Bedmar, 2011).

Pesticides can be of natural, biological and chemical synthesis origin. Natural pesticides can be of mineral or vegetable origin. Among those of mineral origin we can mention sulfur and copper compounds. Among those of vegetable origin, nicotine or pyrethrum. Biological products include those consisting of very specific microorganisms, such as Bacillus thuringiensis, which controls lepidoptera. However, the vast majority of phytosanitary products used today are products obtained by chemical synthesis (CASAFE, 2015).

3. Insecticides

Depending on the chemical structure, a wide variety of families of compounds are considered, which can be divided into two large groups, conventional insecticides and biorational insecticides (CASAFE, 2015).

The first group comprises "modern chemically synthesized" insecticides, which began with the discovery of the insecticidal properties of DDT, which is part of the group of organochlorines - chlorinated hydrocarbons - together with lindane, endosulfan, aldrin, dieldrin and chlordane, currently banned almost everywhere in the world (Zacharia, 2011). In view of their high toxicity to non-target organisms and their bioaccumulation in the food chain, they were replaced by less persistent groups such as organophosphates - esters, amides or thiols derived from phosphoric acid (Spiro & Stigliani, 2004). Finally, among the conventional ones are carbamates-derivatives of carbamic acid, also inhibitors of acetylcholinesterase but in a reversible manner, which makes them less toxic to mammals, and pyrethroids-analogous to the natural pyrethrins. Among the most commonly used synthetic pyrethroids are permethrin, cypermethrin and deltamethrin (Zacharia, 2011).

The second group comprises compounds with a different toxicological profile, called "new generation insecticides" and includes growth regulators and food toxins that act within the insect, in processes such as metamorphosis and digestion, and semiochemicals that influence the interaction between individuals of the same species pheromones and different species-allelomones. These arise as a response to the concern about the negative effects on the environment derived from the massive use of pesticides (Pérez et al., 2013).

According to the Cámara de Sanidad Agropecuaria y Fertilizantes (CASAFE), in the Manual Fitosanitario (2015) there are four large groups of molecules, which act on:

- Nervous-muscular system: To this group belong the conventional organophosphates, carbamates, pyrethroids, neonicotinoids (imidacloprid) and diamide (chlorantraniliprole).

Growth, development and reproduction: It groups biorational insecticides, both growth regulators and semiochemicals.

- Respiration and energy metabolism: Currently in disuse, they include compounds derived from arsenic and fumigants such as methyl bromide.
- Digestive system: They include phytochemical substances isolated from plants and endotoxins from microorganisms that interfere with the normal functioning of the insect's digestive system.

4. Most used pesticides in pest control programs in Caldas

Today there is a great variety of pesticides on the market, where farmers can choose which one to use depending on their needs. Among the most used pesticides in control programs in the department of Caldas are the insecticide the Deltamethrin, Imidacloprid and Chlorantraniliprole.

Deltamethrin, known as a type II pyrethroid, is one of the most widely used pyrethroids in agricultural crops for insect control and affects immature and adult specimens (Rozilawati et al., 2005; EPA, 2013; Bengoa et al., 2014). This substance can enter aquatic ecosystems through direct applications for the control of mosquito vectors of human disease (Rozilawati et al., 2005; Bengoa et al., 2014) or through runoff and drainage from agricultural soils (Schulz, 2001; Reichenberger et al., 2007; Bereswill et al., 2013). Deltamethrin degrades rapidly in sunlight, which could reduce the exposure of some aquatic invertebrates to it (Day, 1989; Wheelock et al., 2005; Lawler et al., 2008). Nevertheless, the presence of Deltamethrin has been demonstrated in water and sediments of aquatic ecosystems (Pawlisz et al., 1998; Amweg et al., 2006; Weston et al., 2006; Feo et al., 2010).

Imidacloprid is an insecticide neonicotinoid, its mode of action is systemic, by ingestion and contact. The mechanism of action in the nervous system binds irreversibly to the nicotinic acetylcholine receptors of insects, causing paralysis and death in a short period of time (Pitti Serrano, 2011). The efficacy of this insecticide is based on the almost total and practically irreversible blockage of the acetylcholine receptor protein in the postsynaptic cell, which interferes with the chemical transmission of the nerve stimulus during synapsis (Cox, 2001; Osorio, 2000). After foliar applications, it has a good residual effect, high light stability and resistance to rain washout. In addition, good translaminar and acropetal distribution has been observed, which allows good control of hidden pests, as well as adequate protection of plant parts that develop after application (Elbert et al., 1991).

Chlorantraniliprole is an insecticide of the ryanodine receptor modulator class, which belongs to a new class of selective insecticides offering a new mode of action to control a number of pests belonging to the order Lepidoptera and some other species of Coleoptera, Diptera and Isoptera. It acts on the muscular activity of insects, immobilizing them and suspending their feeding. This guarantees superior crop protection, since by stopping feeding, significant damage is prevented, even when the pest is still alive (Dupont, 2009).

On the other hand, essential oils are natural products formed by several volatile compounds (Sangwan, 2001). These are formed by complex mixtures originating from the secondary metabolism of plants, which may be located in hairs, vascular system, leaves, stems, flowers or other sites depending on the plant species. Oils are present in low quantities and constitute only a small fraction of the total weight of the plant. The quantity and composition of essential oils varies not only with the type of plant, but in particular with the conditions prevailing during plant growth, such as climate, soil, altitude, etc. (Shaaya and Rafaeli, 2007).

Among the main components of essential oils are phenols, terpenes, acids, esters, acetones, alcohols and aldehydes, which determine the aroma and bioactivity characteristic of the plant from which they originate (Batish et al., 2008). These compounds can be extracted mainly by hydrodistillation, hydrofusion, carbon dioxide extraction, microwave and steam distillation, the latter being the most common (Stefanazzi, 2010; ISO9235, 2013). As for bioassays for the control of insect pests, essential oils from plant products such as marigold, anise and pericón have been used.

5. Some tools used in pesticide effect assessment

Biomarkers are used to evaluate biochemical, cellular, physiological, morphological or behavioral responses, which can be measured at the organism, population or community level, and these responses are reflected in the face of a potential danger from one or more contaminants (Toro, 2011). Its importance lies in facilitating the demonstration of cause/effect relationships and it is a key tool for detecting the impact of pollution on the health of ecosystems (Van der Oost et al., 2003).

In developed countries, different methodologies have been proposed and implemented for the evaluation of the environmental effects produced by contaminants, among them are the following: biotic indices, toxicity tests, evaluation of morphological variations or deformities, genetic variations, and specific biological traits (Bonada et al., 2006; Prat et al., 2012). Therefore, a toxicity test is a complementary tool to detect and evaluate the inherent capacity of an agent to produce toxic effects on living organisms (Chapman et al., 1987; Larraín, 1995; EULA, 1999).

According to Bonada et al. (2007), the use of species traits of aquatic macroinvertebrates as a bioindicator tool has shown promising results. The idea is to replace the list of species with a list of biological characteristics (morphometrics, body shape, life cycle, feeding, reproduction, etc.) and use the combination of these characteristics as bioindicators. These biological traits have proven to be useful in relating variations in indicator organisms to different environmental impacts. An example of this is the studies carried out with members of the Chironomidae family, mainly the genus *Chironomus*, which in the presence of environmental stress can develop body deformities, both in the mouthparts and in other parts of the body (Warwick, 1985; Lenat, 1993; Al-Saffar, 2007; AlShami et al. 2010).

This family comprises one of the most diverse groups of aquatic insects in river systems, playing an important role at all levels of the food chain and with great potential in water source biomonitoring programs (Ferrington, 2008). They are recognized for being opportunistic and fast colonizing species that adapt to fluctuating conditions (Ruse, 1995). Also, Chironomidae larvae are considered resistant to extreme physicochemical conditions, such as changes in pH, temperature, dissolved oxygen, current velocity, salinity and depth (Trivinho, 2011; Viveiro, 2012). The complete larval cycle occurs in the aquatic environment and in direct contact with the sediment and may remain buried.

6. Research objective and chapter outline

The objective of this thesis was to evaluate the letal and sublethal effects of some pesticides (deltamethrin, imidacloprid and chlorantraniliprole) most used in Colombian agriculture, on specimens of *Chironomus columbiensis*. In addition, to evaluate alternatives that can provide a more rational control (i.e., lower environmental toxicological risks) of agricultural pests.

The thesis comprises five experimental chapters with especific objectives, where physiological, reproductive and morphological responses that occur at the individual and population levels are evaluated.

Chapter II evaluates the use of chironomids as test organisms, where a protocol for their rearing under laboratory conditions is established. In addition, information is presented that will allow to broaden the knowledge of their biology.

Chapter III to assess whether sublethal exposure of immature *C. columbiensis* to deltamethrin would elicit within (sublethal)- and trans-generational effects on reproduction (e.g., fecundity and fertility rates) and wing shape. We further evaluated whether the deltamethrin sublethal exposure over three consecutive generations would modify the amplitude responses for the within- and trans-generational effects. By rearing *C. columbiensis* for three consecutive generations, we also evaluated the population recovery after removal of deltamethrin.

Chapter IV to evaluate the susceptibility of larvae of *C. columbiensis* (Diptera: Chironomidae), an aquatic insect used as an indicator of water quality, to imidacloprid. We further evaluated whether the chronic and acute imidacloprid sublethal exposures of *C. columbiensis* larvae would impair adult emergence, wet weight, reproductive success and wing shape.

Chapter V to evaluate the susceptibility of larvae of C. columbiensis (Diptera: Chironomidae), an aquatic insect used as an indicator of water quality, to chlorantraniliprole. In addition, we evaluated whether chronic sublethal exposures to chlorantraniliprole of C. columbiensis larvae would impair adult emergence, wet weight, emergence time, reproductive success, and egg and wing shape.

Chapter VI aimed to evaluate the larvicidal effect of *S. guianensis* and *S. gesnerioides* essential oils on *Aedes aegypti* was evaluated. In addition, whether the application of these essential oils would be selective against the predatory hemiptera *Belostoma anurum.*

7. Articles derived from the thesis

Chapter II: *Chironomus columbiensis* (Diptera: Chironomidae) as test organism for aquatic bioassays: Mass rearing and biological traits

Milton L. Montaño-Campaz [a, b], Lucimar G. Dias [c], Beatriz Toro-Restrepo [c]

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Chapter II: Exposures to deltamethrin on immature *Chironomus columbiensis* drive sublethal and transgenerational effects on their reproduction and wing morphology

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Chapter III: Acute and chronic sublethal exposures to imidacloprid cause sex-dependent changes on the adaptive responses of *Chironomus columbiensis*

Milton L. Montaño-Campaz [a, b], Eugenio E. Oliveira [e,] Tito Bacca [d], Beatriz Toro-Restrepo [c], Lucimar G. Dias [c].

Chapter IV: Exposure to chlorantraniliprole and its combination with imidacloprid reduces reproduction and body mass, and causes shape changes in eggs and wings of *Chironomus columbiensis*

Milton L. Montaño-Campaz [a, b], Eugenio E. Oliveira [e,] Tito Bacca [d], Beatriz Toro-Restrepo [c], Lucimar G. Dias [c].

Chapter V: Chemical composition of essential oils of *Siparuna guianensis* and *Siparuna gesnerioides* and its effect on the *Aedes aegypti* and its predator

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CHAPTER II

Exposures to deltamethrin on immature *Chironomus columbiensis* drive sublethal and transgenerational effects on their reproduction and wing morphology

Exposures to deltamethrin on immature *Chironomus columbiensis* drive sublethal and transgenerational effects on their reproduction and wing morphology

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Abstract

Sublethal exposure to insecticides can trigger unintended responses in non-target insects that may disrupt reproductive and developmental performances of these organisms. Here, we assessed whether sublethal exposure to the pyrethroid insecticide deltamethrin in early life had sublethal and transgenerational effects on the reproduction (i.e., fecundity and fertility) and wing morphology of *Chironomus columbiensis*, an aquatic insect used as a water quality indicator. We first conducted concentration-response bioassays to evaluate the susceptibility of *C. columbiensis* larvae to deltamethrin. Our results revealed that deltamethrin toxicity was approximately 7-fold higher when *C. columbiensis* larvae where exposed to 96 h (LC_{50} = 0.17 [0.15–0.20] µg/L) than to 24 h (LC_{50} = 1.17 [0.97–1.43] µg/L). Furthermore, the sublethal exposures (at LC_1 = 0.02 µg/L or LC_{10} = 0.05 µg/L) of immature *C. columbiensis* resulted in lower fecundity (e.g., reduced eggs production) and morphometric variation wing shapes. Further reduction in fertility rates (quantity of viable eggs) occurred at deltamethrin LC_{10} (0.05 µg/L). Almost 80% of the fecundity was recovered with only a single recovery generation; however, two subsequent recovery generations were not sufficient to fully recover fecundity in *C. columbiensis*. Specimens recovered from 98.5% of wing morphometric variation after two consecutive generations without deltamethrin exposure. Collectively, our findings demonstrates that sublethal exposure to synthetic pyrethroids such as deltamethrin detrimentally affect the reproduction and wing shape of *C. columbiensis*, but also indicate that proper management of these compounds (e.g., concentration and frequency of application) would suffice for these insects' population recovery.

Keywords: Ecotoxicology, Aquatic bioindicators, Pyrethroids, Unintended effects

1. Introduction

Although most of aquatic insects are not the targets of insecticides, these organisms are exposed to pesticides through compounds that enter the aquatic environment from urban and agricultural runoff, spray drift, or direct application (Antwi and Reddy, 2015; Dalhoff et al., 2020; Zhu et al., 2020; Pinto et al., 2021). Therefore, the success of aquatic insects is due largely to their ability to adapt to these stressors, either through genetic assimilation of fitness-related phenotypic variations or through the expression of beneficial traits that are phenotypically neutral under normal conditions (Guedes et al., 2017; Olguín-Jacobson et al., 2021; Meng et al., 2021). Furthermore, sublethal exposures to such organic pollutants have shown to cause alterations physiology, reproductive success and even generate morphological alterations in wing shapes (Valbon et al., 2021; de Oliveira et al., 2021).

Deltamethrin, a type II pyrethroid insecticide used ubiquitously to control garden (urban) and agricultural insect pests and disease vectors (e.g., mosquitoes) (US EPA, 2013), is one of the most common pollutants in aquatic environments, and its indiscriminate use has raised concern for environmental safety due to the serious damage it causes at different levels of the trophic chain (Palmquist et al., 2011; Toumi et al., 2013; Antwi and Reddy, 2015; Guti´errez et al., 2017; Valbon et al., 2018; Montaño-Campaz et al., 2019; Meunier et al., 2020). Despite its rapid degradation in sunlight (photolabile), which may reduce long-term exposure (Day, 1989; Wheelock et al., 2005; Lawler et al., 2008), deltamethrin has been shown to remain in sediment long-term, and may thus be lethal to aquatic populations (Amweg et al., 2006; Weston et al., 2006; Feo et al., 2010; Antwi and Reddy, 2015). Furthermore, Sublethal exposure to deltamethrin can directly affect (positively or detrimentally) *C. columbiensis* population size by altering reproductive performance, and this may have ecological, evolutionary, and epidemiological consequences. Alterations to wing shape may have similar effects, as insect wings are involved in dispersive flights, and wing shape may be a useful phenotypic marker of pyrethroid use in the life history of insects that persisted after pyrethroid application (Nattero et al. 2019, 2021).

Thus, the understanding of undesired impacts of deltamethrin on non-target aquatic insects is a key part of its successful management, particularly for the successful implementation of deltamethrin into the integrated pest management (IPM) in agriculture, forestry, and mosquito control in peridomicile areas. As chironomids are frequently and routinely included in bioassays evaluating the effects of pesticides (Monteiro et al., 2019; Varg et al., 2021; Rodrigues et al., 2015; Majlesi et al., 2020), we examined the Neotropical species

C. columbiensis as a sentinel organism for the lethal and sublethal toxicity of deltamethrin. We selected *C. columbiensis* because these organisms can be grown easily under laboratory conditions, develop relatively quickly, are highly susceptible to agricultural and mining activities (Montaño-Campaz et al., 2019) and play an relevant ecological role in Neotropical freshwater environments (e.g., distribution, abundance, and importance as a prey of young and adult fish) (Taenzler et al., 2007).

Thus, the present study aimed to evaluate whether sublethal exposure of immature *C. columbiensis* to deltamethrin would elicit within (sublethal)- and *trans*-generational effects on reproduction (e.g., fecundity and fertility rates) and wing shape. We further evaluated whether the deltamethrin sublethal exposure over three consecutive generations would modify the amplitude responses for the within- and *trans*-generational effects. By rearing *C. columbiensis* for three consecutive generations, we also evaluated the population recovery after removal of deltamethrin.

2. Materials and methods

2.1. Breeding conditions of *C. columbiensis*

The chironomids used in the study were obtained from a standardized culture established in 2012 at the Zoology laboratory of the University of Caldas (Manizales, Colombia) and that have been reared under controlled conditions in a pesticide-free environment. For the experiments, fresh egg masses were incubated at 23 ± 2 ◦C in glass aquariums with 5 L of semi-soft reconstituted water (pH = 7.2 ± 0.3, electric conductivity = 177 µS/cm2, hardness 138 mg of CaCO3/L) and kept under a 12 h light regimen (Montaño-Campaz et al., 2019). After the hatching of the eggs, individuals from the first larval stage were obtained, which were undergone experimental treatments.

2.2. Acute toxicity bioassays

The first instar larvae of *C. columbiensis* were exposed to different concentrations (i.e., 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56, 5.12 and 7.68 µg/L) of deltamethrin (25 g/L, emulsifiable concentrate, Bayer SA Colombia, Bogota, ´ Colombia.), or to a reconstituted water (i.e., control treatment). These concentrations of deltamethrin were selected after preliminary bioassays with a wide concentration interval, which allowed the selection of the highest concentration unable to kill *C. columbiensis* larvae, and the lowest concentration capable of killing 100% of exposed larvae. The larvae were exposed for 96 h with evaluations at 24 h intervals. Individuals were considered dead when no movement of their appendages was observed after repeated gentle mechanical stimulation with a pipette tip. The experimental unit consisted of groups of 10 *C. columbiensis* larvae that were submitted to 50 mL of de water solution (water + deltamethrin) in 100 mL glass vials. Each treatment was replicated eight times.

2.3. Transgenerational and multigenerational effects of deltamethrin exposure on *C. columbiensis* fecundity and fertility

We exposed the first generation the first instar larvae of *C. columbiensis* (G_0, n = 720, randomly divided into 240 groups) to reconstituted water (control) or to one of two sublethal concentrations (LC_1 = 0.02 µg/L and LC_{10} = 0.05 µg/L) of deltamethrin-contaminated water solution (Fig. 1). The experimental units consisted of groups of 80 larvae that were transferred to glass aquariums (35 × 30 × 20 cm) containing two sheets of crushed disposable towels (as a substrate) and 5 L of water solution (uncontaminated or containing deltamethrin at LC_1 or LC_{10}) via a gentle aeration system. The larvae were fed 0.3 g of TetraMin twice/week until pupation. The larvae were exposed to the treatment throughout immaturity, including during the pupal phase.

The adults were not exposed to any insecticide. For each aquarium, we randomly selected 10 egg masses (or spawnings) to be photographed using a Leica M205C microscope in order to establish a manual record. In order to determine fertility (i.e., % of hatched eggs), we placed the photographed spawnings in plastic tubes (1.5 cm high × 2.5 cm wide), which were closed at the base with porous fabric to allow aeration and exit of the larvae. We left these in the aquariums for five consecutive days, at which time the unhatched eggs were counted using a stereomicroscope, and the percentage of hatched eggs calculated.

In order to assess the transgenerational and multigenerational effects of sublethal exposure to deltamethrin, we conducted the experiment using two other consecutive insect generations (G_1 and G_2), following exposure procedures similar to those described for G_0. We also evaluated any potential recovery from deltamethrin-mediated stresses during immaturity by exposing the offspring of G_0 individuals exposed to deltamethrin LC_{10} (0.05 µg/L) to uncontaminated water across one (G_1) or two (G_1 and G_2) subsequent generations. We also evaluated the recovery potential of the offspring of *C. columbiensis* strains that were exposed to deltamethrin for two consecutive generations (G_0 and G_1).

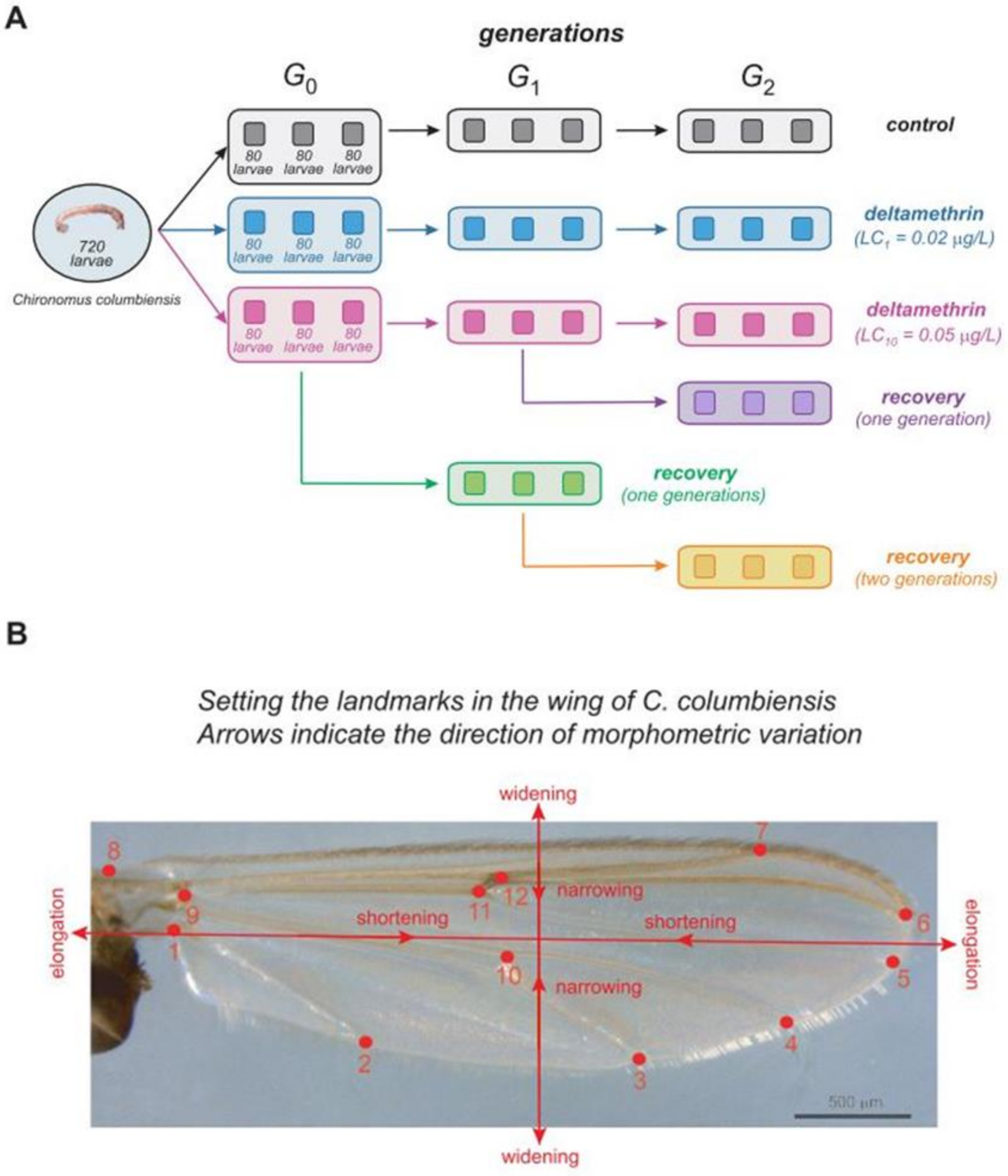


Fig. 1. (***A***) Experimental design used to measure the transgenerational and multigenerational effects of deltamethrin exposures on the reproduction and wing shape of *Chironomus columbiensis*. (***B***) Setting the landmarks in the wing of *C. columbiensis*. Arrows indicate the direction of morphometric variation. Variations in the vertical axis means widening or narrowing, while variations on the horizontal axis means either elongation or shortening.

2.4. Transgenerational and multigenerational effects of deltamethrin exposures on *C. columbiensis* wing shapes

We conducted morphometric analyses using images of the right wings of adult female *C. columbiensis* from each treatment (Fig. 1*B*). The photos were taken using a Leica M205 stereomicroscope equipped with a digital camera. All photos were saved as JPEG files and all specimens were photographed in the same position using clipboard mounts and object covers. Twelve reference points were digitized (Fig. 1*B*) according to the geometric configuration proposed by Montaño-Campaz et al. (2019). Positional homology criteria, relative position consistency, adequate shape coverage, and repeatability were analyzed using tpsDIG2 v2.17 software (Rohlf 2013). X-Y coordinates were obtained, and shape information was extracted using a full Procrustes adjustment (Rohlf and Slice, 1990; Dryden and Mardia, 1998).

We followed the protocol proposed by Palmer and Strobeck (1986), which aids the assessment of measurement error (ME) and ensures the correct procedure for acquiring benchmarks. Analysis of variance (ANOVA) was used to assess possible differences in centroid size, and a Procruste ANOVA was used to evaluate shape differences while considering ANOVA individual variation MS (MS) values with type 1 error. The main patterns of variation in wing shape were visualized using a principal component analysis (PCA) and were calculated from the covariance matrix of the symmetrical component of the average population form (Klingenberg et al., 2002). To statistically assess the differences among treatment groups, a canonical variable analysis (CVA) of the variation in shape was also performed and the Mahalanobis and Procrustes distances were calculated (Campbell and Atchley, 1981).

The CVA is one of the most frequently applied tools for discriminating among groups because it maximizes variation among groups in relation to variation within groups (Campbell and Atchley, 1981). In order to analyze the relationship between the shape and size of the centroid, a multivariate regression was performed that included each group's confidence ellipse (90%) using form and scores with 10,000 iterations. Statistical and morphometric analyses were performed using MorphoJ 2.0 software (Klingenberg, 2011).

2.5. Statistical analysis

The concentration-mortality results obtained in the toxicological bioassays were subjected to a probit analysis using the PROBIT procedure in SAS statistical software (SAS Institute, 2008). For all results obtained during the multigenerational and transgenerational bioassays, we used R software, version 3.6.2 (R Core, 2020) available at the RStudio (R Studio, 2020). We used type II ANOVA tables to evaluate the importance of explanatory variables in all models (generalized linear models, GLM; generalized linear mixed models, GLMMs) using the "Anova" function in the *car* library (Fox and Weisberg, 2018). The data were organized using the *dplyr* library (Wickham et al., 2018), and the figures were constructed using the *sjPlot* (Lüdecke and Schwemmer, 2016) and *ggplot2* (Wickham, 2016) libraries. The fecundity evaluations were fit to GLMMs using the "glmer" function, and the fertility evaluations were fit to GLMs using the "glm" function. The effect of deltamethrin was analyzed using a GLMM with "Poisson" distribution. In this model, the response variable was fecundity (number of eggs per spawning), and treatment, generation, and their interaction were included as explanatory variables. We included aquarium as a random factor because we used 10 spawnings from each of the three replicates (aquariums). The GLM was fitted using a "Quasibinomial" distribution. In this model, the response variable was fertility (proportion of eggs hatched per spawning), and treatment, generation, and their interaction were included as explanatory variables. We evaluated the normality of the residues using q-q graphs and the homogeneity of the variances using Fligner-Killeen tests (Crawley, 2013). We tested for the presence of outliers using Cook distances (Cook distance <1). We constructed all models using the *nlme* library (Pinheiro et al., 2006).

3. Results

3.1. Deltamethrin toxicity to *C. columbiensis* larvae

The results obtained in the concentration-mortality bioassays for 24 h ($n = 720$; $\chi 2 = 3.28$; $P = 0.51$) and 96 h ($n = 720$; $\chi 2 = 5.83$; $P = 0.51$) were fit to a Probit model (Fig. 2). Larvae of *C. columbiensis* were approximately 6.9-fold ($LC_{50} = 0.17$ [0.15–0.20] µg/L) more susceptible to deltamethrin when exposed for 96 h than when exposed for 24 h ($LC_{50} = 1.17$ [0.97–1.43] µg/L) (Table 1, Fig. 2). The bioassays for 96 h exposure also revealed the concentrations of deltamethrin LC_1 (0.02 µg/L) and LC_{10} (0.05 µg/L), which were used in chronic toxicity bioassays (i.e., over the entire immature life of *C. columbiensis*).

Table 1. Toxicity of deltamethrin to first instar larvae of *Chironomus columbiensis* at different exposure periods (i.e., 24 h and 96 h).

Hora	Slope ± SE	LC_1 (95% CI)	LC_{10} (95% CI)	LC_{50} (95% CI)	LC_{80} (95% CI)
24	1.87 ± 0.14	0.07 (0.04-0.09)	0.24 (0.18-0.30)	1.17 (0.97-1.43)	3.30 (2.58-4.47)
96	2.46 ± 0.20	0.02 (0.01-0.03)	0.05 (0.04-0.06)	0.17 (0.15-0.20)	0.38 (0.31- 0.48)

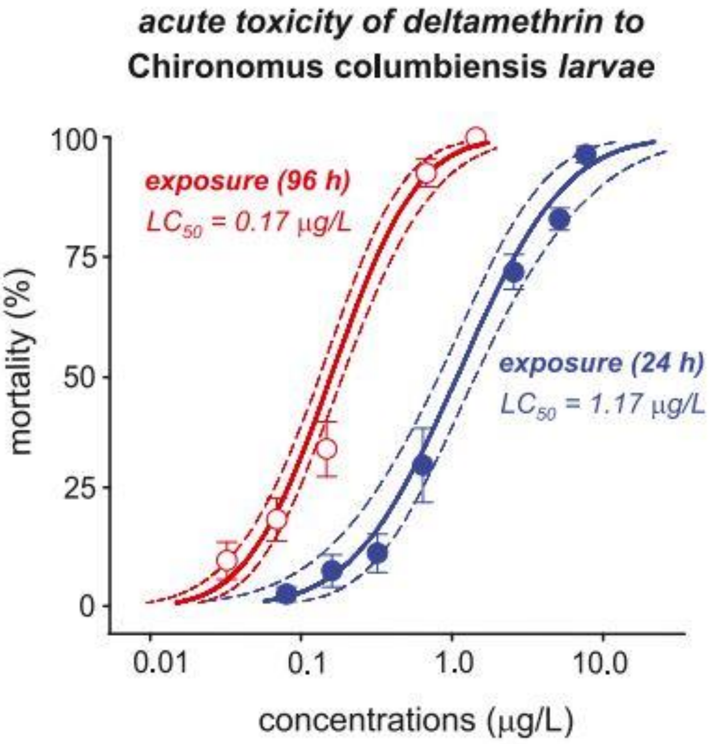
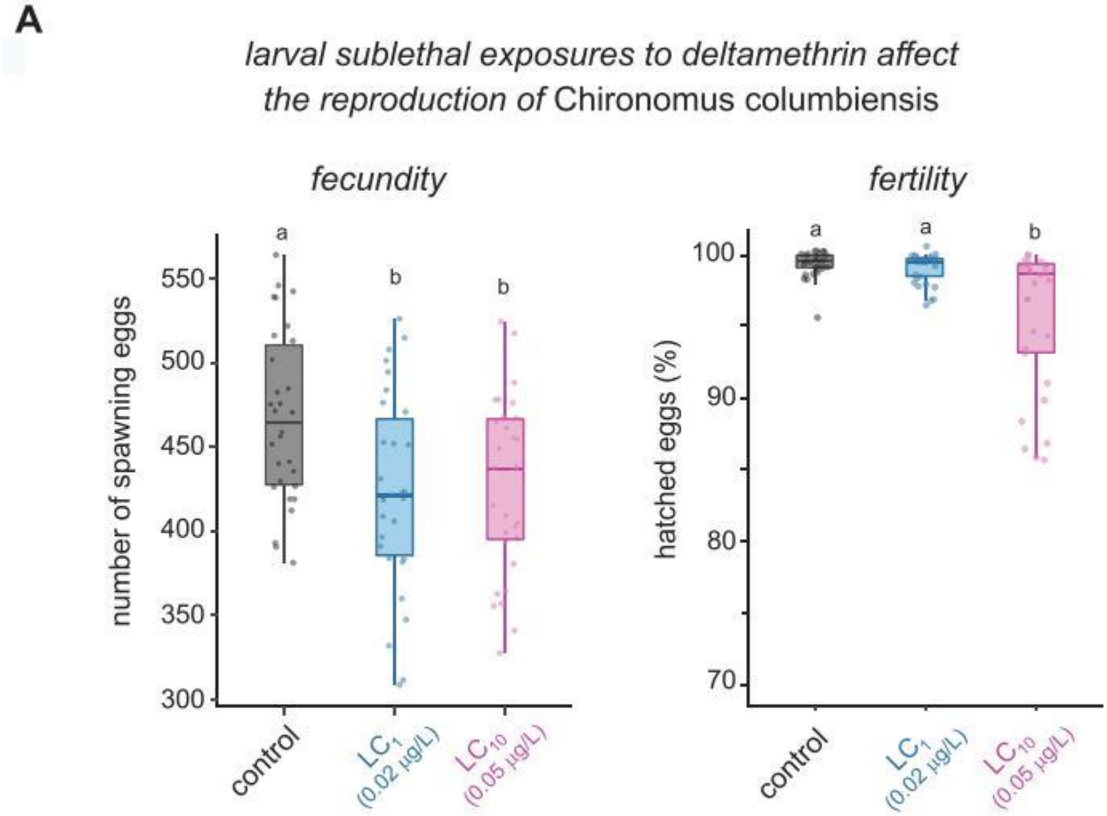


Fig. 2. Toxicity of deltamethrin to *Chironomus columbiensis* larvae. Concentration-response curves for 24 h (blue) and 96 h (red) exposure periods. Dotted lines represent the 95% confidence intervals and symbols shows the mean (±SEM) obtained for eight replicates (aquarium containing 10 *C. columbiensis* larvae). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2. Effects of deltamethrin exposure of immature *C. columbiensis* on their reproduction (fecundity and fertility rates) and wing shape

The fecundity of deltamethrin-exposed individuals was significantly lower than the those recorded for unexposed individuals ($\chi 2$ = 16.97, P = 0.0002), irrespective of the deltamethrin concentration (Fig. 3*A*). The females unexposed to deltamethrin laid approximately 467.5 ± 11.4 eggs per spawning, while the females exposed to deltamethrin at LC_1 (423.4 ± 16.0) or LC_{10} (426.9 ± 16.1) laid fewer eggs per spawning. Fertility rates differed significantly between unexposed females and those that were exposed to deltamethrin LC_{10} ($\chi 2$ = 39.49, $P < 0.0001$, Fig. 3*A*). Unexposed females exhibited a fertility rate of 99.4% ± 0.5%, while females exposed to deltamethrin LC_{10} exhibited a fertility rate of 96.1% ± 0.7%. Females exposed to deltamethrin LC_1 exhibited a 99.0% ± 0.6% (Fig. 3*A*).

Our PCA analysis of wing shape revealed that the first four PCs accounted for 59.0% (PC1: 22.2%; PC2: 15.2%; PC3: 11.1%; PC4: 10.5%) of the total variation in shape (Fig. 3*B*). In order to locate the variation, the average shape was calculated for individuals exposed to deltamethrin (both LC1 and LC_{10}) and those that were not exposed. Populations that were exposed to LC_1 and LC_{10} showed a shortening in the basal part of the wing (landmarks 1 and 9; 8 and 9) and narrowing in the anal and median regions of the wing (landmarks 1 and 2; 2 and 3; 9 and 10; 10 and 3; 10 and 4), and a slight elongation in the apical part of the wing (landmarks 5 and 6) relative to the controls (Fig. 3*B*). The CVA dispersion diagram showed a clear differentiation between populations that were exposed to deltamethrin at either concentration and the population that was not exposed (Fig. 3*B*). Procrustes ANOVA based on centroid size and wing shape revealed similar patterns, with a significant effect of the treatment factor ($P < 0.0001$). In addition, the *P*-values of the Procrustes distance and Mahalanobis distance after the permutation test (10,000 permutations) revealed differences between the three populations ($P < 0.0001$; Table 2).



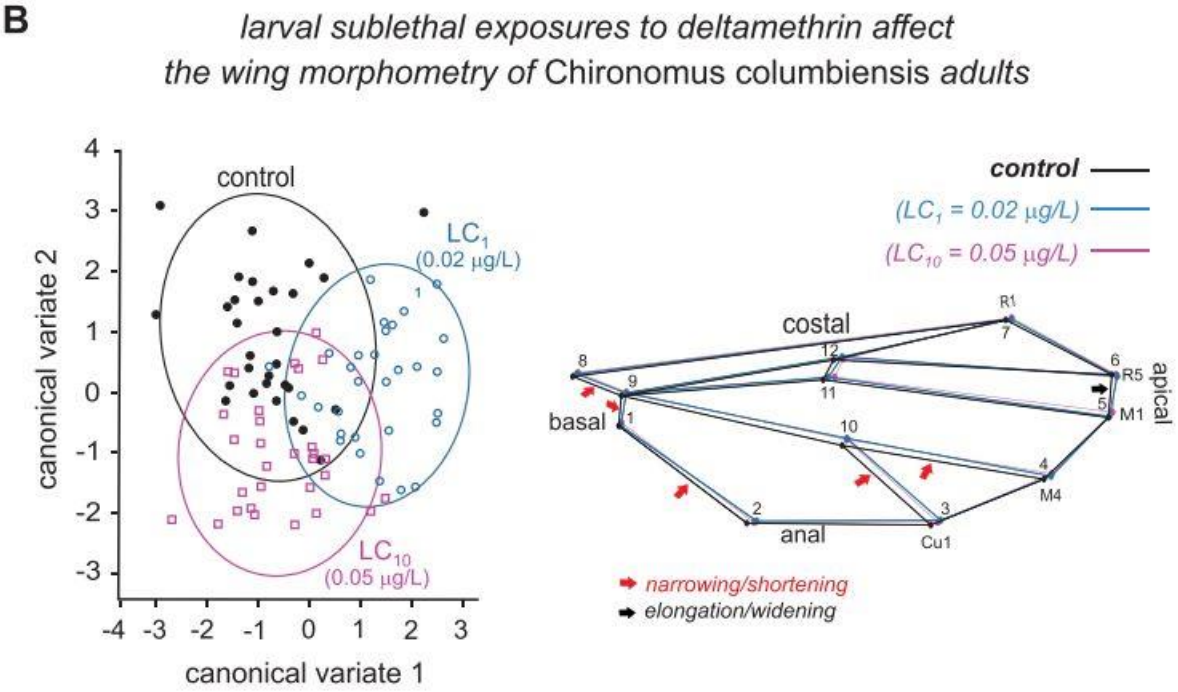
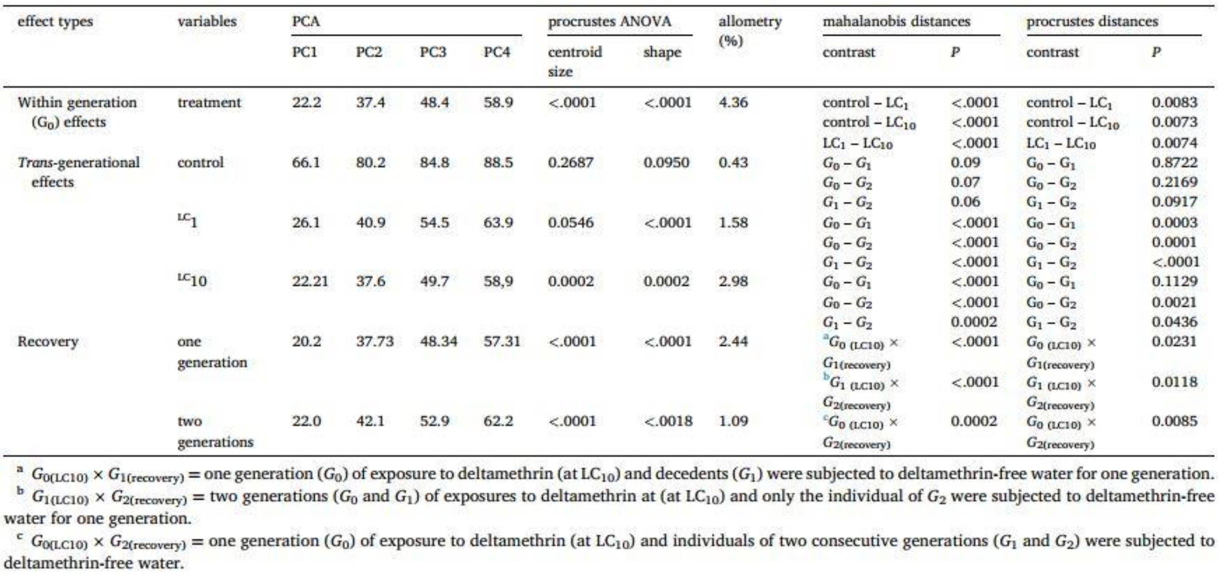


Fig. 3. Effect of immature sublethal exposures to deltamethrin (LC$_1$ = 0.02 µg/L and LC$_{10}$ = 0.05 µg/L) on the reproduction (***A***) and wing shape (***B***) of the *Chironomus columbiensis* adults. (***A***) Fecundity (left panel) and fertility (right panel) rates of *C. columbiensis* adults that faced immature exposures to deltamethrin. Box plots show the median (the line within each box plot) and range of dispersion (lower and upper quartiles). Each symbol represents one spawning replicate used to generate the box plot. Treatments grouped by the same letters indicate no statistical differences by Tukey's honestly significant difference (HSD) test ($P < 0.05$). (***B***) Canonical analysis (left panel) and shape variations (right panel) on wings *C. columbiensis* adults that faced deltamethrin immature exposures. Arrows red indicate the wing portions that were narrowing/shortening ningwhile arrows black indicate those that showed elongations/widening (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

Table 2. Results of geometric morphology analyses on the variation of the wing shape of the *Chironomus columbiensis* according to treatments and generation. D = deltamethrin LC$_{10}$ and R = recovery.



effect types	variables	PCA				procrustes ANOVA		allometry (%)	mahalanobis distances		procrustes distances	
		PC1	PC2	PC3	PC4	centroid size	shape		contrast	*P*	contrast	*P*
Within generation (G_0) effects	treatment	22.2	37.4	48.4	58.9	<.0001	<.0001	4.36	control – LC_1	<.0001	control – LC_1	0.0083
									control – LC_{10}	<.0001	control – LC_{10}	0.0073
									$LC_1 - LC_{10}$	<.0001	$LC_1 - LC_{10}$	0.0074
Trans-generational effects	control	66.1	80.2	84.8	88.5	0.2687	0.0950	0.43	$G_0 - G_1$	0.09	$G_0 - G_1$	0.8722
									$G_0 - G_2$	0.07	$G_0 - G_2$	0.2169
									$G_1 - G_2$	0.06	$G_1 - G_2$	0.0917
	$^{LC}1$	26.1	40.9	54.5	63.9	0.0546	<.0001	1.58	$G_0 - G_1$	<.0001	$G_0 - G_1$	0.0003
									$G_0 - G_2$	<.0001	$G_0 - G_2$	0.0001
									$G_1 - G_2$	<.0001	$G_1 - G_2$	<.0001
	$^{LC}10$	22.21	37.6	49.7	58,9	0.0002	0.0002	2.98	$G_0 - G_1$	<.0001	$G_0 - G_1$	0.1129
									$G_0 - G_2$	<.0001	$G_0 - G_2$	0.0021
									$G_1 - G_2$	0.0002	$G_1 - G_2$	0.0436
Recovery	one generation	20.2	37.73	48.34	57.31	<.0001	<.0001	2.44	$^{a}G_{0\,(LC10)} \times G_{1(recovery)}$	<.0001	$G_{0\,(LC10)} \times G_{1(recovery)}$	0.0231
									$^{b}G_{1\,(LC10)} \times G_{2(recovery)}$	<.0001	$G_{1\,(LC10)} \times G_{2(recovery)}$	0.0118
	two generations	22.0	42.1	52.9	62.2	<.0001	<.0018	1.09	$^{c}G_{0\,(LC10)} \times G_{2(recovery)}$	0.0002	$G_{0\,(LC10)} \times G_{2(recovery)}$	0.0085

[a] $G_{0(LC10)} \times G_{1(recovery)}$ = one generation (G_0) of exposure to deltamethrin (at LC_{10}) and decedents (G_1) were subjected to deltamethrin-free water for one generation.

[b] $G_{1(LC10)} \times G_{2(recovery)}$ = two generations (G_0 and G_1) of exposures to deltamethrin at (at LC_{10}) and only the individual of G_2 were subjected to deltamethrin-free water for one generation.

[c] $G_{0(LC10)} \times G_{2(recovery)}$ = one generation (G_0) of exposure to deltamethrin (at LC_{10}) and individuals of two consecutive generations (G_1 and G_2) were subjected to deltamethrin-free water.

3.3. Transgenerational effects of deltamethrin exposure of immature *C. columbiensis* on their reproduction (fecundity and fertility rates) and wing shape

Our results showed that deltamethrin exposure causes significant transgenerational reductions in fecundity ($\chi2 = 370.53$, $P < 0.001$), and this became more pronounced in individuals that were exposed to insecticide at higher concentration (Fig. 4). The interactions between generations of immature exposure (i.e., G_0, G_1, and G_2) and insecticide concentration (i.e., LC_1 and LC_{10}) also had a significant effect ($\chi2 = 273.35$, $P < 0.001$, Fig. 4). GLMMs revealed significant differences among generations associated with deltamethrin exposure at LC_1 ($\chi2 = 241.14$, $P < 0.001$) and LC_{10} ($\chi2 = 380.51$, $P < 0.001$), and no significant differences among generations for unexposed individuals ($\chi2 = 0.43$, $P = 0.807$, Fig. 4). Interestingly, the populations that were exposed to deltamethrin over two consecutive generations (G_1; LC_1 = 371.13 ± 24.45 eggs per spawning; LC_{10} = 364.04 ± 44.54 eggs per spawning) laid significantly fewer eggs than those that were deltamethrin-exposed for one generation (G_0; LC_1 = 423.4 ± 16.0 eggs per spawning; LC_{10} = 426.9 ± 16.1 eggs per spawning), irrespective of the insecticide concentration. The exposure to deltamethrin in the third consecutive generation (G_2) resulted in further reduction (LC_1 = 345.3 ± 5.1 eggs per spawning; LC_{10} = 327.7 ± 5.1 eggs per spawning) in fecundity rates for the insecticide-exposed populations (Fig. 4). Significant reductions in fertility rates were observed only in populations that were exposed to deltamethrin LC_{10} ($\chi2 = 56.50$, $P < 0.001$), irrespective of the number of generations exposed to the insecticide ($\chi2 = 1.09$, $P = 0.579$) and the interaction between insecticide concentration and exposure generation ($\chi2 = 3.61$, $P = 0.462$).

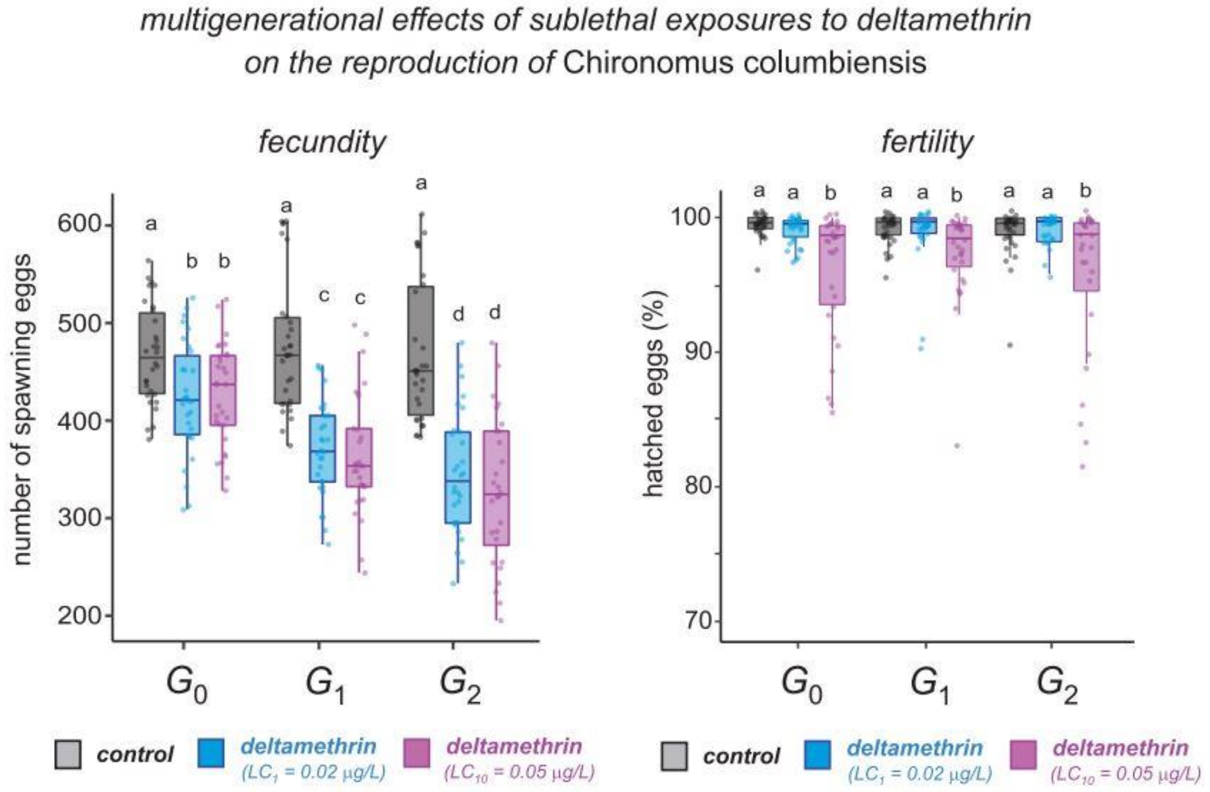
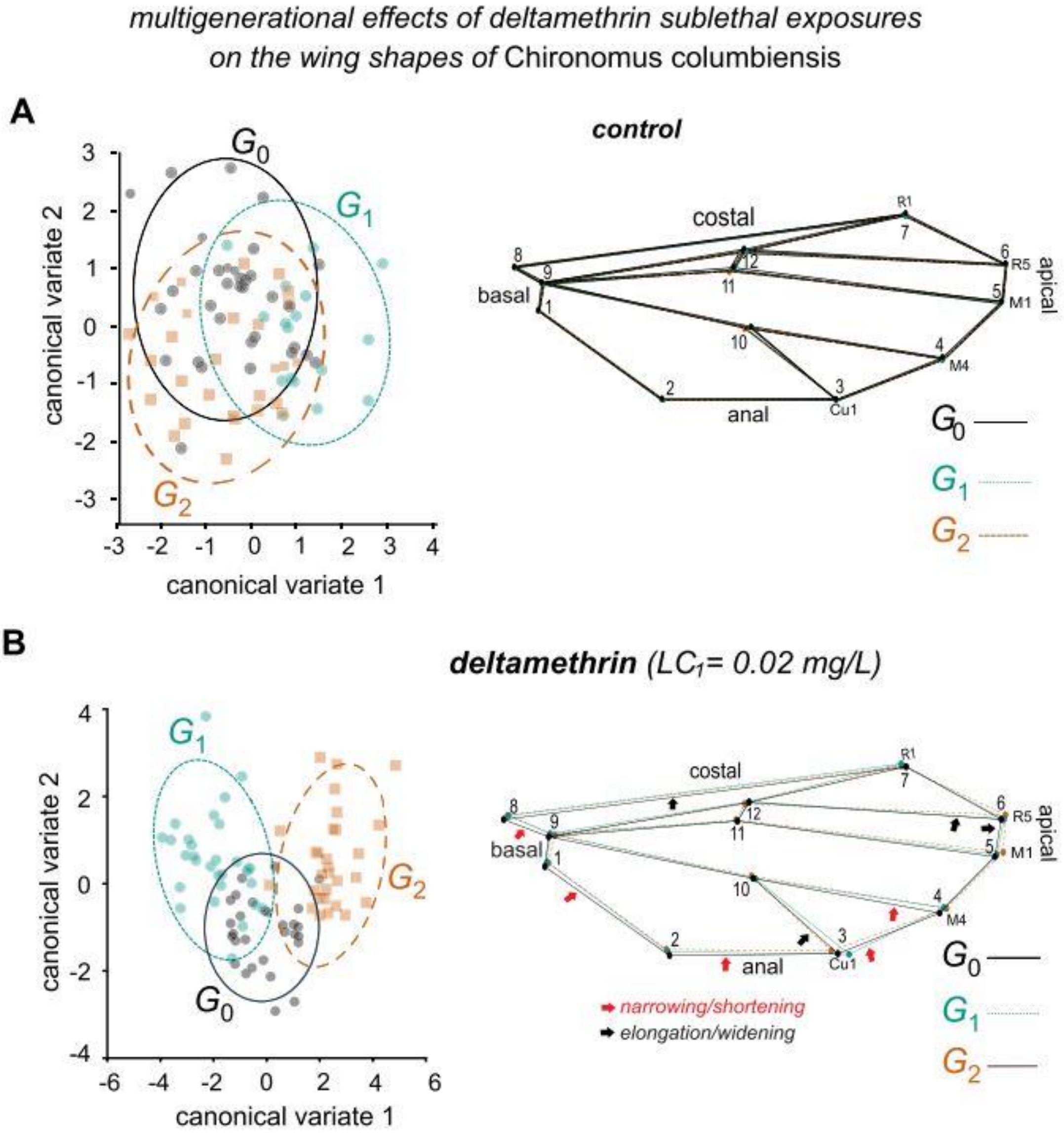


Fig. 4. Transgenerational effects of deltamethrin exposures (LC_1 = 0.02 µg/L and LC_{10} = 0.05 µg/L) on the fecundity (***A***) and fertility (***B***) rates of *Chironomus columbiensis* that faced immature exposure for one or two consecutive generations. Box plots show the median (the line within each box plot) and range of dispersion (lower and upper quartiles). Each symbol represents one spawning replicate used to generate the box plot. In each panel, treatments grouped by the same letters indicate no statistical differences by Tukey's honestly significant difference (HSD) test ($P < 0.05$).

Procrustes ANOVA based on the size of the centroid and the shape of the wing showed differences among the three generations exposed to deltamethrin LC_1 ($P < 0.0001$) and LC_{10} ($P = 0.0002$), but not among individuals that were not exposed to insecticide ($P = 0.0950$) (Table 2, Fig. 5*A*–*C*). According to PCA, the first three factors explained ≥50% of the variation (Table 2). The CVA dispersion diagram for the treatment with deltamethrin at LC_1 showed differences in the shape of the wing for all three generations (Fig. 5*B*). Most specimens of generations G_1 and G_2 exhibited wings with a shortened basal region (landmarks 8 and 9) and a narrow anal region (landmarks 1 and 2; 2 and 3). In addition, a widening of the costal part (landmarks 7 and 8) and an elongation of the apical part of the wing (landmarks: 5 and 6) relative to the specimens exposed in the parental generation (G_0) were observed in these treatments, and this effect was more pronounced in generation G_2 (Fig. 5*B*). However, significant differences between G_1 and G_2 were observed in veins Cu1 and R5. The cubital vein was elongated in G_1 relative to G_0 and G_2. The radial vein was significantly widened in G_2 relative to G_0 and G_1 (Fig. 5*B*).

The CVA dispersion diagram for exposure to deltamethrin at LC_{10} also revealed differences in wing shape among the three generations (Fig. 5*C*). Most specimens in G_1 and G_2 showed elongation of the apical part of the wing (landmarks 5 and 6; 6 and 7) and widening in the coastal region (landmarks 7 and 8; 12 and 7) relative to the specimens exposed in the parental generation (Fig. 5*C*). In addition, specimens in G_1 showed a shortening of the anal part of the wing (landmarks 1 and 2; 2 and 3) and those in G_2 showed an elongation between the cubital and medial veins (landmarks 3 and 4; 4 and 5; Fig. 5*C*).



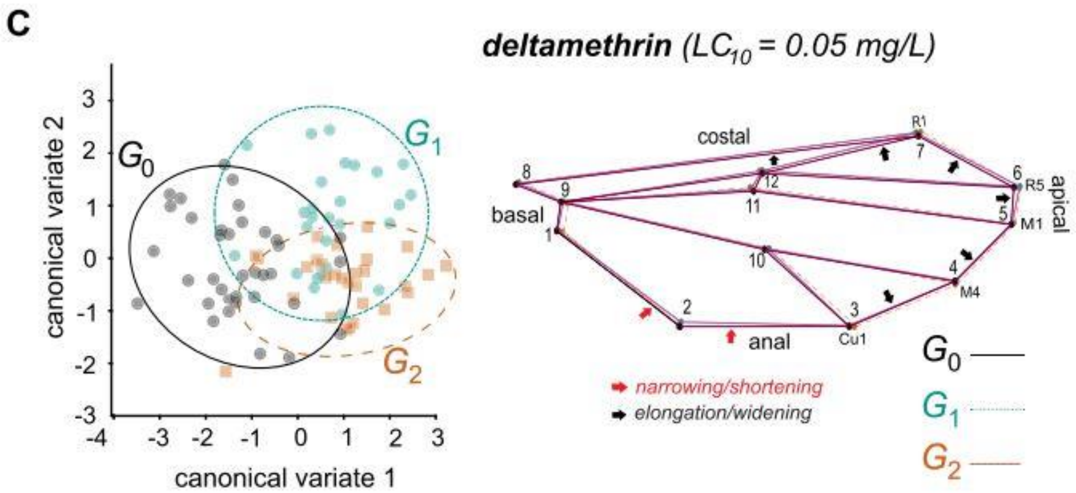
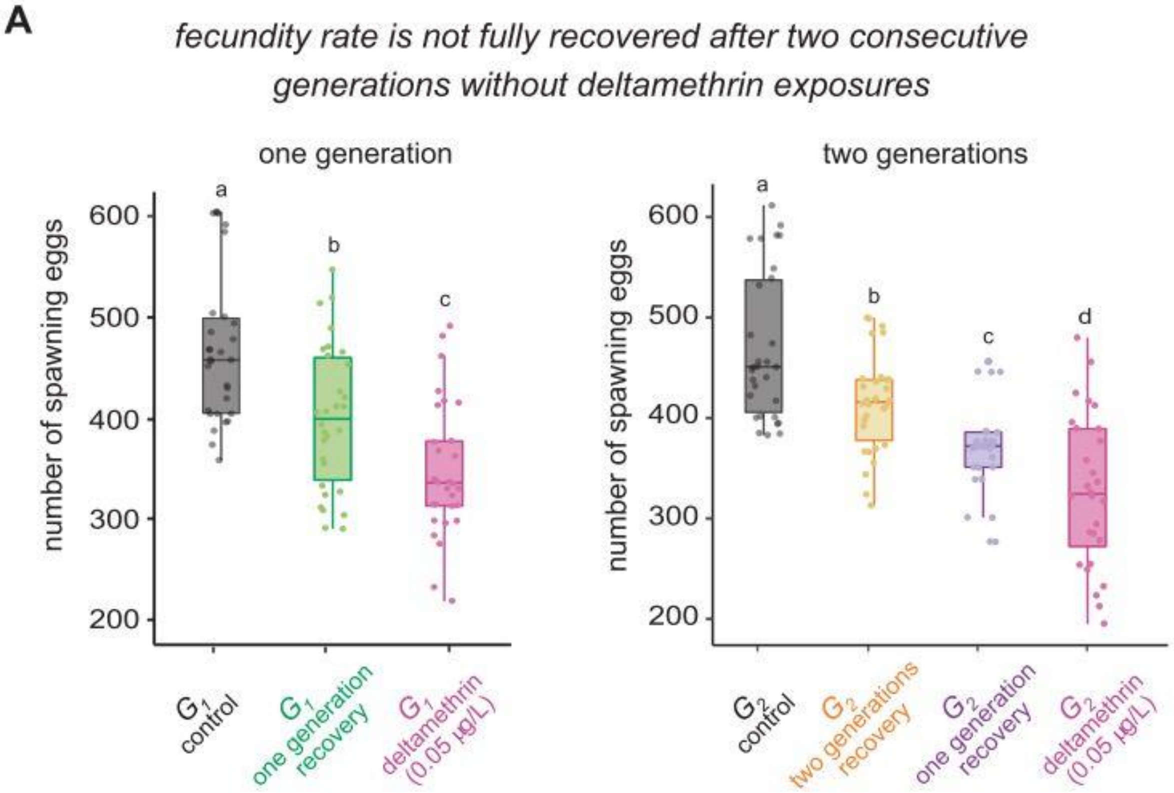


Fig. 5. Transgenerational effects of deltamethrin exposures on the wing shape of *Chironomus columbiensis* that faced immature exposure for one or two consecutive generations. (***A***) Deltamethrin-unexposed insects. (***B***–***C***) Canonical analysis (left panel) and shape variations (right panel) on wings *C. columbiensis* individuals exposed to LC_1 = 0.02 µg/L (***B***) and LC_{10} = 0.05 µg/L (***C***). Red arrows indicate the wing portions that were narrowing/shortening while arrows black indicate those that showed elongations/widening. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

3.4. Ability of *C. columbiensis* to recover from changes in reproduction and wing shape caused by deltamethrin exposure

Our results revealed significant differences ($\chi2$ = 114.28; $P < 0.0001$) among the fecundity rates of individuals that were one generation removed from exposure (one recovery generation) compared to those that were not deltamethrin-exposed (at G_0 or G_1) or those that faced the insecticidal exposure (LC_{10}) at G_0 and G_1 (Fig. 6*A*). Individuals one generation removed from exposure (i.e., at G_1) had higher fecundity rates (413.94 ± 23.1 eggs/spawning) than those that were exposed to deltamethrin over two generations (363.5 ± 23.0 eggs/spawning), but lower than those that were not exposed (478.1 ± 16.5 eggs/spawning; $\chi2$ = 114.24; $P < 0.0001$) (Fig. 6*A*). Our results also showed that even individuals two generations removed from exposure (i.e., deltamethrin exposure at G_0 but not at G_1 and G_2) could not fully recover from the effects of deltamethrin exposure ($\chi2$ = 104.24; $P < 0.0001$; Fig. 6*A*). Interestingly, these individuals had higher fecundity rates (414.17 ± 14.53 eggs/spawning) than individuals that were exposed to deltamethrin over two (374.02 ± 14.44 eggs/spawning) or three (328.68 ± 14.45 eggs/spawning) generations, but lower fecundity rates than individuals that were not exposed to deltamethrin (470.37 ± 10.31 eggs/ spawning) during the three generations (Fig. 6*A*).

Our results also showed that one recovery generation, whether following one or two deltamethrin exposure generations, was sufficient to restore the fertility rates of *C. columbiensis*. Fertility rates in these individuals differed from fertility rates in individuals exposed to deltamethrin for two ($\chi2 = 10.77$; $P = 0.0046$) or three ($\chi2 = 8.01$; $P < 0.0457$) generations (Fig. 6*B*). Individuals that recovered for one (98.5% ± 0.38%) or two (98.0% ± 0.75%) consecutive generations exhibited fertility rates similar to those that were never exposed (99.09% ± 0.23%) (Fig. 6*B*).



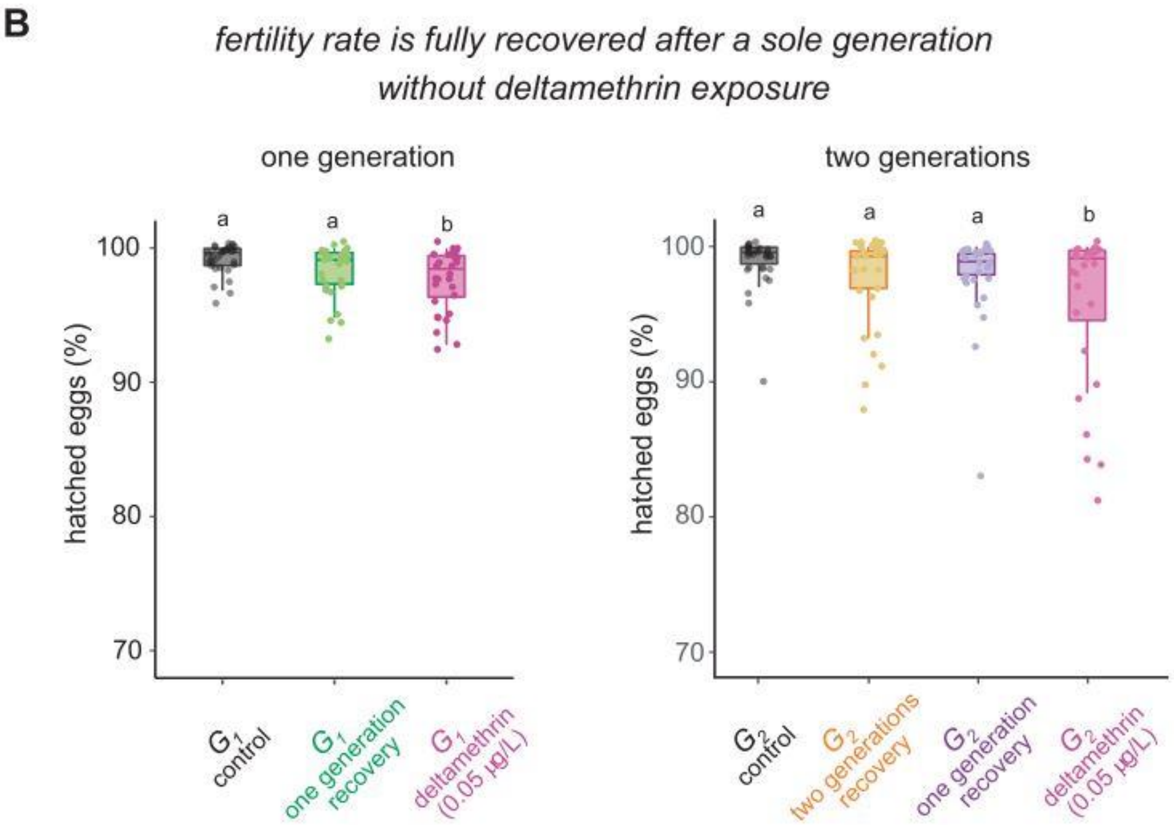


Fig. 6. Recovery on fecundity (***A***) and fertility (***B***) rates of *Chironomus columbiensis* that were subjected to one (left panels) or two (right panels) consecutive recovery generations after being exposed to deltamethrin (LC_{10} = 0.05 µg/L). Box plots show the median (the line within each box plot) and range of dispersion (lower and upper quartiles). Each symbol represents one spawning replicate used to generate the box plot. In each panel, treatments grouped by the same letters indicate no statistical differences by Tukey's honestly significant difference (HSD) test ($P < 0.05$).

The Procrustes ANOVA based on the size of the centroid and the shape of the wing showed that one generation was not sufficient for specimens to fully recover, since the *G*1 specimens differed from both the control specimens and those that were exposed to deltamethrin ($P < 0.0001$; Table 1). The first three PCA factors explained ≥50% of the variation in recovery in G1 and G2 (Table 2). The CVA scatter diagram revealed differences in wing shape among the three treatments (Fig. 7*A*). Most of the specimens that were one generation removed from exposure showed wing shape differences relative to unexposed insects (Fig. 7*A*). However, when recovery was for two consecutive generations, the specimens exhibited wing shapes very similar to those of unexposed insects, particularly in the anal (landmarks 1 and 2) and cubital regions (landmarks 3 and 10) and the median veins (landmarks: 4 and 10; Fig. 7*B*).

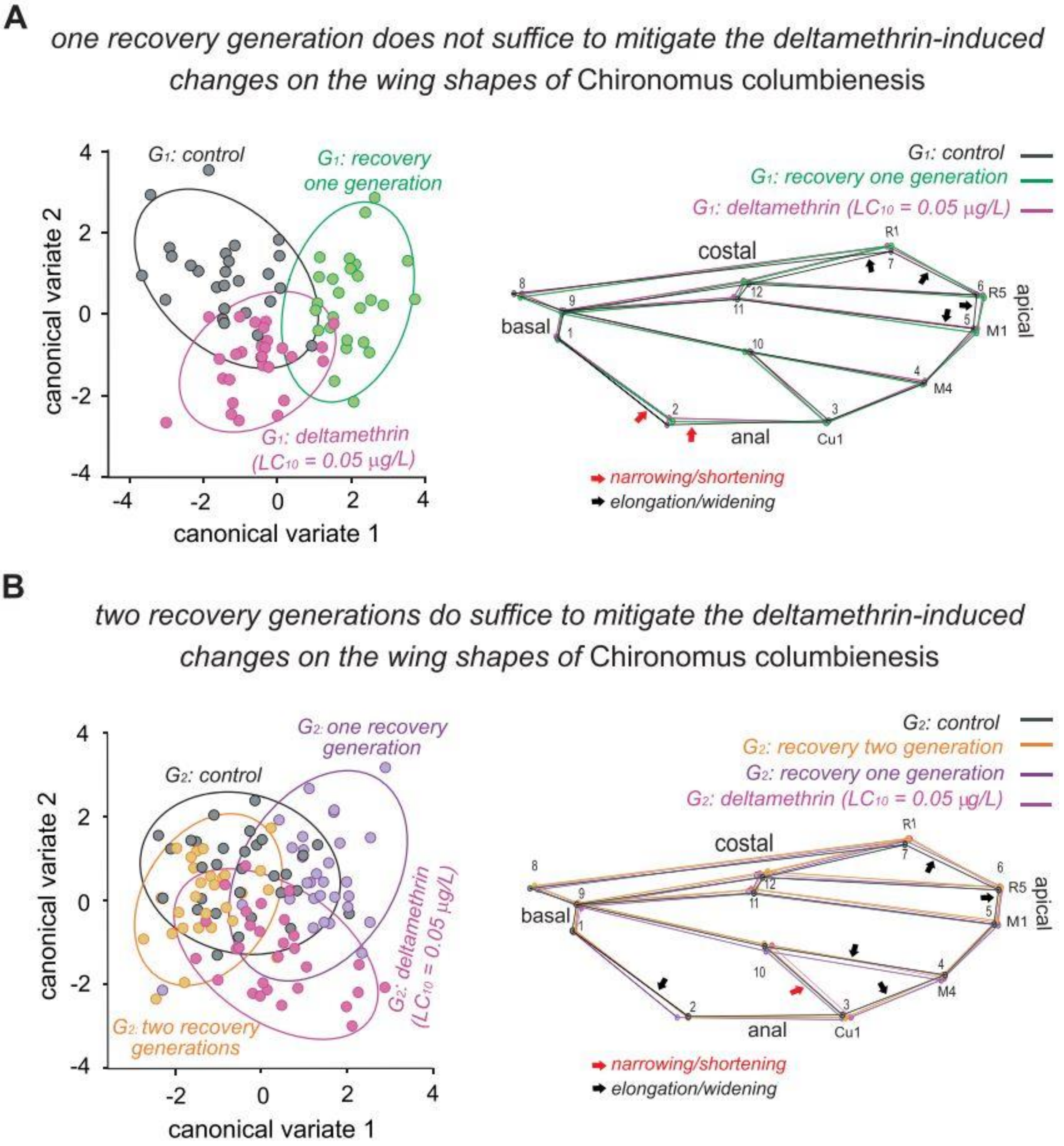


Fig. 7. Recovery on wing shape of *Chironomus columbiensis* that were subjected to one (***A***) or two (***B***) consecutive recovery generations after being exposed to deltamethrin (LC_{10} = 0.05 µg/L). (***A***–***B***) Canonical analysis (left panel) and shape variations (right panel) on wings *C. columbiensis*. Red arrows indicate the wing portions that were narrowing/shortening while arrows black indicate those that showed elongations/widening. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

4. Discussion

Here, we demonstrated that unintentional contamination of water bodies with deltamethrin can lead to sublethal and transgenerational effects on the Neotropical midge *C. columbiensis*. Our findings further revealed that immature *C. columbiensis* can be affected by deltamethrin exposure in time- and concentration-dependent manners. Immature *C. columbiensis* exposed to sublethal concentrations of deltamethrin showed negative effects on their reproduction and wing shape, and carried these undesirable traits to their offspring, also affecting their abilities to respond to other exposures of deltamethrin. Our findings also demonstrate that the proper management of deltamethrin might suffice for *C. columbiensis* population recovery, as fertility and wing shape were recovered after two unexposed generations.

Among other factors (e.g., different substances, species, exposure conditions, and levels of biological organization), the comparison and extrapolation of toxic effects between different exposure durations represent major challenges in ecotoxicology (Jager et al., 2011; Wiberg-Larsen et al., 2016; Dalhoff et al., 2020). Here, as recent investigations have shown in other aquatic non-target arthropods (Valbon et al., 2018; Santos et al., 2018), immature *C. columbiensis* were susceptible to pyrethroids in time- and concentration-dependent manners. The susceptibility ratio (SR50) for the 24 h (LC_{50} = 1.17 µg/L) and 96 h (LC_{50} = 0.17 µg/L) endpoints was approximately 5.8 [4.2–9.8]-fold, and within the susceptibility ratio range described for other aquatic organisms (Prusty et al., 2011; Toumi et al., 2013; Ning et al., 2020). This may relate to potential differences in toxicokinetic (i.e., processes involved in uptake, metabolism, and elimination) and toxicodynamic (i.e., processes happening at compound target sites that may propagate its effects) processes used by these insects to mitigate the action of deltamethrin (Jager et al., 2011; Wiberg-Larsen et al., 2016; Dalhoff et al., 2020).

Toxicokinetic-toxicodynamic processes may contribute to the differential pyrethroid susceptibility of immature *C. columbiensis* compared to other aquatic arthropods. For instance, our findings indicate that *C. columbiensis* was more susceptible than *Daphnia magna* (Toumi et al., 2013), *Eriocheir sinensis* (Ning et al., 2020), *Aedes aegypti* and *Belostoma anurum* (Valbon et al., 2018), but more tolerant than *Callibaetis radiatus* and the backswimmers *Buenoa tarsalis* and *Martarega bentoi* (Guti´ errez et al., 2016; 2017). Recent studies focusing only on type II pyrethroid cypermethrin, attempted to link insecticide toxicity to morphology, toxicokinetic, and toxicodynamic traits across several taxa of aquatic invertebrates, and suggested that toxicodynamic parameters are more relevant than toxicokinetic. This may be because mortality was the only parameter in reduced general unified threshold models of survival assuming stochastic death that was consistently correlated with cypermethrin susceptibility across all investigated species (Dalhoff et al., 2020).

Deltamethrin has been detected in current aquatic environments in concentrations that range from 0.001 µg/L to 0.043 µg/L in water streams nearby rice cultivations (Elfman et al., 2011) and significantly higher concentrations (i.e., from 0.51 µg/L to 2.73 µg/L) in water systems used to irrigate rice fields in Argentina (Medina et al., 2020), which reinforces the relevance of the undesired effects caused by sublethal exposure to deltamethrin in *C. columbiensis*, as the findings described here derived of sublethal concentrations (i.e., LC_1 = 0.02 µg/L and LC_{10} = 0.05 µg/L) that suits within ecologically realistically concentration range. Indeed, it has been shown that sublethal exposure to pyrethroids can modify the metabolic energy redistribution toward detoxification in aquatic insects, and this may in turn decrease the energy availability for other activities such as feeding (Arias et al., 2020), locomotion (Guti´ errez et al., 2017) and reproduction (Arias et al., 2020). Here, we demonstrated that sublethal (LC_{10}) exposure to deltamethrin in immature *C. columbiensis* resulted in severe reduction in fecundity. Although the physiological basis for such detrimental pyrethroid-mediated sublethal effects have not been described in detail in insects, several investigations in mammals (e.g., rodents and humans) have shown that sublethal exposure to pyrethroids are detrimental to the structure and function of gonads (Marettova et al., 2017; Ye and Liu, 2019; Jia et al., 2019; Zhang et al., 2021). This partially explains the reduced reproductive performance described here. Preliminary evidence for such undesired effects has been shown in *Apis* honeybees, in which vitellogenin-producing genes were upregulated in individuals exposed to pyrethroid at sublethal levels (Christen and Fent, 2017).

Here, we also found that offspring of insects exposed to sublethal concentrations of deltamethrin carried over disadvantages in fecundity even after two generations in deltamethrin-free environments. The mechanisms underlying multigenerational effects of sublethal exposure to pyrethroids on insect reproduction are less detailed than those reported for within-generation sublethal effects. Recent investigations, however, have suggested that the mechanisms may derive from selection of individuals with genetic mutations (i.e., pyrethroid resistance) or from disturbances to the gene expression pattern without changes in the underlying DNA sequence (i.e., epigenetics) (Ayyanath et al., 2014; Brevik et al., 2021; Hu et al., 2020, Vandegehuchte et al., 2010). These phenomena (pyrethroid resistance or pyrethroid-mediated epigenetic effects) are expected to have physiological costs that make their expression a disadvantage to individuals in insecticide-free environments. For example, insects can tolerate insecticide exposure through the duplication of detoxification genes and consequent overexpression of detoxification enzymes, but after suspending insecticide exposure, the physiological costs of methylation of extra detoxification gene copies can be an ecological disadvantage (Hunt et al., 2013). Notably, such methylation (and other epigenetic processes) can also be neutral or generate positive responses in insecticide-stressed insects (Ayyanath et al., 2015; Rix et al., 2016).

It has been demonstrated that multigenerational exposures of an insect population to sublethal concentrations of insecticides can favor the abilities of this population to cope with subsequent insecticidal stress (Liang et al., 2012; Rix et al., 2016; Rix and Cutler, 2018). However, such stimulatory responses will depend upon a multitude of factors (e.g., the insecticide type, exposure pathways, generation quantities) and not all compounds can generate such preconditioning traits, even when the subsequent stresses are mediated by similar or identical molecules (Liang et al., 2012; Rix et al., 2016; Rix and Cutler, 2018). Here, similarly to the impacts described for the fecundity recovery, our findings revealed that the worst *C. columbiensis* fecundity rates were obtained in individuals that descend of multiple generations deltamethrin sublethal (LC_{10}) exposures, which indicates that whatever genetic or epigenetic changes in immature *C. columbiensis* elicited by the deltamethrin sublethal exposures were contributing to detrimentally affect the abilities of their descendants to respond to other deltamethrin exposures.

Despite previous investigations having failed to record morphological differences in the wing shape of other *Chironomus* species (Majlesi et al., 2020), wing deformities in *C. columbiensis* have been shown to serve as a useful phenotypic biomarker for environmental pollution caused by agricultural and mining activities (Montaño-Campaz et al., 2019). The present study reinforces this hypothesis, as it revealed that *C. columbiensis* wing shape alterations suffice to capture not only withinand transgenerational deltamethrin-mediated effects but also their recovery. Our results revealed that immature *C. columbiensis* exposed to sublethal concentrations of deltamethrin exhibited altered wing shapes (e.g., shortening in the basal part, slight elongation in the apical part, and narrowing in the anal and median regions). Wing shape alterations mediated by immature sublethal exposures to deltamethrin have also been described for the triatomine *Triatoma infestans* (Natterro et al. 2019, Natterro et al. 2021). Adults of *T. infestans* exposed to sublethal concentrations of deltamethrin exhibited larger, less symmetrical, and less canalized (i.e., lower individual variation within genotypes) wings, which together may impair developmental stability and genetic and environmental canalization (Natterro et al. 2021).

The transgenerational effects of deltamethrin on wing shape were concentration-dependent and more stable when individuals were exposed to higher concentrations (i.e., LC_{10} = 0.05 µg/L). Interestingly, when exposure occurred in only one generation (G_0) and recovery occurred over two generations (G_1 and G_2), *C. columbiensis* wing shapes were very similar to those in unexposed individuals, suggesting that G_2 individuals may have adapted (genetically or epigenetically) to the deltamethrin-elicited stress. Indeed, recent investigations have related the history of pyrethroid spraying (i.e., pyrethroid resistance levels) to wing shapes in *T. infestation* strains (Nattero et al. 2019, 2021), and have shown that DNA methylation affects wing development and chitin formation in *Bombyx mori* (Xu et al., 2020). However, the relationship between this physiological pathway and deltamethrin-elicited transgenerational effects (and recovery) is not yet understood.

4. Conclusions

Despite further investigations aiming to characterize the gene expression and phenotypes over generations need to be conducted before drawing firm conclusion, the unintended intra- and *trans*-generational sublethal stresses (e.g., reduced fecundity and fertility rates, modified wing shape patterns) mediated by deltamethrin on *C. columbiensis* reinforce the potential of pyrethroids to modify the population dynamics of non-target aquatic insects. Furthermore, by collecting evidence for time (i.e., generations) required to recovery of deltamethrin-mediated effects, our efforts highlight the need of adopting proper management (e.g., concentration and frequency of application) strategies to mitigate the unintended effects of deltamethrin on nontarget insects.

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CHAPTER III

Chronic and acute sublethal exposures to imidacloprid cause sex-dependent changes on the adaptive responses of *Chironomus columbiensis*

Chronic and acute sublethal exposures to imidacloprid cause sex-dependent changes on the adaptive responses of *Chironomus columbiensis*

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Abstract

The use of imidacloprid is a common pest control practice in the Neotropical region. However, the potential of imidacloprid to cause unintended sublethal effects on Neotropical aquatic non-target arthropods have not been received adequate attention. Here, we assessed the susceptibility of *Chironomus columbiensis* (Diptera: Chironomidae) larvae to the neonicotinoid imidacloprid and evaluated whether sublethal exposure types would change adaptive responses of *C. columbiensis* males and females. The chronic sublethal exposure consisted of exposing individuals during their entire larval and pupal phases, while the acute sublethal exposures represented a single and of short duration (24h) exposure episode either at the first or at the fourth larval instar. Our results revealed that while chronic sublethal exposure reduced the body mass of males, the acute sublethal exposures of first instar larvae resulted in heavier males than those that were not exposed to imidacloprid. Males or females that survived chronic exposures showed reduced reproduction, while the acute sublethal exposure only affected the reproduction of individuals that were imidacloprid-exposed on their later larval instar. The sublethal exposure types did differentially affect the wing properties of *C. columbiensis* males (e.g., increased size when chronically exposed and highly asymmetric wings when acutely exposed in early larval phase) and females (e.g., highly asymmetric wings when chronically and acutely exposed). Collectively, our findings demonstrated that imidacloprid undesired effects go beyond mortality and its sublethal effects are dependent on the exposure type and the organism developmental phase.

Keywords: undesired insecticidal effects; neonicotinoids; wing asymmetric fluctuations; insect reproductive performances

1. Introduction

Neonicotinoid insecticides represent one of the most effective and sealable molecules used to control insects that attack agricultural crops or transmit diseases to animals, including humans (Casida 2018; Sparks et al. 2020). Among the neonicotinoids, imidacloprid has become the most relevant representant due to its amenability to be used in a variety of methods (e.g., plant sistemicity, seed treatments) and for controlling a broad spectrum of pest insects (Jeschke et al. 2013; Sanchez-Bayo & Tennekes 2020; Frank & Tooker 2020; Malhotra et al. 2021). However, the overreliance on the use of such molecules have led to undesired effects of non-target organisms, especially on those that live on aquatic environments (Morissey et al. 2015; Cavallaro et al. 2017; Queiroz et al. 2021; Pisa et al. 2021; de Souza et al. 2020; Bonmatin et al. 2021).

As imidacloprid can reach the aquatic systems by different events (e.g., runoff from urban and agricultural uses; spray drifts or direct applications), recent investigations (Morissey et al. 2015; Chandran et al. 2018; Queiroz et al. 2021) have indicated the presence of imidacloprid in aquatic ecosystems in concentrations up to 320.0 µg/L, which exceeds for example, the regulatory benchmarks for acute (0.39 µg/L) and chronic (0.01 µg/L) aquatic invertebrate safety in the United States of America (EPA 2022). The adaptive success of aquatic non-target insects will, therefore, rely on how these organisms adapt themselves to such stressors. Genetic assimilation of fitness-related phenotypic adaptions and expression of beneficial traits that are phenotypically neutral under normal conditions have been suggested as the major mechanisms used by these aquatic organisms to mitigate such environmental challenges (Hunn et al. 2019; Queiroz et al. 2021; Wei et al. 2020; Macaulay et al. 2021; Butcherine et al. 2022).

The abilities of these imidacloprid unintended exposures to cause sublethal phenotypical alterations in aquatic non-target organisms may vary with the concentration and length of exposure, which is generally neglected in such ecotoxicological investigations (Tennekes & Sánchez-Bayo 2013; Sánchez-Bayo & Tennekes 2020). Despite the differences methodologies in terms of exposure duration and intensity do not allow a direct comparison, it has been shown that acute or chronical exposures to imidacloprid could modify physiological, morphological, and behavioral responses in non-target aquatic insects (Stoughton et al. 2008; Roessnik et al. 2013; Hunn et al. 2019; Queiroz et al. 2021, Macaulay et al. 2021; Melo et al. 2022). Such modification can be related to subtle alterations on the functions of the nicotinic acetylcholine receptors (nAChR) types, which are the major targets of the neonicotinoid actions (Salgado & Saar 2004; Oliveira et al. 2010,2011; Salgado 2016; Christesen et al. 2021), or alteration on metabolism or other secondary action targets (Chandran et al. 2018; Maloney et al. 2021; Wei et al. 2021; Castellanos et al. 2021).

Here, we evaluated the susceptibility of *Chironomus columbiensis* (Diptera: Chironomidae) to imidacloprid as these aquatic organisms have a wide distribution in the Neotropics (Pape & Thompson 2000; Wülker et al. 1989), ecological importance in aquatic ecosystems in addition to being easily reared under controlled conditions (Montaño-Campaz et al. 2019, 2022 a, b). We further assessed whether different types of sublethal exposures during the immature development would affect the *C. columbiensis* adaptive (e.g., survival, reproduction and wing shape and asymmetric fluctuations) responses

2. Material and methods

2.1 Insect rearing conditions and concentration-mortality bioassays

The chironomids used were obtained from a laboratory strain that has been reared in a pesticide-free environment in the Zoology Laboratory at University of Caldas (Manizales, Colombia). The rearing conditions have been described elsewhere (Montaño-Campaz et al. 2019, 2022). Briefly, recently laid egg masses were collected and placed into glass aquariums containing 5L of semi-soft reconstituted water (pH= 7.2 ± 0.3, electric conductivity = 177 $\mu S/Cm^2$, hardness 138 mg de $CaCO_3$/L). The aquaria were maintained at the temperature of 23 ± 2 °C and under photoperiod of 12h. For the concentration-mortality bioassays, we used newly emerged (< 24h) *C. columbiensis* first instar larvae. Groups of 10 larvae were placed into 100 mL glass vials containing 50 mL imidacloprid (350g of active ingredient (a.i)/L, emulsifiable concentrate, Bayer CropScience SA)-contaminated solution. We used different six concentrations (ranging from 1.2 µg/L up to 170 µg/L), which were defined after a prior bioassay that determined the maximum concentration unable to kill first instar larvae (96h of exposure) and minimum concentration that killed 100% of first instar larvae tested. The individuals were considered dead when were unable to move their appendages after being gently and repeatedly stimulated with a pipette tip. Each treatment was replicated five times. Larvae exposed to 50 mL of semi-soft reconstituted water was considered our control treatment.

2.2. Chronic and acute sublethal exposure bioassays

For the chronic sublethal exposures, we used two sublethal concentrations (LC_1 = 0.59 µg/L and LC_{10} = 1.38 µg/L) previously estimated in the concentration-mortality bioassays. Individuals of *C. columbiensis* faced the exposure to imidacloprid during their entire larval and pupal phases. Groups of 100 newly emerged first instar larvae were placed into glass aquariums

(35 x 30 x 20 cm) that contained two sheets of crushed disposable towels (as a substrate) and 5L of imidacloprid-contaminated water solutions via a gentle aeration system. The control individuals were exposed to reconstituted water only. For each treatment, we use three replicates (i.e., groups of 100 larvae) and we evaluated the mortality during the larval and pupal phases. The larvae larvae were fed with 0.3 g of TetraMin (fish food) twice a week until pupation. The contaminated water was replaced at seven days intervals. For the acute sublethal exposures, we exposed larvae of first or fourth instars to the highest imidacloprid concentration (24.9 µg/L) unable to kill *C. columbiensis* larvae in a 24h exposure period. Here, groups of 100 newly emerged first instar or fourth instar were placed into imidacloprid-containing solutions and once the exposure period were transferred to insecticide-free water solutions until completing their immature stages. The control treatment consisted of individuals of each instar exposed to reconstituted water. Each treatment was replicated three times.

2.3. Chronic and acute sublethal effects on survival, body mass and reproduction of C. columbiensis males and females

2.3.1. Mortality during immature development

In both chronic and acute sublethal exposure bioassays, we daily recorded the number of larvae and pupae died, which allowed the quantification of the total number of *C. columbiensis* males and females that reached adulthood.

2.3.2. Body mass of newly emerged males and females

We used an analytical balance (AS 220.X2 PLUS, RADWAG Balances and Scales, Radom, Poland) to weigh the individual body masses of newly emerged (< 24h) males and females that survived the chronic or acute sublethal exposures. We used 30 couples for each of the treatments in chronic (i.e., imidacloprid-unexposed or individuals facing the imidacloprid LC_1 = 0.59 µg/L or LC_{10} = 1.38 µg/L) and acute (unexposed individuals, first instar or fourth instar larvae that survived 24h of exposure to imidacloprid - 24.9 µg/L) sublethal exposures.

2.3.3. Reproductive performance of males and females

We assessed the potential effects of chronic imidacloprid sublethal exposures on *C. columnenis* reproduction by mating virgin individuals in four different combinations (unexposed couple; exposed female; exposed male; and exposed couple), as previously described elsewhere (Haddi et al. 2016; Santos et al. 2018), and evaluating their fecundity and fertility outcomes. For the fecundity measurements, we randomly selected 30 egg masses (or spawns) of each treatment to be photographed with a stereomicroscope (M205C, Leica Microsystems, Buffalo Grove, IL, USA). To determine fertility (i.e., % hatched eggs), we placed the photographed spawnings in plastic tubes (1.5 cm high × 2.5 cm wide), which were closed at the bottom with porous cloth to facilitate aeration and exit of the larvae. The unhatched eggs were counted after a period of five consecutive days when we calculated the percentage of hatched eggs. Fecundity and fertility rates were also recorded for individuals that faced acute sublethal exposures, but in this scenario, we used only unexposed couples and couples where both males and females were acutely sublethally exposed to imidacloprid.

2.4. Chronic and acute sublethal effects on morphometry and fluctuating asymmetry of *C. columbiensis* wings

In order to quantify potential alterations on the wing form mediated by chronic and acute sublethal exposures to imidacloprid, we conducted morphometric analyzes on images of the right wings of adult males and females. For the asymmetric fluctuation measurements, we used images of the right and left wings of *C. columbiensis* males and females and applied the fluctuating asymmetry approaches previously described elsewhere (Klingenberg 2015; Schaefer et al. 2006).

Photographs were taken with a stereoscopic (Leica M205) equipped with a digital camera. All photos were saved as JPEG files and all specimens were photographed in the same position using slides and coverslips. Twelve reference points (landmarks) per wing were digitized, according to the geometric configuration proposed by Montaño-Campaz et al. (2019; 2022). Positional homology criteria, relative position consistency, adequate shape coverage, and repeatability were analyzed using tpsDIG2 v2.17 software (Rohlf 2013). X-Y coordinates were obtained and shape and size information were extracted using a full Procrustes fit (Dryden & Mardia 1998; Rohlf & Slice 1990). To evaluate the measurement error (ME) we follow the protocol proposed by Palmer & Strobeck (1986), which ensures the correct procedure for the acquisition of reference points. For the case of morphometry, analysis of variance (ANOVA) was used to evaluate the possible differences in the size of the centroid, and a Procruste ANOVA was used to evaluate the differences in shape while considering the values of individual variation (MS) from ANOVA, with type 1 error. The main patterns of variation in wing shape were visualized using principal component analysis (PCA), from the covariance matrix of the symmetric component of the average population shape (Klingenberg et al. 2002). To statistically evaluate the differences between the treatments, a canonical variables analysis

(CVA) of the variation of the form was also performed and the Procrustes distances were calculated (Campbell & Atchley 1981). To analyze the relationship between shape and size of the centroid, a multivariate regression was performed that included the confidence ellipse of each group (90%) using shape and scores with 10,000 iterations. Statistical analyzes were performed with MorphoJ 2.0 software (Klingenberg 2011).

Fluctuating skewness estimates were tested using Procrustes ANOVA (Klingenberg, 2015) by checking individual × side interactions and individual × side mean squares (MS ind × side). The data obtained were subjected to MANOVA statistical tests. Wing size was analyzed by plotting the size of the centroid (Zelditch et al. 2004). The shape was described by Procrustean coordinates, which were scaled to the same size. Size asymmetry was measured as the absolute difference between the centroid sizes of the right and left wings divided by the mean centroid size. Shape asymmetry was measured as the Procrustes distance between the right and left-wing shapes. Centroid sizes and asymmetric shapes were calculated with MorphoJ software (Klingenberg 2011).

2.5. Statistical analysis

Mortality data obtained in the concentration-mortality bioassays were subjected to a probit analysis (PROBIT procedure) available in the SAS statistical software (SAS Institute, 2008). Results obtained for the potential effects of sublethal exposure types on the adaptative traits, we used the R software, version 3.6.2 (R Core 2020) available in RStudio (R Studio 2020). Type II ANOVA tables were used to assess the importance of the explanatory variables in all models (generalized linear models, GLM), by using the "Anova" function in the *car* library (Fox & Weisberg 2018). The data was organized using the *dplyr* library (Wickham et al. 2018), and the figures were built using the sjPlot (Lüdecke & Schwemmer 2016) and ggplot2 (Wickham 2016) libraries. Mortality of immatures, body masses, and reproduction assessments were adjusted to GLMs using the "*glm*" function. The *gamma* distribution was used for the body mass and mortality of larvae and pupa, while a negative × binomial (for adult emergence), poisson (fecundity) and quasi-binomial (fertility) distributions were used in the other analysis. We evaluated the normality of the residuals using q-q plots and the homogeneity of variances using Fligner-Killeen tests (Crawley 2013). We tested for the presence of outliers using Cook's distances (Cook's distance < 1). We built all models using the nlme library (Pinheiro et al. 2006).

3. Results

3.1. Susceptibility of *Chironomus columbiensis* larvae to imidacloprid

The results obtained in the concentration-mortality bioassays fit to a Probit model (n = 250; $\chi^2 = 5.29$; $P = 0.15$) and allowed the estimation of the median lethal concentration (LC_{50}) of 3.86 (3.13 - 4.64) µg/L (Figure 1). These results also allowed the estimation of sublethal concentrations used in the chronic (i.e., $LC_1 = 0.59$ [0.31 – 0.81] µg/L; $LC_{10} = 1.38$ [0.90 – 1.80] µg/L) and acute (24.9 µg/L) sublethal exposure bioassays.

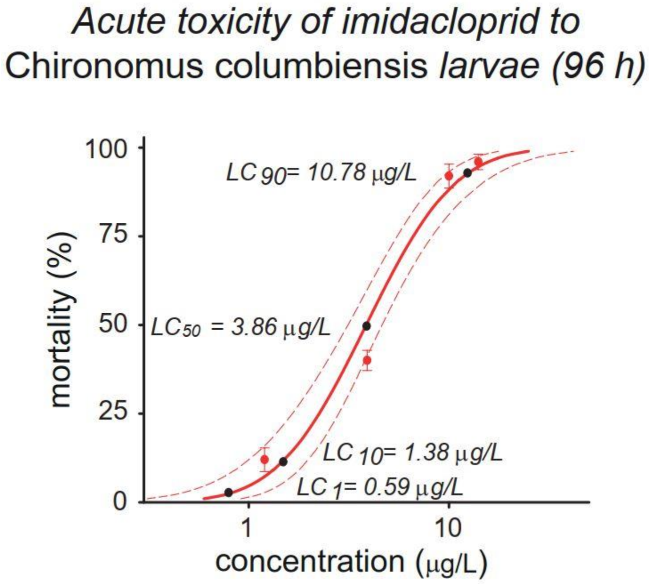


Figure 1. Toxicity (96h of exposure) of imidacloprid to *Chironomus columbiensis* larvae. Dotted lines represent the 95% confidence intervals and symbols shows the mean (± SEM) obtained for eight replicates (aquarium containing 10 *C. columbiensis* larvae).

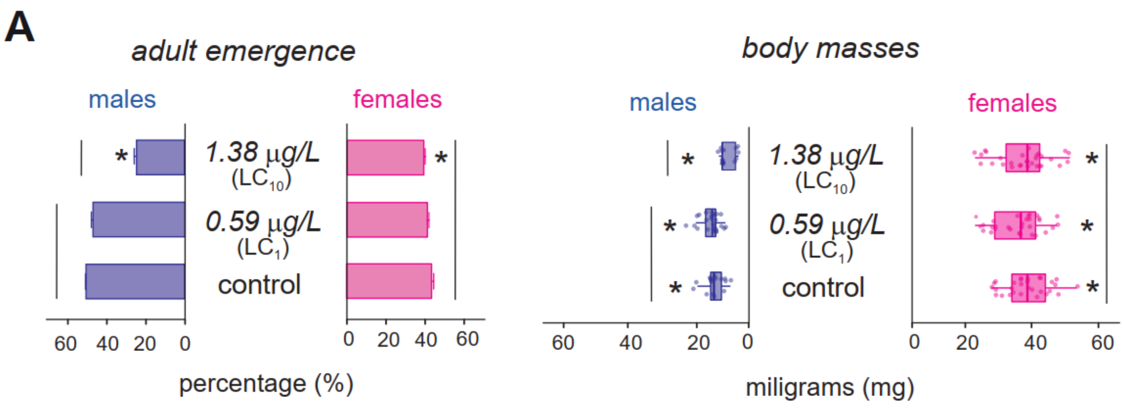
3.2. Chronic sublethal exposure effects on the emergence, body masses and reproduction of *C. columbiensis*

The chronical sublethal exposure of *C. columbiensis* to imidacloprid killed larvae ($\chi^2 = 20.88$, $P < 0.0001$) and pupae ($\chi^2 = 36.20$; $P < 0.0001$) in a concentration-dependent manner (Supplementary Figure 1*A*). The adult emergence was of 94.3 ± 0.67 % for imidacloprid-unexposed insects, which did not differ from the emergence (88.7 ± 0.88 %) recorded to individuals chronically sublethally exposed to 0.59 µg/L (LC_1) of imidacloprid. The chronical sublethal exposure to 1.38 µg/L (LC_{10}) of imidacloprid, however, resulted in significant ($\chi^2 = 459.38$; $P < 0.0001$) reduction on the adult emergence (65.3 ± 1.20 %). The mortality of larvae

(15.3 ± 3.84 %) and pupae (19.6 ± 3.82 %) recorded on the individuals facing the exposure to LC_{10} resulted in a significant ($\chi^2 = 248.4$; $P < 0.0001$) smaller emergence level of males (Figure 2*A*), which unbalanced the sex ratio (1.65 female: 0.61 male) when compared to sex ratio recorded for unexposed individuals (0.86 female: 1.18 male) or individuals (0.87 female: 1.14 male) chronically sublethally exposed to LC_1. The percentage of female's emergence was not affected by the chronic sublethal imidacloprid exposures (Figure 2*A*).

Similar pattern was also recorded for the body masses *C. columbiensis* males and females, where significant ($\chi^2 = 33.33$; $P < 0.0001$) differences were recorded only on males that faced chronical sublethal exposure to LC_{10} (Figure 2*B*). Females were, however, at least 2.8-folder heavier than males in all of three treatments (Figure 2*B*).

Regarding the reproductive responses, the chronical sublethal exposure to imidacloprid reduced the fecundity of *C. columbiensis* couples (χ2 = 20.11, $P = 0.0003$), independent of the insecticide concentration or sex (Figure 2*B*, left panel). Couples where both members were imidacloprid-exposed or couples of only a member (male or female) faced the sublethal exposure exhibited a reduction of approximately 10.9 % in the number of spawning eggs when compared to couples formed by only by unexposed members (Figure 2*B*, left panel). The fertility rates, however, were only significantly ($\chi^2 = 19.3$, $P = 0.004$) reduced in couples that both members faced the sublethal exposure to LC_{10} (Figure 2*B*, right panel). Couples that faced the exposure to the LC_1 produced fertility rates similar (χ2 = 1.71, P = 0.426) to those produced by unexposed couples (Figure 2*B*, right panel)


Chronical sublethal exposure to imidacloprid reduced the survival and body masses of Chironomus columbiensis

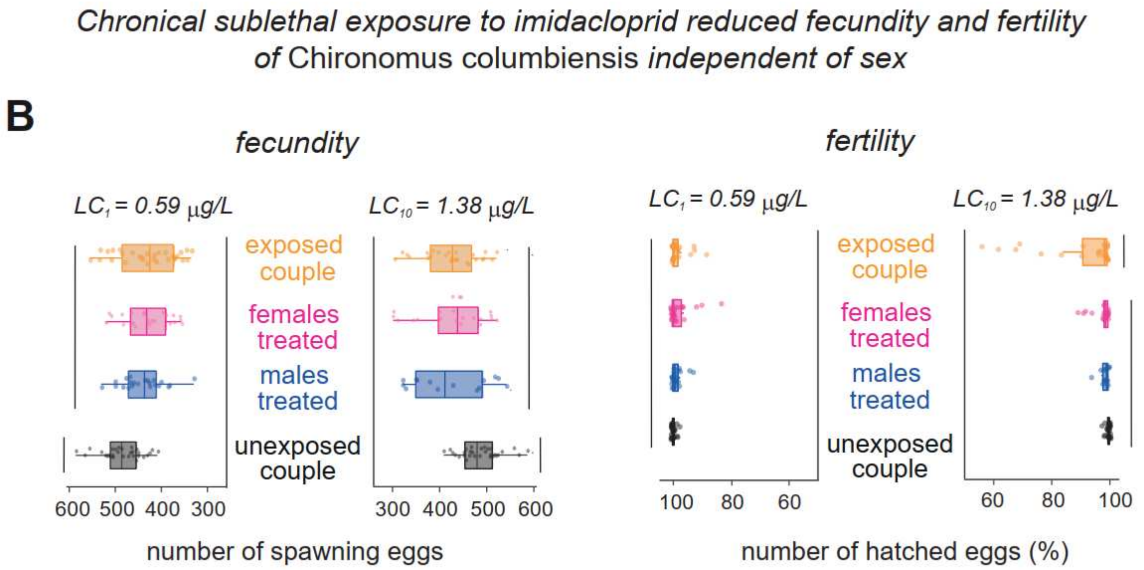


Figure 2. Imidacloprid chronic sublethal effects on the emergence, body mass and reproductive performance (fecundity and fertility) of *Chironomus columbiensis*. The LC_1 (0.59 µg/L) and LC_{10} (1.38 µg/L) were estimated in the 96h exposure bioassays but the exposure time was during the entire immature (larval and pupal) phase. (***A***, ***left panel***) Histograms represent the average number (± standard error, SE) of three replicates. (***A***, ***right panel - B***) Each box plot represents the median (the line within each box plot) and range of dispersion (lower and upper quartiles). Histograms or box plots grouped by the same vertical line indicate no significant differences by Tukey's HSD test ($P < 0.05$).

3.3. Chronic sublethal exposure effects on the wing shapes of *Chironomus columbiensis*

Our PCA analysis of wing morphometry revealed that the top three PCs accounted for 58% (PC1: 30.4%; PC2: 15.4%; PC3: 11.1%) of the total variation in shape and size for females and 60.5% (PC1: 29.9 %; PC2: 19.4 %; PC3: 11.2%) of the total variation in shape and size of males' wings. The procrustes ANOVA conducted for females' results showed significant differences only for the shape, but not for the size among the groups (i.e., unexposed individuals; individuals that faced exposure to imidacloprid LC_1 or LC_{10}) studied, (Table S1). The CVA dispersion diagram (Figure 3*A*, left panel) and the first two PC scores (Supplementary Figure 2A) showed a clear differentiation among the three groups, which highlighted the effect of the sublethal exposure on the wing shapes. In the wing of males, however, the procrustes ANOVA recorded only significant differences in size but not in shape (Table S1). The CVA dispersion diagram (Figure 3A, left panel) and the first two PC scores (Supplementary Figure 2A) showed differentiation between the group exposed to imidacloprid LC_{10} and the group exposed to the control.

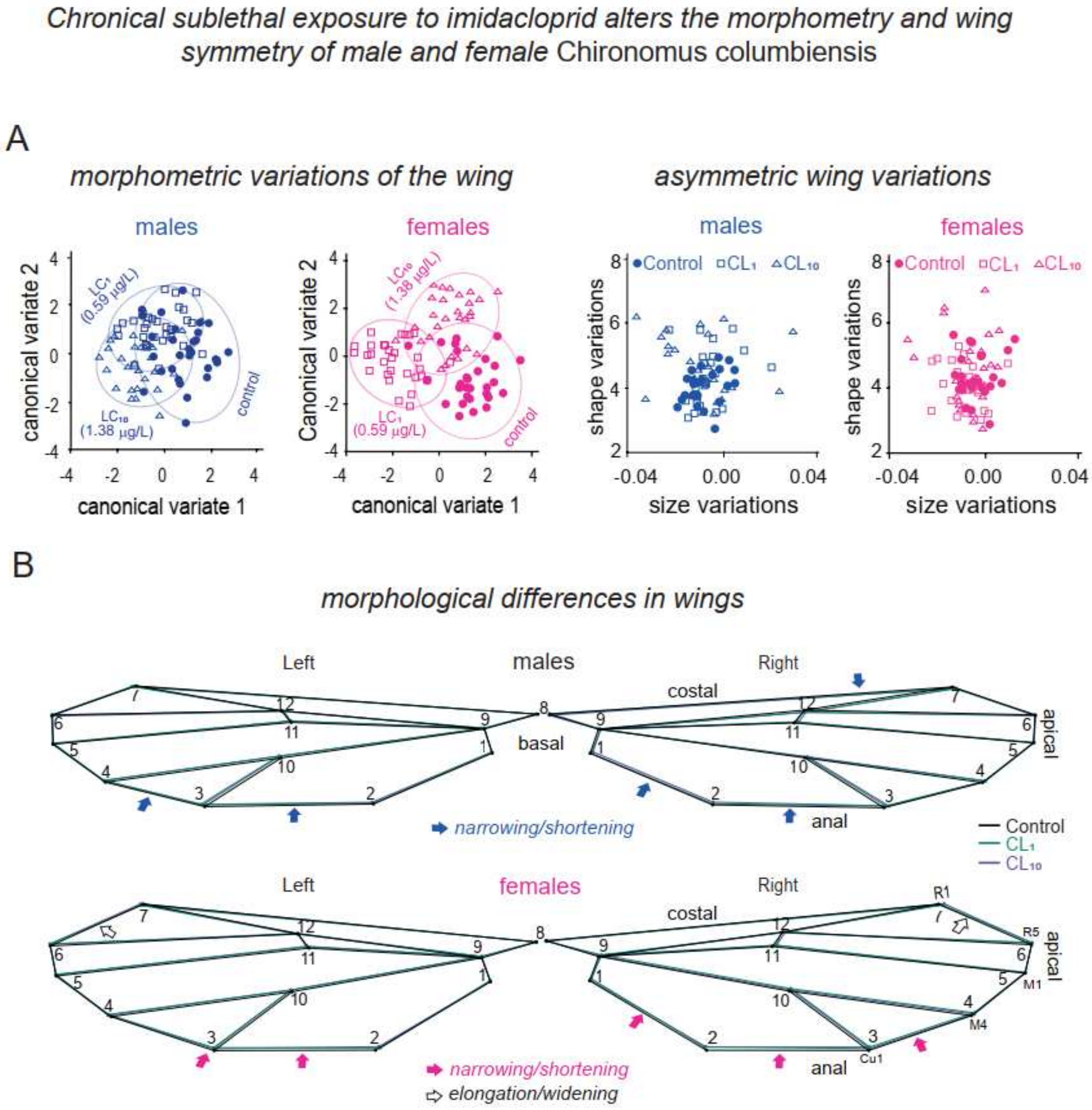
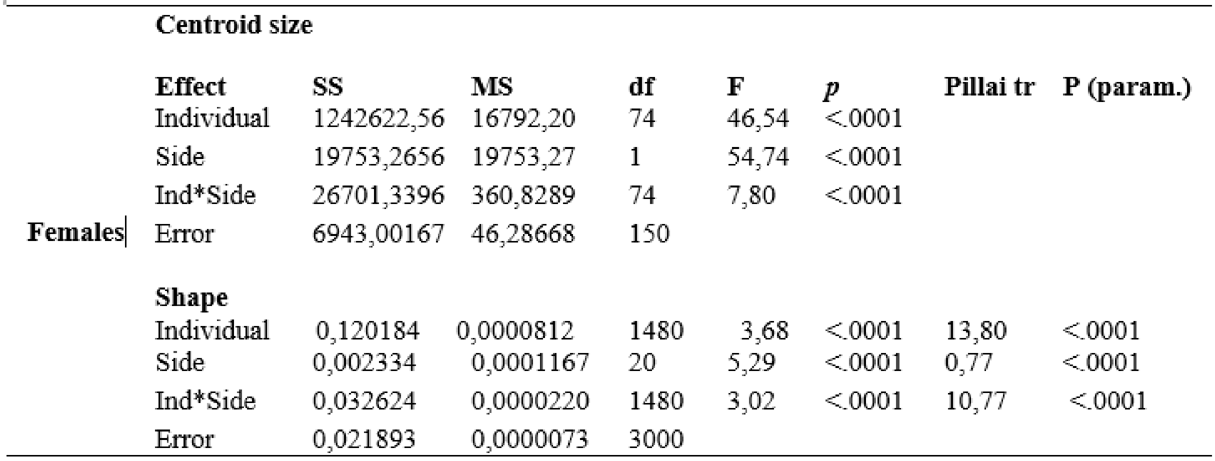

Chronical sublethal exposure to imidacloprid alters the morphometry and wing symmetry of male and female Chironomus columbiensis

Figure 3. Imidacloprid chronic sublethal effects on the wing shapes of *Chironomus columbiensis* males and females. The LC$_1$ (0.59 µg/L) and LC$_{10}$ (1.38 µg/L) were estimated in the 96h exposure bioassays but the exposure time was during the entire immature (larval and pupal) phase. Canonical wing shape analysis (***A, left panel***); Regression of asymmetrical variations in wing shape and size (***A, right panel***); Schematics of average PCs of asymmetric variations in wing shape and size (**B**).

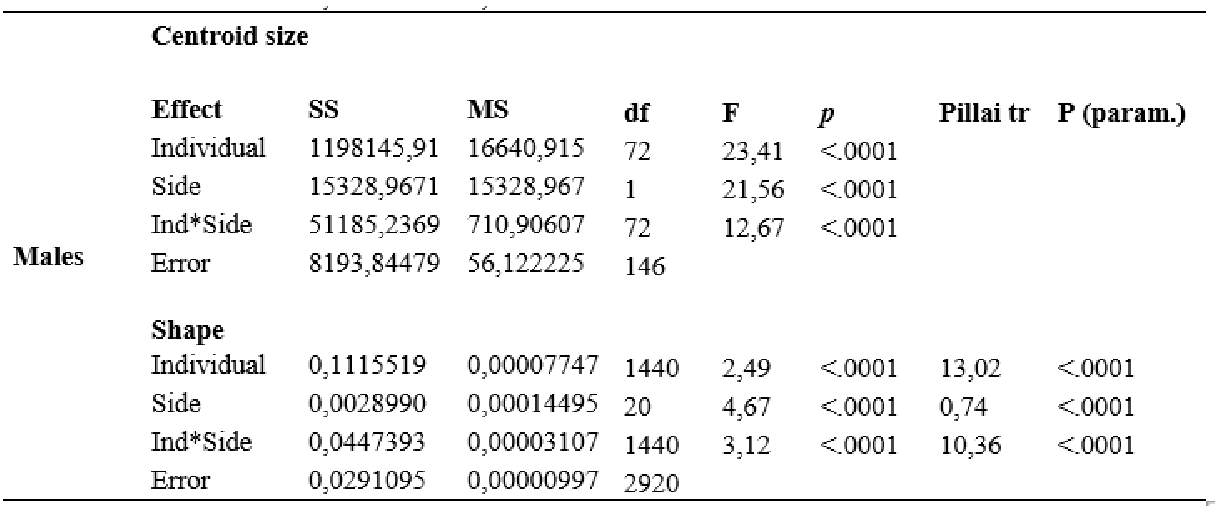
Regarding the fluctuating asymmetry analysis, the measurement error was evaluated in all the populations studied, in order to avoid any type of error associated with the data. Procrustes ANOVA results indicated that fluctuating asymmetry individuals by sides (ind ×

side) mean squares (MS) values exceeded MS error values, implying that there is no measurement error (ME) in the data (Table 2). Regarding FA (ind × side), the results of Procrustes ANOVA (MANOVA) show significant differences between the groups (i.e., unexposed individuals; individuals that faced exposure to imidacloprid LC$_1$ or LC$_{10}$) examined (P < 0.0001), for both females and males (Table 1). Regarding the fluctuating asymmetry between populations and sex, it was observed that the females in all the treatments evaluated had higher levels of AF compared to the male populations (Table 2; Figure 3A, right panel). However, the populations evaluated in the LC$_{10}$ treatment presented the highest levels of AF for both females and males, followed by the LC$_1$ treatment compared to the control (Table 2; Fig. 3*A*, right panel). However, the populations evaluated in the LC$_{10}$ treatment presented the highest levels of AF for both females and males, followed by the LC$_1$ treatment compared to the control (Table 2; Fig. 3*A*, right panel). The principal components analysis showed the variations on average in each of the populations, where the females of the LC$_1$ and LC$_{10}$ treatments presented a reduction in the anal part and a slight increase between the radial vein 1 (R1) and radial 5 (R5) with greater intensity in the right wing compared to the left (red and black arrows), while in the case of males, there was a reduction in the anal and costal part, also with greater intensity in the right wing. compared to the left, showing narrower wings in both sexes (Fig. 3*B*).

Table 1. Results of ANOVA over the effect during the exposure sublethal the life cycle of immature *Chironomus columbiensis* to imidacloprid (LC$_1$ = 0.59 µg/L and LC$_{10}$ = 1.38 µg/L) on the fluctuating asymmetry of females and males: sums of squares (SS), mean squares (MS), degrees of freedom (df), F statistics and parametric P-values for each of the effects.

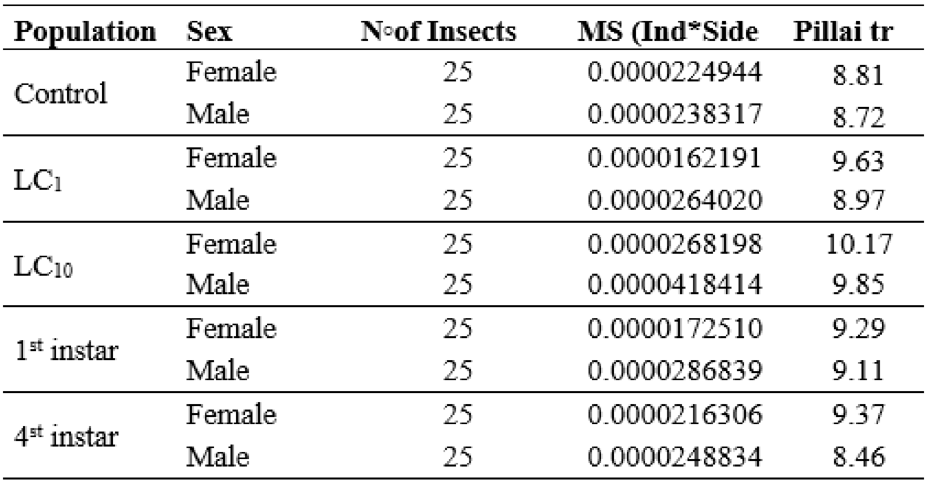


	Centroid size							
Females	**Effect**	**SS**	**MS**	**df**	**F**	***p***	**Pillai tr**	**P (param.)**
	Individual	1242622,56	16792,20	74	46,54	<.0001		
	Side	19753,2656	19753,27	1	54,74	<.0001		
	Ind*Side	26701,3396	360,8289	74	7,80	<.0001		
	Error	6943,00167	46,28668	150				
	Shape							
	Individual	0,120184	0,0000812	1480	3,68	<.0001	13,80	<.0001
	Side	0,002334	0,0001167	20	5,29	<.0001	0,77	<.0001
	Ind*Side	0,032624	0,0000220	1480	3,02	<.0001	10,77	<.0001
	Error	0,021893	0,0000073	3000				



	Effect	SS	MS	df	F	p	Pillai tr	P (param.)
Males	**Centroid size**							
	Effect	**SS**	**MS**	**df**	**F**	***p***	**Pillai tr**	**P (param.)**
	Individual	1198145,91	16640,915	72	23,41	<.0001		
	Side	15328,9671	15328,967	1	21,56	<.0001		
	Ind*Side	51185,2369	710,90607	72	12,67	<.0001		
	Error	8193,84479	56,122225	146				
	Shape							
	Individual	0,1115519	0,00007747	1440	2,49	<.0001	13,02	<.0001
	Side	0,0028990	0,00014495	20	4,67	<.0001	0,74	<.0001
	Ind*Side	0,0447393	0,00003107	1440	3,12	<.0001	10,36	<.0001
	Error	0,0291095	0,00000997	2920				

Table 2. The intensity of fluctuating asymmetry between populations and sex during the exposure sublethal of imidacloprid in *Chironomus columbiensis*. Mean Squares (MS) and Pillai tree data of Procrustes ANOVA of shape.



Population	Sex	N°of Insects	MS (Ind*Side	Pillai tr
Control	Female	25	0.0000224944	8.81
	Male	25	0.0000238317	8.72
LC_1	Female	25	0.0000162191	9.63
	Male	25	0.0000264020	8.97
LC_{10}	Female	25	0.0000268198	10.17
	Male	25	0.0000418414	9.85
1st instar	Female	25	0.0000172510	9.29
	Male	25	0.0000286839	9.11
4st instar	Female	25	0.0000216306	9.37
	Male	25	0.0000248834	8.46

3.4. Acute sublethal exposure effects on the emergence, body masses and reproduction of *Chironomus columbiensis*

The acute sublethal exposure of *C. columbiensis* first or fourth instars did reduce the percentage of emerged males ($\chi^2 = 90.97$, $P < 0.001$) with a most intense effect when the exposure challenge occurred on the first instar (Figure 4A, *left panel*). The percentage of emerged females, however, was significantly ($\chi^2 = 85.58$, $P < 0.001$) reduced only when the exposure challenge occurred on the first instar larvae (Figure 4A, *left panel*). The significant ($\chi^2 = 46.17$, $P < 0.001$) mortality rate during the larval phases (Supplementary Figure 1*B*) of individuals that faced acute sublethal exposure on the first instar contributed to the reduced number of adults. Interestingly, the mortality rate observed during the pupal phase was not affected ($\chi^2 = 2.63$, P = 0.27) by the acute sublethal exposure, independently of the instar that faced such challenge (Supplementary Figure 1*B*). The body mass results obtained for the males and females that faced acute sublethal exposures revealed controversial results (Figure 4*B*). While the acute sublethal exposure on the first instar results in significantly ($\chi^2 = 27.94$, $P < 0.001$) heavier males, the females that faced acute sublethal exposures, independently of when this challenge occur (i.e.., first or fourth instar) results in significantly ($\chi^2 = 19.41$, $P < 0.001$) lighter individuals (Figure 4*A, right panel*). The males that faced acute sublethal exposure on the fourth instar were also lighter that unexposed individuals (Figure 4*A, right panel*). Regarding the reproduction of the individuals that faced the acute sublethal exposures, significant ($\chi^2 = 16.50$, $P < 0.001$) results were recorded only in the fecundity of couples formed by both members that faced this insecticidal challenge at the fourth instar (Figure 4*B*). No significant effect ($\chi2 = 1.7.50$, P = 0.425) was recorded for the fecundity rates, independently of when the acute sublethal exposure occurred (Figure 4*B*)

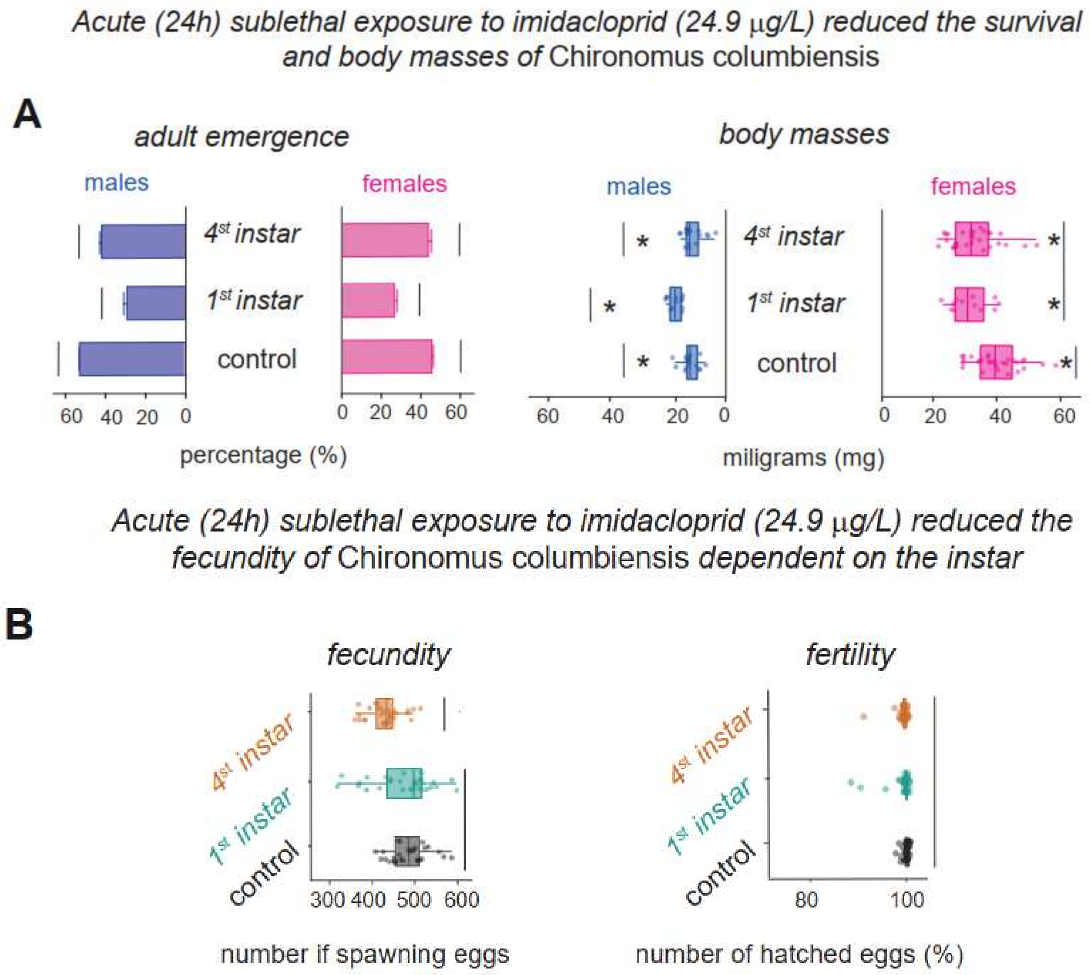
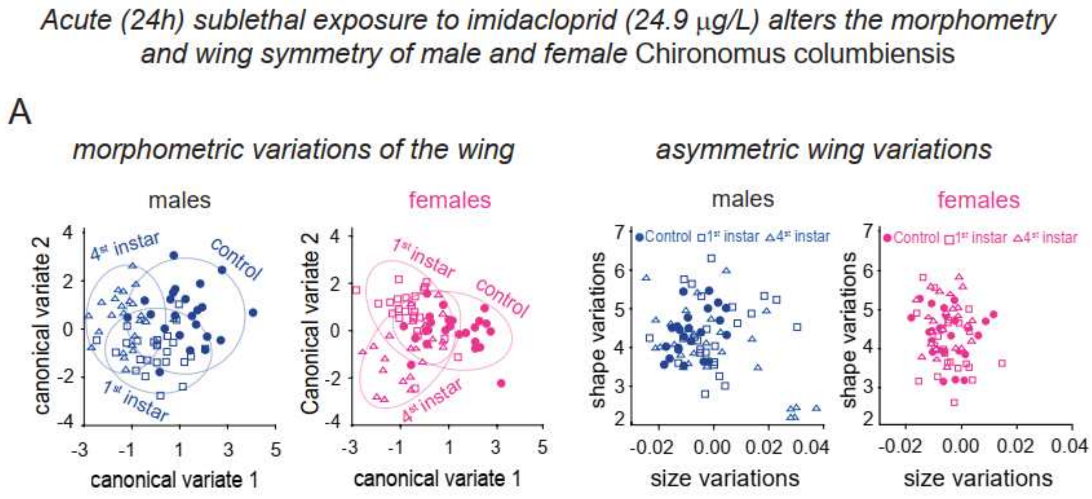


Figure 4. Imidacloprid acute sublethal effects on the emergence, body mass and reproductive performance (fecundity and fertility) of *Chironomus columbiensis*. The insecticidal challenges (24h of exposure to 24.9 µg/L) were applied into groups of first or fourth instar larvae. (***A***, ***left panel***) Histograms represent the average number (± standard error, SE) of three replicates. (***A***, ***right panel - B***) Each box plot represents the median (the line within each box plot) and range of dispersion (lower and upper quartiles). Histograms or box plots grouped by the same vertical line indicate no significant differences by Tukey's HSD test ($P < 0.05$).

3.5. Acute sublethal exposure effects on the wing shapes of *Chironomus columbiensis*

Regarding the total variation in wing shape and size of individuals that faced acute sublethal exposure, the first three PC represented 56% (PC1: 27.4%; PC2: 18.7%; PC3: 9.8%) and 59.5% (PC1: 31.6%; PC2: 15.2%; PC3: 12.7%) of the total variation in shape and size for females and males, respectively. Interestingly, the procrustes ANOVA results for females' wings showed significant differences for size, but not for shape among the groups (i.e., unexposed individuals; individuals exposed on their first, or fourth larval instar) studied (Table S2). The CVA dispersion diagram (Figure 5A, left panel) and the first two PC scores (Supplementary Figure 2B) showed a slight differentiation between the three groups evaluated, which corroborates the effect of the insecticide on the size of the wings. However, in the case of the males' wings, significant differences were found according to the procrustes ANOVA in the size and shape (Table S2). Thee CVA dispersion diagram (Figure 5A, left panel) and the first two PC scores (Supplementary Figure 2B) revealed clear differences among the groups evaluated.



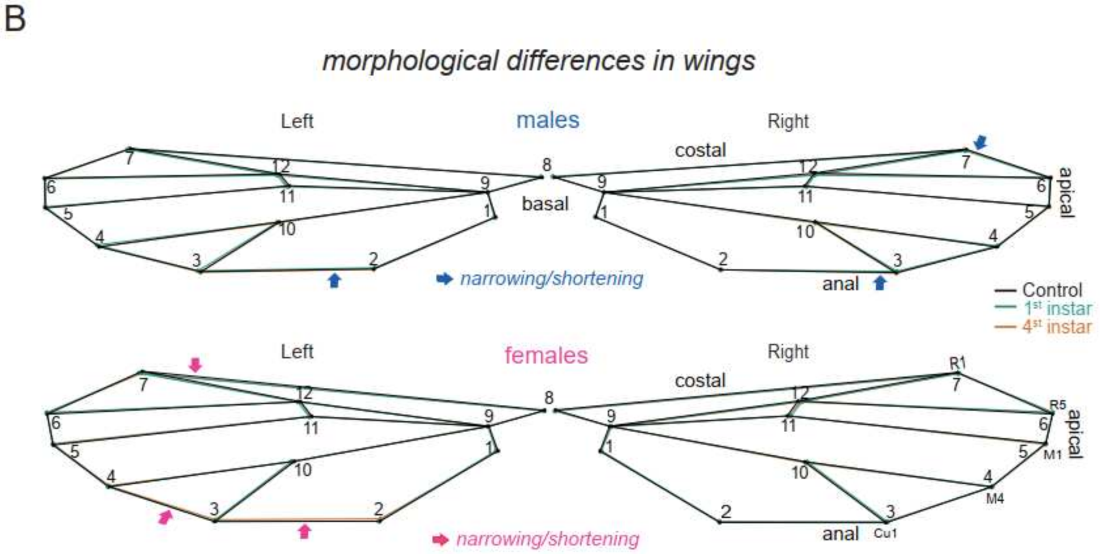


Figure 5. Imidacloprid acute sublethal effects on the wing shapes of *Chironomus columbiensis* males and females. The insecticidal challenges (24h of exposure to 24.9 µg/L) were applied into groups of first or fourth instar larvae. Canonical wing shape analysis (***A, left panel***); Regression of asymmetrical variations in wing shape and size (***A, right panel***); Schematics of average PCs of asymmetric variations in wing shape and size (**B**).

In the fluctuating asymmetry analyses, we assessed the measurement error in all the groups evaluated, attempting to avoid any type of error associated with the data. Procrustes ANOVA results indicated that FA (ind × side) MS values exceeded MS error values, implying that there is no ME in the data (Table 3). Regarding FA (ind × side), the results of the Procrustes ANOVA (MANOVA) showed significant differences between the groups examined ($P < 0.0001$) (Figure 5*A, right panel*) (Table 3). After evaluating the influence of PA in each population by sex, it was observed that the females in all the groups had higher levels of PA compared to the male populations (Table 2; Figure 5A, *right panel*). However, highest levels of AF were recorded for females that faced the acute sublethal exposure on their fourth instar larvae, while for males' AF levels, those males that faced the acute sublethal exposure on their first instar exhibited higher AF levels than those unexposed males (Table 2; Figure 5*A*, *right panel*). The principal components analysis showed the variations on average in each of the populations, where the females that faced imidacloprid exposure on their first or fourth larval instar presented a slight reduction in the anal and costal parts, with greater intensity in the left wing compared to the right (Figure 5*B*, magenta arrows,). Such analysis for males, however, indicated that there was a reduction in the anal and costal part with greater intensity in the right wing compared to the left, showing narrower wings in both sexes (Figure 5*B*, *blue arrows*).

Table 3. Results of ANOVA over the effect of imidacloprid during short-intense sublethal exposure of *Chironomus columbiensis* to imidacloprid (1st instar = 24.9 µg/L and 4st instar = 24.9 µg/L) on the fluctuating asymmetry of females and males: sums of squares (SS), mean squares (MS), degrees of freedom (df), F statistics and parametric P-values for each of the effects.

	Centroid size							
	Effect	**SS**	**MS**	**df**	**F**	***p***	**Pillai tr**	**P (param.)**
Females	Individual	2869165,15	39303,6322	73	149,87	<.0001		
	Side	7280,88415	7280,88415	1	27,76	<.0001		
	Ind*Side	19144,9298	262,259312	73	4,12	<.0001		
	Error	9420,40676	63,651397	148				
	Shape							
	Individual	0,111884	0,0000766	1460	3,75	<.0001	14,14	<.0001
	Side	0,001772	0,0000886	20	4,34	<.0001	0,75	<.0001
	Ind*Side	0,029811	0,0000204	1460	2,65	<.0001	9,95	<.0001
	Error	0,022784	0,0000077	2960				

	Centroid size							
	Effect	**SS**	**MS**	**df**	**F**	***p***	**Pillai tr**	**P (param.)**
Males	Individual	1922344,72	26699,2322	72	50,86	<.0001		
	Side	5656,15749	5656,15749	1	10,78	<.0016		
	Ind*Side	37794,1754	524,919102	72	7,32	<.0001		
	Error	10470,3141	71,714480	146				
	Shape							
	Individual	0,1160999	0,00008062	1440	3,12	<.0001	13,16	<.0001
	Side	0,0035789	0,00017895	20	6,92	<.0001	0,83	<.0001
	Ind*Side	0,0372177	0,00002585	1440	2,67	<.0001	9,60	<.0001
	Error	0,0282490	0,00000967	2920				

4. Discussion

Here, we are presenting how the different types of sublethal exposures to imidacloprid, at concentrations considered safe at 96h exposure bioassays, can modify the adaptive responses (e.g., emergence, wing shapes and reproduction) of *C. columbiensis* males and females. Firstly, we demonstrated that chronic sublethal exposures adversely affected more males than females, as this imidacloprid exposure type killed more immature males, produced lighter adult males and similarly affected the reproductive performance and wing shapes when compared to females. The acute sublethal exposures, in its turn, seems to similarly affect both males and female. Despite the acute sublethal exposure killed more immature males and females when the exposure happened at the first larval instar, we recorded more undesired sublethal effects (e.g., modified wing asymmetries, lighter adult females and reduced fecundity rates) when the acute sublethal effects occurred at the fourth instar.

The potential of imidacloprid to unbalance survival and growth performance of aquatic non-target organisms have been well described (Cavallaro et al. 2017; Sánchez-Bayo et al. 2016; Malhotra et al. 2021). However, how acute (< 96h of exposure at high concentrations) and chronic (low concentrations but for longer exposure periods) imidacloprid sublethal exposures contribute to such sublethal responses has not been detailed exploited. Although initially suggested for the neonicotinoid lethality (Beketov et al. 2008; Tennekes & Sánchez-Bayo 2013; Sánchez-Bayo & Tennekes 2020), it is reasonable to expect that imidacloprid-mediated sublethal effects occurs in a time-cumulative manner i.e., the evaluated effects will increase with exposure time and concentration. The molecular basis for this time-cumulative or delayed toxicity has been attributed to the irreversibility of the compound binding to its receptor (Tennekes & Sanchez-Bayo 2013). However, such proof of concept for the neonicotinoids' major target sites (nAChRs), due to the high number of receptors producing genes (Jones et al. 2005, 2006), which has been shown functionally demonstrated to lead different nAChR subtypes (Salgado & Saar 2004, Oliveira et al. 2010,2011; Salgado 2016). Our adult emergence results reinforce such time-cumulative sublethal toxicity only for *C. columbiensis* males, whose emergence were reduced either with the increase of concentration (for acute exposures) or exposure length (for chronical exposures). For *C. columbiensis* female's emergence, reductions were observed only at acute exposures and when the immature were at first instar larvae. Such sex-dependent effects on adults' emergence are divergent from those described for other chironomid species, where a higher emergence of males was recorded (Cavallaro et al. 2017; Chandran et al. 2018; Fortuin et al. 2021).

Here, despite our results for survival and development growth indicated that *C. columbiensis* was more susceptible to the imidacloprid than few of its closely related species, such as *C. kiinensis* (Liu et al. 2021), *C. riparius* (Maloney et al. 2020,2021; Wei et al. 2021), *C. sancticaroli* (Qeuiroz et al. 2021); *C. xanthus* (Melo et al. 2022) and *C. dilutus* (Raby et al. 2018), methodological differences (e.g., exposure duration, compound concentrations) hindered such direct comparisons and turn the search for physiological explanations a complex task. As discussed above, such differential susceptibilities would initially be expected to relate to differences on the types and densities of nAChR (major targets for neonicotinoid actions) expressed in these chironomid species. However, based on similar results obtained for two closely related chironomid species (Maloney et al. 2021), it is unlikely that the nAChR affinities/densities fully explain these species-specific susceptibilities to imidacloprid in chironomids. Instead, as differences in metabolism (Wei et al. 2021), antioxidant stress responses (Chandran et al. 2018) and actions on secondary targets – e.g., salivary glands (Castellanos et al. 2021, Perez Campos et al. 2021), midgut and optic lobes (Catae et al. 2018) – have shown to shape the imidacloprid actions in insects, it would be reasonable to expect that the combination of several factors (including nAChR affinities/densities) would compose a better explanation.

Indeed, the imidacloprid-mediated sublethal effects have been shown to vary according to the concentration, exposure duration, organismal development, and combination with other stressors (Stoughton et al 2008; Roessnik et al. 2013; Hunn et al. 2019; Queiroz et al. 2021, Macaulay et al. 2021; Melo et al. 2022). Our findings reinforce such statements as males and females responded in different manners when had to faced chronic and acute sublethal exposures to imidacloprid. For instance, the *C. columbiensis* males' body masses responded in different ways when faced with the different types. First, the females' body masses, was not affected by chronical sublethal exposures but significantly reduced by acute sublethal exposures, independent of their developmental phase (i.e., first or forth larval instar), while chronical exposures to 1.38 mg/L (i.e., estimated LC_{10} in a 96h exposure bioassay) reduced the males body masses, but the acute (24h) exposure at the 24.9 mg/L (a not killing concentration) produced heavier males. Indeed, such positive or detrimental sex-biased imidacloprid-mediated sublethal effects have also been recorded for other insect pests (Haddi et al. 2016; Santos et al. 2018; Feng et al. 2019), including chironomids (Langer-Jaesrich et al. 2010; Cavallaro et al. 2017) and may reflect the physiological trade-offs that governs the insect internal energy balance and resource allocations for immature and adult performances (e.g., body mass and size, survival, and reproduction).

Here, we are also presenting those different types of imidacloprid sublethal exposures during the immature phases of *C. columbiensis* can result in modified fecundity and fertility rates. While the chronic exposures reduced fecundity rates independently of the sex and insecticide concentration, the acute exposures reduced fecundity rates when the insecticidal stress happened at the fourth larval instar. Although additional investigations are required to assess precisely what properties of imidacloprid sublethal exposures is contributing to the detriment of *C. columbiensis* reproduction, the reduced reproductive performance described in our study derived from potential alterations on physiological mechanisms (e.g., sperm mobility, sperm storage, stimulation of ovulation/oviposition, and egg protection) of the imidacloprid sublethally challenged organisms (Ge et al. 2010; Yu et al. 2012; Santos et al. 2016; Haddi et al. 2016).

Fluctuation asymmetries and alterations in wing shapes have been suggested as a useful phenotypic biomarker for environmental pollution caused by agricultural insecticides (Rosa et al. 2016; Montano-Campaz et al. 2019; 2022; Nattero et al. 2019, 2021). Here, are also demonstrating detrimental variations in size, shape, and fluctuating asymmetries of *C. columbiensis* wings of males and females stressed by the different imidacloprid sublethal exposures. Considering that chironomid males with low levels of wing fluctuating asymmetries can achieve more matings (McLachlan & Cant 1995; Takamura 1999), and in our case, both males and females exhibited higher levels of fluctuating asymmetry, it is reasonable to expect that part of the reduced reproductive performance described here can be related to reduced mating success of the imidacloprid sublethally challenged individuals. For instance, recent investigation (Christienses et al. 2021) have shown the involvement of nAChR subunits on the bursicon-driven wing expansion, diminishing the viability of adult insects.

5. Conclusions

The present study highlights the relevance of better understanding the unintended effects of imidacloprid on Neotropical aquatic non-target organisms. We demonstrated here that the exposure length, which is generally neglected in the risk assessment evaluations, together with the insecticide concentrations shape the sublethal responses of an aquatic non-target organism (the midges *C. columbiensis*), which unbalance the population equilibrium of insect biota in the aquatic freshwater systems of the Neotropical region. Precisely, our findings demonstrated that *C. columbiensis* males seems to be more affected than females by imidacloprid sublethal challenges, while females seem to suffer more detrimental consequences when need to face imidacloprid acute sublethal challenges.

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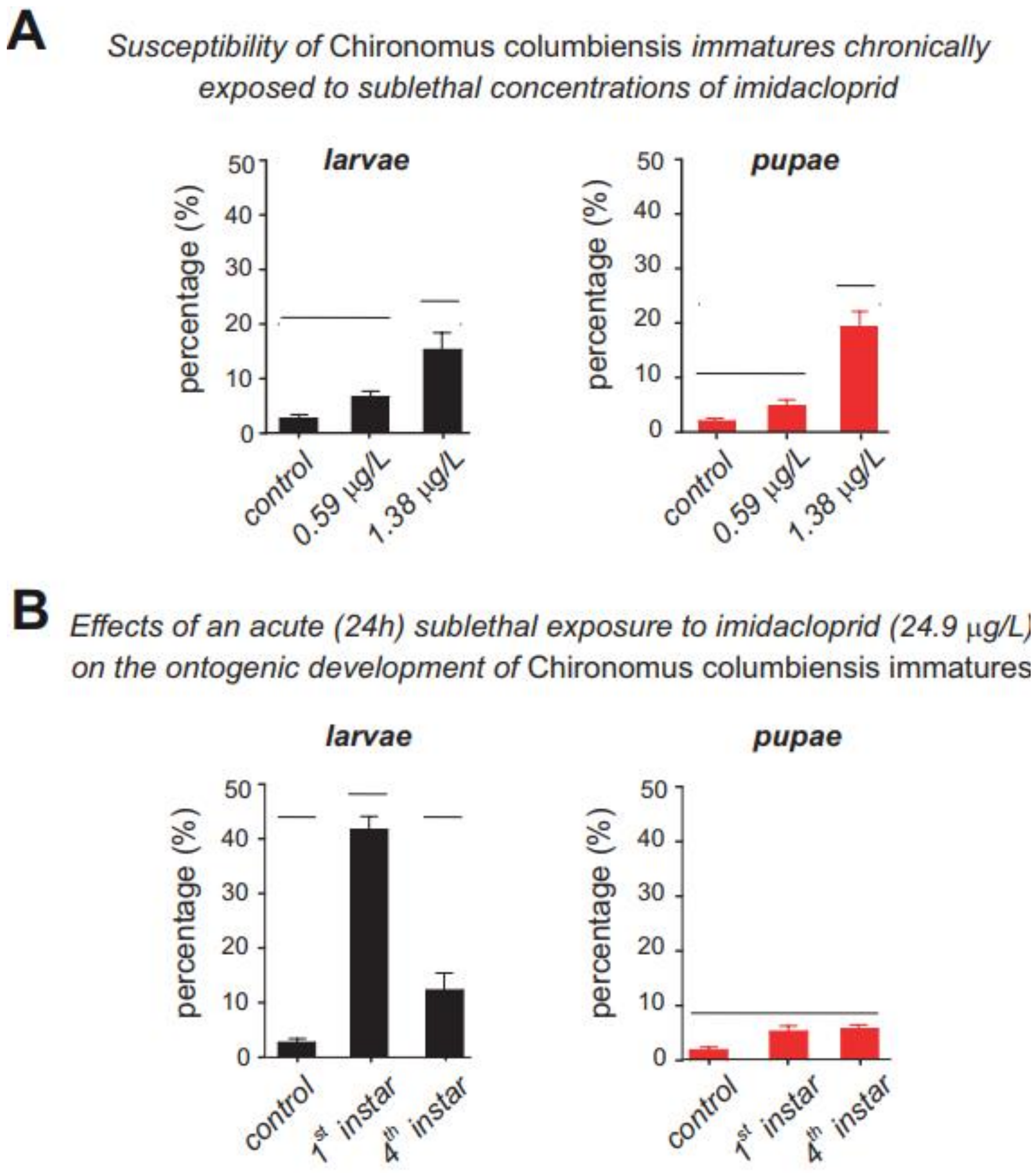
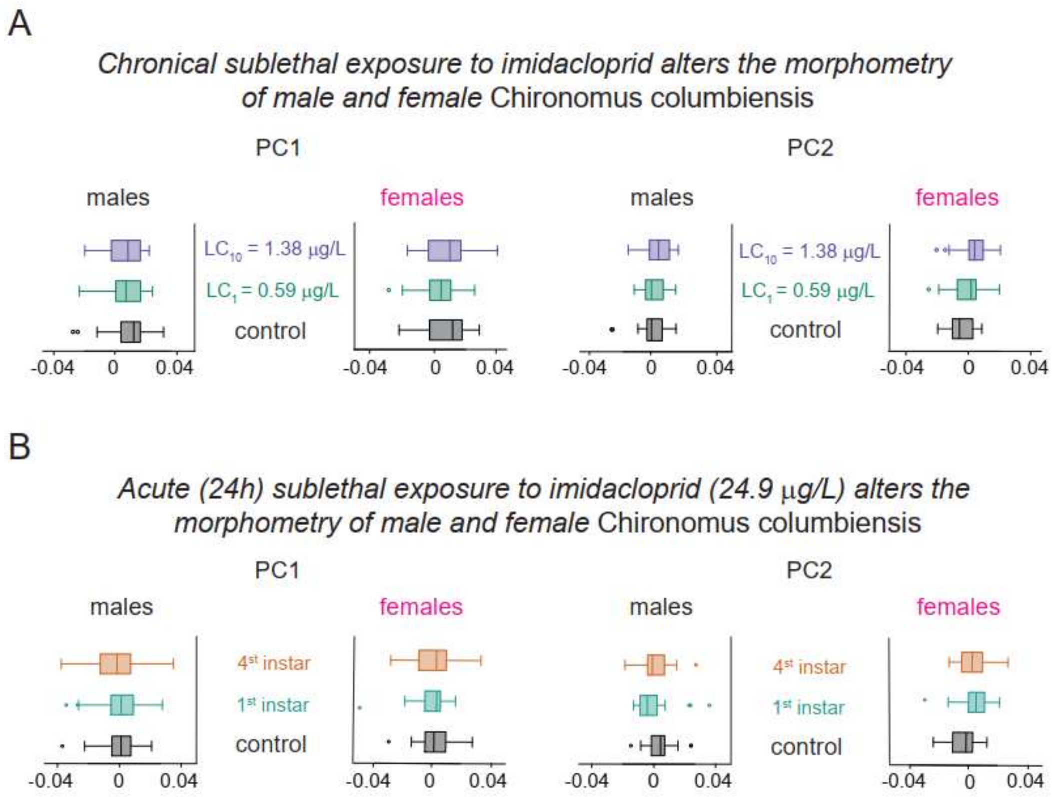
Supporting information

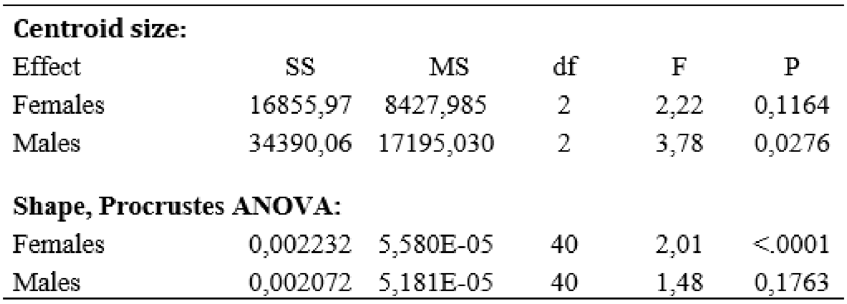


Supplementary Figure 1. The total number of dead larvae and pupae mediated by the imidacloprid chronic (***A***) and acute (***B***) sublethal exposures. The chronic sublethal exposure uses LC_1 (0.59 µg/L) and LC_{10} (1.38 µg/L) estimated in the 96h exposure bioassays but the exposure time was during the entire immature (larval and pupal) phase. The insecticidal challenges (24h of exposure to 24.9 µg/L) were applied into groups of first or fourth instar larvae. Histograms represent the average number (± standard error, SE) of three replicates. Histograms grouped by the same horizontal line indicate no significant differences by Tukey's HSD test ($P < 0.05$).



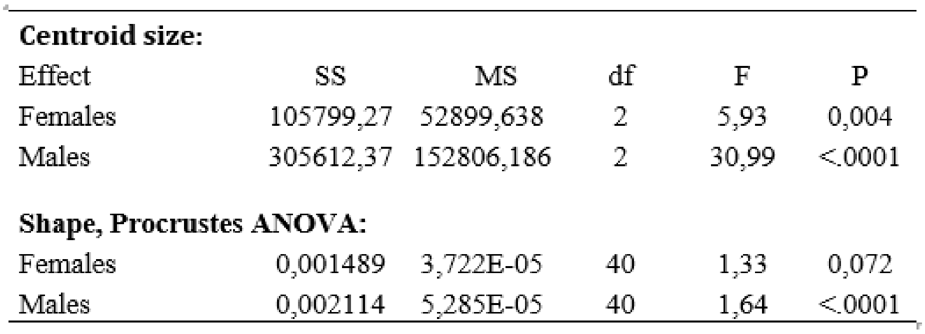
Supplementary Figure 2. Imidacloprid chronic (***A***) and acute (***B***) sublethal exposure effects on the wing shapes of *Chironomus columbiensis* males and females. The chronic sublethal exposure uses LC1 (0.59 µg/L) and LC10 (1.38 µg/L) estimated in the 96h exposure bioassays but the exposure time was during the entire immature (larval and pupal) phase. The insecticidal challenges (24h of exposure to 24.9 µg/L) were applied into groups of first or fourth instar larvae. Histograms represent the average number (± standard error, SE) of three replicates. Box plots grouped by the same vertical line indicate no significant differences by Tukey's HSD test ($P < 0.05$).

Table S1. Results of ANOVA over the effect during the exposure sublethal the life cycle of immature *Chironomus columbiensis* to imidacloprid (LC_1 = 0.59 µg/L and LC_{10} = 1.38 µg/L) on the Morphometric of females and males: sums of squares (SS), mean squares (MS), degrees of freedom (df), F statistics and parametric P-values for each of the effects.



Centroid size:					
Effect	SS	MS	df	F	P
Females	16855,97	8427,985	2	2,22	0,1164
Males	34390,06	17195,030	2	3,78	0,0276
Shape, Procrustes ANOVA:					
Females	0,002232	5,580E-05	40	2,01	<.0001
Males	0,002072	5,181E-05	40	1,48	0,1763

Table S2. Results of ANOVA over the effect of imidacloprid during short-intense sublethal exposure of *Chironomus columbiensis* to imidacloprid (1st instar = 24.9 µg/L and 4st instar = 24.9 µg/L) on the Morphometric of females and males: sums of squares (SS), mean squares (MS), degrees of freedom (df), F statistics and parametric P-values for each of the effects.



Centroid size:					
Effect	SS	MS	df	F	P
Females	105799,27	52899,638	2	5,93	0,004
Males	305612,37	152806,186	2	30,99	<.0001
Shape, Procrustes ANOVA:					
Females	0,001489	3,722E-05	40	1,33	0,072
Males	0,002114	5,285E-05	40	1,64	<.0001

CHAPTER IV

Exposure to chlorantraniliprole and its combination with imidacloprid reduces reproduction and body mass, and causes shape changes in eggs and wings of *Chironomus columbiensis*

Exposure to chlorantraniliprole and its combination with imidacloprid reduces reproduction and body mass, and causes shape changes in eggs and wings of *Chironomus columbiensis*

Milton L. Montaño-Campaz, Eugenio E. Oliveira, Tito Bacca, Beatriz Toro-Restrepo, Lucimar G. Dias

Abstract

The use of insecticides such as diamides and neonicotinoids in pest control is a growing practice, despite the environmental effects. despite limited understanding of the environmental effects. The toxicity of individual pesticides may be underestimated when considering the complex mixtures found in agricultural runoff. Here we evaluated the susceptibility of *Chironomus columbiensis* (Diptera: Chironomidae) larvae to the diamide chlorantraniliprole and assessed whether sublethal exposure types and their combination with imidacloprid would alter emergence, time to initiate emergence, reproduction, body mass, and egg and wing shape of the populations studied. Sublethal exposure consisted of exposing individuals throughout their larval and pupal stages. Chlorantraniliprole (96 h exposure) killed first instar larvae of *C. columbiensis* with a median lethal concentration (LC_{50}) of 1.98 µg/L. Sublethal exposure of chlorantraniliprole (LC_{20} = 0.24 µg/L) reduced adult emergence and body mass, whereas sublethal exposure of chlorantraniliprole (LC_{10} = 0.08 µg/L) and its combination with imidacloprid (LC_{10} = 1.38 µg/L) resulted in a longer time to initiate adult emergence. Males or females that survived sublethal exposures showed reduced reproduction, with males being more affected than females. The types of sublethal exposure differentially affected egg and wing properties of *C. columbiensis* (e.g., shorter and wider eggs when chronically exposed to the combination of the two insecticides and highly asymmetric wings when acutely exposed to chlorantraniliprole). Overall, our findings revealed that sublethal doses of chlorantraniliprole and its combination with imidacloprid negatively influenced the morphology and physiology of *C. columbiensis*, and the use of these compounds in integrated pest management should be given further consideration.

Keywords: Sublethal effects; diamides; insect reproductive performance; egg shape variation; aquatic insects.

1. Introduction

Pesticides are widely used in modern agriculture to improve the quantity and quality of production, leading to increased food security worldwide (Intisar et al., 2022; Anagnostopoulou et al., 2022). In recent years, a large increase in the diversity and quantity of application of these products has been observed worldwide (Stinson et al., 2022; Pisa et al., 2021; Menezes-Oliveira et al., 2018; Bernhardt et al., 2017). However, this increase and their intensive application, especially in developing countries, are negatively affecting the environment and human health (Khan et al., 2021; Guedes et al., 2016; Gontijo et al., 2014). Adicionally, insecticides have constituted one of the most important environmental pollutants threatening biodiversity and ecosystem functioning (Do et al., 2022; Anagnostopoulou et al., 2022).

Classified according to their mode of action, pesticides can be divided into groups, where diamide belongs to a new class that ranks as the fourth most widely used pesticide in the world (Cui et al., 2020). This pesticide targets insect ryanodine receptors, causing Ca^{2+} depletion in insect muscle cells, which affects muscle contraction and ultimately leads to death (Yang et al., 2014; Bentley et al., 2010; Lahm et al., 2 007). Within the diamide class, chlorantraniliprole is one of the most widely used insecticides in agricultural pest control in various types of crops, such as cotton, rice, vegetables, various fruits, etc. (Hannig et al., 2009). This insecticide has a wide range of activity and controls mainly Lepidoptera insect pests, as well as other Coleoptera, Diptera and Hemiptera pests. Chlorantraniliprole is persistent and mobile in soil and aquatic environments and its predicted environmental concentrations in freshwater are 0. 04 ~ 9.12 µg/L (Meng et al., 2022; Song et al., 2019). Studies have reported that chlorantraniliprole can delay the adult emergence of ladybug *Coleomegilla maculata* and *Hippodamia convergens* (Moscardini et al., 2015), reduce the population growth of *Harmonia axyridis* (Nawaz et al., 2017), in addition, it affects the development and reproduction of insects (Cao et al., 2017; Liu et al., 2018; Wu et al., 2018).

Information obtained from a single stressor to infer physiological and morphological effects occurring in the natural environment may underestimate toxicity, as more than one pesticide is often used in an agricultural area (Stinson et al., 2022). These pesticides can enter riverine areas from agricultural and urban areas through runoff, resulting in complex chemical mixtures that have the potential to cause rapid changes in water quality (Stinson et al., 2022; Anagnostopoulou et al., 2022). In addition to chlorantraniliprole, another insecticide widely used worldwide is imidacloprid (Teixeira & Andaloro, 2013; Bakker et al., 2020). Imidacloprid is a neonicotinoid pesticide that targets postsynaptic nicotinic acetylcholine receptors (nAChR), impacting the nervous system (Stinson et al., 2022; Taillebois et al., 2018). Its effects are related to impairment of rapid neurotransmission, memory and learning ability (Taillebois et al., 2018). Taken together, the mechanisms of action of these two pesticides suggest that they would exert toxicity on non-target aquatic invertebrates (Stinson et al., 2022). Multiple stressor studies demonstrate complex nonlinear and often synergistic effects (Spurgeon et al., 2010; Todgham & Stillman, 2013), possibly increasing the effects on organisms compared to those exposed to a single stressor (Stinson et al., 2022; Crain et al., 2008; Yang et al., 2007).

Assessing survival in sensitive model species following acute exposure is a common benchmark for assessing surface water toxicity (Goh et al., 2019); however, this may not represent the ecologically relevant impacts observed in streams impacted by runoff (Montaño-Campaz et al., 2019; Connon et al., 2019; Spurgeon et al., 2010). It is possible that sublethal exposure to chlorantraniliprole and its combination with imidacloprid, may directly affect (positively or detrimentally) the population size of *C. columbiensis* by altering the reproductive performance and properties of its eggs and wings (size and morphology), if these variables are affected, they may bring ecological, evolutionary and epidemiological consequences. *C. columbiensis* is a dipteran that has been included as a sentinel organism for lethal and sublethal effects of pesticides (Montaño-Campaz et al., 2022; Montaño-Campaz et al., 2019). This species was selected because it can be easily cultured under laboratory conditions, develops relatively quickly, is highly susceptible to agricultural and mining activities (Montaño-Campaz et al., 2022; Montano-Campaz et al., 2019), and plays a relevant ecological role in neotropical freshwater environments (e.g., distribution, abundance, and importance as prey for juvenile and adult fish) (Taenzler et al., 2007).

In this study we set out to evaluate the effects of chlorantraniliprole and its combination with imidacloprid (as a pesticide formulation) on the life history of *Chironomus columbiensis*, a neotropical non-target species. Parameters determined in this study included adult emergence, time to adulthood, body mass, reproductive success (fecundity and fertility), and egg and wing shape properties. This work addresses the toxicity of chlorantraniliprole and its combination with imidacloprid in ecologically relevant aquatic organisms. This type of studies is necessary to know the possible physiological and morphological adaptations of non-target insects to these diamides and neonicotinoids to have relevant information for an adequate management of these insecticides for the conservation of these insects.

2. Material and methods

2.1 Insect rearing conditions and concentration-mortality bioassays

The chironomids used were obtained from a laboratory strain that has been reared in a pesticide-free environment in the Zoology Laboratory at Universidad de Caldas (Manizales, Colombia). The rearing conditions have been described elsewhere (Montaño-Campaz et al. 2019, 2022). Briefly, recently laid egg masses were collected and placed into glass aquariums containing 5L of semi-soft reconstituted water (pH= 7.2 ± 0.3, electric conductivity = 177 µS/Cm2, hardness 138 mg de CaCO3/L). The aquaria were maintained at the temperature of 23 ± 2 °C and under photoperiod of 12h. For the concentration-mortality bioassays, we used newly emerged (< 24h) *C. columbiensis* first instar larvae. Groups of 10 larvae were placed into 100 mL glass vials containing 50 mL clorantraniliprole (Coragen SC, DuPont, Bogotá, 200g of active ingredient (a.i)/L). We used different concentrations (ranging from 1.5 µg/L up to 30,5 µg/L). The exposure period was of 96h when mortality was checked. The individuals were considered dead when were unable to move their appendages after being gently and repeatedly stimulated with a pipette tip. Each treatment was replicated five times. Larvae exposed to 50 mL of semi-soft reconstituted water was considered our control treatment.

2.2. Sublethal exposure bioassays

For sublethal exposures, three concentrations (CL_1 = 0.01 µg/L, CL_{10} = 0.08 µg/L and CL_{20} 0.24 µg/L) previously estimated in concentration-mortality bioassays were used. In addition, a combination of two insecticides (chlorantraniliprole: CL_{10} = 0.08 µg/L and imidacloprid: CL_{10} = 1.38 µg/L) was evaluated, the concentration of imidacloprid was previously estimated in concentration-mortality bioassays in Chapter III. Individuals of *C. columbiensis* faced exposure to chlorantraniliprole throughout their larval and pupal stages. Groups of 100 newly emerged first instar larvae were placed in glass aquaria (35 x 30 x 20 cm) containing two sheets of shredded disposable towels (as substrate) and 5L of water solutions contaminated with chlorantraniliprole or the combination, using a gentle aeration system. Control individuals were exposed only to the reconstituted water. Three replicates (i.e., groups of 100 larvae) were used for each treatment. Larvae were fed 0.3 g TetraMin (fish food) twice a week until pupation. Contaminated water was replaced at seven-day intervals. The control treatment consisted of exposing individuals of each instar to the reconstituted water. Each treatment was repeated three times.

2.3. Chronic and acute sublethal effects on survival, development time, body mass and reproduction of *C. columbiensis* males and females

2.3.1. Adult emergence

In the sublethal exposure bioassays, the total number of specimens that reached adulthood was recorded, which made it possible to quantify the total number of males and females of *C. columbiensis* per treatment.

2.3.2. Developmental time

In the sublethal bioassays, we recorded the time that specimens lasted from the first larval instar until the adult stage (onset of emergence) of C. columbiensis by treatment and sex.

2.3.3. Body mass of newly emerged males and females

We used an analytical balance (AS 220.X2 PLUS, RADWAG Balances and Scales, Radom, Poland) to weigh the individual body masses of newly emerged males and females that survived the sublethal exposures. Thirty pairs were used for each of the treatments (i.e., individuals not exposed to chlorantraniliprole or facing sublethal exposures of chlorantraniliprole LC_1 = 0.01 μg/L, LC_{10} = 0.08 μg/L, LC_{20} = 0.24 μg/L or the combination of the two insecticides).

2.3.4. Reproductive performance of males and females

We assessed the potential effects of clorantraniliprole sublethal exposures on *C. columnenis* reproduction by mating virgin individuals in four different combinations (unexposed couple; exposed female; exposed male; and exposed couple), as previously described elsewhere (Haddi et al. 2016; Santos et al. 2018), and evaluating their fecundity and fertility outcomes. For the fecundity measurements, we randomly selected 30 egg masses (or spawns) of each treatment to be photographed with a stereomicroscope (M205C, Leica Microsystems, Buffalo Grove, IL, USA). To determine fertility (i.e., % hatched eggs), we placed the photographed spawnings in plastic tubes (1.5 cm high × 2.5 cm wide), which were closed at the bottom with porous cloth to facilitate aeration and exit of the larvae. The unhatched eggs were counted after a period of five consecutive days when we calculated the percentage of hatched eggs.

2.4. Sublethal effects on morphometry of egg and fluctuating asymmetry of *C. columbiensis* wings

To quantify possible alterations in egg shape mediated by sublethal exposures to chlorantraniliprole, we performed morphometric analyses on egg images for the four different combinations (unexposed pair; exposed female; exposed male; and exposed pair). For asymmetric fluctuation measurements, we used images of the right and left wings of *C. columbiensis* females and applied the fluctuating asymmetry approaches described previously elsewhere (Klingenberg 2015, Schaefer et al. 2006).

Photographs were taken with a stereoscopic (Leica M205) equipped with a digital camera. All photos were saved as JPEG files and all specimens were photographed in the same position using slides and coverslips. Eight landmarks per egg were digitized and twelve reference points (landmarks) per wing were digitized, according to the geometric configuration proposed by Montaño-Campaz et al. (2019; 2022). Positional homology criteria, relative position consistency, adequate shape coverage, and repeatability were analyzed using tpsDIG2 v2.17 software (Rohlf, 2013). X-Y coordinates were obtained and shape and size information were extracted using a full Procrustes fit (Dryden & Mardia, 1998; Rohlf & Slice, 1990). To evaluate the measurement error (ME) we follow the protocol proposed by Palmer & Strobeck (1986), which ensures the correct procedure for the acquisition of reference points. For the case of morphometry, analysis of variance (ANOVA) was used to evaluate the possible differences in the size of the centroid, and a Procruste ANOVA was used to evaluate the differences in shape while considering the values of individual variation (MS) from ANOVA, with type 1 error. The main patterns of variation in wing shape were visualized using principal component analysis (PCA), from the covariance matrix of the symmetric component of the average population shape (Klingenberg et al., 2002). To statistically evaluate the differences between the treatments, a canonical variables analysis (CVA) of the variation of the form was also performed and the Procrustes distances were calculated (Campbell & Atchley, 1981). To analyze the relationship between shape and size of the centroid, a multivariate regression was performed that included the confidence ellipse of each group (90%) using shape and scores with 10,000 iterations. Statistical analyzes were performed with MorphoJ 2.0 software (Klingenberg, 2011).

Fluctuating skewness estimates were tested using Procrustes ANOVA (Klingenberg, 2015) by checking individual × side interactions and individual × side mean squares (MS ind × side). The data obtained were subjected to MANOVA statistical tests. Wing size was analyzed by plotting the size of the centroid (Zelditch et al., 2004). The shape was described by Procrustean coordinates, which were scaled to the same size. Size asymmetry was measured as the absolute difference between the centroid sizes of the right and left wings divided by the mean centroid size. Shape asymmetry was measured as the Procrustes distance between the right and left-wing shapes. Centroid sizes and asymmetric shapes were calculated with MorphoJ software (Klingenberg, 2011).

2.5. Statistical analysis

Mortality data obtained in the concentration-mortality bioassays were subjected to a probit analysis (PROBIT procedure) available in the SAS statistical software (SAS Institute, 2008). Results obtained for the potential effects of sublethal exposure types on the adaptative traits, we used the R software, version 3.6.2 (R Core, 2020) available in RStudio (R Studio, 2020). Type II ANOVA tables were used to assess the importance of the explanatory variables in all models (generalized linear models, GLM), by using the "Anova" function in the car library (Fox & Weisberg, 2018). The data was organized using the dplyr library (Wickham et al., 2018), and the figures were built using the sjPlot (Lüdecke & Schwemmer, 2016) and ggplot2 (Wickham, 2016) libraries. The duration time, body masses, and reproduction assessments were adjusted to GLMs using the "glm" function. The gamma distribution was used for the body mass and the duration time, while a negative × binomial (for adult emergence), poisson (fecundity) and quasi-binomial (fertility) distributions were used in the other analysis. We evaluated the normality of the residuals using q-q plots and the homogeneity of variances using Fligner-Killeen tests (Crawley, 2013). We tested for the presence of outliers using Cook's distances (Cook's distance < 1). We built all models using the nlme library (Pinheiro et al., 2006).

3. Results

3.1. Susceptibility of Chironomus columbiensis larvae to clorantraniliprole

The results obtained in the concentration-mortality bioassays fit to a Probit model ($n = 250$; $\chi^2 = 2.33$; $P = 0.85$) and allowed the estimation of the median lethal concentration (LC_{50}) of 1.98 (1.15 - 3.53) μg/L (Figure 1). These results also allowed the estimation of sublethal concentrations used in the chronic (i.e., $LC_1 = 0.01$ [0.004 – 0.04] μg/L; $LC_{10} = 0.08$ [0.07 – 0.19] μg/L; $LC_{20} = 0.24$ 0.21 – 0.70] μg/L).

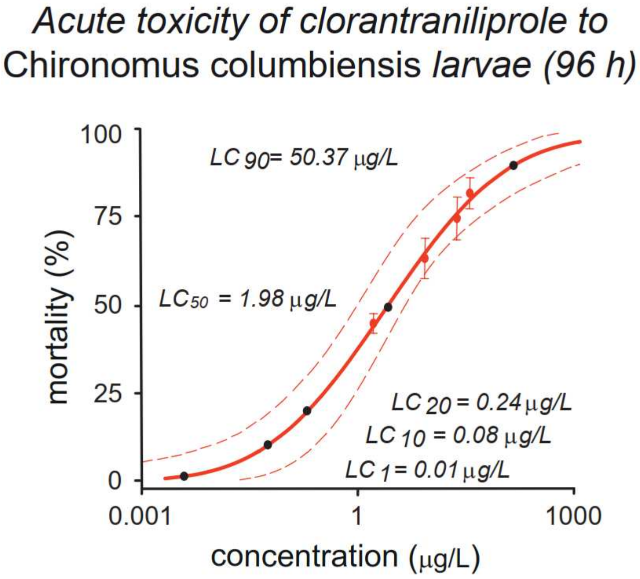


Fig. 1. Toxicity of clorantraniliprole to *Chironomus columbiensis* larvae. Concentration-response curves for 96 h exposure periods. Dotted lines represent the 95% confidence intervals and symbols shows the mean (± SEM) obtained for eight replicates (aquarium containing 10 *C. columbiensis* larvae).

3.2. Effects of sublethal exposure to chlorantraniliprole on emergence, development time, body mass and reproduction of *Chironomus columbiensis*.

The results showed that adult emergence was of 92.8 ± 0.46 % for clorantraniliprole-unexposed insects, which did not differ from the emergence recorded (91.7 ± 0.83 %) to individuals sublethally exposed to 0.01 µg/L (LC_1), nor in the emergence recorded (85.5 ± 0.83 %) in individuals sublethally exposed to 0.08 µg/L (LC_{10}) of chlorantraniliprole. The sublethal exposure to 0.24 µg/L (LC_{20}) of clorantraniliprole, however, resulted in significant ($\chi^2 = 23.41$; $P < 0.0001$) reduction on the adult emergence (79.0 ± 2.59 %), in both males and females (Figure 2A).

A similar pattern was also recorded in the time taken from immature stages (first instar larvae) to adults (onset of emergence), where the results showed that adult emergence began at 12.75 ± 0.63 days for insects not exposed to chlorantraniliprole, which did not differ in the development time (14.50 ± 0.65 days) recorded for individuals sublethally exposed to 0.01 µg/L (CL_1) of chlorantraniliprole. Sublethal exposures to 0.08 µg/L (LC_{10}) and 0.24 µg/L (LC_{20}) chlorantraniliprole, however, produced a significant increase ($\chi 2 = 15.68$; $P < 0.017$) in developmental duration from immature to adult stages (16.25 ± 0.75 and 15.75 ± 0.75 respectively) in both males and females (Figure 2B).

For body masses, significant differences ($\chi 2 = 187.15$; $P < 0.0001$) were recorded for males ($\chi^2 = 134.96$; $P < 0.0001$) as for females facing sublethal exposure to LC_1, LC_{10} or LC_{20} (Figure). However, females were at least 3.3 times heavier than males in all four treatments (Figure 2C).

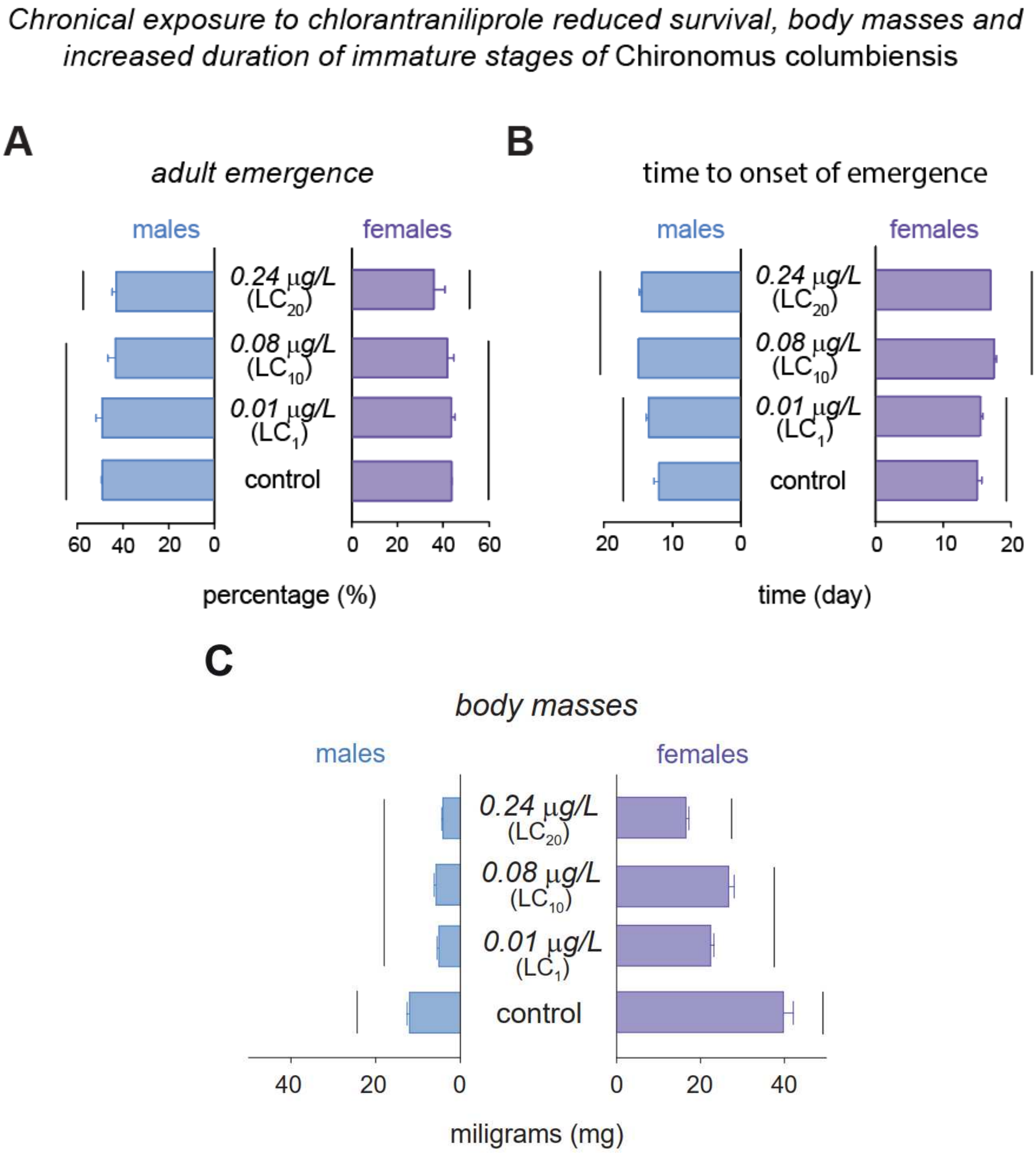

Chronical exposure to chlorantraniliprole reduced survival, body masses and increased duration of immature stages of Chironomus columbiensis

Fig. 2. Sublethal exposure effects on the emergence (A), development time to reach the adult stage and (B) body masses (C) of *Chironomus columbiensis* to clorantraniliprole ($LC_1 = 0.01$ μg/L, $LC_{10} = 0.08$ μg/L and $LC_{20} = 0.24$ μg/L). The results of figures represent the average number (± standard error, SE). The different levels of the lines indicate significant differences by Tukey's HSD test ($P < 0.05$).

Regarding the reproductive responses, the sublethal exposure to clorantraniliprole reduced the fecundity of *C. columbiensis* couples ($\chi^2 = 26.50$, $P < 0.001$), independent of the insecticide concentration or sex (LC_{10}: $\chi^2 = 4.46$, $P = 0.11$; LC_{20}: $\chi^2 = 5.50$, $P = 0.06$) (Figure 3A, left panel). Couples where both members were clorantraniliprole-exposed or couples of only a member (male or female) faced the sublethal exposure exhibited a reduction of approximately 11% in the number of spawning eggs when compared to couples formed by only by unexposed members (Figure 3A left panel).

Fertility rates, however, were only significantly reduced ($\chi^2 = 11.94$, $P = 0.003$) in pairs in which males faced LC_{10} sublethal exposure, as in LC_{20} ($\chi^2 = 20.39$, $P < 0.001$) with females that were not exposed to insecticide (Figure 3B, right panel). Pairs in which both members faced sub-lethal LC_1, LC_{10}, and LC_{20} exposure ($\chi^2 = 4.48$, $P = 0.21$) produced fertility rates similar to those produced by unexposed pairs (Figure 3B, left panel)

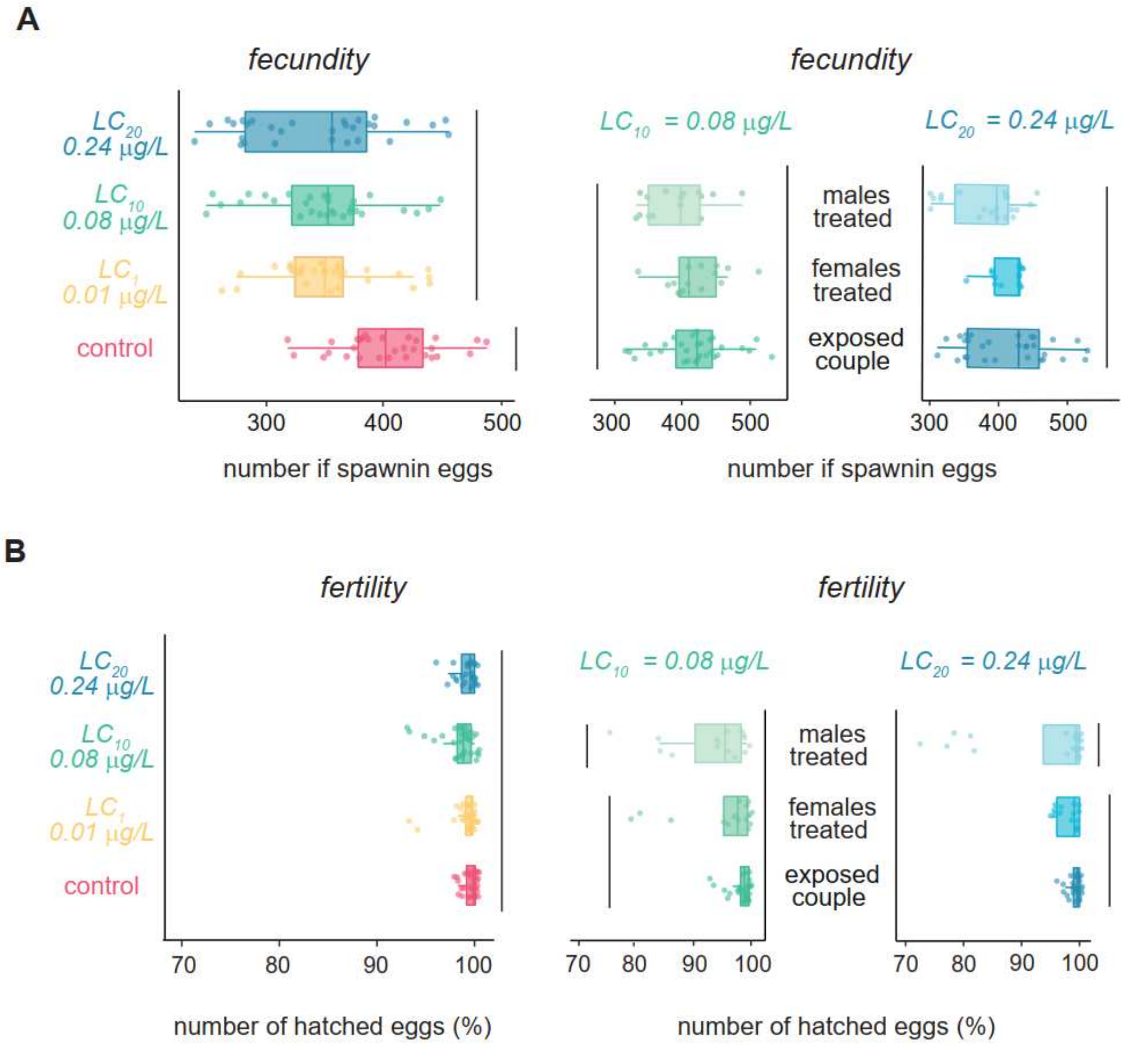

Chronical sublethal exposure to clorantraniliprole reduced fecundity and fertility of Chironomus columbiensis according to sex

Fig. 3. sublethal exposure effects on the reproduction: Fecundity (B, left panel) and fertility (B, right panel) of *Chironomus columbiensis* to clorantraniliprole (LC_1 = 0.01 µg/L, LC_{10} = 0.08 µg/L and LC_{20} = 0.24 µg/L). The results of figures, box plots show the median (the line within each box plot) and range of dispersion (lower and upper quartiles). The different levels of the lines indicate significant differences by Tukey's HSD test ($P < 0.05$).

3.3. Effects of sublethal exposure to chlorantraniliprole on the egg shape of *Chironomus columbiensis*

The PCA by location showed that the first two PC accounted for 87.26 % (PC1: 62.23%; PC2: 25.13%) of the shape variation in *C. columbiensis* eggs, where two groups were clearly reflected (Figure 4A). With the average PCA of the shapes (Figure 4A), it could be observed that one group consisted of elongated but narrower eggs (blue color) while the other group consisted of round eggs (magenta color). Finally, the results of the procrustes ANOVA for the two types of eggs showed significant differences for size ($P < 0.0001$) and shape ($P < 0.0001$) between the groups (i.e., elongated eggs and round eggs), which highlights the existence of the two types of eggs.

Interestingly, the results of the procrustes ANOVA for each of the egg types by treatments (i.e., unexposed individuals; individuals exposed to LC_{10} or LC_{20} concentrations of chlorantraniliprole), showed significant differences in shape ($p = 0.026$) and size ($p = 0.015$) when the egg presented an elongated image (Figure 4B), but no significant differences were found in shape ($p = 0.\ 693$) and size ($p = 0.063$) when the egg had a round image (Figure 4C).

Pairs in which only one member (male or female) faced sublethal exposure to chlorantraniliprole showed significant differences in shape ($p = 0.025$) but not in size ($p = 0.108$) of eggs compared to pairs in which both members were exposed to the LC_{10} concentration (Figure 4D); however, significant differences were found in shape ($p = 0.\ 033$) and size ($p = 0.002$) of eggs in pairs in which only one member (male or female) faced a higher sublethal exposure (LC_{20} = 0.24) of (chlorantraniliprole compared to pairs in which both members were exposed to the insecticide (Figure 4E).

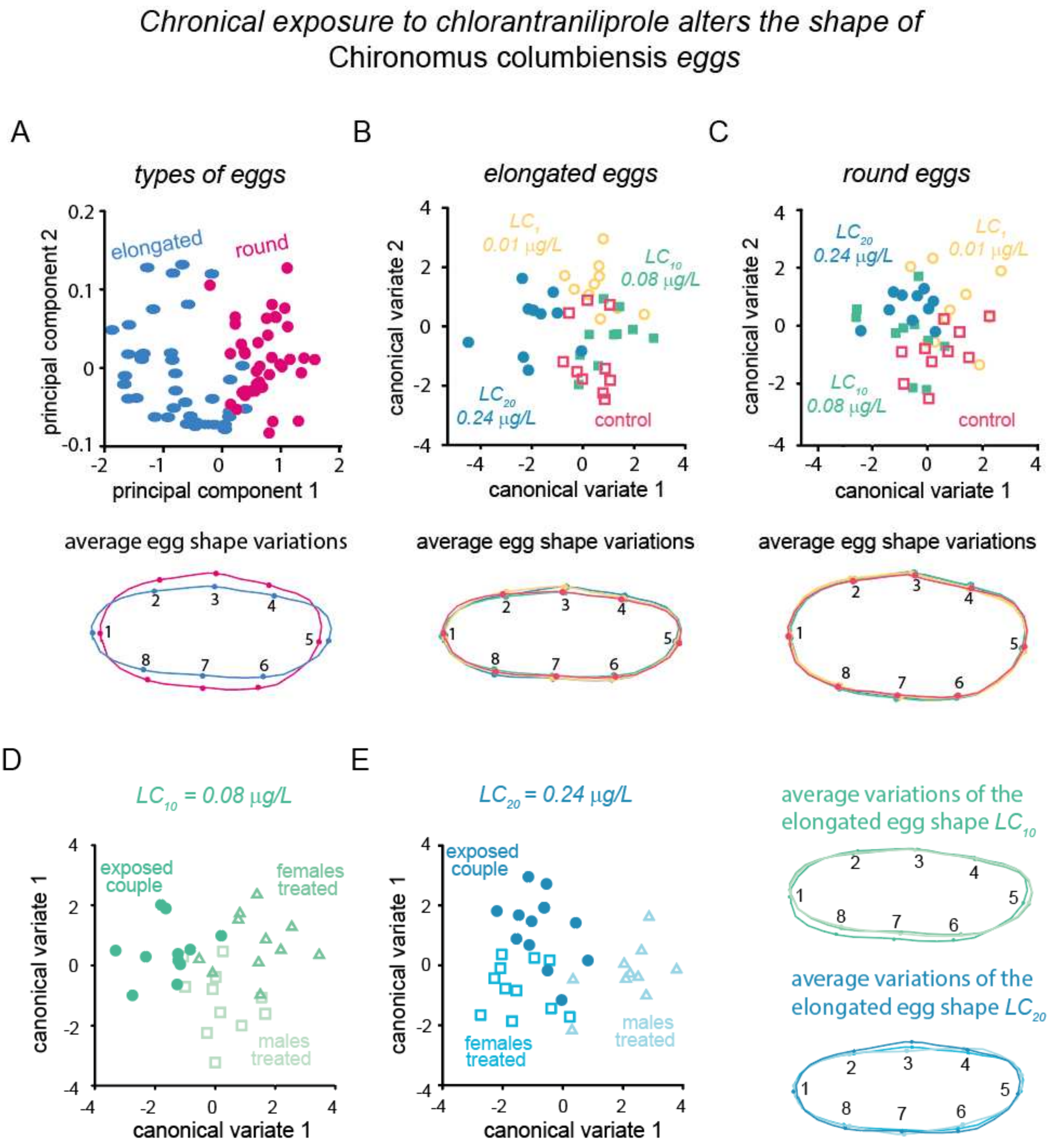
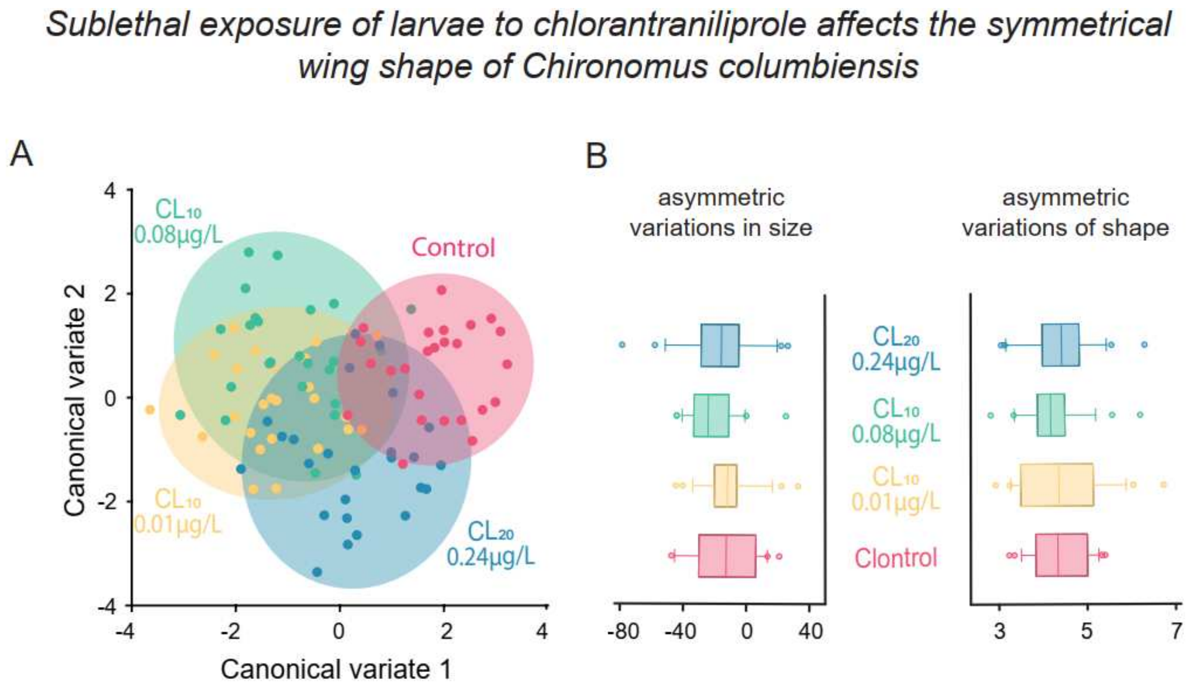


Fig. 4. Sublethal exposure effects on the egg shapes of *Chironomus columbiensis* to clorantraniliprole (LC_1 = 0.01 µg/L, LC_{10} = 0.08 µg/L and LC_{20} = 0.24 µg/L). Principal Component and schematics of average PCs of egg shapes (A); Canonical analysis and schematics of average PCs of the shape of elongated eggs (B); Canonical analysis and schematics of average PCs of the of the shape of round eggs (C); Canonical analysis and schematics of average PCs of the elongated egg shape at LC_{10} = 0.08 µg/L. (D) and Canonical analysis and schematics of average PCs of the elongated egg shape at LC_{20} = 0.24 µg/L. (E).

3.4. Effects of sublethal exposure to chlorantraniliprole on the wing shape of *Chironomus columbiensis*

Regarding the fluctuating asymmetry analysis, the measurement error was evaluated in all the populations studied, in order to avoid any type of error associated with the data. Procrustes ANOVA results indicated that fluctuating asymmetry individuals by sides (ind × side) mean squares (MS) values exceeded MS error values, implying that there is no measurement error (ME) in the data (Table 1). Regarding FA (ind × side), the results of Procrustes ANOVA (MANOVA) show significant differences between the groups (i.e., unexposed individuals; individuals that faced exposure to clorantraniliprole LC$_1$, LC$_{10}$ or LC$_{20}$) examined ($P < 0.0001$) (Figure 5A, Table 2).

After evaluating the influence of Fluctuating Asymmetry between populations, it was observed that individuals exposed to the LC$_{10}$ and LC$_{20}$ concentrations showed the greatest variations in wing size (Figure 5B, left panel), while specimens exposed to the LC$_1$ concentration showed the greatest variations in wing shape compared to the control (Figure 5B, right panel). Principal component analysis showed the variations on average in each of the populations, where specimens exposed to LC$_1$, LC$_{10}$ and LC$_{20}$ treatments showed a reduction between the anal and basal part, with greater intensity in the LC$_1$ treatment compared to the control (Figure 5C).



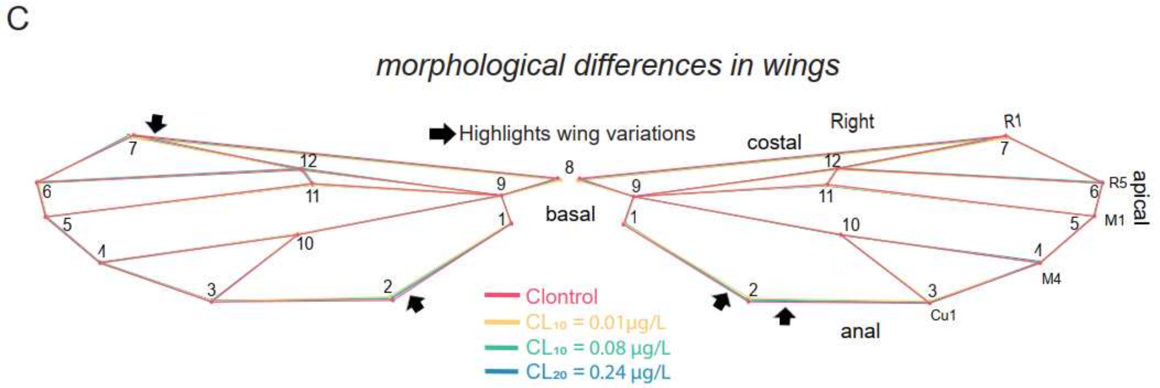
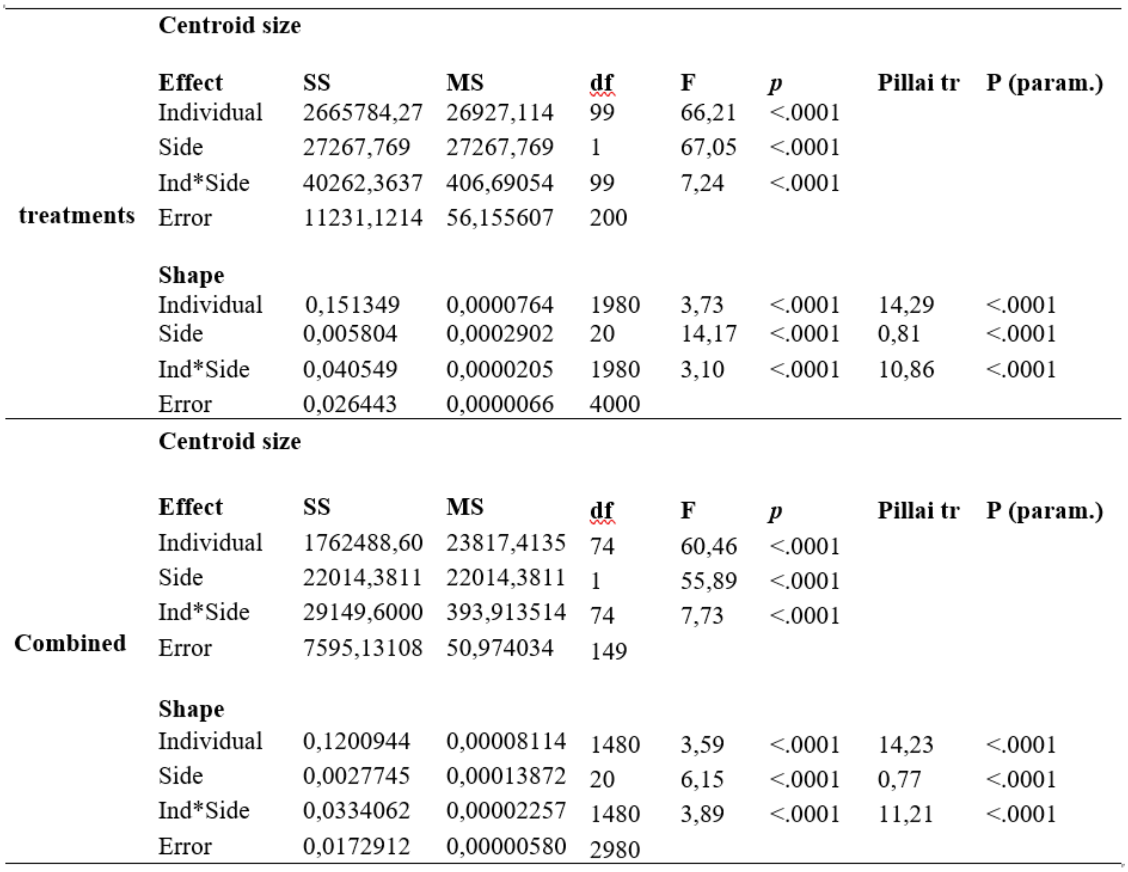


Fig. 5. Sublethal exposure effects on the wing shapes of *Chironomus columbiensis* to clorantraniliprole (LC_1 = 0.01 µg/L, LC_{10} = 0.08 µg/L and LC_{20} = 0.24 µg/L). Canonical wing shape analysis (A); asymmetrical variations in wing shape (B, right panel) and asymmetrical variations in wing size (B, left panel); Schematics of average PCs of asymmetric variations in wing shape and size (C).

Table 1. ANOVA results on the effect of imidacloprid during sublethal exposure of *Chironomus columbiensis* to chlorantraniliprole (LC_1 = 0.01 µg/L, LC_{10} = 0.08 µg/L and LC_{20} = 0.24 µg/L) and its combination with imidacloprid (LC_{10} = 1. 38 µg/L), on fluctuating female asymmetry: sums of squares (SS), mean squares (MS), degrees of freedom (df), F-statistics and parametric P-values for each of the effects. Treatments = chlorantraniliprole concentrations



	Effect	SS	MS	df	F	p	Pillai tr	P (param.)
	Centroid size							
	Effect	**SS**	**MS**	**df**	**F**	***p***	**Pillai tr**	**P (param.)**
	Individual	2665784,27	26927,114	99	66,21	<.0001		
	Side	27267,769	27267,769	1	67,05	<.0001		
	Ind*Side	40262,3637	406,69054	99	7,24	<.0001		
treatments	Error	11231,1214	56,155607	200				
	Shape							
	Individual	0,151349	0,0000764	1980	3,73	<.0001	14,29	<.0001
	Side	0,005804	0,0002902	20	14,17	<.0001	0,81	<.0001
	Ind*Side	0,040549	0,0000205	1980	3,10	<.0001	10,86	<.0001
	Error	0,026443	0,0000066	4000				
	Centroid size							
	Effect	**SS**	**MS**	**df**	**F**	***p***	**Pillai tr**	**P (param.)**
	Individual	1762488,60	23817,4135	74	60,46	<.0001		
	Side	22014,3811	22014,3811	1	55,89	<.0001		
	Ind*Side	29149,6000	393,913514	74	7,73	<.0001		
Combined	Error	7595,13108	50,974034	149				
	Shape							
	Individual	0,1200944	0,00008114	1480	3,59	<.0001	14,23	<.0001
	Side	0,0027745	0,00013872	20	6,15	<.0001	0,77	<.0001
	Ind*Side	0,0334062	0,00002257	1480	3,89	<.0001	11,21	<.0001
	Error	0,0172912	0,00000580	2980				

3.5. Effects of sublethal exposure of chlorantraniliprole combined with imidacloprid on emergence, development time, body mass and reproduction of *Chironomus columbiensis*.

Sublethal exposure of *C. columbiensis* to the combination of chlorantraniliprole and imidacloprid significantly reduced ($\chi^2 = 22.56$; P = 0.002) the percentage of emergence only in females compared to unexposed specimens and those exposed to chlorantraniliprole CL_{10} (Figure 6A). However, for the case of the time it took for specimens to initiate emergence, significant differences ($\chi^2 = 17.73$; P < 0.022) were only found between specimens exposed to the combination of chlorantraniliprole and imidacloprid and those not exposed to the insecticide in both males and females (Figure 6B). Similar results were found for body mass, where specimens exposed to the combination of chlorantraniliprole and imidacloprid only showed significant differences ($\chi^2 = 55.46$; P < 0.0001) in both males ($\chi2 = 21.77$; P < 0.0001) and females with specimens not exposed to the insecticide (Figure 6C).

The effects of sublethal exposure of chlorantraniliprole combined with imidacloprid reducedsurvival, body mass and increased emergence time of Chironomus columbiensis.

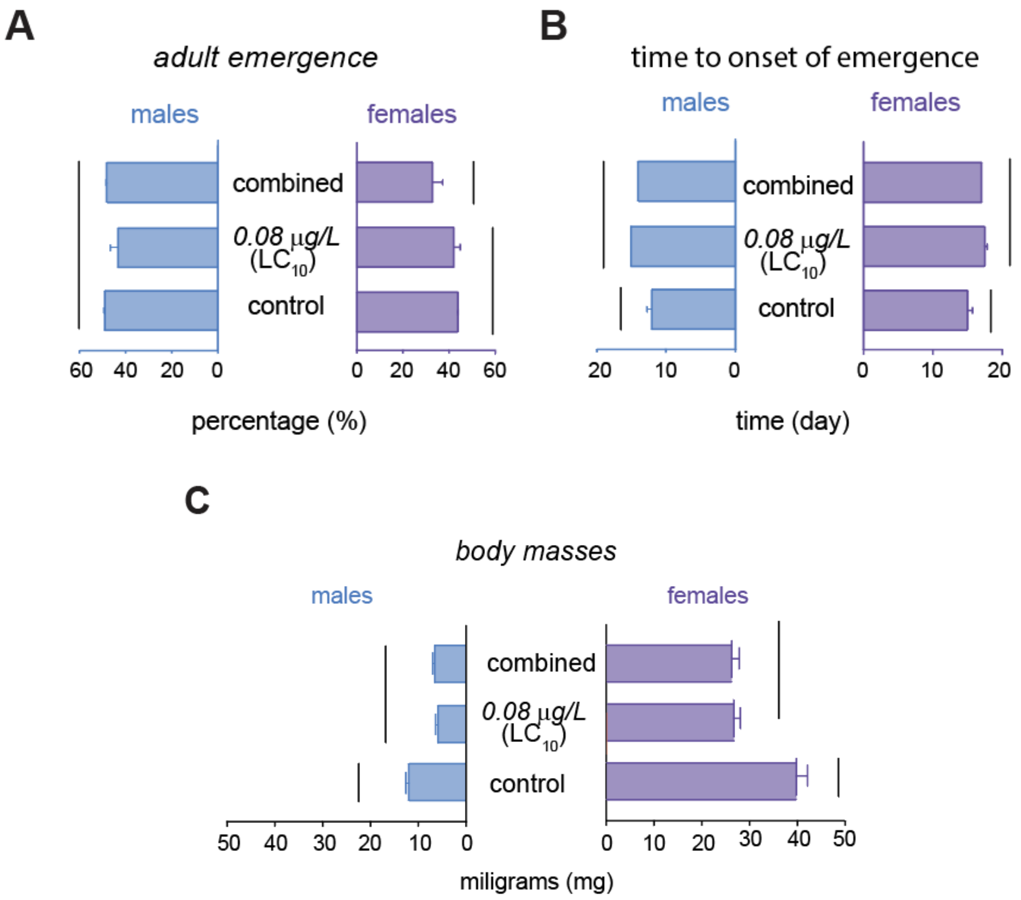


Fig. 6. Effects of sublethal exposure on emergence (A), development time to reach the adult stage and (B) body masses (C) of *Chironomus columbiensis* to chlorantraniliprole (LC_{10} = 0.08 µg/L) combined with imidacloprid (LC_{10} = 1.38 µg/L). The results of figures represent the average number (± standard error, SE). The different levels of the lines indicate significant differences by Tukey's HSD test ($P < 0.05$).

Regarding reproduction, individuals subjected to sublethal exposure to the combination of chlorantraniliprole and imidacloprid, only presented significant differences (χ^2 = 26. 76, P < 0. 001) in fecundity with specimens not exposed to the insecticide (Figure 7A, left panel). The results also showed that males were more affected than females, as only a significant difference (χ^2 = 8. 86, P < 0. 012) was found when crossing male specimens exposed to the insecticide combination and females not exposed to the insecticide compared to when both sexes were exposed to the insecticide combination (Figure 7A, right panel). In terms of fertility, specimens exposed to the chlorantriliprole-imidacloprid combination showed significant differences (χ2 =

11.63, P < 0.001) with specimens not exposed to the insecticide or exposed to chlorantraniliprole alone (Figure 7B).

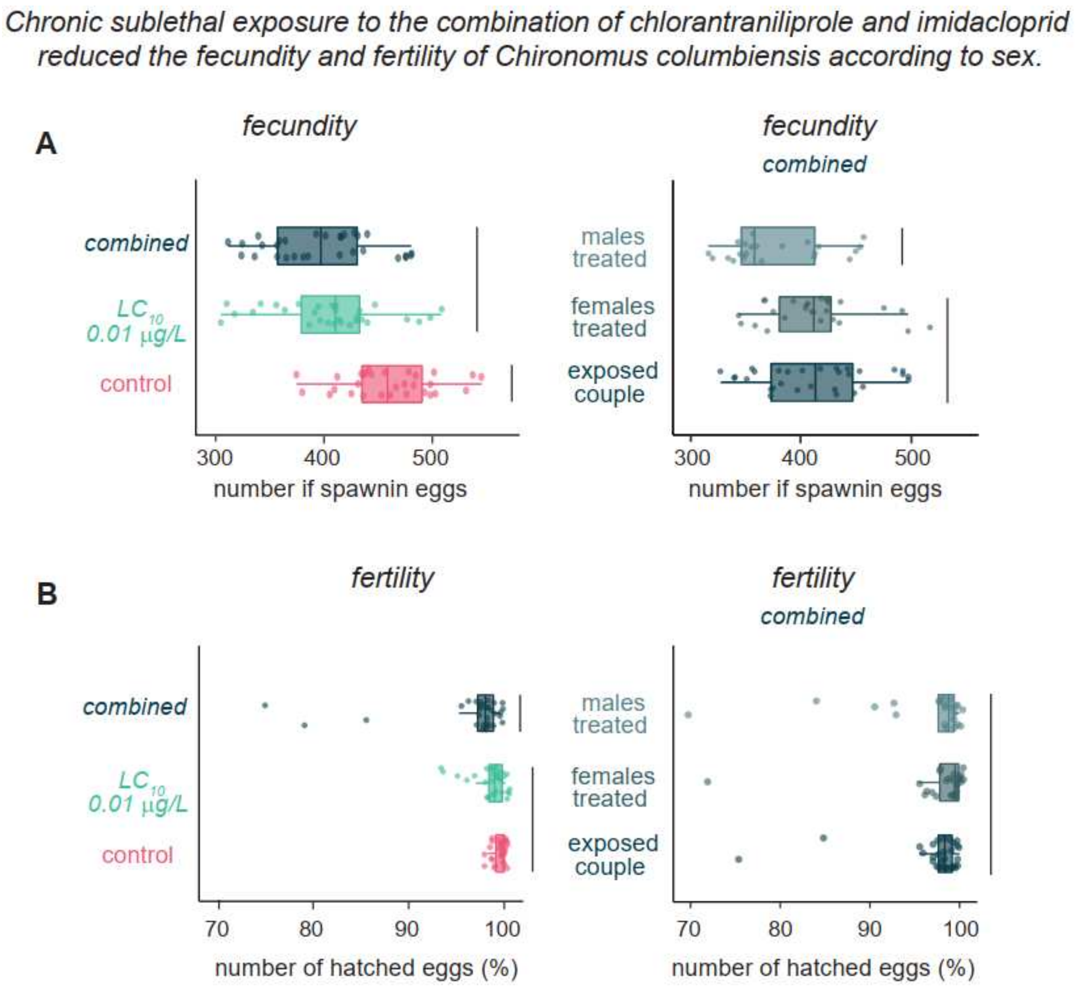


Fig. 7. Sublethal exposure effects on the reproduction: Fecundity (B, left panel) and fertility (B, right panel) of *Chironomus columbiensis* to chlorantraniliprole (LC_{10} = 0.08 µg/L) combined with imidacloprid (LC_{10} = 1.38 µg/L). Box plots show the median (the line within each box plot) and range of dispersion (lower and upper quartiles). The different levels of the lines indicate significant differences by Tukey's HSD test ($P < 0.05$).

3.6. Effects of sublethal exposure of chlorantraniliprole combined with imidacloprid on *Chironomus columbiensis* egg shapes.

PCA by location showed that the first two PCs explained 87.83% (PC1: 54.1%; PC2: 33.73%) of the shape variation for elongated eggs and 69.42% (PC1: 42.1%; PC2: 27.3%) for round eggs. However, Procrustes ANOVA results for eggs from specimens exposed to the three treatments (control, chlorantraniliprole CL_{10} and the chlorantraniliprole-imidacloprid combination), showed significant differences in shape ($p = 0.001$) but not in size ($p = 0.927$) for elongated eggs, but no significant differences in shape ($p = 0.581$) or size ($p = 0.425$) were found for round eggs (Figure 8A). For the case of elongated eggs, the CVA shows a clear differentiation in the shape of the three populations, where the average PCA of the shapes showed that specimens exposed to the combination of the two pesticides presented wider, but shorter eggs, compared to specimens not exposed to insecticide or those exposed to chlorantraniliprole CL$_{10}$ (Figure 8B). In addition, analyses also showed significant differences in shape ($p = 0.0007$) but not in size ($p = 0.223$) when one of the two sexes of the pair exposed to the combination of the two pesticides was crossed with specimens not exposed to the pesticide. Similarly, mean CVA and mean PCA showed clear differences among the three populations (Figure 8C).

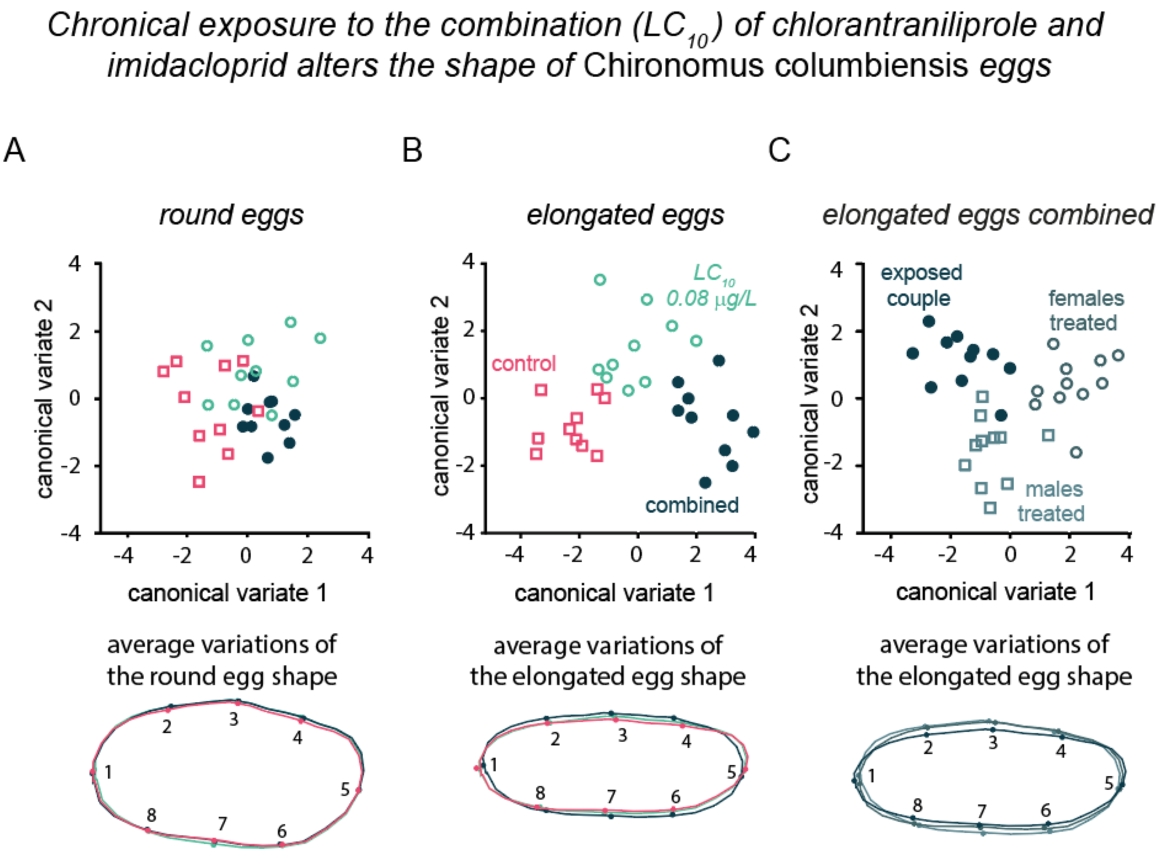


Fig. 8. Sublethal exposure effects on the egg shapes of *Chironomus columbiensis* to chlorantraniliprole (LC$_{10}$ = 0.08 µg/L) combined with imidacloprid (LC$_{10}$ = 1.38 µg/L). Canonical analysis and schematics of average PCs of the shape of round eggs (A); Canonical analysis and schematics of average PCs of the of the shape of elongated eggs (B); Canonical analysis and schematic of the mean PCs of the elongated egg shape,

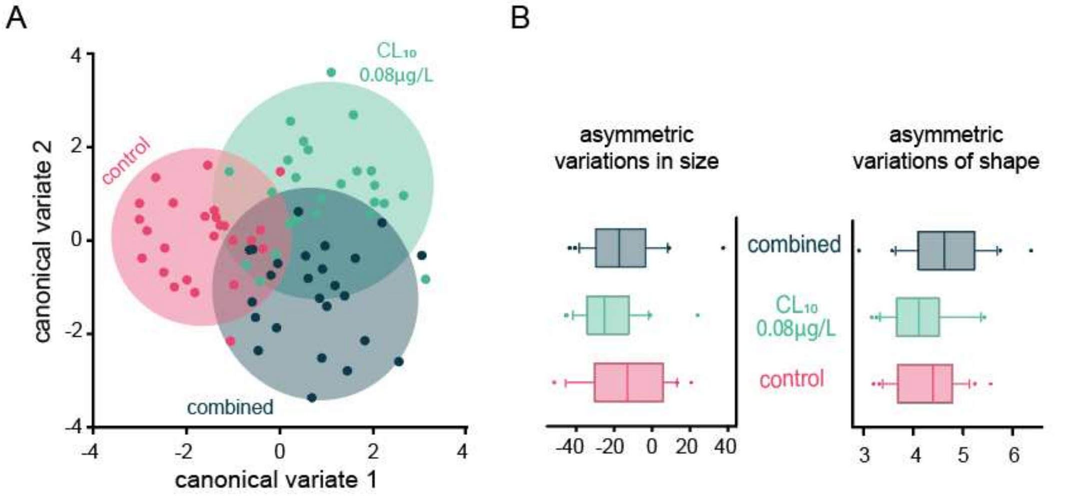
where one of the two sexes of the exposed pair was crossed with unexposed specimens (C).

3.7. Effects of sublethal exposure of chlorantraniliprole combined with imidacloprid on *Chironomus columbiensis*

Regarding the analysis of fluctuating skewness, the measurement error was also evaluated in all the populations studied, in order to avoid any type of error associated with the data. The results of the Procrustes ANOVA indicated that the mean squares (MS) values of the fluctuating skewness of individuals per side (ind × side) exceeded the MS error values, implying that there is no measurement error (ME) in the data (Table 1). As for PA (ind × side), the results of the Procrustes ANOVA (MANOVA) show significant differences between the groups (i.e., unexposed individuals; individuals that faced exposure to chlorantraniliprole LC_{10} or the combination of the two pesticides) examined ($P < 0.0001$) (Figure 9A, Table 1).

After evaluating the influence of Fluctuating Asymmetry among populations, it was observed that individuals exposed to the LC_{10} concentration showed the greatest variations in wing size (Figure 9B, left panel), while specimens exposed to the combination of the two pesticides showed the greatest variations in wing shape compared to the control (Figure 9B, right panel). Principal component analysis showed the variations on average in each of the populations, where specimens exposed to the chlorantraniliprole LC_{10} treatments and the combination of the two insecticides showed a reduction between the anal, basal and apical part, with greater intensity in the combination compared to the control (Figure 9C).

Sublethal exposure of larvae to chlorantraniliprole and combination with imidacloprid affect the symmetrical wings shape of Chironomus columbiensis



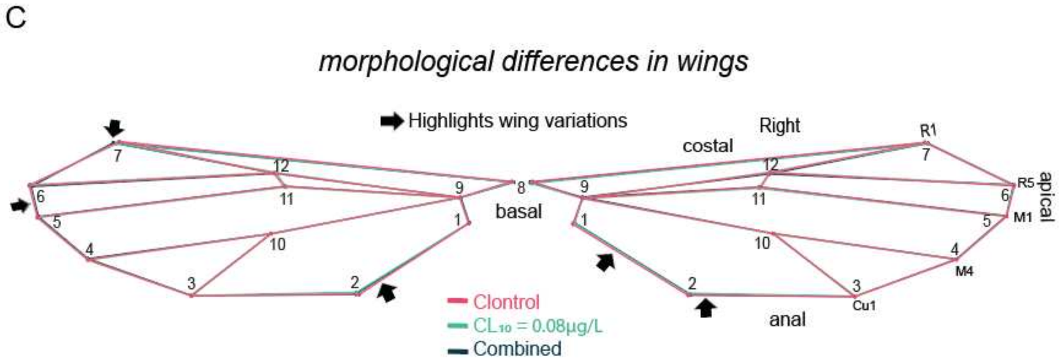


Fig. 9. Sublethal exposure effects on the wing shapes of *Chironomus columbiensis* to clorantraniliprole (LC_{10} = 0.08 µg/L) combined with imidacloprid (LC_{10} = 1.38 µg/L). Canonical wing shape analysis (A); asymmetrical variations in wing shape (B, right panel) and asymmetrical variations in wing size (B, left panel); Schematics of average PCs of asymmetric variations in wing shape and size (C).

4. Discussion

Chlorantraniliprole and its combination with imidacloprid affected important biological and developmental parameters in the non-target invertebrate *C. columbiensis*, such as percent adduct emergence, time to emergence, body mass, reproduction, and egg and larval properties. On its own, chlorantraniliprole was highly toxic to *C. columbiensis*, resulting in significantly reduced emergence, body mass, reproduction and dose-dependent changes in egg and wing shape and size. The high toxicity and rapid action on *C. columbiensis* caused by chlorantraniliprole are similar to those reported on other groups such as *C. riparius* (Rodrigues et al., 2015), *Anticarsia gemmatalis* (e Castro et al., 2021), *Paederus fuscipes* (Khan et al., 2021), *Trichogramma brassicae* (Parsaeyan et al., 2020) and *Daphnia magna* (Stinson et al., 2022). The combination of chlorantraniliprole with imidacloprid resulted in synergism, which resulted in reduced emergence and reproductive success (fecundity and fertility), as well as greater alterations of egg and wing properties (shape and size) in *C. columbiensis*. However, this synergism was not observed in the time to initiate emergence or in the body mass of *C. columbiensis* when combining the two insecticides.

The acute concentration of chlorantraniliprole (LC_{50} = 1.98 µg/L) reported in the present study at 96 h with first instar larvae of *C. columbiensis* is between the range of predicted environmental concentrations in surface waters ranging from 0.04 to 9.12 µg/L (USEPA, 2008, EFSA, 2013), which may be harmful to non-target organisms. As reported by Montaño-Campaz

(Chapter III), chlorantraniliprole was 1.95 times more toxic than imidacloprid (LC_{50} = 3.86 µg/L). The obtained acute toxicity of chlorantraniliprole in *C. columbiensis* is different from that reported for *C. dilutus* (4.0 µg/L) at 96 h (Maloney et al., 2020), *C. riparius* (85.9 µg/L) at 48 h and *Daphnia magna* (11.6 µg/L) at 48 h. The results found indicate that chlorantraniliprole toxicity varies among aquatic invertebrate taxa, even among closely related species (e.g., *C. columbiensis* vs. *C. dilutus*). Nevertheless, the acute toxicities of compounds in different experimental organisms are difficult to compare with precision and accuracy, as the duration and/or methodology are often different. However, the present study indicates that *C. columbiensis* larvae are probably among the most sensitive aquatic invertebrates to insecticides like what was observed with imidacloprid (Montaño-Campaz, Chapter III) and deltamethrin (Montaño-Campaz et al., 2022).

In the present study, chlorantraniliprole was shown to exert sublethal effects on emergence, body mass, reproduction, and egg and wing properties of *C. columbiensis*. Similar results have been reported in other insect groups, where chlorantraniliprole exposure reduces survival and delays oviposition in beneficial organisms such as *Orius insidiosus* (Gontijo et al., 2015), affects the developmental period, fecundity and growth parameters of *Bracon hebetor* (Muslim et al., 2018) and *Chrysoperla carnea* (Gontijo et al. (2014). Also, these insecticides affect feeding, produce lethargy, muscle paralysis and can cause death in insects, including Lepidoptera, Coleoptera, Hemiptera and Diptera (Hannig et al., 2009, Sial & Brunner 2012). Other sublethal effects of this insecticide include disruption of molting, cuticle formation and change in sex ratio (Barbosa et al., 2015; Abbes et al., 2015 and Moscardini et al., 2014).

The mentioned effects may be related to the fact that chlorantranilipro acts on ryanodine receptors where they have the insect muscle as a target organ, stimulating the release of intracellular calcium resulting in the depletion of calcium stored in the sarcoplasmic reticulum, promoting muscle paralysis, cessation of feeding, lethargy and death of individuals (Plata-Rueda et al., 2019; Hannig et al., 2009). As expected, exposure to chlorantranilipro and its combination with imoidacloprid affected *C. columbiensis* developmental rates (delayed emergence time and reduced body mass) with direct consequences in terms of reproduction, morphology and population dynamics. Furthermore, the fact that adult chironomid specimens, exposed at the immature stage (larvae and pupae) to chlorantraniliprole and its combination with imidacloprid, were also significantly smaller in terms of body weight, may be associated with the effects of these insecticides on the balance between emergence time and adult size. Body weight in Diptera is a fitness-related trait, as it is directly related to flight performance, number of eggs in females and sperm count in males (Rodrigues et al., 2015; Ponlawat & Harrington, 2007). Therefore, our results on adult body mass suggest additional detrimental effects on reproduction in chironomid populations, as it could be observed that adult body mass in insecticide-treated populations is comparable to the fecundity rate.

Studies have documented that stress stimuli may also initiate compensatory adjustments in energy metabolism (e.g., increased glycogen, lipid or protein consumption) of organisms to maintain physiological or morphological homeostasis (Bantu et al., 2013; Choi et al., 2001), where high energy demands may be reducing the energy available for growth and reproduction in *C. columbiensis* populations exposed to insecticides. Insects use sophisticated detoxification mechanisms to cope with different toxins introduced into their bodies, for example, studies have reported the involvement of esterase, glutathione-S-transferase and cytochrome P450 in the detoxification process (Mao et al., 2020; Li et al., 2018; Cao et al., 2017). Antioxidant and detoxification enzymes play an important role in the insect immune system (Khan et al., 2021a; Wu et al., 2006). Stress by xenobiotics, such as insecticides, accumulates reactive oxygen species (ROS) that promote a state of oxidative stress (Cullinan & Diehl, 2006). Oxidative damage could induce severe injury in insects (Smith et al., 2013; Wen et al., 2013). For example, chlorantraniliprole exposure significantly decreased SOD, CAT and POD enzyme activities in *Locusta migratoria* (Jia et al., 2016) and also induced SOD and CAT activities in *Bombyx mori* (Mao et al., 2020).

In addition, the effects found in the properties of eggs and wings of *C. columbiensis* could be related to a possible role of chlorantraniliprole as an endocrine disruptor, negatively affecting the mechanism of action of ecdysone. This hormone regulates specific functions such as molting, metamorphosis, diuresis, polyphenism and diapause (Soin & Smagghe, 2007). Ecdysteroids have important functions in arthropod species, including insects (Gilbert et al., 2002). This is because the most typical ecdysteroids, ecdysone and its derivative 20-hydroxydisone, were originally identified as molting hormones that can induce drastic morphological and developmental changes in molting and metamorphosis processes (Spindler et al., 2009). Therefore, the altered ecdysone could be involved in molting processes in immature development, causing developmental changes, variations in reproductive aspects and phenotypic changes in individuals of *C. colombiensis*.

Acute exposure assessments using a single chemical have been an integral part of the regulatory framework, but cannot predict the organism's responses to some environmentally relevant mixtures. Studies have documented the synergistic effects of complex chemical mixtures (Crain et al., 2008; Todgham and Stillman, 2013), however, with the steady increase of new molecules on the market there is a need to further assess the effects these may generate in the ecosystem. The interaction of contaminants in combination with other environmental stressors can result in synergistic, additive and/or antagonistic effects. Studies have documented that the combination of chlorantraniliprole and imidacloprid can alter some genes related to Ca^{2+} homeostasis and signaling in both vertebrates and invertebrates (Stinson et al., 2022; Simon-Delso et al., 2015), which is possibly occurring in the present study with *C. columbiensis*. The development of physiological and morphological assessment assays for use as monitoring and diagnostic tools depends on a clear understanding of the mechanisms underlying a population's response, and more research is needed, particularly for chemicals of emerging interest and their specific mechanisms of activity.

5. Conclusion

The present study revealed that exposure to chlorantraniliptol and its combination with imidacloprid impairs the success and development of the neotropical freshwater invertebrate *C. columbiensis*. The biological properties of *C. columbiensis* evaluated in the present study were useful biomarkers, as they revealed the effects generated by chlorantraniliprole stress and its combination with imidacloprid, reflecting a reduction in their body mass, emergence and reproduction, as well as alterations in egg and wing shape and size. This study may contribute to a better interpretation of the physiological and morphological responses when specimens are exposed to insecticides and contribute to a better assessment of the ecological risk of insecticides in fresh waters.

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CHAPTER V

Chemical composition of essential oils of *Siparuna guianensis* and *Siparuna gesnerioides* and its effect on the *Aedes aegypti* and its predator

Chemical composition of essential oils of *Siparuna guianensis* and *Siparuna gesnerioides* and its effect on the *Aedes aegypti* and its predator

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Abstract

Synthetic insecticides are one of the most widely used methods to vector control, but their use has many environmental problems. Therefore, the application of natural products is an alternative practice, as they can be effective, easily biodegradable and economical. The essential oil obtained from leaves of *Siparuna* (*S. guianensis*) has shown promising larvicidal activity to *A. aegypti* (Diptera: Culicidae), which is one of the major vectors of human pathogens. However, the existence of different chemotypes of essential oils in this species makes it necessary to study the influence of such variation on larvicidal activity. In the present work, the larvicidal effect of *S. guianensis* and *S. gesnerioides* essential oils on susceptible and resistant *A. aegypti* sepae was evaluated. In addition, we evaluated if the application of these essential oils would be selective against the predatory hemiptera *Belostoma anurum*. The chemical composition of the essential oils was characterized showing γ-elemene (45.8%) and germacrene D (43.8%) as major constituents. The essential oils of the *Siparuna*, were toxic to pest insects (*A. aegypti*) with LC_{50} = 0.063 and 0.053 μg/mL of *S. guianensis* and *S. gesnerioides,* respectively, as well as to non-target insects (*B. anuram*). Our results show that despite the toxicity of these essential oils on the non-target organism, they can be a useful biorational tool to replace the use of synthetic insecticides against *A. aegypti*.

Keywords: larvicidal activity; sesquiterpenes; non-target organisms; acetylcholinesterases.

1. Introduction

The *Aedes aegypti* (Diptera: Culicidae) is a vector of yellow fever, dengue, Zika, chikungunya viruses and mayaro fever, which can be found in the American continents (Pless et al., 2017), Africa and Asia (Dickson et al., 2017). The elimination of rearing sites and the application of insecticides in freshwater systems, have been the fundamental strategies to manage the diseases associated with these mosquito vectors of human diseases (Roiz et al., 2018; World, 2013). These strategies have been heavily criticized (Reiter, 2016), especially because of the exaggerated increase in the global prevalence and incidence of these vectors.

Insecticides (e.g., pyrethroids and organophosphorus compounds) despite being effective, several studies have shown that continuous application of these synthetic insecticides can lead to resistant *A. aegypti* populations (Haddi et al., 2017, Cadavid-Restrepo et al., 2012). Similarly, the direct application of these products has led to detrimental effects on human health and the environment, especially non-target fauna (Valbon et al., 2021; Devillers, 2020; Moura and Souza-Santos, 2020; Arias-Estévez et al., 2008). Besides not only insecticide molecules but also metabolites derived from their degradation, can accumulate in sediment particles, increasing the probability of exposure of aquatic organisms to such contaminants (Ccanccapa et al., 2016; Sullivan and Goh, 2008). These concerns have led the scientific community to seek alternatives in disease vector control (Bezerra-Silva et al., 2016).

Biorational products based on botanical species (e.g., extracts and essential oils) have been proposed as suitable alternatives for the control of insect vectors of human disease (Su, 2022; Ahmed, 2019; Chellappandian et al., 2018; Khater, 2012) as these products appear to offer reduced risks compared to synthetic compounds. Several studies have shown that *A. aegypti* can be effectively controlled using plant-based essential oils (Soonwera et al., 2022; Aungtikun & Soonwera, 2021; França et al., 2021; Folly et al., 2021; Marques et al., 2021). Essential oils may present variations in their chemical composition, depending on genetic or environmental factors and the developmental stage of the plant. These variations can interfere with their bioactivity and, therefore, their potential uses. Studies have reported a wide variation in the chemical composition of *Siparuna* essential oils (Diniz et al., 2022; de Souza et al., 2022; Aguiar et al., 2015), these variations represent diversification in the chemical composition of the essential oil, resulting from the interaction between the genetic characteristics of the plant and environmental conditions (Jannuzzi et al., 2011).

The neotropical region exhibits a diverse flora little exploited as a source of biologically active substances. The neotropical plant species *S. guianensis* and *S. gesneroides* (Siparunaceae), also commonly known as negramina, are a relevant example (Diniz et al., 2022). These species are widespread in South America, and products derived from their leaves, bark and flowers have been used in folk medicine (Ferraz et al., 2015; Valentini et al., 2010). However, there is little research on the insecticidal activity of the essential oils, which are associated with the effectiveness of *S. guianensis* essential oil for pest control of *A. aegypti* and *Culex quinquefasciatus* mosquitoes (Aguiar et al., 2015) ticks (Diniz et al., 2022) and *Achroia grisella* and *Galleria mellonella* wax moths (Ferreira et al., 2017). In addition, the determination of lethal and sublethal effects on other insect groups as non-target organisms have not been carried out, although they may be directly or indirectly affected by applications of such products.

Among the aquatic insect fauna, water bugs (Hemiptera: Belostomatidae) are generalist predators and represent a naturally a control agent to mosquito (Valbon et al., 2021; Valbon et al., 2019; Shaalan & Canyon, 2009). Recent research has shown that belostomatids can be behaviorally and physiologically affected by sublethal exposure to insecticides (Valbon et al., 2021; Reegan et al., 2020; Valbon et al., 2018). Other research conducted with predatory aquatic insects has shown that sublethal exposure to insecticides has impaired movements, reflexes, locomotion and, consequently, reduced the attack rate of these organisms (Reegan et al., 2020; Gutiérrez et al., 2017). Therefore, in the present work we evaluated the larvicidal effect of *S. guianensis* and *S. gesnerioides* essential oils on the vector of the human pathogen *A. aegypti* and also evaluated if the application of these essential oils would be selective against the predatory hemiptera *Belostoma anurum*. In addition, we chemically characterized the components of the two S*iparunas* species evaluated.

2. Material and methods

2.1. Collection of plant material and extraction of essential oil.

Leaves of S. *guianensis* and *S. gesnerioide* were collected in the municipality of Norcasia Caldas-Colombia (5°34′27″N 74°53′20″O; altitude 700 m). For each species studied, young and old leaves were collected from different parts of the plants, ensuring the randomness of the sample. The material was placed in bags, identified, and transported to the Zoology Laboratory of the Universidad de Caldas (Manizales, Colombia). The leaves were dried in the shade at room temperature, after drying the leaves were placed in plastic bags and stored until the extraction of the essential oil. The essential oils were obtained in the Kupay laboratory (Colombia) by steam distillation.

2.2. Chemical composition of the essential oil

The identification and semiquantification of essential oil constituents were performed using gas chromatography with a flame ionization detector (GC-FID QP2010SE, Shimadzu, Japan) and gas chromatography coupled to mass spectrometry (GC-MS QP2010SE, Shimadzu, Japan) according to the methodology of Mendes et al. (2017). For these characterizations, the following conditions were adopted: the carrier gas used was He for both detectors with flow rate and linear velocity of 2.80 mL min^{-1} and 50.80 cm sec^{-1} (GC-FID) and 1.98 mL min^{-1} and 50.90 cm sec^{-1} (GC-MS), respectively; injector temperature was 220 °C at a split ratio of 1: 30; fused silica capillary column (30 m x 0.25 mm); Rtx®-5MS stationary phase (0.25 µm film thickness); the oven temperature had the following schedule: initial temperature of 40 °C, which remained for 3 minutes and then the temperature was gradually increased at 3 °C min^{-1} until it reached 180 °C, where it remained for 10 minutes, having a total analysis time of 59.67 min; the temperatures that were used in the FID and MS detectors are 240 and 200 °C, respectively. The samples used were drawn from the vial in a volume of 1 µL of a 1% solution of EO in 95% hexane.

GC-MS analyses were performed in an electron impact equipment with an energy of 70 eV; scan rate of 1000; scan interval of 0.50 fragments sec^{-1} and detected fragments from 29 to 400 (m/z). GC-FID analyses were performed by a flame formed by H_2 and atmospheric air with a temperature of 300 °C. Flow rates of 40 mL min^{-1} and 400 mL min^{-1} were used for H_2 and air, respectively.

The identification of the components of the essential oils was performed by comparing the mass spectra obtained with those available in the spectrophotometer database (Wiley 7, NIST 05, and NIST 05s) and by the retention index (IR). For the IR calculation, a mixture of saturated C7-C40 alkanes (Supelco, USA) submitted under the same chromatographic conditions as the essential oil was used and the adjusted retention time of each compound was obtained using GC-FID. Then, the calculated values for each compound were compared with those in the literature (El-Sayed, 2019; Mallard, 2018; Adams, 2007). The relative percentage of each compound in the EO was calculated by the ratio of the integral area of the peaks to the total area of all constituents in the sample with a relative area above 0.5%.

2.3. *A. aegypti* and *B. anurum* rearing conditions

We used larvae (fourth instar - L4) of the insecticide-susceptible strain of *A. aegypti* (PPCampos, originally collected in Campos dos Goytacazes, State of Rio de Janeiro, Brazil) and larvae (fourth instar - L4) of the insecticide-resistant strain of *A. aegypti* (Oiapoque, originally collected in Rio de Janeiro, Rio de Janeiro State, Brazil) that have been reared in the insectary of the Department of General Biology, UFV (Brazil). In the insectary, larvae were kept in dechlorinated water, being fed daily with turtle food at a controlled temperature (temperature: 25 ± 2 °C, relative humidity: 60 ± 2%, and photoperiod of 12h of light) (25 ± 2 °C) until the L4 stage, which is ideal for conducting larvicidal activity assays, following the methodology previously described in Haddi et al. (2017) and Mendes et al. (2017). As non-target, were reared *B. anurum* insects, BraIN & Phy in the laboratory from adult insects collected from fish farming facilities at the Federal University of Viçosa (UFV, Viçosa, MG, Brazil, 20°45′S, 42°52′W). Field-collected adult insects were maintained in a plastic pot (2L) in pairs (1 male and 1 female) with 1L of dechlorinated water under controlled conditions of temperature (25 ± 2 °C) and photoperiod (12:12 L:D) in the presence of water hyacinth plants (i.e., Eichhornia crassipes (Mart.)) used as a resting and mating shelter. After mating, males with egg pads on their backs were kept in the plastic pot until hatching. After hatching, the first instar nymphs were individualized in glass containers (15 mL) with 10 mL of dechlorinated water to avoid cannibalism, the specimens were fed daily with *A. aegypti* larvae (L4) of the fourth instar until the nymphs reached the 2° instar, which was used in bioassays.

2.4. Larvicidal activity of essential oils against *A. aegypti* larvae

To verify the bioactivity of the essential oils on *A. aegypti* larvae in the fourth instar (L4), tests were conducted at the BraIN & Phy laboratory of UFV, Brazil. For the larvicidal assays, solutions of the two essential oils of *S. guianensis* (0.033, 0.039, 0.049, 0.065, 0.098 and 0.196 [µg/mL]) and *S. gesnerioide* (0.037, 0.046, 0.063, 0.078, 0.096 and 0.131[µg/mL]) were prepared with 3.33 µg/mL of DMSO at 99.9%. Larvae were separated using a Pasteur pipette and subsequently distributed into glass vials containing 50 mL of the dilutions. A total of 100 larvae (L4) per treatment were used, under controlled temperature (25 ± 2 °C) and 12-hour photoperiod. In addition, a DMSO solution (3.33 µg/mL) was prepared as a control. The experimental design consisted of groups of 25 *A. aegypti* larvae that were subjected to 50 mL of aqueous solution (water + essential oil) in 100 mL glass vials. Each treatment was repeated four times. Mortality was checked 24 h after the start of each trial. Larvae that did not show movement or response to stimulation with a Pasteur pipette were considered dead.

In addition, we exposed 4th instar larvae of *A. aegypti* to essential oils of *S. guianensis* (CL_{25} = 0.067 and CL_{80} = 0.112 [µg/mL]) and *S. gesnerioide* CL_{25} = 0.056 and CL_{80} = 0.109

[µg/mL]) with 3.33 µg/mL of 99.9% DMSO. For bioassays, 25 individuals were placed in 50 mL of aqueous solution (water + essential oil) in 100 mL glass vials. For each treatment, we used 100 fourth instar larvae (i.e., four groups of 25 larvae as replicates). Insect mortality was assessed at 24 h after the start of the experiment. All toxicity bioassays were conducted at controlled temperature (25 ± 2 °C), humidity (60 ± 2 %) and photoperiod (12 h light phase).

2.5.Toxicity bioassays with essential oils on the non-target *organism B. anurum*

We exposed *B. anurum* nymphs 2st instar to essential oils of *S. guianensis* (CL_{25} = 0.063, CL_{50} = 0.078 and CL_{75} = 0.096 [µg/mL]) and *S. gesnerioide* CL_{25} = 0.053, CL_{50} = 0.070 and CL_{75} = 0.092 [µg/mL]) with 3.33 µg/mL of 99.9% DMSO. Additionally, *B. anurum* nymphs were exposed to concentration of *S. guianensis* oils (CL_{25} = 0.063 µg/mL) combined with concentration of *S. gesnerioide* oils (CL_{25} = 0.063 µg/mL) and 3.33 µg/mL of 99.9% DMSO. For bioassays, one individual was placed in 15 mL of aqueous solution (water + essential oil) in 20 mL glass vials to avoid cannibalism. For each treatment, we used 30 2nd instar nymphs (i.e., three groups of 10 individualized nymphs as replicates). In the control groups, insects were maintained in 99.9% DMSO (3.33 µg/mL) solution. Insect mortality was assessed at 24 h after the start of the experiment. All toxicity bioassays were conducted at controlled temperature (25 ± 2 °C), humidity (60 ± 2 %) and photoperiod (12 h light phase).

2.6. Statistical analysis

The concentration-mortality results obtained in the toxicological bioassays with *A. aegypti* larvae were subjected to a probit analysis using the PROBIT procedure in SAS statistical software (SAS Institute, 2008). Data from the bioassay of *B. anurum* nymphs with different plant extracts were subjected to analysis of variance (ANOVA) and the percentage mortality was compared by Tukey's HSD test ($P < 0.05$), using SigmaPlot 12.5 software.

3. Results

3.1. Yield and chemical composition of essential oils

S. gesnerioides presented the highest yield of extracted essential oil (0.384%) compared to *S. guianensis* (0.21 %). Both oils presented a viscous liquid of bright yellow color, *sui generis* odor less dense than water. Qualitative and semi-quantitative variations in chemical composition were observed between the essential oils of *S. guianensis* and *S. gesnerioides*. Six compounds were identified for *S. guianensis* (Table 1, Figure 1A), which represented 100% of the essential oil composition. For *S. gesnerioides*, nine compounds were identified (Table 2, Figure 1B), which represented 94.8% of the essential oil composition. All oils presented more than 94% of sesquiterpenes in their composition and very low contents of monoterpenes.

Table 1. Chemical composition, concentrations (%), and terpene classification for the essential oil of *S. guianensis.*

N	Compounda	tretention	Arel (%)b	Terpenic classificationc
1	α-copaene	28,632	4,7	HS
2	β-bourbonene	29,002	5,5	HS
3	β-caryophyllene	30,484	4,6	HS
4	germacrene D	33,212	31,9	HS
5	γ-elemene	33,924	45,8	HS
6	Δ-cadinene	35,046	7,5	HS
	Total Identified		100,0	

[a]Compounds listed in the elution order using Rtx®-5MS column. [b]Compounds with a relative area > 2% were identified. [c]Terpenic classification: hydrocarbon sesquiterpene (HS).

Table 2. Chemical composition, concentrations (%) and terpene classification for the essential oil of *S. gesnerioides*

N	Compounda	tretention	Arel (%)b	Terpenic classificationc
1	β-pinene	10,318	3,0	HM
2	α-copaene	28,636	6,7	HS
3	β-caryophyllene	30,479	6,6	HS
4	α-bergamotene	31,306	10,7	HS
5	α-humulene	31,927	3,7	HS
6	germacrene D	33,263	43,8	HS
7	aromadendrene	33,383	6,1	HS
8	α-amorphene	34,586	2,8	HS
9	Δ-cadinene	35,068	11,4	HS
	Total Identified		94,8	

[a]Compounds listed in the elution order using Rtx®-5MS column. [b]Compounds with a relative area > 2% were identified. [c]Terpenic classification: hydrocarbon monoterpene (HM) and hydrocarbon sesquiterpene (HS).

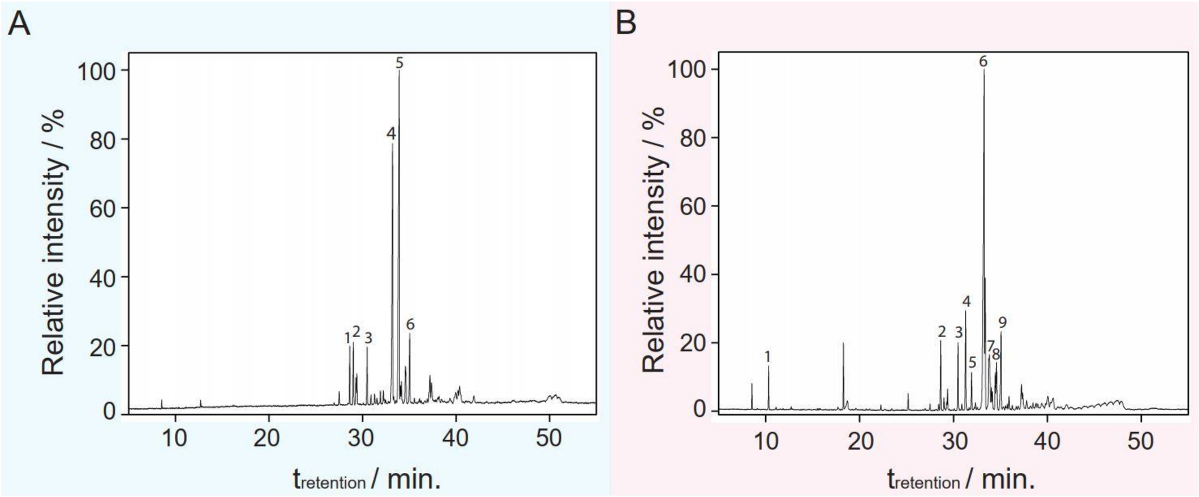


Figure 1. Composition and identification of essential oils of *S. guianensis* (A) and *S. genericides* (B) evaluated by gas chromatography, with the chemical constituents present in each of the oils defined comparatively by GC-MS mass spectrometry.

3.2. Toxicity of S. guianensis and S. genericides against A. aegypti larvae.

The concentration-mortality results obtained for *S. guianensis* (n = 700; $\chi2 = 3.89$; P = 0.27) and *S. genericides* (n = 700; $\chi2 = 5.76$; P = 0.12) on *A. aegypti* larvae after a 24h exposure was successfully fitted to a Probit model (Figura 2), which allowed estimation of the desired toxicological endpoint (i.e. LC_{50}) for each of the *Siparunas* essential oils. *A. aegypti* larvae were approximately 0.008-fold (LC50 = 0.070 [0.066-0.074] μg/L) more susceptible to *S. genericides* oil than to *S. guianensis* oil (LC_{50} = 0.078 [0.073-0.082] μg/L) (Table 3, Figura 2).

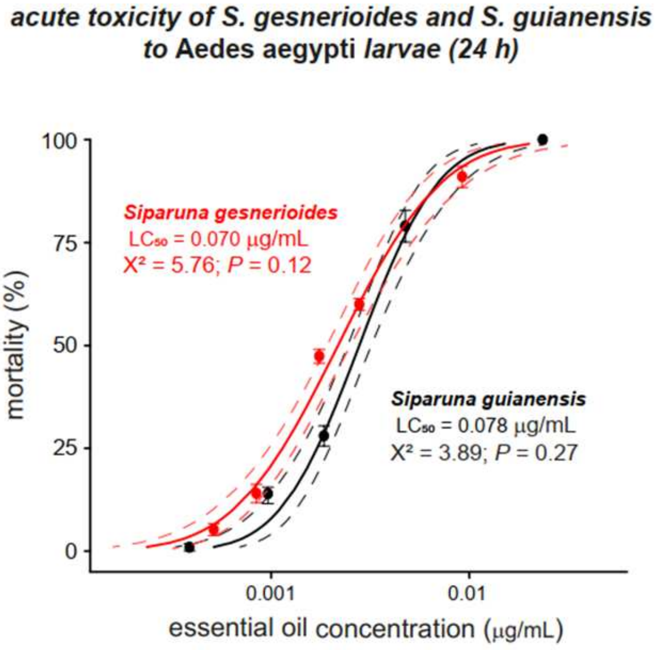
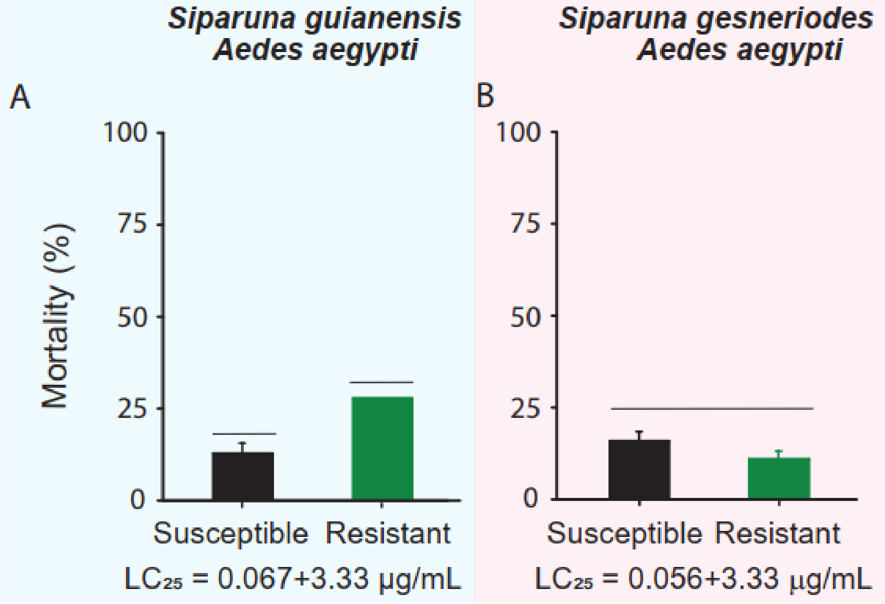


Fig. 2. Toxicity of *Siparunas* essential oils to *Aedes aegypti* larvae during a 24-h exposure period. Concentration-response curves for *Siparuna guianensis* (black) and *Siparuna gengericides* (red). Dotted lines represent 95% confidence intervals and symbols show the mean (±SEM) obtained for four replicates (aquarium with 25 *A. aegypti* larvae).

Table 3. Toxicity of essential oils of Siparuna guianensis and Siparuna gengericides to fourth instar larvae (L4) of *Aedes aegypti* over a 24-h exposure period.

Species	Hours	Slope ± SE	LC_{25} (95% CI)	LC_{50} (95% CI)	LC_{75} (95% CI)
S. guianensis	24	5.42 ± 0.56	0.063 (0.06-0.07)	0.078 (0.07-0.08)	0.096 (0.09-0.10)
S. gengericides	24	5.55 ± 0.43	0.053 (0.05-0.06)	0.070 (0.07-0.07)	0.092 (0.09-0.10)

The *Siparuna* essential oils studied were highly toxic to susceptible and resistant populations of fourth instar larvae of *A. aegypti* (Fig. 3). When the populations were exposed to 0.067 µg/mL (CL_{25}) concentration of *S. guianensis* essential oil, it presented a higher mortality in the resistant *A. aegypti* larvae population compared to the susceptible population (Fig. 3A); unlike the populations exposed to 0.056 µg/mL (CL_{25}) of *S. gesnerodes* essential oil, which showed no significant difference in the mortality of susceptible and resistant *A. aegypti* populations (Fig. 3B). However, no significant differences were found in susceptible and resistant populations of *A. aegypti* larvae when exposed to the LC_{80} of *S. guianensis* and *S. gesnerodes* essential oils (Fig. 3C-D).



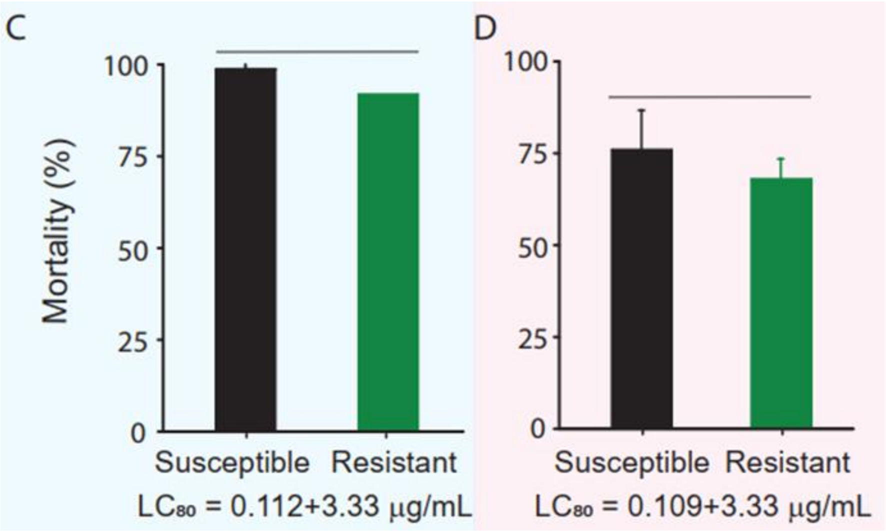
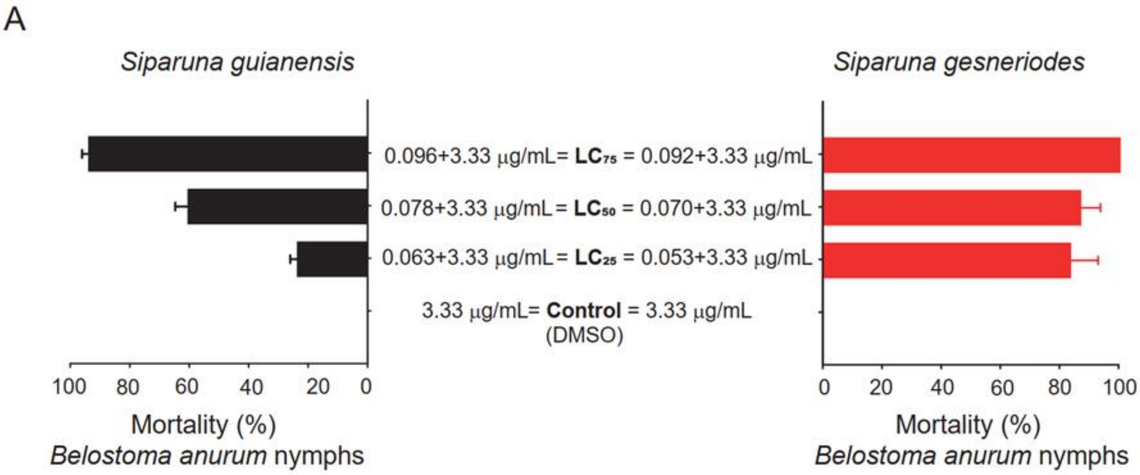


Fig. 3. Percent mortality of susceptible and resistant *A. aegypti* larvae exposed to *S. gianensi* essential oils CL_{25} (A) and CL_{80} (C) and *S. gesneroides* essential oils CL_{25} (B) and CL_{80} (D) during 24 h of exposure. Each bar represents the result of four replicates of CL_{25} and CL_{80} obtained for each *Siparuna* species. Lines indicate significant differences by Tukey's HSD test ($P < 0.05$).

3.3. Toxicity of *S. guianensis* and *S. genericides* to *B. anurum* nymphs

B. anurum nymphs were adversely affected when exposed to *Siparuna* essential oils (Figure 4A). *S. genericides* essential oil (LC_{25} = 83.3%, LC_{50} = 86.7% and LC_{75} = 100%) had the greatest negative effects on the non-target insect *B. anurum* compared to *S. guianensis* essential oil (LC25 = 23.3%, LC_{50} = 60% and LC_{75} = 93%) which at its lowest concentration (LC_{25}) had 1.7% less mortality compared to *A. aegypti*. When the CL_{25} concentrations of the oils of the two *Siparuna* species were combined, a synergism was observed, reflected in higher mortality of *B. anurum* (Figure 4B).



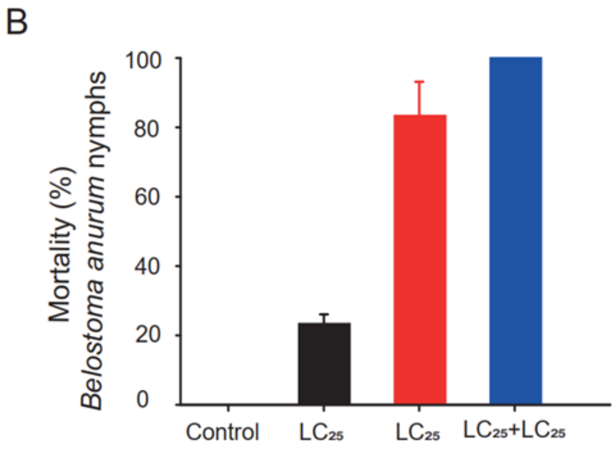


Fig. 4. Percentage mortality of the non-target predatory insect *Belostoma anurum* exposed for 24 h to essential oils of *Siparuna guianensis* (black) and *Siparuna genericides* (red) (Figure 4A). Combination of *Siparuna guianensis* essential oil (CL25) and *Siparuna genericides* essential oil (CL$_{25}$) (Figure 4B). Each bar represents the result of three replicates of LC$_{25}$; LC$_{50}$ and LC$_{75}$ obtained for each *Siparuna* species. Concentrations are expressed in µL of essential oil/mL of water.

4. Discussion

The management of the mosquito that transmits different diseases *A. aegypti* is a very challenging task, as many populations have developed high levels of resistance to multiple groups of insecticides (Bass et al., 2014). In this study, we have demonstrated the potential of S. guianensis and S. gesnerioides essential oil to control killing fourth instar larvae of A. aegypti and tested the safety of these essential oils on second instar nymphs of B. anurum. Our results revealed that the concentrations of *Siparunas* essential oils that kill insect pests (A. aegypti), can be equally toxic on non-target insects (*B. anurum*). Furthermore, it was interesting to note that the concentration of *S. guianensis* and *S. gesnerioides* essential oil that killed 80% of the susceptible *A. aegypti* population also killed more than 80% of the resistant *A. aegypti* population.

In recent years, plant-derived insecticides have emerged as relevant alternatives in pest management programs. Within the genus *Siparuna*, several species with toxic activity against arthropod pests of agricultural and medical importance have been reported (Diniz et al., 2022; de Souza et al., 2022; da Silva et al., 2020; Vila et al., 2002). Regarding the safety and potential of the *Siparuna* essential oils, promising results have been reported for its use in the control of insect pests that attack crops, beekeeping facilities and/or those that transmit animal diseases (de Souza et al., 2022; Diniz et al., 2022; Aguiar et al., 2015; Ferreira et al., 2017; Lourenço et al., 2018). The essential oil from the leaves of *S. guianensis* and *S. gesnerioides* tested on *A. aegypti* showed an LC_{50} lower than 0.08 µg/ml. This result is valuable, as Paulraj & Ignacimuthu (2022), after analyzing large amounts of data on essential oils as larvicides, noted that only 42% of the reported oil data had $LC_{50} < 50$ ppm, while 6% of them showed $LC_{50} < 10$ ppm.

The results found in the present study are consistent with data found in the literature on the oil extraction yield of *Siparuna* leaves, with values ranging from 0.10 to 0.61% (Ferreira et al., 2017; Valentini et al., 2010). The observed concentration of the main constituents in the *S. guianensis* essential oils studied was different from that reported by Diniz (2022), de Souza et al. (2022), Aguiar et al. (2015), Andrade et al. (2013) and Fischer et al. (2005), who also found that these oils have varying amounts of the major compounds. For the case of *S. gesneroides*, no studies related to essential oils were found, meaning that this is the first report of the species.

GC-MS analysis showed that γ-elemene (45.8%) and germacrene D (31.9%) for *S. guianensis*, and germacrene D (43.8%) and Δ-cadinene D (11.4%) for *S. gesnerioides* were the main components of the essential oil extracted from the leaves of these two species. Most of the essential oil compounds found in *S. gesneroides* were different from those of *S. guianensis*. Of the nine compounds reported in the essential oil of *S. gesneroides*, four (α-copaene, β-caryophyllene, germacrene D and Δ-cadinene) share with *S. guianensis* presenting high differences in their concentrations. These changes in the composition and concentration of the essential oils of the species of *Siparuna* may be due to physiological and genetic aspects, since the two species were collected in the same area. The differences in their composition and concentrations, may be explaining the small variations recorded in the mortality of *A. aegypti* as in *B. anurum*; since although the main modes of action so far have not been fully explained, it has been shown that essential oils rich in terpenes disrupt the functions of octopamine, tyramine and GABA A receptors (Peng et al., 2015; Bergougnoux et al., 2013; Blenau and Thamm, 2011; Enan, 2005;). In addition, they can inhibit the actions of acetylcholinesterase (Salgado, 2017; Miyazawa et al., 2016; Lopez and Pascual-Villalobos, 2010; Rattan, 2010).

Germacrene-D is a common sesquiterpene in *Siparuna* as in other plant species (Casiglia et al., 2017; Noge and Becerra, 2009; Young et al., 2007), and was found in our study as one of the two main components detected in the essential oils of the two *Siparuna* species. Studies have demonstrated the efficacy of essential oils rich in germacrene-D compounds as insecticides on mosquitoes and agricultural pests (Bruce et al., 2005; Gregg et al., 2016; Ravi

Kiran and Sita Devi, 2007). Similarly, it has been shown that this compound can serve as a mediator in volatile interactions between plants and insects (Stranden et al., 2002, Stranden et al., 2003). In addition, studies with bean weevils *Acanthoscelides obtectus* and *Zabrotes subfasciatus* (Jumbo et al., 2022), *Litopenaeus vannamei*, *Crassostrea rhizophorae* and *Electrophorus electricus* (Martins et al., 2021) have shown that this compound successfully interacts with acetylcholinesterase (AChE) and transient receptor potential channels in arthropods.

Another important compound was γ-elemene, which has been included in patented formulations to control insects, targeting several types of receptors (Patent US20080075796 A1 2008), but its specific mode of action has never been explained in detail (Benelli et al., 2018). Studies have reported the toxicological efficacy presented by γ-elemene-rich essential oils on the tobacco budworm *Spodoptera litura* (Benelli et al., 2018), the chestnut meal weevil *Tribolium castaneum* (Liang et al., 2016), on *Spodoptera littoralis* (Jaramillo-Colorado et al., 2015) and the library louse *Liposcelis bostrychophila* Badonell (Liu et al. 2012). In addition, this compound is one of the main volatile components of the rhizome of Curcuma wenyujin, one of the most famous traditional Chinese remedies for cancer treatment, and are promising candidates for the development of new drugs against leishmaniasis (de Lima Nunes et al., 2021).

Before reaching conclusions on the precise contribution of the constituents of *Siparuna* essential oils in insecticidal activity, it is of great importance to study their main compounds separately, since essential oils are usually a complex of molecules that can act cooperatively to exert their toxicity. However, the toxicity of essential oils is not always exerted by the most abundant constituent, as the less abundant ones may act on specific neuronal targets, playing relevant roles in the toxicity of these alternative compounds (Liu et al., 2021; Wang et al., 2021).

According to some authors, the essential oils of Siparunas shows reduced toxicological threats to non-target organisms (Lourenço et al., 2018; Ferreira et al., 2017;). However, in the present studies, the essential oils of *S. guianensis* and *S. gesnerioides* were equally toxic on both the pest insect and non-target insect. Appropriate pest control products are not expected to kill or cause (sub-lethal) side effects in non-target organisms, however, there are some reports showing how harmful these products, which are often considered safe, can be (Parreira et al., 2018; Ndakidemi et al., 2016; Kimbaris et al., 2010). Despite the results found with non-target organisms, the essential oils of *S. guianensis* and *S. gesnerioides* are shown to be effective as a pest control tool on *A. aegypti* larvae.

5. Conclusion

The present study shows that the essential oils of *S. guianensis* and *S. gesnerioides* are rich in sesquiterpenes (e.g., γ-elemene and germacrene D), with insecticidal and repellent actions against insects. Although further studies are required on the effects of these essential oils on agricultural pest insects (e.g., phytophagous bugs and *Drosophila suzukii*), beneficial (e.g., parasitoids, pollinators), our results highlight their potential to serve as an alternative tool for the control of pests (e.g., A. aegypti) which is a mosquito that can carry dengue virus, yellow fever, chikungunya, Zika fever and Mayaro fever.

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CHAPTER VI

General discussion

General discussion

In the last decades, the intensification of agricultural production has forced the rapid increase in the use of pesticides that generally end up in the aquatic environment (Anagnostopoulou et al., 2022; Fu et al., 2022). These pesticides have generated great concern, due to the great impact they have on the biodiversity of natural ecosystems (Kadiru et al., 2022; Intisar et al., 2022; Anagnostopoulou et al., 2022; Fu et al., 2022; Do et al., 2022). Among other consequences, these substances cause the decline of natural populations of non-target insects, altering ecosystem functioning (Kuechle et al., 2022; Barmentlo et al., 2021; Sánchez-Bayo & Wyckhuys, 2021; Sánchez-Bayo & Wyckhuys, 2019). Among the ecologically important non-target organisms that are strongly affected by the excessive use of chemicals are chironomids, which have been widely used as biological models to assess anthropogenic disturbance (Montaño-Campaz et al., 2022; Phillips et al., 2022; Melo et al., 2022; Lencioni et al., 2021; Wei et al., 2021). In this work, using *Chironomus columbiensis* as a study model, we have collected a wide variety of responses at both the individual and population level, induced by pesticides such as deltamethrin, imidacloprid and chlorantraniliprole. Although the negative effects of these insecticides on various insects have been previously reported (Zhang et al., 2022; Wang et al., 2022; Macaulay et al., 2021; Barmentlo et al., 2021; Merga & Van den Brink, 2021). little is known about the physiological and morphological level responses of this group.

This thesis presents results that support the idea that physiological and morphological conditions such as reproduction, body mass, wing and egg properties are highly sensitive to agricultural disturbance by insecticides (Montaño-Campaz 2022; Montaño-Campaz 2019). This work puts forward the idea that the reproductive success, body mass, and wing and egg properties of *C. columbiensis*, is an excellent way to assess the effects of insecticide contamination under controlled conditions, and these responses can contribute to our understanding of the underlying mechanisms of insect species loss in nature (Montaño-Campaz 2022), as our findings revealed that the unwanted effects of these insecticides go beyond mortality and that their sublethal actions depend on the type of exposure and the developmental stage of the organism.

This work demonstrated that the insecticides deltamethrin, imidacloprid and chlorantraniliprole alter reproductive success (fecundity and fertility) and generate changes in the wing properties (morphological variations) of *C. columbiensis*, which can be transmitted from one generation to the next as also discuted to Montaño-Campaz (2022). These effects may be related to possible differences in the toxicokinetic and toxicodynamic processes used by these insects to mitigate the action of insecticides (Jager et al., 2011; Wiberg-Larsen et al., 2016; Dalhoff et al., 2020). Toxicokinetic-toxicodynamic processes may contribute to differential susceptibility to insecticides in immature *C. columbiensis* compared to other aquatic arthropods. Recent studies have attempted to relate insecticide toxicity to morphology, toxicokinetics, and toxicodynamic traits in several aquatic invertebrate taxa, and suggested that toxicodynamic parameters are more relevant than toxicokinetics (Dalhoff et al., 2020).

This study corroborates what has been proposed by other authors, regarding that sublethal exposure to pesticides can alter the redistribution of metabolic energy toward detoxification in aquatic insects, and this can in turn decrease energy availability for other activities, such as feeding (Arias et al., 2020), locomotion (Gutierrez et al., 2017), and reproduction (Arias et al., 2020). Although the physiological basis of these detrimental insecticide-mediated sublethal effects has not been described in detail in insects, several investigations in mammals (e.g., rodents and humans) have shown that sublethal exposure of these molecules is detrimental to the structure and function of the gonads (Ye & Liu, 2019; Jia et al., 2019; Zhang et al., 2021). Likewise, preliminary evidence of unwanted sublethal effects has been shown in *Apis* bees, in which vitellogenin-producing genes were up-regulated in insecticide-exposed individuals (Christen & Fent, 2017).

Another phenomenon that may be exerting a sublethal negative effect on the morphology of *C. columbiensis* is the alteration of ecdysone receptors. This hormone when altered can interfere during molting processes in larval development, causing phenotypic abnormalities (Soin & Smagghe, 2007; Goretti et al., 2019). Ecdysone receptor (EcR), a known biomarker of endocrine disruption, and insulin receptor (InR) are two key genes involved in the control of insect development (Lin & Smagghe, 2019). Exposure of these insects to different pollutants as is in these case insecticides can cause changes in the regulation of both genes, suggesting that pollutants can impede the action of these hormones, possibly by disrupting their gene signaling cascades (Mamon et al., 2016; Aramborou et al., 2019; Aramborou et al., 2020).

In general terms, this work presents novel data and information about the response of chironomids to stress induced by pesticides (deltamethrin, imidacloprid and deltamethrin). In addition, biorational alternatives (essential oils of Siparuna Guianensis and Siparuna Gesneroides) were evaluated as alternatives in pest control. The great capacity of organisms to respond to environmental stress factors, given by the flexibility of a great variety of physiological and morphological mechanisms, was evidenced. It is of great relevance to know the effects of these substances in insects of ecological and economic importance, since they are fundamental for the functioning of natural ecosystems. Understanding the possible responses that occur at the individual level (i.e., morphology, physiology) is key to solving questions about phenomena that occur at evolutionary levels (e.g., adaptation to new environments, phenotypic plasticity and extinction).

1. Final considerations

The results presented in this thesis contribute to several fields of research (discussed above) and provide evidence on different stress response mechanisms used by *C. columbiensis* to cope with pesticide stressors. Assessing the physiology and morphology of individuals in different processes (e.g., emergence, body mass, reproduction, shape and size variations) is key in a world where anthropogenic activities are major drivers of environmental change. The use of chemical compounds such as deltamethrin, imidacloprid and chlorantraniliprole has serious impacts on the population and individual conditions of the non-target aquatic insect *C. columbiensis* (Montaño-Campaz et al., 2019; Montaño-Campaz et al., 2022). The use of these products has been reported to contribute to macroinvertebrate depletion in many regions of the world (Anagnostopoulou et al., 2022; Du et al., 2022; Fu et al., 2022).

Although the results of the characterization of the essential oils of *Siparuna guanensis* and *Siparuna gesneroide* presented in this thesis coincide in part with those reported in other works (Valbon et al.., 2021; Devillers, 2020; Moura and Souza-Santos, 2020; Arias-Estévez et al., 2008), the toxicological effects on *Aedes aegypti* evidence the high toxicity and efficacy of these oils, which differs from those reported by Haddi et al. (2017) and Cadavid-Restrepo et al. (2012). Despite intensive research on plant natural products and insect-plant chemical interactions during the last decades, few such products have been commercialized in recent years worldwide (Isman et al., 2011). These products have long been implemented as potential alternatives to conventional synthetic insecticides, possibly because natural products would have fewer impacts on the environment and human health compared to many conventional pesticides, which have had demonstrable adverse effects on non-target organisms, and even at the ecosystem level. Unfortunately, this delineation is not as clear as it is often presented in the literature, as some natural plant products used for pest control can be quite hazardous to humans as well as to non-target species.

Some of the chemical compounds evaluated in this work presented sex-, instar- and exposure time-dependent effects, which have been reported for other insect groups exposed to these contaminants (Ju et al., 2022; Shang et al., 2021; Cribiu et al., 2020). It is likely that the variation between sexes, instar and exposure time is a self-generated effect of the insecticide on these organisms, as some factors were controlled in the laboratory (Montaño-Campaz et al., 2022). Therefore, the physiological and morphological mechanisms of stress response exhibited by insects are vital to understand how contamination alters population dynamics. Thus, an in-depth understanding of how protective mechanisms differ between sexes and the effectiveness of such protection would require studies that evaluate more variables (e.g., metabolic pathways involved, gene expressions, behavior, antioxidants, etc.).

Assessing the effect of contaminants on individual and population responses (e.g., physiology and morphology) at the laboratory level is vitally important to understanding the role of environmental stressors on individuals and populations. Assessing these responses directly in the field would be optimal, as they would represent all possible variations to which organisms are exposed. However, information obtained under laboratory conditions is essential and necessary when trying to accurately assess the effects of single factors, such as differences in chemical concentrations on insect biological responses, since under natural conditions they are difficult to predict (Angert et al., 2011; Gutiérrez, 2020).

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