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Efecto de fipronil en núcleos de abejas

Effect of fipronil in cores of bees

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EFECTO DE FIPRONIL EN NÚCLEOS DE ABEJAS

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TABLA DE CONTENIDOS

ÍNDICE DE FIGURAS Y TABLAS

Página

EFECTO DE FIPRONIL EN NÚCLEOS DE ABEJAS

EFFECT OF FIPRONIL IN CORES OF BEES

Palabras adicionales: Fenilpirazol, Neurosistémico, morfología, melisopalinología, pecoreo

RESUMEN

Las abejas desempeñan un rol ecológico fundamental como polinizadores de muchas especies vegetales. A pesar de su importancia, el uso irracional de agroquímicos podría poner en peligro estos insectos. En este estudio, el objetivo fue investigar si la exposición subletal a fipronil influye negativamente en la morfología, producción de miel y hábitos de pecoreo de las abejas. Se utilizaron seis colmenas las cuales se alimentaron con suplemento de agua azucarada durante el otoño-invierno 2015 y otras tres con cantidades conocidas de fipronil (0.00125 µ g por abeja). Se permitió que las seis colmenas se alimentaran libremente de lo que había presente en el lugar del experimento. Las tres colonias tratadas sobrevivieron a la dosis subletal de fipronil, sin embargo al final del invierno, la exposición subletal al insecticida en comparación con las colonias control produjo una disminución de la longitud de la antena izquierda, del área del ala anterior derecha y de la longitud de las abejas. Las colmenas expuestas disminuyeron significativamente su producción de miel entre la temporada 2014-2015 y 2015-2016. En los periodos de cosecha (diciembre y marzo) la disminución fue en promedio superior al 50% (diciembre 57,2% y marzo 53,9%). Las colmenas tratadas con fipronil evidenciaron una disminución en la recolección de polen de Brassica (Raps) distante a más de un kilómetro de las colmenas, de una temporada a otra. Además en el mismo periodo aumentó la participación en la miel de polen de las familias Fabaceae y Poaceae, que se encontraban más cercanas a las colmenas. La exposición no parece comprometer la inmunidad de las abejas melíferas hacia la infección patógena. Se concluye que cuando las abejas se exponen a una dosis subletal de fipronil desarrollan malformaciones morfo anatómicas y disminuyen la producción de miel de la colmena, presentando una variación en sus hábitos de alimentación.

SUMMARY

Bees play a fundamental ecological role as pollinators of many plant species. Despite its importance, the irrational use of agrochemicals could endanger these insects. In this study, the objective was to investigate whether the sublethal exposure to fipronil negatively influences the morphology, honey production and beekeeping habits of bees. Six hives were used which were fed with sugar water supplementation during autumn-winter 2015 and three with known amounts of fipronil (0.00125 μg per bee). The six hives were allowed to feed freely from what was present at the experiment site. The three colonies treated survived the sublethal dose of fipronil, however at the end of winter, sublethal exposure to the insecticide compared to control colonies resulted in a decrease in the length of the left antenna, the right anterior wing area, and the Length of the bees. Exposed hives significantly decreased their honey production between the 2014-2015 and 2015-2016 seasons. In the harvest periods (December and March) the decrease was on average over 50% (December 57.2% and March 53.9%). The hives treated with fipronil evidenced a decrease in the collection of pollen from Brassica (Raps) distant to more than a kilometer of the hives, from season to season. In addition, in the same period increased participation in the pollen honey of the families Fabaceae and Poaceae, which were closer to the hives. The exposure does not seem to compromise the immunity of honey bees to pathogenic infection. It is concluded that when bees are exposed to a sublethal dose of fipronil, they develop anatomical morphological malformations and decrease the honey production of the hive, presenting a variation in their eating habits.

INTRODUCCIÓN

A través de la polinización de las plantas, las abejas (*Apis mellifera* L., Hymenoptera, Apoidea) desempeñan un rol fundamental en la mantención de la biodiversidad global y la producción de muchos de los cultivos alimentarios más importantes del planeta (Klein *et al.*, 2007; Bradbear, 2009; Breeze *et al.*, 2011). La contribución de los polinizadores a la producción de cultivos alimenticios destinados para el consumo humano, en términos de capital, se ha estimado en 153.000 millones de euros en todo el mundo; lo que representa aproximadamente el 9,5% del valor total de la producción de alimentos consumidos por la humanidad (Gallaia *et al*., 2009). Según la Organización de las Naciones Unidas para la Agricultura y la Alimentación (FAO), entre las 100 especies de plantas que conforman los alimentos más consumidos por el hombre y que aportan el 90% de los alimentos en el mundo, un 71% de estas son polinizadas por las abejas melíferas (FAO, 2005; UNEP 2010). Existen numerosos reportes sobre la importancia de la polinización de plantas debido a la actividad de forrajeo de las abejas, en la cual las colonias recogen polen, néctar, agua y resina (Young *et al.*, 2007; Klein et al., 2007; Eilers *et al*., 2011; Sushil *et al*., 2013).

Las abejas en su actividad de pecoreo visitan diferentes especies vegetales en busca de su alimento, en este proceso se embadurnan de los diversos granos de polen de las plantas que visitan (Navarrete *et al*., 2016). Los granos de polen presentan una morfología bien definida y conservativa lo que permite la identificación de la familia botánica a la cual pertenece y en algunos casos, la especie de la cual procede (García *et al*., 2015). Debido a lo anterior, es posible realizar un análisis físico-.químico para determinar el origen botánico y geográfico de la miel mediante su composición polínica. El procedimiento para la determinación del contenido polínico de la miel se denomina melisopalinología (Soria *et al*., 2004; Corvucci *et al*., 2015) y los resultados obtenidos también se utilizan para correlacionar los parámetros climáticos *in situ* como la lluvia y la temperatura (Bilisik *et al*., 2008). El conocer el contenido polínico de la miel permite visualizar el contexto de factores externos que influyen en los polinizadores y las redes de polinización (Jens *et al*., 2008; Selva Singh Richard *et al*., 2011; Nascimento y Nascimento, 2012). Por otra parte, el polen recogido por las abejas puede contener residuos de plaguicidas, que se utilizan en la mayoría de los cultivos alimentarios, lo que puede afectar la salud de las abejas (Mullin *et al.*, 2010). Cualquier alteración en la salud de las abejas implica una reducción en su capacidad biológica, longevidad y polinización lo

que produce una reducción en el rendimiento de los cultivos (Loos *et al.*, 2010; Potts *et al.*, 2010; Jansen *et al.*, 2011). La capacidad de los polinizadores para resistir las enfermedades y parásitos parece estar influenciada por varios factores presentes en su vida cotidiana, estado nutricional y exposición a sustancias químicas tóxicas (Genersch *et al*., 2010).

Las evidencias sugieren que muchas poblaciones de abejas silvestres y domesticadas, están disminuyendo en todo el mundo (Nguyen *et al.*, 2009; Holmstrup *et al.*, 2010; Johnson *et al.* 2010; Moritz *et al.*, 2010; Calderone, 2012; Gill *et al.*, 2012; Biondi *et al.*, 2013; González-Varo *et al.*, 2013; Sanchez-Bayo *et al.*, 2014; Goulson *et al.*, 2015). Este declive poblacional es probablemente debido a múltiples presiones simultáneas sobre el ecosistema que incluye la pérdida de hábitat, factores ambientales, patógenos y el impacto de los plaguicidas (Mullin *et al.*, 2010; Potts *et al.*, 2010; Kluser *et al.*, 2011; Vidau *et al.*, 2011; Martínez *et al.*, 2012; Botías *et al.*, 2013; Schmehl *et al.*, 2014; Goulson *et al.*, 2015; Calatayud-Vernich *et al.*, 2016). Los plaguicidas pueden afectar también negativamente a las abejas indirectamente, haciéndolas más susceptibles a plagas y patógenos (Pettis *et al*., 2004; Vidau *et al.*, 2011; Goulson *et al.*, 2015).

En la actualidad, las poblaciones de abejas se exponen a menudo insecticidas del grupo de los neonicotinoides y fenilpirazoles, que son compuestos neurotóxicos sistémicos de uso agrícola intensivo en todo el mundo contra plagas de insectos (Mullin et al., 2010, Vidau et al, 2011; Sánchez - Bayo y col., 2014). El insecticida fipronil, de la familia química fenilpirazol, se utiliza ampliamente contra plagas de artrópodos en cultivos de todo el mundo (Mullin *et al*., 2010). Este insecticida actúa sobre el sistema nervioso de los insectos mediante el bloqueo de los canales de cloro regulados por el ácido gamma-aminobutírico y el glutamato (Barbara *et al.*, 2005; Gunasekara *et al.*, 2007; Thompson, 2010). Desde finales de 2013, la Unión Europea restringió por dos años su uso. Esta decisión se apoyó en un estudio realizado por la Autoridad Europea de Seguridad Alimentaria (EFSA), que concluyó que fipronil constituye un grave riesgo para las poblaciones de abejas (EC, 2013; EFSA, 2013). Según la Autoridad Europea de Seguridad Alimentaria, las semillas tratadas con Fipronil son un riesgo para las poblaciones de abejas.

Se ha estudiado que Fipronil afecta la movilidad de *A. mellifera* provocando un aumento en el consumo de agua y un deterioro progresivo de la capacidad olfativa de las abejas (Aliouane *et*

al., 2009). De hecho, las dosis subletales de fenilpirazoles inducen múltiples efectos negativos como alteraciones de comportamiento o fisiológicos en abejas y otros artrópodos beneficiosos para el ecosistema y el hombre (Desneux *et al*., 2007). Además el mecanismo de acción de fipronil sobre la señalización neuronal de las abejas puede resultar potencialmente en su mortalidad (Gunasekara *et al*., 2007). Igualmente existen antecedentes de que las abejas melíferas hay sinergias negativas entre los insecticidas neonicotinoides y los fenilpirazoles como el fipronil (Vidau *et al*., 2011). La permanencia documentada de este insecticida es de 120 días después de la aplicación mientras que los residuos de sus metabolitos se calcularon en 0,047 μg g^{-1} de suelo (Cummings *et al.*, 2006). Por ejemplo las concentraciones de fipronil en suelo sobre arrozales aumentaron hasta los tres días después del trasplante, luego disminuyeron lentamente hacia el día 14, y finalmente permanecieron bastante estables en el tiempo (Kasai et al., 2016).

En Chile, fipronil está autorizado por el Servicio Agrícola y Ganadero (SAG) para los cultivos de trigo (*Triticum* spp), ballica (*Lolium perenne* L.) y maíz (*Zea mays* L.). Según estimaciones de la Oficina de Estudios y Políticas Agrarias (ODEPA) estos cultivos para la temporada 2015-2016, en la que se realizó la presente investigación, ocuparon una superficie sembrada de 285.297 hectáreas para trigo y 101.740 hectáreas para maíz. La superficie cultivada de trigo y maíz en conjunto, representa un 52,71% de la estimación de superficie sembrada a nivel nacional para el año agrícola 2015-2016 (ODEPA, 2016). Por lo anterior, las colmenas de abejas melíferas en las cercanías de estos cultivos tendrán una alta probabilidad de quedar expuestas a fipronil, debido a que el polen recolectado por las abejas puede llevar consigo altos niveles de residuos de plaguicidas (Mullin *et al.*, 2010). Sin embargo, y a pesar de los antecedentes existentes sobre el tema, aún se continúan utilizando en la agricultura a nivel nacional y mundial plaguicidas sistémicos neurotóxicos; como fipronil, cuyo potencial efecto en la muerte de las colonias de abejas, así como en sus hábitos de pecoreo y el desarrollo de la colmena, es aún desconocido.

HIPOTESIS

La hipótesis puesta a prueba, sustentada en la fundamentación precedente, es que la exposición de colmenas de abejas melíferas (*Apis melífera L.*) a dosis subletales del insecticida neurotóxico Fipronil influye negativamente en la morfología y producción de miel, junto con producir una variación en los hábitos de pecoreo de las abejas.

OBJETIVOS

Objetivo general

Estudiar el efecto neurotóxico de la exposición a dosis subletales del insecticida sistémico, Fipronil, en el desarrollo, producción y sobrevivencia de los núcleos de abejas melíferas (*Apis melífera* L.).

Objetivos específicos

Determinar la existencia de variaciones morfológicas en abejas melíferas expuestas a dosis subletales de Fipronil por medio de un análisis morfo anatómico.

Evaluar la influencia de la exposición al insecticida Fipronil en los hábitos de pecoreo de la colmena por medio del análisis del polen constitutivo y volumen de la miel producida.

CAPÍTULO 1

Enviado a la revista Bulletin of Insectology.

SUBLETHAL EXPOSURE TO FIPRONIL AFFECTS THE MORPHOLOGY AND DEVELOPMENT OF HONEYBEES (*APIS MELLIFERA* L.)

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ABSTRACT

Honeybees play an important 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 fipronil affects the morphology and causes any abnormal development of bee colonies during the winter. Six hives that were used, were fed with sugar water (Sugar and water ratio, 2:1) during the experiment; three of them were also fed with known amounts of fipronil (0.00125 μg per bee). Six colonies are allowed to feed freely from the environment. Bees that were exposed to sublethal doses of fipronil for six consecutive months (May to October 2015), had abnormal development of wings and antennae. Colonies that survived this dose of fipronil exhibited abnormal development during the winter, but this exposure does not appear to compromise the honeybee's immunity to pathogenic infection. We conclude that when bees exposed to the sublethal dose of fipronil during winter, they developed an abnormal growth in relation to the length of the left antenna, the area of the right wing and the size of the honeybee.

Keywords: Neurosystem pesticide, phenylpyrazole, fipronil, honey bees, morphology

INTRODUCTION

Through pollination of plants, honeybees (*Apis mellifera* L, Hymenoptera, Apoidea) 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) among the 100 most consumed species of food crops, which provide 90% of food in the world, 71% are pollinated by honeybees (FAO, 2005; UNEP 2010). Any alteration in the health of bees implies a reduction in its biological capacity, longevity, pollination that lead to reduction in crop yield (Loos *et al.*, 2010; Potts *et al*, 2010; Jansen *et al.*, 2011). Overwhelming evidence suggests that many wild and managed populations of bees are currently declining worldwide (Nguyen *et al*., 2009; Holmstrup *et al*., 2010; Johnson *et al*., 2010; Moritz *et al.*, 2010; Calderone, 2012; Gill *et al*., 2012; Biondi *et al*., 2013; González-Varo *et al*., 2013; Sanchez-Bayo *et al*., 2014; Goulson *et al*., 2015). This decline is likely due to multiple simultaneous pressures, including habitat loss, climate change, pathogens and the impact of pesticides exposure (Williams *et al*., 2010; Mullin *et al*., 2010; Potts *et al*., 2010; Kluger *et al*., 2011; Vidal *et al*., 2011; Martínez *et al*., 2012; Botías *et al*., 2013; Schmehl *et al*., 2014; Goulson *et al*., 2015; Calatayud-Vernich *et al*., 2016).

Pesticides can adversely affect honeybees also indirectly, such as, making them more susceptible to pests and pathogens (Pettis *et al*., 2004; Vidau *et al*., 2011; Goulson *et al*., 2015). Currently, the honeybees populations are often exposed to the neonicotinoids and the phenylpyrazoles insecticides, which are systemic neurotoxic compounds of intensive agricultural use worldwide of the against insect pests (Mullin *et al*., 2010; Johnson *et al*., 2010; Vidau *et al*., 2011; Sanchez-Bayo *et al*., 2014) and they are linked to adverse effects to honeybees (Pedraza *et al*., 2013; Nicodemo *et al*., 2014; Lunardi *et al*., 2017). Since late 2013, the European Union restricted for two years the use of Fipronil. This decision was supported on a study carried on by the European Food Safety Authority (EFSA) which concluded that fipronil pose a serious risk to the honeybee 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; Gunasekara *et al*., 2007; Thompson, 2010). It has been studied 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 behavioral or physiological alterations in bees and other beneficial arthropods (Desneux *et al*., 2007). In honeybees there is negative synergies between neonicotinoid insecticides and phenylpyrazoles such as fipronil on *Ceranae* something here is missing (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 of soil (Cummings *et al*., 2006). The concentration of fipronil in soil, on rice, increased three days after transplanting, and decreased slowly during the next14 days; after that, and it remained fairly stable (0.241 μg kg−1) (Kasai *et al*., 2016). However, pesticides of the systemic neurotoxic type of pesticides are still being used in agriculture at the local and global levels, such as fipronil, whose potential effect on the death of bee colonies and developmental habits of 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 the honeybees of a Carnica race because it corresponds to one of the most used in Chile due to their good yields and high adaptability (Montenegro *et al*., 2009). A total of 25 hives were established (between 26 and 29 September 2014), 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 experiment the number of breeding frames in the hive, the number of sealed brood count and the amount of honey produced. The six used hives were used were fed with sugar water (Sugar and water ratio, 2:1) during the experiment, three of them were also fed with 0.00125 μg of fipronil per bee, (the LD_{50} of fipronil of 0.00417 μg per bee). In addition, all colonies were allowed to feed freely on the environment, according to the modified method of Lu *et al*., 2014. Fipronil feeding was conducted during the months of May and October 2015.

Description of the study area

The trial was established in the area of Llano Verde (37 ° 43 'S, 72 ° 22' W, 184 m above sea level) at a distance of 10 kilometers from the city of Los Angeles, capital of the province of Biobio, Chile. The climate in the area is characterized by maintaining 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).

Morphological Analysis

A total of six colonies were subjected to morphological analysis. 60 workers were analyzed per sample, 30 workers set obtained before the trial and 30 after the establishment of test workers. Bees were dried and three morphological characters: size of each insect (mm), size of the left antenna (mm length), and wide of the right forewing (mm^2 wing area) were evaluated. 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 by means of 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 biweekly evaluation provides objective measures of breeding livestock between each colony. 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.

Tabla 1.1. Chronological characterization of the observed events in the bees colonies studied.

Date	Event
26-29 September 2014	Assembling 25 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.
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.
July-November, 2015	Winter hive strength monitoring.
December 2nd, 2015	Last count of sealed brood and recollection of bees to morphological measurement.
December, 2015	Collecting honey and counting frames.

Source: Elaborated with own data.

Analysis of data

An analysis of variance were carried out by means of ANOVA procedure and the means comparison by using the Tukey, alpha 0,05 (Windows InfoStat, 2015).

RESULTS

The treated 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 1.2). In the untreated control, treatment there were no significant differences in the size of the left antenna (Figure 1.1) and the size of the right forewing (Figure 1.2).. The exposure to fipronil decreased the size of the right forewing (Figure 1.2). The experiment started with healthy bees in both the treated and the untreated treatments. The body length was also significantly reduced on exposed bees (Figure 1.3). Were 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.

Tabla 1.2. ANOVA and Tukey test in *Apis mellifera*

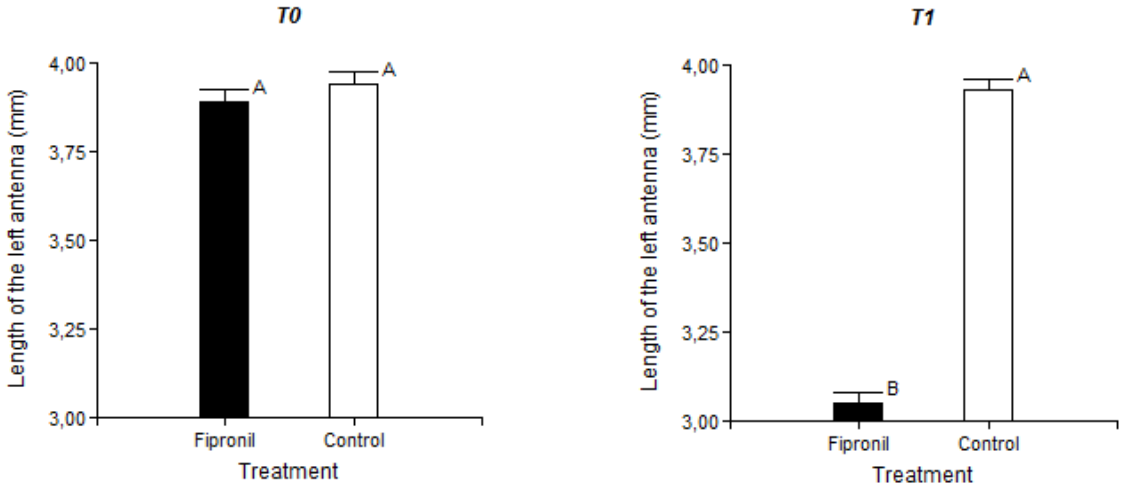
Analysis of Variance (SC Type I)						
	Length of the left antenna		Area Of the right forewing		Bee length	
	T0	T1	T0	T1	T0	T1
N	180	180	180	180	180	180
p-valor (Treatment)	0,3168	<0,0001 ***	0,1893	<0,0001 ***	0,3290	<0,0001 ***

Tukey Test												
	Length of the left antenna				Area Of the right forewing				Bee length			
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

F: Fipronil; C: Control. Stockings with a common are not significantly different (p> 0.05);

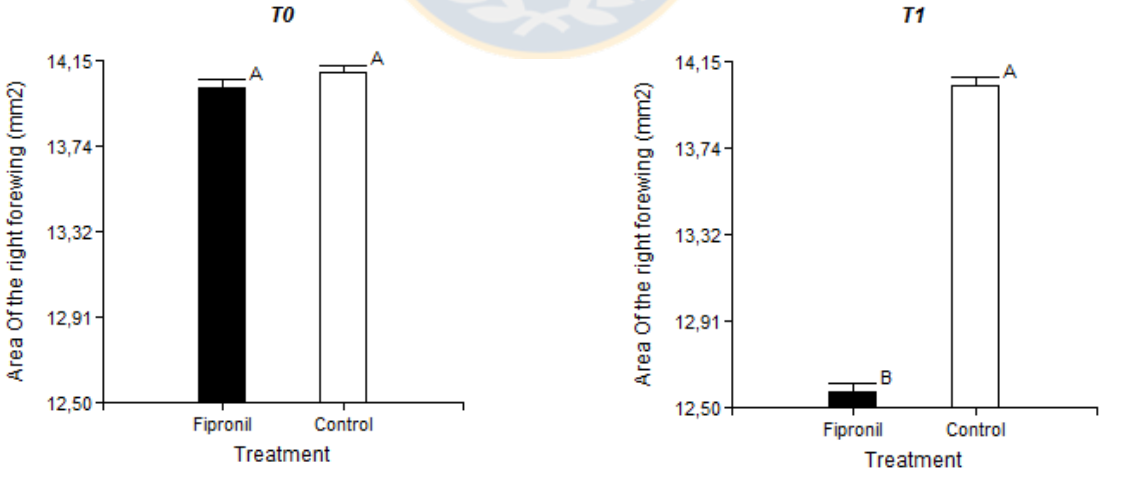
***: Significantly different. Alpha: 0,05. (Source: Elaborated with own data).

Figura 1.1. Length of the left antenna of *Apis mellifera* non-exposed or exposed during treatment days to fipronil at the dose 0.00125 µg per bee



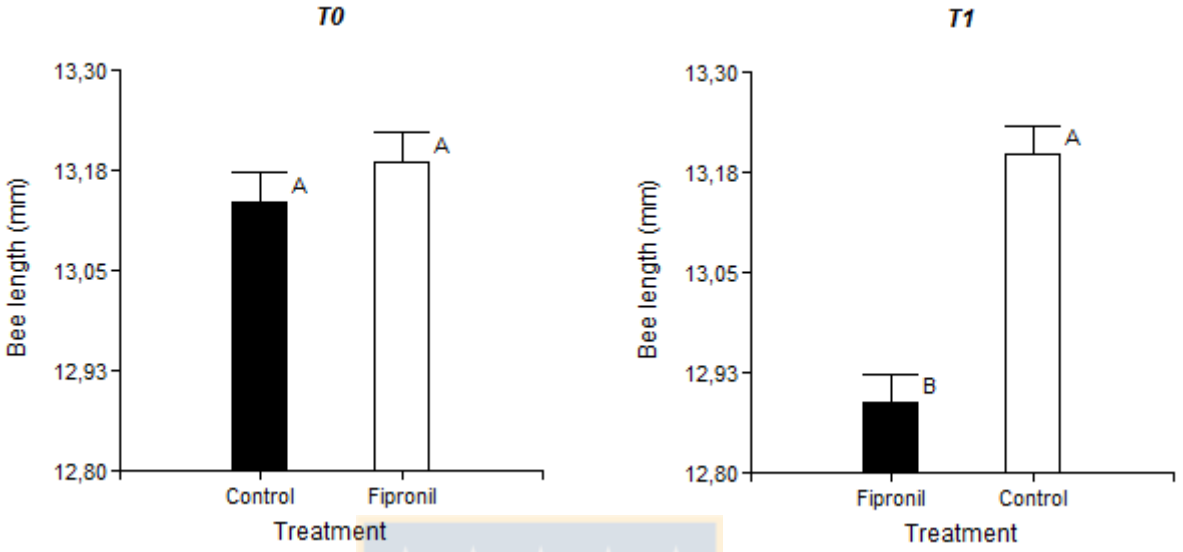
The lines above the bars indicate the standard deviation error of the mean. T0: 5/1/2015; T1: 12/2/2015. (Source: Elaborated with own data).

Figura 1.2. Area Of the right forewing of *Apis mellifera* non exposed or exposed during treatment days to fipronil at the dose 0.00125 µg per bee



The lines above the bars indicate the standard deviation error of the mean. T0: 5/1/2015; T1: 12/2/2015. (Source: Elaborated with own data).

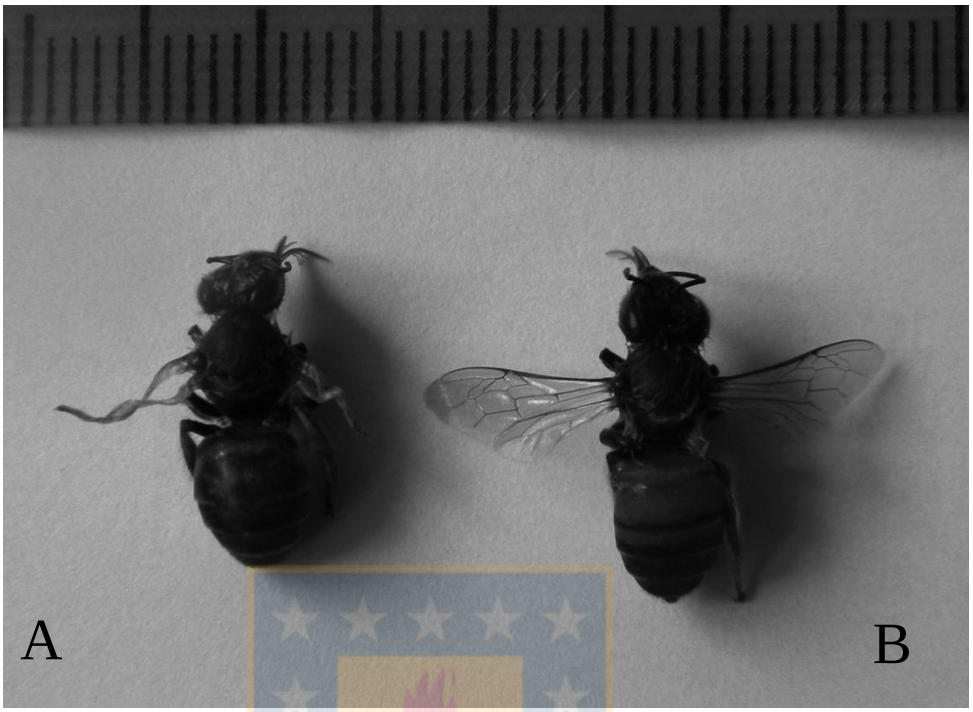
Figura 1.3. Bee length of *Apis mellifera* non exposed or exposed during treatment days to fipronil at the dose 0.00125 µg per bee



The lines above the bars indicate the standard deviation error of the mean. T0: 5/1/2015; T1: 12/2/2015. (Source: Elaborated with own data).

To morphological parameters we were considered a random sample of working bees in the hive. Sublethal doses of fipronil caused severe malformations mainly on the wings the wings (Figure 1.4). The number of bees with abnormalities increased from May to October 2015. In early October, bees with no visible abnormalities began to be found inside and near the hives, and we observed that they were unable to fly. They treated to fly out of the hive but fell to the ground and were unable to undertake normal flight.

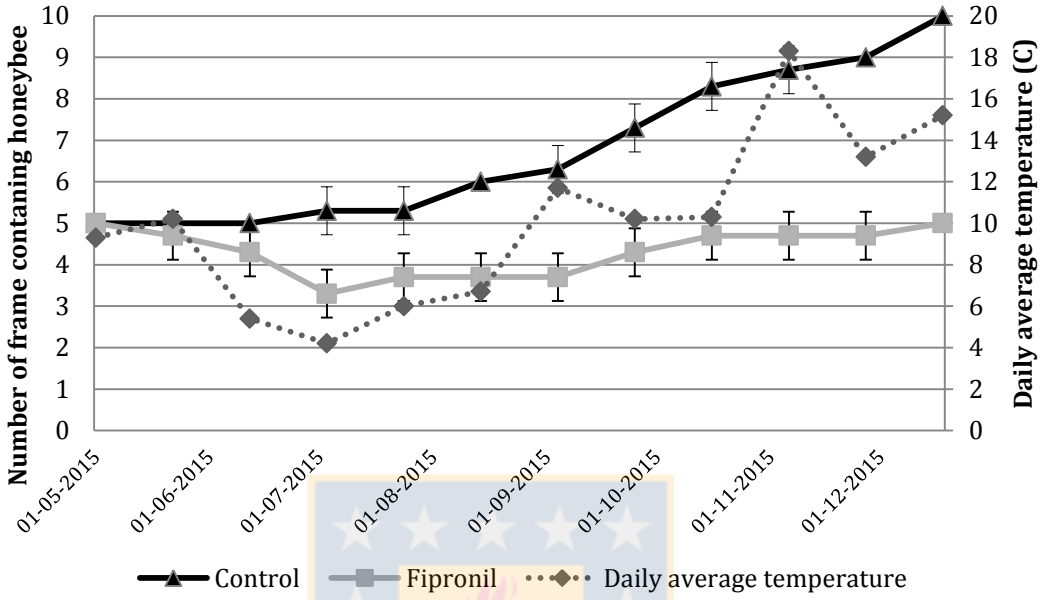
Figura 1.4.Aspect of the bees and abnormal development



A) Exposed to dose 0.00125 µg per bee of fipronil. B) Untreated bee. (Source: Elaborated with own data).

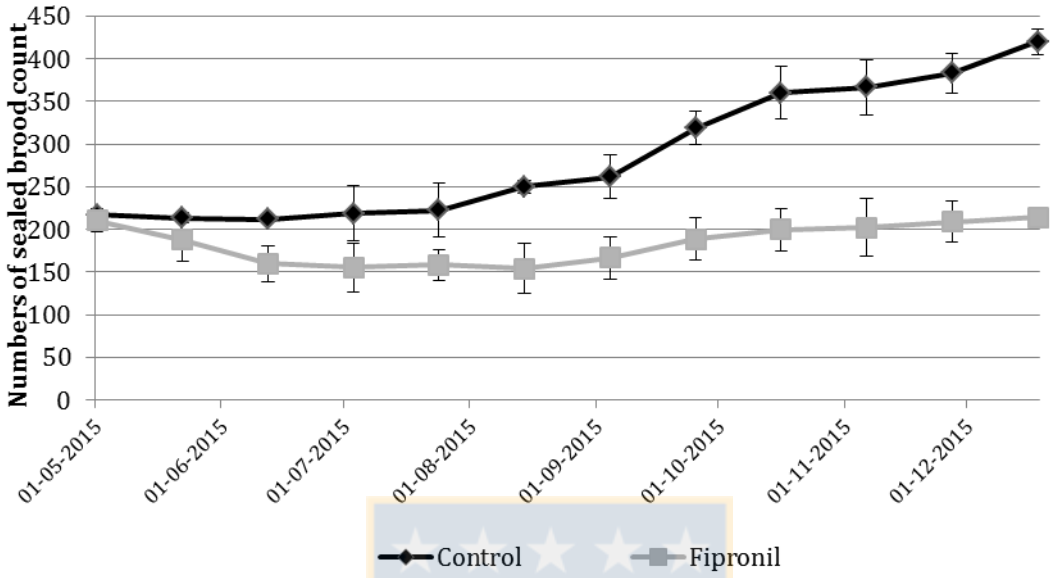
From May to December 2015, the number of of sealed brood per hive was counted every 15 days. As temperatures began to decrease in late May 2015, we observed a steady decrease of bee cluster size in fipronil treated colonies; while the untreated control maintained its size. While such decline in the fipronil 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 decrease 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 mortality in neither group until the arrival of autumn or winter. Fipronil hives began to show signs of weakness throughout December 2015; this is due to increased deaths in the hives. There was no loss of hives did not occur because to increased feeding of the hive in the spring. Figure 1.6 shows the progression of sealed brood in control and fipronil treatment with different evolution during the experiment.

Figura 1.5. Average numbers of the frame (standard deviations shown as error bars) containing honeybees for control and fipronil treated colonies and the corresponding daily average temperature



At AgrometINIA agro-meteorological network recorded from May to December 2015 (Source: Elaborated with own data and AgrometINIA data http://agromet.inia.cl/estaciones.php#estaciones).

Figura 1.6. 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). However, sealed brood counts were increased for all colonies from August to December, but did not necessarily produce significant differences (Pearson 2-tails, $p < 0.0001$). (Source: Elaborated with own data).

DISCUSSION

Even though most of the studies on the effect sublethal exposure are generally represented by behavioral 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 (Figure 1.1 and Figure 1.2) and a smaller body size in bees (Figure 1.3). These abnormalities could be present at the beginning of the pupae stage, since there are records of toxicity bioassays performed in Africanized Honey Bees that show that the pupal abnormalities in relation to the control were statistically significant, resulting in corporal malformations such as absence of head and extremities (Silva *et al*., 2015). The malformations may be due to the influence of fipronil on fat tissue during the post-embryonic development of the bee, as this tissue acts on hormone transport-related proteins that are important for metamorphosis, such as hexamerins (Locke, 1998; Martins and Bitondi, 2012). Fipronil acts as an antagonist of the GABA and 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 honeybee.

Malformations in the antennae of honey bees cause problems in the development of hives, because their role in the daily life of bees is vital, by reason of they perform multiple functions such as sensitivity to humidity, air pressure, temperature, odor, near-field sound vibrations, gustatory stimuli, tactile contact and substrate vibrations (Suwannapong *et al*., 2011). Tactile cues play an important 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 1.2), 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, favoring a lower memory performance, leading to a reduction in Learning outcomes necessary for the survival of the colony (Decourtye *et al*., 2005). In addition, the ability of honey bees exposed to insecticide to fly to the hive after feeding would be impaired by the ingestion of solutions contaminated with fipronil, because according to Decourtye *et al*. (2011) bees reduce the number of foraging trips. Along with the above, we observed that when measuring the area of the left hind wing in the bees of hives exposed to fipronil, it decreased significantly by 10.3% on average (Table 1.2). The reduction of the wing area would harm the bees in their flight capacity, which together with a lower performance in their memory, would be causing their ability to carry out the foraging activities is limited. Previously, Henry *et al*. (2012) demonstrated the detrimental effects on spatial orientation capacities in forage bees produced by insecticides such as fipronil and thiamethoxam (neonicotinoid). On the other hand, since adult bees have abnormal developmental characteristics of their antennae and wings, they are potentially disadvantaged in terms of their carrying capacity. If it is also considered that the transmission of the location of the food sources is done through a dance performed by the exploring bees, smaller wings can potentially intervene in a negative way in the communication of this information and limit their capacities of flight. It has been studied that exposure to fipronil may lead to behaviors that reduce the efficiency of foraging and also lead to reductions in the proportion of active bees in hive flights (Pisa *et al*., 2014). 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.

With the beginning of spring, fipronil-treated hives have an average of 3.7 active breeding frames, while control hives have 6.3 breeding frames on average (Figure 1.5). As mentioned above, exposure to fipronil reduces the number of foraging trips of the bee, which in our trial would limit the potential for collecting pollen and nectar for colonies in hives exposed to fipronil, would explain in turn, the low production of breeding frames. Considering the growth potential of hives in terms of the creation of breeding frames, we have 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 1.5). Control hives potentially possess a greater capacity for growth as a colony, which allows them to have a greater number of workers who play the role of seeking food, which favors foraging activities and increases the total carrying capacity of hives. On the other hand, it has been demonstrated in-vitro that the presence of 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 for the present trial in in-vivo conditions, which would suggest that the mechanisms that affect the development of the bees exposed to the pesticide in controlled conditions would follow the same tendency in function of the colonization of breeding frames and Growth in hives in an field experimental setting. The sublethal levels in the μg kg-1 range of fipronil have been suspected of impairing the performance of bees by inhibiting their learning and foraging activities (Kadar and Faucon 2006), which in our experiment would be explaining differences in 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 (Figure 1.5 and Figure 1.6) can be explained by the limiting effect of fipronil on colony development because in in vitro conditions , Sublethal doses of fipronil have limited the number of larvae and pupae to develop properly in hives (Silva *et al*., 2015).

From the point of view of the conformation of the population constituting a colony of honeybees, it must be considered that this normally varies according to the annual seasonality. However, it must be considered that if adverse changes in feeding and in the health of the hive

enhance an increase in population mortality, the trend towards a collapse of the hive also increases. If this increase in mortality occurs between spring and summer, its effect is not easily visible, because as there are abundant sources of food available, there is also the construction of breeding frames. But if this increase in 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 influence of fipronil in the colonies exposed to the insecticide. Fipronil can induce an insecticidal action in numerous species, because the ligand-controlled chloride channel has been identified as its target (Narahashi *et al*., 2010), which can not only potentially affect the development of the pupae, but also Limit its occurrence. In the autumn and winter period, the sealed brood in hives exposed to fipronil, by early July, showed a significant decrease in an average of 26.5% with respect to the initial value, while in the control hives in the same Period, a mean value was maintained without significant statistical differences, although with a wide variability among the hives (Figure 1.6).

When spring arrives, with increasing availability of pollen and nectar due to flowering and temperature, there is a slight increase in the number of sealed brood in hives exposed to fipronil, but without significant differences (Figure 1.6). In control hives the number of sealed offspring increased by an average of 37.4% between September and October, which would show that over the availability of food present in the ecosystem, it is the insecticidal action of fipronil which does not allow the Normal growth of the colony. It is important that the beekeeper detect the abnormal behavior in bees that allows him to take preventive measures against the potential exposure to this type of insecticide, frequently used as herbicides and insecticides of broad spectrum nationally and internationally. 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).

We conclude that, although there was no evidence of a collapse in fipronil exposed hives during the winter season, they presented an abnormal growth with respect to the untreated individuals of the population, evidenced by observable anatomical malformations and deficit

development of the hive. When bees are exposed to the dose of 0.00125 µg / bee of fipronil, during six consecutive months, they produce an abnormal length of development of the left antenna, the right wing area and the size of the bees in previously healthy colonies. The survival of colonies with sublethal doses of fipronil produces individuals with deteriorated development during the winter, but the exposure does not seem to compromise the immunity of the honeybees towards the pathogenic infection. The development of control colonies and the observation of the absence of massive deadly symptoms in these colonies shows that under normal conditions, the hive survives the winter and there is no evidence of a decrease in immunity to pathogens. The mechanisms by which sub-lethal exposure of fipronil in honeybees causes abnormal development in their hives, seasonally, needs to be clarified.

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CAPÍTULO 2

Estado del artículo: en preparación.

SUBLETHAL EXPOSURE TO FIPRONIL AFFECTS HONEY ORIGIN, PRODUCTION AND FORAGING BEHAVIOUR OF HONEY BEES (*APIS MELLIFERA* L.)

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ABSTRACT

Honeybees play a very important role in pollinating multiple crops of food and agriculture. Rational use of chemicals that are harmful to pollinators is essential to sustainable production over time. In the present study, we investigated the effects of fipronil on honey origin, production and foraging behavior of honeybees to seek an explanation for bee decline. Six hives that were used were fed sugar-water during the experiment, three of which are also fed with known amounts of fipronil default (0.00125 μg per bee). In addition, to the six colonies are allowed to feed freely on the environment. Exposed hives significantly decreased their honey production between the 2014-2015 season and 2015-2016. In the harvesting periods (December and March) the decrease was over 50% on average (December 57.2% and March 53.9%). The hives treated with fipronil evidenced a decrease in the collection of pollen from Brassica (Raps) distant to more than a kilometer of the hives, from season to season. While in the same period the representation in the pollen honey of the families Fabaceae and Poaceae, represented by species close to the hives increased. We conclude that when honeybees were exposed to the sublethal dose of fipronil produce a decrease in honey production of the hive and a variation in its foraging habits.

Keywords: Honeybee, phenylpyrazole, fipronil, foraging, melissopalynology

INTRODUCTION

The foraging of honey bees (*Apis mellifera* Linneo, Hymenoptera, Apoidea) played an important role by in maintaining global biodiversity and production of food crops (Klein *et al*, 2007; Bradbear, 2009; Breeze *et al*, 2011). The contribution of pollinators to the production of food crops used directly for human consumption in terms of capital has been estimated at 153 billion euros worldwide, corresponding to about 9.5% of the total value of food production consumed by mankind (Gallai *et al*., 2009). Pollination by bees represents 71% of the 100 species most consumed food crops, providing 90% of food in the world (FAO, 2005; UNEP 2010). For example, honey bees have a key role in increasing the seed production of three Brassica vegetables: Chinese cabbage, broccoli and kohlrabi (Sushil *et al*., 2013). There are numerous reports of importance for pollination of plants by the foraging activity in which colonies of bees collect pollen, nectar, water and resin (Young *et al*, 2007).

The Honeybees in their foragein activity visit different plant species in search of their food, in this process they are impregnated with the characteristic and diverse pollen grains of the plants they visit (Navarrete *et al*., 2016). Due to the above, it is possible to perform an analysis to determine the botanical and geographical origin of the honey through its pollen composition, the procedure for determining the pollen content of the honey is called Melissopalynology (Soria *et al*., 2004; Corvucci *et al*., 2015). Melissopalynology is also used to evaluate correlations with in situ climatic parameters such as rainfall and temperature (Bilisik *et al*., 2008). In order to understand the pollen content of the honey, it is possible to visualize the context of external factors that influence pollinators and pollination networks (Jens *et al*., 2008; Selva Singh Richard *et al*., 2011; Nascimento and Nascimento, 2012).

The pollen collected by bees can contain high levels of multiple pesticide residues, which are present in most food crops in their environment, which can affect the health of bees (Mullin *et al.*, 2010). The ability of pollinators to resist disease and parasites seem to be influenced by several factors which are constantly exposed, particularly their nutritional status and exposure to toxic chemicals (Genersch *et al*., 2010). At present, systemic pesticides neurotoxic of intensive use of the crops in the whole world against plagues, as the neonicotinoids and the phenylpyrazoles expose often to the bees and hive (Vidau *et al*, 2011; Sanchez-Bayo *et al*, 2014). The phenylpyrazole insecticide fipronil (5-amino-1-(2,6-dichloro-α,α,α-trifluoro-p-

tolyl)-4-trifluoromethylsulfinylpyrazole-3-carbonitrile) is amply used against arthropod pests on crops worldwide (Mullin *et al*, 2010). Fipronil pesticide-treated seed supposed a risk of the population of honeybees, according to European Food Safety Authority EFSA (EC, 2013; EFSA, 2013). The action on neuronal signaling of fipronil can potentially results in mortality the honey bee (Gunasekara *et al*, 2007). In the present study, we investigated the effects of fipronil on honey origin, production and foraging behavior of honeybees to seek an explanation for bee decline.

MATERIALS AND METHODS

We used a replicated split-plot design consisting of two treatments with three honey bee hives in each case. We used the honeybees of a Carnica race because it corresponds to one of the most used in Chile due to their good yields and high adaptability (Montenegro *et al*., 2009). A total of 25 hives were established (between 26 and 29 September 2014), 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 experiment the number of breeding frames in the hive, the number of sealed brood count and the amount of honey produced. The six used hives were used were fed with sugar water (Sugar and water ratio, 2:1) during the experiment, three of them were also fed with 0.00125 µg of fipronil per bee, (the LD_{50} of fipronil of 0.00417 µg per bee). In addition, all colonies were allowed to feed freely on the environment, according to the modified method of Lu *et al*., 2014. Fipronil feeding was conducted during the months of May and October 2015.

Description of the study area and crop map

The trial was established in the area of Llano Verde (37 ° 43 'S, 72 ° 22' W, 184 m above sea level) at a distance of 10 kilometers from the city of Los Angeles, capital of the province of Biobio, Chile. The climate in the area is characterized by maintaining 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). To facilitate melissopalynological analysis is made a map of the adjacent

areas to hives, to identify crops occupying a larger area. For the map is considered a distance of 1 km since the establishment of the hives. However, some more remote areas that might be of interest to the foraging activity of the hives were identified. The coordinates of the most important crops were identified by GPS, to consider the distance to the hive.

Analysis melisopalinological

A harvest of a representative sample of about 1 kg of honey obtained from each hive is performed. To remove and identify the pollen from the honey, the Chilean standard will be used "Designation of botanical origin by melisopalinological essay" National Standards Institute (INN) made official in 2005. The standard consists of 10 g. of honey dissolved in 20 mL of distilled water and heated to 40 ° C water, then centrifuged for 10 minutes at 2,500 rpm, and then the supernatant is removed and added 5 mL of distilled water. The sample is centrifuged again for five min. and then the sediment is fixed on a slide for the identification and counting of pollen grains under an optical microscope is removed. He shall have a minimum of 1,200 grains per sample according to standardized national protocol (Montenegro et al., 2006). Analyses were performed at the Laboratory of Palynology and Plant Ecology, University of Concepción, Campus Los Angeles.

Extraction and quantification of honey

The process of extracting honey from each hive was conducted by extracting honey avoiding any contamination. Frames with mature honey were selected. To determine if the honey mature runoff test was performed, whether of honey slips will be considered immature. The percentage of capping layer (wax) in each frame, which must have at least a 75% capping was also considered. Once they collected the capping layer in each frame, were removed bees and were placed in a plastic box for extraction. The frames were filled with honey, and these, in turn, are protected by the wax cap that protects, so the first job was to remove the wax layer to reach the honey. The wax is removed with a spatula heated to about 65 ° C. Once uncapping part, this was introduced into the centrifuge than by pressure exerted by movement, honey just decanting and out the bottom of the appliance. Subsequently the honey each hive was weighed to estimate the approximate amount of honey produced during the period from March to October 2015. It is considered that the quantified honey is not pure because it has some impurities (mainly wax) which it is separated from the final product in the decanting process.

Tabla 2.1. Chronological characterization of the observed events in the bees colonies studied

Date	Event
26-29 September 2014	Assembling 25 new 10-frame Langstroth pine honey bee hives.
December, 2014	Weighing the first harvest honey. Preliminary selection of ten honey bee hives in study site according to its production of honey. Honey extraction for melipolinological analysis.
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 monitoring and sublethal fipronil dosing for five consecutive months.
May	Recollection of data previous to fipronil application.
May-November, 2015	The monitoring hives biweekly.
June 1st, 2015	Autumn hive strength monitoring and monitoring date without the observation of dead bees.
July-November, 2015	Winter hive strength monitoring.
December, 2015	Honey extraction for melipolinological analysis. Weighing of the honey.
March, 2016	End of the harvest season and final collecting honey.

Source: Elaborated with own data.

Analysis of data

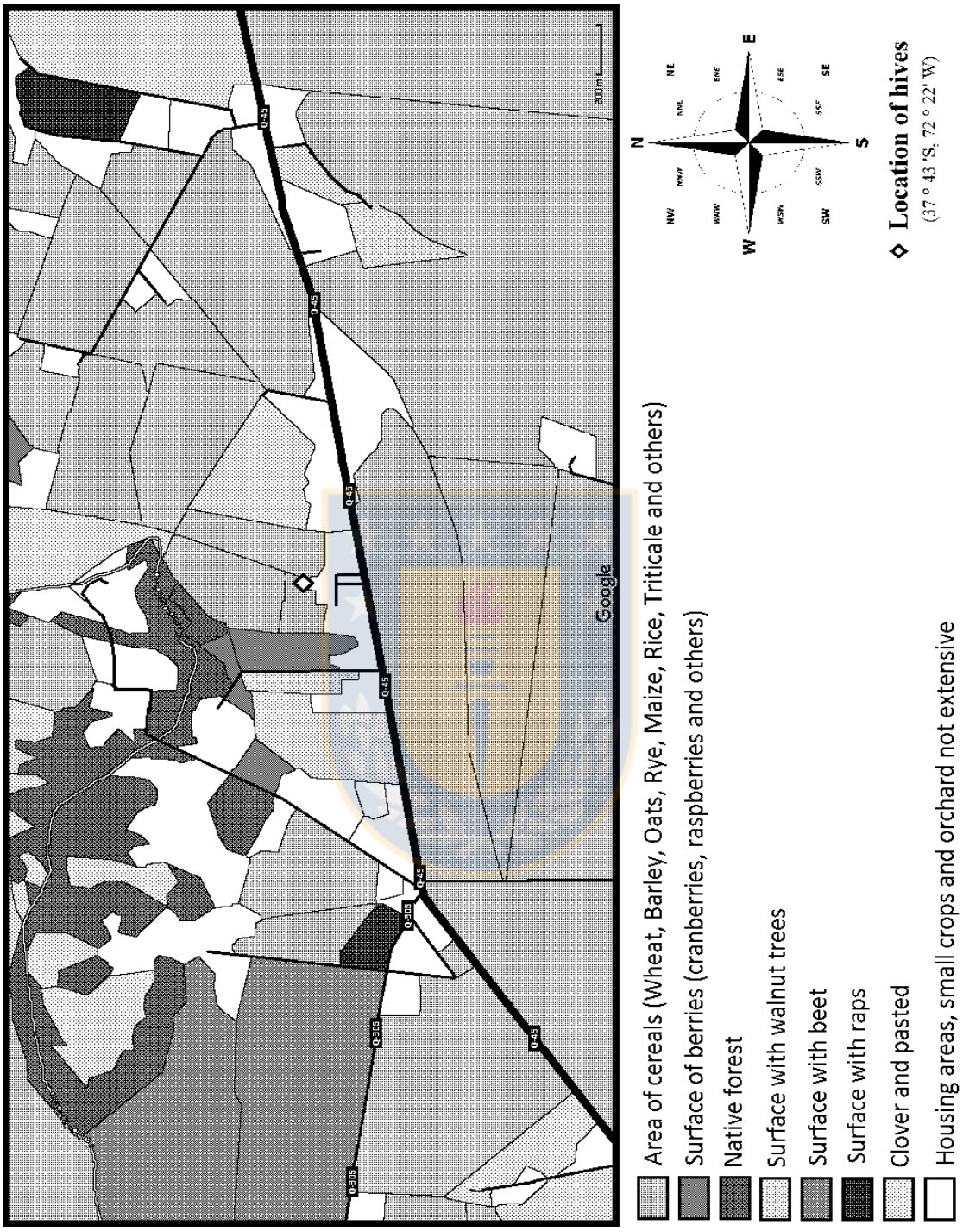
An analysis of variance were carried out by means of ANOVA procedure and the means comparison by using the Tukey (Windows InfoStat, 2015).

RESULTS

The timeline of this experiment, including the dates of observed events, is shown in table 2.1. The area in which this experiment set, found a large number of annual plants in the family of grasses, such as wheat (*Triticum spp*) and oats (*Avena sativa L.*), widely grown around the world (Figure 2.1). In the area, there are several houses. In these areas there are a lot of fruit trees; also they can be found trees to serve as windbreaks such as pine (*Pinus radiata D. Don*) and eucalyptus (*Eucaliptus spp*). In the vicinity there is also an area with native forest on the banks of the estuary Pichidiuto. This forest is mainly composed of Quillay (*Quillaja saponaria* Mol.), Maitén (*Maytenus boaria* Mol.), Peumo (*Cryptocarya alba* Mol.), Boldo (*Peumus boldus* Mol.), Lingue (*Persea lingue* Nees) and Arrayán (*Luma apiculata* (DC.) Burret). In grassland areas the most important herbaceous species for bees are grass pig (*Hypochaeris*

radicata L.), white clover (*Trifolium repens L.*), seven veins (*Plantago lanceolata L.*), black nightshade (*Prunella vulgaris L.*) and trefoil (*Lotus uliginosus Schkuhr*). In artificial meadows or grassed areas, the most important grass species are the red clover (*Trifolium pratense L.*) and white clover. Within crop species most important in the honey is raps (*Brassica napus* L. ssp oleifera.); and to a lesser extent apple (*Malus pumila Mill.*), guindo (Prunus avium L.), cherry (*Prunus cerasus* L.) and plum (*Prunus domestica* L.). Other species mellific value in the area, but in small quantities are the Canelo (*Drimys winteri* JR *et* Forster), Maqui (*Aristotelia chilensis* (Mol.) Stuntz), murta (*Ugni molinae* Turzc.) Radal (*Lomatia hirsuta* (Lam.) Diels. Macbr ex.), chilco (*Fuchsia magellanica* Lam.), Matico (*Buddleja globosa* Hope), Hazel (*Gevuina avellana* Mol.)

Figura 2.1. Map of the study area



In the lower right corner equivalence scale is observed. The map depicts the established crops during the months of September and November 2015. (Source: Elaborated with own data and map edited from Google Maps 2015). https://www.google.cl/maps/@-37.4333294,-72.2249444,14z).

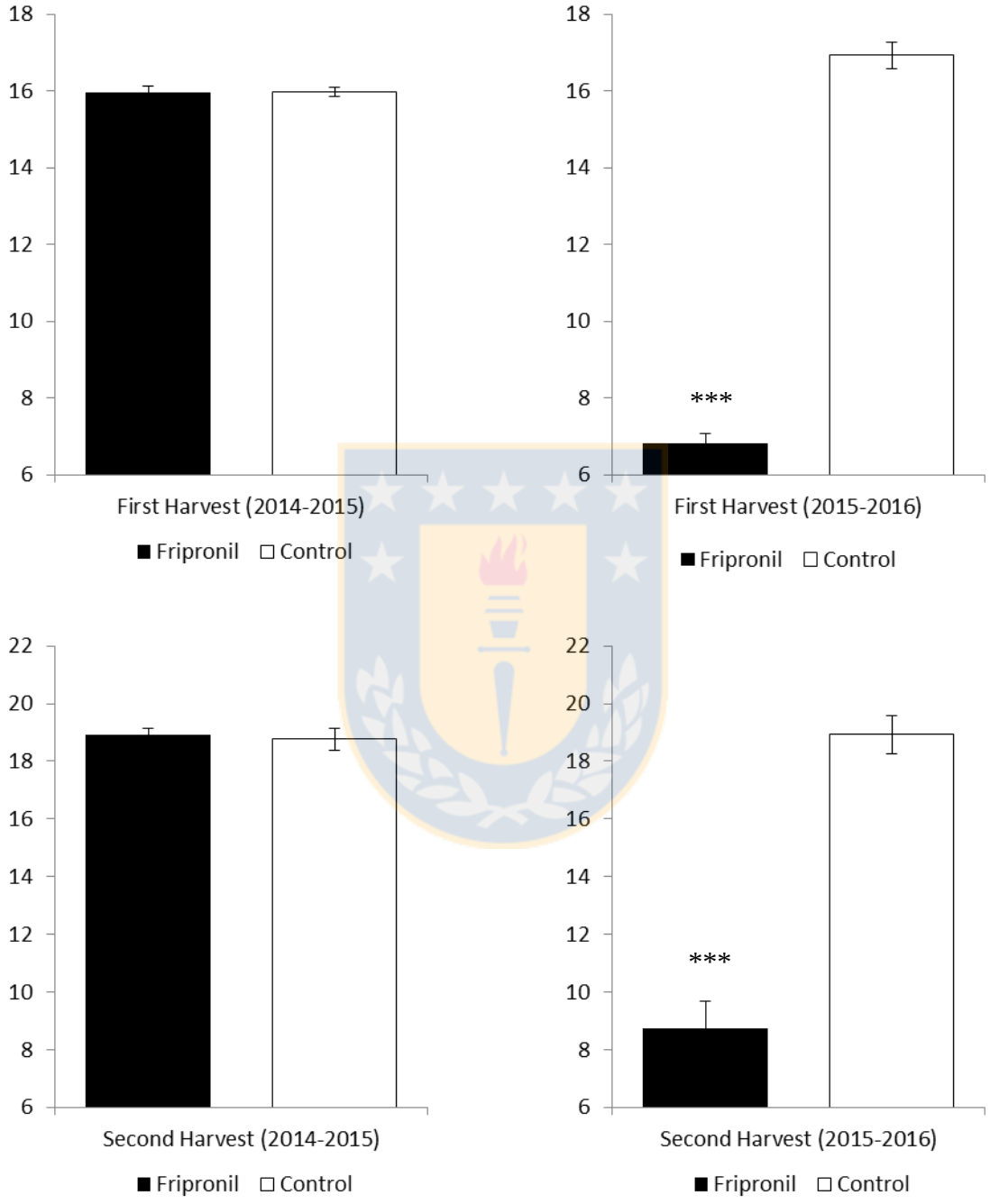
Tabla 2.2. Main species present for coverage area and flowering season

Scientific name	Common name	Flowering (Months)	
Aristotelia chilensis (Mol.) Stuntz	Maqui	OCT	NOV
Avena sativa L.	Oats	NOV	DEC
Brassica napus L. ssp oleifera	Raps	OCT	NOV
Buddleja globosa Hope	Matico	OCT	JAN
Conium maculatum	Hemlock	NOV	DEC
Cryptocarya alba Mol.	Peumo	OCT	NOV
Chrysanthemum	Chrysanths	MAR	APR
Drimys winteri JR et Forster	Canelo	OCT	NOV
Echium vulgare	Viper's-bugloss	DEC	APR
Eucaliptus spp	Eucalyptus	AUG	SEP
Fuchsia magellanica Lam.	Chilco	NOV	JAN
Gevuina avellana Mol.	Hazel	JAN	APR
Hypochaeris radicata L.	Grass pig	DEC	APR
Lomatia hirsuta (Lam.) Diels. Macbr ex.	Radal	OCT	OCT
Lotus uliginosus Schkuhr	Trefoil	NOV	DEC
Luma apiculata (DC.) Burret	Arrayán	NOV	DEC
Malus pumila Mill.	Apple	SEP	OCT
Maytenus boaria Mol.	Maitén	SEP	NOV
Modiola caroliniana	Bristly-fruited mallow	DEC	JAN
Persea lingue Nees	Lingue	OCT	JAN
Peumus boldus Mol.	Boldo	DIC	JAN
Pinus radiata D. Don	Pine	SEP	OCT

Plantago lanceolata L.	Seven veins	DEC	JAN
Prunella vulgaris L.	Black nightshade	NOV	JAN
Prunus avium L.	Guindo	SEP	OCT
Prunus cerasus L.	Cherry	SEP	OCT
Prunus domestica L	Plum	SEP	OCT
Quillaja saponaria Mol.	Quillay	DEC	JAN
Taraxacum officinale	Dandelion	NOV	APR
Trifolium pratense L.	Red clover	NOV	FEB
Trifolium repens L.	White clover	NOV	FEB
Triticum spp	Wheat	SEP	OCT
Ugni molinae Turzc.	Murta	DEC	DEC
Vaccinium spp.	Cranberries	SEP	OCT

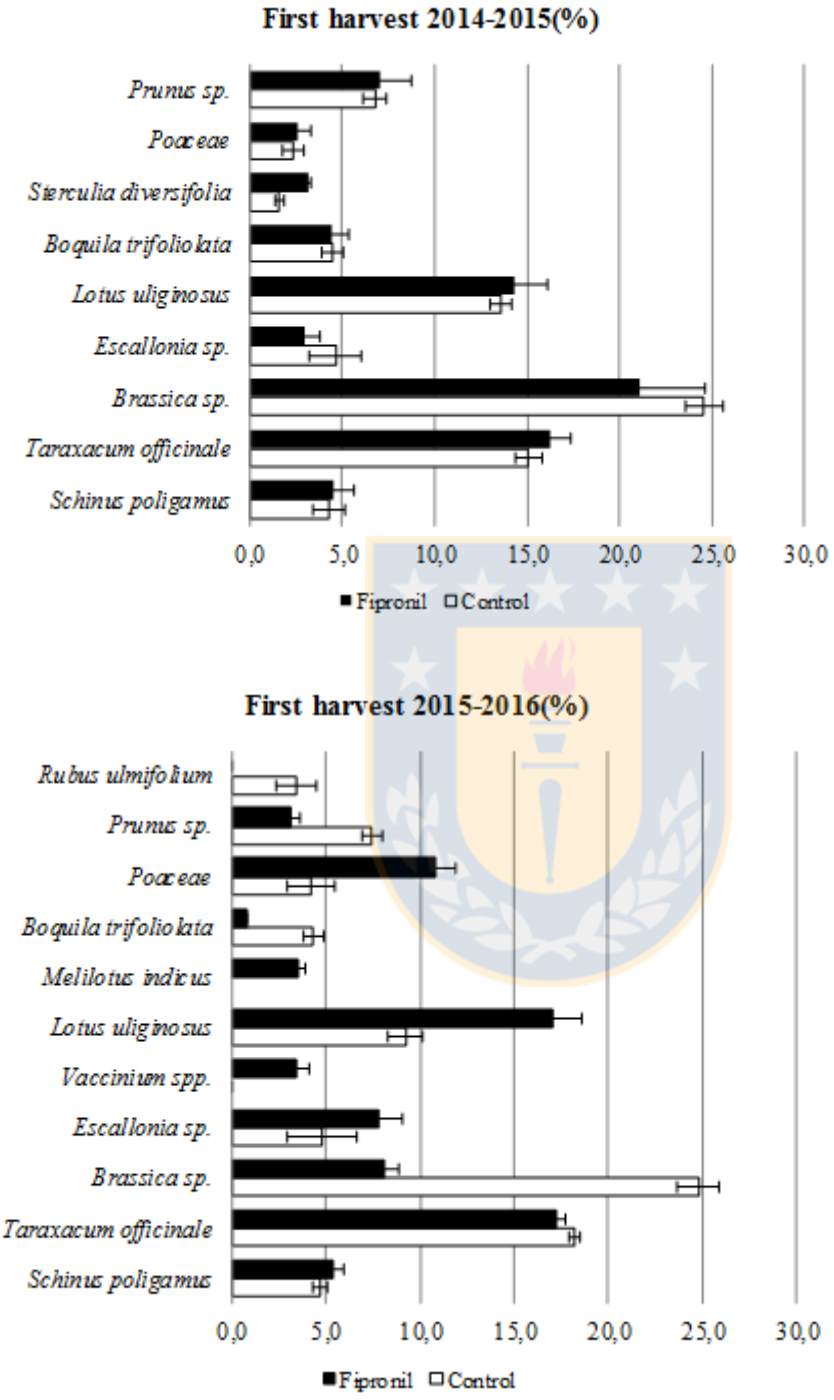
. The table presents the species that are mostly found in the study area. The flowering months are represented with the first three letters of the month. (Source: Elaborated with own data).

Figura 2.2. Production of honey in hives of *Apis mellifera* non exposed or exposed during treatment days to fipronil at the dose 0.00125 μg per bee



First Harvest (December); Second Harvest (March). The lines above the bars indicate the standard deviation of the mean. ***: Significantly different. Alpha: 0,05. (Source: Elaborated with own data).

Figura 2.3. Results of melisopolinological analysis



We present the species that represent more than 2% representativeness within the analysis. Significantly different. Alpha: 0,05.

The melisopalinological analysis made to the honeys produced in the harvest (First Harvest in December); of the seasons 2014-2015 and 2015-2016 were expected variations for polyfloral honeys (Figure 2.3). Pollen grains representing 18 families (Anacardiaceae, Apiaceae, Boraginaceae, Brassicaceae, Convolvulaceae, Escaloniaceae, Ericaceae, Fabaceae, Gunneraceae, Lardizabalaceae, Malvaceae, Myrtaceae, Poaceae, Quillajaceae, Rosaceae, Verbenaceae and Vitaceae). In some cases the determination allowed to reach species level in the determination, but in others it was only possible to reach determination at the family level (Figure 2.3).

DISCUSSION AND CONCLUSIONS

We will evaluate the sublethal effects of fipronil on the development of bee hives and their foraging activities; considering that the morphology of bees may be relevant for risk assessment, although most studies on sublethal effects are generally represented by behavioral traits in honey bees (Muñoz-Capponi *et al*., 2017). Hives exposed to sublethal doses of fipronil decreased their honey production significantly at both harvest times (First Harvest in December and Second Harvest March); In both cases the decrease was more than 50% on average (First Harvest 57.2% and Second Harvest 53.9%). While the variations of the hives that were not exposed to fipronil did not suffer statistically significant differences between both harvest times. Foraging activity in bee hives is affected by the amount of pollen needed for the hive (Weidenmuller and Tautz 2002); and the strength of the colony and the breeding activity within the hive (Amdam *et al*. 2009). In the hives it was found that the application of sublethal doses of fipronil produced a lower number average of sealed brood count (Muñoz-Capponi *et al*., 2017). Were found Positive correlations between sealed brood area and foraging activity as well as in hive bee number (Abou-Shaara *et al.* 2013). Considering the above, we propose that in having a hive with fewer individuals these can not perform the foraging activity in the same way as hives not exposed to sublethal doses of fipronil; for that reason the hives diminish their ability to produce honey from one season to the next.

The hives in the study area have flowering of melliferous species between the months of August to April (Table 2.2). During these nine months the bees can find flowers in the sector for their food, notwithstanding the foregoing in the winter and autumn periods the number of flowers available is less than the beehives need for their development, so that beekeepers have

the Need to incorporate supplementary feeding into hives. In terms of feeding behaviour and activity, bees with exposure to fipronil might not be foraging on treated crops in the same way as they would do on untreated crops (Colin *et al*. 2004). In beehives bees can be classified according to foraging activities in two, bees explorers and reticent bees. The exploring bees are in charge of finding the best food resource and the reticent wait in the hive for the information provided by the exploring bees to carry out the forage activities (Abou-Shaara, 2014). The exploring bees transmit information about feeding sources to reticent bees through a dance; therefore any factor that affects this communication of information directly influences the collection capabilities of the hive.

In the study area at least 34 plant species were found attractive for the collection of pollen and nectar for the beehives, which gave them multiple options for feeding the bees (Table 2.2). As documented by bees in their foraging activity, they need to visit a large number of flowers in order to meet the feeding needs of the hive (Corbet *et al*., 1991). However, there are flowers that are considered highly attractive for bees such as *Brassica napus* (Abrol, 2007), this is demonstrated in our study since in the season 2014-2015 Brassica pollen was the most represented after the melisopalinological analysis in the honey (Figure 2.3).

The nearest plantation of *Brassica napus* was at a distance of 1, 33 kilometers from the hives, which indicates that in spite of having other species closer, they preferred their flowers to obtain their food resources. This is consistent with what was documented previously, because *Brassica napus* has abundant food resources (Mesquida et al., 1988) and a high interest related to its fragrance (Mussury and Fernandes, 2000), which makes it an attractive species for *Apis mellifera*. In the 2014-2015 season the bees without treatment of fipronil maintained the tendency of harvesting being again the pollen grain of Brassica the one with greater presence in the honey after the analysis melisopalinological (figure 2.3). In hives treated with sub-lethal doses of fipronil the situation was different, since the percentage representation of Brassica was 8.1% on average, which is less than half of the previous season in the same hives, and one Third of the presence of control hives.

Bees exposed to sublethal doses of fipronil undergo morphological variations compared to unexposed bees (Muñoz-Capponi *et al*., 2017); these morphological variations could influence peccaries, because scout bees might not transmit the co-ordinates of feeding sources to reticent

bees in a good way. A decrease in the size of the wings can directly influence the communication of the bees and the activities of harvesting of honey. Bees with short wings were reported to have smaller flying ability than large ones (Mostajeran *et al*. 2006); this directly influences its capacity to load and collect nectar. Bees with damaged wings they fly closer to the hive and had less foraging trips than healthy ones (Higginson *et al*., 2011). Bees exposed to fipronil maintain their preferences for pollen and nectar collection, which is evidenced by a presence of similar pollen in honey from season to season; however, the percentage representation by taxa in honey varies with the presence of fipronil. The abundant percentage representation of Brassica pollen (Raps), distant more than 1 kilometer from hives, decreases in honey samples from nuclei exposed to sublethal doses of fipronil, from one season to another, to less than half. Comparatively, in these same nuclei, the pollen grains with the highest percentage representation in the honey correspond to those coming from vegetation closest to the hives exposed to the pesticide, belonging to the families Fabaceae and Poaceae represented by species close to hives such as *Lotus uliginosus* (Figure 2.1).

We conclude that when bees are exposed to sublethal of fipronil at a dose of 0.00125 µg / bee for six consecutive months produce a decrease in honey production of the hive and a variation in its foraging habits. After the melisopalinological analysis it can be observed that the honey produced by the hives treated with fipronil varies their habits of collection of pollen and nectar. Bees exposed to fipronil preferably collect pollen and nectar from the same plant species as control hives if pollen is considered to be the most representative of honey; However, the representation of the pollen that is more distant as Brassica sp. And those closest to the hives of the families Fabaceae and Poaceae increase. Finally, it is necessary to point out that the mechanisms by which sub-lethal exposure to fipronil of honey bees, which causes variation in nectar and pollen collection habits in hives through seasonal succession, have not been detected in the Present investigation. The mechanisms that cause a decrease in the growth rates of the hive and its production of honey must also be clarified.

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CONCLUSIONES

Se concluye que cuando las colmenas de *Apis mellifera* se exponen a dosis subletales del neurotóxico sistémico fipronil, con una dosis de 0,00125 µg / abeja durante seis meses consecutivos, se producen variaciones morfológicas en las abejas y alteraciones en el desarrollo de la colmena. La exposición influye negativamente en la longitud de la antena izquierda, del área del ala anterior derecha y de la longitud de las abejas en colonias previamente sanas.

La supervivencia de colonias con dosis subletales de fipronil produce individuos con desarrollo deteriorado durante el invierno, pero la exposición no parece comprometer la inmunidad de las abejas melíferas hacia la infección patógena. No se aprecia en las colmenas expuestas a fipronil y control una disminución de la inmunidad a patógenos, como *Varroa destructor* o *Nosema ceranae*, debido a que no se encontró la presencia durante todo el desarrollo del ensayo en ninguna de las colmenas estudiadas. El desarrollo de colonias de control y expuestas a fipronil, y la ausencia de síntomas mortales masivos en las colonias muestran que en las condiciones que se realizó el ensayo las colmenas sobreviven al invierno.

A partir del análisis melisopalinológico se observa que la caracterización polínica de la miel, producida por las colmenas tratadas con fipronil, varía en relación con las colmenas control. Posiblemente esto se debe a una variación en sus hábitos de recolección de polen y néctar. Las abejas expuestas al fipronil mantienen sus preferencias de recolección de polen y néctar, lo que se evidencia en una presencia de polen similar en la miel de una temporada a otra; no obstante esto, la representación porcentual por taxones, en la miel, varía con la presencia de fipronil. La representación porcentual en abundancia del polen de *Brassica* (Raps), distante a más de 1 kilómetro de las colmenas, disminuye en las muestras de miel de los núcleos expuestos a dosis subletales de fipronil, de una temporada a otra, a menos de la mitad. Comparativamente, en estos mismos núcleos, los granos de polen con mayor representatividad porcentual en la miel corresponden a aquellos provenientes de vegetación más cercana a las colmenas expuestas al pesticida, pertenecientes a las familias Fabaceae y Poaceae.

Finalmente, es necesario señalar que los mecanismos por los cuales la exposición sub-letal al fipronil de abejas melíferas, que causa variación en los hábitos de recolección de néctar y polen en las colmenas a través de la sucesión estacional, no han sido detectados en la presente

investigación. Solo se ha relacionado la alteración morfológica con la capacidad de vuelo y orientación. Así también, deben aclararse los mecanismos que causan una disminución en las tasas de crecimiento de las colmenas, en su producción de miel y en el desarrollo anormal de las abejas melíferas. Una propuesta nueva de estudio, a futuro.

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