Mealybug insects as models of a previously undescribed overwintering behavior

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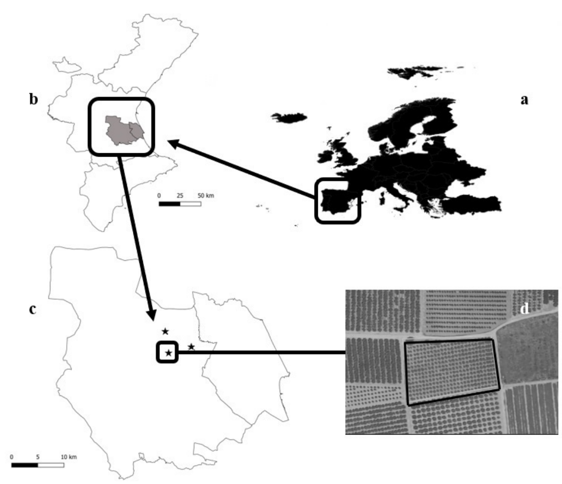
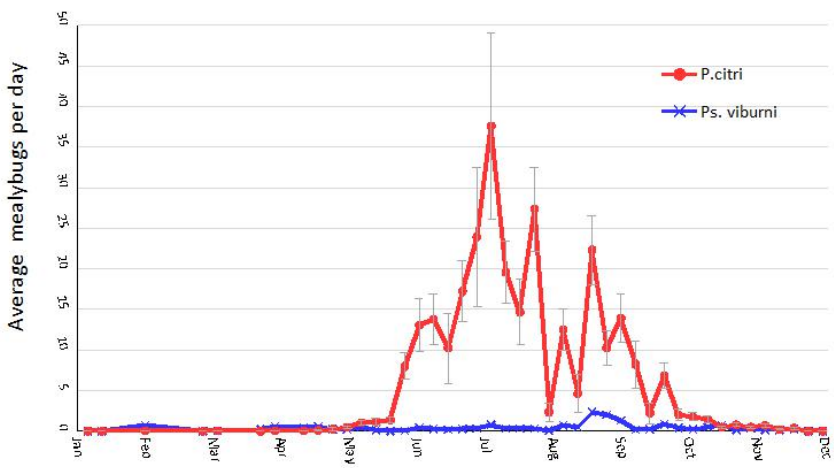
Abstract

Insects, whose body temperature depends on ambient temperature, often take refuge under the soil surface to survive unfavorable winter conditions. Considering the strategies used to cope with cold winter temperatures insects are classified into several groups. Here we propose a new group of insects with a previously unreported overwintering strategy taking as a model the mealybug insect family. Using specifically designed sampling methods we found that these sap-sucking insects, which spend half of the year feeding and reproducing on the plant canopy, move to the rhizosphere during the winter to feed on tree roots and reproduce. Our results show that the preferred area to overwinter for the main female mealybug species is within 1 meter around a fruit tree trunk, where more than 25000 mealybug males per square meter can emerge every spring. This unique adaptive strategy allows these aboveground herbivores to turn into belowground root feeders during the winter.




Average mealybugs per day
P.citri
Ps. viburni
0
5
10
15
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25
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Jan
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Mar
Apr
May
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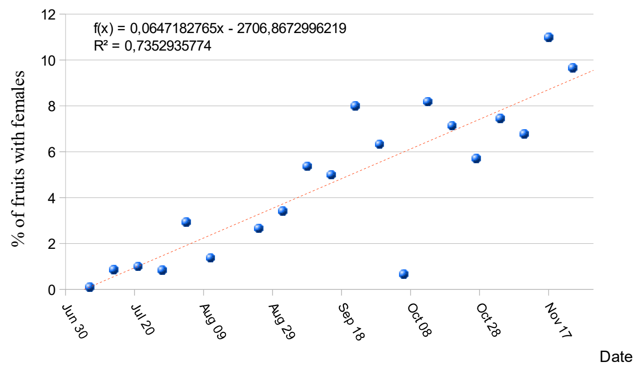
f(x) = 0,0647182765x - 2706,8672996219
R² = 0,7352935774
% of fruits with females
0
2
4
6
8
10
12
Jun 30
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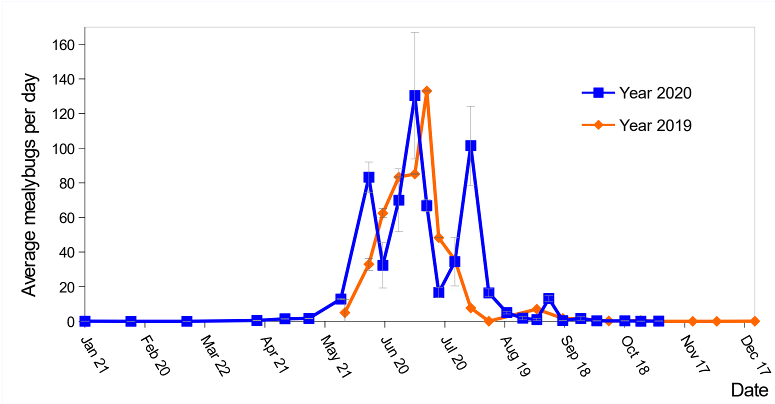
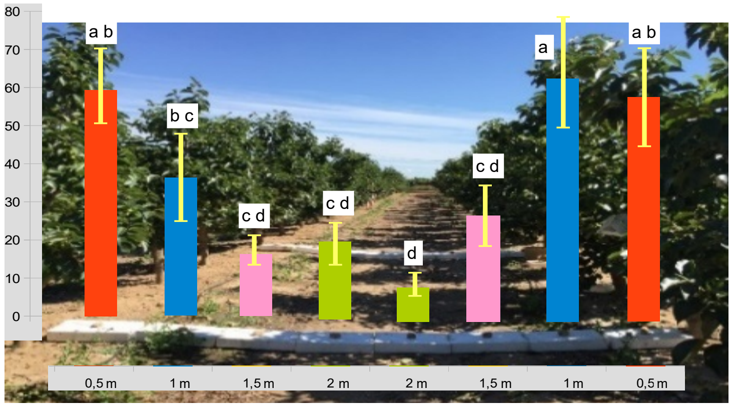
Aug 09
Aug 29
Sep 18
Oct 08
Oct 28
Nov 17
Date

Gráfico Fig 5_2



Page 1


80
70
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INTRODUCTION

Insects, as cold-blooded ectothermic animals, have limited ability to regulate their body temperature which depends largely on ambient temperature. so insects use behavior to adjust their temperature to some degree (Capinera, 2008). In temperate zones temperature drops during the winter months trigger different responses on insects for surviving the coldness. Insects overwintering without migrating often require special habitats to survive unfavorable environmental conditions (Bale, 1993).

The insect winter ecology has traditionally divided into two main groups the several overwintering strategies that can be observed: “freeze tolerant” and “freeze avoiding”. Some overwintering insects replace water in their bodies with glycerol or polyhydroxyl alcohols, which act as antifreeze to make them freeze tolerant (Bale & Hayward, 2010) while many other insects partially avoid the cold by hiding in protected, warmer locations such as leaf litter, the bark of certain trees or under the soil surface (e.g. Karg, 2004). The group of insects showing the ability to overwinter in the ground can be further split into two subgroups: 1) insects that have evolved to enter in the diapause phase (usually pupal diapause) as soon as they penetrate into the ground and find a suitable place and 2) insects that can feed within the rhizosphere before or during the overwintering phase.

Many examples can be found for the first group of insects such as the medfly, *Ceratitis capitata,* whose larvae, upon arrival at ground level, transform immediately to pupal stage and remain in diapause until the climate is suitable for adult emergence (Bento et al., 2010). Further examples are the potato beetle, *Leptinotarsa decemlineata* (Ferro et al., 1999) or the beet armyworm *Spodoptera exigua* (Zheng et al., 2011).

Examples of the aforementioned second group are more scarce (or less known). The lupine ghost moth, *Hepialus californicus,* whose larvae develop and feed on the lupine root, has one generation per year. Pupation occurs in winter and pupal diapause lasts until the adult emergence in the spring (Nielsen et al., 2000). Another species, the grape root borer, *Vitacea polistiformis*, feeds on the roots of grape vines. The life cycle takes two years to complete and almost all of it is spent as larvae. Flying adults emerge in the beginning of the summer after an obligate pupal diapause (Williams & Snow, 1991). A third example is the cabbage root fly, *Delia radicum*, whose larvae feed on the roots and stems of cabbage plants. After overwintering as pupae in the soil adult flies emerge in the spring (Nottingham, 1988).

Species of the last mentioned group are obligated insect root herbivores and their flying adults emerge for mating after a pupal diapause phase. However there is a particular family of insects showing a different pattern of behavior than any other, the mealybugs (Hemiptera: Pseudococcidae). They are sap-sucking herbivorous that feed on leaves, stems and twigs of trees, shrubs and herbs from spring to the end of the autumn (Hardy et al., 2008). Females of several mealybug species have been reported to migrate during the winter from the plant canopy toward the basal parts of the trunks, mostly on citrus trees and vineyards (Longo, 1986; Franco, 1992; Geiger & Daane, 2001; Cid et al., 2010). However, until now, the presence of mealybugs at ground level was considered accidental and insignificant being considered the tree canopy or the trunk the main overwintering areas (Rivnay, 1961; Klein, 1994; Franco et al., 2000; Bignell et al., 2018).

In this study, mealybugs were chosen as a model for a new group of insects with a previously undescribed behavior: insects that spend half of their lifecycle feeding on the aerial parts of the plant and change their main developing environment for that of the subterranean rhizosphere environment to overwinter while continuing to feed and develop. In other words, an adaptive strategy that allows some aboveground insect herbivores to turn into belowground herbivores to survive and reproduce during the winter. We conducted specifically designed field experiments in order to show that the rhizosphere represents an essential environmental medium during the winter months for mealybugs' life cycle in which they continue their reproductive process. Additionally we aimed to determine mealybugs' preferred root areas to overwinter. The large amount of flying mealybugs captured in spring while emerging from the soil show that we are facing a new overwintering strategy.

MATERIALS AND METHODS

Studied organisms

Mealybugs are a large family of *Coccoidea* insects comprising more than 2000 species worldwide distributed (Ben-Dov, 1994). They are sexually dimorphic: the adult female mealybug is wingless and is covered with a white-cottony wax. However, male mealybugs are smaller and do exhibit a radical change during their life cycle, changing from wingless, ovoid nymphs to wasp-like flying adults (Ben-Dov, 1994; Jahn et al., 2003).

Mealybugs are sap-sucking insects feeding on many species of shrubs, trees and grasses. Some of them, such as sugarcane, pineapple, cotton, pear, mulberry, sunflower, cacti, gardenias and orchids are economically important agricultural and ornamental plants (Ben-Dov, 1994; Jahn et al., 2003; Hardy et al., 2008; Arif et al., 2012). Nevertheless, mealybugs feeding on subtropical fruit trees and vineyards are the most widely studied due to their wide distribution and severe pest effects on these plants. Citrus are probably the most cited, but mealybugs have been also described as a serious pest on mango, papaya and persimmon trees (i.e. Franco et al., 2000; Jahn et al., 2003; Wakgari & Giliomee, 2004; Karar et al., 2010; Cid et al., 2010; Pacheco da Silva et al., 2016).

The occasional appearance of mealybugs on the soil surface or underground have been associated with the fruit tree dropping (Rivnay, 1961; Panis, 1986). Accordingly, female progeny have been described to migrate during the spring from the bottom part of the plants towards the fruit tree canopy (Longo, 1986; Panis, 1986; Franco, 1992, Cid et al., 2010). However, studies trying to find overwintering mealybugs belowground, on adventitious plant roots and on fruit tree roots, did not obtain any result in this respect (Klein, 1994; Franco et al., 2000; Cid et al., 2010; Bignell et al., 2018). Thus authors assumed that ground presence was accidental and irrelevant.

As a model for this study we used the binome mealybugs-persimmon fruit trees. The oriental persimmon tree, *Diospyros kaki*, native of China, has become widespread in the Mediterranean basin during last decades (Giunchi et al., 1988; Orobal et al., 2012; FAOSTAT, 2019) and in many countries such as the Republic of Korea, Japan, Brazil, Chile, United States and Australia (Wakgari & Giliomee, 2004; Pacheco da Silva et al., 2016; Bignell et al., 2018; FAOSTAT, 2019; Ames, 2021). In most of these countries mealybugs have been reported as a serious pest, and this is also the case in eastern Spain (Alonso-Muñoz et al., 2004; Tena et al., 2015), the region where this study was performed. In Spain, the citrus mealybug *Planococcus citri* and the obscure mealybug *Pseudococcus viburni* have been cited as the most abundant mealybugs in persimmon orchards (Romero-Colomer et al., 2010; García-Martínez et al., 2017), however recent studies have shown that in some areas these species have been outcompeted by the newly arrived *Pseudococcus longispinus,* the long-tailed mealybug (Plata et al., 2022). *P. citri*, the most widely studied species, can complete 5 generations per year in spanish citrus/persimmon growing areas and its lower developmental threshold temperature is 8,3 ºC (Martínez-Ferrer el al., 2003).

Study area and sampling stages

Field studies were performed in Valencia Region, a traditional Spanish agriculture area located in the western Mediterranean basin. The landscape of the study area is composed mainly of small citrus and persimmon orchards, of <1 ha spread over an extension of about 160,000 ha in an almost continuous belt, 300 km long from north to south and about 60 km wide. The area has a typical Mediterranean coastal climate with temperatures seldom below 0ºC during winter due to the influence of the sea.

The persimmon production area has been increasing since 1998 upto about 18,000 ha in 2019, replacing in many cases the previous citrus orchards (Orobal et al. 2012; MAPA, 2020). In the spanish citrus groves mealybugs have been considered one of the main pests for many years, especially the citrus mealybug *P. citri* (Gómez-Menor, 1937; Ripollés, 1990). In the Valencia Region mealybugs are so common and widely distributed in fruit trees and ornamental plants that they have a local name, namely “cotonet”, different from the spanish names, “cochinilla algodonosa” or “chanchito blanco”.

From 2018 to 2020 we conducted 3 consecutive field experiments. The survey was scheduled in progressive form and the results of the first experiment during the first year were the basis for the design of the sampling protocol for the second experiment that started in 2019. Similarly, the results of the second experiment were the basis for the sampling protocol for the third experiment. Initially, we sampled 3 persimmon orchards belonging to 3 villages, namely Alginet, Sollana and Algemesí, all about 20 km apart (see Fig 1). This was the prior trial before the start of the so called “belowground experiments” in order to have a picture of the mealybug species composition in the area of study, their relative abundance and population dynamics. In the second year we selected one orchard for the specifically designed ground sampling and in the third year we adapted the methodology and dates for the sampling of insect rhizosphere distribution. Therefore the sampling process was divided into 3 stages: 1) Sampling for species identification and abundance from 2018 to 2020. 2) Sampling for evaluating belowground mealybugs from 2019 to 2020. 3) Sampling to determine mealybugs distribution in the rhizosphere during 2020. All orchards were cultivated under integrated pest management according to EU rules and no treatments against mealybugs were performed. Orchards had been under identical farming practice for at least the previous 5 years.

Tree canopy mealybugs

Two mealybug sampling methods were used on the canopy of persimmon trees: 1) delta traps for males and 2) direct visual observation of fruits for females. Delta traps consist of a plastic trap, folded into a triangle with a suspension hook hung on a branch and with a sticky insert where female sex pheromones were placed. Traps, baited with *P. citri* (OpenNatur, Pherobank®) and *P. viburni* female sex pheromones (OpenNatur, Pherobank®) were placed in the canopy of two trees in every targeted orchard. These pheromones were selected on the basis of previous results of other studies in fruit orchards of the Valencia Region (García-Martínez et al., 2017). Fruits visual inspection of calyx and fruit surface was performed from the fruit set (phenology 77, developing fruit according to the BBCH scale) up to the end of the sampling period or until fruit harvest if that was the case. Fruits of citrus and persimmon trees are described to attract and concentrate mealybugs (Franco et al., 2000; Franco et al., 2004; Martínez-Ferrer, 2003). For each sampling day, 100 persimmon fruits were randomly selected from 20 trees (that is 6000 fruits visually examined per day unless fruit harvesting had started). When present, mealybugs were annotated and adult females identified to species level. The taxonomic identification of the mealybug species is based almost exclusively on the morphological characteristics of the adult female (Yang & Sadof, 1995, Beltrà & Soto, 2012).

Both the fruit observation and the traps collection were performed on the same day (if fruits were available), weekly from May to November and once per month during the coldest period for this region i.e. December to April for the 3 years. Traps were replaced at the same time they were collected. The trap sampling during the second and third year was intended to compare captures and seasonal population dynamics with those obtained from belowground sampling during the same period. In the laboratory traps were examined and mealybug males captured were identified to species level and counted. Adult females on the fruits were also identified to species level and counted.

Taking into account the sharp decline in the abundance of mealybugs on fruits and traps at the beginning of the winter and the low level of mealybugs observed on the leaves, branches or tree trunks we assumed that, at least part of them, were overwintering in the rhizosphere. This fostered the idea of developing sampling methods to evaluate the presence of mealybugs belowground. We use the results of these pre-assays to determine the sampling period and the best pheromone for capturing mealybugs overwintering in the ground.

Belowground mealybugs

For this experiment we selected the orchard with the highest levels of mealybugs during the first year of canopy sampling, located in Sollana. In light of the data obtained from the previous experiment the sampling period started in June 2019 and ended in October 2020.

Initially we dug holes roughly 0,2 x 0,2 meters in size at distances varying from the tree trunk of the examined trees until we found the roots and gently removed the soil around them. We only observed 5 female mealybugs in some of the roots, which confirmed their at ground level presence (as observed by other authors) and 2 more attached to the roots of a *Parietaria* spp spontaneous plant. However, this method is very costly, laborious and difficult to standardize since we don’t know previously where the roots are located in the ground. In addition some mealybugs can be accidentally removed with the soil. Similar problems were described by Bignell et al. 2018 using this mealybug sampling method.

We then tested a new, specifically designed, sampling method using hard plastic trays (50 x 30 cm and 10 cm) which were placed in an upside-down position on the soil surface after removing leaf litter or any grass presence. At the top of the inside part of the trays we attached a 10 × 25 cm sticky plastic trap baited with *P. citri* pheromones. This pheromone was selected taking into account the results of the previous experiment showing that the vast majority of the mealybugs in this area are *P. citri*. Trays were then placed 25 cm away from the persimmon tree trunk. To avoid the lateral entrance of any insects the lateral borders of the trays were covered and sealed with loose soil. The experiment was replicated in 2 trees for the period 2019-2020 and in 4 trees in 2020, all of them spaced about 30 meters apart. Since ants can tend and/or atack mealybugs (Pekas et al., 2011; Offenberg, 2015; Mani et al., 2016) and thus interfering in the experiment we also checked that there was no ants present near the trays. Traps were collected and replaced weekly from May to November and at least once per month during the rest of the year. In the laboratory all traps were examined and mealybugs identified and counted.

Mealybug root preference

We used the results of the previous experiment confirming the belowground abundance of mealybugs and the period of male emergence from the ground to design an experiment to evaluate the preferred root area (or root size) by mealybugs to feed and overwinter.

From June to August 2020 we placed on the ground 8 plastic trays side-by-side forming a continuous line that filled the gap between two opposite trees of two contiguous field rows. Trays were equal to that described in the previous experiment. The experiment was replicated twice with 10 meters apart between tray lines (Fig 2). Traps were collected and replaced every week. We also checked there were no ants surrounding the trays. In the laboratory all traps were examined and male mealybugs identified and counted.

Data analysis

To standardize the results the number of insects captured in all traps (at tree canopy and ground level) was divided by the number of days that each particular trap was in the field to obtain the average number of mealybugs captured per day.

The number of insects captured in the trap trays was correlated with the ground surface covered by one tray (0,15 m^2) to obtain the estimated average number of mealybugs emerging per square meter.

All data were submitted for ANOVA (Analysis of Variance) at P= 0.05 level, followed by a Multiple Range Test for statistical analysis. Comparisons between the means were made using Fisher's Least Significant Difference (LSD). Data was log-transformed when necessary before ANOVA to stabilize the variance. Statistical analyses were performed using Statgraphics® software (Statgraphics Centurion XVI).

RESULTS

Tree canopy mealybugs

A total of 1.048 delta traps were collected over 3 years. The total number of males captured were 46965 for *P. citri* compared to 2869 for *P. viburni*, meaning that more than 94% of the total mealybug males captured in the pheromone traps were *P. citri*.

Over the 3 years, the captures of *P. citri* mealybugs in traps started at the end of May and reached their highest peak in July before decreasing from September to almost nil during autumn and winter. The seasonal population dynamic of *P. viburni* was very different, with captures distributed all along the year, a peak of captures observed in September and no clear overwintering period. In order to compare with results of the second experiment we analyzed the trap captures separately for the period 2019-2020 (Fig 3).

A total of 28.400 fruits were examined. Based on adult females observed, three mealybug species were identified on persimmon fruits: *Planococcus citri*, *Pseudococcus viburni* and *Pseudococcus longispinus*. The relative abundance of mealybugs feeding on persimmon fruits (female stages) was: 98 % *P. citri*, 2% *P. viburni*, and <0.1% *P. longispinus*. There were no statistical differences between the years ($F_{2,1045}$ = 0,10; P = 0,90). In our study, *P. citri* and *P. viburni* coexisted in most trees (76%) while *P. longispinus* was only found in one of the studied orchards.

The presence of adult females on the fruits steadily increased from the beginning of July until the end of November. The percentage of fruits in which one or more mealybugs were observed is shown in Fig 4. Young females were assumed to be *P. citri* when direct field identification to species level was not possible. Most years fruit harvest was performed at the end of November or at the beginning of December, therefore data collected in December were excluded from figure 4.

Mealybug belowground survival

During the two years, 2019 and 2020, a total of 22930 mealybug males were captured from the sticky traps placed inside the trays. Considering the size of the trays, this means that per square meter and year an estimated average of 25478 ± 1525 mealybug males can emerge every spring.

Captures reached the highest peak in July and decreased from August onwards to almost zero during the autumn and winter (Fig 5). Consequently, we stopped the sampling in

October 2020. As expected, seasonal dynamics were fairly similar to those shown by the captures with Delta pheromone traps placed in the tree canopy. Curiously, for the trap trays in which the experiment took two years the capture of mealybugs during the second year did not decrease i.e. 7156 in 2019 and 8913 in 2020, considering the same time period.

There were no statistical differences between captures in trays in any of the years when we considered the same time period ($F_{3,72}$ = 0,18; P = 0,91).

Mealybug root preference

We captured 28801 mealybugs from the 112 trap trays collected (56 traps per line). The number of mealybugs showed a decreasing gradient from the tree vicinity towards the center of the row (Fig 6). The average of captures in the two trap trays closer to each of the tree trunks (within 1,25 m in diameter) was 55,1 ± 6,1 insects per day while in those placed at larger distances the average was 18,3 ± 2,9. There were statistical differences between both groups ($F_{1,110}$ = 29,64; P < 0.0001). The higher numbers of insects in the traps located next to the tree trunks show that, for overwintering, mealybugs prefer larger tree roots.

DISCUSSION

Considering the small size of the trap trays randomly placed on the ground surface and the surprisingly large amount of flying mealybug males captured every year while emerging from the soil we can infer how big the population of mealybugs overwintering belowground is. Aditionally, we should consider that the sex ratio for *P. citri* is about 1:1 (James, 1937) and that the estimated amount of males that can emerge during spring within 1 meter diameter around a persimmon tree trunk is higher than 25000 insects per square meter.

Previous field works evaluating the mealybug life cycle in temperate climates wrongly assumed that the tree trunk, or some other areas within the tree canopy were considered the main overwintering sites (see Franco, 1992; Geiger & Daane, 2001; Cid et al., 2010; Bignell et al., 2018). However, the results of our novel, specific sampling method give a clear insight into the importance of the role played by the rhizosphere in the life-cycle of these insects. In fact, our results suggest that these insects spend about half of the year belowground. If we compare the captures of pheromone traps placed on the same tree but in the plant canopy during the spring and beginning of the summer, we can observe that the average number of insects captured per day is even higher in the ground traps (more than twice as much most days). Taking into account that the action radius estimated in the field for this pheromone is about 150 meters (Branco et al., 2006) and that part of the insects captured in the tree canopy most likely come from the belowground emerging population, we can conclude that most mealybugs are overwintering in the rhizosphere. Moreover, the results of our third experiment indicate that ground overwintering mealybugs seem to prefer bigger roots near the tree trunk to feed and reproduce rather than the smaller ones further away. A large majority of mealybugs appear to choose the roots closer to the tree trunk nevertheless, even at 2 meters away from the tree trunk, the maximum distance between tree rows, more than 18 mealybugs per day were captured. This means that virtually all the field orchard rhizosphere area contains overwintering mealybugs.

Captures of aerial pheromone traps showed two peaks, the main one in September and a smaller one in October. Other authors found similar peaks of captures in Valencia Region (see Martínez-Ferrer el al., 2003). However, captures of the trap trays were almost zero during these periods which means that insects in the canopy traps during the end of the summer and autumn originate from new generations of females that developed in the aerial plant zone. The highest female mealybug populations on the fruits were observed in October and November. The gap between the peaks of capture for males and females has been previously described by other authors (Balachowsky, 1939). One possible reasons is that female mealybugs begin to release the sex pheromones and are ready to mate the first day after molting but the duration of the pre-oviposition period take several weeks (Nelson-Rees, 1961; Mendel et al., 2012).

Some mealybug species such as *P. citri* were thought to have facultatively parthenogenesis (Myers, 1932). That could partially explain the high numbers of males emerging during the spring of the second year below the trays. However the most recent intensive studies have demonstrated that all female mealybug species studied, *P. citri* included, produced no viable offspring from unmated females (Borges da Silva et al., 2010; Waterworth et al., 2011). Since no adult males have been observed to look for females at the ground level the only possible explanation is that gravid females arrive at the rhizosphere during the autumn either by walking from the trunk, being transported by ants (Pekas et al., 2011), or by falling from the tree canopy (with or without fruits). However, we carefully checked that there were no ants, or ant nests, below or around the sampling trays which suggest that this is not the main way. Besides, it is important to bear in mind that female mealybugs have been reported to survive up to 120 days under laboratory conditions (Nelson-Rees, 1960).

Much is now known about the ways in which insects cope with, and respond to, low temperatures. Studies concerning insect overwintering responses are mostly based on areas where subzero temperatures are common (e.g. Udaka et al., 2014; Sinclair et al., 2015) however overwintering in temperate climates has been less studied. In the case of the mealybugs, the overwintering strategy of the particular group of insects that can feed, complete their life cycle and reproduce both in the tree canopy area during warmer seasons of the year and in the soil during the winter has not been previously recorded. In fact, one of the first assumptions of researchers focusing on winter ecology i.e. “overwintering insects cannot feed” (see Sinclair, 2015) is not true for these insects. Feeding on the roots during the winter is a strategy for mealybugs to maintain their energy reserves at a time at which they minimize their cold exposure. This drastic change in habitat and feeding source during half of the year while continuing the reproduction process (i.e. complete at least one generation) under the "new belowground conditions" is, to our knowledge, a pattern that has not previously been reported for any other group of insects. In fact, none of the main groups for which insect winter ecology have traditionally classified the known overwintering strategies fits with the mealybug's winter behavior. Therefore, the discovery of this winter survival strategy converts mealybugs, or at least some mealybug species inhabiting temperate zones, into a model for a new ecological group with a different overwintering behavior than any other described group showing cold behavioral avoidance.

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