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Can the ladybird predator *Scymnus nubilus* contribute to control of the aphid *Aphis frangulae*, a pest threatening the Macaronesia endemic *Frangula azorica*?

Abstract

Aphis frangulae (Hemiptera: Aphididae) is a major pest of the laurel forest endemic *Frangula azorica* (Rosales: Rhamnaceae) produced in nursery conditions by the Forestry Services of the Azores (Portugal). The suitability of *A. frangulae* for the development and reproduction of a potential biological control agent, *Scymnus nubilus* (Coleoptera: Coccinellidae), was assessed under laboratory conditions (25±1° C, 75±5% relative humidity, 16L:8D light regime). The predation potential of *S. nubilus* was also assessed. *Scymnus nubilus* 4th instar larvae and pupae successfully completed development in 9.0±0.2 days. The 4th instar larvae ate 15.1 aphids per day, corresponding to 1.52 mg of biomass ingested. On *A. frangulae*, *S. nubilus* females took 5.5±0.3 days to start oviposition and an average of 135±12 eggs were laid per female over the first 15 days of oviposition. Field tests showed that *S. nubilus* 4th instar larvae were more efficient in controlling the pest in closed systems (isolated aphid colonies) and the effect was more pronounced at high predator densities within 3 days. In open systems, the aphid natural control was higher than initially expected. This work highlights the role of the large aphidophagous guild present in forestry nurseries. The results of this study show that *A. frangulae* is an essential prey species for *S. nubilus* and therefore the predator can be used in pest management programs against this pest. However, further studies focusing on different biological control tactics (inundative or inoculative) are required to assess more accurately the effectiveness of *S. nubilus* as a biological control agent against *A. frangulae*.

Keywords: Biological control; ladybird, aphid; *Scymnus nubilus*; *Aphis frangulae*; *Frangula azorica.*

Introduction

Aphis frangulae Kaltenbach (Hemiptera: Aphididae) is an aphid species with a wide distribution across Palearctic and Nearctic regions. This oligophagous aphid has species of the genera *Frangula* and *Rhamnus* as primary host plants. However, it is polyphagous with regard to secondary hosts, which includes *Capsella bursa-pastoris* (L.) Medik. (Brassicales: Brassicaceae), *Cirsium monspessulanum* subsp. *ferox* (Coss.) Talavera, *Eupatorium cannabinum* L. (Asterales: Asteraceae), *Chamerion angustifolium* (L.) Scop. (Myrtales: Onagraceae), *Epilobium parviflorum* Schreb. (Myrtales: Onagraceae), *Hypericum perforatum* L. (Malpighiales: Hypericaceae), *Lysimachia vulgaris* L. (Ericales: Primulaceae), *Nigella arvensis* L. (Ranunculales: Ranunculaceae), *Rumex crispus* L. (Polygonales: Polygonaceae) and *Solanum tuberosum* L. (Solanales: Solanaceae) (Blackman and Eastop 2007).

Aphis frangulae has been recognized as a major phytosanitary problem to *Frangula azorica* V. Grubow (Rosales: Rhamnaceae) when mass reared under nursery conditions in the facilities of the Forestry Services of the Azores (Portugal). *Frangula azorica* is an endemic plant species of the Laurel Forests from the Macaronesia region. The mass reared plants are used under the scope of conservation projects, including the restoration of endangered native Laurel Forests (e.g. Triantis et al. 2011; Norder et al. 2020), assisting the preservation of the Azorean bullfinch *Pyrrhula murina* Godman (Passeriformes: Fringillidae), one of the most endangered bird species in Europe (SPEA 2007; Ceia et al. 2011).

Mass rearing of *F. azorica* is compromised after irreversible damage of young shoots by *A. frangulae*. Additionally, the honeydew produced by aphids accumulating over the leaves promotes mold growth which limits the photosynthetic capacity of the plants. Due to certification requirements, pest chemical control is restricted and therefore environmentally friendly alternatives are required to control aphid outbreaks. Biological control of pest populations in forestry systems is a fundamental strategy for sustainable production, providing a safe alternative to chemical control (Kenis et al. 2019).

The classical approach of introducing exotic species to control herbivorous pests, commonly used in the past, poses important challenges for conservation biology (Soares et al. 2018; Rondoni et al. 2021; Soares et al. in press). A possible alternative could be the application of mass-reared native natural enemies, through an inoculative or inundative strategy. During previous studies, we recorded a potential candidate to limit *A. frangulae* populations, the aphidophagous ladybird predator *Scymnus interruptus* (Goeze) (Coleoptera: Coccinellidae) (Rosagro et al. 2020). Of the two most abundant aphidophagous species present in the Azores, *Scymnus nubilus* Mulsant (Coleoptera: Coccinellidae) and *S. interruptus* (Soares et al. 2021a, b), previous experiments have shown that the former is much more easily maintained under controlled conditions (e.g., Borges et al. 2013; Sebastião et al. 2015). *Scymnus nubilus* is a widely distributed species, recorded in Palearctic, Afrotropical, Australian and Oriental zoogeographical regions (Kovář 2007). The species of the genus *Scymnus* are among the least studied ladybirds in the world. However, a few studies have demonstrated the potential of this group as natural enemies and important biological control agents in their native regions (e.g., Tawfik et al. 1973, Rosagro et al. 2020).

Considering the biological and ecological similarity of these phylogenetically closely related species, *S. nubilus* was selected for testing as a biological control agent against aphids infesting the endemic plant species produced in the forestry nurseries. However, no information is available on the suitability of *A. frangulae* as a food source for *S. nubilus*. From the ecophysiological point of view, the suitability of prey can be divided into two main groups (sensu Hodek and Evans 2012): (i) essential foods ensure the completion of larval development and oviposition and (ii) alternative foods serve only as a source of energy and thus prolong survival in comparison to starvation. This study aims: (1) to determine the seasonal abundance of the aphid *A. frangulae* on *F. azorica* reared in forestry nurseries, (2) to assess the ecophysiological suitability of *A. frangulae* as prey for the ladybird predator *S. nubilus*, and (3) to test the efficacy of the 4th larval stage of *S. nubilus* as a biological control agent.

Materials and Methods

Laboratory experiments

Laboratory stock population

A laboratory stock population of *S. nubilus,* collected in coastal prairies (37°44'38"N 25°42'42"W) of S. Miguel Island (Portugal), was maintained and renewed each year during the spring season with new field-collected individuals. The ladybirds were provided with a mixed diet of the fresh aphids *Aphis fabae* Scopoli and *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) reared on *Vicia faba* L. (Fabales: Fabaceae) and complemented with honey and pollen. The laboratory stock population was reared at 25±1ºC, 75±5% relative humidity and a light regime of 16L:8D.

Development and reproduction

To test the suitability of *A. frangulae* for *S. nubilus*, in the laboratory we first attempted to set-up the trophic chain of *F. azorica* – *A. frangulae*. However, this task was unsuccessful. We therefore provided an alternative aphid species, *A. fabae*, a highly suitable prey species for the predator (Borges, I. *pers. obs*.), during the earlier stages of larval development. In this way, it was possible to ensure sufficient *A. frangulae* for the experiments by bringing infested plants from the forestry nurseries in Nordeste county

(S. Miguel Island). For the development experiments, *S. nubilus* adults from the laboratory population were sexed and coupled. Each couple was kept in a plastic box (2 cm Ø x 1 cm height) with *A. fabae* to obtain eggs. Newly hatched larvae were transferred individually into new boxes and fed with *A. fabae ad libitum*. Every day the boxes were checked for the presence of exuviae, as evidence of moulting. When larvae moulted into the 4th larval stage, a single diet of *A. frangulae* was provided from that point on (n=30). The developmental time from the 4th larval stage to the adult stage was determined, as well as the sex ratio and the weight of males (N=13) and females (N=13) upon emergence. To assess the reproductive performance, newly emerged adults were coupled (N=10 couples), kept in plastic boxes (2 cm Ø x 1 cm height) and fed with *A. frangulae*. Daily observations were carried out to determine the pre-oviposition time and the number of eggs laid in the first 15 days of oviposition. The eggs were checked on a daily basis for egg hatching to determine the fertility, that is, the number of hatched larvae.

Feeding parameters

Predators of the 4th larval stage were obtained as described in the previous section. Newly moulted larvae were fed for 12 hours with *A. fabae* then starved for the next 12 hours, prior to performing the 24 hours consumption test. Because the rates of dehydration and mortality of the aphid in 24 hours were considerably high, it was decided to establish two 12-hour feeding periods in which 10 fresh aphids with an average of 2 mg of biomass were provided to the larvae. According to Borges (2008) the food quantity provided was *ad libitum*. Additionally, a control test was performed to determine aphid weight loss due to dehydration (PW_d) to correct the prey biomass ingested (BI) and assess aphid natural mortality. For this purpose, 10 *A. frangulae* apterous females were kept in plastic boxes for 12 hours. The initial and final weights of the aphids were recorded, as well as a count of remaining aphids alive at the end of the control test. The feeding method of *S. nubilus* larvae allowed easy identification of the preyed-upon aphids because they are sucked in rather than chewed (i.e. in contrast to larger aphidophagous ladybirds). Aphid prey may still be alive after being injured by predator feeding activity. Thus, in this study, the voracity was assessed not as the number of dead aphids but instead, as the number of predated aphids that were readily identifiable. The initial and final weights of the aphids (PW_i and PW_f, respectively) and predator larvae (LW_i and LW_f, respectively) in the consumption test (N=12) were recorded to determine the biomass ingested ($BI = PW_i - PW_f - PW_d$), relative growth rate (RGR: percent weight growth of predator compared to initial weight) and conversion efficiency (CE: percent weight growth of predator compared to biomass ingested) according to Borges (2008).

Field studies

Population dynamics of A. frangulae

The abundance of *A. frangulae* was recorded every week, from March to September, in the Nordeste county forestry nurseries in 2014 and 2015. Fifty 10 to 50 cm plants of *F. azorica* were randomly selected from all trays with potted plants, and carefully checked for the presence of the aphid *A. frangulae*. The number of aphids (observation dates) on each plant was recorded and a total value was calculated per sampling event. The infestation levels were calculated as the number of plants infested with aphids divided by the total number of plants observed. To determine the lifespan of *A. frangulae* colonies, ten colonies at an early stage of their development were selected and followed until their collapse. The number of aphids in each colony was recorded on a weekly basis.

In situ efficacy of S. nubilus against A. frangulae

In situ experiments were designed to assess the potential of the ladybird predator 4th larval instar to control *A. frangulae*. The experiments were done in the nurseries of the Forestry

Services of the Azores, in Nordeste county. Several colonies of *A. frangulae* of different size were selected. Four treatments were established for the following aphid colony typologies: i) aphid colony in a closed system using a sleeve cage (Insect Rearing Sleeve, Dimensions: L70 x W30 cm, Net Weight: 66 grams, Main Material: Woven Mesh | Nylon Mesh Size: 104 x 94 | 300 μm aperture), ii) aphid colony + predator larvae in a closed system using a sleeve cage, iii) aphid colony + predator larvae in an open system and iv) aphid colony in an open system (control). For each of the four treatments, the aphid density effect was further tested on the basis of the results obtained in the consumption tests under laboratory conditions. The number of larvae released in each aphid colony aimed to ensure the following aphid/larvae ratios: i) low predator density (LPD) i.e., one predator larva per number of aphids required for the development of the predator 4th larval instar ($N_{aphids/larvae}$ = 60), ii) high predator density (HPD) e.g. one predator larva per number of aphids consumed in 24 hours ($N_{aphids/larvae}$ = 15) and iii) medium predator density (MPD) e.g. one predator larva per an intermediate number of aphids used in the LPD and HPD treatments ($N_{aphids/larvae}$ = 30). There were 10 replicates for each of the 12 treatments. The aphid colonies were tagged, and the number of aphids were counted (observation dates) after 3 and 10 days. Twelve-hour old predator larvae in the 4th instar were obtained as described in section 1.2.2 and similarly starved for 12 hours prior to being used in the field trials. The larvae were transported to the Forestry Services and inoculated carefully into the plants infested with aphids, using a fine brush.

Statistical analysis

To compare female and male weights, after normality of data was confirmed by the Kolmogorov-Smirnov test, the student t-test was performed assuming non-homogeneous variances. To compare the variation of aphid colony size (%) for the three predator/prey densities (LPD, MPD and HPD) and aphid colony typology, the Kruskal-Wallis nonparametric ANOVA was used. Pairwise multiple comparisons, using the Mann-Whitney U-test, were performed applying the Bonferroni correction. Mean values were considered significantly different when $P < 0.05$. The values shown next to the means are the standard errors. SPSS v. 25 was used to perform the statistical analyses (IBM Corp 2017).

Results

Laboratory experiments

Development and reproduction

Scymnus nubilus larvae fed *ad libitum* with *A. frangulae* took 9.0±0.2 days to develop from the 4th instar to the adult stage, and the duration of the 4th larval stage was 4 days. Female ladybirds were heavier than males (1.29±0.06 mg vs. 1.06±0.03 mg) (t-test: $t=3.849$; $df=17.2$; $p<0.05$). The proportion of males obtained was slightly higher than that of females (F:M=43%). Adult females took 5.5±0.3 days to start oviposition and laid an average of 135±12 eggs in the first 15 days of oviposition. Most of the eggs were fertile (84.9%) but 2.7% of the larvae failed to successfully hatch.

Feeding parameters

The feeding parameters of *S. nubilus* larvae fed *A. frangulae* are presented in Table I. In 24 hours, larvae ingested 15.1±1.0 aphids which corresponded to 1.52 ± 0.04 mg of prey biomass ingested that, in turn, allowed larvae to increase their weight by approximately 50%.

Field studies

Population dynamics of A. frangulae

There was considerable variation in the abundance of *A. frangulae* between years. In 2015, an outbreak was observed in July, whereas in 2014 aphid population levels remained at lower levels (Fig. 1). Over the course of this study, an average of 19.8% of *F. azorica* plants were infested with *A. frangulae* and the aphid colonies lasted for 5.7±0.7 weeks.

In situ efficacy of S. nubilus against A. frangulae

Predator larval impact on aphid colonies tended to increase with predator density and was more pronounced after 3 days in closed systems (Fig. 2). It was in the open systems that higher levels of aphid suppression were observed. Except for the HPD treatment after 10 days (HPD, after 10 days: $\chi^2 = 9.245$, df = 3, p = 0.026) no significant differences were detected between treatments (HPD, after 3 days: $\chi^2 = 7.688$, df = 3, p = 0.053; MPD, after 3 days: $\chi^2 = 4.398$, df = 3, p = 0.222; MPD, after 10 days: $\chi^2 = 6.148$, df = 3, p = 0.105; LPD, after 3 days: $\chi^2 = 1.534$, df = 3, p = 0.674; LPD, after 10 days: $\chi^2 = 2.025$, df = 3, p = 0.567) (Fig. 2). Pairwise multiple comparisons between treatments of high predator density (HPD), after 10 days, reveals significant differences between aphid colony size variation (%) of the closed system without predator larvae and the open system with predator larvae (U = 14.2, p = 0.035). No significant differences were found between the other treatments (open system with predator larvae *vs* open system without predator larvae: U = 5.18, p = 1; open system with predator larvae *vs* closed system with predator larvae: U = 2.13, p = 0.198; open system without predator larvae *vs* closed system with predator larvae: U = 1.28, p = 1; open system without predator larvae *vs* closed system without predator larvae: U = 1.90, p = 0.344 and closed system with predator larvae *vs* closed system without predator larvae: U = -0.62, p = 1) (Fig. 2).

Discussion

Our study provides evidence of the potential of *S. nubilus* as a natural enemy against *A. frangulae*, under an inoculative/inundative IPM strategy. The most remarkable thing is that these two species, with worldwide distributions, have been the subject of few studies concerning their biology and ecology, especially in forest systems. Aphid pest outbreaks compromise production of the endemic plants used for native habitat restoration. *Aphis frangulae* showed considerable population density oscillation between years. The outbreak of *A. frangulae* was more evident in 2015 than 2014, although, even in 2014 we observed severe damage to the young shoots of the host plant and the presence of mold covering the leaves. The population dynamics and lifespan of *A. frangulae* colonies follows the shooting of the young leaves. Aphid colony growth was typically sigmoidal and successful colonies were those that survived initial stages of slow growth to reach the exponential growth phase. This occurs with other aphid species (Borges et al. 2011; Dixon 2000), including *Aphis spiraecola* Patch and *Cinara juniperi* (De Geer) (Hemiptera: Aphididae) infesting, respectively, *Viburnum treleasei* Gand. (Dipsacales: Adroxaceae), and *Juniperus brevifolia* (Seub.) Antoine (Pinales: Cupressaceae), both endemic plants reared in the same forestry nurseries (Rosagro et al. 2020). The lifespan of the colonies of *A. frangulae* was 5.7±0.7 weeks, similar to that of *A. spiraecola* and lower than that of *C. juniperi* (Rosagro 2020). These lifespans are similar to those of other successful aphid colonies which, in general, stand for 6–8 weeks (Dixon 1998).

The results of this study indicate that *A. frangulae* is an essential prey (sensu Hodek and Evans 2012) for *S. nubilus* and therefore the predator has the potential to be used in pest management programs. *Aphis frangulae* allowed predator 4th instar larvae to successfully complete development and adults to reproduce. It is not possible to assert that the aphid is equally suitable as a food source for all immature stages, as we did not provide the first three larval stages of *S. nubilus* with *A. frangulae*. However, once our goal was achieved to propose this natural enemy for use within the scope of an inundative or inoculative strategy, we opted to mass rear the first larval stages under laboratory conditions on *A. fabae*, a suitable aphid already tested, and then to release the 4th stage in the forestry nurseries. Rosagro et al (2020) studied two other aphid pests present in the forestry nurseries as food resource for *S. nubilus*, i.e., *A. spiraecola* and *C. juniperi*. With the former, development from the 4th larval stage to the adult stage took 7.8±0.2 days, females weighed 1.19±0.03 mg, and males weighed 0.99±0.02 mg, whereas with the latter the values were 8.2±0.2 days, 1.24±0.03 mg and 0.92±0.03 mg, respectively. On either of these diets, predator fertility in the first 15 days of oviposition was much lower than with *A. frangulae*. Thus, considering the aphid species available for the ladybird predator in the forestry nurseries, *A. frangulae* seems to be the most suitable prey. On *Rhopalosiphum padi* (L.) (Hemiptera: Aphididae) (Borges et al. 2013), an aphid species that *S. nubilus* is commonly associated with in Azorean corn field stands, the ladybird took a similar time to complete development from the 4th larval instar to adult, i.e., 8.5±0.1 days, but female adult weight was slightly higher (males: 1.05±0.02 and females: 1.37±0.03). Pre-oviposition time was slightly shorter, at 4.9±0.3 days and females laid considerably more eggs, 185.1±13.4 eggs.

Although the voracity of *S. nubilus* is much lower than that of larger ladybird species, its predation effect on aphid colonies can be enhanced firstly by arriving at earlier stages of prey colony development, that is, with reduced aphid densities and, secondly, by extending their predation effect in older colonies during the declining phase. Our results indicate that a single 4th instar larva can eat 15 *A. frangulae* per day. On *R. padi*, the predator larvae consumed fewer prey items, 8.7±0.7 aphids (due to aphid species larger size) but ingested a similar amount of prey biomass, 1.7±0.2 mg (Borges 2008).

Larvae gained 0.45±0.06 mg with a feeding efficiency of 25.8±2.06 %, values lower than the ones obtained with *A. frangulae*.

To our knowledge, there are no experimental studies on the potential of *Scymnus* spp against pests of forestry systems in Europe, but they are recognized as useful natural enemies (Wermelinger 2021). Our *in situ* experiments reveal that *S. nubilus* can play an important role in these systems, at least in forestry nurseries. The results of our experiments showed that after approximately one week, predator larvae pupated and in the absence of predation pressure, the aphid colonies increased their size, mainly in the closed systems, that is, in the treatments where sleeves were used. As field experiments did not extend after the emergence of the adults, it is not possible to ascertain if *S. nubilus* would be an efficient predator in an *A. frangulae* outbreak.

Pest control levels in open systems were higher than initially expected. This result highlights the important role of the aphidophagous guild present in forestry nurseries. However, the control of aphid populations in the nurseries can be supplemented by additional natural enemies. Indeed, during our field work we have recorded other natural enemies with the potential to be used in IPM programs, namely *Aphidius colemani* Viereck, *Binodoxys angelicae* (Halliday) (Hymenoptera: Braconidae), *Aphidoletes aphidimyza* (Rondani) (Diptera: Cecidomyiidae), and also syrphids that contribute to natural control of aphid pests. Thus, further studies focusing on different biological control approaches (inundative or inoculative), and on biological interactions among aphidophagous guild members, are required to more accurately assess the potential role of *S. nubilus* as a biological control agent against *A. frangulae*.

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Table 1. Feeding parameters of *S. nubilus* 4th instar larvae fed *A. frangulae*.

Parameter	Mean ± SE
Larval weight $_{initial}$ (mg)	1.25 ± 0.06
Larval weight $_{final}$ (mg)	1.83 ± 0.07
Weight gain (mg)	0.58 ± 0.06
Voracity (aphids consumed)	15.09 ± 0.27
Biomass ingested (mg)	1.52 ± 0.04
Relative growth rate (%)	48.35 ± 1.85
Conversion efficiency (%)	40.25 ± 1.04

Fig. 1. Population dynamics of *A. frangulae* at Nordeste county nursery in 2014 and 2015. Total abundance (A) and mean number of aphids (± SE) of *A. frangulae* recorded on 50 plants of *F. azorica*.

Fig. 2. Aphid colony size variation (mean percentage ± SE) in open and closed systems, with or without predator larvae at low (LPD), medium (MPD) and high (HPD) predator/prey densities after 3 and 10 days. For each treatment, different letters on the upper part of the histograms indicate significant differences ($P < 0.05$).

Fig. 1

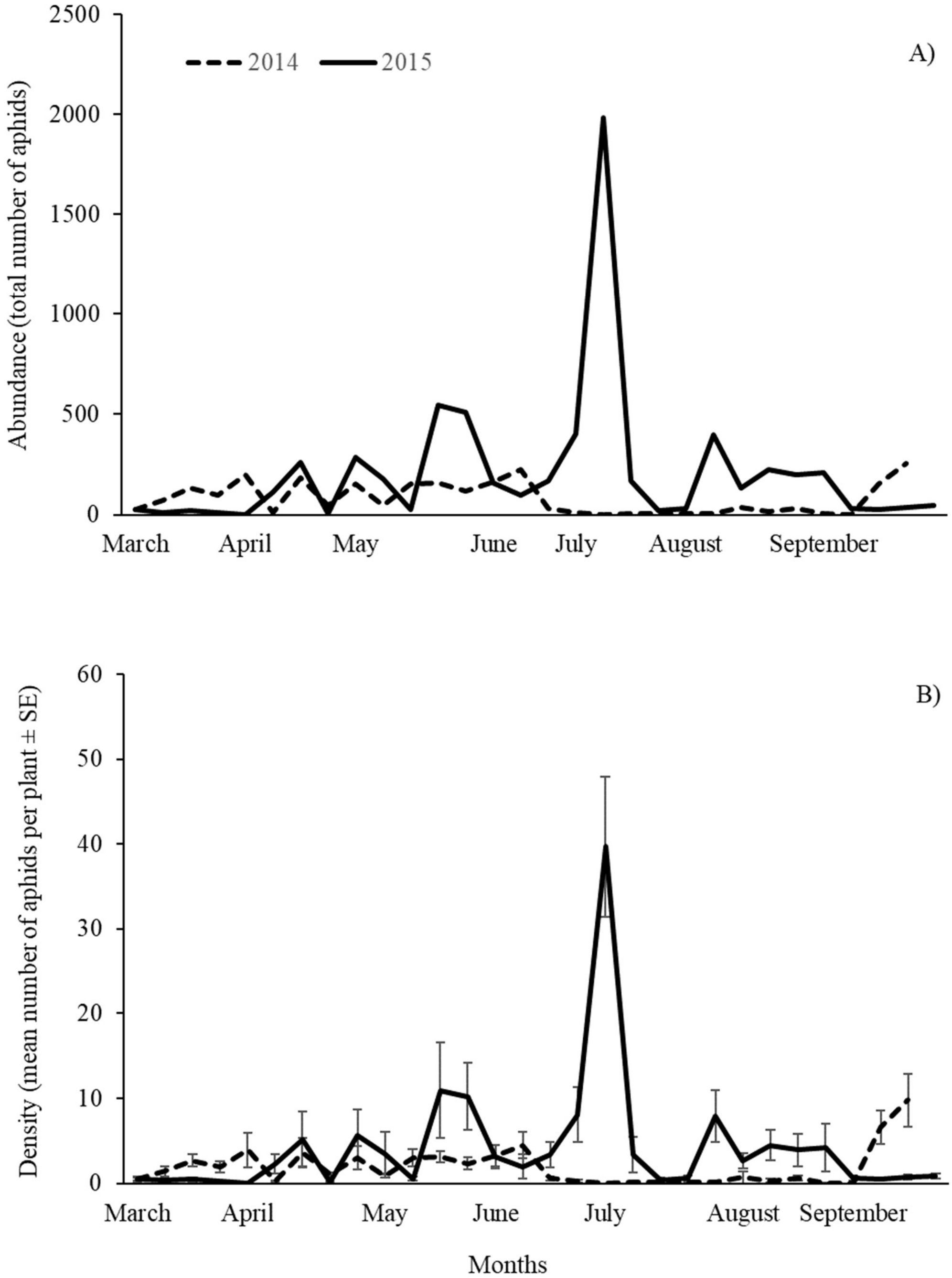
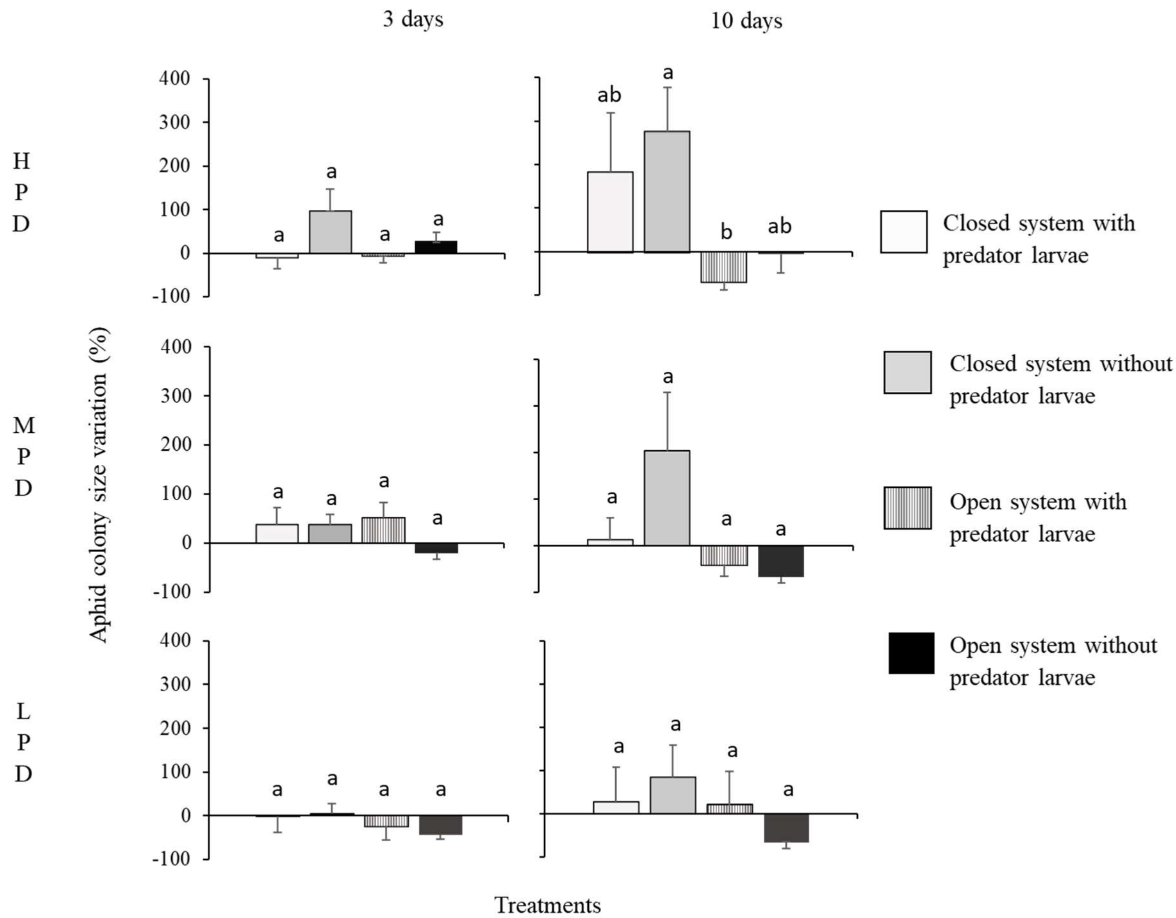

2014
2015
A)
Abundance (total number of aphids)
0
500
1000
1500
2000
2500
March
April
May
June
July
August
September
B)
Density (mean number of aphids per plant ± SE)
0
10
20
30
40
50
60
March
April
May
June
July
August
September
Months

Fig. 2


3 days
10 days
H P D
M P D
L P D
Aphid colony size variation (%)
400
300
200
100
0
-100
a
ab
b
Closed system with predator larvae
Closed system without predator larvae
Open system with predator larvae
Open system without predator larvae
Treatments