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Sublethal exposure to fipronil affects the morphology and development of honey bees, *Apis mellifera*

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Abstract

Honey bees play a major role in agriculture because they pollinate most crops. Despite its importance, the non-rational use of agrochemicals could endanger the bee populations. In this study, the objective was to investigate if the sublethal exposure to the fipronil insecticide affects the morphology and causes any abnormal development of bee colonies during the winter of the southern hemisphere. Six hives were fed with sugar water (Sugar and tap water ratio, 2:1) during the experiment; three of them were also fed with known amounts of fipronil (dose 0.025 µg g^{-1}). The six colonies were allowed to feed freely in the surroundings. Bees that were exposed to a sublethal dose of fipronil for six consecutive months (May to October 2015) had abnormal development of body size, wings and antennae. During the autumn and winter period, number of sealed offspring and brood frames in hives exposed to fipronil showed a significant decrease with respect to the initial value. We conclude to exposure to the subletal dose of fipronil during the winter season, this insecticide caused abnormal growth in the exposed bees and hives, in comparison with the untreated ones, developed an abnormal growth of the left antenna, the area of the right wing and the size of the honey bee.

Key words: neurosystem pesticide, phenylpyrazole, fipronil, honey bees, morphology.

Introduction

Through pollination of plants, honey bees (*Apis mellifera* L., Hymenoptera Apidae) play a vital role in maintaining global biodiversity and sustaining the production of many of the most important food crops (Klein *et al.*, 2007; Bradbear, 2009; Breeze *et al.*, 2011). According to the Food and Agriculture Organization (FAO - www.fao.org), among the 100 most human-consumed species of food crops, which provide 90% of food in the world, 71% are pollinated by honey bees (UNEP, 2010). Any alteration in the health of bees implies a reduction in pollination services, its fitness, longevity that may lead to a reduction in crop yield (Loos *et al.*, 2010; Potts *et al.*, 2010; Jansen *et al.*, 2011). Overwhelming evidence suggests that the pollination services are currently declining worldwide (Nguyen *et al.*, 2009; Holmstrup *et al.*, 2010; Calderone, 2012; Biondi *et al.*, 2013). This decline is likely due to multiple simultaneous pressures like habitat loss (vanEngelsdorp *et al.*, 2010; vanEngelsdorp *et al.*, 2011; Goulson et al., 2015), climate and global change pressures (Le Conte and Navajas, 2008; González-Varo *et al.*, 2013), pathogens (Martínez *et al.*, 2012; Botías *et al.*, 2013) nutrition and the impact of pesticides exposure (Johnson *et al.*, 2010; Gill *et al.*, 2012; Schmehl *et al.*, 2014; Calatayud-Vernich *et al.*, 2016).

Pesticides can adversely affect honey bees also indirectly, such as making them more susceptible to pests and pathogens (Vidau *et al.*, 2011). Currently, the honey bees populations are often exposed to the neonicotinoids and the phenylpyrazoles insecticides, which are systemic neurotoxic compounds of intensive agricultural use worldwide against insect pests (Vidau *et al.*, 2011; Sanchez-Bayo *et al.*, 2014), and they are linked to adverse effects to honey bees (Lunardi *et al.*, 2017). Since late 2013, the European Union restricted for two years the use of fipronil. This decision was supported by a study carried on by the European Food Safety Authority (EFSA) which concluded that fipronil poses a grave risk to the honey bee populations (EC, 2013; EFSA, 2013).

The insecticide fipronil acts on the nervous system of insects by blocking the chloride channels gated by gamma-aminobutyric acid and glutamate (Barbara *et al.*, 2005; Thompson, 2010). It has been demonstrated that fipronil affects the mobility of *A. mellifera*, and lead to an increase in water consumption and progressive deterioration of the olfactory ability of bees (El Hassani *et al.*, 2005; Aliouane *et al.*, 2009). In fact, sublethal doses of phenylpyrazoles induce multiple effects such as behavioural or physiological alterations in bees and other beneficial arthropods (Desneux *et al.*, 2007; Renzi *et al.*, 2016). In honey bees, there are negative synergies between neonicotinoid insecticides and phenylpyrazoles such as fipronil (Vidau *et al.*, 2011), however, fipronil is still in use. The documented concentration of fipronil and its metabolites after 120 d of its field application was 0.047 µg g^{-1} of soil (Cummings *et al.*, 2006). The concentration of fipronil in the soil, on rice, increased three days after transplanting, and decreased slowly during the next 14 days; after that, and it remained relatively stable (0.241 µg kg^{-1}) (Kasai *et al.*, 2016). The European Commission has approved restrictions on the use of the insecticide fipronil in Europe, in countries such as France is no longer used. In Chile during the period in which the research was carried out the use of insecticides with fipronil as an active ingredient had authorized by the Servicio Agrícola y Ganadero (SAG) for use in the treatment of seeds in wheat, ray grass and corn crops (SAG, 2018). In the agricultural practice in the country is used to control wireworms (*Agriotes* spp.) and whiteworms (*Diloboderus abderus* Sturm, *Dyscinetus gagates* Burmeister, *Cyclocephala* spp.), without there being greater restrictions on its use in the period. However, systemic and neurotoxic insecticides, such as fipronil, are currently being used in agriculture at the local and global levels, despite that the potential effect on the death of bee colonies and adverse effects on the hive are still unknown. The aim of this study was to investigate whether sublethal exposure of fipronil in hives of honey bees causes variations in morphology and abnormal development of the colonies during the season.

Materials and methods

We used a replicated split-plot design consisting of two treatments with three honey bee hives in each case. We used *Apis mellifera carnica* Pollmann because it corresponds to one of the most used in Chile due to their good honey yields and high adaptability (Montenegro *et al*., 2009). The hives were established (between 26 and 29 September 2014 in early spring), with unfertilized queen season. The hives were monitored weekly and managed using the standard techniques of beekeeping. After the end of the harvest season (March 18, 2015), six hives were selected for the experiment. It was considered for the selection of the number of breeding frames in the hive, the number of sealed brood count and the amount of honey produced. Starting from May 1st 2015, we administered 50 µg of fipronil (0.025 µg g^{-1}) {(RS)-5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4- (trifluoromethylsulfinyl)-1H-pyrazole-3-carbonitrile, CAS# 120068-37-3} in 2 litre (0.528 gallons) of sugar water (ratio, 2:1) to the treated colonies each week, respectively, for twenty two consecutive weeks ending on October 2nd 2015 (table 1). Assuming each colony consisted of 40,000 bees at any given day in autumn and winter, we administered 0.00125 µg bee^{-1} of either fipronil to three treated hives for 22 consecutive weeks. This dosage is far below the oral LD50 of 0.00417 µg bee^{-1} for fipronil (EFSA, 2013). In addition, all colonies were allowed to feed freely on the environment, according to the modified method of Lu *et al*., 2014. Control colonies were given fipronil-free sugar water (ratio, 2:1) throughout the experimental period. During the fipronil experimental period of the sugar water it was completely consumed at the end of each week in the 22-week.

Description of the study area

The trial was established in the area of Llano Verde (37°43'S 72°22'W, 184 m above the average sea level) at a distance of 10 km from the city of Los Angeles, capital of the province of Bío Bío, Chile. The climate in the area is characterized by having all months with average temperatures below 22 °C and at least four months averaging above 10 °C; during the winter, the rains are much more abundant, in comparison to the summer season, and according to Köppen and Geiger the climate is classified as Csb. The average annual temperature in the city of Los Angeles is 13.6 °C and average rainfall is 1207 mm per year (Dirección Meteorológica de Chile, 2014). The most cultivated cereal with which potentially seeds can be treated with fipronil in Chile is wheat, which represents more than 40% of the national production of cereal, which is concentrated between the region of Araucania and Biobio, where the province of Biobio is located in the middle of that zone (ODEPA, 2018).

Morphological analysis

A total of six colonies were subjected to morphological analysis. 60 workers were analyzed per hive, a set of 30 workers in May 1st 2015 and 30 in October 2nd 2015 obtained. The bees were kept in 70% Ethyl Alcohol. Three morphological characters were measured: size of each insect (mm), size of the left antenna (mm length), and width of the right forewing (mm^2 wing area). We chose the size of bees as a parameter because plays an important role in the carrying capacity of bees, in addition to habitually body size is related to foraging ability

Table 1. Chronological characterization of the observed events in the bees colonies studied.

Date	Event
26-29 September 2014	Assembling new 10-frame Langstroth pine honey bee hives
March, 2015	End of the harvest season, collecting honey, counting frames and sealed brood count
April, 2015	Selection of six honey bee hives in study site and apiary set up
April 30th, 2015	All six hives contained, at least, five frames of capped brood
May 1st, 2015	Initially sealed brood count and sublethal fipronil dosing for five consecutive months (Fipronil dose 0.025 µg g^{-1})
May	Recollection of bees previous to fipronil application
May-December, 2015	The monitoring strength of honey bee hives biweekly
June 1st, 2015	Autumn hive strength monitoring and monitoring date without the observation of dead bees
October 2nd, 2015	Recollection of bees to morphological measurement
July-November, 2015	Winter hive strength monitoring
December 2nd, 2015	Last count of sealed brood
December, 2015	Collecting honey and counting frames

(Johnson, 1990). One of the two honey bee antennas was randomly selected, because they have important roles in their daily life such as primarily as an odour receptor and secondarily as a taste receptor (Suwannapong *et al*., 2011). The honey bee has two sets of wings, of the four wings one was selected at random for the measurement, the wings were selected because of the importance of the role of flight in the forage activity of bees (Suwannapong *et al*., 2011). The dissections were carried out according to the methodology established by Ruttner *et al*., 1978.

Determination of the survival and development of the colony

We evaluate the growth of the colony over time using the modified breeding evaluation of Emsen (2005). The frames in each hive were scored cumulatively since the beginning of the experiment to the area covered by "sealed brood". Sealed brood is the bee pupal stage of development. Therefore, this bi-weekly evaluation provides objective measures of breeding livestock in each colony. For the measurement, the method of digital photography was used. Measurements of the worker's breeding area were determined by measuring the sealed brood to the nearest cm^2 using Adobe Photoshop CC 2015. The number of offspring is estimated by dividing the face of each side frame in 32 squares (each square containing approximately 100 cells). The frames in each hive were visually scored to estimate the number of breeding places covered by the face of the frame. This method is based on the estimation of the capped and uncapped brood.

Analysis of data

An analysis of variance was performed using the ANOVA procedure to compare length of the left antenna, area of the right forewing, bee length and numbers of the frame and sealed brood count. The comparison of means was made by using the Tukey test (alpha 0.05, Windows InfoStat, 2015), to compare length of the left antenna, area of the right forewing and bee lengthy prior to the application of fipronil (May 1st 2015) and upon completion of its application (October 2nd 2015).

Results

The experiment started with healthy bees in both the treated and the untreated treatments. The hives bees showed a significant reduction in the length of the left antenna, area of the right forewing and bee length, in comparison with the untreated ones (table 2). In the untreated control, there were no significant differences in the size of the left antenna (figure 1) and the size of the right forewing (figure 2). The exposure to fipronil decreased the size of the right forewing (figure 2). The body length was also significantly reduced on exposed bees (figure 3). We found a tendency to decrease in size with respect to the initial conditions of the experiment in fipronil exposed bees, but this significant difference is not easy to observe with the naked eye.

To evaluate morphological parameters we considered a random sample of 200 working bees in the hive. Sublethal doses of fipronil caused severe malformations mainly on the wings (figure 4). In early October live bees had no visible abnormalities, but began to be found bees inside and near the hives, and we observed that they were unable to fly. They tried to fly out of the hive but fell to the ground and were unable to undertake normal flight. The number of bees with abnormalities increased in bees dead, 1 out of 4 in exposed hives with fipronil possesses a visible malformation. For the measurement of morphological characters, bees with visible abnormalities were not considered, only those that were visible healthy were considered.

From May to December 2015, the number of sealed brood per hive was counted every 15 days. As temperatures began to decrease in late May 2015, we observed a steady decline of bee cluster size in fipronil treated colonies; while the untreated control maintained its size. While such decline in the fipronil colonies was slightly reversed in July 2015, the untreated control hives started to increase quickly in spring (figure 5). The number of the frame for fipronil hives decreased from May to June ($p < 0.001$), however, this decline changed in July-December and hives grew slowly. We found honey bee colonies in both control and fipronil treated groups progressed differently and observed no acute morbidity or

Table 2. ANOVA and Tukey test in *A. mellifera*. T0: May 1st 2015; T1: October 2nd 2015. F: Fipronil; C: Control. Stockings with a common are not significantly different ($p > 0.05$); ***: Significantly different. Alpha: 0.05. Fipronil dose 0.025 µg g^{-1}.

ANOVA	Length of the left antenna				Area of the right forewing				Bee length			
	T0		T1		T0		T1		T0		T1	
N	180		180		180		180		180		180	
p-value (Treatment)	0.3168		<0.0001 ***		0.1893		<0.0001 ***		0.3290		<0.0001 ***	
Tukey Test	**Length of the left antenna**				**Area of the right forewing**				**Bee length**			
	T0		T1		T0		T1		T0		T1	
DMS	0.09852		0.07591		0.10669		0.10450		0.10422		0.09617	
Error	0.1134		0.0673		0.1330		0.1276		0.1269		0.1081	
Treatment	F	C	F	C	F	C	F	C	F	C	F	C
Mean	3.89	3.94	3.05	3.93	14.02	14.09	12.57	14.04	13.13	13.19	12.89	13.20
Letter	A	A	B ***	A	A	A	B ***	A	A	A	B ***	A

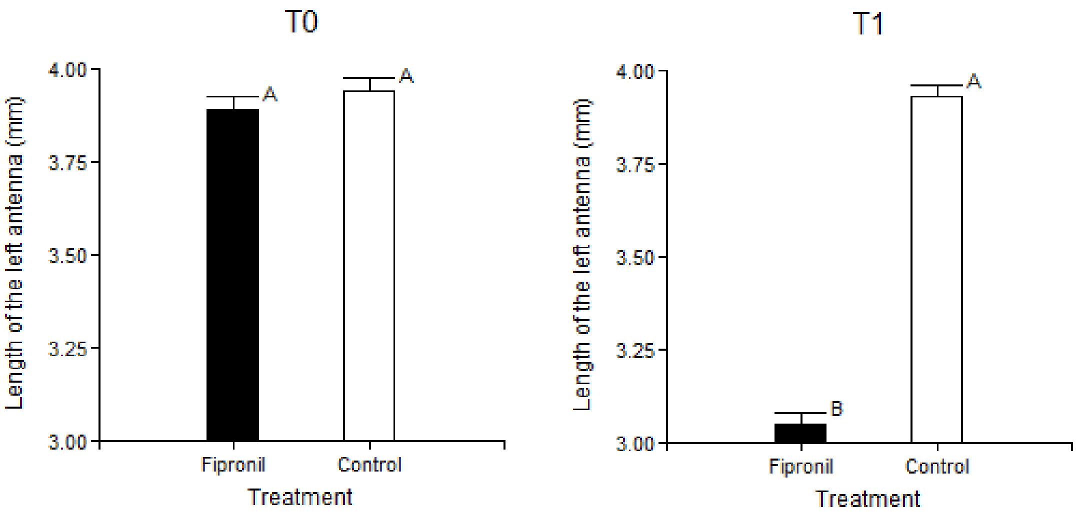

Figure 1. Length of the left antenna of *A. mellifera* non-exposed or exposed during treatment days to fipronil. The lines above the bars indicate the standard deviation error of the mean. T0: May 1st 2015; T1: October 2nd 2015. Fipronil dose 0.025 μg g^{-1}.

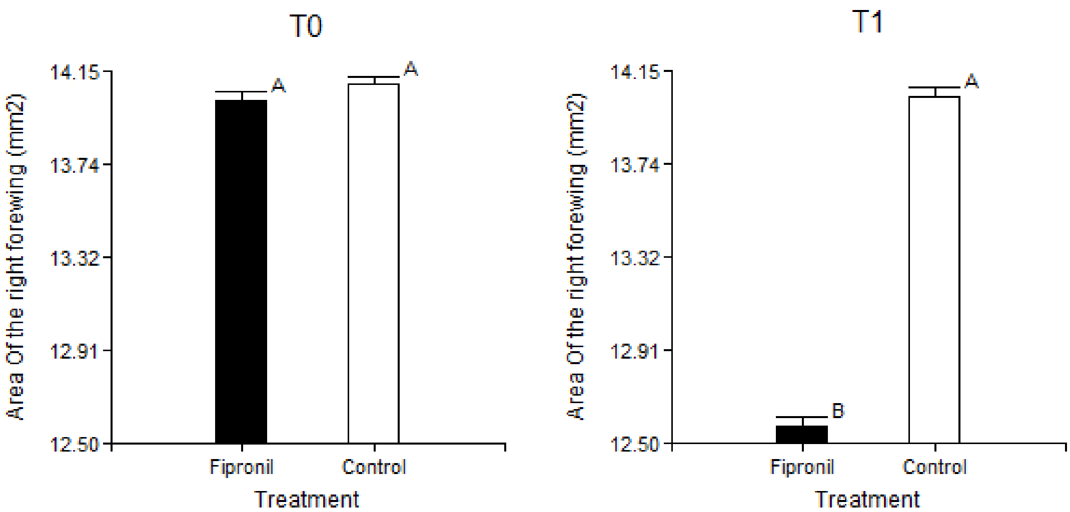

Figure 2. Area of the right forewing of *A. mellifera* non exposed or exposed during treatment days to fipronil. The lines above the bars indicate the standard deviation error of the mean. T0: May 1st 2015; T1: October 2nd 2015. Fipronil dose 0.025 μg g^{-1}.

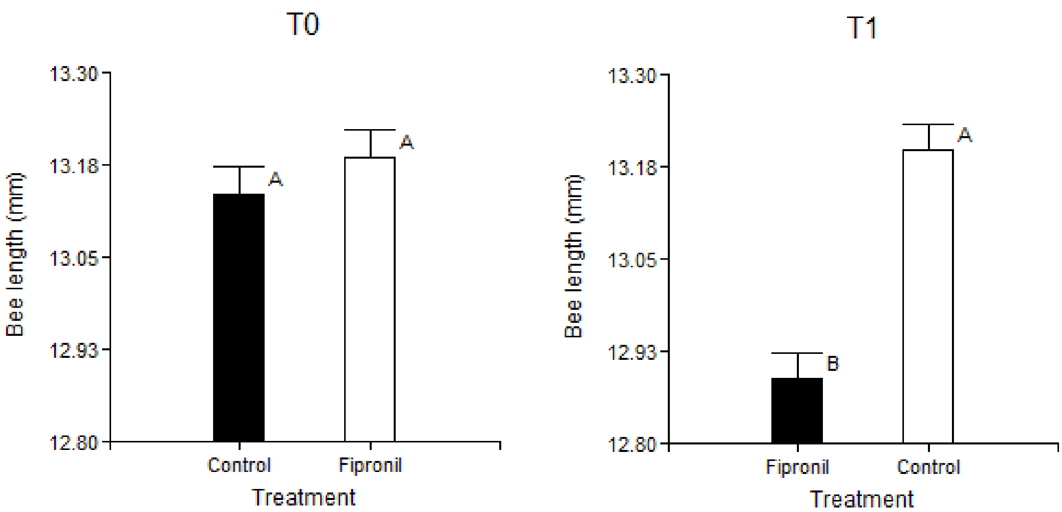

Figure 3. Bee length of *A. mellifera* non exposed or exposed during treatment days to fipronil. The lines above the bars indicate the standard deviation error of the mean. T0: May 1st 2015; T1: October 2nd 2015. Fipronil dose 0.025 μg g^{-1}.

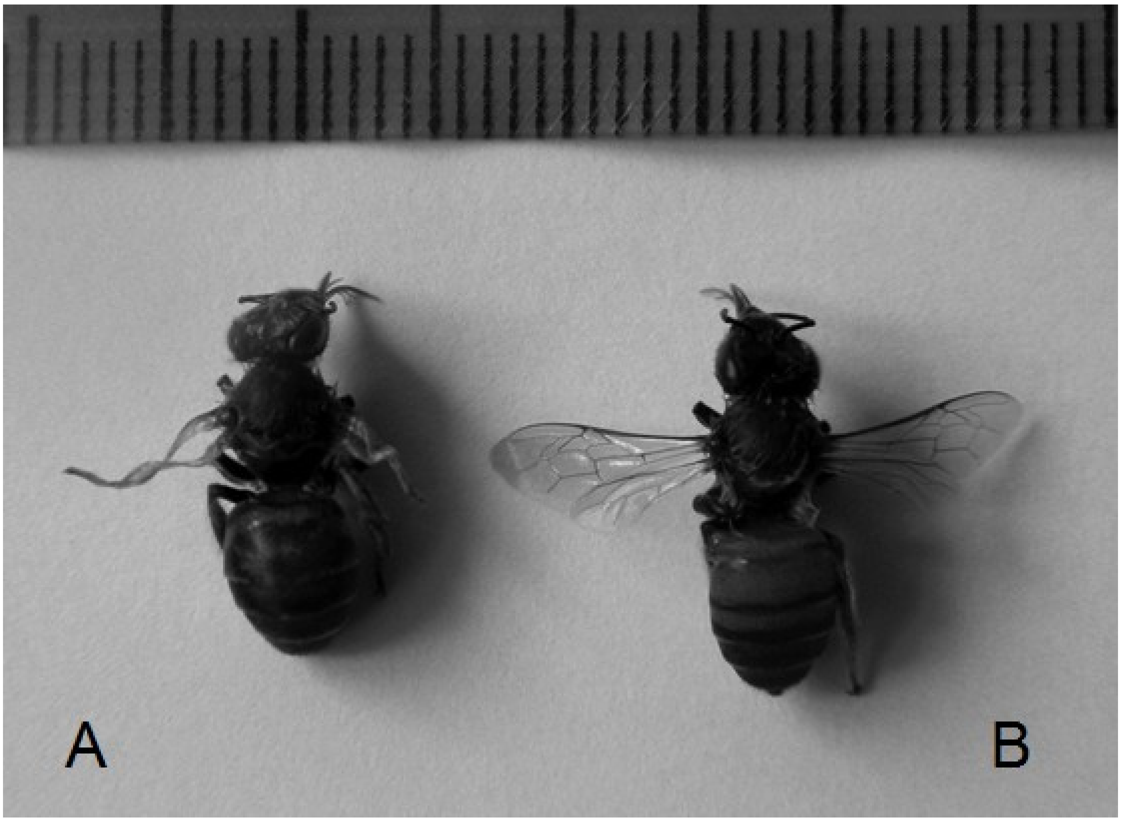

Figure 4. Aspect of the bees and abnormal development. A) Exposed to dose 0.025 µg g^{-1} of fipronil. B) Untreated bee.

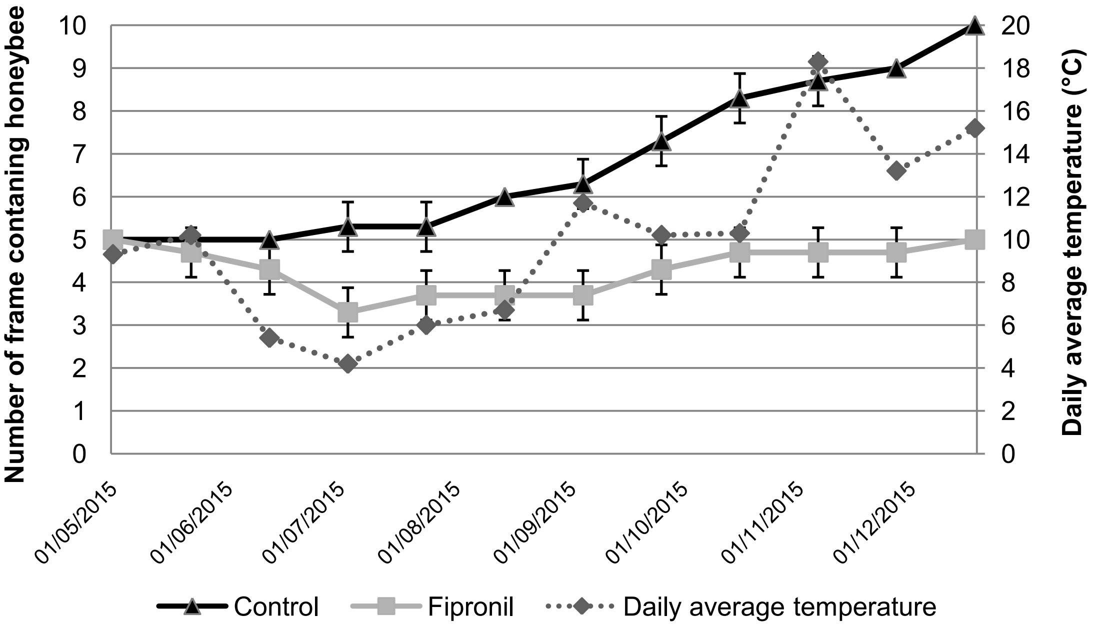

Figure 5. Average numbers of the frame (standard deviations shown as error bars) containing honey bees for control and fipronil treated colonies and the corresponding daily average temperature at AgrometINIA agro-meteorological network recorded from May to December 2015 (http://agromet.inia.cl/estaciones.php#estaciones).

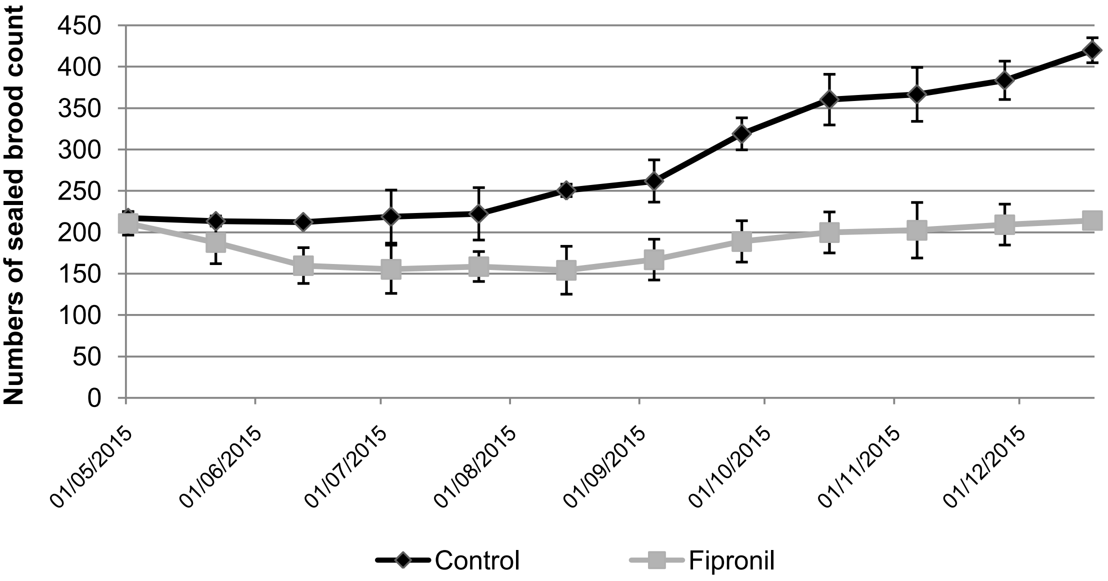

Figure 6. Average numbers of sealed brood count (standard deviations shown as error bars) for control and fipronil treated colonies during the dosing period (May to October 2015). Sealed brood counts among treatments are significantly different (one-way ANOVA).

mortality in neither group until the arrival of autumn or winter. Fipronil hives began to show signs of weakness throughout December 2015; this is probably due to the increased deaths in the hives. There was no loss of hives perhaps due to the increased feeding of the hive in the spring. Figure 6 shows the progression of sealed brood in control and fipronil treatment with different evolution during the experiment.

Discussion and conclusions

Even though most of the studies on the effect of sublethal exposure are generally represented by behavioural traits in honey bees, we consider that the assessment of fipronil effects on the morphology of bees could be relevant to risk assessment. According to our results, the sublethal exposure to fipronil in honey bees produces an abnormal development of the antennae and wings (figures 1 and 2) and a smaller body size in bees (figure 3). These abnormalities could be present at the beginning of the pupal stage. There are previous reports of adverse effects resulting in corporal malformations such as the absence of the head and appendices (Silva *et al*., 2015). The malformations may be due to the influence of fipronil on fat tissue during the postembryonic development of the bee, as this tissue acts on hormone transport-related proteins that are necessary for metamorphosis, such as hexamerins (Locke, 1998; Martins and Bitondi, 2012). The fat body is in direct contact with the haemocoel. If we assume that insecticides and natural metabolites are present in the haemocoel, it is expected that the action and interaction of the insecticide molecules with the fatty body is very fast, increasing under low temperature conditions (Locke, 1998; Medrzycki *et al*., 2010). Fipronil acts as an antagonist of the GABA and glutamate-gated chloride (GluCl) receptors, blocking the inhibitory networks in the bees' brain (Barbara *et al*., 2005), which could lead to problems in their communication, potentially leading to the death of the honey bee.

Malformations in the antennae of honey bees could cause problems in the development of hives, because their role in the daily life of bees is vital: sensitivity to humidity, air pressure, temperature, odour, near-field sound vibrations, gustatory stimuli, tactile contact and substrate vibrations (Suwannapong *et al*., 2011). Tactile cues play a major role in the life of the hive. Adult bees use mechanical stimuli for intraspecific communication and the construction of new cells (Kevan, 1987). Due to this, the results of our investigation regarding the reduction of the average length of the left antenna by 21.5% in bees of hives exposed to fipronil (table 2), should represent a limitation in the capacities to perceive the environment, affecting their social communication. Exposure to fipronil in honey bees also alters responses to the olfactory learning procedure (Aliouane *et al*., 2009) and decreases the success of information acquisition, favouring a lower memory performance, leading to a reduction in learning outcomes necessary for the survival of the colony (Decourtye *et al*., 2005). Also, the ability of the exposed honey bees to fly toward the hive after feeding would be impaired by the ingestion of solutions contaminated with fipronil. According to Decourtye *et al*. (2011) bees fipronil-exposed reduce the number of foraging trips. The area of the left hind wing in the bees of hives exposed to fipronil decreased significantly by 10.3% on average (table 2). Both, the reduction of the wing's area and the adverse effects on their memory, may severely decrease their ability to carry out the foraging activities. Henry *et al*. (2012) demonstrated the detrimental effects on spatial orientation capacities in forage bees produced by exposure to insecticides such as thiamethoxam. The adverse effects of insecticide exposure on the normal development of antennae and wings are disadvantageous regarding their carrying capacity. The communication about the location of the food sources is done through a dance performed by the exploring bees; thus, smaller wings may limit this capacity and also reduce the probability to find food. It has been reported that exposure to fipronil may lead to behaviours that lessen the efficiency of foraging and also result in reductions in the proportion of active bees in hive flights (Pisa *et al*., 2015). Finally, at the end of the experiment period, abnormalities in the size of bees exposed to sublethal doses of fipronil were also found (table 2), but this difference in size, statistically significant, is not easy to assess visually. It has been investigated that the body size in bees is key in the fitness of the colony and is related to environmental and food factors, at least 75.5% of the size variations in worker bees are attributed to food collection necessities and not for phylogenetic effects (Pignata and Diniz-Filho, 1996). According Greenleaf *et al*. (2007), after studying 62 species of bees, they determined that a relationship can be established between the size of the bee's body and feeding distances, where a smaller size of the bee supposes a smaller feeding distance. In *Melipona mandacaia* Smith the existence of a relationship between the range of alimentation of individuals and the hive and their body size is known (Kuhn-Neto *et al*., 2009), this relationship would also be observed in our research in the hives of bees exposed to fipronil.

At the beginning of spring, fipronil-treated hives had an average of 3.7 active breeding frames, while untreated control hives had 6.3 breeding frames, on average (figure 5). According to Decourtye *et al*. (2011) oral treatment of 0.003 µg of fipronil per bee reduced the number of foraging trips; this directly limit the potential for collecting pollen and nectar for colonies in hives, thus reducing honey yield. Considering the growth potential of hives regarding the creation of breeding frames, we observed that in the first summer crop (December), hives exposed to fipronil manage to maintain only five breeding frames while control hives get to reach 10 breeding frames for the same period (figure 5). Non exposed hives to fipronil displayed a greater capacity for growth, which allows them to have more workers who play the role of seeking food, which favours foraging activities and increases the total carrying capacity of hives. It has been demonstrated *in-vitro* that the exposure to sublethal doses of fipronil reduces the number of hatching eggs and also reduces the area occupied by the eggs of worker bees in hives (Silva *et al*., 2015). A similar result was obtained in *in-vivo* conditions, which would suggest that the mechanisms that affect the development of the bees exposed to the pesticide would follow the same tendency in function of the colonization of breeding frames and growth in hives under field conditions. The exposure to chronic sublethal doses of fipronil have been suspected of impairing the performance of bees by inhibiting their learning and foraging activities (Decourtye *et al*., 2003; Pedraza *et al*., 2013), which in our experiment would be explaining differences in the creation of breeding frames between control hives and exposed to fipronil. Along with this, the creation of new breeding frames would also be limited by the number of individuals born alive in the hive. The decrease in the number of sealed offspring and brood frames in the autumn-winter period (figures 5 and 6) can be explained by the limiting effect of fipronil on colony development. Sublethal doses of fipronil have limited the number of larvae and pupae to develop properly in hives (Silva *et al*., 2015).

The population of a bee hive varies constantly during each season of the year. However, if adverse effects cause changes in the normal feeding and the health of the hive, the probability of population mortality and the collapse of the hive, also increases. If this increase in mortality occurs between spring and summer, its effect may not easily visible, because there are abundant sources of food and breeding frames. But if mortality occurs from autumn to winter, when food availability decreases, it can effectively cause a collapse in the colony (vanEngelsdorp *et al*., 2011). If we consider that at the beginning of the trial, all the hives had a similar number of sealed brood and breeding frames in the hives, then the ability to cope and survive the winter would be mainly determined by the impact of fipronil in the exposed colonies. Fipronil induce an insecticidal action by affecting the ligand-controlled chloride channel (Narahashi *et al*., 2010), which can not only potentially affect the development of the pupae, but also limit its occurrence. During the autumn and winter periods, the sealed brood in hives exposed to fipronil, by early July, showed a significant decrease (about 26.5%) with respect to the initial value, while in the control hives there were not significant changes, although a wide variability was observed among hives (figure 6).

When spring arrives, the availability of pollen and nectar also increase due to flowering, and there is a slight increase in the number of sealed brood in hives, so the consequences of exposure to fipronil, are mitigated (figure 6). In control hives, the number of sealed offspring increased by an average of 37.4%, between September and October, thus, the observed negative impact on the treated colonies is attributed to fipronil exposure. It is important for beekeepers and crop growers to understand the adverse effects that some pesticides may cause to the wellbeing of bees in order to avoid their use. These adverse effects are detected through abnormalities in the forage activities of the bee, such as stumbling, showing lack of coordination, staying still, lying on its back or remaining still beating its wings (Vidau *et al*., 2011).

The detrimental effects on bees produced by exposure to seed treatment insecticides such as fipronil and neonicotinoids, it is necessary to develop a new control strategy for wireworms and whiteworms, a measure is the application of an Integrated Pest Management (IPM). According to Barsics *et al*. (2013) it is possible to establish strategies for wireworms realizing a identifying the areas of high risk, planting sensible crops in areas with little risk and locating areas with populations wireworms over thresholds, which could reduce the use of insecticides.

Although there was no evidence of a collapse in fipronil exposed hives during the winter season, this insecticide caused abnormal growth in the exposed bees, in comparison with the untreated ones. When bees are exposed to the dose of 0.025 μg g^{-1} of fipronil, during six consecutive months, the left antenna, the right wing area and the size of the bees in previously healthy colonies, are severely affected in their development. The survival of colonies with sublethal doses of fipronil produces individuals with deteriorated development during the winter. The mechanism by which sublethal exposure of fipronil in honey bees causes abnormal development in their hives, seasonally, needs to be clarified.

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