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Rearing the predatory coccinellid *Cryptolaemus montrouzieri* with factitious diets modifies its relationship with ants and reduces its efficacy as biocontrol agent

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La dieta artificial del coccinélido depredador *Cryptolaemus montrouzieri* modifica su relación con las hormigas y reduce su eficacia como agente de control biológico

RESUMEN

La presencia de hormigas que se alimentan de la melaza que excretan los hemípteros dificulta el control biológico de los pseudocóccidos mediante el uso del depredador especialista *Cryptolaemus montrouzieri* (Mulsant). Este coccinélido es criado masivamente, utilizando dietas artificiales, y comercializado por varias compañías e insectarios. Las hormigas atacan a las larvas de este coccinélido depredador cuando compiten por los recursos alimenticios. En este trabajo se plantea si este comportamiento agresivo de las hormigas depende de la dieta proporcionada a las larvas de *C. montrouzieri* durante su cría masiva. Para ello, examinamos el comportamiento de la hormiga *Lasius grandis* (Forel), la especie de hormiga más abundante y ampliamente distribuida en los cítricos españoles, hacia larvas de *C. montrouzieri* criadas con una dieta artificial, huevos de *Ephestia kuehniella* (Zeller), o con su presa natural, ninfas de *Planococcus citri* (Risso), tanto en laboratorio como en ensayos de campo. El ensayo de campo confirmó que la presencia de *L. grandis* reduce la eficacia de las larvas de *C. montrouzieri* como agentes de control biológico de *P. citri*. Nuestros resultados de campo y de laboratorio también mostraron que las hormigas eran más agresivas hacia las larvas de *C. montrouzieri* criadas con huevos de *E. kuehniella* que sobre las ninfas de *P. citri*. Las larvas criadas con huevos de *E. kuehniella*: i) fueron atacadas por las hormigas con mayor frecuencia, ii) abandonaron las colonias de pseudocóccidos antes, iii) depredaron menos pseudocóccidos y iii) murieron con mayor frecuencia que las larvas criadas con ninfas de *P. citri*. En general, nuestros resultados demuestran que la dieta de la cría afecta a la relación entre *C. montrouzieri* y las hormigas. Por lo tanto, se debería mejorar el manejo de las hormigas y / o las dietas para criar *C. montrouzieri* en masa para mejorar el control biológico de los pseudocóccidos por este depredador.

PALABRAS CLAVE: plagas invasoras, control biológico, mielato, mecanismos de defensa, *Lasius grandis, Ephestia kuehniella, Planococcus citri*

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Rearing the predatory coccinelid *Cryptolaemus montrouzieri* with factitious diets modifies its relationship with ants and reduces its efficacy as biocontrol agent

ABSTRACT

The presence of honeydew-seeking ants hinders the biological control of mealybugs using the specialist predator *Cryptolaemus montrouzieri* (Mulsant), which is reared and commercialized by several companies and insectaries. Ants are aggressive towards the larvae of this predatory coccinellid as they compete for food resources. We hypothesized that antagonism from ants may depend on the rearing diet provided to *C. montrouzieri* larvae. Here, we examined the behavior of the ant *Lasius grandis* (Forel), the most abundant and widely distributed ant species in Spanish citrus, towards larvae of *C. montrouzieri* reared on a factitious diet, *Ephestia kuehniella* (Zeller) eggs, or on its natural prey, *Planococcus citri* (Risso) nymphs, in both laboratory and field assays. The field assay confirmed that the presence of *L. grandis* reduced the efficacy of *C. montrouzieri* larvae as biological control of *P. citri.* Our field and laboratory results also showed that ants were more aggressive towards *C. montrouzieri* larvae reared on *E. kuehniella* eggs than on *P. citri* nymphs. Larvae reared on *E. kuehniella* were attacked by ants more frequently, left mealybug colonies earlier, preyed lower number of mealybugs and died more frequently than larvae reared on *P. citri*. Overall, our results demonstrate that the rearing diet interfere the relationship between *C. montrouzieri* and ants. Therefore, ant management and/or diets to mass rear *C. montrouzieri* should be analyzed to enhance the biological control of mealybugs by this predator.

KEYWORDS: invasive pests, honeydew, defense mechanisms, *Lasius grandis*, *Ephestia kuehniella*, *Planococcus citri*

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1. INTRODUCTION

Mealybugs (Hemiptera: Pseudococcidae) are one of the main phloem-feeding pests in numerous crops worldwide (Williams and Watson, 1988; Charles, 1993; Ben-Dov, 1994; Blumberg *et al.*, 1995; Miller *et al.*, 2002; Roques *et al.*, 2009; Pellizzari and Germain, 2010). They are considered key pests in grapes, citrus, ornamental plants and some horticultural crops under greenhouse conditions (McKenzie, 1967; Daane *et al.*, 2008; Peri and Kapranas, 2012; Cranshaw and Shetlar, 2017). Mealybugs suck phloem fluids from different organs of the host plants reducing their vigor (Daane *et al.*, 2008). When largely accumulated, they can cause physiological and morphological damages to the infested plants; examples can be stunted growth (McKenzie, 1967; Neuenschwander *et al.*, 1989), leaf yellowing (Culik and Gullan, 2005), leaf defoliation (Nwanze, 1982; Daane *et al.*, 2008; Cranshaw and Shetlar, 2017), fruit distortions (Tena *et al.*, 2017; Martinez-Blay *et al.*, 2017; Perez-Rodriguez *et al.*, 2017; Tena *et al.*, 2018) or in severe cases, the death of the plant (McKenzie, 1967; Mani and Shivaraju, 2016). Some mealybug species can also transmit virus to plants (McKenzie, 1967; Culik and Gullan, 2005; Daane *et al.*, 2008; Cooper *et al.*, 2008). In addition to their feeding habits, these hemipterans excrete honeydew abundantly (McKenzie, 1967; Itioka and Inoue, 1996). Honeydew on plant surfaces supports the growth of black sooty mold fungi, which can also reduce the productivity and marketability of infested crops (McKenzie, 1967; Mani and Shivaraju, 2016). Mealybugs are also typical invasive pests due to their small size and cryptic behavior (Miller et al. 2002; Pellizzari and Germain 2010).

The invasive nature, severe damages and the difficulties presented by the chemical control of mealybugs have made them a principal target of biological control programs (Miller et al. 2002; Moore 1988). Biological control is one of the prioritized methods in formulating an integrated pest management approach especially in Europe (directive order number 2009/128/EC). Biological control of mealybugs has used natural enemies ranging from generalist predators to specialist parasitoids (DeBach, 1964; Fisher *et al.*, 1999; Cock *et al.*, 2015). One of the most of the successful group of biological control agents is parasitoids of family Encyrtidae. Apart from parasitoids, the coccinellid *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinelidae) is also a well-known and successful example of a specialist predator of mealybugs (Bartlett, 1978; Moore, 1988).

However, the presence of tending ants, which is sometimes overlooked and underappreciated (Strysky and Eubanks, 2007), should be considered in biological control of mealybugs (Itioka and Inoue, 1996; Tollerup *et al.*, 2004; Beltrà *et al.*, 2015; Beltrà *et al.*, 2017). In a mutualistic association, ants protect mealybugs from natural enemies and, in exchange, they feed on honeydew. Honeydew contains carbohydrates and other nutritional substances that mealybugs obtained from feeding on their host plants (Styrsky and Eubanks, 2007; Daane *et al.*, 2008; Vantaux *et al.*, 2012). For ants, this is a good food resource which explains their tending behavior to mealybug colonies and other honeydew-producing hemipterans such as aphids, coccids, whiteflies and planthoppers (McKenzie, 1967; Sakata, 1994; Yao *et al.*, 2000; Quieroz and Oliveira, 2001; Styrsky and Eubanks, 2007; Vantaux *et al.*, 2012; Cranshaw and Shetlar, 2017). Under some conditions, ants also acquire protein by preying on them (McKenzie, 1967; Majerus *et al.*, 2007; Vantaux *et al.*, 2012). In exchange, ants prevent their hemipteran partners from the factors that could impair with their availability and activity. These include the removal of sources of fungal infections, such as exuviae, dead bodies and honeydew (Bach, 1991; Quieroz and Oliveira, 2001; Majerus *et al.*, 2007; Vanek and Potter, 2010; Vantaux *et al.*, 2012), and relocation to suitable feeding sites when the quality of a host plant deteriorates (Vanek and Potter, 2010; Vantaux *et al.*, 2012). Ants also restrain competitions with other non-honeydew producing herbivores (Styrsky and Eubanks, 2006; Marras *et al.*, 2008; Cooper *et al.*, 2008; Nygard *et al.*, 2008; Vantaux *et al.*, 2012; Calabuig Gomar *et al.*, 2014; Sagata and Gibbs, 2016). Nevertheless, the most important benefit that attending ants could offer to these hemipterans is the protection from their natural enemies (predators and parasitoids) (Bartlett, 1961; Rosen, 1967; Bach, 1991; Sloggett and Majerus, 2000; Yao *et al.*, 2000; Quieroz and Oliveira, 2001; Styrsky and Eubanks, 2007; Majerus *et al.*, 2007; Nelson and Daane, 2007; Marras *et al.*, 2008; Vanek and Potter, 2010; Vantaux *et al.*, 2012; Calabuig Gomar *et al.*, 2014; Zhou *et al.*, 2015; Cranshaw and Shetlar, 2017). This association generally leads to increase in density and persistence for longer periods of time of mealybug colonies when ants are present (Bartlett, 1961; Buckley and Gullan, 1991; Itioka and Inoue, 1996; Daane *et al.*, 2003; Tollerup *et al.*, 2004; Nelson and Daane, 2007; Daane *et al.*, 2007; Marras *et al.*, 2008; Yoo *et al.*, 2013; Zhou *et al.*, 2015; Beltrà *et al.*, 2017). This occurs because ant may drive natural enemies away from the colony, kill or feed them (Rosen, 1967; Sloggett and Majerus, 2003; Majerus *et al.*, 2007).

Natural enemies of ant-tended honeydew-producing hemipterans have adapted evolutionary and ecological responses against ant aggressiveness. Some predatory coccinellids use behavioral, physical and chemical defense mechanisms when they come into conflict with ants to be able to feed on their hemipteran preys (Majerus *et al.*, 2007; Vantaux *et al.*, 2012). Among these ladybirds, the specialist and successful mealybug predator *C. montrouzieri* was claimed to effectively mimic mealybugs while foraging on them. This mimicry is considered one of the main reasons to use *C. montrouzieri* when mealybugs are tended by ants (Flint and Dreistadt, 1998; Daane *et al.*, 2007; Daane *et al.*, 2008; Hodek *et al.*, 2012). However, Marras *et al.* (2008) and Mansour *et al.* (2012) reported that the larvae of this predatory coccinellid were also attacked by ants that disrupted the foraging activities of the coccinellid. Therefore, it is unclear under which conditions the larvae of *C. montrouzieri* are detected and attacked by ants.

Nowadays, *C. montrouzieri* is mass reared and released in the field to control mealybugs in different "augmentative biological control" programs (Hodek, 1973; Flint and Dreistadt, 1998; Fisher *et al.*, 1999; Maes *et al.*, 2014a; Maes *et al.*, 2014b; Maes *et al.*, 2014c; Maes *et al.*, 2015; Mani and Shivaraju, 2016). Traditionally, *C. montrouzieri* was mass-produced using mealybugs reared on plant materials like potato sprouts or pumpkins. However, due to the laborious work, including the time requirement and seasonal availability of these plant materials in establishing this rearing system in the commercialization of *C. montrouzieri*, several studies explored alternative diets to decrease its production costs. At present, factitious food sources such as eggs of different Lepidopteran species are used to rear them (Hodek *et al.*, 2012; Maes *et al.*, 2014a; Wu *et al.*, 2014; Xie *et al.*, 2016; Sun *et al.*, 2017). However, it is unknown whether using an artificial diet could affect the interaction between *C. montrouzieri* and ants.

Here, we used the citrus crop to test whether ants are more aggressive towards *C. montrouzieri* larvae reared on a factitious diet than on mealybugs. To verify this presumption, we had examined the behavior of *Lasius grandis* (Forel) (Hymenoptera: Formicidae), the most abundant and widely distributed ant in Spanish citrus (Pekas *et al.*, 2011), towards larvae of *C. montrouzieri* reared on either *Ephestia kuehniella* Zell. (Lepidoptera: Pyralidae) eggs or *Planococcus citri* Risso (Hemiptera: Pseudococcidae) under both laboratory and field conditions.

2. MATERIALS AND METHODS

2.1. Insect colonies

Planococcus citri were obtained from the State Insectary of Valencia (Spain) and were reared on green beans kept in plastic boxes (30.5 x 24.5 x 20 cm) with a hole covered with muslin on top under laboratory conditions (at 23 ± 3°C, natural daylight).

Cryptolaemus montrouzieri were obtained from Biobest Group N.V. (Belgium) as adults. Upon arrival, 30 couples were individualized in plastic Petri dishes (9 x 2.5 cm) and were provided with moistened cotton and three pieces of oviposition substrate (Rolta®Soft synthetic polyester wadding of 1 x 1 cm). Either *E. kuehniella* eggs from Koppert Biological Systems (Netherlands) or *P. citri* nymphs reared on green beans were provided as food depending on the treatment. The couples were maintained in a climatic chamber at 25 ± 1°C, 75 ± 5% HR, photoperiod 14:10. All foods used were offered *ad libitum* and renewed every 3-5 days. *Cryptolaemus montrouzieri* eggs were collected every 3-5 days from the oviposition substrate. Eggs laid by the couples fed on the same diet were all gathered in one Petri plate (measurement: 9 x 2.5 cm) with the oviposition substrate. After egg collection, oviposition substrates were renewed. Newly emerged first-instar larvae were isolated individually into plastic Petri dishes (measurement: 5.5 x 1.5 cm). They were reared with the same food and water provisions as the adults depending on the treatment. This procedure was derived from Maes *et al.* (2014a). Larvae were 15 ± 5 days old when they were used in the experiments, which means they were in the third and fourth instar. Larvae were starved for 24 hours (only access to water) before the experiments.

16 queenless colony fragments of the ant *Lasius grandis* were collected from IVIA orchards one week before the laboratory assay started. Each colony fragment was confined in plastic boxes (measurement: 38.5 x 32 x 25 cm), which had inner walls lined with a mixture of petroleum jelly and mineral oil (at 1:4 ratio) that hindered ants to escape. These colony fragments, comprising of ~150-200 workers each, were maintained in the laboratory at 23 ± 3°C, natural daylight. On the day of collection, each of these colonies was provided with honey on a piece of aluminum paper and freeze-killed Mediterranean fruit flies (*Ceratitis capitata)* as diet in an *ad libitum* manner. Test tubes (measurement: 10 x 1.5 cm), half-filled with distilled water, was used to simulate a real anthill. A piece of cotton was placed over the water to avoid spilling while the tube rests horizontally inside the rearing boxes for ants. The tubes were covered with

aluminum paper, which created dark and humid conditions inside, allowing ants to establish their colony. These ant colonies were starved for 48 hours before the implementation of the laboratory assay.

2.2. Laboratory assay

To determine whether the rearing diet of *C. montrouzieri* can affect its interactions with ants, we observed the behavior of *L. grandis* and *C. montrouzieri* larvae reared on different diets under laboratory conditions. After starving ant colonies, one green bean infested with 20 *P. citri* (2nd instar to pre-ovipositional females) was placed in each of the boxes with ants. After 48 hours in contact, one larva of *C. montrouzieri* reared either on *E. kuehniella* eggs or *P. citri* nymphs was introduced in each box with ant-tended mealybugs.

Three behaviors of *L. grandis* were observed when they came in contact with *C. montrouzieri* larvae: i) “quick encounter” , when they stroked their antennae on the larva’s body for a very short time – less than five seconds – before ignoring it; ii) “encounter and ignore” when stroked their antennae on the larva’s body for more than five seconds before ignoring them); and, iii) “attack” when, after stroking their antennae, they stung the larva’s body with the tip of their abdomen and/or start removing the wax filaments using their mouthparts. These behaviors were closely monitored within the first hour after the introduction of *C. montrouzieri* larvae. During one-hour period, we also recorded: i) “time at which ants detected the larvae” (the first observation when ants “encountered and ignored” the larvae); ii) “time at which ants attacked the larvae”; and iii) “number of ants involved per encounter or attack” to the larvae.

The behavioral responses of *C. montrouzieri* larvae were also measured: i) “time at which the larvae left the mealybug-infested bean" within the first hour of introduction; ii) the number of mealybugs that were preyed after 24 hours of introduction; and iii) the mortality of the *C. montrouzieri* larvae after 24 hours.

These observations were based on Bach (1991) and Daane *et al.* (2007). Each treatment was replicated 20 times, twice per day. Equal numbers of each treatment were tested each day, randomizing the order and ant colony of testing between days in both experiments to account for potential temporal and spatial effects.

2.3. Field assay

To confirm the previous results obtained in the lab, we carried out a field assay in a citrus orchard from Instituto Valenciano de Investigaciones Agrarias (Spain) in trees with and without ants. Within the orchard, 16 citrus trees (Var. Navelate) were selected, eight trees had *L. grandis* colonies and, in the other eight trees, ants were excluded using a similar methodology than Pekas *et al.* (2011). For this, 30-45 cm on the trunk base of each tree was divided into three strips (top, middle and bottom) measuring 10-15 cm each. The top and bottom strips where wrapped with tape and then sprayed with a sticky coating aerosol (Tanglefoot®Tangle-Trap®). The middle strip was cleared from ants while setting up the top and bottom strips.

One plastic box (measurement: 38.5 x 32 x 25 cm) with four holes (0.5 cm diameter) in one side to allow the entry of ants and the exit of *C. montrouzieri* larvae was used as arena. Boxes were placed in the base of each tree trunk (treatment with ants) or in the middle strip (treatment without ants). Each box contained one green bean infested with 100-200 *P. citri* nymphs of different instars. The bean was laid on top of two pillars of clay. The boxes were covered with a mesh to avoid any external interference and tied up to the tree to prevent them from being blown by the wind. The boxes with the infested beans were in contact with ants for 24 hours before the experiment started.

After these 24 hours, one *C. montrouzieri* larva reared on either *E. kuehniella* eggs or *P. citri* nymphs was introduced in each box either with or without ant-tended mealybugs. In total, there were four treatments (two rearing diets × two ant densities). The behaviors of both *L. grandis* and *C. montrouzieri* were recorded within the first six hours after larvae introduction. The following information was obtained: i) "number of ants" present before and six hours after the introduction of the larvae; ii) "time at which the larvae left the mealybug-infested bean"; and, iv) "time at which the larvae left or was removed from the arena". The arenas were observed during five minutes with one-hour interval for the first 6 hours after the introduction of predatory coccinellid larvae. Ant detection and ant attack were not included in these observations because all the larvae in both diets were detected and attacked by *L. grandis* within the first five minutes of observation. All observations were carried out between 8:30 am and 16:30 pm approx.

Each treatment was replicated 28 times, twice per day. Equal numbers of each treatment were tested each day, randomizing the order and tree of testing between days in both experiments to account for potential temporal and spatial effects.

2.4. Statistical analysis

The effect of *C. montrouzieri* diet on i) the time at which the larvae were detected by ants; ii) the time at which the larvae were attacked by ants; and iii) the time at which the larvae left the mealybug colony were represented by Kaplan–Meier survivorship curves and analyzed by a Likelihood ratio test using the “coxph” function of the “Survival” package of R (Crawley, 2013).

We used one-way ANOVA, assuming to have normally distributed error variances, to determine whether the mean number of ants that encountered or attacked *C. montrouzieri* larvae was affected by the diet provided to the larvae in the laboratory assay. The same analysis was carried out to determine whether the number of ants presents before and six hours after introducing *C. montrouzieri* larvae were the same in both treatments in the field assay. The normality assumption was assessed using Shapiro’s test, and the homoscedasticity assumption was assessed with the Levene test.

Proportional and count data were analyzed with generalized linear models (GLMs). Initially, we assumed a Poisson error variance for count data (number of preyed mealybugs) and a binomial error variance for proportional data (*C. montrouzieri* mortality, and ratio of larvae that remained in the colony or arena). We assessed the assumed error structures by a heterogeneity factor equal to the residual deviance divided by the residual degrees of freedom. If we detected an over- or under-dispersion, we reevaluated the significance of the explanatory variables using an F test after rescaling the statistical model by a Pearson’s chi-square divided by the residual degrees of freedom (Crawley, 2007). We present the means of untransformed proportion and count data (in preference to less intuitive statistics such as the back-transformed means of logit-transformed data). All data analyses were performed with the R freeware statistical package version 3.5.1 (http://www.R-project.org/).

3. RESULTS

3.1. Laboratory assay

3.1.1. Ant behavior

3.1.1.1. Detection of *C. montrouzieri* larvae

During the 60 minutes of observation, *L. grandis* detected all the *C. montrouzieri* larvae in both treatments (*C. montrouzieri* reared on *E. kuehniella* eggs or *P. citri* nymphs) (Figure 1). After 10 minutes in the arena, 95% of the larvae had been already detected by the ants. The time at which *C. montrouzieri* larvae was detected by the ants was independent on the diet provided to the larvae (*Likelihood ratio test*$_1$ = 0.52; *P* = 0.50).

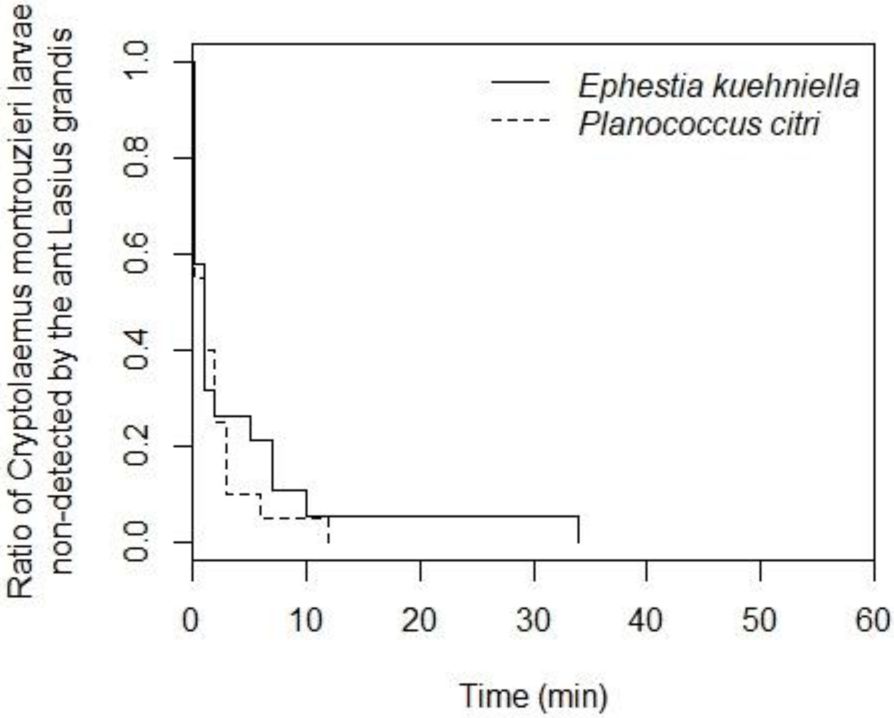

Figure 1. Ratio of *Cryptolaemus montrouzieri* larvae non-detected by the ant *Lasius grandis* when searching in a colony of *Planococcus citri* tended by ants. *Cryptolaemus montrouzieri* had been reared on either the artificial diet *Ephestia kuehniella* eggs or its natural prey *Planococcus citri*.

3.1.1.2. Attack of *C. montrouzieri* larvae

Most *C. montrouzieri* larvae were attacked by the ants during the 60 minutes of observation (Figure 2). *Cryptolaemus montrouzieri* larvae reared on the artificial diet *E. kuehniella* eggs were attacked by the ants earlier than larvae reared on its natural prey *P. citri* nymphs (*Likelihood ratio test*$_1$ = 5.5; *P* = 0.02). After four minutes in contact with the ants, 50% of the larvae reared on *E. kuehniella* eggs had been attacked by the ants, whereas only 22% of the larvae reared on *P. citri* nymphs had been attacked.

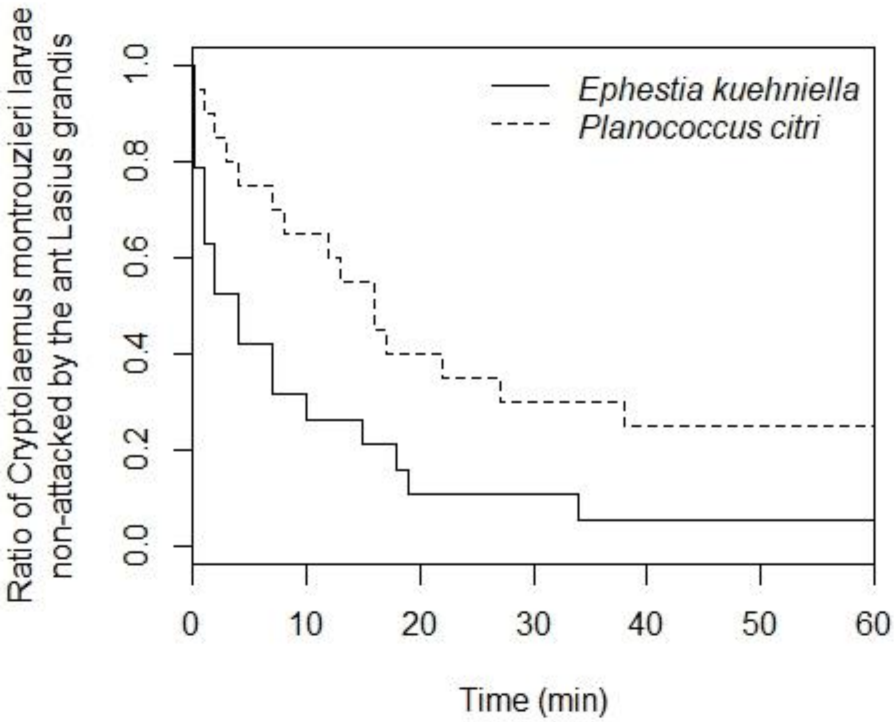

Figure 2. Ratio of *Cryptolaemus montrouzieri* larvae non-attacked by the ant *Lasius grandis* when searching in a colony of *Planococcus citri* tended by ants. *Cryptolaemus montrouzieri* had been reared on either the artificial diet *Ephestia kuehniella* eggs or its natural prey *Planococcus citri*.

3.1.1.3. Number of ants attacking *C. montrouzieri* larvae

The mean number of ants that "had quick encounters with larvae" ($F_{1,\,37}$ = 2.7; *P* = 0.11), "encountered and ignored the larvae" ($F_{1,\,32}$ = 3.7; *P* = 0.06) and "attacked the larvae" ($F_{1,\,31}$ = 0.44; *P* = 0.51) were independent on the diet provided to rear *C. montrouzieri* larvae (Table 1).

Table 1. Mean number (± SE) of Lasius grandis involved in each encounter and attack to *Cryptolaemus montrouzieri* larvae.

Ant Behavior	**Diet**	**Mean number of ants / encounters**
Quick encounter	*E. kuehniella*	2.9 ± 0.42
	P. citri	2.5 ± 0.36
Encounter and ignore	*E. kuehniella*	1.4 ± 0.09
	P. citri	1.6 ± 0.09
Attack	*E. kuehniella*	1.1 ± 0.03
	P. citri	1.3 ± 0.09

3.1.2. *Cryptolaemus montrouzieri* behavior

3.1.2.1. Time spent in the mealybug colony

Cryptolaemus montrouzieri larvae reared on *E. kuehniella* eggs left the mealybug colony tended by ants earlier than larvae reared on *P. citri* nymphs (*Likelihood ratio test*$_1$ = 13.3; *P* = 0.0003) (Figure 3). After 22 minutes in contact with ants, 50% of the larvae reared on *E. kuehniella eggs* had left the colony whereas only 15% of the larvae reared on *P. citri* nymphs had left it.

3.1.2.2. Predatory potential of *C. montrouzieri*

The number of mealybugs that were preyed by *C. montrouzieri* larvae during 24 hours in mealybug colonies tended by ants was significantly lower when the larvae had previously fed on *E. kuehniella* eggs (1.8 ± 0.44 mealybugs preyed) than on *P. citri* nymphs (4.8 ± 0.98) ($F_{1,\,37}$ = 9.3; *P* = 0.0042).

3.1.2.3. Mortality of *C. montrouzieri*

The mortality (ratio) of *C. montrouzieri* larvae after 24 hours in contact with a mealybug colony tended by ants was significantly higher when the larvae had previously fed on *E. kuehniella* eggs (0.37 ± 0.11) than on *P. citri* nymphs (0.10 ± 0.07) (χ_1^2 = 38; *P* = 0.04).

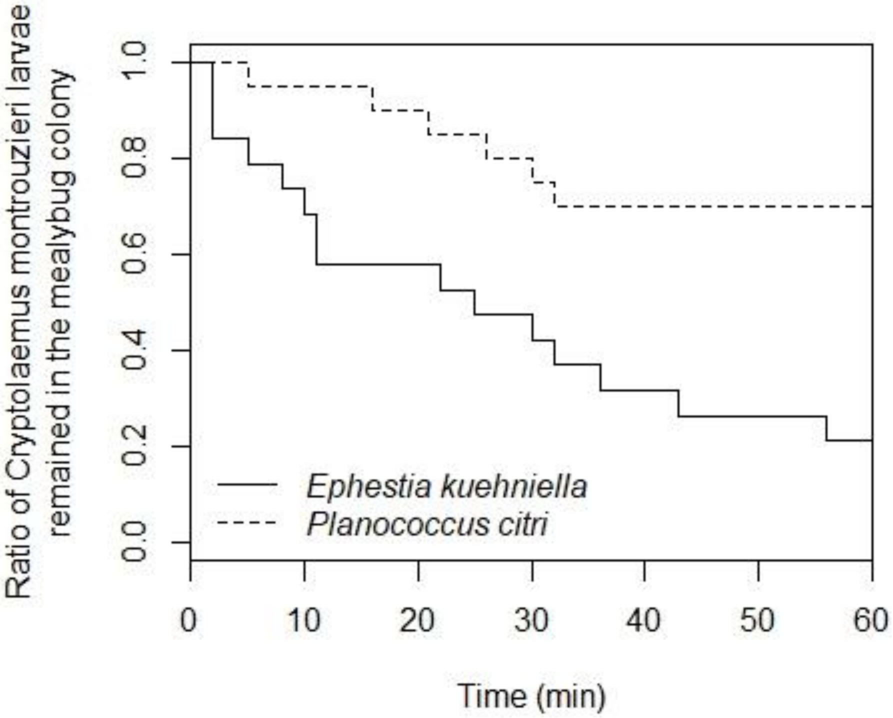

Figure 3. Ratio of *Cryptolaemus montrouzieri* larvae remaining in a mealybug colony tended by the ant *Lasius grandis*. *Cryptolaemus montrouzieri* had been reared on either the artificial diet *Ephestia kuehniella* eggs or its natural prey *Planococcus citri*.

3.2. Field assay

3.2.1. Ant activity

The number of *L. grandis* ants tending mealybug colonies was similar in both treatments (*C. montrouzieri* larvae reared on either *E. kuehniella* eggs or *P. citri* nymphs) before the experiment started ($F_{1,54} = 0.01$; $P = 0.93$) and six hours after the larvae were introduced ($F_{1,54} = 0.01$; $P = 0.91$).

3.2.2. *Cryptolaemus montrouzieri* larvae behavior

After six hours searching in the arenas, the ratio of *C. montrouzieri* larvae that remained in mealybug colonies decreased with the presence of ants ($\chi_1^2 = 93.9$; $P < 0.0001$) and depended on the diet used to rear the larvae ($\chi_1^2 = 81.5$; $P = 0.0004$) (Figure 4). This ratio was significantly lower when the larvae were reared on *E. kuehniella* eggs than on *P. citri* nymphs. The interaction between both factors (ants and diet) was not significant ($\chi_1^2 = 80.3$; $P = 0.28$).

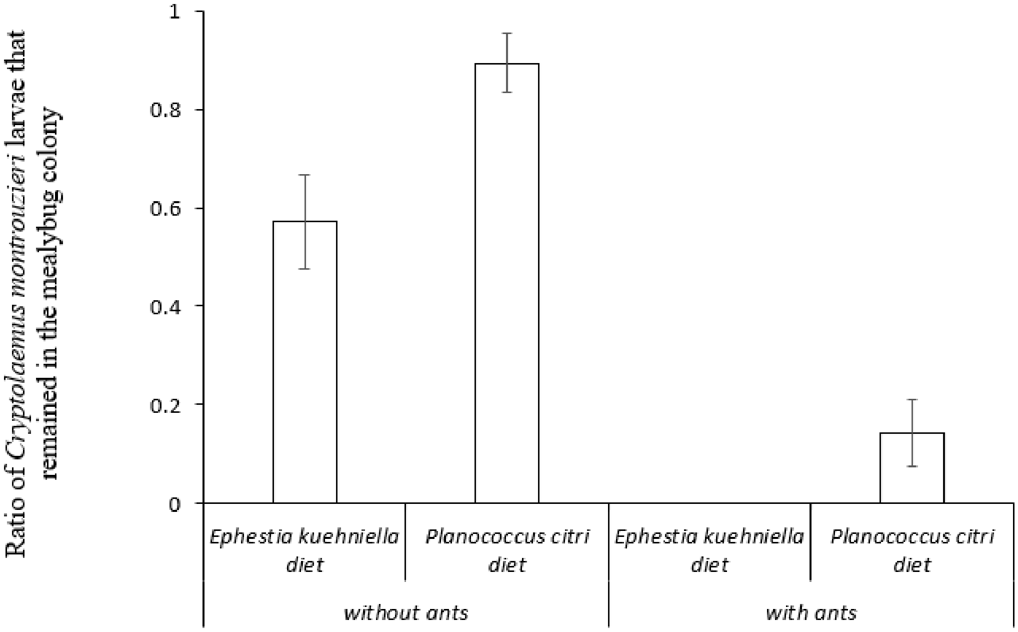

Figure 4. Ratio of *Cryptolaemus montrouzieri* larvae that remained in the mealybug colony. *Cryptolaemus montrouzieri* had been reared on either the artificial diet *Ephestia kuehniella* eggs or its natural prey *Planococcus citri*.

After six hours searching in the arenas without ants, more than 95% of the *C. montrouzieri* larvae remained inside the arenas independently on the diet provided to rear the larvae. In the arenas with ant-tended colonies, however, the ratio of *C. montrouzieri* larvae that remained in the arena was significantly lower when *C. montrouzieri* larvae had been reared on *E. kuehniella* eggs than on *P. citri* nymphs (χ_1^2 = 66.6; P = 0.002) (Figure 5).

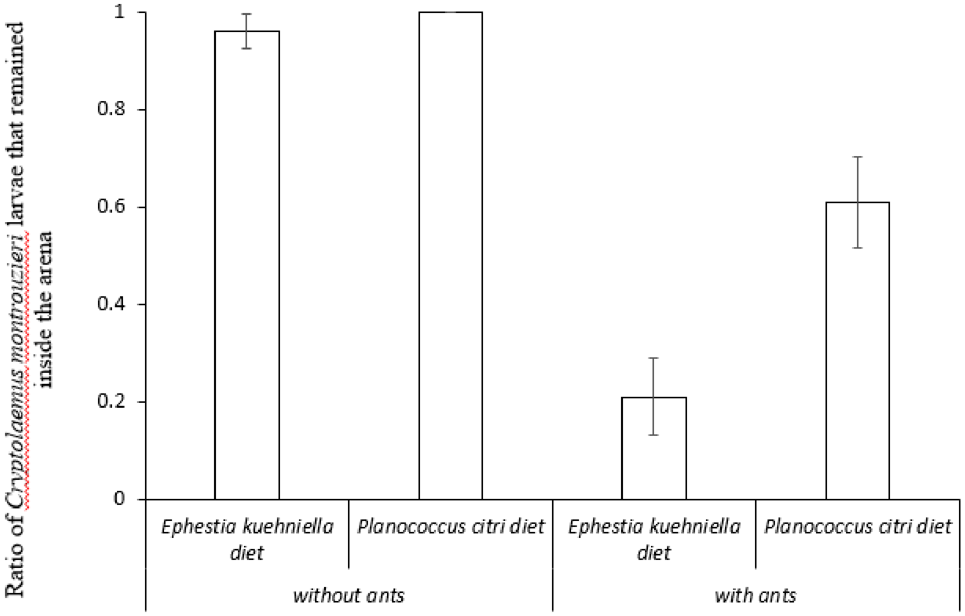

Figure 5. Ratio of *Cryptolaemus montrouzieri* larvae that remained inside the arena. *Cryptolaemus montrouzieri* had been reared on either the artificial diet *Ephestia kuehniella* eggs or its natural prey *Planococcus citri*.

4. DISCUSSION

Our study confirms that ants hinder biological control of mealybugs by the specialist predator *C. montrouzieri*. Many larvae of this coccinellid disappeared from the arena when they were searching on *P. citri* colonies tended by the ant *L. grandis* in the field assay. During our observations, ants removed some waxes of the larvae, killed some of the larvae and carried them away from the arenas to their nest in the base of the citrus trees. These observations, together with the fact that any larva left the arena in the colonies without ants, suggest that ants might take *C. montrouzieri* larvae away from mealybug colonies, reducing their efficacy as biological control agents. Bach (1991) obtained similar results under field conditions with the soft scale *Coccus viridis* (Green) (Hemiptera: Coccidae) and the ant *Pheidole megacephala* (Fabr.) on *Pluchea indica*. In her study, she observed that most *C. montrouzieri* larvae were killed and removed from the plant by ants within three hours of observation. Overall, our result highlights the importance of managing ants to control mealybugs, especially in citrus where *P. citri* is highly tended by different species of ants, including *L. grandis*, *Pheidole pallidula* (Nylander) and *Linepithema humile* Mayr (Pekas *et al.*, 2011; Tena *et al*., 2013).

Rearing *C. montrouzieri* on *E. kuehniella* eggs reduces the efficacy of this biological control agent when it is released in crops where mealybugs are tended by ants. Our laboratory and field results demonstrate that the ant *L. grandis* was more aggressive towards larvae reared on this alternative prey than on *P. citri* nymphs. Although the number of ants involved per attack was similar in both rearing diets, larvae reared on *E. kuehniella* eggs were attacked by ants and left mealybug colonies earlier than those reared on *P. citri* nymphs in the laboratory assay. The same occurred in the field, where only ~20% of the larvae reared on *E. kuehniella* eggs remained in the arena whereas more than 60% of the larvae reared on *P. citri* remained. Finally, and likely a consequence of the previous observations, larvae reared on *E. kuehniella* eggs preyed less mealybugs and died more frequently than when they were reared on *P. citri* nymphs. To our knowledge, this is the first study that has analyzed the effect of the factitious diet apart from the natural ones in the interaction between ants and coccinellids.

Majerus et al. (2007) and Vantaux *et al.* (2012) reviewed the behavioral, physical and chemical traits that allowed coccinellids to attack hemipteran colonies tended by ants. During our laboratory and field observations, we also observed some of these behaviors when *C.*

montrouzieri larvae were detected by ants. First, *C. montrouzieri* larvae tended to run away from ants after when they were attacked, leaving the mealybug colony. Second, some of the larvae of this biological control agent remained motionless when they were detected. This behavior has been suggested to mimic their preys (Daane *et al*, 2007). Moreover, Völkl (1995) mentioned that coccinellids may produce volatiles mimicking the scent of their prey if ants ignore them after detection while in a motionless behavior for instance. Since *C. montrouzieri* larvae reared on *E. kuehniella* eggs tended to leave mealybug colonies more frequently than those reared on *P. citri*, the diet might have affected the volatiles produced by the larvae. Further research is necessary to prove this hypothesis. For this, it would be necessary to check and compare the chemical components of the wax covering of the *C. montrouzieri* larvae reared in both diets. The analysis should include hydrocarbons and lipids that are present in the covering as well as the degree of stickiness of wax filaments. Another non-exclusive hypothesis could be that *C. montrouzieri* larvae do not synthesize the chemical profile that allows them to mimic mealybugs but instead, impregnate their body with waxes and honeydew from the *P. citri* colony where larvae feed.

5. CONCLUSION

Overall, our study had demonstrated the effect of rearing diet in the relationship between *C. montrouzieri* and ants. This result should be taken into consideration by companies or private institutions that produce not only *C. montrouzieri* but also other coccinellids that feed on hemipterans which are tended by ants. These insectaries and companies should develop new rearing systems to improve the efficacy of *C. montrouzieri* as biological control agent. A potential solution could be a diet based on mixtures of different preys, including *P. citri*.

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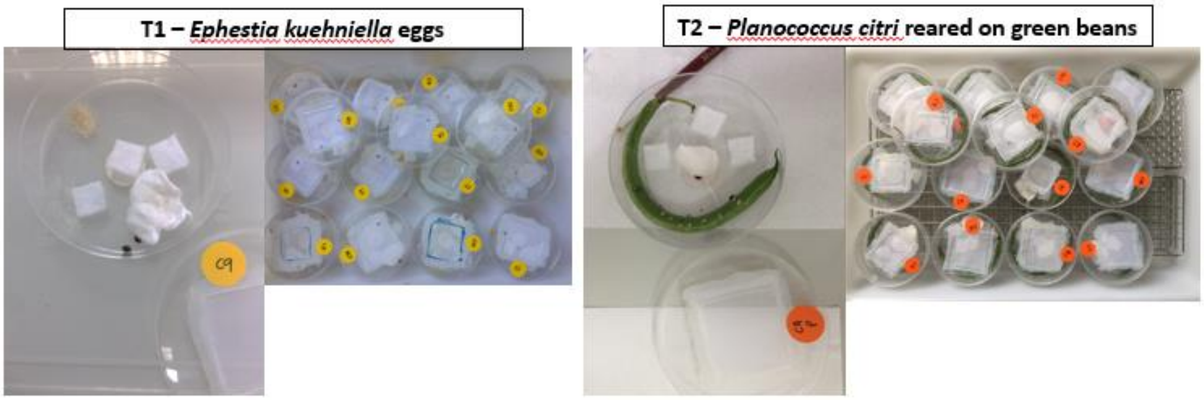
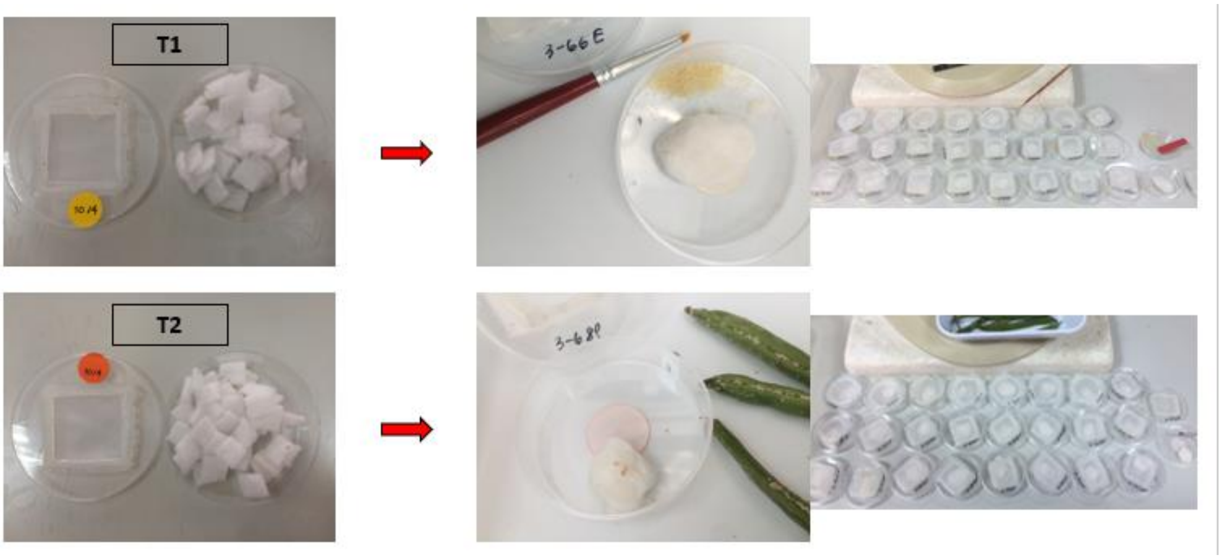
7. APPENDICES

Appendix 1. Schematic rearing procedure of *Cryptolaemus montrouzieri* in either *Ephestia kuehniella* eggs and *Planococcus citri* nymphs: A) pairing and mating of adults and B) egg collection to isolation of larvae.



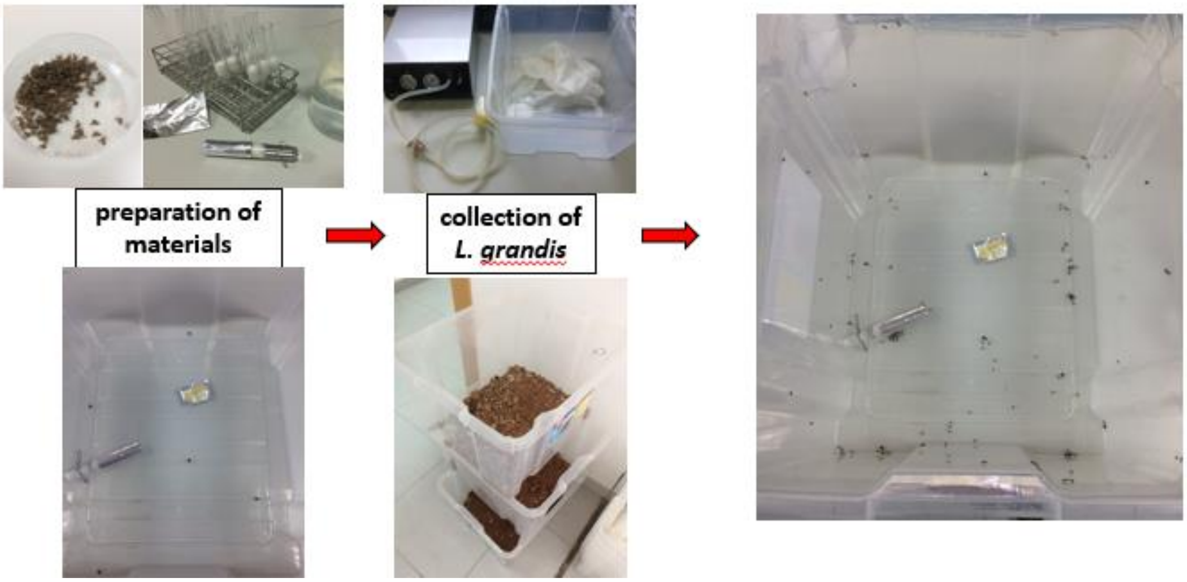
A



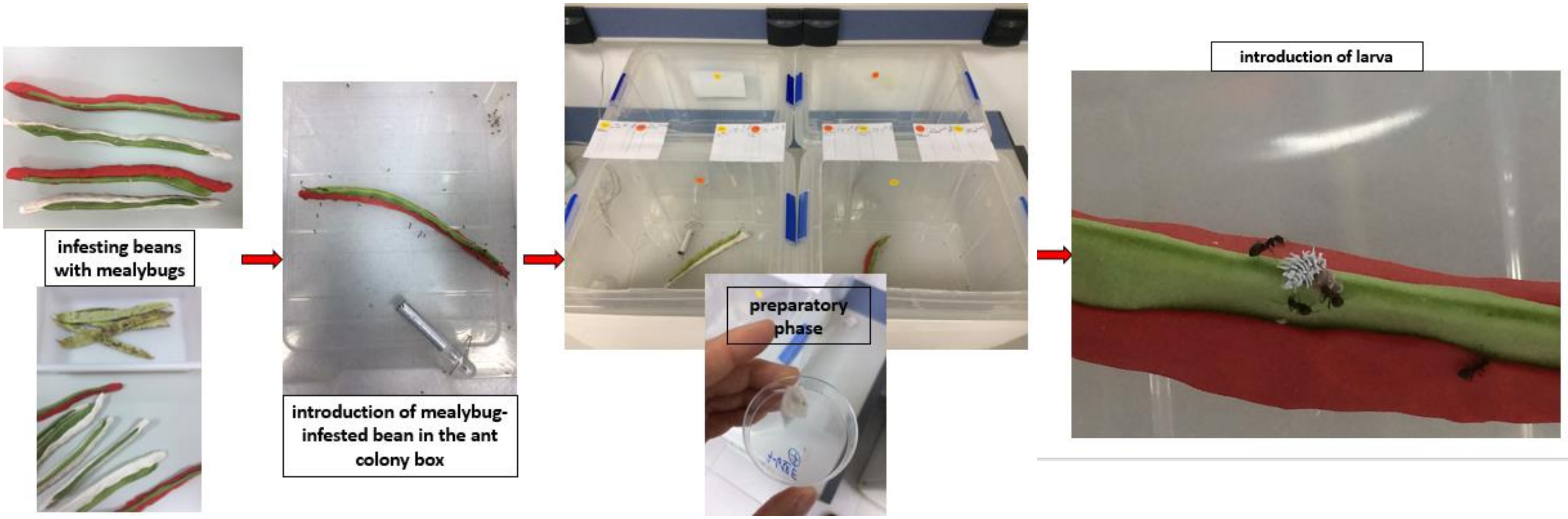
B

Appendix 2. Schematic rearing procedure of *Planococcus citri* in green beans.

Appendix 3. Schematic rearing procedure of *Lasius grandis* in the laboratory.



Appendix 4. Schematic diagram in the establishment of the laboratory assay. *Cryptolaemus montrouzieri* larvae either reared on *Ephestia kuehniella* eggs or *Planococcus citri* nymphs were introduced to mealybug colonies in the ant colony boxes.



Appendix 5. Set-up of the field assay. *Cryptolaemus montrouzieri* larvae either reared on *Ephestia kuehniella* eggs or *Planococcus citri* nymphs were introduced to ant-tended (A) and ant-excluded (B) arenas to monitor their behavior with and without *Lasius grandis*.