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EFECTOS SUBLETALES DE INSECTICIDAS SOBRE ENEMIGOS NATURALES DEL CHANCHITO BLANCO DE LA VID

POR

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Contents

General abstract

Pest management of economic importance is mainly based on the use of synthetic insecticides. Given the appearance of environmental and toxicological problems associated with the indiscriminate use of insecticides, integrated pest management has been developed, which combines different tools, such as chemical and biological control, among others. Biological control involves the use of natural enemies, such as predators and parasitoids, to reduce populations of a target pest. In particular, biological control requires the use of selective insecticides that are compatible with the latter.

The obscure mealybug (*Pseudococcus viburni*) is a pest of economic importance in fruit trees, being the main responsible for the rejection of apples for export, whose biological control is mainly done by the parasitoid *Acerophagus flavidulus* and the predator *Cryptolaemus montrouzieri*.

The intensive use of insecticides in the control of apple tree pests may be causing negative effects not only on the target pests, such as the woolly apple aphid and the obscure mealybug, but also on the natural enemies. Insecticides produce lethal and sublethal effects on natural enemies, affecting their behavior and physiology. Thus, we were interested in determining the sublethal effects of insecticides on *Acerophagus flavidulus* and *Cryptolaemus montrouzieri*, the main natural enemies of the obscure mealybug, *Pseudococcus viburni*.

First, the acute toxicity of the insecticide growth regulators buprofezin and pyriproxyfen, the neonicotinoids acetamiprid and thiacloprid and the diamides chlorantraniliprole and cyantraniliprole was evaluated on adults of *Aphelinus mali*, parasitoid of the woolly apple aphid, *Eriosoma lanigerum*, and on adults of *A. flavidulus*, as well as on larvae and adults of *C. montrouzieri*. Subsequently, the behavior of *A. flavidulus* and *C. montrouzieri* facing increasing densities of *P. viburni* was evaluated to determine the effect of buprofezin, pyriproxyfen and acetamiprid on the parasitism of *A. flavidulus* and the functional response of *C. montrouzieri*, determining the variation of attack rate (a) and handling time (T_h). Finally, parameters associated with the development of *A. flavidulus* were estimated, such as emergence, clutch size, development time, secondary sex ratio and longevity. Regarding *C. montrouzieri*, survival and development time were estimated.

Neonicotinoid insecticides were moderately harmful to harmful on both parasitoids and harmless to slightly harmful on larvae and adults of *C. montrouzieri*. Buprofezin, pyriproxyfen and chlorantraniliprole seem to be safe to both parasitoids and predators evaluated. However, cyantraniliprole was less compatible than chlorantraniliprole for all natural enemies evaluated. Regarding parasitism of *A. flavidulus*, buprofezin and pyriproxyfen applied at the label rate induced lower sublethal effects than acetamiprid applied at a very low rate. Acetamiprid reduced parasitism but did not affect other aspects of *A. flavidulus* development, such as longevity, clutch size and secondary sex ratio. All three insecticides evaluated had detrimental effects on the functional response of *C. montrouzieri* by reducing the biological control of *P. viburni*. Both growth regulators, buprofezin and pyriproxyfen, to different extents, had a detrimental effect on the immature stages of the predator, decreasing their attack rate and/or handling time of their prey. In fact, both affected egg and larval survival, although no impact was observed on the development time of the first larval stages. Acetamiprid was very harmful to the immature stages, affecting their predation and also the survival of neonate larvae. Our results would have an adverse impact on apple integrated pest management programs, especially with growth regulator insecticides applied on immature stages of *C. montrouzieri* and the neonicotinoid acetamiprid on *A. flavidulus*, as it would only be compatible when very low residue levels are reached in the field.

Keywords: Sublethal effects, Insecticides, Neonicotinoids, Insect gorwth regulators, Toxicity, Biological control, Integrated Pest Control, Functional response, *Acerophagus flavidulus*, *Cryptolaemus montrouzieri*

Resumen general

El manejo de plagas de importancia económica se basa principalmente en el uso de insecticidas sintéticos. Dada la aparición de problemas medioambientales y toxicológicos asociados al uso indiscriminado de insecticidas, se desarrolla el manejo integrado de plagas, el cual combina diferentes herramientas, tales como el control químico y biológico, entre otras. El control biológico involucra el uso de enemigos naturales, tales como depredadores y parasitoides, para reducir poblaciones de una plaga objetivo. En particular, el control biológico requiere del uso de insecticidas selectivos que logren ser compatibles con éste último.

El chanchito blanco (*Pseudococcus viburni*) es una plaga de importancia económica en frutales, siendo el principal responsable de rechazos de manzanas de exportación, cuyo control biológico, lo efectúan principalmente, el parasitoide *Acerophagus flavidulus* y el depredador *Cryptolaemus montrouzieri*.

El uso intensivo de insecticidas en el control de plagas del manzano, puede estar ocasionando efectos negativos no tan sólo en las plagas objetivo, como pulgón lanígero y chanchito blanco, sino también en los enemigos naturales. Los insecticidas generan efectos letales y subletales sobre los enemigos naturales, afectando su comportamiento y fisiología. Así, surge el interés de determinar los efectos subletales de insecticidas sobre *Acerophagus flavidulus* y *Cryptolaemus montrouzieri*, principales enemigos naturales del chanchito blanco de la vid, *Pseudococcus viburni*.

En primer lugar, se evaluó la toxicidad aguda de los reguladores de crecimiento buprofezin y piriproxifen, de los neonicotinoides acetamiprid y tiacloprid y de las diamidas clorantraniliprol y ciantraniliprol, sobre adultos de *Aphelinus mali*, parasitoide del pulgón lanígero del manzano, *Eriosoma lanigerum* y sobre adultos de *A. flavidulus*, como en larvas y adultos de *C. montrouzieri*. Posteriormente, se evaluó el comportamiento de *A. flavidulus* y *C. montrouzieri* frente a densidades crecientes de *P. viburni*, para determinar el efecto de buprofezin, piriproxifen y acetamiprid sobre el parasitismo de *A. flavidulus* y la respuesta funcional de *C. montrouzieri*, determinándose la variación de la tasa de ataque (a) y el tiempo de manipulación (T_h). Finalmente, se estimaron parámetros asociados al desarrollo de *A. flavidulus*, como emergencia, tamaño de camada, tiempo de desarrollo, razón sexual secundaria y longevidad. En el caso de *C. montrouzieri*, la sobrevivencia y tiempo de desarrollo.

Los insecticidas neonicotinoides fueron moderadamente dañinos a dañinos en ambos parasitoides e inocuos y ligeramente tóxicos en larvas y adultos de *C. montrouzieri*. Buprofezin, piriproxifen y clorantraniliprol serían seguros, tanto para los parasitoides como para los depredadores evaluados. Sin embargo, ciantraniliprol siempre fue menos compatible que clorantraniliprol en todos los enemigos naturales evaluados. En cuanto al parasitimo de *A. flavidulus*, buprofezin y piriproxifen aplicados a la dosis de etiqueta, indujeron menores efectos subletales que acetamiprid aplicado a una bajísima dosis. Acetamiprid redujo el parasitismo pero no afectó otros aspectos del desarrollo de *A. flavidulus*, como longevidad, tamaño de camada y razón sexual secundaria. Los tres insecticidas evaluados tuvieron efectos perjudiciales en la respuesta funcional de *C. montrouzieri* reduciendo el control biológico de *P. viburni*. Ambos reguladores de crecimiento, buprofezin y piriproxifen, en diferentes grados, tuvieron un efecto detrimental sobre los estadíos inmaduros del depredador, disminuyendo su tasa de ataque y/o el tiempo de manipulación de sus presas. De hecho, ambos afectaron la supervivencia de los huevos y de las larvas, aunque no se observó ningún impacto en el tiempo de desarrollo de los primeros estadíos larvarios. Acetamiprid resultó ser muy perjudicial para los estadíos inmaduros, afectando su depredación y también la supervivencia de las larvas neonatas. Nuestros resultados tendrían un impacto adverso en los programas de manejo integrado de plagas de manzano, especialmente con los insecticidas reguladores de crecimiento aplicados en estadíos inmaduros de *C. montrouzieri* y del neonicotinoide acetamiprid sobre *A. flavidulus*, ya que sólo sería compatible cuando se alcanzasen niveles de residuos muy bajos en el campo.

Palabras claves: Efectos subletales, Insecticidas, Reguladores de crecimiento, Neonicotinoides, Toxicidad, Control biológico, Manejo integrado de plagas, Respuesta funcional, *Acerophagus flavidulus*, *Cryptolaemus montrouzieri*

1. General Introduction

1.1 Pests of economic importance in fruticulture

A fruit system of global importance, such as apple trees, is interesting to study, since it is a species with a high insecticide load during the growing season, added to the fact that it is one of the main crops in temperate zones, including Chile. In our country, this species reaches about 26.000 ha (ODEPA, 2021) distributed mainly in the central-south zone, achieving an exported production close to 590.000 tons (Pefaur, 2021).

Given the increasing demands of foreign markets, such as the private standards of retail companies (Nature's Choice, TESCO) and Resolution 2009/128/EC, which establishes that all member countries of the European Community must have integrated pest management programs in course, the issue is becoming more relevant for the agricultural industry.

The phytosanitary aspect is a priority for apple trees, since it is the third most exported species in our country, after cherries and table grapes (Pefaur, 2021). The most common pests associated with apple trees are the codling moth (*Cydia pomonella* L), San Jose scale (*Diaspidiotus perniciosus* Comstock), the woolly apple aphid (*Eriosoma lanigerum* Hausmann) and the obscure mealybug (*Pseudococcus viburni* Signoret). Until a few years ago, the last two pests were classified as secondary pests, however, given the high pressure of insecticides, they have become primary pests, forcing the adoption of continuous measures to keep them below the economic threshold.

The woolly apple aphid (WAA) (Blommers, 1994; González, 1998) is considered a cosmopolitan pest that has spread to virtually all areas of apple cultivation. The origin is unknown, but it is probably from North America (Blackman and Eastop, 2000). It has a small body, which does not exceed 2 mm; it has a covering of filamentous, waxy wooliness, which is produced by waxy glands. Its body is purple or orange, depending on the state in which it is found. This pest forms colonies on its hosts, both on the aerial part of the apple tree, as well as on the roots, excreting honeydew where fungi could form and causing damage to the roots, trunk and branches, staining leaves and fruit with honeydew secretions (González, 1984, 1998; Artigas, 1994). Aphid females hibernate in cracked places such as lignified trunks and roots and then migrate to the upper parts of the tree (González, 2001). Also they move between the trees that touch each other, colonizing a large part of the canopy, mainly the axils and shoots of the plant (Brown and Schmitt, 1994; Lordan et al., 2015).

Generally, aphids have an annual life cycle that includes several parthenogenetic generations and in North America, it has another alternate host, the American elm (*Ulmus* spp.), where it develops sexually (Blackman and Eastop, 2000). In our country, it was believed that this species was anholocyclic, but Lavandero et al. (2009), using genetic markers, suggested that *E. lanigerum* could present sexual reproduction in fields of central Chile.

On the other hand, mealybugs are another important pest of chilean apples. Mealybugs in general are small polyphagous soft-bodied insects belonging to the family Pseudococcidae. Their status as an agricultural pest is due to the different types of damage they cause to crops: i) trophic damage due to its direct feeding on plants; ii) cosmetic damage due to its presence and by the honeydew and/or sooty mould produced by its feeding; iii) transmission of toxins and viruses and iv) its quarantine status for many markets, producing a high impact on fruit exporting countries (Mathulwe et al., 2021).

Mealybugs were responsible for 45% of the rejections of fruit trade in Chile, with apple standing out as the main fruit rejected by pseudococcids (Moore, 2012). The most common species found in apples is *Pseudococcus viburni*, mostly known as the obscure mealybug (OMB). It is considered a quarantine pest in several countries, such as Mexico, Colombia, South Korea, New Zealand (Moore, 2012). The life cycle of female mealybugs consists of an egg, often laid inside a filamentous ovisac, 3 nymphal stages, and a wingless neotenic adult stage (Franco et al., 2009; Daane et al., 2012). The life cycle of the male includes 4 nymphal stages, of which the third instar is considered the prepupal stage and the fourth instar the pupal stage; followed by the fully developed adult (Borges da Silva et al., 2010; Franco et al., 2009). Reproduction can occur sexually, parthenogenetically or by facultative parthenogenesis (Borges da Silva et al., 2010; Waterworth et al., 2012). Mealybugs are dispersed by walking inside and between plants by human activities and wind. They feed in hidden areas of the host plant, a behavior that protects them from natural enemies and contact insecticide applications. Currently, the use of synthetic insecticides is the most widely used strategy to control mealybugs. However, many of the insecticides used have a low efficiency considering the cryptic habit of the pest and its waxy cover.

In general, selective insecticide applications in apple orchards are aimed at controlling the codling moth, which is the most economically important pest worldwide. However, WAA and OMB are also pests that must be controlled.

Broad-spectrum insecticides are generally used and a single or twice sprays per season can kept them below the economic threshold, but it does not always provide reliable results.

The use of biological control as the introduction of natural enemies or cultural control like the management of weeds which are reservoir of other pests and beneficial arthropods for controlling the aforementioned pests, also help to manage their populations on apple orchards. This is what growers worldwide are trying to establish, the integrated pest management (IPM), giving greater emphasis to the use of chemical insecticides selective to target pests, as well as the introduction of biological products, plant extracts and biological control with beneficial organisms (Kogan, 1998; Radcliffe et al., 2009).

A suitable mechanism for controlling important pests is to employ insecticides that are effective and compatible with natural enemies. An Integrated pest management (IPM) considers all available pest control techniques and subsequent integration of appropriate measures that discourage the development of pest populations and keep pesticides and other interventions to levels that are economically justified and reduce or minimize risks to human health and the environment. (FAO, 2021). According to the International Organization of Biological Control (IOBC), IPM is the part of Integrated Production (IP) focusing on pests, pathogens and weeds. The IP is a concept of sustainable agriculture based on agro-ecology and a system approach that aims at contributing to sustainable, resilient, profitable and robust farming systems (IOBC, 2021). Integrated pest management proposes the integration of biological and chemical control, since it has been proved that agroecosystem management based exclusively on the intensive use of chemical insecticides tends to be unstable (Jones et al., 2009; Ragsdale et al., 2011; Biondi et al., 2018).

1.2 Chemical control

As mentioned above, the use of synthetic insecticides is unsustainable on its own, but it is still the most widely used pest control method. Currently, the most frequently used insecticides in apple trees are selective, with less residual effect and new modes of action than the broad-spectrum insecticides, especially neonicotinoids, insect growth regulators and diamides.

Although neonicotinoids are increasingly restricted in several markets, especially in the EU, due to their adverse effect on bees and pollinators (Chensheng et al., 2014; Kassiotis et al., 2014; Laycock et al., 2014), they are the most widely insecticide group used to control pest nowadays (Jeshcke, 2021).

Neonicotinoids are a group of neurotoxic insecticides, specifically acetylcholine receptor antagonists (Blacquière et al., 2012; Fogel et al., 2013), which were discovered in the 1970's. Their main products are imidacloprid, thiamethoxam acetamiprid, and tiachloprid. They are easily absorbed by plants and act rapidly on sap-sucking insects. Their main mode of action is systemic (by ingestion) and very little by contact (Casida, 2018). They have high efficacy on larval stages and eggs. Given their systemic action, their specificity and low relative toxicity to mammals and the environment, they have been considered a good replacement for organophosphate insecticides in IPM systems (Fogel et al., 2013).

Insect growth regulators (IGRs) are a group of insecticides which acts as juvenile hormone mimics, disrupting and preventing metamorphosis such as pyriproxyfen (Tunaz and Uygun, 2004; IRAC, 2021) and inhibiting the chitin biosynthesis, as buprofezin.

Pyriproxyfen is a potent juvenile hormone (JH) mimic affecting the hormonal balance in insects, suppressing embryogenesis, metamorphosis and adult formation. Both pyriproxyfen and buprofezin were considered harmless to natural enemies as compared with conventional insecticides (Horowitz, 2020). The main formulations of pyriproxyfen are applied to crops by spraying. When sprayed to plants, pyriproxyfen behaves as a translaminar insecticide. This means that after absorption of the insecticide by the upper surface of the leaves, a reservoir is formed within the leaf tissues allowing a residual activity as well as a potential exposure via the lower surface of the leaves (Devillers, 2020).

By the other hand, buprofezin is thiadiazine-like compound that has both contact and fumigant activities and affects the nymph stages of the target pest without altering the adult stage (Ghanim and Ishaaya, 2011). Vapor phase and contact toxicity seem to be major factors contributing to the potency of buprofezin. In some cases a weak translaminar activity was observed, suggesting that buprofezin acts in part by ingestion (De Cock et al., 1990).

According to IRAC (2021), buprofezin belongs to Type 1 growth regulator insecticides which have an incompletely defined mode of action leading to inhibition of chitin biosynthesis in various insect species, including scale insects, plant hoppers, and whiteflies (De Cock and Degheele, 1998) and it has been very effective against two important mealybugs, *Planococcus ficus* and *Planococcus* citri. Also, this IGR is quite harmless for beneficial insects, natural enemies, fish and mammals (Ke et al., 2011). Generally, pyriproxyfen is used for lepidopteran and scale control pests and buprofezin is used for early season control of mealybugs and scales in apple orchards.

Among the newer chemical groups are anthranilic diamides newer muscular contraction disruptors, such as chlorantraniliprole and cyantraniliprole (Lahm et al., 2005, 2007; Selby et al., 2013) which activate the ryanodin receptor that controls calcium release in insect muscles. Affected individuals are paralyzed, quickly stop eating and die within one to three days. The two most relevant active ingredients are chlorantraniliprole and its analog cyantraniliprole. These type of insecticides have a toxic effect on eggs and larvae, with the main mode of action being ingestion and, secondly, topical action. These insecticides are attractive for products without cross-resistance to other known modes of action (Jeanguenat, 2013). Both molecules are recommended to control codling moth in apple orchards and had been tested in natural enemies of apple pests being more harmless chlorantraniliprole when compared to cyantraniliprole at recommended rates and they have been harmless to *Aphelinus mali*, *Cryptolaemus montrouzieri*, *Hippodamia convergens*, *Chrysoperla carnea*, among others (Mills et al., 2015; Radrigán-Navarro et al., 2021).

As it was mentioned before, IPM proposes the integration of chemical and biological control, as it has been shown that management based solely on the intensified use of chemical insecticides tends to be fragile. Thus, biological control emerges as the keystone within IPM programs.

1.3 Biological Control

Biological control is a component of an integrated pest management strategy defined as ‘the use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less damaging than it would otherwise be’ (Eilenberg et al., 2001). These living organisms can be natural enemies, such as predators, parasitoids, pathogens and competitors, which reduce populations of a target pest, where man plays an active role (Hajek et al., 2015). Although all insect species are also suppressed by naturally occurring organisms and environmental factors, with no human input (Marchiori, 2020).There are relationships which include direct and indirect interactions between populations of target organisms (used in the broadest sense to include animal pests, weeds and disease-causing organisms), biological control agents, humans and their resources. Interactions are considered direct when they involve consumption or interference competition, but indirect when they are mediated by a third species and do not involve direct contact as can occur through processes such as resource competition, apparent competition or induced resistance (Wooton, 1994). Hence, biological control is the indirect positive effect of a biological control agent on humans that is mediated by direct or indirect negative effects of that agent on populations of one or more target species (Heimpel and Mills, 2017).

1.4 Natural enemies of apple pests

Among the natural enemies of two relevant pests present in apple orchards, as the woolly apple aphid (WAA) and the obscure mealybug (OMB), parasitoids and predators stand out, respectively. The WAA has predators from Syrphidae, Chrysopidae and Coccinellidae families (Gontijo et al., 2012). The most important natural enemy of the WAA, *Aphelinus mali* Haldeman (Hymenoptera: Chalcidoidea: Aphelinidae) is an endoparasitoid from North America (Asante 1997, Mols and Boers 2001, Wearing et al., 2010; Gontijo et al., 2012). The parasitoid is about 1 mm in size and shiny black with a light yellow ring (Noyes and Valentine, 1989). It causes a chronic infestation on the WAA, with a decrease in pest population size as parasitism increases (Beers et al., 2007). Females are mostly synovigenic, their eggs developing and maturing continuously during their life cycle (Viggiani 1984; Godfray, 1994). The latter only parasitize aphids on the aerial part of trees and oviposit one or more eggs in the abdomen, only one of which hatches (Asante, 1997). This parasitoid is koinobiont, that is, it allows its host to continue growing until the parasitoid larva kills it (Godfray, 1994).

Four or five days after oviposition, and depending on whether it is fertilized, it will generate a female or male individual (Asante and Danthanarayana, 1993). Upon hatching of the parasitoid egg, a larva originates inside the aphid and feeds on the aphid, transforming into a pupa and emerging from the aphid mummy, leaving a small perforation in its abdomen (Asante, 1997).

The development of *A. mali* inside the aphid varies according to temperature: between 45-50 days in spring and 20 days in the middle of the summer season in Chile (González 1984, 2001). This parasitoid was successfully introduced into the country from Uruguay, previously brought from the United States, in 1921. The control of the WAA using *A. mali* was the first case in which farmers corroborated the advantages of biological control as a useful and economically efficient tool in Chile (Rojas, 2005).

Regarding the natural enemies of the obscure mealybug (OMB), *Pseudococcus viburni* (Signoret) (Hemiptera: Pseudococcidae) in Chile, the parasitoid *Acerophagus flavidulus* (Brethés) (Hymenoptera: Encyrtidae) is the most relevant (González, 2011; Charles, 2011; Daane et al., 2008). *Acerophagus flavidulus* is a specialist, gregarious, koinobiont endoparasitoid of the family Encyrtidae native to Argentina and Chile (Karamaouna and Copland, 2000, 2008; Noyes, 2003). This is a small wasp that possesses remarkable ability to locate obscure mealybugs in crevices under bark, being able to oviposit on small individuals up to females (Karamaouna and Copland, 2000). In Chile, it is the most frequent and important natural enemy of the OMB (Prado et al., 2000).

Parasitized mealybugs lose mobility and acquire a yellowish color. Parasitoids emerge from these mummies through small circular holes in the body, leaving an empty yellowish structure that is very fragile to the touch (Ripa et al., 1992). Its biological cycle develops between 12 and 15 days at 25°C average.

As for the most important predators of *P. viburni*, *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) is the most relevant. This predator is native to Australia (Mani, 2018) and has been used in augmentative releases against mealybugs in many crops and countries (Kairo et al., 2013; Mani, 2018). In Chile, it was introduced from US in 1931 and later, from Australia, in 1995-1996 (Rojas, 2005), not only to control *P. viburni*, but also to control diverse mealybugs.

Both larvae and adults are voracious feeders, which prey on all stages of the mealybug hosts. Adult *C. montrouzieri* locate their prey using visual and chemical stimuli and larvae perceive prey only when there is actual physical contact. The wax secretions and honeydew produced by hosts mealybugs act as attractants as well as oviposition stimulants for *C. montrouzieri* (Kairo et al., 2013).

A sex ratio of 1:1 is common and adults have a pre-mating period of 5–7 days. Females mate repeatedly throughout their life and may receive spermatozoa from 3 to 4 males at a time. Frequent multiple matings help keep the population of *C. montrouzieri* genetically diversified (Kauffman, 1996). Adult longevity ranges between 50 and 110 days under controlled temperature (25–30°C). The average fecundity of *C. montrouzieri* is 211 eggs per female, although a maximum fecundity of 500 eggs per female has been reported (Kairo et al., 2013). It is a very good biological control of the obscure mealybug with frequent releases in apple orchards.

The aforementioned beneficial insects are not exempt from the effects of synthetic insecticides that are applied every season in apple orchards. When receiving these applications or their drift, even when they are not the target, these natural enemies may suffer consequences, such as lethal and sublethal effects after an application of chemical control in the orchard.

Direct and indirect effects of insecticides on beneficial arthropods have been reported, mainly on natural enemies and pollinating agents (Desneux et al., 2007). Thus, to develop effective IPM strategies, using both natural enemies and selective insecticides, the secondary effects of the latter on natural enemies must be considered (Desneux et al., 2007; Stark et al., 2007).

1.5 Lethal effects of insecticides on natural enemies

For years, the classical method to estimate the side effects of insecticides on natural enemies, both parasitoids and predators, has been the determination of the median lethal dose (LD_{50}) or the median lethal concentration (LC_{50}), taking into account only the lethal effects of insecticides at the individual level (Desneux et al., 2007, Hanson and Stark, 2012).

Lethal effects of insecticides have been reported on parasitoids, as is the case of *A. mali,* where neonicotinoids such as imidacloprid and thiacloprid caused higher mortality compared to diamide-type insecticides (Cohen, 1996; Gontijo, 2011; Ateyat et al.,, 2012; Mills et al., 2016, Ebadollahi et al., 2020).

In the case of other aphelinids, such as *Aphytis melinus*, Biondi et al. (2015), observed mortality of less than 40%, and in *Encarsia formosa*, pyriproxyfen and buprofezin were found to be innocuous (Wang et al., 2016).

Concerning parasitoids of the family Encyrtidae, such as *Anagyrus kamali*, imidacloprid caused 100% mortality compared to 18 and 21% mortality produced by pyriproxyfen and buprofezin, respectively (Juarez-Maya et al., 2020). Suma and Mazzeo (2008) found similar results in *Anagyrus pseudococci* with pyriproxyfen and buprofezin and Mgocheki and Addison (2009), in *Anagyrus* sp. nov. near *pseudococci*, resulting innocuous for both parasitoids, according to the toxicological classification of the IOBC.

In *Cryptolaemus montrouzieri*, pyriproxyfen caused low mortality to the predator (El Aalaoui et al., 2021). However, applications of imidacloprid were much more toxic than pyriproxyfen; buprofezin was found to be harmless on *C. montrouzieri* after 24 hours.

The lethal effects are those effects at the level of the individual, which are based on the average lethal dose (LD_{50}) which correspond to (*i*) a known amount of a toxicant (insecticide) per kilogram of weight of the individual (mg insecticide/kg body weight) or (*ii*) amount of a toxicant (insecticide) per animal (mg insecticide/animal). In both cases, the amount received by the animal (insect) is known.

By the contrary, the mean lethal concentration (LC_{50}) corresponds to the amount of a toxicant in an environmental medium, such as water, soil or air, and the amount that actually penetrates the body of the insect or animal is not known. Both parameters ultimately measure the concentration or dose of a toxicant that is capable of killing 50% of the exposed individuals in a population (Stark and Banks, 2003).

However, the latter can induce multiple sublethal effects on those individuals that survive exposure to a certain insecticide (Desneux et al., 2007), and can have a strong impact on the population dynamics of natural enemies (Stark and Banks, 2003).

1.6 Sublethal effects of insecticides on natural enemies

These are the effects on the behavior and physiology of organisms that manage to survive exposure to insecticides under a certain dose/concentration ratio (Desneux et al., 2007). Sublethal doses involve (*i*) physiological effects: the rate of development of different stages of the beneficial insect, adult longevity, immune system, fecundity and sex ratio, among others (Desneux et al., 2007; Suma et al., 2009; Biondi et al., 2015) and (*ii*) behavioral effects: alterations in mobility, orientation towards the pest or food source, feeding and oviposition (Desneux et al., 2007; Biondi et al., 2012), among others.

1.6.1. Development effects

Regarding the effect of sublethal doses, Gontijo (2011) had reported the effects of a sublethal dose of 0.1x of the maximum recommended dose for codling moth of chlorantraniliprole cyantraniliprole (anthranilinic diamide)., which reduced the population of the parasitoid *A. mali* by less than 25%. For the parasitoid of the OMB, *A. flavidulus,* the neonicotinoid acetamiprid at the reduced concentration of 0.005x of the label rate, caused certain sublethal effects on this parasitoid, reducing its parasitism (Chapter 2, Table 1). This could be due to the fact that acetamiprid is neurotoxic and since it acts by ingestion and contact, the effect on the adult may have been more acute during the time it had contact with the insecticide residues, inducing a reduction of its ability to parasitize *P. viburni*. For an encyrtid parasitoid of the citrus mealybug, *Planococcus citri*, *Leptomastix dactylopii* (Howard) another formulation of acetamiprid (SG) at its label rate showed more detrimental effects on parasitism and emergence rate, but not on sex ratio of the progeny (Cloyd and Dickinson, 2006) than acetamiprid at a wettable powder (WP) formulation.

Honeydew excreted by aphids feeding on soybean plants reared from neonicotinoid coated seeds did not result toxic to the parasitic wasp *Aphelinus glycinis* but reduced the longevity of *Aphelinus certus* (Calvo-Agudo et al., 2021).

Speaking about IGRs, laboratory studies testing the effect of exposure to pesticide residues on leaves of sprayed citrus showed that buprofezin did not alter the progeny production of the parasitoid *Leptomastix dactylopii,* but adversely affected *Anagyrus pseudococci* progeny (Campos et al., 2008), both parasitoids of the citrus mealybug, *Planococcus citrus*. This situation was completely different when buprofezin was applied to mummies of *Planococcus ficcus* parasitized by *Anagyrus* sp. near *pseudococci* (Girault) and *Coccidoxenoides perminutus* (Timberlake), where the development time was longer (Mgocheki and Addison, 2009).

Regarding the predator, *C. montrouzieri*, sublethal effects has been seen with a reduced concentration of 10% of acetamiprid on *C. montrouzieri* eggs, which did not affect the hatching of first instar larvae; however, the survival of neonate larvae was practically absent (Chapter 3, Table 3). Even though neonicotinoids exhibit the same mode of action targeting the nicotinic acetylcholine receptor, ovicidal activity and effects on embryos is variable between compounds of this chemical group (Hoffmann et al., 2008). Youn et al. (2003) observed no larvae emergence when dipping the eggs of the predator *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae) in acetamiprid and imidacloprid, while another insecticide belonging to this family, thiametoxam, did not cause any deleterious effect. This may have due to the mode of action of acetamiprid which is systemic with translaminar activity having both contact and ingestion action (Lewis et al., 2016). However, studies conducted by Fogel, 2013, on another aphid predatory coccinellid, *Eriopis connexa*, showed that a rate of 50% acetamiprid induced a lengthening of the embryogenesis of the predator eggs.

When *C. montrouzieri* larvae either treated with pyriproxyfen or fed with treated prey reached the pupal stage but most of them died during the metamorphosis and those that survived were sterile (Planes et al., 2013). Compounds developed to disrupt metamorphosis- juvenile hormone analogs, as pyriproxyfen, ensure that no reproductive adults are formed or development of adults with certain morphogenetic abnormalities that reduce their reproductive potential. Adults may be sterile or possess abnormally developed genitalia, which hinders the mating process or the capacity to produce fertile offspring (Masih and Ahmad, 2019).

The beginning periods of metamorphosis and embryogenesis in insects were the most sensitive to juvenile hormone analogs (JHAs), thus embryogenesis was disrupted when young eggs were treated with JHAs (Tunaz and Uygun, 2004).

Pyriproxyfen was harmless to second and third instar of *C. montrouzieri* (Radrigán- Navarro et al., 2021) and to eggs, it could be classified as slightly harmful at 10% of the recommended label rate. Cabral et al. (2008) reported on other coccinellid predator, *Coccinella undecimpunctata,* that survival of first and second instars was reduced when eggs were treated with buprofezin. In a further study on the predatory coccinellid, *Harmonia axyridis*, He et al. (2018) observed that eggs exposed to concentrations of buprofezin recommended for field and greenhouse pest control reduced embryonic and larval survival on *H. axyridis.*

By the other hand, Barbosa et al. (2018) found that females of *Tenuisvalvae notata* (Mulsant) (Coleptera: Coccinellidade) exposed to pyriproxyfen residues laid non-viable eggs for 4-5 days after 12 or 24 h the cessation of exposure. There were not differences in fecundity with lufenuron and pyriproxyfen, but egg viability was reduced with pyriproxyfen residues (Barbosa et al., 2018). Likewise, pyriproxyfen was moderately harmful to eggs of *Harmonia axyridis* Pallas (He et al., 2018) and deformed eggs of the F1 generation of *H. axyridis* treated with pyriproxyfen were observed by Ghanim and Ishaaya (2011). The aforementioned indicates differences between rates, formulations and spray methodologies among laboratory studies on coccinellid species. Regarding the other IGR, pyriproxyfen is an effective juvenile hormone analogue (JHA) that affects the hormonal balance in insects and suppresses egg-hatching and adult formation (Ghanim and Ishaaya, 2011; Ishaaya et al., 1994).

Regarding to the secondary sex ratio (male/female ratio), two main causes have been detected which would alter this ratio in offspring of adults exposed to insecticides: (*i*) effect on egg fertilization and (*ii*) the survival rate of the sexes when exposure to insecticides is prior to the adult stage. Both fecundity and sex ratio can be altered by insecticides because they can produce deformations in the ovaries and testes of their offspring (Schneider et al.,2004).

In biological control, most studies on oviposition have proven the direct relationship between oviposition and parasitism rate, with consequent regulation and efficient pest control (Desneux et al., 2007).

In this study, parasitism was not affected by buprofezin, but it was reduced with residues of pyriproxyfen at the density of six obscure mealybugs evaluated on adults of *A. flavidulus* (Chapter 2, Table 1).

Similar results were reported in *Leptomastix dactylopii* (Howard), parasitoid of the citrus mealybug, *Planococcus citri*, where parasitism was reduced with pyriproxyfen at the label rate (Rothwangl et al., 2004; Cloyd and Dickinson, 2006). By the contrary, buprofezin applied at the label rate onto adults of *L. dactylopii* decreased parasitism and emergence rate (Cloyd and Dickinson, 2006).

Changes in parasitism by the parasitoid have been determined as the density of a pest increases. This phenomenon is called functional response, which represents the change in the number of hosts parasitized or predated by a parasitoid/predator in response to a change in host/prey density (Fernández-Arhex et al., 2004) The functional response was studied by Solomon (1949) and, later Holling (1959, 1966) developed the mathematical model (three types of functional response). In type I, the relationship between host density and number of host parasitized is linear, whereas in type II, the response curve rises in a negatively accelerating manner to a plateau. In type III the response curve rises in a sigmoid manner to a plateau.

The oviposition behavior of specialist natural enemies, such as parasitoids or specialist predators, is determined as a type II functional response, in which host mortality related to oviposition, decreases with host/prey density (Holling 1959). It has been reported that insecticides could affect the attack rate (a) and handling time (T_h) exerted by natural enemies against their host/prey, decreasing the biological control of pests (Umoru and Powell 2002; Řezáč et al., 2010; Gholamzadehchitgar et al., 2014; Reegan et al., 2020; El Aalaoui et al., 2021). Therefore, the interest of studying the possible effects of sublethal concentrations of neonicotinoid and insect growth regulator insecticides on the functional response of natural enemies was raised, in order to describe if indeed, these sublethal concentrations could alter the biological control carried out by beneficial arthropods and to see if they really are compatible or not with chemical control.

The attack rate and handling time- parameters of the functional response of the parasitoid *Lisiphlebus fabarum* exposed to a sublethal concentration of imidacloprid were decreased compared to the control (Jam et al., 2018). In predatory coccinellids such as *Serangium japonicum* and *Coccinella semptenpunctata*, neonicotinoid insecticides, such as imidacloprid and thiamethoxam, decreased the voracity of the predator and decreased the attack rate and increased the handling time of the latter, respectively (Skouras et al., 2017; Afza et al., 2021).

Something similar was observed in *Harmonia axyridis*, when exposed to LC_{50} of thiamethoxam, decreasing its predation. With a high concentration of imidacloprid, the predator decreased its attack rate and increased handling time (Dai et al., 2020).

On the other hand, El Aalaoui and Sbaghi (2021) tested a sublethal dose of pyriproxyfen on the functional response of *C. montrouzieri,* finding a type II response, which did not affect adults, but they did obtain a lower attack rate and a high handling time. A similar situation was reported for buprofezin on another predatory coccinellid, *Coccinella semptenpunctata*, which decreased its attack rate and raised handling time (Sultan et al., 2019).

Finally, Rasheed et al. (2020) found that the feeding potential of larvae and adults of *H. axyridis* was significantly reduced when third instar larvae were treated with chlorpyrifos.

1.6.2. Behavior effects

In general, the mobility of beneficial arthropods is assessed according to direct insecticide intoxication as a result of a knock-down effect, uncoordinated movements, tremors and abdominal retraction (Desneux et al., 2007). Tappert et al. (2017) showed that female parasitoids treated with the neonicotinoid imidacloprid lost their ability to locate hosts by using olfactory cues. Limited mobility may also deter adult feeding, crucial for energy acquisition, longevity and reproduction of many synovigenic parasitoid species (Jervis et al., 2008; Tang et al., 2014; Zhang et al., 2014).

Lately, secondary effects have been observed in beneficial insects, such as the disruption in the detection of kairomones (semiochemicals produced by one organism that provoke a reaction in another organism of a different species, which is favorable to the recipient and not to the emitter), generally to warn of the presence of a food source, host or prey (Delpuech et al., 2005). In addition, a repellent or irritant effect of insecticides has been reported, which induces insects to move away from treated areas, increasing their mobility, which is not necessarily associated with an increase in the efficiency of the natural enemy, as in C. *septempunctata* in deltamethrin treated plots compared to untreated plots up to three days after the spray application (Wiles and Jepson, 1994) and in *H. axyridis,* another coccinellid exposed to sublethal dose of beta-cypermethrin promoted an increase in walking rate, walking distance and walking duration (Xiao et al., 2017).

As mentioned above, the study of the potential sublethal effects associated with development and behavior of neonicotinoid, growth regulators on the parasitoid *A. flavidulus* and the predator *C. montrouzieri*, has not been studied deeply. Hence the potential effects that acetamiprid and the IGRs, buprofezin and pyriproxyfen could exert on both beneficial arthropods and their potential impact on the biological control of the obscure mealybug remain unknown.

The first step is to assess the acute toxicity of lethal (minimum field recommended rate) and sublethal (dilutions from minimum field recommended rate) concentrations of six insecticides frequently used for the main apple pests in pome fruit orchards in Chile, on adults of the parasitoids *A. mali* and *A. flavidulus*, and also on larvae and adults of the predator *C. montrouzieri.*

After determining the sublethal concentrations for these natural enemies, their effect on developmental and behavioral variables will be evaluated. For the parasitoid, *A. flavidulus*, the parasitism, emergence rate, clutch size, development time, longevity and secondary sex ratio on to increasing densities of their host, *P. viburni* will be evaluated under laboratory conditions.

Finally, we will determine sublethal effects of the neonicotinoid acetamiprid and two insect growth regulators, buprofezin and pyriproxyfen on the immature stages (eggs and third instar larvae) of *C. montrouzieri* in topical and residual application laboratory tests. The effects of these insecticides on the functional response on the third instar of *C. montrouzieri* on *P. viburni* nymphs will be assessed. Besides, it will be determined how residues of these insecticides will affect the survival and development of *C. montrouzieri* eggs.

This research will provide the necessary data to build integrated pest management programs of pome fruit orchards, attempting to optimize programs that combine the use of natural enemies with chemical insecticides, knowing the phenology of natural enemies, controlling the target pest without harming the beneficial arthropods, such as *A. flavidulus* and *C. montrouzieri*, allowing sustainable pest control.

General Hypothesis

A sublethal concentration of two growth regulator insecticides, buprofezin and pyriproxyfen and the neonicotinoid acetamiprid, will decrease parasitism and predation, as well as the development of natural enemies of the obscure mealybug, *Pseudococcus viburni*.

Specific hypotheses

1. Growth regulator insecticides and anthranilinic diamides cause lower mortality than neonicotinoid insecticides in natural enemies of the obscure mealybug and woolly apple aphid.
2. Growth regulator insecticides buprofezin and pyriproxyfen have no effect on parasitism and development of *Acerophagus flavidulus* while the neonicotinoid acetamiprid, reduces parasitism and delays development of *A. flavidulus*.
3. Growth regulator insecticides buprofezin and pyriproxyfen and the neonicotinoid acetamiprid have an effect on the functional response type II of *Cryptolaemus montrouzieri* larvae.
4. Sublethal doses of growth regulator insecticides buprofezin and pyriproxyfen and the neonicotinoid acetamiprid, do not allow the predator *Cryptolaemus montrouzieri* to complete its development.

Specific objectives

1. To determine the acute toxicity of lethal and sublethal concentrations of growth regulator insecticides, neonicotinoids and diamides in adults of parasitoids *Aphelinus mali* and *Acerophagus flavidulus* and in larvae and adults of *Cryptolaemus montrouzieri*.

2. To describe the functional response type of *C. montrouzieri* treated with a sublethal dose of buprofezin, pyriproxyfen and acetamiprid facing increasing densities of obscure mealybugs.

3. To determine the effect of a sublethal dose of buprofezin, pyriproxyfen and acetamiprid on the parasitism, emergence rate, clutch size, longevity, development time and secondary sex ratio of *A. flavidulus* facing increasing densities of obscure mealybugs.

4. To determine the effect of a sublethal dose of buprofezin, pyriproxyfen and acetamiprid on the survival and development time of *C. montrouzieri*.

It is worth to mention that the general hypothesis involves natural enemies of the obscure mealybug (OMB) and the impact of lethal and sublethal concentrations of neonicotinoid and insect growth regulator insecticides.

Each specific hypothesis turns out into a new chapter (manuscript) of this PhD thesis.

The first chapter includes studies on the acute toxicity of lethal and sublethal concentrations of neonicotinoid, insect growth regulator and diamide insecticides on natural enemies of the woolly apple aphid and the obscure mealybug.

The parasitoid of the WAA, *Aphelinus mali* was included in the first chapter cause it was part of my first PhD thesis project and results were interesting to be published on a scientific journal. Then, the committee members accept a new thesis which just envolved the obscure mealybug natural enemies, *Acerophagus flavidulus* and *Cryptolaemus montrouzieri*.

After the first chapter, the thesis is going to refer just to the impact of sublethal concentrations of neonicotinoid and insect growth regulators insecticides on natural enemies of the obscure mealybug (OMB), *A. flavidulus* and *C. montrouzieri*.

The second chapter is about sublethal effects of neonicotinoid and insect growth regulator insecticides on *Acerophagus flavidulus,* parasitoid of the obscure mealybug, *Pseudococcus viburni.*

Finally, the third chapter concerns the sublethal effects of insecticides on the functional response, survival and development of the coccinellid predator, *Cryptolaemus montrouzieri.*

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Chapter 1

Acute toxicity of lethal and sublethal concentrations of neonicotinoid, insect growth regulator and diamide insecticides on natural enemies of the woolly apple aphid and the obscure mealybug.

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Chapter 2

Sublethal effects of neonicotinoid and insect growth regulator insecticides on *Acerophagus flavidulus*, parasitoid of the obscure mealybug, *Pseudococcus viburni*

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Sublethal effects of neonicotinoid and insect growth regulator insecticides on *Acerophagus flavidulus*, parasitoid of the obscure mealybug, *Pseudococcus viburni*

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Abstract

Insecticides used to control apple pests interfere with beneficial arthropods.This study determined the sublethal effects of three insecticides, IGRs - buprofezin and pyriproxyfen - and the neonicotinoid acetamiprid used in apple orchards on adults of *Acerophagus flavidulus* (Brethés), parasitoid of the obscure mealybug, *Pseudococcus viburni* (Signoret) *A. flavidulus* was exposed to insecticide residues of the minimum recommended rate of buprofezin and pyriproxyfen (1x) and 0.005x of acetamiprid on apple leaves under laboratory conditions. Each female parasitoid was in contact with insecticide residues for 24 h and then allowed to parasitize three mealybug densities (2-4-6) per parasitoid for 24 h. Parasitism, emergence rate, clutch size, development time, longevity and secondary sex ratio were evaluated under each insecticide treatment and mealybug density. Growth regulators, such as buprofezin and pyriproxyfen, applied at the labeled rate (1x), induced less sublethal effects than acetamiprid applied at a low rate (0.005x) on *A. flavidulus*. Pyriproxyfen and acetamiprid reduced parasitism but they did not affect other aspects of development such as emergence rate, clutch size, development time, longevity and secondary sex ratio. Our data suggest that buprofezin and pyriproxyfen are more compatible with *A. flavidulus* than acetamiprid, which could be integrated with parasitoid activity only when reaches low residue levels in the field.

Key words Behavior, development, parasitism, integrated pest management

Introduction

Apple is an important fruit crop in many regions around the world, including central Chile. Problems caused by phloem-feeding pests, such as the obscure mealybug *Pseudococcus viburni* Signoret (Hemiptera: Pseudococcidae), have become increasingly important due to their quarantine status in various countries (González 2011). In apple orchards, regular applications of broad-spectrum insecticides may be the key factor explaining the limited success of some natural enemies. The use of more selective and reduced-risk insecticides is a relevant strategy to improve the integration of chemical and biological control, enhancing the latter, as the basis for integrated pest management (IPM) (Jones et al. 2009; Weddle et al. 2009). Insecticides used to control codling moth and other pests in apple orchards can disrupt biological control (Beers et al 2016; Radrigán-Navarro et al. 2021). Some of these are wide-spectrum neurotoxic insecticides, such as the neonicotinoids thiacloprid and acetamiprid (Casida 2018) while other are more selective insect growth regulators (IGRs), such as buprofezin and pyriproxyfen (Tunaz and Uygun 2004). Among the natural enemies of *P. viburni* in Chile (González 2011; Charles 2011), the parasitoid *Acerophagus flavidulus* Brethés (Hymenoptera: Encyrtidae) is the most important and efficient. *A. flavidulus* is a specialist, gregarious, koinobiont endoparasitoid native to Argentina and Chile (Karamaouna and Copland 2000; Noyes 2003). To develop effective IPM strategies, using both natural enemies and selective insecticides, the secondary effects of the latter on natural enemies must be considered (Stark et al. 2007; Desneux et al. 2007). Sublethal effects could induce multiple reactions on those individuals that survive exposure to a certain insecticide (Desneux et al. 2007). These sublethal effects could affect their behavior and development (Desneux et al. 2004, 2006, 2007). On encyrtid parasitoids of mealybugs sublethal effects have been reported affecting their parasitism, emergence rate, development time and longevity (Rothwangl et al. 2004; Cloyd & Dickinson 2006; Mgocheki and Addison 2009; Suma et al. 2009; Karmakar and Shera 2018).

The aim of the study is to provide the necessary data to build integrated pest management programs of pome fruit orchards. We exposed the parasitoids to insecticide residues on apple leaves under laboratory conditions, in order to evaluate the parasitism, emergence rate, clutch size, development time, longevity and secondary sex ratio on to increasing densities of their host, *P. viburni*.

Material and Methods

Insect material

Colonies of *P. viburni* were reared in butternut squash (*Cucurbita moschata* D.) inside plastic containers with orifices and blue cloth on its top for ventilation and to avoid mealybug escapes. These containers were kept in a room chamber with controlled conditions at 23± 5 °C, 50 ± 10 % RH and in complete darkness.

The parasitoid *A. flavidulus* was reared at Xilema SpA, a biological control company located in Quillota, Chile. Parasitoid mummies were sent to our laboratories and were maintained in a CONVIRON® chamber at 25 ± 0.1 °C, 50 ± 5 % RH and a photoperiod of 16/8 L/ D until the emergence of adults. A drop of 125 *u*L of a diet (organic honey, water and agar) was given on each petri dish to feed the newly emerged parasitoids for 2 to 3 days and to allow mating.

Application of insecticides

The IGR buprofezin (Applaud™ 25 WP, Anasac, Chile) was used at the minimum label rate for obscure mealybug on apples (80 $g{\cdot}L^{-1}$) and pyriproxyfen (Delico™ 100 EC, Anasac, Chile) was applied at the label rate for codling moth on apples (50 $ml{\cdot}L^{-1}$). A neonicotinoid insecticide, acetamiprid (Hurricane 75 WP, Anasac, Chile) at 0.005x of the label rate (0.6 $mg{\cdot}L^{-1}$) to control *P. viburni* on apples was tested too. These concentrations were defined as ´sublethal´ concentrations in previous studies (Radrigán-Navarro et al. 2021). A volume of 2 mL of each insecticide concentration was applied at 4.5 kPa with a Potter Precision Laboratory Spray Tower (BURKARD SCIENTIFIC, UK) on an apple leaf disk with the lower surface facing up on a pad of moist cotton inside a 30 mL plastic cup (Beers et al. 2009). Distilled water was used as a control.

Once the treated surface dried, one adult of *A. flavidulus,* which were ≤ 72 h old, was placed in the bioassay arena, which had a lid with a small orifice (≈ 5 mm in diameter) on the top covered by Micropore™ tape (3M®, US). Every lid contained 125 *u*L of a diet for parasitoids. Plastic cups were maintained in a CONVIRON® chamber with the same conditions previously described for parasitoid mummies. After this period, each parasitoid was placed in a 90 mm diameter plastic petri dish which contained 2, 4 and 6 third instar nymphs of *P. viburni*.

During 24 hours, the parasitoid could oviposit on to the *P. viburni* offered.

After this time, the parasitoid was killed to observe its terminalia under a stereoscopic magnifying glass to identify the presence of ovipositor. Sexing after the experiment was performed because it was difficult to manipulate and distinguish females before the experiment without producing damage. Petri dishes containing *P. viburni* were sealed again and maintained under the same controlled conditions in the chamber. The parasitism rate (number of mummies per number of mealybug nymphs offered) was measured at day 14 and mummies were individually separated in 0.5 mL Eppendorf tubes awaiting the emergence of parasitoids. In the lid of each Eppendorf tube, a 125 *u*L of diet was placed for feeding the newly emerged parasitoids. The sublethal effects were evaluated daily until the death of all individuals. Emergence rate (number of emerged mummies per number of mummies), clutch size (number of emerged parasitoids per mummy) and development time (days from oviposition to adult emergence) were measured. Newly emerged parasitoids were separated into new Eppendorf tubes to determine their longevity and secondary sex ratio. The experimental unit was one female of *A. flavidulus* in a fixed density of *P. viburni* nymphs. For each treatment and mealybug density, between 27- 54 experimental units were used. These whole bioassays were replicated three times.

Statiscal analysis

Generalized linear models (GLM) with binomial distribution were used to analyze data of parasitism rate, emergency rate and secondary sex ratio and Poisson distribution was used to analyze clutch size. Development time and longevity were analyzed using a two-way ANOVA, because the assumptions of homoscedasticity and normality were fulfilled. Effects of insecticides, mealybug densities and their interaction were evaluated. If significant differences ($p < 0.05$) were found, they were evaluated with multiple comparisons Tukey test, using the “multcomp” and “emmeans” R packages. All analyses were performed using the statistical software R (R Development Core Team 2020).

Results

For the parasitism rate a significant interaction was found between insecticide treatment and mealybug density (χ^2 = 12.66, df = 6, 11; P ≤ 0.05). Parasitism rate at the host density of 6 mealybugs was lower for pyriproxyfen and acetamiprid in relation with the control (Table 1). The main effects of insecticide treatment (χ^2 = 37.62, df = 3, 11, P ≤ 0.001) and mealybug density (χ^2 = 15.46, df = 2, 11; P ≤ 0.001) were significant too.

For the emergence rate also the interaction between insecticides and mealybug density was significant (χ^2 = 21.46, df = 6, 11; P ≤ 0.05), but no significant differences between combinations of treatment were detected by the Tukey test (Table 1). The main effects of insecticide treatment (χ^2 = 6.81, df = 3, 11; P = 0.08) and mealybug density (χ^2 = 5.41, df = 3, 11; P = 0.07) were not significant.

For the clutch size the insecticide treatment was significant (χ^2 = 19.75, df = 3, 11, P ≤ 0.001), with buprofezin showing a higher clutch size compared with acetamiprid and the control (Table 1). Similarly, the clutch size was significantly affected by mealybug density (χ^2 = 6.26, df = 2, 11; P ≤ 0.05). Density of six mealybugs increased the number of emerged parasitoids per mummy in relation to the density of four, but not two mealybugs (Table 1). No interaction between insecticide treatment and mealybug density was found (χ^2 = 5.83, df = 6, 11; P = 0.44).

For the development time only the insecticide treatment was significant (F = 3.25, df = 3, 11, P ≤ 0.05), with buprofezin showing a shorter development time compared with the control (Table 2). No effects of density of mealybugs (F = 2.45, df = 2, 11, P = 0.09) or interaction between main factors was found (F = 1.51, df = 6, 11, P = 0.18).

The longevity was not affected by the insecticide treatment (F = 1.42, df = 3, 11; p = 0.24) nor by mealybug density (F = 1.92; df = 2, 11; p = 0.15); and no interaction between main factors was found (F = 0.47, df = 6, 11; p = 0.83) (Table 2).

There was no significant effect of the insecticide treatments (χ^2 = 0.38, df = 3, 11; P =0.095) neither interaction (χ^2 = 9.17, df = 6, 11; P =0.16) on the secondary sex ratio. However, the mealybug density was significant (χ^2 = 8.04, df = 2, 11; P ≤ 0.05), with densities of six and two mealybugs showing lower number of males compared to the intermediate density of four mealybugs (Table 2).

Discussion

It had been reported that IGRs at the label rate are slightly harmful to *A. flavidulus* (Radrigán-Navarro et al. 2021). Nevertheless, the parasitism was not affected by buprofezin, but it was reduced with residues of pyriproxyfen at the highest mealybug density evaluated. Similar results were reported in *Leptomastix dactylopii* (Howard), parasitoid of the citrus mealybug, *Planococcus citri,* where parasitism was reduced with pyriproxyfen at the label rate (Rothwangl et al. 2004; Cloyd and Dickinson 2006). By the contrary, buprofezin applied at the label rate onto adults of *L. dactylopii* decreased parasitism and emergence rate (Cloyd and Dickinson 2006). This could be explained by the different formulation (40 SC vs 25 WP) and water volume (0.8 mL vs 2 mL) used in our study, that resulted in a lower amount of active ingredient per parasitoid. As it was mentioned by Suma et al. (2009), the highest label rate of buprofezin and pyriproxyfen sprayed on adults of *L. dactylopii* did not affect the emergence rate of survivor females exposed to these insecticides for 72 h. In our study, buprofezin, besides being innocuous on parasitism, emergence rate and clutch size, the development time on its host *P. viburni* was significantly shorter than in the control. This situation was completely different when the same insecticide was applied to mummies of *P. ficcus* parasitized by *Anagyrus* sp. near *pseudococci* (Girault) and *Coccidoxenoides perminutus* (Timberlake), where the development time was longer (Mgocheki and Addison 2009).

It is important to mention that parasitism was always lower as host density increased, which may be due to the fact that at low densities of *P. viburni*, the parasitoid did not have to make great efforts to parasitize it; however, the effect of host density only begins to be observed at the highest density, which would be interesting to continue evaluating to confirm if the parasitism rate continues to decrease as the pest increases, which would have an effect on the biological control of this pest at high densities.

The effect of the sub-lethal concentrations of insecticides evaluated was relatively low, in terms of parasitism rate, since the individuals that had contact with the residues were the females and not the larvae (immature stages inside the mummy), which is why it is thought that it did not affect other developmental parameters, such as the viability of eggs and larvae.

In all studies previously mentioned, the sex ratio was not affected by either IGRs, as we have reported.

On the other hand, acetamiprid was harmful to *A. flavidulus* in terms of acute toxicity even at 0.1x of the label rate (Radrigán-Navarro et al. 2021). However, at the reduced concentration tested of 0.005x of the label rate, it caused certain sublethal effects on this parasitoid, reducing its parasitism, especially at the highest mealybug density tested. This could be due to the fact that acetamiprid is neurotoxic and since it acts by ingestion and contact, the effect on the adult may have been more acute during the time it had contact with the insecticide residues, inducing a reduction of its ability to parasitize *P. viburni*. It has been shown that the uptake of neonicotinoids may impair the host finding ability of females of *Microplitis croceipes* (Cresson), parasitoid of the lepidopteran pest, *Helicoverpa zea*, which occur after they feed on extrafloral nectar from cotton plants (Stapel et al. 2000).

Besides, females of the encyrtid *Avetianella longoi* (Siscaro), parasitoid of the cerambycid *Phoracantha semipunctata* which were fed with accumulated imidacloprid in the nectar of treated eucalyptus trees, had a significantly lower rate of parasitization (Paine et al. 2011). For *L. dactylopii*, another formulation of acetamiprid (SG) at its label rate showed more detrimental effects on parasitism and emergence rate, but not on sex ratio of the progeny (Cloyd and Dickinson 2006).

In an integrated pest management, it is important to consider the insecticide to be used, its formulation, the stage of development of the parasitoid, since these factors will determine their sublethal effects at the level of parasitism, emergence rate, clutch size, development time, longevity, or sex ratio. Considering the above, IGRs at the minimum label rate would be more compatible with *A. flavidulus* than the neonicotinoid acetamiprid, especially buprofezin. This could be due to the rapid dissipation of the latter in apples, as demonstrated by González (2009) with the same commercial product. Thus, buprofezin would be compatible with this parasitoid one day after treatment. Our results suggest that even with an acetamiprid concentration of 0.005x, parasitism of *A. flavidulus* is affected. Therefore, parasitoids should be released once chemical residues are below sublethal levels. Considering the natural degradation of acetamiprid in apples and pears (González 2009; Galietta et al. 2011; Lazić et al. 2019) a period of 20-25 days after chemical spraying of the insecticide is suggested before parasitoids can be 'safely released'. Although the insecticide dissipation references cited are on fruit and not apple tree leaves, as the arena used in our bioassays, the difference in persistence of the insecticides mentioned is clearly described.

This study provides the first evidence of certain sublethal effects of selective insecticides such as IGRs and conventional insecticides such as neonicotinoids on *A. flavidulus*. The next step would be to predict the effects of these insecticides based on field data, which could allow the use of insecticides that cause minimal impact on this beneficial arthropod.

Conclusions

This study determined the sublethal effects of three insecticides, IGRs - buprofezin and pyriproxyfen -and the neonicotinoid acetamiprid used in apple orchards on adults of *A. flavidulus*, parasitoid of the obscure mealybug, *P. viburni*. Growth regulators, such as buprofezin and pyriproxyfen, applied at the labeled rate, induced less sublethal effects than acetamiprid applied at a very low rate on *A. flavidulus*.

Acetamiprid reduced parasitism but it did not affect other aspects of development such as longevity, clutch size and secondary sex ratio, at a very low concentration. This would indicate its compatibility with *A. flavidulus* only when very low residue levels are reached in the field.

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Author Contributions CRN and EFC designed the studies. CRN performed the bioassays. CRN analyzed the data and wrote the manuscript. CRN and EFC reviewed and approved the manuscript before submission.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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Figures and Tables

Table 1. Mean parasitism rate, emergence rate and clutch size of the parasitoid *A. flavidulus*, at three densities of *P. viburni*. Different letters within each column indicate that the values are statistically significant ($P \leq 0.05$).

Insecticide	Mealybug density	Parasitism rate		Emergence rate		Clutch size	
		N^a	Mean ± SE	N^b	Mean ± SE	N^c	Mean ± SE
Control	2	54/66	0.82 ± 0.006	6/54	0.11 ± 0.002	26/6	4.33 ± 0.57
Buprofezin		41/58	0.71 ± 0.008	10/41	0.18 ± 0.004	43/10	4.30 ± 0.26
Pyriproxyfen		43/56	0.77 ± 0.008	8/43	0.19 ± 0.004	38/8	4.75 ± 0.42
Acetamiprid		52/84	0.62 ± 0.006	10/52	0.19 ± 0.004	26/10	2.60 ± 0.20
Control	4	111/164	0.68 ± 0.003	14/111	0.13 ± 0.001	45/14	3.21 ± 0.16
Buprofezin		134/216	0.62 ± 0.002	17/134	0.23 ± 0.002	74/17	4.35 ± 0.11
Pyriproxyfen		116/200	0.58 ± 0.002	17/116	0.15 ± 0.001	53/17	3.12 ± 0.10
Acetamiprid		89/152	0.59 ± 0.003	15/89	0.17 ± 0.002	34/15	2.27 ± 0.10
Control	6	116/162	0.72 ± 0.003a	20/116	0.17 ± 0.001	68/20	3.40 ± 0.12
Buprofezin		148/216	0.69 ± 0.002ab	9/148	0.15 ± 0.001	50/9	5.56 ± 0.35
Pyriproxyfen		95/180	0.53 ± 0.003bc	2/95	0.02 ± 0.0002	9/2	4.50 ± 1.77
Acetamiprid		83/186	0.45 ± 0.003c	16/83	0.19 ± 0.002	56/16	3.50 ± 0.14

N^a = number of parasitized hosts per total offered mealybugs (Parasitism rate)

N^b = number of emerged mummies per total parasitized hosts (Emergence rate)

N^c = number of emerged parasitoids (offspring) per total successful parasitized hosts

Table 2. Development time, longevity and secondary sex ratio of the parasitoid *A. flavidulus*, at three densities of *P. viburni*.

Insecticide	Mealybug density		Development Time (days)	Longevity (days)		Sex ratio (Males/Total)
		N[a]	Mean ± SE	Mean ± SE	N[b]	Mean ± SE
Control	2	26	20,83 ± 0.27	3,72 ± 0.54	24	0,16 ± 0.04
Buprofezin		43	18,70 ± 0.09	4,13 ± 0.14	9	0,13 ± 0.03
Pyriproxyfen		38	19,72 ± 0.12	3,75 ± 0.22	25	0,34 ± 0.05
Acetamiprid		26	20,69 ± 0.17	3,71 ± 0.14	23	0,29 ± 0.04
Control	4	45	19,52 ± 0.09	4,64 ± 0.14	31	0,16 ± 0.01
Buprofezin		74	20,06 ± 0.10	4,55 ± 0.10	46	0,45 ± 0.03
Pyriproxyfen		53	20.00 ± 0.12	4,10 ± 0.10	19	0,26 ± 0.02
Acetamiprid		34	20,3 ± 0.14	6,06 ± 0.25	22	0,24 ± 0.03
Control	6	68	21,60 ± 0.13	4,15 ± 0.09	53	0,26 ± 0.02
Buprofezin		50	19,67 ± 0.24	4,90 ± 0.20	48	0,07 ± 0.01
Pyriproxyfen		9	20.00 ± 0.00	3,79 ± 0.86	8	0.00 ± 0.00
Acetamiprid		56	20,94 ± 0.13	5,16 ± 0.26	49	0,18 ± 0.02

N[a] = offspring

N[b] = offspring identified (male-female), presence or absence ovipositor

Chapter 3

Sublethal effects of insecticides on the functional response, survival and development of the coccinellid predator *Cryptolaemus montrouzieri*

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Sublethal effects of insecticides on the functional response, survival and development of the coccinellid predator *Cryptolaemus montrouzieri*

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ABSTRACT

Insecticides may disrupt biological control of coccinellid predators. We investigated the sublethal effects of reduced concentrations of insecticides buprofezin, pyriproxyfen and acetamiprid on the functional response of *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) preying on *Pseudococcus viburni* Signoret (Hemiptera: Pseudococcidae) nymphs. Type of functional response, attack rate (*a*) and handling time (T_h) of the predator were estimated using six prey densities. In addition, survival and development time were evaluated by exposing the predator eggs to direct insecticide spraying. The third instar of *C. montrouzieri* had Type II functional response regardless of the insecticide. Both insect growth regulators, buprofezin and pyriproxyfen and the neonicotinoid acetamiprid had a deleterious effect on *C. montrouzieri* immature stages, decreasing their attack rate and/or handling time of their prey. Survival was drastically reduced with buprofezin and acetamiprid: no eggs reached the adulthood and only 1% managed to complete the development cycle, respectively. With pyriproxyfen, 15% of the eggs completed their development cycle.

Finally, the development time of *C. montrouzieri* under the three insecticides showed no differences in the first larval instars. However, all eggs treated with buprofezin failed to molt to the third instar. Upon pupal stage, a significant increase in development time was observed on acetamiprid and pyriproxyfen with respect to the control. These results would not be compatible with IPM programs. Further studies should be conducted on different developmental stages of *C. montrouzieri*, as well as using other routes of exposure to insecticides in order to determine their selectivity for *C. montrouzieri* and its prey, *P. viburni*.

Keywords: mealybug destroyer, non-target effects, attack rate, handling time, Kaplan-Meier.

1. Introduction

Integrated pest management (IPM) promotes the integration of different control methods, among which the chemical and biological control are main components. Reduction of insecticide applications and enhancement of biological control can reduce pest populations below economic damage levels taking into account the activity of natural enemies in different agricultural crops (Ali et al., 2019; Hill et al., 2017). Therefore, the use of more selective and reduced-risk insecticides can reduce the disruption of biological control of agricultural pests (Roubos et al., 2014; Radrigán-Navarro et al., 2021).

In this context, the assessment of possible non target effects of insecticides on beneficial arthropods is crucial for effective IPM strategies. The effects of pesticides on natural enemies and beneficial organisms could be lethal and sublethal. Lethal effects include the acute toxicity or mortality of any toxicant on organisms and the sublethal effects are either physiological or behavioral effects on individuals that survive exposure to a certain dose/concentration of a pesticide (Desneux et al., 2007). A sublethal dose/concentration is defined as inducing no statistically significant mortality in the natural enemy population (Desneux et al., 2007), but producing a reduction in the biological control action of the natural enemy (Biondi et al., 2013; Bueno et al., 2017).

For risk assessment, insecticides are generally evaluated on non-target organisms at the label rate suggested by the manufacturer. However, new research is focusing on the effects of lower concentrations of pesticide residues on natural enemies occurring in the field after pesticide applications (Tan et al., 2012; He et al., 2013; Guedes et al., 2016; Müller, 2018).

Most of the insecticides used to control important economic pests are wide-spectrum neurotoxic insecticides, such as pyrethroids and neonicotinoids (Casida, 2018). In recent years, chemical control practices are shifting towards more selective and safer insecticides for non-target organisms. However, neonicotinoids are still the most used group of insecticides worldwide (Jeschke, 2021). Several studies have recognized the negative impacts of neonicotinoids on natural enemies and pollinators even at low doses (Desneux et al., 2007; Cloyd and Bethke, 2011; Decourtye et al., 2013; Ricupero et al., 2020). On the contrary, insect growth regulators (IGRs), such as buprofezin and pyriproxyfen (Tunaz and Uygun, 2004) are considered more selective insecticides for non-target organisms, although harmful effects have been reported, especially on immature stages of predatory insects (Ruiz-Sánchez et al., 2010; Bueno and Freitas, 2014; He et al., 2018; Suárez-López et al., 2020).

The functional response is the number of prey successfully attacked per predator as a function of prey density (Solomon, 1949). Holling (1959), described three types of functional response. Generally, parasitoids and specific predators showed a type II response, which is an asymptotic curve that decelerates constantly as prey number increase due to the time it takes the predator to manipulate its prey. It has been reported that insecticides could affect the attack rate (a) and handling time (T_h) exerted by natural enemies against their prey, decreasing the biological control of pests (Řezáč et al., 2010; Gholamzadehchitgar et al., 2014).

Among predators affected by insecticide residues are Coccinellidae, which play a key role controlling several pests across multiple agricultural crops (Obrycki and Kring, 1998; Obrycki et al., 2009; Khan et al., 2012; Ali et al., 2014; Maes et al., 2014; Erkilic et al., 2015).

In particular, the ‘mealybug destroyer’, *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae), contributes in suppressing mealybug (Pseudococcidae) pests in citrus, vineyards, avocado, cotton and several ornamental species (Kairo et al., 2013; Mani, 2018). This coccinellid predator is native from Australia and it has been introduced to many countries for biological control of mealybug pests (Solangi et al., 2012; Kairo et al., 2013; Mani, 2018). In Chile, it was introduced in 1931 (Rojas, 2005), but have a limited biological control effect. At present, this predator is used in inundative releases for the control of mealybug species on several fruit crops, such as *Pseudococcus viburni* (Signoret) (Hemiptera: Pseudococcidae) on pome fruits and vineyards.

To the best of our knowledge, few studies have investigated the effect of sublethal concentrations of neonicotinoid insecticides and insect growth regulators (IGRs) on the functional response of *C. montrouzieri*. Similarly, insecticides affect the survival and development of predatory natural enemies, especially coccinellids (Fogel et al., 2013; He et al., 2018; Li et al., 2015; Planes et al., 2013).

Thus, the aim of this study is to determine sublethal effects of two insect growth regulators, buprofezin and pyriproxyfen and the neonicotinoid acetamiprid on the immature stages (eggs and third instar larvae) of *C. montrouzieri* in topical and residual application laboratory tests. We will assess the effects of these insecticides on the functional response of the third instar larvae feeding on *P. viburni* nymphs. Besides, we will determine how residues of these insecticides affect the survival and development of eggs of *C. montrouzieri*. This research attempts to contribute in optimizing IPM programs that combine the use of natural enemies with chemical insecticides.

2. Materials and methods

2.1. Biological materials

Individuals of *C. montrouzieri* were reared at Xilema SpA, a biological control company located in Quillota, Chile. Third instar larvae and newly eclosed adults were sent to laboratories of Universidad de Talca (UTAL), Talca, Chile. All insects were maintained in a CONVIRON® chamber at 25 ± 0.1 °C, 50 ± 5 % RH and a photoperiod of 16: 8 L: D.

Laboratory colonies of *P. viburni* were established by egg masses, nymphs and adults provided by Xilema SpA. The mealybugs were reared on butternut squash (*Cucurbita moschata* L.) inside a room chamber with controlled conditions at 23 ± 1 °C, 50 ± 10 % RH and complete darkness. *C. montrouzieri* larvae used in predation bioassays were not fed until the experiment, which was performed the same day at their arrival to the laboratory. Adults used for the survival bioassays were fed *ad libitum* with *P. viburni* egg masses and nymphs that were renewed daily.

2.2. Insecticides

Commercially available insecticides Applaud 25 WP (0.2 g/L buprofezin, Anasac, Chile), Delico 10 EC (0.05 mL/L pyriproxyfen, Anasac, Chile) and Hurricane 70 WP (0.012 g/L acetamiprid, Anasac, Chile) were used in the tests.

Sublethal concentrations used correspond to 25% of the label rate for buprofezin and 10% for pyriproxyfen and acetamiprid, which were shown in previous studies to do not cause acute lethal effects (Radrigán-Navarro et al., 2021). The suspensions and emulsions were prepared with distilled water 20 min before the start of each test.

2.3. Sublethal effects on the functional response

The functional response to prey density of the third instar larvae of *C. montrouzieri* was estimated with the application of sublethal concentrations of buprofezin, pyriproxyfen and acetamiprid. A volume of 2 mL of each insecticide concentration was applied at 0.045 MPa with a Potter precision laboratory spray tower (Burkard Scientific, Uxbridge, UK) on an apple leaf disk with the lower surface facing up on a pad of moist cotton inside a 30 mL plastic cup (Beers et al., 2009). Distilled water was used as a control. After the residues were dried, each third instar of *C. montrouzieri* (L3) was transferred to a cup to be in contact with the insecticides residues for 24 h. After this period, each larva was singularly tested by offering six prey densities of mealybug nymphs (Xilema SpA, Chile) inside a ventilated Petri dish (9 cm in diameter) for 24h. The following prey densities were chosen: 4, 8, 12, 16 and 20 nymphs/larva/24 h. Predators and mealybugs were maintained in a growth chamber at the previous controlled environmental conditions for 24 h.

At the end of the experiment, each predator larva was removed and the number of non-eaten prey (i.e., residual nymphs) was counted under a binocular to quantify the predator voracity. The number of eaten prey was obtained through the difference between the number of offered prey and the number of non-eaten prey. Between 10 and 15 *C. montrouzieri* larvae were tested per each prey density and per each concentration/ insecticide combination, including a control treatment, with distilled water.

The experiment was replicated three times.

2.4. Sublethal effects on survival and development

Newly emerged *C. montrouzieri* adults (about 60) were brought to UTAL laboratories. They were kept in a growth room with controlled conditions mentioned above for 7 days until they were mature for mating (Xie et al., 2017). They were kept on a butternut squash with *P. viburni* colonies for feeding and egg laying, because *C. montrouzieri* females lay their eggs on *P. viburni* egg masses.

After this period, we seek for *C. montrouzieri* eggs with a NIKON stereoscopic binocular and individual eggs were transferred with a fine brush into 6 cm petri dishes. A volume of 2 mL of each insecticide concentration was applied at 0.045 MPa with a Potter precision laboratory spray tower (Burkard Scientific, Uxbridge, UK). Distilled water was used as a control. Egg hatching was checked daily and the survival of the larvae and their molts were recorded until adulthood was complete. Throughout this period, larvae were fed *ad libitum* with mealybug egg masses and nymphs, which were renewed daily. The complete development cycle of *C. montrouzieri* was followed, from egg, first, second, third, and fourth instar; pupa and adult under the three insecticides and the control. Survival and developmental time of each stage was recorded. Each insecticide was applied on 20-40 eggs and the whole bioassay was replicated three times. The experiments were performed separately, first buprofezin, and then pyriproxyfen and acetamiprid including distilled water as control.

2.5. Data analysis

The type of functional response (FR) of *C. montrouzieri* to the prey densities of the mealybug was estimated by the relationship between proportional consumption of prey and prey density, which was modelled using a second order logistic regression (Crawley, 2007). The sign and significance of the coefficients was used to distinguish the shape of the FR curve, with significant and negative values indicating type II, and significant and positive values indicating type III. There was no prey replacement during the experiment, so the type of FR was determined from random predator model proposed by Rogers (1972) (Eq. (1)) (Trexler et al., 1988; Juliano, 2001)

$$N_e = N_0(1 - \exp(a(N_e h - \mathrm{T}))) \quad (1)$$

where N_e is the number of prey eaten, N_0 is the initial density of prey, a is the attack rate coefficient, h is the handling time and T is the total time available for predation (days). Modelling was performed in the R package *frair* (Pritchard, 2014), which utilizes maximum likelihood estimation within the bbmle package (Bolker, 2014) and a modified version of Eq. (1) with an additional Lambert W function to make the equation solvable (Eq. (2)).

$$N_e = N_0 - \mathrm{lambert}W \\ (a \cdot h \cdot N_0 \cdot \exp(-a(\mathrm{T} - N_0 h)))/(a \cdot h) \quad (2)$$

Curves were bootstrapped to visualize variability (n = 999), and the parameters *a* and *h* compared between treatments (insecticides and respective controls) with the function frair_compare (Juliano, 2001; Paterson et al., 2014). The functional response analyses were performed separately, first buprofezin, then pyriproxyfen and finally acetamiprid, including distilled water as control.

To estimate survival for each development stage of *C. montrouzieri*, the survival data was submitted to a Kaplan- Meier survival analysis and treatments were compared by Log-rank test at $P \leq 0.05$. The survival analyses were performed separately, first buprofezin, and then pyriproxyfen and acetamiprid including distilled water as control.

The development time of each stage of *C. montrouzieri* from egg to adult was analyzed with a generalized linear model (GLM) with Poisson distribution.

The larval period (egg to fourth instar) and the total development time (egg to adult) was analyzed with an ANOVA and a Kruskall Wallis test, respectively.

When statistical differences existed between treatment sets (P < 0.05), Tukey HSD test was used to separate means. The analyses were performed separately, first buprofezin, and then pyriproxyfen and acetamiprid including distilled water as control.

All statistical analyses were carried out in R version 4.1.0 (R Core Team, 2021).

3. Results

3.1. Sublethal effects on the functional response

The results obtained for the functional responses of the third instar larvae of the predator *C. montrouzieri* when consuming mealybug nymphs showed type II responses, in both the control and insecticide treatments. The estimate for the density parameter in every case was negative and significant ($P < 0.001$): for buprofezin (-0.0743 ± 0.006); pyriproxyfen (-0.089 ± 0.006) and for acetamiprid (-0.1159 ± 0.05). The consumption of mealybug nymphs never exceed 92% of the lowest density (four mealybugs), representing 3.69 nymphs per day (Figure 1).

Predation rate of third instar larvae of *C. montrouzieri* on mealybug nymphs showed higher attack rate (*a*) in the control compared to buprofezin and pyriproxyfen (Table 1). On the other hand, handling time (*h*) did not differ in the pyriproxyfen and acetamiprid treatments, lasting between 90 and 144 min, respectively. In contrast, buprofezin residues on larvae shortened the handling time by 40 minutes compared to the control treatment (Table 1).

3.2. Sublethal effects on the survival and development time

Survival was always higher in the control treatments in relation with the treatments with the three insecticides (Table 2, 3). With buprofezin, no eggs reached the adulthood failing to molt to the third instar, so survival analysis from that instar onwards could not be performed (Table 2). A similar situation was observed with the neonicotinoid, acetamiprid, since only 1% of the eggs managed to complete the development cycle.

With pyriproxyfen, 15% of the eggs completed their development cycle. In all cases, the probability of survival was lower and statistically different from the control (Table 3).

Regarding the duration of the different developmental stages of *C. montrouzieri* no differences were observed between the control and the buprofezin treatment for the first ($\chi^2 = 0.005$, $P = 0.94$) and second ($\chi^2 = 1.04$, $P = 0.3$) larval instars. However, all eggs treated with buprofezin failed to molt to third instar (L3) (Table 4). Acetamiprid showed a longer development time during fourth instar (L4) than the control, although this difference was based in a single individual ($\chi^2 = 16.65$, $P < 0.001$). On the other hand, upon pupal stage, a significant increase in development time was observed on both insecticides with respect to the control ($\chi^2 = 11.89$, $P < 0.05$) (Table 4).

4. Discussion

The results obtained in functional response tests with sublethal concentrations of buprofezin, pyriproxyfen and acetamiprid on *C. montrouzieri* larvae indicate that this predator present a type II response curve when feeding on nymphs of *P. viburni*. This type of curve is frequent in parasitoids and some predators. This is the case of *C. montrouzieri* which is a predator of mealybugs of the Pseudococcidae family, such as *Planococcus citri* (Risso), *Maconellicoccus hirsutus* (Green) and *Pseudococcus viburni* (Signoret) (De Bortolo et al., 2014; Torres and Marcano, 2015; Mani, 2018). It is important to mention that functional response may vary with the developmental stage of the predator. For instance, the adult female of *C. montrouzieri* showed a functional response type II when fed with *M. hirsutus* (Torres and Marcano, 2015) and *P. citri* (Pakyari et al., 2016), while second, third and fourth instar fed on *M. hirsutus* showed a type III functional response (Torres and Marcano, 2015). In this study, all insecticides had detrimental effects on functional response, either on attack rate (*a*) or handling time (T_h). It is important to emphasize that the parameters of *a* and T_h were not measured directly, but correspond to mathematical estimates, so these values must be carefully and biologically interpreted in context. However, buprofezin residues on larvae shortened the handling time compared to the control. Perhaps we are facing a case of hormesis which is a stimulatory effect associated with low (sublethal) doses of compounds that are toxic at higher doses, and is characterized by a reversal in response between low and high doses of a stressor (Guedes et al., 2016).

Probably, buprofezin is causing certain acceleration in the behavior of the predator before the larva molts to the next stage or detoxifying processes develop under a sublethal concentration of this IGR.

In contrast, Sultan et al. (2019), showed that first and second instar of *Coccinella semptempunctata* (L.) (Coleoptera: Coccinellidae) had longer handling time and lower attack rate than the control. Our results are not conclusive, and further investigation is needed.

Moreover, pyriproxyfen bioassays of El Aalaoui and Sbaghi (2021) performed in adults of *C. montrouzieri* showed the same type II FR curve and a decrease in the attack rate as was seen in our study. However, they reported a reduction in the handling time, which was not seen in C. *montrouzieri* third instar.

The studies of sublethal concentrations of neonicotinoid insecticides, imidacloprid and thiamethoxam on the functional response of *Serangium japonicum* Chapin (Coleoptera: Coccinellidae), an egg predator of *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) resulted in a type II curve (He et al., 2012; Yao et al., 2015) which is consistent with our study, with residues of acetamiprid. An increased handling time also was observed in adults exposed to dry residues of imidacloprid (He et al., 2012) and to thiamethoxam by a systemic exposure route (Yao et al., 2015).

Although statistical differences were observed between the handling times (T_h) of acetamiprid and its control, these are practically the same, which implies that these results should be taken with caution, since the effect of this nicotinoid on *C. montrouzieri* third instar may be overestimated.

Accordingly, the three insecticides tested caused alterations in the functional response. This would mean (i) that *P. viburni* had effective defense strategies, which were not overcome by *C. montrouzieri* larvae, or (ii) that *P. viburni* did not present any defense strategy, such that the predator consumed *ad libitum,* without the need to enhance prey intake at all (Almeida et al., 2007). This situation began to be observed in the larger densities of mealybugs offered, when the number of nymphs consumed stabilized at an average of eleven nymphs despite the fact the prey offered were sixteen and twenty, respectively in the control.

It is important to mention that coccinellids are digestive-limited predators, so the limitation of maximum consumption rate was shown because of predator satiation (Papanikolaou et al., 2014). This should be examined to confirm if it occurs in immature stages as well.
It is well known that immature stages of natural enemies are frequently more susceptible than adults toward pesticides (Barbosa et al., 2018; Silva et al., 2021). This could be associated to a thinner and more permeable cuticle of immature stages or less active enzymatic detoxifying processes in comparison to adult stage (Stark et al., 2004). It is known that the egg chorion exerts a protective barrier against external factors such as pesticides.

Although the chorion surface layer is a sclerotized protein membrane providing mechanic resistance with non-permeable properties (Nation, 2008). Although, some chemicals can pass through it affecting (i) the embryo development (ii) or, in the case of successful hatching, the individual may die when feeding the insecticide-contaminated chorion (Trisyono et al., 2000; Consoli et al., 2001; Galvan et al., 2005; Rimoldi et al., 2008; Fogel et al., 2013). With buprofezin, this must have probably been the most likely cause of the premature death of first instar larvae, since no differences were observed at the time of emergence, but rather, when they were about to molt to second instar. Similar results were found by Cabral et al. (2008), on *Coccinella undecimpunctata* L (Coleoptera: Coccinellidae), where survival of first and second instars was reduced when eggs were treated with buprofezin. Also, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) eggs reduced their embryonic and subsequent larval survival when exposed to concentrations of buprofezin recommended for field and greenhouse pest control (He et al., 2018).

Similarly, pyriproxyfen has been to be detrimental to some coccinellid species. (Hattingh and Tate, 1995). Barbosa et al. (2018) found that females of the striped mealybug predator *Tenuisvalvae notate* (Cockerell) (Coleoptera: Coccinellidae) exposed to pyriproxyfen residues laid non-viable eggs for 4-5 days after cessation of exposure for 12 or 24 h. After the exposure, females recover but the egg viability was reduced in 66% with pyriproxyfen residues.

As for buprofezin, acetamiprid did not affect egg hatching but the mortality of *C. montrouzieri* neonate larvae was practically complete.

Similar results were reported on immature stages of *Eriopis connexa* (Germar) (Coleoptera: Coccinellidae) under 50% of the field rate of acetamiprid, where neonate larvae that were able to emerge showed a significant reduction of survivorship (Fogel et al., 2013).

Regarding the development time of *C. montrouzieri*, when buprofezin and pyriproxyfen were sprayed on eggs, no differences were found in the development time of larvae and pupae, which is in agreement with the report of He et al. (2018) on *H. axyridis*.

In contrast, with acetamiprid, a much longer development time was seen, but only one pupa reached the adult stage, which is not representative. However, studies conducted by Fogel (2013) on *E. connexa*, showed that a rate of 50% acetamiprid induced a lengthening of the embryogenesis of the predator eggs.

When comparing larval period (F= 0.113; df =2/5.761; p=0.894) and total development time (KW χ^2= 2.264; df= 2; p=0.323) between acetamiprid, pyriproxyfen and control, no significant differences were observed, respectively. However, additional studies should provide greater understanding of the physiological and biochemical processes involved in the differential toxicity of buprofezin, pyriproxyfen and acetamiprid to *C. montrouzieri*.

5. Conclusions

As we have seen, *C. montrouzieri* is a good predator of the obscure mealybug, *P. viburni*. The sublethal concentrations of the insecticides buprofezin, pyriproxyfen and acetamiprid have detrimental effects on the predator functional response, reducing the biological control of *P. viburni*.

Both IGRs, buprofezin and pyriproxyfen, to different extents, had a deleterious effect on *C. montrouzieri* immature stages, decreasing their attack rate and/or handling time of their prey.

Indeed, both IGRs affected egg and larval survival, although no impact was observed on developmental time in early larval stages. Acetamiprid is also very harmful to immature stages, affecting their predation behavior and also neonate larval survival. These results would have an adverse impact on IPM programs.

More studies should be conducted using different developmental stages of *C. montrouzieri* as well as other methods of exposure to insecticides to examine their selectivity to *C. montrouzieri* and its prey, *P. viburni*.

Author contributions

All authors contributed to the study conception and design. **Conception and design of study**: Radrigán-Navarro, C., Estay, S.A. and Fuentes-Contreras, E. **Acquisition of data:** Radrigán-Navarro, C. **Analysis and/or interpretation of data:** Radrigán-Navarro, C., Estay, S.A. and Fuentes-Contreras, E. **Drafting the manuscript:** Radrigán-Navarro, C., Estay, S.A. and Fuentes-Contreras, E. **Revising the manuscript critically for important intellectual content**: Radrigán-Navarro, C., Estay, S.A. and Fuentes-Contreras, E. **Approval of the version of the manuscript to be published:** Radrigán-Navarro, C., Estay, S.A. and Fuentes-Contreras, E.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Tables and Figures

Table 1. Functional response parameter estimates for *C. montrouzieri* third instar feeding on *P. viburni* nymphs as prey extracted from Roger´s equation.

Treatment	*a*	SE	z	p	*h*	SE	z	p
Control	2.86 (2.16- 3.69) a	0.2486	11.49	< 0.01	0.063 (0.047- 0.079) a	0.0041	15.377	< 0.01
Buprofezin	1.702 (1.32- 2.15) b	0.128	13.27	< 0.01	0.036 (0.016-0.05) b	0.004	72.815	< 0.01

Treatment	*a*	SE	z	p	*h*	SE	z	p
Control	2.81 (2.176- 3.82) a	0.2479	11.329	< 0.01	0.064 (0.048- 0.08)	0.00424	15.03	< 0.01
Pyriproxyfen	2.13 (1.635- 2.833) b	0.1781	11.963	< 0.01	0.062 (0.046- 0.078)	0.00475	13.181	< 0.01

Treatment	*a*	SE	z	p	*h*	SE	z	p
Control	3.13 (2.313- 4.25)	0.272	11.494	< 0.01	0.094 (0.074- 0.113) a	0.004	22.496	< 0.01
Acetamiprid	2.49 (1.89- 3.28)	0.2184	11.414	< 0.01	0.109 (0.091- 0.128) b	0.005	21.638	< 0.01

Different letters in the same column indicate significant differences ($P < 0.05$) with *frair* test.

Table 2. Survival probability of *C. montrouzieri* eggs following the exposure of sublethal concentrations of buprofezin.

		Survival probability					
	n	First Instar	Second instar	Third instar	Fourth instar	Pupa	Adult
Control	146	0.81 ± 0.03 a	0.52 ± 0.04 a	0.44 ± 0.04 a	0.33 ± 0.04	0.32 ± 0.04	0.32 ± 0.04
Buprofezin	104	0.77 ± 0.04 a	0.19 ± 0.04 b	- b	-	-	-
χ^2		0.7	12.7	8.8	-	-	-
P		0.4	< 0.001	< 0.01	-	-	-

Values within columns followed by a different letter are significantly different according to Log Rank test ($P \leq 0.05$). Values are mean ± SE. n: number of eggs tested.

Table 3. Survival probability of *C. montrouzieri* eggs following the exposure of sublethal concentrations of pyriproxyfen and acetamiprid.

		Survival probability					
	n	First Instar	Second instar	Third instar	Fourth instar	Pupa	Adult
Control	73	0.92 ± 0.03 a	0.64 ± 0.06 a	0.59 ± 0.06 a	0.53 ± 0.06 a	0.49 ± 0.06 a	0.49 ± 0.06 a
Pyriproxyfen	99	0.44 ± 0.05 b	0.15 ± 0.04 b	0.09 ± 0.03 a	0.09 ± 0.04 a	0.07 ± 0.02 b	0.07 ± 0.02 b
Acetamiprid	92	0.84 ± 0.04 a	0.01 ± 0.01 c	0.01 ± 0.01 b	0.01 ± 0.01 b	0.01 ± 0.01 c	0.01 ± 0.01 c
χ^2		54	23.4	48	32.9	53.3	53.3
P		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

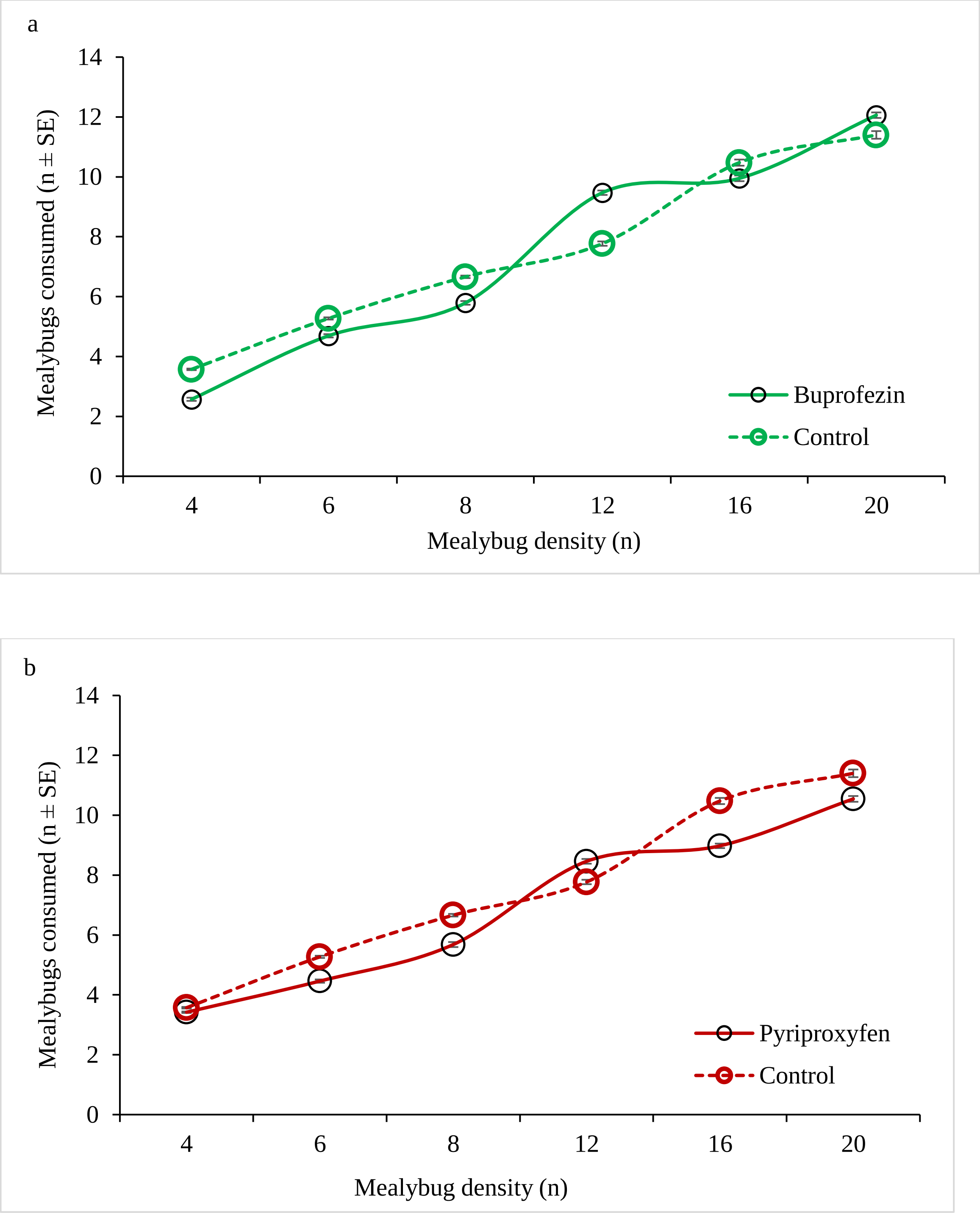
Values within columns followed by a different letter are significantly different according to Log Rank test ($P \leq 0.05$). Values are mean ± SE. n: number of eggs tested.

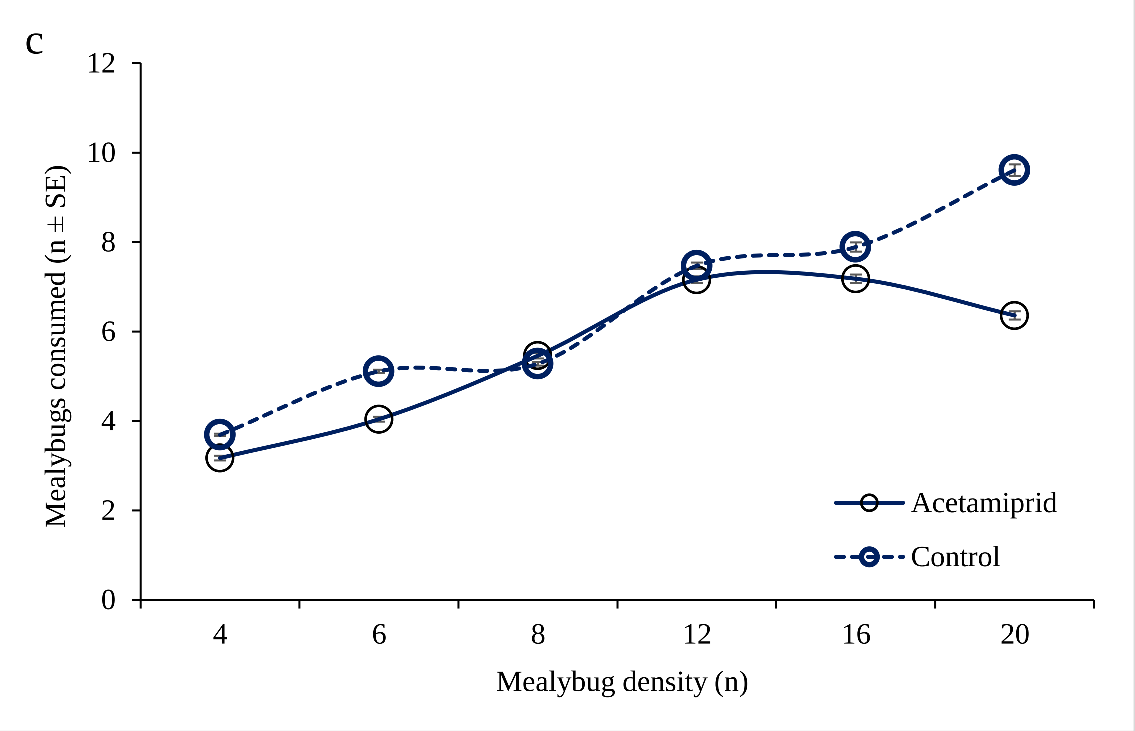
Table 4. Duration of immature development in days (mean ± SE) of *C. montrouzieri* when eggs were sprayed with buprofezin, pyriproxyfen and acetamiprid.

Treatment	Duration (days) Developmental Stage							
	Egg	1st instar	2nd instar	3rd instar	4th instar	Pupa	Larval period	Total time (T_t)
Control	3.78 ± 0.86	5.43 ± 1.29	3.86 ± 0.78	4.23 ± 0.89	11.08 ± 2.45	9.26 ± 1.74	17.3 ± 3.82	37.65 ± 8
Buprofezin	3.8 ± 0.74	4.85 ± 1.13	-	-	-	-	-	-
Control	2.37 ± 1.03	5.34 ± 1.38	3.65 ± 1.03	4.13 ± 1.45	5.28 ± 2.51 a	3.81 ± 2.02 a	15.49 ± 4.89	24.58 ± 9.42
Pyriproxyfen	2.65 ± 0.92	4.93 ± 1.59	2.38 ± 0.71	6.17 ± 1.75	2.17 ± 0.52 a	6.17 ± 1.82 b	16.11 ± 4.98	24.45 ± 7.32
Acetamiprid	2.70 ± 0.66	7 ± 0.00	4 ± 0.00	4 ± 0.00	10 ± 0.00 b	10 ± 0.00 b	17.70 ± 0.66	37.70 ± 0.66

Means followed by different letters were significantly different within columns by Tukey HSD test

Figure 1. Roger´s type II functional responses of *C. montrouzieri* third instar feeding on *P. viburni* nymphs (a) buprofezin (b) pyriproxyfen (c) acetamiprid with their respective control treatment.




c
12
10
8
6
4
2
0
Mealybugs consumed (n ± SE)
4
6
8
12
16
20
Mealybug density (n)
Acetamiprid
Control

5. General Discussion

As predicted in the general hypothesis, the sublethal concentration of two growth regulator insecticides, buprofezin and pyriproxyfen and the neonicotinoid acetamiprid, have not reduced parasitism, predation and development of the two natural enemies of *Pseudococcus viburni, A. flavidulus and C. montrouzieri* in an equivalent manner.

For each natural enemy, different results were observed, depending on their stage of development, concentration and method of application.

As it was seen on the first chapter, growth regulator insecticides and anthranilinic diamides cause lower mortality than neonicotinoid insecticides in natural enemies of the obscure mealybug and woolly apple aphid, as it was predicted in the first specific hypothesis.

Overall, various sublethal concentrations of IGR and neonicotinoid inecticides exerted different effects on the two natural enemies of *P. viburni.*

First, these are two beneficial arthropods of different kinds: *A. flavidulus* is a parasitoid and *C. montrouzieri* is a predator.

Each natural enemy was exposed to residues or direct applications of these concentrations at different stages of development: the parasitoid only to adult females; the predator to adults, larvae and eggs. This led to differential effects of these insecticides. For example, label concentrations of both growth regulators practically did not affect parasitism of *A. flavidulus* females nor their development, but did affect predation on larval stages of *C. montrouzieri*. Likewise, lower concentrations of IGR on immature stages of the predator caused relevant sublethal effects, such as the interruption of development, decreased predation (attack rate) and increased the handling time (T_h) of its prey, when received by the third instar of *C. montrouzieri*. As for the neonicotinoid, acetamiprid, when residues of 0.05% of the label concentration were applied, almost no effect on parasitism and no effect on their development were observed. However, when the concentration was increased to 10% of the label rate and was applied residually or directly to immature stages of the predator, the effects on predation and development were significant, reducing almost completely the survival of *C. montrouzieri* eggs. However, such concentration failed to alter the development time of the different stages and instars of the predator.

Thus, it is evident that the type of natural enemy, parasitoid or predator, its stage of development (adult or immature) as well as the body size of each beneficial arthropod influences the behavior (parasitism/predation) and development in response to different sublethal concentrations of insecticides, applied residually or directly.

For instance, parasitism was reduced only when100% pyriproxyfen and 0.05% acetamiprid, respectively, were applied as dry residues on *A. flavidulus* adults, at the highest density of mealybugs offered. Concerning other variables evaluated, such as clutch size and development time, it was observed that with 100% of buprofezin, both variables were favored by the insecticide with respect to the control, suggesting some accelerating effect of buprofezin, exerting a hormesis-type effect (Guedes et al., 2014, 2016).

Finally, both IGRs at commercial doses and the neonicotinoid acetamiprid at a very low concentration were not able to alter either the longevity of adults emerged from *A. flavidulus* parasitized mealybugs that were in contact with their residues or altered the secondary sex ratio. However, the density of mealybugs offered did show that at low and high densities, there was a prevalence of females relative to males, which is what has been reported in the literature for *A. flavidulus* (Karamaouna and Copland, 2000, 2008).

Insect growth regulators at the recommended concentrations for apple tree pests are not as harmful compared to the neonicotinoid, acetamiprid, which could be inferred given the mode of action of the IGR, since it was applied to adults of *A. flavidulus*.

The concentration applied did not affect parasitism in the case of buprofezin; even the clutch size and development time was lower with this insecticide. Few negative effects were observed with acetamiprid, but these must have been because the concentration applied was so low (0.005% of the recommended rate), that it did not exert deleterious effects on parasitoid developmental parameters. This means that buprofezin and pyriproxyfen act in a different way on *A. flavidulus* adults and that pyriproxyfen can alter the parasitism but not the parasitoid development. As a recommendation, if biological control release is to be used, OMB mummies parasitized with *A. flavidulus* should not be released until 10 to 15 days when acetamiprid residues reach the levels evaluated in this study. However, there would be no problem in releasing after one application of the two IGRs evaluated.

A more complete study with respect to the increasing host density, in this case of OMB, is determinant to study, since it would be possible to confirm the hypothesis that these insecticides **decrease** the functional response of this parasitoid, which could have consequences in the biological control of the OMB. In addition, the results found in this study could be validated whether at low and high host densities this parasitoid, for example, is predisposed to lay more females than males, which would favor biological control, since the females are the ones who oviposit on the mealybugs.

Regarding the effect of sublethal concentrations of insecticides buprofezin, pyriproxyfen and acetamiprid on the type II functional response of larvae of *C. montrouzieri*, it can be confirmed that the insecticides evaluated, did have deleterious effects on the third instar of the predator, being different according to the insecticide applied.

When applied residually to third instar larvae, they affected the functional response, decreasing the attack rate with IGRs, buprofezin, and pyriproxyfen. On the contrary, buprofezin shortened the handling time of the prey, *P. viburni*. This result may be related to detoxification processes seen in other insects tolerant to insecticides (Yang et al., 2016). The mode of action is not well understood, but primary enzymes such as cytochrome P450 monooxygenases (P450), glutathione S-transferases (GST), and esterases (EST) are involved in detoxifying toxicants (Feyereisen et al., 1999; Panini et al., 2016; Wei et al., 2020). Mainly, the expression gene of the P450 is associated with resistance to buprofezin (Datta et al., 2021). Zhang et al. (2016) studied the P450-mediated metabolic detoxification of xenobiotics might be an essential mechanism for buprofezin resistance in *Laodelphax striatellus* (Hemiptera: Delphacidae). In the planthopper, *Sogatella furcifera* (Hemiptera: Delphacidae), enzyme activities of P450 monooxygenase and GST were increased significantly in the buprofezin resistant strain (Bup-R) compared with the Bup-S strain (Ali et al., 2019). These metabolic processes may relate to the shortened handling time observed in *C. montrouzieri* larvae while predating *P. viburni* nymphs. Further work must be performed to describe this predator behavior certainly.

With acetamiprid, the handling time was almost the same, showing significant differences which must be accurately observed, because their confidence interval overlap.

Speaking of the development effects on *C. montrouzieri* eggs, it can be established that the three insecticides evaluated did not allow to complete the development cycle of the predator. When the same concentrations were applied directly to the predator's eggs, buprofezin and acetamiprid reduced by 75% to 100% its capacity to complete its development, without becoming adults. The egg only completes some larval stages and dies. It is known that the first two larval stages, although predatory, are not the most voracious. Therefore, biological control is disrupted to a lesser or greater degree, depending on the insecticide applied.

Regarding to the predator *C. montrouzieri*, larvae should not be released when any of the three insecticide concentrations evaluated in this study were applied. And if we add to this the fact that adults of the predator lay eggs, the effect on the biological control of the OMB pest will be practically null.

Now, the research must continue. It remains to extrapolate these results to the effect on the populations of these natural enemies and not only to make conjectures about what would happen with biological control, but to actually quantify it. And finally, to carry out greenhouse and field studies to verify what has been observed under controlled conditions.

This study is the first step towards an integrated pest management of pome pests, which aims to achieve a desired balance of releasing natural enemies, *A. flavidulus* and *C. montrouzieri* and using insecticide applications, if they are really necessary, to keep pests of economic importance in apples, such as OMB, under the threshold of economic damage.

6. General Conclusions

1. Neonicotinoids such as thiacloprid and acetamiprid were moderately harmful to harmful especially on the parasitoids, *Aphelinus mali* and *Acerophagus flavidulus* and harmless and slightly harmful on larvae and adults of *C. montrouzieri*.
2. Growth regulators as buprofezin and pyriproxyfen, and the diamide chlorantraniliprole seem to be safe for adult parasitoids and for larvae and adults of *C. montrouzieri*. The newer diamide cyantraniliprole was less compatible than chlorantraniliprole with the natural enemies evaluated in terms of acute toxicity.
3. Growth regulators, such as buprofezin and pyriproxyfen, applied at the labeled rate, induced less sublethal effects than acetamiprid applied at a very low rate on *A. flavidulus*. Acetamiprid reduced parasitism but it did not affect other aspects of development such as longevity, clutch size and secondary sex ratio, at a very low concentration. This would indicate its compatibility with *A. flavidulus* only when very low residue levels are reached in the field.
4. The sublethal concentrations of the insecticides buprofezin, pyriproxyfen and acetamiprid have detrimental effects on *C. montrouzieri* functional response, thus eventually reducing the biological control of *P. viburni*.
5. Both insect growth regulators, buprofezin and pyriproxyfen, to different extents, had a deleterious effect on *C. montrouzieri* immature stages, decreasing their attack rate and/or handling time of their prey.

6. Both insect growth regulators affected egg and larval survival, although no impact was observed on developmental time in early larval stages of *C. montrouzieri*.
7. Acetamiprid is also very harmful to immature stages of the predator, affecting their predation behavior and also neonate larval survival.

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