



# Sugar provisioning for ants enhances biological control of mealybugs in citrus

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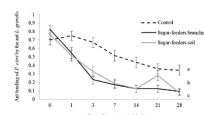
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## HIGHLIGHTS

- Ant tending was reduced within 1-day of sugar provisioning.
- Sugar provisioning for ants disrupted their mutualistic association in citrus.
- Ant activity and ant tending decreased when sugar-feeders were provided.
- Parasitism rates increased in mealybug's colonies when sugar-feeders were provided on branches.

## GRAPHICAL ABSTRACT



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## ABSTRACT

In many agroecosystems, the implementation of an integrated pest management (IPM) program against mealybugs (Hemiptera: Pseudococcidae) is undermined by the presence of ants (Hymenoptera: Formicidae). In a mutualistic association, ants obtain carbohydrates from the mealybugs in the form of honeydew and, in exchange, protect the mealybugs against their natural enemies. Ants however also need protein in their diet. Thus, they can also prey on the mealybugs, especially when their carbohydrates requirements are met. This study aims to assess whether providing ants with sugar-feeders (representing *ad libitum* sources of carbohydrates) i) alters their mutualistic relationships with the mealybugs (ant tending), ii) increases the parasitism of mealybugs, iii) increases ant predation rate and iv) alters the protein and carbohydrate profile in ant workers. For this study, we selected a citrus orchard with high infestation levels of the mealybug *Planococcus citri* tended by the ant *Lasius grandis*. Our results showed that sugar provisioning reduced ant activity and ant tending, resulting in an increase of mealybug parasitism rates. Ants showed a tendency to prey more sentinel preys in trees with sugar-feeders, even though the differences were not significant. The gut content in terms of carbohydrates and proteins of ant workers did not differ among trees with and without sugar-feeders. Overall, our results show that sugar provisioning can be used for improving pest management strategies against mealybugs; while further research is necessary to corroborate that predation by ants of honeydew producers increases when sugar-feeders are provided.

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## 1. Introduction

Mealybugs (Hemiptera: Pseudococcidae) are major agricultural pests worldwide (Miller et al., 2002; Franco et al., 2009; Mani and Shivaraju, 2016). They are phloem feeders and can reduce plant vigour, fruit quality and crop yield. Moreover, they excrete honeydew that promotes the growth of black sooty mold (Franco et al., 2004; Gullan and Martin, 2009; Pérez-Rodríguez et al., 2017). Biological control, using predators and parasitoids, can be an effective strategy to control mealybug populations (Moore, 1988; Roltsch et al., 2006; Mani and Shivaraju, 2016; Shylesha and Mani, 2016; Hajek and Eilenberg, 2018; van Lenteren et al., 2018; Andreason et al., 2019). Its use has increased during the last decades in several crops (Neuenschwander, 2001; Muniappan et al., 2006; Roltsch et al., 2006; Amarasekare et al., 2009; Ahmad et al., 2011; Rodrigues-Silva et al., 2017). However, the success of parasitoids and predators in controlling mealybugs can be counteracted by the presence of ants (Cudjoe et al., 1993; González-Hernández et al., 1999; Daane et al., 2007; Mgocheki, and Addison, 2009; Zhou et al., 2014).

In a mutualistic association with the pest, ants obtain honeydew (carbohydrates) by tending mealybugs and, in return, they improve hygiene of the mealybug colony (when ants remove dead individuals or exuviae), transport them to new feeding sites and, more importantly, protect them against natural enemies (Helms and Vinson, 2002; Daane et al., 2007; Cheng et al., 2015; Marques et al., 2018; Xu et al., 2020). Ants have disrupted several biological control programs against mealybugs by disrupting, attacking or killing parasitoids and predators (Campos et al., 2006; Feng et al., 2015; Tanga et al., 2016; Fanani et al., 2020). For instance, the ant species *Lasius niger* (Latreille) (Hymenoptera: Formicidae) reduced the parasitism of the citrus mealybug *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae) by 50% (Campos et al., 2006). A similar reduction in the parasitism was observed in the cassava mealybug *Phenacoccus manihoti* Matile-Ferrer (Hemiptera: Pseudococcidae) when it was tended by three different ant species (Cudjoe et al., 1993).

Ant species that establish mutualistic association with hemipterans that excrete honeydew also need to feed on protein sources for colony growth, which especially applies to the queen and brood (Beattie, 1985). Depending on the ant species, proteins are obtained from preying and/or scavenging (Carroll and Janzen, 1973; Stradling, 1978; Cerdà and Dejean, 2011) and its demand is determined by carbohydrate availability (Kay, 2004). An increase in carbohydrate availability promotes workers activity and aggression towards protein-rich prey (Ness et al., 2009; Kay et al., 2010). In this context, ants might tend honeydew producers, such as mealybugs, but may also prey upon them or other arthropods when there is a surplus of carbohydrates (Engel et al., 2001; Pekas et al., 2011). Due to the fact that ants prey on arthropod pests and can be highly effective in regulating their populations, ants have long been used as biocontrol agents (Offenberg, 2015).

In Mediterranean citrus, *Lasius grandis* Forel (Hymenoptera: Formicidae) is a dominant ant species and has a notable preference for *P. citri* honeydew over other honeydew producers such as whiteflies, aphids or coccids (Pekas et al., 2011). *Lasius grandis* workers ascend to the tree canopy from April to November, when *P. citri* populations are at their peak and establish mutualistic associations (Martínez-Ferrer, 2003; Pekas et al., 2011). The provisioning of alternative sugar sources with sugar-feeders has been shown to disrupt the mutualistic association between ants and honeydew producers (Carabali-Banguero et al., 2013; Nagy et al., 2013; Beltrà et al., 2017; Wäckers et al., 2017). For instance, the provisioning of alternative sugar sources reduced ant tending and colony size of aphids (Nagy et al., 2013; Wäckers et al., 2017). Similarly, sugar provisioning was effective in reducing ant tending of the vine mealybug, *Planococcus ficus* (Signoret) (Beltrà et al., 2017), of the pineapple mealybug, *Dysmicoccus brevipes* (Cockerell) (Hemiptera: Pseudococcidae) by the predaceous fire ant *Solenopsis geminata* (Fabricius) (Carabali-Banguero et al., 2013) or by the big-headed ant, *Pheidole megacephala* (Hymenoptera: Formicidae) (Win et al., 2018).

Here, we tested whether sugar provisioning with sugar-feeders i) disrupts the mutualistic association between the ant *L. grandis* and the citrus mealybug *P. citri* and as a consequence ii) enhances the biological control of the mealybug; iii) whether this provision also affects the carbohydrate:protein content of *L. grandis* ants, and iv) whether sugar feeders increase ants predation in the citrus canopy. For this purpose, the sugar-feeders were installed either on the soil or at the trunk of citrus trees of a commercial citrus orchard. Then, we measured i) the ant activity and ant tending of mealybug colonies in citrus canopies; ii) parasitism of mealybugs; iii) protein and carbohydrate content in ant workers; and iv) predation rates by ants upon sentinel prey.

## 2. Materials and methods

### 2.1. Sampling site

The study was conducted in a citrus orchard located in the main citrus production area of eastern Spain (X: 39.594327, Y: -0.426612). It was selected based on the high infestation levels of *P. citri* and the presence of the dominant ant *L. grandis*. It was a 12 years old production orchard (~330 trees / ha) with a 6 × 5 m planting pattern. The citrus cultivar grown was a “navel powell” variety grafted on “citrange Carrizo” rootstock. The insecticides chlorpyrifos-methyl and spirotetramat were sprayed three and two months respectively, prior to our experiment against *Pezothrips kellyanus* (Bagnall) (Thysanoptera: Thripidae) and California red scale *Aonidiella aurantii* (Hemiptera: Diaspididae) (Maskell).

### 2.2. Experimental design

The orchard was divided in four blocks. Each block contained 48 trees (4 × 12 trees) and was divided in three plots (4 × 4 trees) corresponding to the three treatments, i.e. sugar-feeders placed on the soil, sugar-feeders placed at the branches and control (=no sugars) randomly assigned within the block. In the sugar-feeders in the soil treatment, one sugar-feeder was placed at ~20 cm from the trunk of each tree. In sugar-feeders on the branches treatment, one sugar-feeder per tree was placed above the trunk, at the first division of the three main branches (at ~0.4 m height). Finally, the control treatment included trees without sugar-feeders. Observations and samplings took place in the four central trees of each plot (i.e., 16 trees per treatment). Sugar-feeders were installed on August 1st, once *P. citri* had colonized the fruits and after confirming the presence of *L. grandis* and *P. citri* in all of the trees.

### 2.3. Description of the sugar-feeders

Sugar-feeders consisted of 250 mL plastic pouches sealed at the top with a perforated lid 28 mm in diameter. Sugars were available through a cotton wick 15 cm long; approximately 10 cm of the wick was inside the pouch whereas the rest was exiting the pouch through the perforated lid at the top. The part of the wick outside the pouch was covered with a cylindrical plastic mesh (openings size 25 × 28 mm), adjusted on the lid and effectively excluding the larger sugar-feeding arthropods, such as wasps and honeybees, yet allowing ants to feed on the sugar (see Wäckers et al., 2017). The sugar used was Biogluc® (Belgoscuc, Belgium), a ready to use sugar solution diluted 1:1 with water. This resulted in a 35.7% (w/w) sugar solution containing fructose (37.5%), glucose (34.5%), sucrose (25%), maltose (2%) and oligosaccharides (1%).

### 2.4. Effect of artificial sugar-feeders on ant activity and parasitism rates

We measured ant activity on the tree canopy and on the sugar-feeders. On the same days that ant activity was assessed, we also recorded ant tending on the *P. citri* colonies. Parasitism of the mealybug was assessed the last day of the assay.

#### 2.4.1. Ant activity in citrus canopy and at sugar-feeders

Ant activity on the canopy was defined as the number of ants crossing a section, located 20 cm above the sugar dispensers, during one minute (Pekas et al., 2011). Given that ant activity can vary throughout the day, assessments took place between 9:00 and 13:00. Ants crossing the section were identified to species *in situ*. Ant activity assessments took place before sugar-feeders were installed as well as, 1, 3, 7, 14, 21 and 28 days after installation.

Ant activity on the sugar-feeders was defined as the number of ants present within the grid surrounding the sugar wick during one minute. Assessments took place at 7, 14, 21 and 28 days after sugar provisioning.

Ant activity on the canopy prior to sugar provisioning was analyzed using a generalized linear model (GLM) with a quasi Poisson error distribution. Following sugar provisioning, ant activity on the citrus canopy and at the sugar-feeders was analyzed using a generalized linear mixed effect model (GLMM) with a Poisson error distribution. Treatment was the fixed-effect while tree was nested within block and time were the random effects. We used the *glht* function from the *multcomp* package to perform Tukey HSD tests for post-hoc pairwise comparisons (Hothorn et al., 2008).

#### 2.4.2. Ant tending and parasitism on *Planococcus citri* colonies

Depending on availability, three to five colonies of *P. citri* were selected and labelled on each of the four central trees per treatment and replicate (~64 colonies per treatment). Sampled trees were the same as those in which ant activity was recorded. The number of ants on each mealybug colony was recorded. We considered as a colony when more than three mealybug individuals were clustered. Ant tending on the *P. citri* colonies was defined as the ratio of mealybug colonies per tree tended by at least one ant and the mean number of ants per mealybug colony per tree (absolute-ant tending). Ant tending and absolute-ant tending were recorded one day prior to sugar provisioning as well as 1, 3, 7, 14, 21 and 28 days following installation.

Differences between treatments in ant tending (i.e., the ratio of mealybug colonies tended by ants) and absolute ant tending were analyzed prior to the sugar-feeder installation using a generalized linear model with a quasibinomial and quasi Poisson error distribution. After sugar provisioning, ant tending was analyzed using a generalized linear mixed effect model with a binomial error distribution and absolute ant tending with a Poisson error distribution. Treatment was the fixed-effect and tree nested within block and time the random effects. We used the *glht* function from the *multcomp* package to perform Tukey HSD tests for post-hoc pairwise comparisons.

In order to determine the mealybug parasitism, a destructive sampling took place the last day (28th day after sugar provisioning) of the study. The labelled mealybug colonies were collected, placed individually in sealed bags and transported in a portable refrigerator with cooling elements to the laboratory where mealybugs were observed under a binocular for signs of parasitism. Parasitism rates were calculated as the number of mealybugs parasitized (with a puncture or mummified body) or with an emergence hole out of the total mealybugs stages suitable for parasitism. Parasitism rates were analyzed with a generalized linear model with quasibinomial distribution. We used the *glht* function from the *multcomp* package to perform Tukey HSD tests for post-hoc pairwise comparisons.

#### 2.5. Predatory ant activity on the soil surface and tree branches

*In the soil.* To determine if sugar provisioning affected the predatory activity of the ants, four Petri dishes each one containing ten pupae of *Ceratitis capitata* (6 cm Ø x 1 cm height), were placed in each of the four central trees (i.e., 640 pupae per treatment and 1,920 in total). *Ceratitis capitata* pupae were used as sentinel prey because they are immobile, are present in citrus and ants are reported prying upon them (ref). Four holes (0.5 cm Ø) were perforated laterally on the petri dishes for allowing ants to enter and were filled with a layer of sterilized sand to

simulate the soil. The petri dishes were placed at 40 cm from the trunk and facing each other with the trunk at the center. *Ceratitis capitata* pupae were obtained from a rearing established at IVIA and renewed yearly. The predation experiment was carried out the 8th and the 22nd day after sugar provisioning. Predation was evaluated 24 h later by counting the number of pupae that had disappeared. During the experiment, several ants were observed leaving the Petri dishes with mealy pupae in their mouth and were identified *in situ* to the species level.

*On the tree branches.* Since *L. grandis* ants were not observed foraging inside the Petri dishes located in the soil, predation rates were also measured on tree branches (where *L. grandis* was observed foraging) the 22nd day of the trial. Therefore, two Petri dishes with the same characteristics as explained above were placed on the two main branches closest to the trunk basis (one per branch). Ant predation on branches was evaluated 72 h later and ants were identified *in situ* at the species level.

Ant predation on the soil and on the tree branches were analyzed using a generalized linear mixed effect model with a binomial error distribution. Treatment was the fixed-effect and tree nested within block. We used the *glht* function from the *multcomp* package to perform Tukey HSD tests for post-hoc pairwise comparisons.

#### 2.6. Protein and carbohydrate content of ant workers

After identifying the two dominant ant species that preyed upon *C. capitata* pupae, between 31 and 51 ants of *L. grandis* and *Aphaenogaster iberica* Emery (Hymenoptera: Formicidae) were collected per treatment the 22nd day after sugar provisioning. *Aphaenogaster iberica* workers that were foraging on the ground close to the tree (~80 cm from the trunk) and *L. grandis* that were foraging on the tree trunk were aspirated, placed individually in Eppendorf tubes and kept in a portable refrigerator with cooling elements. Once in the lab, they were kept at -20 °C until their protein and carbohydrate (fructose, sucrose and glycogen) levels were analysed following the protocol described by Foray et al. (2012) and Urbaneja-Bernat et al. (2019). Eppendorf tubes with individual ants were vortexed for 10 s in 1 mL warm (60 °C) autoclaved deionized water to remove carbohydrates from the outside of the ant bodies. Afterwards, ants were transferred to new Eppendorf tubes and kept on ice. Each ant was crashed in an aqueous buffer solution and centrifuged at 10,000 rpm during 5 min at 4 °C. In order to analyse the protein content, during those 5 min 250 µL Bradford microassay reagent was added. After 10 min 5 µL of the supernatant was taken and the absorbance at 595 nm was measured in a 96-well plate reader (Biochrom® Anthos 2010, Biochrom, UK). The rest of the sample was kept at -20 °C for measuring carbohydrate content. The absorbance values were compared with absorbance values of known protein using bovine serum albumin [0, 1, 2, 4, 6, 8, and 10 µg].

Ants carbohydrate content was measured using a quantitative anthrone assay (modified from Olson et al., 2001; Lee et al., 2004; Wyckhuys et al., 2008; Foray et al., 2012). Particularly, we measured fructose, sucrose and glycogen content. To determine the carbohydrate content, each sample was homogenized in a vortex mixer and 20 µL of 20% sodium sulfate and 5 µL of aqueous buffer solution were added. This solution was mixed with 1500 µL of chloroform/ methanol (1:2 v/v) and homogenized in a vortex mixer. The homogenate was centrifuged for 15 min at 13,000 rpm and 4 °C and the supernatant, containing all soluble sugars, was transferred to an Eppendorf tube. The white pellet, containing insoluble high molecular weight sugars such as glycogen, was kept on ice until used to determine glycogen level. The supernatant was used to determine fructose and sucrose. First, to determine fructose content 100 µL of anthrone was added in each sample. After 15 min at room temperature the absorbance at 620 nm was measured in a 96-well plate reader (Biochrom® Anthos 2010, Biochrom, UK). The absorbance values were compared with absorbance values of known fructose. After these 15 min, samples were incubated at 65 °C during 2 h (hot anthrone

test) and the absorbance was recorded at 625 nm. To determine glycogen content, 200  $\mu$ L of anthrone reagent was added to the previous remaining white pellet and incubated at 90 °C for 3 min. Next, the tubes were cooled on ice for 15 min and the absorbance read as above at 620 nm.

Carbohydrate and protein concentration for each ant species were compared among treatments using ANOVA with treatment as factor and ant as replicate. All analyses were performed with R studio (Version 1.2.5042, Rstudio, Inc., <https://www.rstudio.com>).

### 3. Results

#### 3.1. Ant activity on the citrus canopy and sugar-feeders

A total of 6,690 ants were observed ascending or descending the citrus canopy throughout the study. From this total, 98.6% were *L. grandis*, 1.1% *Pheidole pallidula* (Nylander), 0.2% *Aphaenogaster iberica* Emery and 0.1% *Plagiolepis* spp. The number of ants ascending and descending the canopy was similar in the three treatments before the sugar-feeders were placed in the field (GLM quasiPoisson:  $F_{2,47} = 1.41$ ;  $P = 0.25$ ) (Fig. 1).

After sugar provisioning, ant activity differed among treatments (GLMM Poisson:  $\chi^2 = 109.54$ ;  $P < 0.0001$ ). The level of ant activity decreased significantly in the trees with sugar provisioning compared to the control trees; the lowest ant activity level was registered in the trees with the sugar-feeders placed on the branches. Ant activity started decreasing as soon as from day 1 after sugar provisioning and remained significantly lower as compared to the control up to the end of the observations (day 28 after sugar provisioning) ( $\chi^2 = 5.27$ ;  $P < 0.0001$ ).

The number of ants present on the sugar-feeders was similar in both treatments (soil and branches) throughout the assay (GLMM Poisson:  $\chi^2 = 1.01$ ;  $P = 0.31$ ). (Fig. 2).

#### 3.2. Ant tending and parasitism on *Planococcus citri* colonies

Ant tending on *P. citri* colonies (ratio of mealybug colonies tended by ants) was similar in the three treatments before sugar provisioning (GLM quasibinomial:  $F_{2, 47} = 0.38$ ;  $P = 0.25$ ) (Fig. 3). However, after sugar provisioning, ant tending differed significantly among treatments (GLMM Binomial:  $\chi^2 = 88.93$ ;  $P < 0.0001$ ). The ratio of colonies tended by ants was higher in control trees than in trees with sugar-feeders ( $P < 0.0001$ ). Significant differences were also found among trees with sugar-feeders located in branches and soil ( $P = 0.01$ ).

Absolute ant tending on *P. citri* colonies was similar in the three treatments before sugar provisioning (GLM quasiPoisson:  $F_{2, 47} = 1.25$ ;

$P = 0.29$ ) (Fig. 4). However, after sugar provisioning, ant tending differed among treatments (GLMM Poisson:  $\chi^2 = 29.64$ ;  $P < 0.0001$ ). Absolute ant tending was higher in control trees than in trees with sugar-feeders ( $P < 0.0001$ ). Significant differences were also found among trees with sugar-feeders located in soil and branches.

Parasitism of *P. citri* differed among treatments (GLM quasibinomial:  $F_{2, 46} = 4.16$ ;  $P = 0.01$ ) and was significantly higher in trees with sugar-feeders on the branches ( $0.58 \pm 0.09$ ) than in trees with sugar-feeders on the soil ( $0.44 \pm 0.07$ ) or control trees ( $0.32 \pm 0.05$ ) ( $P = 0.005$ ). There were no significant differences in parasitism rates between trees with sugar-feeders in soil and controls.

#### 3.3. Predatory ant activity

**In the soil.** Eight days after sugar provisioning, more than 90% of the *C. capitata* pupae (sentinel prey) placed on the soil during 24 h had been preyed (Fig. 5). The percentage of *C. capitata* pupae preyed differed among treatments (GLMM Binomial:  $\chi^2 = 6.13$ ;  $P = 0.047$ ). The percentage of pupae preyed was higher in trees with sugar-feeders installed on the soil than in trees with sugar-feeders on branches ( $P = 0.04$ ). No significant differences were found among control trees and trees with sugar-feeders.

*Aphaenogaster iberica* was the only ant species recorded preying upon and carrying pupae in its mouthparts during the observations. No other insects were observed inside the petri dishes.

Twenty-two days after sugar provisioning, between 60 and 75% of the *C. capitata* pupae placed on the soil during 24 h had been preyed (Fig. 5). The percentage of *C. capitata* pupae preyed upon did not differ among treatments (GLMM Binomial:  $\chi^2 = 2.79$ ;  $P = 0.25$ ). *Aphaenogaster iberica* was again the only ant species recorded preying and carrying pupae in the soil.

**On the tree branches.** Twenty-two days after sugar provisioning, the percentage of *C. capitata* pupae preyed upon during 72 h on the branches was almost twice as high in trees with sugar-feeders (41%) when compared to the control trees (22%). However, this difference was not significant (GLMM Binomial:  $\chi^2 = 3.02$ ;  $P = 0.22$ ), nor were the other comparisons (Fig. 6). *Lasius grandis* was the only species observed foraging and carrying pupae in its mouth parts.

#### 3.4. Protein and carbohydrate content of ant workers

*Lasius grandis* and *A. iberica* workers had markedly different profiles regarding protein and carbohydrate content. *Aphaenogaster iberica* contained almost double of proteins and half of sugars compared to *L. grandis* (Table 1). When comparing treatments, there were no

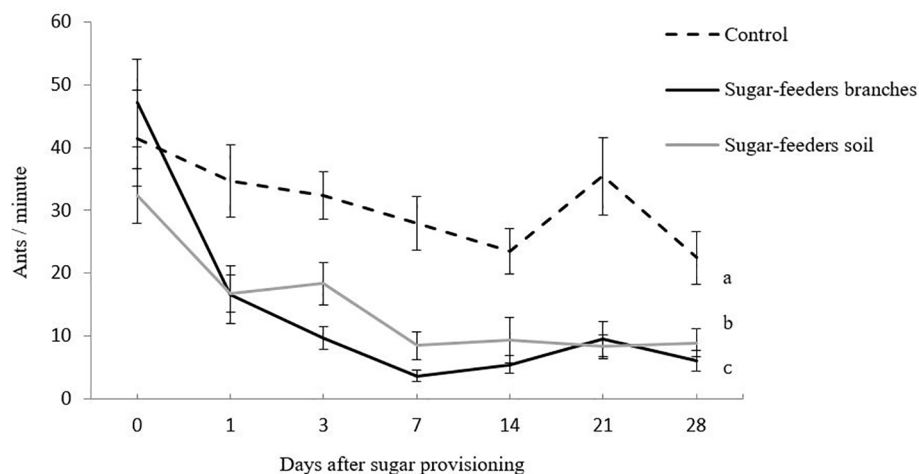


Fig. 1. Mean number of ants ascending and descending to citrus trees without and with sugar-feeders in the branches or in the soil. Different letters indicate significant differences among treatments ( $P < 0.0001$ ).



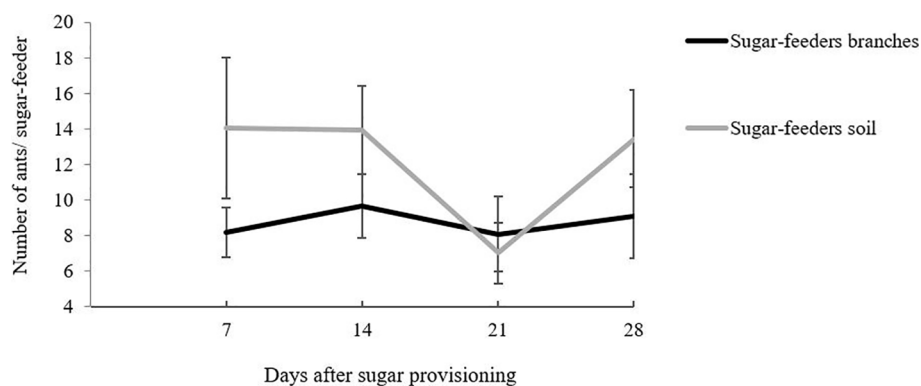


Fig. 2. Mean number of ants per sugar-feeder on both sugar treatments (soil and branches).

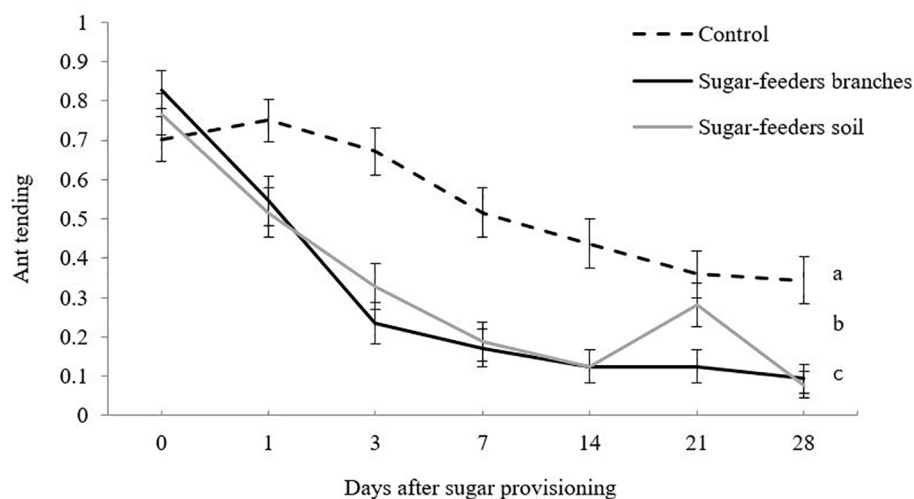


Fig. 3. Ratio of mealybug colonies attended by ants in the sugar-feeders treatments and control. Different letters show significant differences between treatments ( $P < 0.0001$ ).

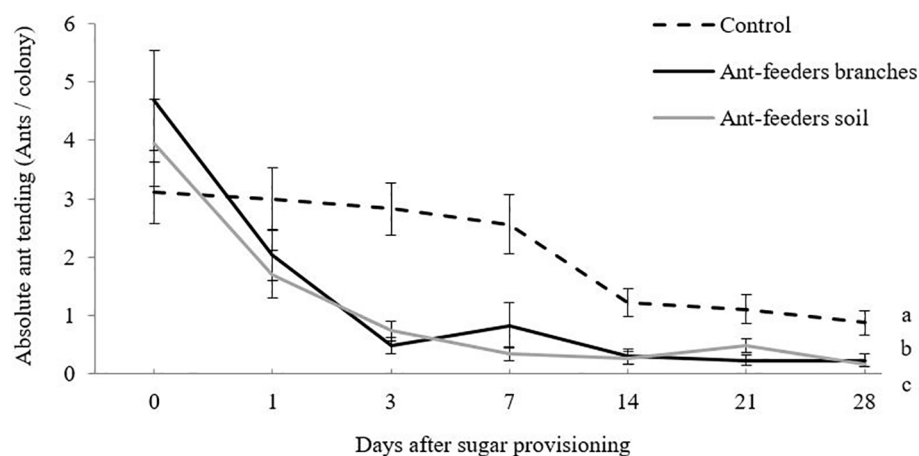


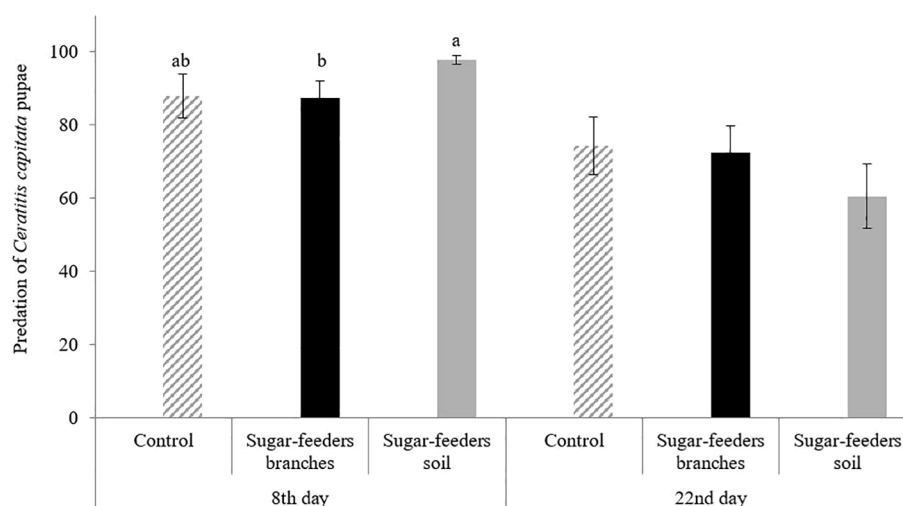
Fig. 4. Mean number of ants per mealybug colony in the sugar treatments (soil and branches) and control. Different letters show significant differences between treatments ( $P < 0.01$ ).

statistically significant differences in protein or carbohydrate content neither for *A. ibérica* nor for *L. grandis* (Table 1).

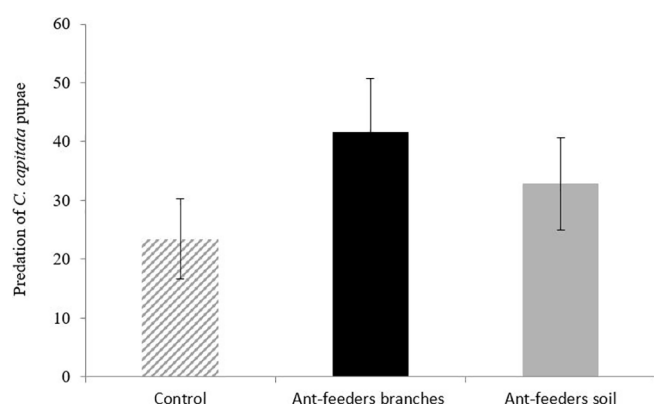
#### 4. Discussion

Our study demonstrated that sugar-feeders containing the sugar

solution Biogluc© disrupted the association between *L. grandis* and *P. citri* at end of the summer, a moment when ant activity and *P. citri* infestation in navel oranges are peaking (Calabuig et al., 2014; Martínez-Ferrer, 2003; Pekas et al., 2011). *Planococcus citri* is present in the trunk and branches of citrus trees during spring and moves to the fruits in summer. Then, forms colonies that are heavily tended by ants and



**Fig. 5.** Percentage of *Ceratitis capitata* pupae predated during 24 h by ants when Petri dishes were placed on the soil A) at the 8th day after sugar provisioning and B) at the 22nd day of the study. Different letters show significant differences between treatments ( $P = 0.047$ ).



**Fig. 6.** Percentage of *Ceratitis capitata* pupae predated by ants when Petri dishes were placed at branches after 72 h.

**Table 1**

Carbohydrate (fructose, sucrose and glycogen) and protein content (mean  $\mu\text{g} \pm \text{SE}$ ) of the ants A) *Lasius grandis* and B) *Aphaenogaster iberica* collected in trees with and without sugar-feeders.

	Control	Ant-feeders branches	Ant-feeders soil	F value	df	P value
<b>(A)</b>						
Fructose	3.19 $\pm$ 0.63	4.31 $\pm$ 0.13	2.51 $\pm$ 0.63	1.04	2, 118	0.36
Sucrose	17.51 $\pm$ 4.25	22.80 $\pm$ 6.06	10.35 $\pm$ 2.21	1.51	2, 118	0.23
Glycogen	15.70 $\pm$ 3.47	15.20 $\pm$ 2.84	12.60 $\pm$ 2.84	0.24	2, 118	0.78
Protein	10.93 $\pm$ 0.74	12.57 $\pm$ 1.57	9.42 $\pm$ 0.64	1.75	2, 118	0.17
<b>(B)</b>						
Fructose	3.09 $\pm$ 0.05	2.46 $\pm$ 0.92	3.74 $\pm$ 0.55	0.9	2, 127	0.41
Sucrose	6.24 $\pm$ 0.99	5.55 $\pm$ 1.42	6.35 $\pm$ 1.01	1.18	2, 127	0.31
Glycogen	14.99 $\pm$ 2.99	9.99 $\pm$ 2.19	16.06 $\pm$ 2.79	0.13	2, 127	0.88
Protein	30.85 $\pm$ 2.39	26.74 $\pm$ 2.79	25.66 $\pm$ 2.07	1.4	2, 127	0.25

excrete large amount of honeydew upon which sooty mould grows, causing severe economic damage by degrading fruit value (Martínez-Ferrer, 2003). Our results showed that, the provisioning of sugar-feeders during that period can represent a sustainable alternative to the use of insecticides for managing infestations of *P. citri*.

Parasitism rates by the parasitoid *A. vladimiri* in *P. citri* colonies increased twofold when ants were provided easy and prolific sugar access from the sugar-feeders placed in the canopy. Without their ant bodyguards, the mealybug colonies are more exposed to their natural enemies. Parasitoids have more opportunities to oviposit when they are less likely to be attacked by ants. When ants attack or chase away parasitoids, this results in the parasitoids reducing their host searching bouts while increasing the time handling hosts until successful oviposition. Fanani et al. (2020) reported that *Anagyrus lopezi* (Santos) (Hymenoptera: Encyrtidae) spent seven minutes to find the host in ant-excluded treatment, whereas in ants present this ranged 24 to 45 min. As result, *A. lopezi* laid ten times more eggs in colonies without than with ants. Moreover, it has been recently demonstrated that *A. vladimiri* females tend to sting a lower number of mealybugs in colonies tended by ants, even when those have recently abandoned the colony (Mouratidis et al., 2020). This is because the parasitoid is able to detect the cuticular hydrocarbon footprint that ants leave when searching for food (Mouratidis et al., 2020). Therefore, ants can disrupt parasitoid efficacy even when they are not present in the mealybug colony. Finally, ant tending alters the composition and quality of the honeydew produced by plant feeders that acts as a host-searching kairomone for parasitoids (Bouchard and Cloutier, 1985; Romeis and Zebitz, 1997; Tranfaglia and Dfga, 2013). It is also worth to mention that the highest parasitism rates were obtained in tree colonies where sugar-feeders were located among the branches. Similar results were obtained in a previous study (Wackers et al. 2017) where the reduction in the size of *Aphis spiraecola* (Hemiptera: Aphididae) colonies was faster when sugars were provided in the branches than in the soil. Therefore, we would recommend providing sugar-feeders on the branches that apparently will also benefit the biological control of other honeydew producing pests in citrus.

The provisioning of sugar-feeders has already been used to reduce ant attendance in citrus and other crops (Offenberg, 2001; Carabali-Banguero et al., 2013; Nagy et al., 2013; Win et al., 2018). Moreover, at least two studies have used Biogluc® as sugar source (Beltrà et al., 2017; Wäckers et al., 2017). Further studies should test whether sugar-feeders containing Biogluc® can be used in other periods of the year and with other dominant ant species present in other citrus growing areas such as the Argentine ant, *Linepithema humile* (Mayr).

Engel et al (2001) showed that when ants lose interest in a honeydew

producer in the presence of alternative sugars sources, this can carry a double cost to the deserted honeydew producers. Not only do they lose their protection, they may also now suffer predation from their previous bodyguards. To test if this possible change in predation, we used *C. capitata* pupae as sentinel prey, offered to two different ant species: *L. grandis* and the endemic Iberian Peninsula species *A. iberica*. The latter preyed upon pupae located on the soil whereas the former preyed upon pupae located on the branches. This segregation means both ant species occupy different ecological niches. Whereas *L. grandis* is frequently found foraging on tree canopies (Paris and Espadaler, 2009; Pekas et al., 2011), *A. iberica* forages on the soil and has rarely been observed on the citrus canopies (Martínez et al., 1997, 2002). *Aphaenogaster iberica* is a scavenging, insectivorous ant species but it is also recorded as an elaiosome-bearing seeds collector (Boulay et al., 2005; Warren et al., 2020). In our study, in contrast to *L. grandis*, *A. iberica* preyed on almost all medfly pupae within 24 h. The role of *A. iberica* in the control of insect pests in citrus remains largely unexplored. Therefore, future research should evaluate the potential use of this ant species as biological control agent against citrus pests that spend part of their life cycle in the soil, such as *C. capitata* (Urbaneja et al., 2006), the mealybug *Delottococcus aberiae* (De Lotto) (Hemiptera: Pseudococcidae) (Martínez-Blay et al., 2018; Pérez-Rodríguez et al., 2018) or thrips pupae (Navarro-Campos et al., 2012).

Although ants fed on the sentinel prey both on the soil and on the branches, there were not significant differences among treatments. We expected increased predation in trees with sugar-feeders because, when carbohydrates are over-abundant, ant colonies may re-establish the protein intake by predation to maintain their carbohydrate:protein balance (Offenberg, 2001). The similar level of predation on sentinel placed on the soil, with and without sugar-feeders can be explained by the fact that this niche was dominated by *A. iberica*, whose diet and behaviour, are less dependent on carbohydrate sources as compared to *L. grandis*. Our biochemical analyses support this hypothesis because the protein content in *A. iberica* was twofold higher than the carbohydrate content. When the sentinel prey was placed on the branches, predation was double in trees with sugar-feeders placed also on the branches than on the control trees. This result suggests that further studies are necessary to implement this strategy. For example, in our study we placed the sentinel prey on the soil and branches at the same time and trees. For future experiments, we would suggest to place the sentinel prey only on the branches to avoid distraction from other potential protein sources. Moreover, while the medfly pupae that occur in the soil, proved to be a highly appreciated prey for the terricolous *A. iberica*, perhaps they do not constitute an ideal prey item for the mainly arboreal *L. grandis*.

Overall, our results demonstrate that sugar provisioning disrupted the mutualistic relationship between *L. grandis* and *P. citri* in citrus. Sugar provisioning reduced ant activity in the canopy of citrus trees. This reduction of ant activity resulted in lower levels of ants tending mealybug colonies and higher parasitism levels. Between the two strategies tested here, we would recommend to place the sugar-feeder among the branches of trees at the beginning of August. In this month, *P. citri* establish colonies in fruit and *L. grandis* is active searching in the canopy. Further research will be necessary to evaluate whether the provision of sugar-feeders increase predation by ants in citrus. In this respect, our study also highlights the importance of scavenging insectivorous ant species can have as generalist predators of citrus key pests that spend part of their life cycle in the soil.

#### CRediT authorship contribution statement

**J. Pérez-Rodríguez:** Data collection, data analyses and writing - original draft. **A. Pekas:** Conceptualization, experimental design, data analysis and writing. **A. Tena:** Conceptualization, experimental design, data analysis and writing. **F.L. Wäckers:** Conceptualization and writing.

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