VIVIANA LORENA BOHÓRQUEZ ZAPATA

SYNERGISTIC AND UNINTENDED EFFECTS OF THE COMBINATION BETWEEN IMIDACLOPRID AND SODIUM CHLORIDE (NaCl) ON *Belostoma anurum* (HEMIPTERA: BELOSTOMATIDAE)

Dissertation presented to the Universidade Federal de Viçosa, as part of the requeriments of the Entomology Graduate Program, to obtain the title of *Magister Scientiae*.

Adviser: Eugênio Eduardo de Oliveira

Co-advisers: Lucimar Gomes Dias
Sabrina H. Araujo

VIÇOSA-MINAS GERAIS
2020

Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

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Bohórquez Zapata, Viviana Lorena, 1986-
Synergistic and unintended effects ofthe combination between imidacloprid and sodium chloride (NaCl) on *Belostoma anurum* (Hemiptera: Belostomatidae) / Viviana Lorena Bohórquez Zapata. - Viçosa, MG, 2020.
62 f. : il. (algumas color.) ; 29 cm.

Texto em inglês.
Orientador: Eugênio Eduardo de Oliveira.
Dissertação (mestrado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Insetos aquáticos. 2. Toxicologia ambiental. 3. Insetos aquáticos - Comportamento. 4. Inseticidas - Toxicologia. I. Universidade Federal de Viçosa. Departamento de Entomologia. Programa de Pós-Graduação em Entomologia. II. Título.

CDD 22. ed. 595.7

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APPROVED: October 30, 2020.

Assent:

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ACKNOWLEDGMENTS

I thank God for life, health, for the opportunity to have allowed me to study and for giving me the strength to fight the challenges of day to day.

I thank my mother María Rocío Zapata and my father José Gilberto Bohórquez for their love and support, I thank my uncle Mario for being the best of friends, for his love and unconditional support, I thank my cousin-brother James for accompanying me in this walk and my family in general, thank you.

I thank the Federal University of Viçosa and the Entomology department for having the opportunity to study.

I thank the Professor and advisor Eugênio E. Oliveira for opening the doors of his laboratory to me, for the trust, guidance and support in the development of my work, I also thank his wife and daughter for their affection.

I thank the professor Lucimar Gomes Dias for her friendship, for believing in me, and helping me make my dream of studying in Brazil come true. I also thank you for your time and contributions received in this work.

I thank the Dra. Sabrina Helena da Cruz Araujo for her collaboration in the development of laboratory experiments and for the shared moments.

I thank Professor Beatriz Edilma Toro for her wise advice, for her friendship, for her affection and for always being unconditionally.

I thank Dr. Luis Oswaldo Viteri Jumbo for transmitting his knowledge to me, for helping me with the experiments in the laboratory, for his friendship and for his willingness to help.

I thank the entire team of the laboratory "BrainPhy" for the learnings, and the time shared inside and outside the laboratory.

I thank Professor Gustavo Martins and the companions from the insectary for their help with the mosquito larvae and for the moments we share.

I thank Mr. Zé fish farming technician for the collaboration i have always received in the capture of organisms.

I thank Dra. Laura Machuca for the help with the TeXstudio.

I thank my friends in Colombia who were always aware of my process through distance, for their love, affection and support.

I thank the people i met and became my friends in Viçosa, and who were present supporting and collaborating with me during this time.

To the Federal University of Viçosa, for the opportunity to complete the postgraduate course.

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Financing Code 001.

To the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), to granting the scholarship.

To the Fundação de Amparo a Pesquisa de Minas Gerais (FAPEMIG), for granting the scholarship.

Thank you very much!

RESUMO

BOHÓRQUEZ ZAPATA, Viviana Lorena, M.Sc., Universidade Federal de Viçosa, outubro de 2020. **Efeitos sinergísticos e não intencionais da combinação entre imidaclopride e cloreto de sódio (NaCl) em *Belostoma anurum* (Hemiptera: Belostomatide)**. Orientador: Eugênio E. Oliveira. Coorientadoras: Lucimar Gomes Dias e Sabrina Helena da Cruz Araujo.

Nas últimas décadas, o uso de agrotóxicos para o controle de pragas nas lavouras tem se intensificado, sendo o imidaclopride um dos mais aplicados em diversos países. O uso indiscriminado desses compostos está causando efeitos negativos em diferentes ecossitemas aquáticos e terrestres, assim como na fauna associada a estes, incluindo organismos não alvos como os insetos aquáticos. Este grupo de organismos compreendem o grupo mais representativo dos ecossistemas dulceaquícolas, com um papel relevante na cadeia trófica desses ambientes e qualquer efeito letal ou subletal de compostos tóxicos a esta comunidade poderia gerar um desequilíbrio na biodiversidade aquática. Por isso o objetivo deste trabalho foi determinar os efeitos sinergísticos da combinação entre imidaclopride e cloreto de sódio (NaCl) em *Belostoma anurum* (Hemiptera: Belostomatidae) e as possíveis alterações na predação quando os indivíduos foram expostos a concentrações subletais. Foram realizados experimentos com insetos de segundo ínstar de *B. anurum* e com varias séries onde as ninfas foram expostas a diferentes concentrações por um período de 24 e 96 horas. As seguintes concentrações de imidaclopride foram usadas no experimento: 0,125; 0,375; 12,5; 18,75; 37,5; 125; e 375 mg/L. O imidaclopride foi usado em combinação com NaCl nas concentrações: 0,03; 0,09; 0,135; 0,18; 0,27; 0,3; 0,36 mg/L por um período de 24 horas. Uma maior susceptibilidade de ninfas de *B. anurum* ao imidaclopride foi observada para o tempo de exposicao de 96h, quando comparado a registrada para a exposição de 24h, o que indica que o efeito do inseticida é dependente do periodo de exposição. Para a combinação sinergística de imidaclopride + NaCl (0,5% w/v de cloreto de sódio), uma suscetibilidade mais elevada foi apresentada com o uso de concentrações mais baixas em comparação com o imidaclopride sozinho por 24 e 96 horas de exposição. Os experimentos de predação foram realizados a partir do CL_{10} obtido na primeira serie de experimentos. As ninfas de *B. anurum* foram expostas a quatro tratamentos: NaCl: solução de cloreto de sódio a 0,5% w/v, imidaclopride: 0,14 mg/L (CL_{10}), e imidaclopride 0,14 mg/L (CL_{10}) + NaCl (0.5% w/v) e um controle (água destilada). Foram utilizadas densidades de 3, 6 e 9 larvas (L4) de *Aedes aegypti* (Diptera) e se avaliaram as habilidades de predação tanto no primeiro dia apos a exposição, quanto nos proximos 3 dias consecutivos. Como resultado, foi registrado que para o primeiro dia o número médio de larvas consumidas por avaliação é dependente da densidade de larvas, com maiores densidades com 9 e 6 larvas diferindo nas densidades de 3 larvas, as quais foram expostas á combinação de imidaclopride + NaCl 0,5%, que antecedeu uma menor quantidade de presas em relação ao tratamento controle. Os resultados obtidos indicam que o imidaclopride sozinho ou em combinação com NaCl 0.5% w/v pode causar declínios populacionais e consequentemente, potenciais desequilíbrios nas comunidades de insetos aquáticos.

Palavras-chave: Ecotoxicologia. Inseticida. Predador aquático.

ABSTRACT

BOHÓRQUEZ ZAPATA, Viviana Lorena, M.Sc., Universidade Federal de Viçosa, October, 2020. **Synergistic and unintended effects of the combination between imidacloprid and sodium chloride (NaCl) on** *Belostoma anurum* **(Hemiptera: Belostomatidae)**. Adviser: Eugênio Eduardo de Oliveira. Co-advisers: Lucimar Gomes Dias and Sabrina Helena da Cruz Araujo.

In recent decades, the use of agrochemicals for or control of pests in crops has intensified, being imidacloprid one of the most applied in various countries. The indiscriminate use of these compounds is causing negative effects in different aquatic and terrestrial ecosystems, as well as the fauna associated with them, including non-target organisms such as aquatic insects. This group of organisms comprises the most representative group of freshwater ecosystems, with a relevant role in the trophic chain of those environments and any lethal or sublethal effect of toxic compost on this community can generate an imbalance in aquatic biodiversity. The objective of this work is it was to determine the susceptibility of *Belostoma anurum* (Hemiptera: Belostomatidae) exposed to imidacloprid and imidacloprid in combination with NaCl (Sodium chloride at 0.5 % w/v) and possible alterations in predation when exposed to sublethal concentrations. Experiments were carried out with insects of second instar of *B. anurum* and with several series where nymphs were exposed to different concentrations for a period of 24 and 96 hours. The following concentrations of imidacloprid were used in the experiment: 0.125; 0.375; 12.5; 18.75; 37.5; 125; and 375 mg/L. The imidacloprid was used in combination with NaCl in the concentrations: 0.03; 0.09; 0.135; 0.18; 0.27; 0.3; 0.36 mg/L for a period of 24 hours. A greater susceptibility of nymphs of *B. anurum* to imidacloprid was observed for the exposure time of 96h, when compared to recorded for the 24h exposure, which indicates that the effect of the insecticide is dependent on the exposure period. For the synergistic combination of imidacloprid + NaCl (0.5% w/v sodium chloride) higher susceptibility was presented using lower concentrations compared to imidacloprid alone for both 24 and 96 hours of exposure. The predation experiments were carried out from the LC_{10} obtained in the first series of experiments. Nymphs of *B. anurum* were exposed to three treatments: NaCl: sodium chloride solution at 0.5% w/v, imidacloprid: 0.14 mg/L (LC_{10}), and imidacloprid 0.14 mg/L (LC_{10}) + NaCl (0.5 % w/v) and a control (distilled water). Densities of 3, 6 and 9 larvae (L4) of *B. anurum* (Diptera) were used and predation abilities were assessed the first day and the 3 consecutive days after exposure. As a result, it was recorded that for the first day average number of larvae consumed per evaluation is dependent on the density of larvae, with higher densities of 9 and 6 larvae differing from the densities of 3 larvae, as well as for four exposures to the combination of imidacloprid + NaCl 0.5 %, which precedes a lower amount of prey in relation to the treatment control. The results obtained indicate that imidacloprid alone or in combination with NaCl 0.5 % w/v can cause communities declines and potential imbalances in insect populations, mortality being higher with the synergistic effect of the combination of imidacloprid + NaCl 0.5 %.

Keywords: Ecotoxicology. Insecticide. Aquatic predator.

SUMMARY

1. INTRODUCTION

Neonicotinoids are one of the most important chemical classes of insecticides due to their high efficacy against a wide spectrum of insects and the versatility of their use, together with fact that they are powerful to low dose. Are a group of systemic insecticides that are applied as foliar spray, granules, or on seeds in a wide range of agricultural crops and therefore can be absorbed by the plant, meaning the entire plant becomes toxic to the target pest and other organisms (Kobashi et al., 2017). The imidacloprid is one of the most used pesticides to protect crops from pest insects like aphids, leafhoppers, and whiteflies (Thunnissen et al., 2020). This substance non-volatile and highly soluble in water, which have become one of the most widely used classes of insecticides with a global market share of more than 25% (Jeschke et al., 2011).

Imidacloprid (IMI) was the first neonicotinoid launched in 1991 and has become one of the main products in many pest controls, being in Brazil the tenth most sold active ingredient in 2013, with approximately 8 thousand tons of compound sold (Vieira et al., 2018).

These chemicals mimic the neurotransmitter acetylcholine and are highly neurotoxic to insects, they act as neurotoxic, especially in invertebrates, because it is chemically related to nicotinic acetylcholine receptor (nAChR) agonists, nicotine and epibatidine (Tennekes and Sánchez-Bayo, 2013; Eng et al., 2017; de Lima e Silva et al., 2017).

This substance presents a risk of water contamination due to spray drift, leaching and runoff (Morrissey et al., 2015; Wettstein et al., 2016). It has been identified as a contaminant of concern for freshwater ecosystems due to its frequent occurrence at concentrations that can exert toxic effects on populations and communities of aquatic invertebrates (Rico et al., 2018). Dijk et al. (2013) found that the abundance of freshwater macroinvertebrates was negatively correlated with higher measured concentrations of imidacloprid.

In addition to the above, an alternative that has been developed empirically has been adopted in agriculture for some time now and consists of applying NaCl (0.5% w/v) to insecticides in order to reduce the negative impacts caused these substances to the environment. It is believed that with the application of NaCl (0.5% w/v) reduces the application of the insecticide by 70%, having a superior performance compared to the normal application, since according to the observations made in the field the pest insects were more attracted to salt and thus greater control of soybean pest insects was exerted (Corso and Gazzoni, 1998).

Recent studies where the combination of imidacloprid with NaCl (0.5% w/v) has been used in soybean pest insects and it was determined that this combination increases insect mortality and at the same time increased fertility and fecundity Rodrigues et al. (2020) but to date there is no information that allows us to know the effect of this combination on aquatic insects.

Tropical freshwater ecosystems are important as over a million species depend on them for their survival. However human population growth and deforestation are having a negative impact on continental aquatic biodiversity, leading to a rapid deterioration of fresh water (Cumberlidge et al., 2009).

In adition, the world production and the increase of pesticide application have given a progressive increase during the last two decades, where a large part of the applied pesticide is released into the environment. Causing problems, such as toxicity of non-target organisms and with economic importance, that comply an important role within ecosystems such as pollination in the case of bees (Tišler et al., 2009; Brandt et al., 2016; Zhu et al., 2017; Gregorc et al., 2018).

Macroinvertebrates are normally present in rivers, which represent several trophic levels, and where one of their ecological functions is to participate in the nutrient cycle, besides they present sensitivity to pollution and most of them have wide distribution areas, at least in aquatic stages, have long life cycles and are therefore good bioindicators, on water quality (Fierro et al., 2017; Svensson et al., 2017; Patang et al., 2018; Goncharov et al., 2020). Therefore, it has been used the macroinvertebrates in ecotoxicological studies for evaluate the effects of pesticides in laboratories conditions to generate information on that will allow the determination of water quality criteria and safe concentrations of contaminants (Pestana et al., 2009).

In freshwater ecosystems there is a great variety of species, some species being more sensitive than others, in the case of Hemiptera organisms. This order includes numerous predatory species that feed from other aquatic invertebrates, and also from the pupae of flies, insects that fall to the surface of the water to tadpoles (Płaska et al., 2016). Within the Hemiptera group is the Belostomatidae family, which are promising biological control agents against immature mosquitoes (Nabaneeta Saha, 2007). The organisms of *Belostoma anurum* feed on mosquito larvae, including the *Culex quinquefasciatus*, and the yellow fever mosquito *Aedes aegypti* (both Diptera: Culicidae) then the fact that *B. anurum* is feeding these mosquitoes allows the reduction of tropical diseases generated by these vectors (Valbon et al., 2019).

The aim of this study was evaluate the susceptibility of *B. anurum* (Hemiptera: Belostomatidae), an important predator of mosquito larvae to the insecticide imidacloprid alone and mixed with sodium chloride (NaCl). In adition, the sublethal effects of imidacloprid alone and mixed with sodium chloride (NaCl) on predation in *B. anurum*, was evaluated when it fed on larvae of *A. aegypti* (Diptera: Culicidae) (L4) in three densities (3, 6 and 9 larvae).

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Chapter 1

Susceptibility of *Belostoma anurum* (Hemiptera: Belostomatidae) to imidacloprid alone and mixed with sodium chloride (NaCl)

Susceptibility of *Belostoma anurum* (Hemiptera: Belostomatidae) to imidacloprid alone and mixed with sodium chloride (NaCl)

Abstract

Neonicotinoids have been one of the most widely used substances in the field of agriculture for a last decades. Among the most widely used is imidacloprid, which has been adapted in many countries to control pests that attack different crops. The problem with the use of these substances is that are also affecting non-target organisms, which have important functions within ecosystems. So, it is important to know what happens when substances such as imidacloprid reach rivers, lakes and lagoons. Benthic macroinvertebrates, which are bioindicators and help determine the state in which these ecosystems, besides an important function within the trophic link since feed and serve as food for other organisms. The objective of this work is to determine the mortality and survival of *Belostoma anurum* (Hemiptera: Belostomatidae) exposed to imidacloprid in combination with NaCl (0.5% w/v sodium chloride). Several experiments were carried out in different sets, for the first and second sets various concentrations of imidacloprid were used for both 24 and 96 hours respectively (between 0.12 and 375 mg/L), and for the third set were used concentrations between 0.03 and 0.36 mg/L of imidacloprid with the addition of NaCl: 0.5% w/v (sodium chloride solution) for a time of 24 hours, all sets were worked with second instar nymphs of *Belostoma anurum* of a rearing that was already established in the laboratory. The results obtained showed that there was a lower survival for *Belostoma anurum* nymphs that were subjected for a time of 96 hours compared to 24 hours in the same concentrations. The combination of imidacloprid

NaCl (0.5% w/v) sodium chloride with respect to imidacloprid for 24 and 96 hours presented higher mortality in lower concentrations compared to those used for imidacloprid alone. Therefore, it can be considered that *Belostoma anurum* nymphs are more sensitive when exposed to combination imidacloprid + NaCl.

Key words: Aquatic insects. Ecotoxicology. Insecticide.

1.1 Introduction

The insecticide imidacloprid (IMI) is the most sold and used insecticide, its sales and its constant use increase gradually, it has been found that it has possible downstream effects. Recent studies have highlighted its negative impacts on aquatic arthropods, it has high solubility in water and it presents environmental persistence, therefore it has a high risk since it promotes serious effects on non-target organisms compared to other neonicotinoids (Chang et al., 2020).

Neonicotinoids are neuroactive insecticides causing toxicity for the target species in the farming. This insecticides perform mainly agonistically at nicotinic acetylcholine receptors (nAChR) affect nerve transmission in the insect's causing a deterioration in normal nerve impulses (Navarro-Roldán et al., 2019).

In Brazil, in addition to using imidacloprid to control pests, they adopted an empirical practice in soybean cultures that consists in implementing sodium chloride (NaCl) in the concentration of 0.5% (w/v) to insecticide solutions, in order to reduce the amounts of pesticides by half (50%) without losing efficiency in the application (Ramos et al., 2019; Rodrigues et al., 2020).

The risk of pesticides is that can enter the aquatic ecosystem, as natural water sources, through natural processes such as surface runoff, rain erosion, infiltration, degradation, volatilization and atmospheric sedimentation Dijk et al. (2013); Das et al. (2020), thus alter diversity fauna existing in water bodies.

The macroinvertebrates aquatics, are indicators of quality of water, and present high functional and taxonomic diversity, rapid, and predicts the response to environment changes of natural and anthropogenic origin. These organisms are also bioindicartors of aquatic health status and play an essential role in aquatic habitat foodwebs (Ghosh and Biswas, 2018; Bonada et al., 2006).

Among the aquatic insects are the organisms of the Hemiptera order of the Belostomatidae family, which feed on a variety of organisms, including mosquitoes, which are the generators of diseases in human health (Shin-ya, 2018; Valbon et al., 2018). The objective of this work is to determine the susceptibility of *B. anurum* (Hemiptera Belostomatidae) to imidacloprid alone and mixed with sodium chloride (NaCl).

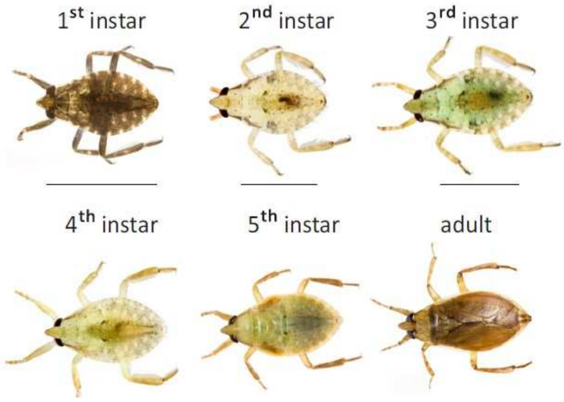

Figure 1.1: Each immature (i.e., 1st to 5th instars) and adult of *Belostoma anurum*. The scale bar is equivalent to 5 mm in for the instar (Valbon et al., 2019).

1.2 Material & Methods

1.2.1 Insects

Belostoma anurum: The insects for the experiments were obtained from a stock colony of *Belostoma anurum* insects that was already established in Invertebrate Physiology and Neurobiology Laboratory at the Federal University of Viçosa (UFV, Viçosa, MG) under controlled conditions ($27 \pm 2°C, 60 \pm 20\%$ relative humidity (RH), 12 h photophase) (Valbon et al., 2018).

The organisms from the first to the third instar were individualized in glass flasks (30 ml). Each bottle contained distilled water. The insects were fed with larvae of *Aedes aegypti* (Diptera: Culicidae). Adults and nymphs of *Belostoma anurum* of fourth, and fifth instar were transferred to larger plastic containers (100 ml) to complete the development. During this time the insects were fed with *Buenoa* spp. and *Martarega* spp. (Hemiptera: Notonectidae) and with fish (*Poecilia reticulata*). Each organism was individualized to avoid cannibalism (Figure 1.1).

Aedes aegypti: To feed nymphs from first to third instar of *Belostoma* *anurum* were used, fourth instar larvae of *Aedes aegypti* (Culicidae), which were obtained from a colony maintained in the insectary of the Department of General Biology of the Federal University of Viçosa (Viçosa, MG, Brazil). The larvae were fed with commercial food "Fed turtle food" (Reptolife, Alcon Pet, Camburiú, SC, Brazil) and were kept over a controlled temperature ($25 \pm 2°C$), and a photoperiod (Marriel et al., 2016).

1.2.2 Insecticide- Imidacloprid

To bioassays was used, the commercial formulation of the neonicoticonoid insecticide imidacloprid (EVIDENCE® 700 WG) 1-(6-Chloro-3-pyridylmenthyl)-N-nitroimidazolidin-2-yilideneamine (IMIDACLORIDO) (70% m/m) other ingredients (30% m/m) Bayer SA. São Paulo, SP, Brazil.

Glass containers (beakers) were used to carry out the imidacloprid concentrations, using distilled water and all the protection elements for the safe handling of this chemical component.

1.2.3 Concentration-mortality bioassay

For bioassay with Imidacloprid (24 hours)

Second instar nymphs of *B. anurum* (24-72h after ecdise) were used, which were individually exposed to concentrations of 0.125; 0.375; 12.5; 18.75; 37.5; 125; 375; mg/L of imidacloprid, each organism was placed in a 250 mL glass container containing 100 mL of solution. The containers were covered with microscopic mesh fabric.

For each concentration and the control treatment, three repetitions were made each with a minimum of 10 insects, for control treatment was made with distilled. Mortality was evaluated 24h after exposure. Insects without movement after mechanical stimulus were considered dead.

For bioassay with Imidacloprid (96 hours)

Second instar nymphs of *B. anurum* (0 - 72h after ecdise) were used, which were individually exposed to concentrations of 0.125; 0.375; 12.5; 18.75; 37.5; 125; 375; mg/L of imidacloprid, each organism was placed in a 250 mL glass container containing 100 mL of solution. The containers were covered with microscopic mesh fabric. For each concentration and the control treatment, three repetitions were made each with a minimum of 10 insects, for control treatment was made with distilled. Mortality was evaluated every 24h until 96h exposure of the respective concentrations was completed. Insects without movement after mechanical stimulus were considered dead.

Imidacloprid in combination with NaCl (0.5 % w/v) sodium chloride concentration mortality bioassay for 24 hours.

Second instar nymphs of *B. anurum* (0 - 72h after ecdise) were used which were individually exposed to concentrations of 0.03; 0.09; 0.135; 0.18; 0.27; 0.3; 0.36 mg/L each with the combination of NaCl: (0.5 % w/v) (sodium chloride solution) for 24h in 250 mL glass containers containing 100 mL of solution and covered by microscopic mesh tissue. For each concentration and the control treatment, three repetitions were made each with a minimum of 10 insects, for control treatment was made with distilled. Mortality was evaluated 24h after exposure. Insects without movement after mechanical stimulus were considered dead.

1.2.4 Statistic analysis

To analyze the concentration-mortality curve was determined using the PROC PROBIT procedure (SAS Institute, 2010). And with the data obtained from the bioassay, the graphs were made for the Teste-t analysis using

SigmaPlot 12.0 software (Systat Software, San Jose, Califórnia, USA).

1.3 Results

1.3.1 Concentration-mortality

For bioassay with Imidacloprid (24 hours)

The results obtained for the estimation of the concentration-mortality curve for 24h of *B. anurum* were adequate for the Probit model. It can be seen that a low value of χ^2 (0.0437) and the value of P (> 0.05) equal to 0.9976 was obtained, the LC_{50} value was 0.338 and LC_{10} (0.00053 mg/L) of imidacloprid (Figure 1.2).

For bioassay with Imidacloprid (96 hours)

For the estimation of the concentration-mortality curve to 96h for *B. anurum*, the results were adequate for the Probit model. It gave to see that a low value of χ^2 (3.354) was obtained and the value of P (> 0.05) equal to 0.645, and the value of LC_{50} was 0.0055 mg/L of imidacloprid (Figure 1.3).

Imidacloprid in combination with NaCl (0.5 % w/v) sodium chloride concentration-mortality bioassay for 24 hours

The results obtained for the estimation of the concentration-mortality curve for 24h of *B. anurum* were adequate for the Probit model. It can be seen that a low value of χ^2 (5.6389) and the value of P (> 0.05) equal to 0.1306 was obtained, the LC_{50} value was 0.12 mg/L of imidacloprid. (Figure 1.4).

The LC_{50} and LC_{10} values were 0.12 mg/L and 0.0405 mg/L of imidacloprid, respectively.

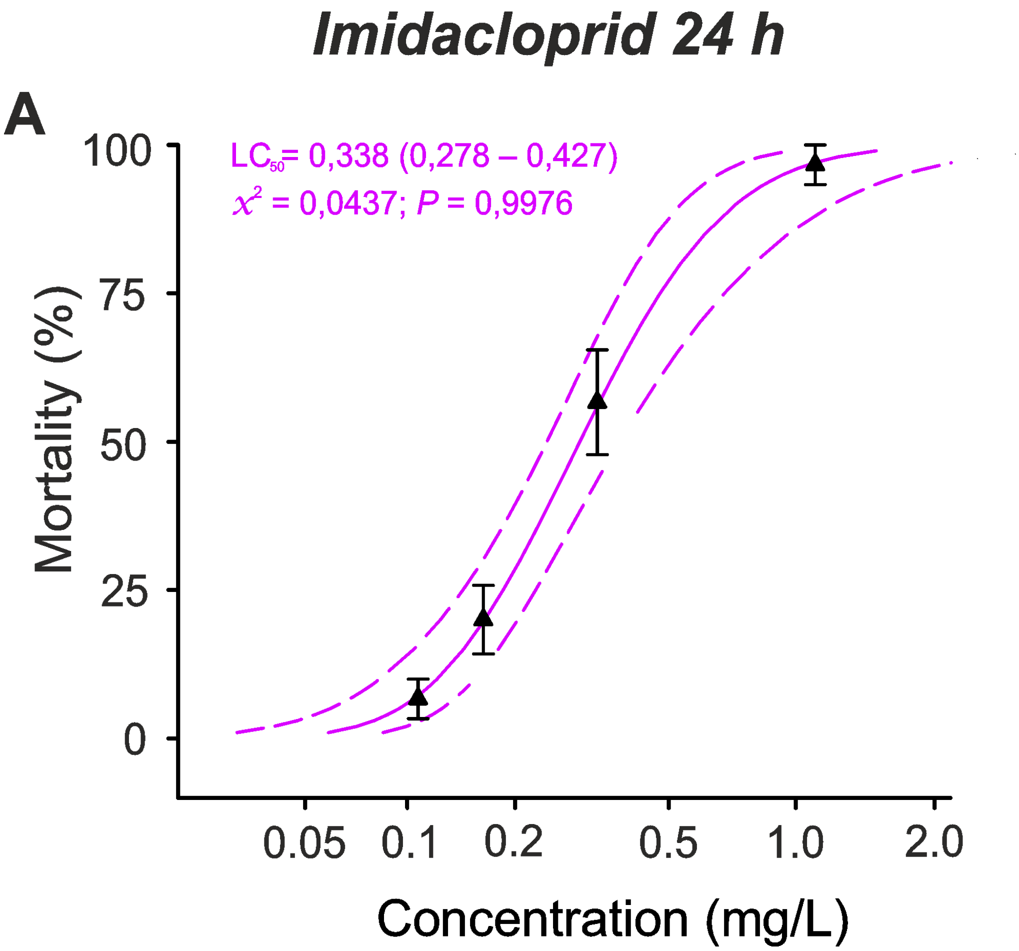

Figure 1.2: Concentration-mortality curve of *B. anurum* nymphs after 24 h exposure to Imidacloprid. Lethal concentration (LC) values were estimated based on the concentration-mortality bioassay using Probit analysis. Dotted lines show 95% confidence intervals. The points represent the mean mortality (±*SE*) of each treatment performed.

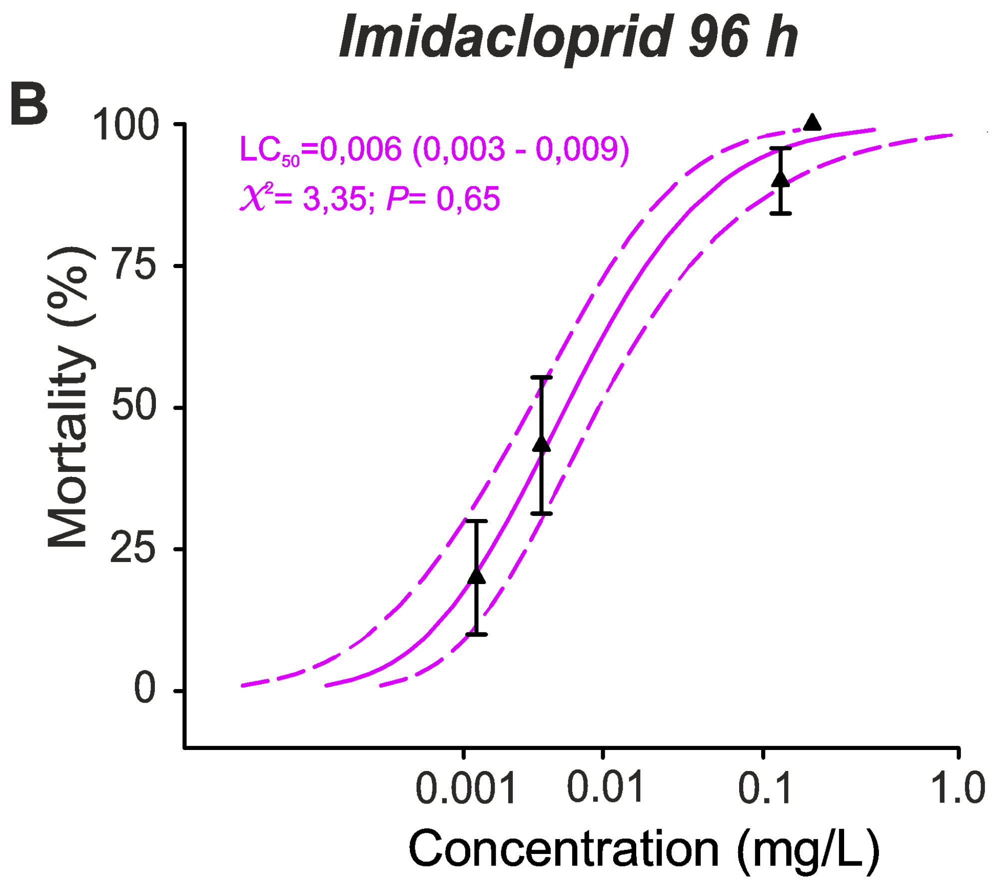

Figure 1.3: Mortality-concentration curve of *B. anurum* nymphs after 96 h exposure to imidacloprid. Lethal concentration (LC) values were estimated based on the concentration mortality bioassay using Probit analysis. Dotted lines show 95% confidence intervals. The points represent the mean mortality ($\pm SE$) of each treatment performed.

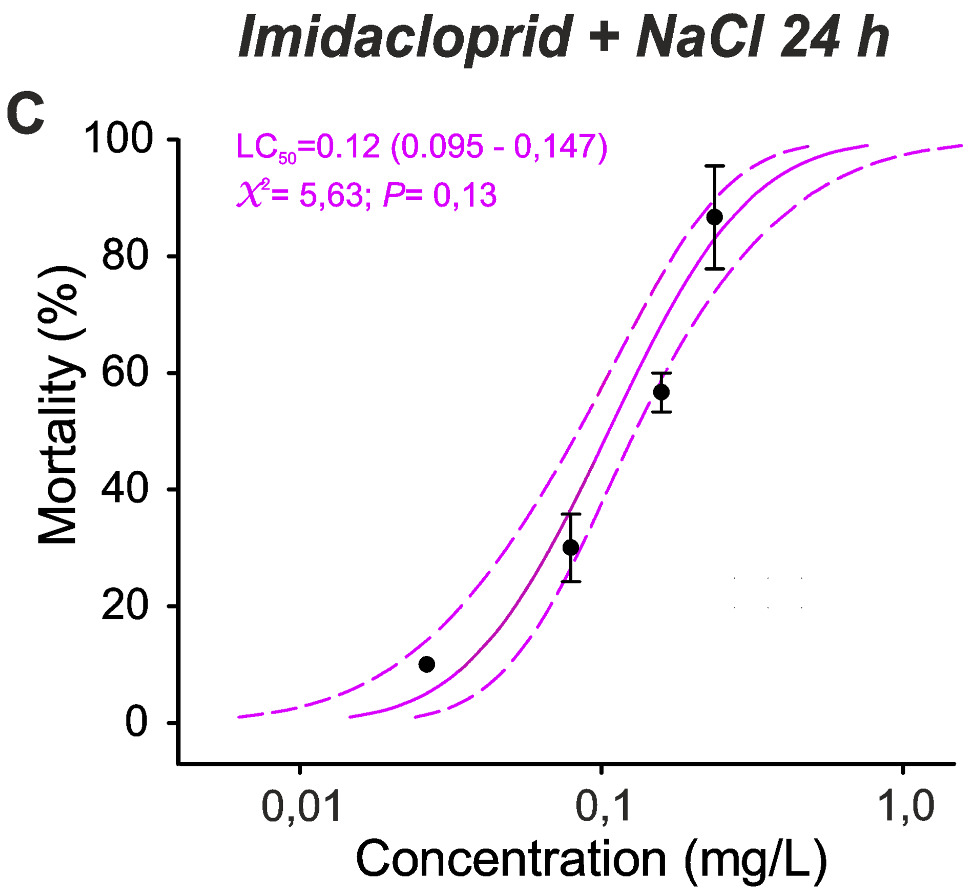

Figure 1.4: Concentration-mortality curve of *B. anurum* nymphs after 24 h exposure to imidacloprid in combination with NaCl (0.5 % w/v) sodium chloride. Lethal concentration (LC) values were estimated based on the concentration-mortality bioassay using Probit analysis. Dotted lines show 95% confidence intervals. The points represent the mean mortality (±*SE*) of each treatment tested.

1.4 Discussion

The present study showed that the nymphs of *Belostoma anurum* presented susceptibility to imidacloprid, where a decrease in survival was observed when were exposed to different concentrations of the insecticide. These results are consistent with others studies, (Jinguji et al., 2012; Nakanishi et al., 2018; Sumon et al., 2018)that reported neocotinoid insecticides such as imidacloprid cause mortality to non-target aquatic organisms. These alterations in aquatic organisms in the long term as in the case of insects of *Belostoma anurum* can cause an imbalance in the ecosystem since it is an important predator for the control of vectors such as *Aedes aegypti*, a mosquito that transmits yellow fever (Valbon et al., 2019).

Lansdell and Millar (2000); Matsuda et al. (2001); Shimomura et al. (2004) referenced that the imidacloprid, being a neonicotinoid, is chemically related to agonists of acetylcholine receptor (nAChR) and in insects these receptors are limited to the nervous system, altering the transmission of nerve impulses.

Neonicotinoids are neuroactive insecticides which act on nicotinic acetylcholine receptors (nAChR) on the post synaptic membrane, disrupting neural transmission in the central nervous system of insects (Cresswell, 2010; de Lima e Silva et al., 2017; Vieira et al., 2018).

The binding of imidacloprid to nAChRs elicits a continuous electric impulse that results in hyperactivity and overstimulation of the neurons, this process eventually leads to the death of the neuron. When more chemical molecules bind to other nAChRs it leads to damage and eventually death of the organism (Thunnissen et al., 2020). Probably the susceptibility presented by *B. anurum* is due to the effect that the neocotinoid caused on the nervous system of organisms.

Stoughton et al. (2008) mentioned that solubility of imidacloprid is relatively high water (0.51–0.61 g/L at 20) associated potential transport into surface water and to leach into ground water, slow degradation (half-life in water = 31–46 days), and tendency to persist in soil (half-life in soil = 69–997 days. The cumulative effects of neonicotinoids even in lower concentrations have toxic effects if maintained for a long period (Roessink et al., 2013). These results are agrees with the present study since as the days of exposure increased to imidacloprid the susceptibility of *B. anurum* organisms increased compared to 24 hour vs. 96 hour experiments.

Stoughton et al. (2008) mentioned that differences in phylogeny, physiology, and life history can result in different sensitivities to the same toxin and, therefore, be more useful in predicting associated risk of imidacloprid in natural aquatic animals. Researchers exposed aquatic organisms to imidacloprid, *Hyalella azteca* and *Chironomus tentans* presented lower susceptibility to imidacloprid, the LC_{50} for *Hyalella azteca* was 65.43 mg/l and for *Chironomus tentans* was 5.75 mg/L at 96 hours of exposition and in our study *B. anurum* presented LC_{50} 0.0055 mg/L for the same exposure time, presenting greater susceptibility to imidacloprid.

The increased susceptibility of *B. anurum* nymphs when in combination of imidacloprid with NaCl (0.5 % w/v) can also be attributed to the synergism of the two substances when the overall effect is greater than the sum of their individual effect (Ramos et al., 2019). Synergism helps decrease the use of active ingredients while maintaining the same level of activity against organisms (Giorio et al., 2017). According to Rodrigues et al. (2020) this is probably due to the fact that in the osmoregulation and neurophysiology process, sodium ions (Na +) play important roles, in addition, a hyperarousal of the nervous system of insects can occur causing mortality in organisms when it is combining with imidacloprid.

In conclusion, the combination of imidacloprid with NaCl (0.5 % w/v) generated the increased susceptibility of *B. anurum*, because probably the afectation of its nervous system caused by increased sodio ions.

1.5 Acknowledgment

This work was supported by the National Council of Scientific and Technological Development (CNPq), the Minas Gerais State Foundation for Research Aid (FAPEMIG), the Coordination for the Improvement of Higher Education Personnel (CAPES, Financial code 001)

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Chapter 2

Effect of sublethal doses of imidacloprid alone and mixed with sodium chloride (NaCl) on the predatory ability of *Belostoma anurum* (Hemiptera: Belostomatidae)

Effect of sublethal doses of imidacloprid alone and mixed with sodium chloride (NaCl) on the predatory ability of *Belostoma anurum* (Hemiptera: Belostomatidae)

Abstract

Benthic macroinvertebrates are important organisms within communities since, are essential in food chains, occupying different levels, besides to serving as food for many specie. The pesticides in water sources can promote an imbalance in the levels of food chains, it is the case of the predators organisms as Belostomatidae (Hemiptera) that feed on insects such as mosquitoes vectors. Therefore, the objective of this work is to determine the sublethal effects of imidacloprid alone and mixed with sodium chloride (NaCl) on predation in *B. anurum*, when it feeds on larvae of *A. aegypti* (Diptera: Culicidae) (L4) in three densities (3, 6 and 9 larvae). This work was carried out with LC_{10} concentrations already determined from the concentration-mortality. Were used second instar nymphs of *Belostoma anurum* and we did three treatments: NaCl (sodium chloride solution) at 0.5% w/v, imidaclopride 0.14 mg /L (LC_{10}), imidaclopride 0.14 mg/L (LC_{10}) + NaCl (0.5% w/v) and a control (distilled water). Predation evaluations consumed larvae every 40 minutes, for six hours a day (nine evaluations), larval density was restored at the end of each evaluation, this experiment was performed for 4 days. Glass jars were used and each one contained a *B. anurum* organism with *A. aegypti larvae* (L4) in different densities (3, 6, 9 larvae) per glass pot. The results obtained indicate that for the first day the average number of larvae consumed per evaluation is dependent on the densities of larvae offered. The depreation being higher with 9 and 6 larvae, as opposed to the densities of 3 larvae, which were exposed to the combination of NaCl + imidacloprid. In the fourth day the organisms that were exposed to the combination of NaCl + imidacloprid had a different behavior with respect to the other treatments.

Key words: Insecticide. Aquatic environment. Behaviour effects.

2.1 Introduction

In recent years, the use of insecticides in agricultural activities has increased, the complex mixtures of these chemical compounds have promoted a series of alterations in ecosystems, including aquatic environments. In the case of rivers, a large number of chemical compounds originated by anthropogens activities are receive daily, where mixtures occur complexes of potentially hazardous compounds in the aquatic environ (Kuzmanović et al., 2016; Ghorab, 2018).

Neonicotinoids have become one of the most commercialized and used insecticide classes worldwide, causing adverse effects on ecosystems and biodiversity asociated (Kobashi et al., 2017). The use of neonicotinoids, as imidacloprid, in the pest control could generated a indirect contamination in the aquatic habitats and to contributed the impoverishment of aquatic biodiversity. In Brasil, Imidacloprid molecules have been detected rivers, lakes and lagoons, due to the intensification of agricultural activities with Imidacloprid molecules have already been detected (Vieira et al., 2018).

The neonicotinoids causing lethality through ingestion or spraying on non-target organisms, also due to natural phenomena these substances reach surface waters such as runoff and leaching, which are determined by climatic conditions (Gupta et al., 2002; Bonmatin et al., 2014; Vijver and van den Brink, 2014) facilitating insecticides reach the freswater and generating disturbances.

The aquatic macroinvertebrates, particularly soil-dwelling species, are more likely to be at long-term risk from exposure to these insecticides accumulated in this habitat (Hayasaka et al., 2019). In general, these aquatic macroinvertebrates have a long useful life (from several weeks to years), and therefore are indicative of changes in water qualities by reflecting cumulative effects of present and past short- and long-term conditions of environmental stressors and they have numerous traits that make them excellent bioindicators in many aquatic ecosystems (Johnson et al., 2013; Gleason and Rooney, 2017; Ghosh and Biswas, 2018).

Benthic macroinvertebrates participate in the conversion of vegetal and waste into animal tissues in the aquatic decomposition chain and in riparian zones, being fundamental in aquatic food webs whose interruption can cause changes in the energy supply for the entire ecosystem (Svensson et al., 2017; Chellaiah and Yule, 2018; Gonzalez et al., 2020).

Miranda et al. (2011) exposed to neocotinoids various non-target organisms such as mayfly larvae *Epeorus longimanus*, black fly *Simulium vittatum*, mosquito larvae *(Aedes aegypti, Culex quinquefasciatus, Aedes albopictus)*, fleas of tested water *(Chydorus sphaericus, Daphnia magna)* and ostracods. Where they presented a high sensitivity when exposed to the neocotinoid, being the least sensitive *D. magna*.

In this context, the use of neonicotinoids, such as imidaclopride, can cause mortality or physiological and behavioral alterations in macroinvertebrates and, consequently, cause the interruption of energy flow, altering biodiversity and the ecological function of organisms and ecosystem services (Edwards, 2002; Chagnon et al., 2014; Kobashi et al., 2017).

The aquatic macroinvertebrates have also gained prominence as predators of insects of vector importance such as *Aedes aegypti* (Valbon et al., 2018). Rückert et al. (2017) reported that mosquitoes *A. aegypti* has attracted worldwide attention due to its vector capacity, since it can currently cause diseases such as dengue, yellow fever, zica, chicungunya infecting and transmitting various combinations of these viruses simultaneously.

The Hemiptera have fulfills an important function within aquatic ecosystems as biological control agents, since are organisms that prey larvae of *A. aegypti* helping to regulate the mosquito population (Aditya, 2004; R and JS, 2006; Sareein et al., 2019).

In the case of *B. anurum* which feeds of *A. aegypti* (Diptera: Culicidae) larvae, allowing the reduction of this population of mosquitoes, therefore an alteration in predation can increase diseases and deaths in different parts of the world caused by this vector (Valbon et al., 2018).

Finally, the neonicotinoids can affect lethally or sublethally the insects highlights that sublethal insecticides doses affect certain behaviors in organisms such as predatory capacity in non-target organisms (Gutiérrez et al., 2017).

This study was carried out to determine the sublethal effects of imidacloprid alone and mixed with sodium chloride (NaCl) on predation in *B. anurum*, when it feeds on larvae of *A. aegypti* (Diptera: Culicidae) (L4) in three densities (3, 6 and 9 larvae).

2.2 Material & Methods

2.2.1 Insects

Belostoma anurum: The insects for the experiments were obtained from a stock colony of *B. anurum* insects that was already established in Invertebrate Physiology and Neurobiology Laboratory at the Federal University of Viçosa (UFV, Viçosa, MG) under controlled conditions (27 ± 2C, 60 ± 20% relative humidity (RH), 12 h photophase) (Valbon et al., 2018).

The organisms from the first to the third instar were individualized in glass flasks (30 ml). Each bottle contained distilled water. The insects were fed with larvae of *Aedes aegypti* (Diptera: Culicidae). Adults and nymphs of *Belostoma anurum* of fourth, and fifth instar were transferred to larger plastic containers (100 ml) to complete the development. During this time the insects were fed with *Buenoa* spp. and *Martarega* spp. (Hemiptera: Notonectidae) and with fish (*Poecilia reticulata*). Each organism was individualized to avoid cannibalism.

Aedes aegypti: To feed nymphs from first to third instar of *B. anurum* were used, fourth instar larvae of *A. aegypti* (Culicidae), which were obtained from a colony maintained in the insectary of the Department of General Biology of the Federal University of Viçosa (Viçosa, MG, Brazil). The larvae were fed with commercial food "Fed turtle food" (Reptolife, Alcon Pet, Camburiú, SC, Brazil) and were kept over a controlled temperature (25 ± 2 C), and a photoperiod (12 h) (Marriel et al., 2016).

2.2.2 Insecticide- Imidacloprid

To bioassays was used, the commercial formulation of the neonicoticonoid insecticide imidacloprid (EVIDENCE® 700 WG) 1-(6-chloro-3-pyridylmenthyl)-N-nitroimidazolidin-2-yilideneamine

(IMIDACLORIDO) (70% m/m) other ingredients (30% m/m) Bayer SA. São Paulo, SP, Brazil.

Glass containers (beakers) were used to carry out the imidacloprid concentrations, using distilled water and all the protection elements for the safe handling of this chemical component.

2.2.3 Predation bioassay

To carry out this bioassay, second instar nymphs of *B. anurum* (24 - 72h old) were used, three trataments and a control were carried out as follows:

- Imidacloprid: 0.14 mg/L (LC_{10})
- Imidacloprid + NaCl: 0.14 mg/L (LC_{10}) of imidacloprid + (0.5% w/v) sodium chloride solution
- NaCl: (0.5% w/v) sodium chloride solution
- Control: distilled water

These concentrations were obtained after performing the concentration-mortality bi-test. Each of the nymphs were individually exposed in 250 mL glass containers containing 100 mL of solution. The containers were covered with microscopic mesh fabric. One insect per glass pot was used, 15 pots per treatment and three repetitions were made. The evaluation was carried out at 24h after exposure, those individuals that did not respond to mechanical stimulus performed with a Pasteur pipette were considered dead and the living organisms were used for the predation bioassay.

The nymphs of *B. anurum* that remained alive from the survival bioassay were transferred to 100 mL glass flasks and allowed to acclimatize for one hour in distilled water, then L4 *A. aegypti* larvae were placed at different densities (3, 6, 9 larvae) in each of the jars, the evaluations were carried out every 40 minutes, during 6 hours (9 evaluations per day) and the number of larvae was replaced at the end of each evaluation, until completing evaluation days. These insects were kept under controlled laboratory conditions (27 ± 2C, 60 ± 20% relative humidity (RH), 12 h photophase) in the Invertebrate Physiology and Neurobiology Laboratory at the Federal University of Viçosa.

2.2.4 Statistic analysis

The larval predation data were subjected to an analysis of variance with repetitive mean in time to determine the effect of imidacloprid, and imidacloprid in combination with NaCl (0.5% w/v), the density and the recovery time (SAS Institute, 2010). The factors studied were insecticide and density, and larvae predated as pseudo-repetition. The number of larvae predated every 40 min were used as repeated means over time.

2.3 Results

2.3.1 Predation

We have that for the analysis of variance with repetitive mean in time for the first day of evaluation it had an effect on variables such, as time (F = (92, 8) = 21.07; P = <.0001), time and density (F=92, 8)=16,27; P = <.0001), interaction between the insecticide and density (F = (267.43, 24) = 2.98; P = <.0001) (Table 2.1).

Table 2.1: Analysis of variance with repeated measures over the predation effect in the first day of *B. anurum* exposed to imidacropid + salt (density 6-9 larvae of *Aedes aegypti*)

Source of variation	**df**			**F**	***P***
Subject effects					
Insecticide (I)	3			2.90	0.039*
Density (D)	1			2.61	0.109
Insecticide * density (I*D)	3			3.25	0.025*
Error	99				
Within Subject Effects					
Time	8			23.65	<.0001*
Time*Insecticide	24			0.94	0.541
Time*Density	8			14.40	<.0001*
Time*Density*Insecticide	24			3.10	<.0001*
Error	792				
	Df_{den}	**Df_{num}**	**Wilks' lambda**	**F**	***P***
Between factors					
Time (T)	92.00	8	0.353	21.07	<.0001*
Time*Insecticide	267.43	24	0.819	0.79	0.744
Time*Density	92.00	8	414	16.27	<.0001*
T*Insecticide*Density	267.43	24	0.503	2.98	<.0001*

**Significant to P<0.05*

For the first day, in the imidacloprid treatment alone, the average number (Figure 2.1) of larvae consumed per evaluation was higher for the densities 9 and 6 larvae per 100 ml, unlike the densities of 3 larvae, in which *B. anurum* was exposed.

All the other depredator, that were exposed to the other treatments did not differ in terms of the average number of larvae consumed in each evaluation. The waterbug that were exposed to the combination of NaCl + imidacloprid predated a smaller amount of prey with respect to the control insects, in the intermediate densities, in terms of a higher density of larvae. Predators exposed to imidacloprid reduced predatory ability in the number of larvae consumed over time.

In the densities of 6 and 9 larvae/100 mL, the mean number of larvae consumed in the first evaluation was significantly higher than in the other evaluations. Although the average number of larvae predated by bedbugs that were exposed in NaCl (0.5% w/v) did not show differences with the control organisms. For the larvae densities of 3 and 6 larvae / 100 mL, it resulted in a lower quantity of predated larvae in the higher larvae density. Similar trend for larvae exposed to imidacloprid alone.

The analysis of variance with repeated measurement time over the 4 days showed an effect on time (F (96.3) = 44.23; P = $<$.0001), time and insecticide (F (233.79, 9) = 2,61; P = (0.0068), interaction between time, insecticide and density (F (233.79, 9) = 2.07; P = $<$0.33) (Table 2.2).

Table 2.2: Analysis of variance with repeated measures over the predation effect in the four days of *B. anurum* exposed to imidacloprid + salt (density 3-9 larvae of *Aedes aegypti*)

Source of variation	**df**			**F**	***P***
Subject effects					
Insecticide (I)	3			0.13	0.9391
Density (D)	1			32.84	<.0.0001*
Insecticide * density (I*D)	3			4.46	0.0056*
Error	98				
Within Subject Effects					
Time	3			65.63	<.0001*
Time*Insecticide	9			3.30	0.0008*
Time*Density	3			3.65	0.0131*
Time*Density*Insecticide	9			1.85	0.0600
Error	294				
	Df_{den}	**Df_{num}**	**Wilks' lambda**	**F**	***P***
Between factors					
Time (T)	96	3	0.419	44.23	<.0001*
Time*Insecticide	233.79	9	0.791	2.61	0.0068*
Time*Density	96	3	0.921	2.71	0.05
T*Insecticide*Density	233.79	9	0.829	2.07	<.033*

**Significant to P<0.05*

For the case of the four-day bioassay in the low prey density condition, the individuals exposed to this combination of imidacloprid + NaCl (0.5 % w/v) presented a better predation performance (Figure 2.2) (A). Meanwhile, the intermediary condition of prey (Figure 2.2) (B), the individuals of this treatment predated significantly less than in the other treatments, in the condition of high prey density (Figure 2.2) (C) the individuals exposed to imidacloprid and NaCl in this combination predated significantly less than in the individuals belonging to the control.

In summary the results for predation during the four days (Figure 2.2) individuals who were exposed to the combination of imidacloprid + NaCl (0.5% w/v) presented different results in relation to the other treatments (Figure 2.2) showed that the conditions of low and intermediate density of larvae, the predators that were exposed to NaCl 0.5 % w/v and imidacloprid presented similar predation patterns to the control individuals.

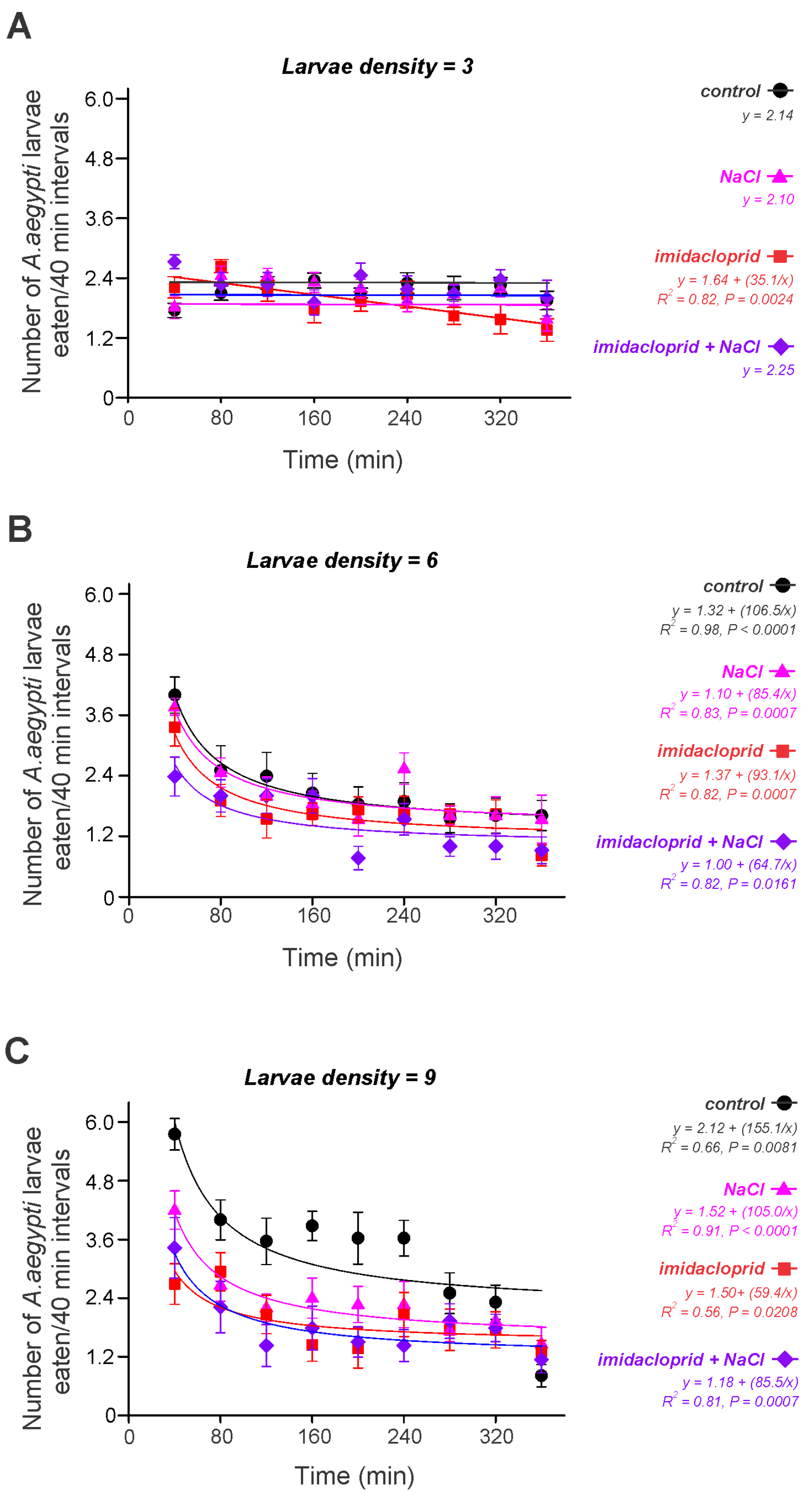

Figure 2.1: Number of *Aedes aegypti* larvae ($\pm SE$) consumed at 40 minute intervals, at different densities predated by *B. anurum* em densidade three (A), six (B), and nine (C).

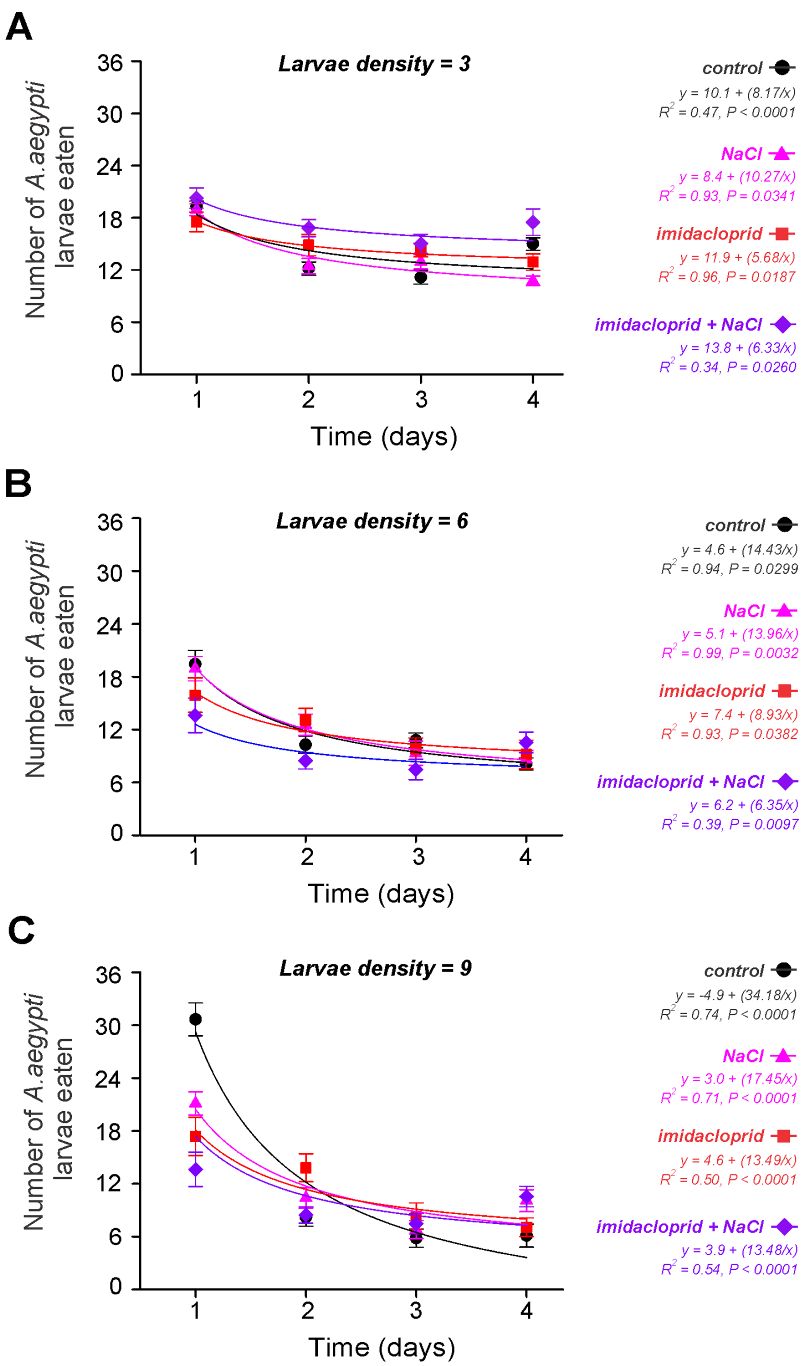

Figure 2.2: Number of *Aedes aegypti* larvae (±*SE*) consumed in four days at different densities, predated by *B. anurum* em densidade three (A), six (B), and nine (C).

2.4 Discussion

In this study, we detected that imidacloprid has an effect on the predatory ability of *Belostoma anurum*. Authors as Song et al. (1997); Alexander et al. (2007); Kreutzweiser et al. (2008); Colombo et al. (2013) make reference that although neocothionoids are one of the most used substances in the field of agriculture worldwide, which according to studies carried out in different aquatic communities has caused effects on non-target aquatic organisms.

The results about on the predatory capacity of the first day coincided with the studies carried out by Gutiérrez et al. (2017); Valbon et al. (2018) where exposed insects of the order Hemiptera to insecticides and with three larval densities of *A. aegypti* (3, 6, 9) there was greater predation of larvae for densities 6 and 9 with respect to density 3.

The less predation in the nymphs of *Belostoma anurum* in the first day for the NaCl + imidacloprid treatment with respect to the control, in the intermediate and higher density, his result may be due to the regulation of key body fluids in the freshwater organisms and when an imbalance occurs it generates a greater expenditure of energy necessary to maintain osmotic balance. Tyree et al. (2016) then it may be that when encountering ions NaCl and imidacloprid molecules, organisms reduce the energy consumption required to capture prey and focus on staying regulated.

The results about the predatory ability of *B. anurum* for the 24h in all desities studied (3, 6 and 9), can be attributed to the fact that there are various defense mechanisms of insects against insecticides, such as behavioral evasion, thickening of the cuticle, which reduces the penetration of the insecticide; increased excretion of insecticides; increased activity of detoxifying enzymes (metabolic resistance); and modifications of insecticide target sites that reduce or eliminate insecticide sensitivity

(Castañeda et al., 2011).

We found that with the passing of the days the treatment with imidacloprid presented a reduction in predatory larvae, probably due to the fact that when there is an influence of insecticides on the organisms, there are disturbances between the predator-prey interactions due to the physiological stress that these substances generate in organisms (Lefcort et al., 2000; Relyea and Mills, 2001). Afza et al. (2019) Also found that neonicotinoid exposition can produce sublethal effects in organisms and cause alterations in important functions such as predation.

The presence of imidacloprid causes a reduction in the predatory ability of *B. anurum* when it is exposed to sublethal concentrations over time, which can cause alterations within aquatic food webs. Predation plays a very important role within the structure of the community, and the negative effects caused by it in a local habitat can affect both the abundance and biomass of the prey community (Wesner, 2010). These sublethal alterations at the predators organisms of mosquitoes can generate an imbalance in the comunity and a reduction in the bioecological services produced by these predators.

2.5 Acknowledgment

This work was supported by the National Council of Scientific and Technological Development (CNPq), the Minas Gerais State Foundation for Research Aid (FAPEMIG), the Coordination for the Improvement of Higher Education Personnel (CAPES, Financial code 001)

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GENERAL CONCLUSIONS

The combination of imidacloprid with NaCl (0.5 % w/v) generated the increased susceptibility of *B. anurum*, because probably the afectation of its nervous system caused by increased sodio ions. More detailed studies to confirm the mechanism of action of Imidacloprid alone and combined with NaCl in aquatic insects would be helpful.

Sublethal doses of imidacloprid (LC_{10}) affected the predation ability of *B. anurum*, demonstrating that this insecticide can affect the trophic levels in the aquatic food chain.