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Additional Information

Field evaluation of *Cryptolaemus montrouzieri* (Mulsant) (Coleoptera: Coccinellidae) as biological control agent of the citrus invasive pest *Delottococcus aberiae* De Lotto (Hemiptera: Pseudococcidae)

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Abstract

Delottococcus aberiae (Hemiptera: Pseudococcidae) is one of the latest invasive mealybugs in Europe. It causes severe fruit distortions and reduced fruit size leading into important economic losses in citrus. *Cryptolaemus montrouzieri* (Coleoptera: Coccinellidae) is one of the most worldwide used predators of mealybugs in biological control programs. However, its impact on *D. aberiae* is still unknown. Here, we conducted a two-year field study in order to describe the population dynamics of *D. aberiae* and *C. montrouzieri* and evaluate the effect of the predator on *D. aberiae* using different approaches. Our results showed that *C. montrouzieri* and *D. aberiae* had simultaneously two main population peaks per year: early spring and summer. Although *D. aberiae* and *C. montrouzieri* seasonal trends were synchronised, *C. montrouzieri* did neither reduce mealybug density within the same year nor avoid fruit damage, which occurs early in the season. Moreover, *D. aberiae* population growth rates were not correlated with *C. montrouzieri* density. On contrary, the increase of *D. aberiae* in the second year was negatively correlated with the density of *C. montrouzieri* in summer of the previous year. Overall, our data demonstrate that *C. montrouzieri* cannot control *D. aberiae* but it could contribute to regulate the mealybug once a successful parasitoid is introduced in Spain.

Keywords: conservation biological control, prey-predator, coccinellid, mealybug.

Introduction

Delottococcus aberiae De Lotto (Hemiptera: Pseudococcidae) is one of the latest exotic mealybug species introduced in citrus in the Mediterranean basin (Beltrà et al., 2012; Pérez-Rodríguez et al., 2017). It was first recorded in 2009 in the region of “Les Valls”, Valencia (eastern Spain) and since then, *D. aberiae* has been spreading to the surrounding areas becoming a significant citrus pest (Pérez-Rodríguez et al. 2017, Tena et al. 2017, Martínez-Blay et al. 2018b). Unlike other species of citrus inhabiting mealybug, *D. aberiae* causes severe fruit distortion and size reduction leading to major economic losses (Pérez-Rodríguez et al., 2017). The damaging period ranges between April and July, during the fruit set, when *D. aberiae* alters the fruit cell division while feeding (Martínez-Blay et al., 2018). Generally, *D. aberiae* is distributed in the whole tree canopy, but during the damaging period it shows an aggregation pattern in fruits (Pérez-Rodríguez et al. 2017). In addition to this, *D. aberiae* gravid females also descend down the trunk to the soil searching for shelter in the subterranean crown in the early spring (Franco et al., 2000; Martínez-Blay et al., 2018b). Once in the soil, they lay the ovisacs and emerging nymphs climb to the upper part of the tree (Martínez-Blay et al., 2018a).

Current Integrated Pest Management (IPM) of *D. aberiae* relies on the application of broad-spectrum insecticides such as chlorpyrifos. Recently the Environmental Economic Injury Levels for *D. aberiae* has been established in 12% of fruit occupation. When more than 12% of fruits out of 275 are occupied by *D. aberiae* a chemical treatment is needed (Pérez-Rodríguez et al. 2017). This is because native parasitoids, the most important group of biological control agents of mealybugs, cannot develop in *D. aberiae* and the role of predators is poorly known (Tena et al., 2016). Recently, the soil predatory mite *Gaeolaelaps aculeifer* (Acari: Laelapidae) has been recorded as a potential predator of young instars of *D. aberiae* in the soil because it feeds on the first instar under semi-field conditions (Pérez-Rodríguez et al., 2018) but the impact of other generalist predators is still unknown.

Among mealybug predators, *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) has been used worldwide in many biological control programs (Kairo et al., 2000; Stiling 2004).

It is native to the Australasian Zoogeographic Region and it was introduced to more than 64 countries to suppress important mealybug pests (Kairo et al. 2013). In the citrus-growing areas of the Mediterranean Basin such as France, Greece, Italy, Spain or Turkey, *C. montrouzieri* has been introduced (classical biological control) and/or inoculatively released in spring to control *Planococcus citri* Risso (Hemiptera: Pseudococcidae) (Garrido 1986; Ripollés et al. 1994; Spicciareui et al. 1994; Katsoyannos 1996; Franco et al., 2004; Villalba et al., 2006; Mani 2018). The larvae and adult of *C. montrouzieri* are voracious feeders and prey on all instars of *P. citri*. However, the earlier instars prefer to feed on eggs and young nymphs while older instars feed mostly on mealybugs of any size and show a higher predation rate (Copland et al., 1985; Mani, 1986; Mani 2018). For instance, a fourth instar larva can feed up to 3,330 eggs or 250 young instars of *P. citri* at 28°C (Hussey & Scopes, 1985; Ilyas & Khan, 2009). Adult *C. montrouzieri* become smaller or even infertile as prey increase in scarcity (Torres & Marcano 2015) and engage in cannibalism when prey are absent (Mani 2018).

In order to locate their prey, *C. montrouzieri* larvae use physical contact whereas adults require visual and chemical stimuli (Heidari & Copland, 1992; Kairo 2004). The wax secretions and honeydew produced by mealybugs act as attractants as well as oviposition stimulants for *C. montrouzieri* females (Merlin et al. 1996), which are able to lay viable eggs as long as they can prey on mealybugs (Doney & Walter 2012). A single adult female lays about 400 eggs in 40 days of oviposition (Bhat et al., 1981) and this quantity can increase if multiple mating occurs (Jiaquin et al., 2014).

Under Mediterranean conditions, *C. montrouzieri* has four generations per year (Hussey & Scopes, 1985) and it can overwinter depending on the climatic conditions (Kaneko 2017; Toorani et al., 2017). Apart from *P. citri*, it has been observed feeding on other mealybug species, as well as soft scales (Hemiptera: Coccidae), aphids (Hemiptera: Aphididae) and white flies (Hemiptera: Aleyrodidae) (Osborne et al., 2004; Amal et al. 2010). In the main citrus producing areas of Spain, *C. montrouzieri* follows a prey-predator population dynamic with its main prey *P. citri* (Llorens 1990; Martínez 2006; Villalba 2006).

During the last years, *C. montrouzieri* has been observed feeding on *D. aberiae*. However, the impact of this predator on the population of this invasive mealybug is still unknown. For this reason, here we sampled nine citrus orchards during two consecutive years in order to: i) describe damages produced by *D. aberiae* throughout the year and ii) the population dynamics of *D. aberiae* and *C. montrouzieri*; iii) evaluate the effect of *C. montrouzieri* density on the population levels of *D. aberiae* within the same year and among years; and iv) evaluate the effect of *C. montrouzieri* as well as the mean temperature per day and citrus variety on *D. aberiae* population growth rates. We used these data to discuss the potential of *C. montrouzieri* as biological control agent of *D. aberiae*.

2. Material and methods

2.1 Sampling sites and sampling procedure

The study was carried out in the core of the citrus producing area of eastern Spain (Valencian Community). A total of nine orchards were sampled during the years 2014 and 2015. Five orchards belonged to *Citrus reticulata* Blanco: three were Clemenules and two were Oroval variety. The remaining four consisted of *Citrus sinensis* (L.) Osbeck: Nave Late variety. Sampled orchards ranged from 0.4 to 3 ha and all of them were drop-irrigated. The sampling area was pesticide-free during the assay.

Between eight to ten trees were sampled twice a month from March to November and monthly from December to February. Trees were selected at the beginning of the assay because they were infested the previous years. In each tree, two different strata were sampled: canopy and trunk. In the canopy four 30-cm long twigs with flowers or fruits or both were collected and each twig belonged to a different cardinal orientation (i.e., one twig randomly selected in each orientation). All samples of a tree were placed in individual plastic bags, enclosed, and transported to the laboratory. Within the next 24 h, twigs and four leaves and a maximum of eight flowers or fruits per twig were examined under a stereomicroscope. Leaves and flowers or fruits were selected at random within the twig. All individuals of *D. aberiae* and *C.*

montrouzieri were counted. In the trunk strata, sampling consisted of visual counts during 2 min. All *D. aberiae* and *C. montrouzieri* individuals present on the trunk and main branches of the trees (until 60 cm in height) were recorded. During fruit formation, we also recorded whether the fruit was damaged by *D. aberiae*. We considered damaged fruit as those with a size reduction or deformation that completely depreciated them from a commercial view (Pérez-Rodríguez *et al.*, 2017).

2. Data analyses

2.1 Prey-Predator dynamics and impact of *C. montrouzieri* on *D. aberiae*.

In order to represent the population dynamics of *D. aberiae* and *C. montrouzieri*, sampling data of the different strata were averaged per tree and afterward per orchard. The ratio of fruit damaged by *D. aberiae* was also averaged per tree and afterward per orchard. The maximums of *C. montrouzieri* density throughout the two years of the study were correlated using Kendall tests. The same analysis was used to determine correlations between the maximums of *C. montrouzieri* density and the fluctuations of *D. aberiae* density among generations (or maximum peaks). All analyses were conducted using the software R version 3.4.4 (The R Foundation for Statistical Computing).

2.2 Population growth rates

Population growth was expressed as the intrinsic rate of growth, r , calculated as $\ln(N_{t+1}/N_t)$, in which N represents the total number all developmental *D. aberiae* stages per tree at time t and $t + 1$. r was expressed normally using the units d^{-1} by dividing $\ln(N_{t+1}/N_t)$ by the period of time in days between the sampling date t and the subsequent sampling date $t + 1$. The relative density of *C. montrouzieri* was calculated as $C. montrouzieri / (D. aberiae + C. montrouzieri)$, which is the fraction of *C. montrouzieri* in an isolated predator-prey community (per tree).

A linear mixed-effects model was used to quantify the effect of *C. montrouzieri* on *D. aberiae* population growth. The model incorporated predator density, temperature, and citrus

species as main effects, and orchard and tree as nested random effects to account for repeated measures. The data were also analyzed for any delayed effects by plotting the slope of *D. aberiae* population growth and *C. montrouzieri* density as a function of increasing the magnitude of time (incremental increase in sampling period) between measuring *C. montrouzieri* density and the associated *D. aberiae* population growth rate. Thus, as the time delay becomes more extreme (considered as increasing values along an x -axis), this represents a larger delay in the effect of *C. montrouzieri* on *D. aberiae*. For example, a 365-day delay correlates *D. aberiae* population growth with *C. montrouzieri* densities sampled one year prior. Citrus varieties were analyzed separated in this variation of the model because sampling periods differed between varieties. All analyses were conducted using nlme:lme in R version 3.4.4 (The R Foundation for Statistical Computing).

3. Results

3.1. Prey-predator population dynamics and impact of *C. montrouzieri* on *D. aberiae*

The population densities of *D. aberiae* and *C. montrouzieri* had two main maximum peaks both years and their populations were synchronized except in summer of 2014, when the mealybug peaked earlier than the predator (Figure 1). The first peak was at the end of May and the second varied between July and August.

The maximum number of *C. montrouzieri* in the first and second peak had a positive correlation both years (2014: $Z = 2.04$; $df = 1, 70$; $P = 0.042$; $\tau = 0.19$; 2015: $Z = 2.69$; $df = 1, 70$ $P = 0.007$ $\tau = 0.27$) (Table 1). Other correlations between different peaks of *C. montrouzieri* were not significant (Table 1).

When we analysed the effect of *C. montrouzieri* on the variation of *D. aberiae* populations, the number of predators in the second peak of 2014 had a negative relationship with the increase of *D. aberiae* in 2015 [increase between the first (May) and second peak (July-August); $Z = -3.75$; $df = 1, 70$ $P < 0.001$ $\tau = -0.31$] (Table 2). Other correlations between the

variation of the number of *D. aberiae* and the number of *C. montrouzieri* were not significant (Table 2).

3.2. Fruit damaged by *D. aberiae*

During both years, the ratio of fruit that could be categorized as damaged by *D. aberiae* increased exponentially from the end of May until the end of June (Figure 2). The last week of June around 0.7 of the fruit was damaged both years.

3.3. Analyses of population growth rates

Changes in *D. aberiae* population growth rate were associated with changes in temperature and *C. montrouzieri* density, but not citrus variety (Figure 3; Table 3). *Delotococcus aberiae* population growth rate tended to slightly decrease with temperature increase, whereas it increased with *C. montrouzieri* density. Similarly, analyses of a subset of the data that encompassed from 1 May to 30 September (outbreak and decline of peak *D. aberiae* densities) exhibited similar trends, except that *D. aberiae* population growth rate was not associated with changes in *C. montrouzieri* density in 2014 (Table 3).

Figure 4 summarizes the analysis of delayed predator effects on *D. aberiae* population growth rates over time. While the slope was positive when *C. montrouzieri* density was delayed 15 days, it tended to be negative latter on. Fourteen out the 22 (64%) slopes were negative after this period of delay.

4. Discussion

Our two-field year study showed that the seasonal abundance of the coccinellid predator *C. montrouzieri* was synchronized with that of the invasive mealybug *D. aberiae*. The mealybug and the predator had two main peaks per year; the first one was in spring and the second in summer. The populations of the *D. aberiae* and *C. montrouzieri* were synchronised in both peaks in the two years of the study. This prey-predator synchrony, as well as the absence of

alternative mealybug species, suggests that *C. montrouzieri* is able to locate and prey upon *D. aberiae* in the field just a few years after the mealybug established in Europe. It is worth mentioning that, although *P. citri* is abundant and widely distributed in Mediterranean citrus (Tena & Garcia-Marí 2011), *D. aberiae* was the main mealybug species in the sampled orchards. In fact, only *Pseudococcus longispinus* (Targioni Tozzetti) (Hemiptera: Pseudococcidae) was recovered occasionally in one of the orchards.

The population levels of *D. aberiae* and *C. montrouzieri* tended also to overlap, contrary to other prey-predator dynamics. Generally, predators peak a few weeks after their prey, showing a temporal mismatch between population peaks (Holling 1965; Tansky 1978; Martin & Ruan 2001; Begon et al., 2006; Baigent 2010). The reason of this overlap might be due to the preference of *C. montrouzieri* adults of laying their eggs in mealybug ovisacs, just before the new generation of the prey emerge and peak (Whitcomb 1940; Bodenheimer 1951; Merlin et al., 1996). In fact, *C. montrouzieri* adult females withhold their eggs in the oviducts delaying their oviposition when there are not mealybug ovisacs (Maes et al., 2014).

Although *D. aberiae* and *C. montrouzieri* seasonal trends were synchronised, our results show that *C. montrouzieri* was not able to avoid fruit damage by the mealybug. Damages produced by *D. aberiae* were observed already in May and increased exponentially in June. At the end of June, almost 70% of the fruit was already damaged both years, which is around 4-5 times higher than the proposed Environmental Economic Injury Level for oranges (Pérez-Rodríguez et al., 2017). Therefore, *C. montrouzieri* could not avoid the damages produced by *D. aberiae*. Two reasons might explain this lack of damage control. First, the population levels of the predator might be still too low to avoid the damages. However, it does not seem the most plausible explanation because *C. montrouzieri* is a voracious feeder that can prey up to 3,300 *P. citri* eggs or 300 mealybug nymphs during their life and the relation prey/predator was close to 1/50 during May and June both years. Another reason is that *C. montrouzieri* is ineffective at low prey densities and contrary to adults, its larvae need physical contact for prey detection (Moore 1988; García & O'Neil 2000; Kairo et al., 2013; Torres & Marcano, 2015).

Similarly, *C. montrouzieri* was not able to regulate the populations of *D. aberiae* within the same year. Density fluctuations of the mealybug between spring and summer, as well as its population growth rates were independent of the density of the coccinellid. One possible explanation could be that *D. aberiae* is an unsuitable prey for *C. montrouzieri*. The nutritional value of some preys can be insufficient to allow the development of their predators, or even can contain toxic components that cause the death of the predator (Hodek et al., 2012). It is unlikely that *D. aberiae* is a toxic prey for the coccinellid because, as shown in Table 1, *C. montrouzieri* population increased within the same year in orchards where other mealybugs were not present. The nutritional value of *D. aberiae* as prey for *C. montrouzieri*, however, needs to be evaluated because coccinellids are not able to distinguish between adequate food and a less suitable prey (gustatory discrimination) (Nielsen et al. 2002; Snyder & Clevenger 2004; Nedvêd & Salvucci 2008; Hodel et al., 2012). Therefore, *D. aberiae* could be a less suitable prey for *C. montrouzieri* than other citrus mealybugs as *P. citri*, which density levels within the same year depend on the coccinellid (Martínez-Ferrer, 2006). Other possible factor that might explain this lack of regulation is temperature. The coccinellid is adapted to tropical temperatures and needs a minimum temperature of 21°C to feed or lay eggs and it is unable to complete development between 0 and 17°C (Kairo et al., 2013). Although the minimum temperature threshold of *D. aberiae* is unknown, the mealybug might have a lower threshold than *C. montrouzieri* because it is active during the Mediterranean winter and a male flight occurs in February (Martínez-Blay, 2018a, b). These different requirements of temperature might contribute to explain the lack of regulation within the same year. Finally, down-up regulation maybe more important than up-down regulation and mask the effect of *C. montrouzieri*. As mealybug, *D. aberiae* is likely a phloem feeder. In citrus, the two highest concentrations of amino acids in the phloem, occurs in early spring (March) and the fruit setting period June-July (Moreno & García-Martínez 1983; Yang and Sadof 1995; Sétamou et al., 2016), during the two main peaks of *D. aberiae*. The increase of amino acids might accelerate *D. aberiae* development and its reproduction.

Another detrimental factor that could disrupt the prey-predator dynamic between *C. montrouzieri* and *D. aberiae* is the presence of ants in citrus. In a mutualistic association, ants obtain carbohydrates and other nutrients from the mealybug honeydew in exchange of providing protection against their natural enemies (Tollerup *et al.*, 2004; Styrsky and Eubanks, 2007; Majerus *et al.*, 2007; Nelson and Daane, 2007; Tena *et al.*, 2013; Calabuig Gomar *et al.*, 2014; Zhou *et al.*, 2015; Beltrà *et al.*, 2017; Cranshaw and Shetlar, 2017). In Spanish citrus orchards, the two dominant ants species are *Lasius grandis* (Forel) (Hymenoptera: Formicidae) and *Pheidole pallidula* (Nyl) (Hymenoptera: Formicidae). They attend the main citrus mealybug species *P. citri* (Pekas *et al.*, 2011; Calabuig *et al.*, 2013) and have been reported attacking *C. montrouzieri* larvae (Mansour 2012; Kairo & Paraiso, 2013). However, it is unclear whether *L. grandis* and *P. pallidula* disrupt the biological control of *D. aberiae* because we observed them attending *D. aberiae* only occasionally (JPR, personal observations).

In spite of the lack of correlation between *C. montrouzieri* and *D. aberiae* populations within the same year, the density of *C. montrouzieri* in the summer of 2014 was negatively related with the increase of *D. aberiae* density in 2015. *Cryptolaemus montrouzieri* has been largely considered in conservation biological control programmes of other mealybug species because it complements the action of parasitoids (Quayle, 1941; Prokopenko 1982; Longo & Benfatto 1987; Moore & Hattigh 2004; Martínez-Ferrer 2003; Kairo *et al.*, 2013). This result suggests that *C. montrouzieri* could contribute to regulate *D. aberiae* once a successful parasitoid is introduced in Spain. Nowadays, biological control of *D. aberiae* is restricted because the mealybug encapsulates the eggs of the native or naturalized parasitoids in Spanish citrus (Tena *et al.*, 2017). Recently, two new parasitoid species of *D. aberiae* have been described in South Africa, its origin area (Beltrà *et al.*, 2015; Guerrieri & Cascone, 2018). Due to the lack of native parasitoids and the low effect of *C. montrouzieri* present herein, their introduction within a classical biological control program might contribute to the biological control of *D. aberiae*. In the meanwhile, augmentative or inoculative releases of *C. montrouzieri* larvae during winter, or before the first main peak, could be tested. Third instar nymphs and

adult females of *D. aberiae* tend to migrate to the trunk and soil in February and March. Since the trunk is a drain of mealybugs these months, third instar larvae of *C. montrouzieri* could be released in its base. The third instar is more voracious than previous instars and feeds on immature and adult instars of mealybugs (Rosas-García et al., 2009; Torres & Marcano, 2015).

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Conflict of interest

The authors declare that they have no conflict of interest.

Table 1. Correlation between the densities of the predatory coccinellid *Cryptolaemus montrouzieri* in different maximum peaks of 2014 and 2015. Significant *P* values are presented in boldface (N = 71 trees).

<i>C. montrouzieri</i> density		Z	P	T	slope
(x-Axis)	(y-Axis)				
	2 nd peak 2014	2.04	0.04	0.19	1.29
1 st peak 2014	1 st peak 2015	1.62	0.11	0.16	0.16
	2 nd peak 2015	0.05	0.96	0.01	-0.12
2 nd peak 2014	1 st peak 2015	-0.43	0.67	-0.04	-0.001
	2 nd peak 2015	-1.24	0.21	-0.12	-0.04
1 st peak 2015	2 nd peak 2015	2.69	0.01	0.27	0.46

Table 2. Effect of *Cryptolaemus montrouzieri* density on *Delottococcus aberiae* increase among different maximum peaks of 2014 and 2015. Significant *P* values are presented in boldface. The significant interactions are graphically represented in Fig. 2.

<i>C. montrouzieri</i> density	Population fluctuations of <i>D. aberiae</i>	Z	P	T	slope
1 st peak 2014	2 nd peak 2014 - 1 st peak 2014	-1.43	0.15	-0.13	-13.52
	1 st peak 2015 - 2 nd peak 2014	0.35	0.72	0.032	9.83
	2 nd peak 2015 - 1 st peak 2015	-1.62	0.11	-0.14	-14.30
2 nd peak 2014	1 st peak 2015-2 nd peak 2014	-1.13	0.26	-0.10	-1.68
	2 nd peak 2015-1 st peak 2015	-3.75	<0.001	-0.31	-3.50
3 rd peak 2015	2 nd peak 2015-1 st peak 2015	-0.79	0.43	-0.07	0.09

Table 3. Effect of the density of *C. montrouzieri*, mean temperature per day and citrus variety on *Delottococcus aberiae* population growth rates during 2014–2015. Significant *P* values are presented in boldface and represented graphically in Fig. 3.

Time period	Factor	df	F-value	P-value
2014–2015	<i>C. montrouzieri</i>	1, 2502	10.32	0.001
	temperature	1, 2502	35.67	<0.0001
	citrus variety	1, 6	1.880	0.219
May–Sep 2014	<i>C. montrouzieri</i>	1, 749	1.500	0.221
	temperature	1, 749	53.00	< 0.0001
	citrus variety	1, 6	1.134	0.328
May–Sep 2015	<i>C. montrouzieri</i>	1, 647	17.05	<0.0001
	temperature	1, 647	9.939	0.002
	citrus variety	1, 6	3.356	0.117

Figure legends

Figure 1. Population density (mean \pm SE) of the invasive mealybug *Delottococcus aberiae* and its predatory coccinellid *Cryptolaemus montrouzieri* in nine citrus orchards from Spain in 2014 and 2015.

Figure 2. Fruit damaged (mean ratio \pm SE) by *Delottococcus aberiae* in nine citrus orchards from Spain throughout 2014 and 2015.

Figure 3. a) Population density and b) population growth rate of *Delottococcus aberiae* represented per day and tree. Different colours represent mean daily temperature. c) Population growth rate of *D. aberiae* as a function of relative density of *Cryptolaemus montrouzieri* and d) mean temperature during 2014 and 2015. Dashed line plots the slope β_1 from the linear mixed models presented in Table 3.

Figure 4. Slope (β_1) of *Delottococcus aberiae* population growth rate (r) vs. the relative *Cryptolaemus montrouzieri* density as a function of offsetting the temporal relationship between *D. aberiae* r and *C. montrouzieri* density from 2014–2015. The x -axis maps incremental delays in sampling periods. Slopes significantly different than zero are indicated by open circles. The dashed line represents no correlation between *C. montrouzieri* and *D. aberiae* population density.

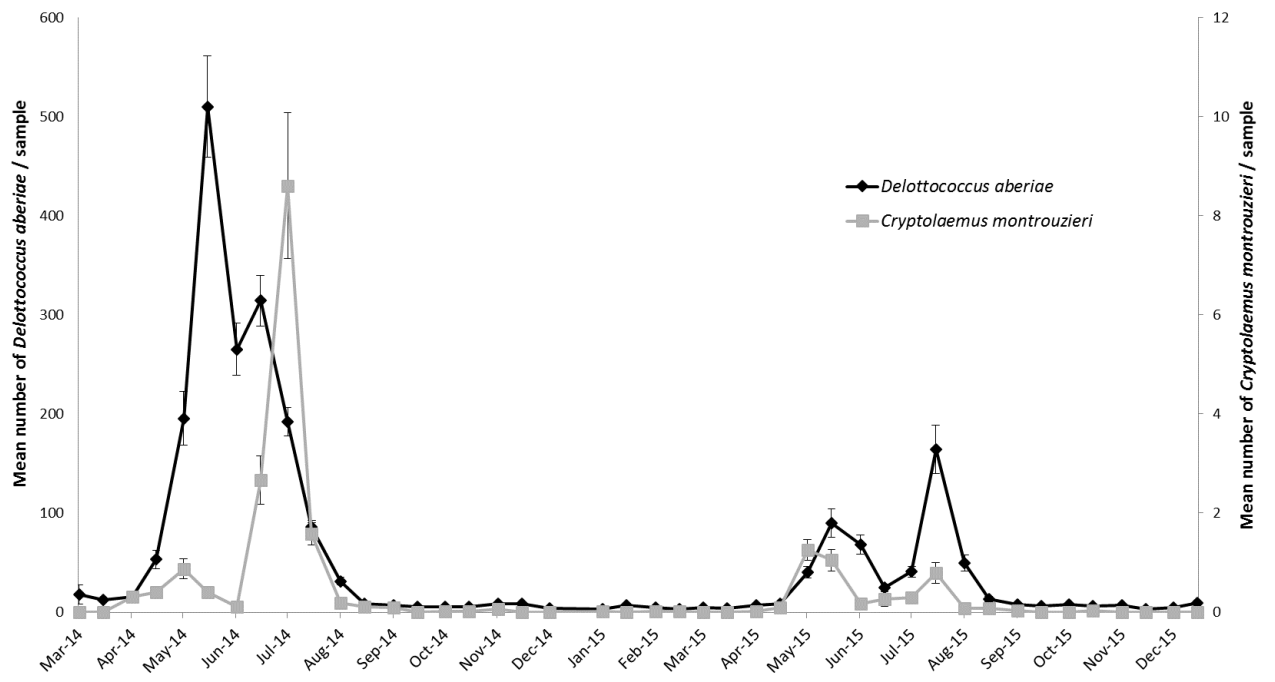


Fig. 1

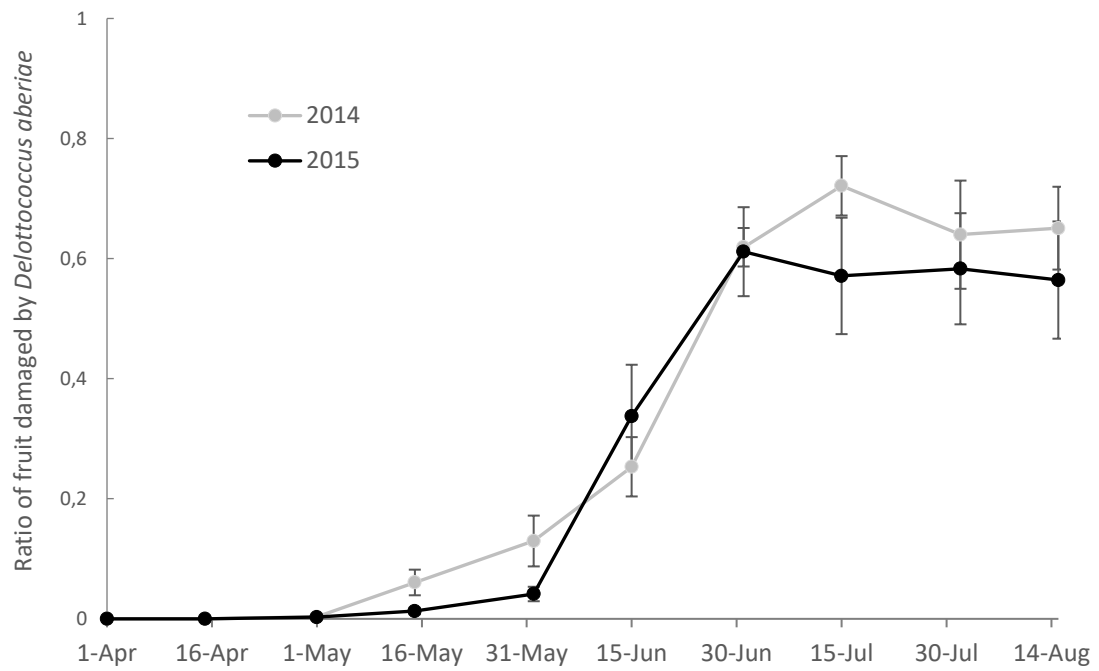


Fig. 2

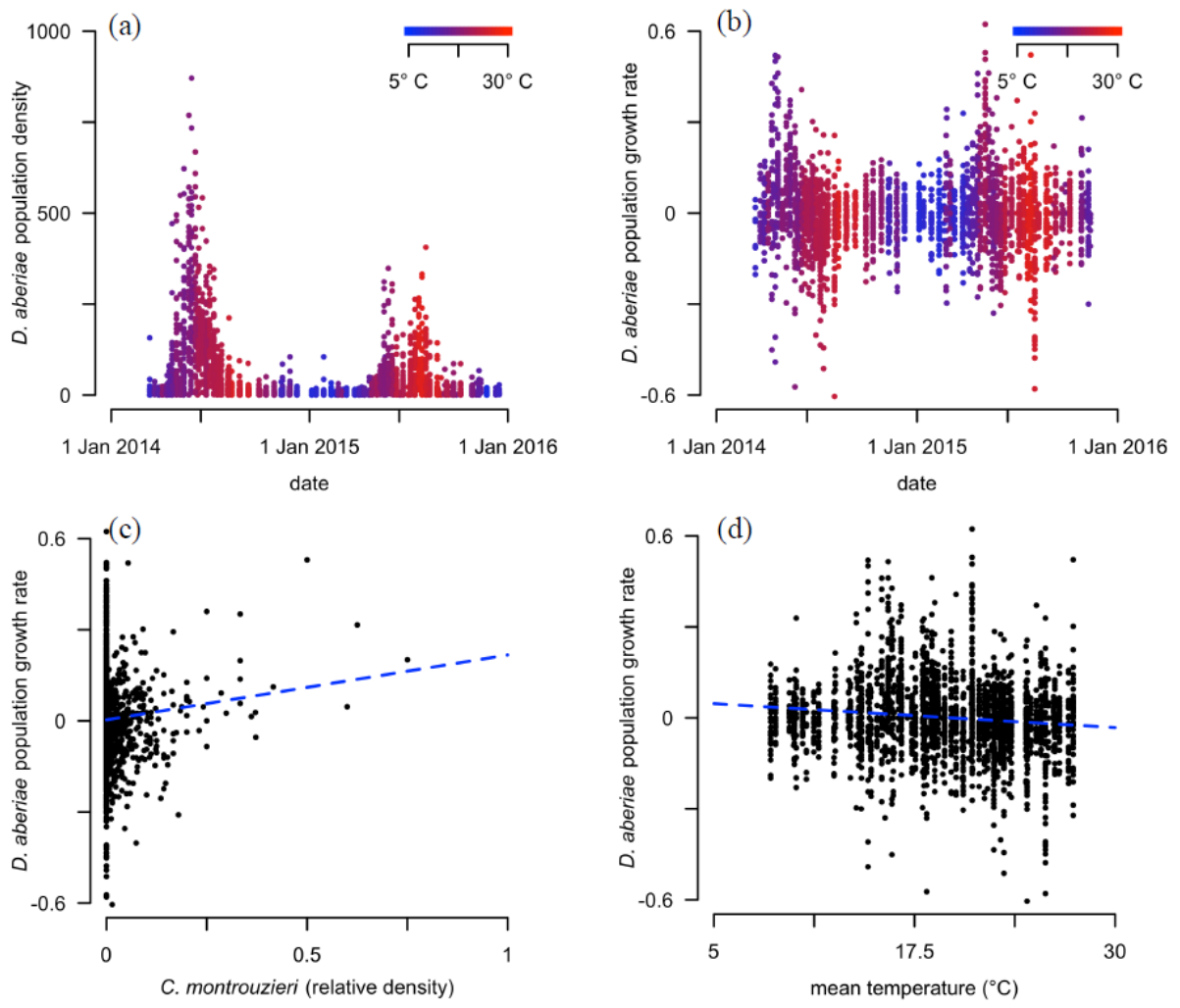


Fig. 3

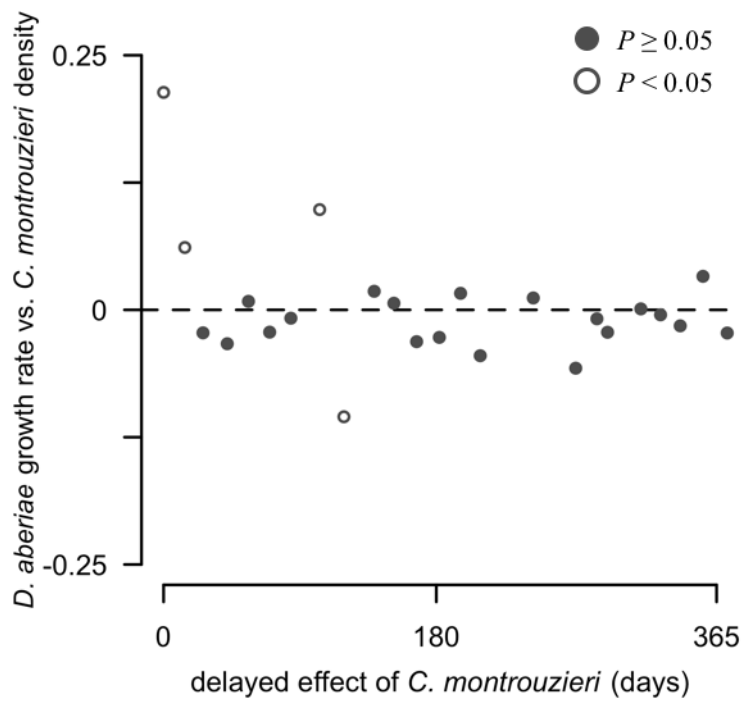


Fig. 4.