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Evaluation of residue levels of imidacloprid and thiamethoxam after foliar application to the citrus varieties Lane Late, Valencia Late, Rohde Summer and Nules

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ABSTRACT

Neonicotinoids are used to protect citrus trees against pests. Dissipation and persistence of neonicotinoids in pollen and nectar of citrus trees after foliar applications and their potential exposure to pollinators have not been well characterized. Field studies were conducted using three orange and one mandarin varieties to compare the imidacloprid and thiamethoxam residue levels and their decline in pollen and nectar after treatments in pre-bloom close to flowering period and their persistence one year after treatment. The possible risk to honeybees was assessed.

In nectar, thiamethoxam and imidacloprid residues were between 61 and 99% lower than in pollen, depending on the citrus variety or/and the days after treatment when applied close to blooming. At the end of the flowering period, imidacloprid in pollen and nectar was no detected in the mandarin variety after treatment in pre-bloom, whereas for thiamethoxam, no residues were detected in nectar but 10 ng g^{-1} was detected in pollen. There were no quantifiable levels of residues for either neonicotinoids in pollen or nectar during the flowering period of the following year.

Neonicotinoid residue levels and their decline in nectar and pollen in citrus depended on the timing of applications relative to flowering and on the citrus variety. The absence of neonicotinoid residues one year out after foliar applications in all varieties assayed demonstrated that none of the neonicotinoids tested were persistent. The results could be different in other citrus varieties, and therefore, also the exposure assessment for managed pollinators.

Keywords: citrus variety, neonicotinoids, foliar spraying, residues in pollen and nectar, pollinators.

1. INTRODUCTION

The citrus agrosystem is very rich and varied in terms of pests and natural enemies. In Mediterranean citrus trees, two leaf flushes on new growth are produced per year, in spring and summer. The timing of foliar applications of neonicotinoids in citrus trees is linked to the presence of pests that feed on this new growth. The most important sap-sucking pests that feed on tender shoots are aphids, whiteflies, the citrus leaf-miner *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae) and the psyllid vectors of Huanglongbing (HLB), the main disease of citrus. Currently, where HLB vectors are present, the psyllids *Diaphorina citri* Kuwayama and *Trioza erytreae* Del Guercio (Hemiptera: Psyllidae) are treated with systemic broad-spectrum neonicotinoid insecticides that are highly mobile within plant tissues (Grafton-Cardwell et al. 2013, Boina et al. 2015). Due to their systemic action, neonicotinoids applied in soil are absorbed by roots and transported to the aerial surface through the xylem to apical tissues including new growth, mostly affecting sap-sucking insects. Foliar spraying involves the absorption of the pesticides, which can cross the epidermal barrier (cuticle) or enter through the stomata of the leaf, whose opening depends on the level of transpiration regulated by environmental factors (light, temperature, water balance). The risk of exposure to beneficial insects and pollinators via pollen or nectar may differ with the application method, the number of applications, the time of application, the time period following treatment and the crop type. The large diversity of citrus varieties also implies differences in the aerial parts such as the surface of leaves, their lipid cover, the chemical and physical properties of the cuticle and the foliage area. These factors may affect foliar absorption and distribution in aerial tissues, and eventually translocation to pollen and nectar. The variation in systemic movement of neonicotinoids and their potential exposure to pollinators via pollen and nectar, depending on citrus variety, has not been evidenced.

Neonicotinoids have been identified as agents involved in the loss of bees. This has led to a plethora of studies that have focused on the effect of neonicotinoids on honeybees (Decourtye et al. 2004, Iwasa et al. 2004, Suchail et al. 2004, Henry et al. 2012, Brandt et al. 2016). Nevertheless, pollinators are

threatened by a complex interplay of stressors such as pathogens, parasites, climate change or incorrect management of the beehive (Henry et al. 2012, Brandt et al. 2016, Hernando et al. 2018).

To the best of our knowledge, no information is available on neonicotinoid residues in pollen and nectar after foliar treatment of citrus trees in Mediterranean conditions, where the climate can influence the uptake, distribution and dissipation in/on the plants. The purpose of this work is to evaluate the dissipation of imidacloprid and thiamethoxam in pollen and nectar from several orange and mandarin cultivars after foliar application of commercial formulations during the pre-bloom period (close to the flowering period). The persistence of residues from post-bloom application into the following flowering season and from pre-bloom application into the flowers growing one year or more after treatment was also evaluated. This study provides reliable data on both neonicotinoid residues in pollen and nectar of citrus varieties under realistic agricultural practices that could give information about the periods of highest exposure risk for pollinators (honeybees) after feeding on nectar and pollen contaminated with neonicotinoids. Field studies were conducted on three varieties of orange (Lane Late, Valencia Late and Rohde Summer) and one variety of mandarin (Nules) to compare the level of residues and the decline in these levels in pollen and nectar during blooming after foliar application.

2. MATERIAL AND METHODS

2.1. Field experiments – orchards with citrus varieties

The field studies were carried out in six commercial citrus orchards (orchards 1 to 6) located in northeastern Spain (**Table 1**). Three varieties of orange (*Citrus sinensis L.* cv. 'Lane Late' navel in orchards 2 and 5, cv. 'Valencia Late' in orchard 3 and 'Rohde Summer' navel in orchard 6) and one variety of mandarin (*Citrus clementina* Hort. ex Tanaka. cv 'Nules' in orchards 1 and 4) were included in this study. Trees were grafted on citrange Carrizo rootstock. The orchards had 400-455 trees per ha, with a tree spacing of 5-5.5 x 4 m and a drip irrigation system. In each grove, foliar spraying was applied in a randomized design with three treatments (four replicates per treatment) applied as a single application at the maximum label rate recommended at least 8-13 days before blooming. Each replicate

consisted of 30 (5x6) trees (600-660 m^2) that had not been treated with thiamethoxam or imidacloprid at least five years prior to the actual assay. The treatments were: a) Confidor 20 LS from Bayer (imidacloprid (20% [SL] p/v), 0.075% concentration, b) Actara 25 WG from Syngenta (thiamethoxam 25% [WG] p/p), 0.030% concentration and c) water (control). An adjuvant, Mojante Oro no iónico™ (Químicas Oro, SA, Valencia, Spain, alkyl polyglycol ether 20%), was added to all treatments at 0.5% to facilitate product penetration in the leaves. These commercial products were selected for this study because both contain imidacloprid or thiamethoxam (the neonicotinoids most commonly used in citrus) without other agrochemical products. In each orchard, vegetation volume was estimated based on Tree Row Volume, which determines rates based on the assumptions that each tree row is a rectangular box whose volume could be used to calculate the volume space occupied by foliage per unit of ground surface (m^3 of foliage per ha) (Byers 1978). Orchards 1, 2, 4 and 5 had a vegetation volume approximately of 10,800 m^3 ha^{-1}, orchards 3 and 6 about 6,800 m3 ha^{-1}. Spraying was performed with a 600L-air-blast sprayer (Gaysa, Murcia, Spain), at a pressure of 8-10 bars, speed of the tractor 2.2-2.7 km h^{-1} and 1,140-1,500 L ha^{-1}. The concentration of imidacloprid and thiamethoxam was 0.17-0.22 kg ha^{-1} and 0.08-0.11 kg ha^{-1}, respectively.

Sprays were performed linked to the presence of sap-sucking pests that feed on tender shoots in citrus trees: in spring before flowering (pre-bloom), in spring after flowering (post-bloom) and in summer (post-bloom). Orange and mandarin citrus only bloom once a year, in spring. We divided our experiments in two classes: “Persistence”, that refers to residues of neonicotinoids in pollen and nectar of flowers of the following year after the treatments (418, 336 and 224 days after treatment (dat)), and "Dissipation", that refers to neonicotinoid residues in pollen and nectar of flowers of the same year of the treatment (8-37 dat). For dissipation experiments, three sampling events were performed, at 10%, 35% and 75% of flowering period (8-37 dat, depending on the orchard). For persistence experiments, only one sampling event was performed (418, 336 and 224 dat, depending on the orchard and treatment in pre or post-bloom). All these data are summarized in detail in **Table 1**.

2.2. Sampling of pollen and nectar

Flower samples were taken only from the 12 central trees of each replicate plot to avoid contamination by drift from adjacent treatments. At least 300 flowers per replicate in mandarin and 150 flowers in orange varieties, selected randomly, were needed to obtain each sample of pollen or nectar. Each pollen sample consisted of 2 g of anthers cut with scissors from flowers and transferred to vials. Each nectar sample consisted of 200 µL collected from the flower´s nectaries using a graduated microcapillary tube inserted into a bulb dispenser. The nectar in the tube was transferred to a vial. Both pollen and nectar samples were immediately refrigerated in iceboxes (4-5ºC) and transported to the laboratory where they were stored at -20 Cº in a freezer until analysis. Nectar and pollen were collected from different flowers. To evaluate the dissipation of residues associated with the pre-bloom treatment, samplings were performed during the blossom period in 2016 for mandarins and in 2017 for orange trees (**Table 1**). To evaluate the persistence of the treatment applied during the previous year (in pre- and post-bloom during 2015), monitoring of residues was carried out in the following season of bloom (2016) at the dates indicated in **Table 1**. Dissipation and persistence were used to determine the potential for exposure to neonicotinoid residues for pollinators. Information about climatic conditions (temperature (ºC), relative humidity (%) and rainfall (mm)) is included in **Figure S1** (supporting information).

2.3. Residue analysis

Residue analysis of neonicotinoids was done using a generic extraction method and quantification was performed with liquid chromatography and mass spectrometry (LC-MS) using an Agilent system with a Model 1200 chromatograph and a Model 6410 triple quadrupole analyser (Agilent Technologies, Palo Alto, CA, USA). LC analysis was performed in reversed-phase with a F5 column of 100 x 3 mm i.d. and 2.6µm, 100Å particle size (Kinetex F5, Phenomenex, Torrance, CA, USA). The mobile phase A was 0.1% formic acid in LC grade water and mobile phase B was LC grade acetonitrile (ACN). The gradient used was as follows: 95% of A, decreased to 70% in 3 min, to 50% in 2 min, up to 2% in 3 min, and finally back to initial conditions in 4 min. A post-run time of 5 min was done before the next injection. The column was thermostatted at 25ºC. The flow rate was set at 0.35 mL/min and the injection volume was 10 µL. The system used an electrospray ion source (ESI) operating in positive mode in the

following conditions: drying gas temperature (300ºC), drying gas flow (10 L/min), pressure of the nebuliser (40 psi) and capillary voltage (4000 V). Nitrogen gas was used in the nebuliser and in the collision cell. Identification of neonicotinoid residues in samples of pollen and nectar was based on the detection of two selected reaction monitoring (SRMs transitions) The optimal SRM transitions for residue identification and quantification are included in **Table S1** (supporting information). The most intense SRM was selected as the quantifier transition (SRM1), while the second most intense SRM was chosen as the qualifier transition (SRM2).

Pollen samples were extracted using a modified QuEChERS method (AOAC 2007). Pollen sample (0.5g) was weighed into a 30 mL polypropylene (PP) tube, 2 mL of MilliQ water and 30 µL of internal standard were added; the PP tube provided with a ceramic homogeniser was agitated in an automatic shaker for 2 min, in horizontal position. 2.5 mL of ACN was added and the tube was agitated during 1 min. A mixture of 2.5 g of anhydrous magnesium sulphate and 0.6 g of sodium acetate was added and the sample was immediately shaken on a mechanical shaker. After shaking and centrifugation for 5 min at 4500 rpm and 4ºC, an aliquot of the extract (2 mL) was transferred into a 10 mL PP centrifuge tube. 200 mg of a mixture of sorbents PSA (primary secondary amine), C18 and graphitised carbon black in an equivalent proportion 1:1:1, w/w was added and the sample was shaken again for 2 min, in vertical position. As a final step, and after centrifugation for 5 min at 4500 rpm, an aliquot of cleaned extract (1.5 mL) was evaporated to dryness in a vacuum evaporator (Genevac EZ-2, Ipswich, U. K.) and reconstituted in ACN:water (1:9). The extracts were filtered through a nylon filter 0.22 µm (Phenomenex, Torrance, CA, USA) before LC-MS analysis. Recovery was from 90 to 112% and from 97% to 115% with a relative standard deviation RSD<15% for thiamethoxam and imidacloprid, respectively (from 1 to 300 ng g^{-1}), being acceptable values, within the 70-120% range and with an associated precision of RSD < 20%.

Nectar samples were diluted before direct analysis with LC-MS system (Martel et al. 2013). In a screw-cap vial, a volume of 100 µL of nectar was weighed and diluted with 850 µL of a solution of MilliQ

168 water (0.05% formic acid) and ACN (9:1, v/v). In addition, 50 µL of internal standard (1000ng mL^{-1})
169 was added. The mixture was homogenised in a vortex for 2 min. The extracts were filtered through a
170 nylon filter 0.22 µm before LC-MS analysis. Thiamethoxam-d3 was used as the internal standard (IS)
171 and surrogate in the residue analysis of samples of pollen and nectar.

172 For quantitative residue analysis, calibration standards were prepared by spiking working standard
173 solutions and the IS into of blank samples of pollen and nectar collected from experimental orchards
174 with no neonicotinoid treatment. Linearity was checked with correlation coefficients above 0.990 in the
175 range from MQL (method quantification limit, 1 ng g^{-1}) to 350 ng g^{-1} (in pollen) or 100 ng g^{-1} (in nectar).
176 IQL (instrumental quantification limit) was established as ten times the standard deviation (10 × SD)
177 (Corley 2003) of the measurement at a concentration level of 1 ng g^{-1}. Standard deviation (SD) was
178 calculated from the repeated measurements (n = 7) of 1 ng g^{-1} spiking level, multiplied by Student's
179 value for a 99% confidence level and six degrees of freedom. IQLs in pollen were 0.6 and 0.9 ng g^{-1} and
180 in nectar were 0.5 and 0.6 ng g^{-1}, for thiamethoxam and imidacloprid, respectively.
181

182 **2.4. Assessment of risk for honeybees**

183 To relate the neonicotinoid concentrations obtained to the risk for honeybees, ETR_{acute} values (acute
184 exposure to toxicity ratio) were calculated when neonicotinoids were applied during the pre-bloom
185 period close to the flowering. ETR_{acute} is the ratio between the amount of neonicotinoid ingested by
186 honeybee and the LD_{50}.
187

188 **2.5. Statistical analysis**

189 Due to the mobility of neonicotinoids in the plant, the nectar and pollen cannot be considered as
190 independent variables. Therefore, we conducted a multivariate analysis of variance (MANOVA) to
191 determine the effect of flowering period (dat) and citrus variety on neonicotinoids in both matrixes,
192 pollen and nectar, considering the flowering period (dat) and the citrus variety as within subject
193 independent variables. When MANOVA showed a significant effect, we analyzed the effect of

194 flowering period (dat) and the citrus variety on the neonicotinoid residues in each matrix individually,
195 pollen and nectar, using an ANOVA. Regression analysis was carried out to evaluate the relationship
196 between pollen or nectar residue levels and the number of days after treatment (dat). Independent
197 samples t-tests were used to determine whether there were significant differences between pollen and
198 nectar residue levels of samples collected during the flowering period. Analysis of variance coupled
199 with LSD multiple comparisons range test were used to evaluate the significance of differences (at
200 α=0.95) of residue levels between different citrus varieties and between different flowering periods,
201 after verification of variance was confirmed. Samples with no detectable residues were scored 0.1 ng g^{-1}
202 (EPA 2000). Statistical analysis was performed using Statgraphics Centurion XVII (Statpoint
203 Technologies 2014) software.

204

205 **3. RESULTS**

206 **3.1. Persistence: Monitoring residue levels in the blooming season of the year following**
207 **treatment**

208 No detectable residues of either neonicotinoid were obtained from the pollen and nectar samples
209 collected 336 and 224 days after treatments in post-bloom for the orange varieties 'Lane Late' navel and
210 'Valencia Late' respectively. Similarly, no detectable neonicotinoid residues were found in pollen and
211 nectar samples of the mandarin variety 'Nules' 418 days after the pre-bloom treatment.

212 **3.2. Dissipation: Monitoring residue levels during the flowering period associated with pre-**
213 **bloom treatment**

214 The ranges and average value of the neonicotinoid concentrations found in pollen and nectar after
215 treatment when application were made during the pre-bloom period are presented in **Table 2.**

216 Based on pooled data, independent of the citrus variety and sample times, the imidacloprid residue
217 (average concentration ± standard deviation) levels in pollen samples (155.5 ± 93.4 ng g^{-1}) were
218 significantly higher than thiamethoxam residues levels (105.6 ± 67.9 ng g^{-1}) (n = 68, t = -2.69; p =

0.009). Furthermore, there were no significant differences between Nules and navel varieties for imidacloprid or thiamethoxam residues in pollen ($p > 0.05$) for pooled data over the three sample periods. In nectar, the average concentration ± standard deviation of imidacloprid (32.0 ± 24.7 ng g^{-1}) was also higher than the average concentration of thiamethoxam (12.6 ± 11.5 ng g^{-1}) ($p < 0.0001$).

For each insecticide, MANOVA analysis indicate that residues in nectar and pollen were significantly affected by flowering period (dat), the citrus variety, and the interaction of both variables (Thiamethoxam: dat: Wilk's $\lambda = 0.01$; $F_{2,23} = 92.32$; $P<0.0001$; variety: Wilk's $\lambda = 0.2$; $F_{2,23} = 12.98$; $P<0.0001$; dat*variety: Wilk's $\lambda = 0.06$; $F_{4,23} = 16.81$; $P<0.0001$; Imidacloprid: dat: Wilk's $\lambda = 0.008$; $F_{2,21} = 94.7$; $P<0.0001$; variety: Wilk's $\lambda = 0.39$; $F_{2,21} = 5.48$; $P=0.001$; dat*variety: Wilk's $\lambda = 0.07$; $F_{4,21} = 12.63$; $P<0.0001$)).

In pollen samples, thiamethoxam and imidacloprid concentrations followed a similar pattern with time, which fitted into a second-degree polynomial equation (R^2 from 0.9834 to 0.6341). The goodness of fit of the three sets of data to this curve indicates that a similar rise and fall of concentration occurs for all three varieties (**Figures 1 and 2)**. This curve describes the predominant processes that occur over time after pesticide application: an initial penetration in the leaf followed by continuous translocation to the pollen and then metabolism in the plant/pollen, with a consequent decline of pesticide residues. Using these second-degree polynomial regression curves, we predicted the time in days after treatment (dat) to reach the maximum and complete dissipation of thiamethoxam and imidacloprid residue levels in each orchard. The highest concentrations were estimated to occur on 17 dat and 18 dat for Nules (orchard 4), 22 dat and 23 dat for Lane Late (orchard 5) and 19 dat and 16 dat for Rohde Summer (orchard 6), for thiamethoxam and imidacloprid, respectively. The time in which the average concentration dropped below detectable residue levels for thiamethoxam was 38 dat, 37 dat and 32 dat for the varieties Nules, Lane Late and Rohde Summer, respectively. Dissipation of imidacloprid concentrations to below detectable levels was estimated to occur after 37 dat, 38 dat and 32 dat for the varieties Nules, Lane Late and Rohde Summer, respectively.

Differences in pollen residue levels were observed among citrus varieties. At the beginning of the flowering period (10% bloom), neonicotinoid residue levels detected in pollen from Nules were significantly higher than in the two varieties of navels ($F = 15.14$, $df = 10$, $p = 0.0019$ and $F= 16.15$, $df =9$, $p=0.016$) for thiamethoxam and imidacloprid, respectively (**Table 2**). Conversely, at the end of flowering period (75% bloom), thiamethoxam and imidacloprid residues levels were significantly lower for Nules than for the navel varieties ($F = 13.59$, $df = 11$, $p = 0.0019$ and $F = 26.04$, $df = 9$, $p = 0.0003$, respectively). At the end of the flowering period, thiamethoxam and imidacloprid displayed similar dissipation rates in Lane Late, whereas in Nules and Rohde Summer imidacloprid, underwent faster dissipation than thiamethoxam (Fig 1 and 2).

For pooled data over the three varieties, thiamethoxam and imidacloprid residue levels in nectar were lower than in pollen collected at the same time after treatment ($n = 67$, $t = 7.83$, $p < 0.0001$ and $n = 63$, $t = 7.11$, $p < 0.0001$). This represented a decrease from 74 to 99% depending on the variety at the end of bloom period, or from 61 to 95%, depending on the flowering period in the variety with the highest difference in concentration between pollen and nectar (Lane late).

In nectar, residues of thiamethoxam and imidacloprid followed a similar pattern that fit a simple first order model (SFO) (R^2 from 0.6052 to 0.944), independent of citrus variety (**Figures 3 and 4)**. There was a decrease in concentration with time from the first sampling. Across all citrus varieties, imidacloprid residue levels detected in nectar were at least two times higher than thiamethoxam levels (**Table 2**).

At the beginning of the flowering period (10% bloom), thiamethoxam and imidacloprid residues were detected in nectar samples, with significantly higher residue levels in samples collected from Nules than in samples collected from navel varieties ($F = 5.42$, $df = 10$, $p = 0.0325$ and $F = 4.69$, $df = 9$; $p = 0.0511$ for thiamethoxam and imidacloprid, respectively) (**Table 2**). At the end of the flowering period (75% bloom), thiamethoxam and imidacloprid were both below detectable residue levels in Nules. In Lane Late, thiamethoxam and imidacloprid residues levels in nectar decreased to 2.1 ng g^{-1} and 4.6 ng g^{-1}, respectively, and in Rohde Summer, decreased to 9.8 ng g^{-1} and 24.1 ng g^{-1}, respectively. The absence

of a maximum concentration in nectar, such as that seen in pollen, can be explained by assuming that the uptake process in nectar was rapid and essentially complete before the first sampling interval.

In general, dissipation rate of imidacloprid in pollen and nectar from 35% to 75% of bloom period seems to be faster than those of thiamethoxam for all citrus varieties assayed (Table 2; Figs. 2 and 4).

3.3. **Assessment of risk for honeybees**

Evaluation of potential risk of foraging and nurse bees was carried out relating the maximum value of the residues found at each sampling period for each orchard (**Table 2**) with the LD_{50} for bees, taking into account a maximum likely intake of pollen and nectar. We assumed that the sugar content in the nectar of citrus flowers is 25% (Byrne et al. 2014) and according to EFSA (EFSA 2013a, EFSA 2013b), for forager bees the maximum intake of nectar is 512 mg day^{-1} and for nurse bees 160 mg day^{-1} of nectar and 12 mg day^{-1} of pollen.

In most cases, the ETR_{acute} values obtained for imidacloprid treatments were higher than those treated with thiamethoxam (**Fig 5**). In the case of imidacloprid, the ETR_{acute} was > 1 until 35 dat, so there was a risk of acute toxicity for foragers and nurses until that date, except for Nules, where the risk is shorter than 35 dat. In the case of thiamethoxam, the ETR_{acute} was < 1 for foragers from 27 dat and from 20 dat for nurse bees, so from 27 dat there was no risk of acute toxicity calculated in this way.

4. DISCUSSION

4.1. Persistence

No residues in pollen and nectar were detected in any orchards (mandarin and orange) between 7 and 14 months after a foliar treatment.

Our results differ from a study that evaluated residues in citrus trees from treatments applied to the soil, where imidacloprid residues in nectar and pollen were detected 232 days after a pre-bloom soil imidacloprid treatment (Byrne et al. 2014). It should be noted that the dose applied to the soil by Byrne

et al. (2014) (0.56 kg ha^{-1}, maximum rate on product label for soil application) was higher than those applied to leaves in our study (0.171-0.225 kg ha^{-1}). In addition, the high persistence of imidacloprid in soil (half-life ranges from 107 to 1,250 days (Goulson 2013, Bonmatin et al. 2015), depending on soil type and its microbial activity) and its high water solubility make it available to the roots for a long period of time, resulting in a continuous roots uptake and translocation to other parts of the plant over time. The foliar dissipation half-lives of neonicotinoids are much shorter than the soil half-lives. From studies on grapes, cabbage and cotton (Buchholz and Nauen 2001, Arora et al. 2009) leaves, the foliar dissipation half-lives of neonicotinoids are also short. In the case of grape leaves, imidacloprid residues dissipated with a half-life of 2.35 to 2.97 days (Arora et al. 2009) which is very short compared with the half-life of these compounds in soil. Nevertheless, to the best of our knowledge, there was no prior data on the half-life of neonicotinoids in leaf for citrus trees.

Therefore, soil application of neonicotinoids may result in more persistent presence of neonicotinoids residues in plant than foliar application. Under the conditions of the recommended use on the product label, it seems that foliar application results in faster translocation and/or metabolization of imidacloprid or thiamethoxam in citrus nectar and pollen than soil application

4.2. Dissipation

Several factors influence in the efficacy of insecticide application by hydraulic sprayers. The most important are droplet size, relative humidity, time elapsed between spray product application and rainfall, product formulation and the leaf structure (Yu et al. 2009, Aryal and Neuner 2010, Xu et al. 2011, Decaro et al. 2016, Lasmar and Cunha 2016). In addition, stomatal pore size and density (Reed and Hirano 1931) and the composition of the epicuticular wax that covers the leaf and the surface roughness depend on the plant species (Wang et al. 2014). All of these parameters affect the penetration of foliar-applied pesticides and also cause differences among plant varieties within the same species.

In our experiment, the same hydraulic sprayer was use in all treatment and so that, the application efficiency could be considered the same in all orchards. On the date of insecticide application and on

later days, the climate conditions were similar among the experimental orchards (**Figure S1)**. During 2016, there were some rain events in the mandarin orchards, which could have led to a lower retention of the treatment on the leaf and therefore to a greater leaching effect. However, in this study, it seems that the levels of leaching occurring through rainfall were insignificant, because the imidacloprid and thiamethoxam residue levels in pollen and nectar detected in mandarins at the beginning of the flowering period were higher than those observed in the orange varieties. These facts could be accounted for by assuming that more foliar penetration by neonicotinoids occurs in mandarins than in oranges, and as a consequence, an initial higher concentration of neonicotinoids in mandarin than in oranges was observed. Nevertheless, biotic factors such as those related to bloom (data and duration of bloom) must be taken into account because experiments of mandarin and oranges varieties were carried out in different years. Differences in the penetration of pesticide into leaf after foliar treatment using the same treatment solution and at the same time and site (the same environmental conditions) in different plant species has been shown by others (Bentson 1990).

The physicochemical properties of the neonicotinoids also influence their absorption and translocation in plants. Both thiamethoxam and imidacloprid have low volatility and are hydrophilic (log Kow value of < 1.8); they are delivered in undissociated form and can cross membranes easily and move through the xylem (Burken and Schnoor 1988. Both compounds have a similar molecular size and molecular weight (291.7 and 255.7 Da). Their water solubilities are 0.61 and 4.1 g L^{-1} for imidacloprid and thiamethoxam, respectively, which is consistent with the greater mobility of thiamethoxam versus imidacloprid, especially in pollen, if we take into account that the application dose of imidacloprid was double that of thiamethoxam.

We consistently found higher concentrations of both neonicotinoids in pollen than in nectar. This difference has also been observed in other plants, such as pumpkin where 73.5 to 88.8% less in nectar than in pollen were obtained (Dively and Kamel 2012). Byrne et al. (2014) also reported that imidacloprid residue levels detected in nectar were lower than those observed in pollen.

In this study, it was observed that in nectar, imidacloprid and thiamethoxam residue levels decreased after the first sampling event (8-13 dat), whereas in pollen the reduction in residue levels began 16 to 22 dat and continued until the end of the flowering period. These results contrast with those obtained by Byrne et al. (2014), where in nectar samples after imidacloprid treatment applied to the soil, a higher neonicotinoid residue level was detected at the second sampling (57-62 dat) compared to the first sampling event (50-55 dat). This difference from the behavior of residues in pollen obtained in this study (**Figs 1 and 2**) may be because our first sampling could have been taken after the maximum absorption in nectar, and we sampled it in the decline period, which would demonstrate a more rapid degradation in this aqueous medium.

In addition, it was observed that the dissipation rate of thiamethoxam and imidacloprid was faster for the mandarin variety in comparison with orange varieties. In the Nules variety, at 37 dat imidacloprid residues were not detected both in pollen and nectar, and similar findings were obtained for thiamethoxam residues in nectar as well. Then, difference in concentration profile with time occurs in orange and mandarin. This could indicate that extrapolations of residue concentrations from one crop to another is not reliable (Sappingon et al. 2018).

4.3. **Assessment of risk for honeybees and other pollinators**

Our results showed that potential exposure of thiamethoxam and imidacloprid residues in pollen and nectar to honeybees depends on the timing of application relative to the flowering period. No risk was obtained 7 months after neonicotinoids application because no residues were detected and the MQL of the validation method in this study was below the concentration that would indicate a risk of adverse effect at this time. When treatments were applied 8-13 days prior to bloom, the ETR_{acute} was >1 indicating a potential risk of mortality for both for foragers and nurses and only at the end of flowering the ETR_{acute} was < 1 (Fig. 5). However, in the calculation of this ETR_{acute} it is not taken into account that honeybees do not normally consume the nectar or pollen they collect until these materials are processed

in their hives (Blatt and Roces 2002, Harano and Nakamura 2006). The LD_{50} used to calculate ETR_{acute} is a measure obtained in the laboratory after an adult worker bee ingests the pesticide in a sugar solution. These risks have been calculated assuming that bees have received that dose only once, under conditions in which the bees ingest the material immediately instead of holding it for delivery to the colony. In addition, processed of stored pollen and nectar in their hives, depending of the conditions, could lead to a degradation of pesticides. Since honey bees metabolize neonicotinoids with half-lives of one hour or less (Suchail et al. 2004), spreading the same dose out over time would greatly reduce the potential risk. In addition, other effects such as synergism with other pesticides, dilution or concentration of neonicotinoids by feeding pollen or nectar from other uncontaminated or contaminated plants and interaction between bees in the colony are not considered. A colony level field study would help to understand the significance of these findings for honey bee health.

It is worth noting that the effect of sub lethal doses of neonicotinoids involves physiological and behavior modifications of honeybees. They do not directly cause the death of the individual bee or the collapse of the colony (van der Sluijs et al. 2013).

In honeybees, at doses of 0.21 and 2.16 ng bee^{-1} imidacloprid disrupts waggle dancing and sucrose responsiveness (Eiri and Nieh 2012). Sub lethal doses of imidacloprid were also found to have cytotoxic activity in the Malpighian tubules (De Almeida Rossi et al. 2013). Exposure to thiamethoxam has also been shown to result in morphological impairment of the bee brain and midgut (Oliveira et al. 2013). Studies based in chronical exposures of 2.87 ppb of thiamethoxam conducted on *Osmia bicornis* (Sandrock et al. 2014) showed that this chronic sublethal exposure had no effect on the longevity but it resulted in fewer nests (22% lower), fewer total broad cells (43.7% lower), and relative offspring mortality was almost two-folder higher. The chronic exposure of 2.4 ppb of thiamethoxam as a field-realistic exposure on *Bombus terrestris* (Stanley et al. 2016) showed that can have impact on both foraging ability and homing success.

Levels of 0.7 ppb in sugar water and 6 ppb in pollen of imidacloprid during two weeks reduced *B. terrestris* workers foraging efficiency (Feltham et al. 2014) and reduced queen production by 85%

(Whitehorn et al. 2012). At exposure to imidacloprid at 10 ppb in sugar water, Gill et al (2012) found a lower foraging of worker bumblebees, and at exposure levels below 10 ppb caused adverse effects on their reproduction (Laycock et al. 2012).

In this work, the concentration of imidacloprid and thiamethoxam in nectar of the orange varieties was on average above 4 and 2.87 ppb respectively during the entire flowering period sampled. In the Nules variety this concentration was above 10 and 2.87 ppb respectively during the first flowering period. Therefore, according to literature , if one pollinator had exclusively fed on the nectar of these flowers during the entire flowering period, it would have been chronically exposed to the product.

4. CONCLUSIONS

Foliar application of neonicotinoids in citrus trees is linked to the presence of pests that feed on tender shoots. These tender shoots occur with the flushes of new growth in spring and summer. In this work, the absence of imidacloprid and thiamethoxam residues levels at 224 days after its foliar application in summer or at least 336 days after its foliar application in spring, indicates that foliar neonicotinoids do not persist and do not pose a lethal risk to bees and other pollinators at those dat. Conversely, imidacloprid and thiamethoxam were detected within the floral nectar and pollen of citrus trees treated pre-bloom close to the flowering period. These neonicotinoid residues levels depended on the timing of treatments relative to flowering period and on the citrus variety, and therefore, these factors are crucial to determine the risk to pollinators that can potentially feed on them. In general, for pre-bloom treatments, neonicotinoid residues in pollen lasted the full flowering period and posed risk to honeybees on the basis of the calculation of the ETR_{acute} value given in this study. Nevertheless, the obtained ETR_{acute} values in this study are not applicable to evaluate the impact of this exposure at the bee colony level. Dissipation of these insecticides in nectar was faster than for pollen, and at the end of flowering period, they were almost undectable. The uptake of both neonicotinoids was higher in mandarin than in the other orange varieties. In addition, higher amounts of these pesticides were translocated to pollen

and nectar of mandarin. Regarding dissipation rates from pollen and nectar, a faster degradation of imidacloprid and thiamethoxam was observed in mandarin than in other orange varieties. The results obtained in this work could be different in other citrus varieties not tested in this trial and should be interpreted with caution when performing exposure assessments for managed pollinators.

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Figure Legends

Fig 1. Thiamethoxam residues in pollen (ng g^{-1}) versus time after treatment (days) on citrus varieties Nules (Orchard 4), Lane Late (Orchard 5) and Rohde Summer (Orchard 6). Fit equations: Orchard 4: $y=-0.5465\ x^2 + 19.519\ x + 35.95$ ($R^2= 0.9396$); Orchard 5: $y=-0.9455\ x^2+ 42.282\ x-280.22$ ($R^2= 0.9705$); Orchard 6: $y= -0.6423\ x^2 +24.294\ x -126.67$ ($R^2=0.6341$).

Fig 2. Imidacloprid residues in pollen (ng g^{-1}) versus time after treatment (days) on citrus varieties Nules (Orchard 4), Lane Late (Orchard 5) and Rohde Summer (Orchard 6). Fit equations: Orchard 4: $y=-0.9084\ x^2 + 33.353\ x + 9.6607$ ($R^2= 0.9834$); Orchard 5: $y=-1.2634\ x^2+ 59.413\ x-445.95$ ($R^2= 0.9454$); Orchard 6: $-0.7989\ x^2 +26.659\ x -44.522$ ($R^2=0.6785$).

Fig 3. Thiamethoxam residues in nectar (ng g^{-1}) versus time after treatment (days) on citrus varieties Nules (Orchard 4), Lane Late (Orchard 5) and Rohde Summer (Orchard 6). Fit equations: Orchard 4: $y=98.843e^{-0.19x}$ ($R^2=0.8343$); Orchard 5: $y=70.158\ e^{-0.095x}$ ($R^2=0.8264$); Orchard 6: $y=50.983e^{-0.065x}$ ($R^2=0.6052$).

Fig 4. Imidacloprid residues in nectar (ng g^{-1}) versus time after treatment (days) on citrus varieties Nules (Orchard 4), Lane Late (Orchard 5) and Rohde Summer (Orchard 6). Fit equations: Orchard 4: $y=706.4\ e^{-0.221x}$ ($R^2=0.944$); Orchard 5: $y=208.92\ e^{-0.104x}$ ($R^2=0.8495$); Orchard 6: $y=132.08\ e^{-0.069x}$ ($R^2=0.7302$).

Fig 5. Acute exposure to toxicity ratio (ETR_{acute}) for forager and nurse bees in the citrus groves at each time of flowering for each neonicotinoid calculated for the maximum diary intake of nectar and pollen. The oral LD_{50} for thiamethoxam and imidacloprid is 5.0 ng bee^{-1} and 3.7 ng bee^{-1}, respectively. According to the EFSA, consumption of pollen and nectar by a worker honeybee was 32-128 mg of sugar day^{-1} and by nurse bee 35-40 mg of sugar day^{-1} and 6.5-12 mg day^{-1} of pollen[16,17]. A sugar content of 25% in citrus flower nectar was assumed[15]. (F): Forager; (N): Nurse.

570 Fig S1. Average temperature (ºC), rainfall (mm) and relative humidity (%) in the citrus orchards in the
571 studied period. Source: Xarxa Agrometeorològica de Catalunya (Orchard 1: Aldover Station ($40^0$52'47
572 N; $0^0$29'57 E). Orchards 2-6: Alcanar Station ($40^0$33'13 N; $0^0$30'55 E)).

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574

1 **TABLES**

2 **Table 1.** Geolocation of orchards, date of application, date of sampling, treatment dose and varieties of citrus.

Experiments	**Persistence**			**Dissipation**		
Citrus trees	Mandarin	Oranges		Mandarin	Oranges	
Orchard	orchard 1[a]	orchard 2[a]	orchard 3[a]	orchard 4[b]	orchard 5[b]	orchard 6[b]
Geolocation of orchards	$40^0$30'35 N; $0^0$29'35 E	$40^0$32'24 N; $0^0$27'08 E	$40^0$30'54 N; $0^0$29'51 E	$40^0$32'06 N; $0^0$26'48 E	$40^0$52'01 N; $0^0$31'17 E	$40^0$33'19 N; $0^0$25'39 E
Date of application	2 Apr 2015 (pre-bloom)	13 May 2015 (post-bloom)	10 Sept 2015 (post-bloom)	26 Apr 2016 (pre-bloom)	29 Mar 2017 (pre-bloom)	29 Mar 2017 (pre-bloom)
Date of sampling (Days after treatment)	23 May 2016 (418)	13 Apr 2016 (336)	21 Apr 2016 (224)	4 May 2016 (8) 18 May 2016 (22) 2 Jun 2016 (37)	11 Apr 2017 (13) 24 Apr 2017 (22) 3 May 2017 (35)	11 Apr 2017 (13) 18 Apr 2017 (20) 25 Apr 2017 (27)
Species	*C. clementina* Hort. ex Tanaka. cv 'Nules'	*C. sinensis L.* cv. 'Lane Late'	*C. sinensis L.* cv. 'Valencia Late'	*C. clementina* Hort. ex Tanaka. cv 'Nules'	*C. sinensis L.* cv. 'Lane Late'	*C. sinensis L.* cv. 'Rohde Summer'
Imidacloprid a.i. ha^{-1} (Kg)	0.225	0.225	0.171	0.225	0.21	0.19
Thiamethoxam a.i. ha^{-1} (Kg)	0.113	0.113	0.085	0.113	0.105	0.095

3 [a] Orchards 1, 2 and 3: experiments for evaluating persistence after pre- and post-bloom treatments

4 [b] Orchards 4, 5 and 6: experiments for evaluating dissipation after pre-bloom treatments

5 a.i active ingredient

6

Table 2. Thiamethoxam and imidacloprid (ng g^{-1}) residue levels measured in pollen and nectar samples after pre-bloom treatments

		Thiamethoxam						Imidacloprid					
		Pollen			Nectar			Pollen			Nectar		
Citrus variety (orchard)	**% flowering period* (days after treatment)**	**mean**	**max**	**min**	**mean**	**max**	**min**	**mean**	**max**	**min**	**mean**	**max**	**min**
Nules (4)	10% (8)	157.1**a**	180.1	111.1	35.1**a**	41.9	25.6	218.4**a**	242.8	197.3	77.5**a**	95.9	53.3
Lane Late (5)	10% (13)	109.7**b**	113.2	105.9	16.8**b**	22.1	13.4	112.9**c**	119.9	106.8	43.5**b**	54.5	34.8
Rohde Summer (6)	10% (13)	80.6**b**	89.7	70.6	23.9**ab**	31.7	15.6	167.0**b**	197.3	124.7	62.3**ab**	73.6	50.2
Nules (4)	35% (22)	200.9**a**	236.2	170.6	1.5**b**	3.0	0.1	303.8**a**	323.9	270.6	13.4**b**	15.4	11.6
Lane Late (5)	35% (22)	192.4**a**	193.8	190.7	13.1**a**	19.0	8.8	249.7**b**	284.2	211.4	32.5**a**	37.9	23.9
Rohde Summer (6)	35% (20)	97.8**b**	116.2	72.0	13.1**a**	16.6	11.3	169.1**c**	190.7	154.5	27.5**a**	29.8	25.7
Nules (4)	75% (37)	10.0**b**	12.1	5.7	0.1**c**			0.1**b**			0.1**c**		
Lane Late (5)	75% (35)	41.4**a**	62.3	17.1	2.2**b**	3.1	1.7	85.8**a**	93.5	72.7	4.6**b**	6.6	3.2
Rohde Summer (6)	75% (27)	61.0**a**	72.5	47.5	9.8**a**	12.5	5.7	92.8**a**	127.9	64.8	24.1**a**	30.6	17.1

Means within a column within the same percentage of flowering followed by the same letter are not statistically significant (LSD test $p<0.05$).

*Estimated percentage of fully open flowers in the citrus orchard

No detected scored as 0.1 ng g^{-1}

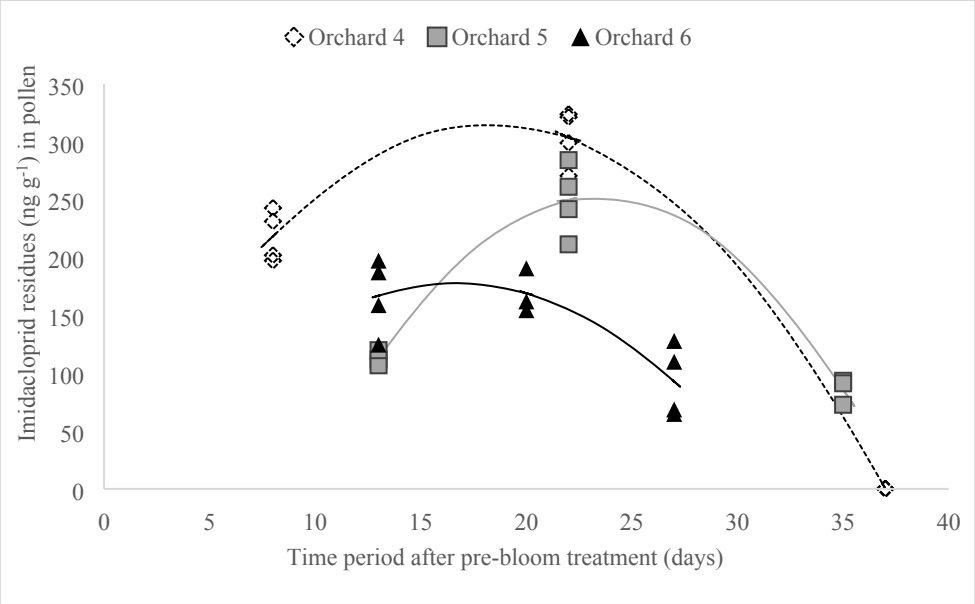
Orchard 4
Orchard 5
Orchard 6
Imidacloprid residues (ng g-1) in pollen
350
300
250
200
150
100
50
0
0
5
10
15
20
25
30
35
40
Time period after pre-bloom treatment (days)

Fig 1

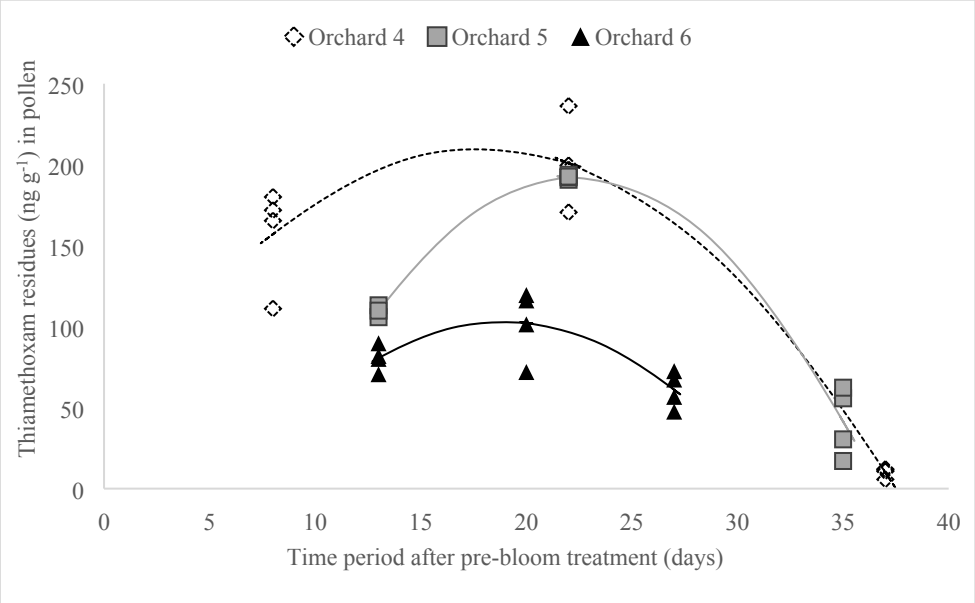
Orchard 4
Orchard 5
Orchard 6
Thiamethoxam residues (ng g-1) in pollen
250
200
150
100
50
0
0
5
10
15
20
25
30
35
40
Time period after pre-bloom treatment (days)

Fig 2

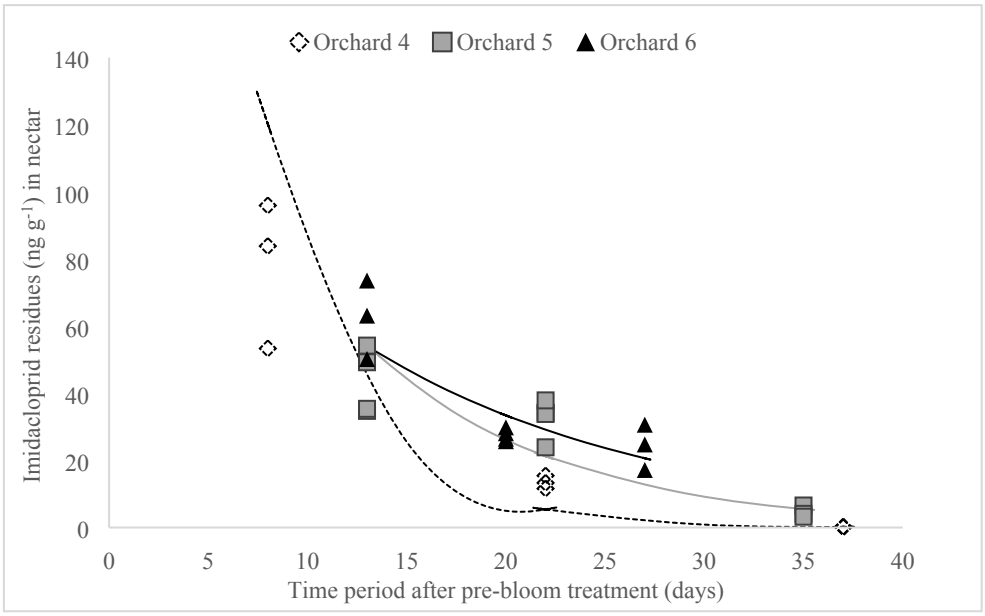
Orchard 4
Orchard 5
Orchard 6
Imidacloprid residues (ng g-1) in nectar
Time period after pre-bloom treatment (days)
0
20
40
60
80
100
120
140
0
5
10
15
20
25
30
35
40

Fig 3.

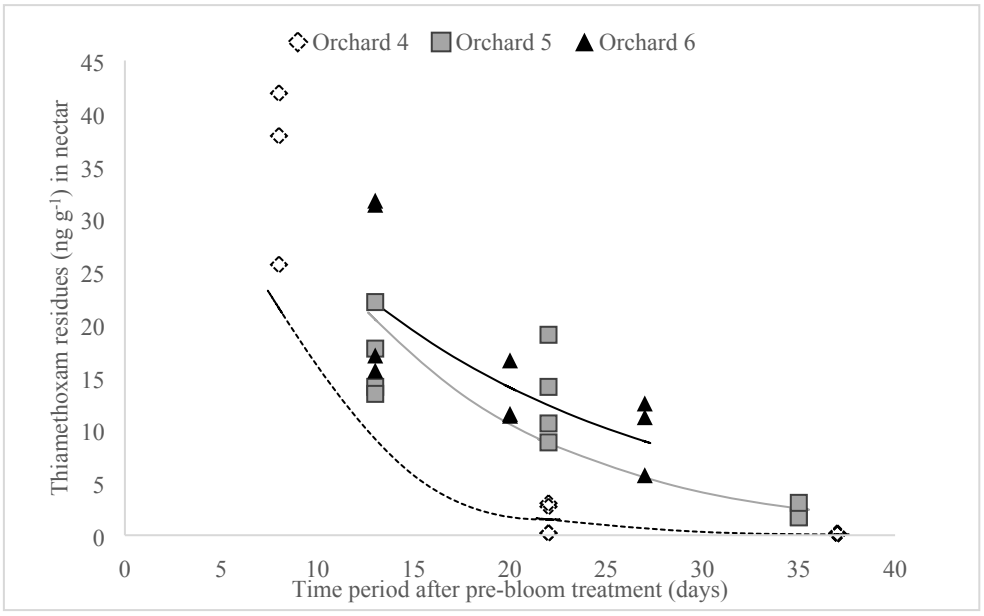
Orchard 4
Orchard 5
Orchard 6
Thiamethoxam residues (ng g-1) in nectar
Time period after pre-bloom treatment (days)
0
5
10
15
20
25
30
35
40
45
0
5
10
15
20
25
30
35
40

Fig 4.

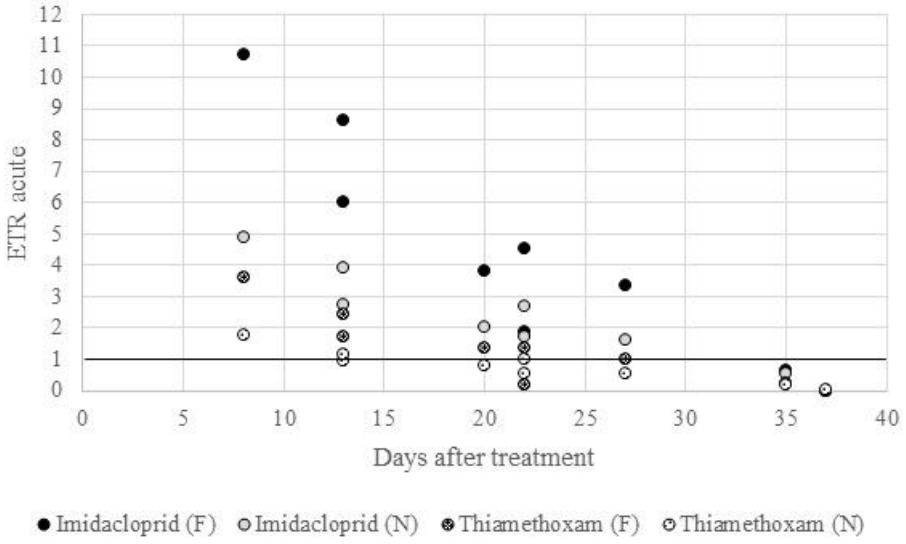
ETR acute
Days after treatment
Imidacloprid (F)
Imidacloprid (N)
Thiamethoxam (F)
Thiamethoxam (N)

Fig 5.

1 **Table S1**. Optimized transitions for MS/MS analysis

Compound name	Precursor Ion	Fragmentor (V)	Product Ion 1	Collision Energy 1 (V)	Product Ion 2	Collision Energy 2 (V)
Thiamethoxam	292	80	211	5	181	20
Imidacloprid	256	10	175	20	209	10
Thiamethoxam d3	295	60	214	15	184	20

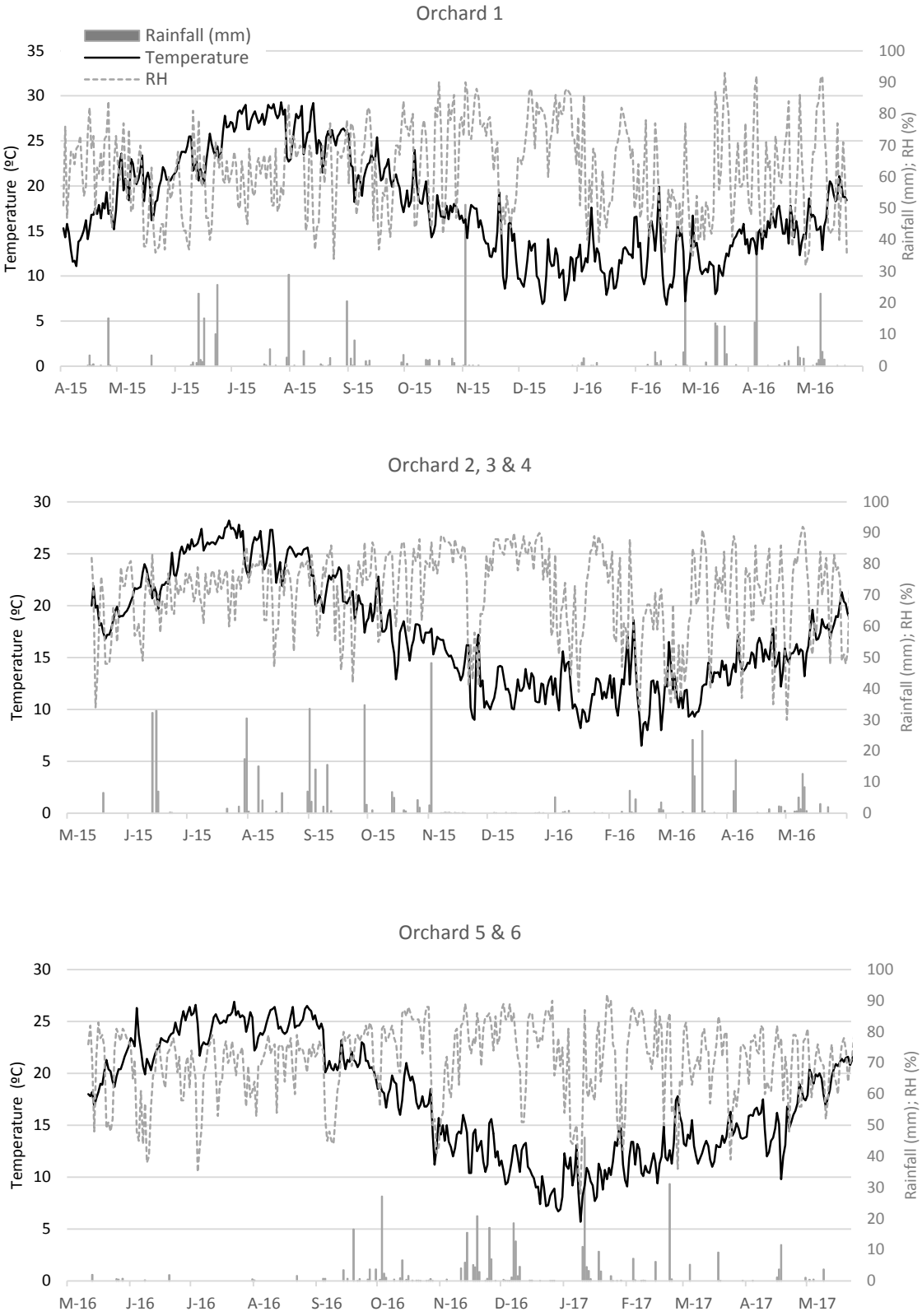
Orchard 1
Rainfall (mm)
Temperature
RH
Temperature (ºC)
Rainfall (mm); RH (%)
A-15
M-15
J-15
J-15
A-15
S-15
O-15
N-15
D-15
J-16
F-16
M-16
A-16
M-16
Orchard 2, 3 & 4
Temperature (ºC)
Rainfall (mm); RH (%)
M-15
J-15
J-15
A-15
S-15
O-15
N-15
D-15
J-16
F-16
M-16
A-16
M-16
Orchard 5 & 6
Temperature (ºC)
Rainfall (mm); RH (%)
M-16
J-16
J-16
A-16
S-16
O-16
N-16
D-16
J-17
F-17
M-17
A-17
M-17

Figure S1.