Effect of multi-specific releases of *Scymnus nubilus* Mulsant (Coleoptera: Coccinellidae) and *Aphidius colemani* Viereck (Hymenoptera: Braconidae) on biological control of *Myzus persicae* Sulzer (Hemiptera: Aphididae) populations

Dissertação de Mestrado

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Mestrado em
Biodiversidade e Biotecnologia

SICUT AURORA SCIENTIA LUCET
UNIVERSIDADE DOS AÇORES

Ponta Delgada
2020

Efeito das interações multiespecíficas de *Scymnus nubilus* Mulsant (Coleoptera: Coccinellidae) e *Aphidius colemani* Viereck (Hymenoptera: Braconidae) no controlo biológico de populações de *Myzus persicae* Sulzer (Hemiptera: Aphididae)

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Dissertação de Mestrado submetida como requisito parcial para obtenção do grau de
Mestre em Biodiversidade e Biotecnologia

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Master Thesis

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Master's Dissertation submitted as a partial requirement to obtain the
Master degree in Biodiversity and Biotechnology

Acknowledgments

I would like to express my deepest thanks to my supervisors, Professor António Onofre Costa Miranda Soares and Doctor Isabel Marisa Mateus Borges for all the help, support and guidance provided to me throughout this process, but especially in these last months of work.

To my co-supervisor Professor William E. Snyder for his suggestions in carrying out this work.

To Manuel Fernando Almeida, laboratory technician for his work in sowing the plants used in the experiments.

To my father and sister and the rest of the family for their understanding due to my absence and constant support.

Also to my best friends Andreia and Francisco for their support because even far away, they were always close.

And last but not least, to my guardian angel, whom I had the privilege of calling my mother and who I know has given me immense strength to achieve it!

Thank you all from the heart!

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Resumo

Na Região Autónoma dos Açores, a Direção Regional dos Recursos Florestais é responsável pela produção em massa de plantas endémicas da Macaronésia com a finalidade de restaurar habitats naturais. No entanto, as plantas produzidas nos viveiros florestais são negativamente impactadas pela presença de afídeos.

Os afídeos (Hemiptera: Aphididae) são, do ponto de vista fitossanitário, dos mais importantes problemas, dado que são capazes de afetar sistemas florestais e agrícolas, pois vivem em colónias de elevadas densidades, infligindo aos seus hospedeiros diversos efeitos, quer diretos quer indiretos. Com este estudo pretendemos contribuir, para o controlo biológico daquelas populações, avaliaando até que ponto as interações bióticas entre *Scymnus nubilus* (Coleoptera: Coccinellidae) e *Aphidius colemani* Viereck (Hymenoptera: Braconidae), numa estratégia de largadas multiespecíficas, poderão gerar impacto positivos, ou negativos, no controlo de populações de afídeos, em particular sobre *Myzus persicae* Sulzer como modelo biológico.

Assim, foram realizados trabalhos laboratoriais para avaliar (i) o efeito das interações bióticas entre fêmeas adultas de *A. colemani* e o 4º instar larvar de *S. nubilus* e (ii) a preferência alimentar do 4º instar larvar de *S. nubilus* entre afídeos parasitados vs não parasitados.

Os nossos resultados indicam que *S. nubilus* poder-se-á constituir como um eficiente agente de controlo biológico, especialmente em tratamentos conspecíficos, dado que a larva consome em média 50 ninfas de *M. persicae* em 24h. Nos testes interespecíficos verificou-se que *S. nubilus* interfere com o nível de parasitismo e demonstrou consumir afídeos parasitados e/ou interferência na atividade parasitária. Nos testes de preferência alimentar de *S. nubilus*, este demonstrou uma preferência tendencial por afídeos parasitados, o que pode dever-se à menor mobilidade e capacidade de defesa dos afídeos parasitados.

Será importante num futuro próximo estudar estas interações em condições de campo.

Palavras-chave: controlo biológico, *S. nubilus*, *A. colemani*, interações bióticas, predação intraguilde, preferência alimentar.

Abstract

In the Azores, the Regional Directorate for Forestry Resources is responsible for the mass production of Macaronesia endemic plants in nurseries conditions, which are later used in restoration programs of native forestery habitats. However, almost of the plants species in the nurseries are heavily infested by aphid species affecting negatively their hosts.

The aphids (Hemiptera: Aphididae), from the phytosanitary point of view, major problem, capable of affecting forest and agricultural systems because they live in colonies densely populated, inflicting direct and direct and indirect damages on their hosts. With this study we intend to explore the extent to which it is possible to implement a program of biological control to control aphids populations under a multi-specific release of two biological control agents, and using *Myzus persicae* Sulzer as target model. To this purpose, we evaluate the effects of biotic interactions between *Scymnus nubilus* (Coleoptera: Coccinellidae) and *Aphidius colemani* Viereck (Hymenoptera: Braconidae).

This study has two major objectives; to assess (i) the effect of biotic interactions in intraspecific and interspecific treatments with 4th larval stage of *S. nubilus* and females of *A. colemani* and (ii) feeding preference of parasitized and non-parasitized aphids of *M. persicae* by 4th larval instar *S. nubilus*.

Our results indicate that *S. nubilus* may be a suitable biological control agent, especially in conspecific treatments with two predators and a prey density of 80 aphis per larvae, once they consumed on average 50 nymph of *M. persicae* in 24h.

In heterospecific tests, it was found that *S. nubilus* interferes with the level of parasitism and has shown to consume parasitized aphids and / or interfere with parasitic activity.

In the food preference tests of *S. nubilus*, we showed a tendency towards parasitized aphids, which may be due to the lesser mobility and defense capacity of the parasitized aphids.

Keywords: biological control, *S. nubilus*, *A. colemani*, biotic interactions, intraguild predation, food preference.

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1. Introduction

The Azorean Forestry Services are responsible for the production of about 80,000 endemic plants for reforestation of the endangered native Azorean Laurisilva forest and is only allowed to use the fungicide compound SCORE 250 EC (difenoconazole 250 g / L) to meet the certification requirements. In this condition, the plants are vulnerable to infestation by several species of aphids. Indeed, the Regional Directorate of Forestry Resources was concerned about the levels of aphid infestation in a particular plant species, *Frangula azorica* because the Services lacked alternatives to chemical control measures. After contacts with the University of the Azores, it came to the knowledge of the Forestry Services that Biological Control could be an option. Thus, to promote the biological control of aphids present in the endemic plants produced in the Forestry nurseries, a collaborative research project was designed to meet this goal, PICA. The results obtained in this first project, in particular the high biodiversity of aphid pests and biological control agents found in the forestry nurseries, represented only the first step of a research line evolving to new projects, PICA II and PICONIA. The present master thesis is integrated in the latter project and focuses on the biotic interactions between biological control agents targeting the same aphid pest.

1.1 Aphid pests and their economic importance

According to van Emdem and Harrington (2007), aphids are considered to be one of the most important phytophagous insects in the field of agriculture and forestry, mainly for their reproductive capacity and parthenogenetic dispersion, thus becoming pests when their populations present themselves with high abundance values (Teulon and Stufkens, 2002; Messing *et al.* 2007).

When these feed on the phloem of plants through their oral appendices (Vilcinskas, 2016), about 50% of viruses are transmitted to plants (Ng and Perry, 2004; Stufkens and Teulon, 2001).

Significant economic losses occur due to the damage caused by many species of aphids in forest trees, fruits and ornamental plantations (Stufkens and Teulon, 2003; Sopow *et al.* 2017). Considered one of the most important pests of agricultural and horticultural crops in the world, the aphid *Myzus persicae* Sulzer (Hemiptera: Aphididae), green in color, is an example of a pest capable of transmitting more than 100 viruses and attacking approximately 40 plant families (van Emden and Harrington, 2007).

It has the ability to hibernate when it is in the egg state on peach, its main host or to maintain itself throughout the year in potatoes, beets, brassicas, cereals, peas, grazing clovers, tomatoes, among many other secondary hosts. parthenogenetically (Cameron and Fletcher, 2005).

Alikhani *et al*. (2013) states that causes such as yellowing of premature death of leaves, stunting, leaf ripple, twisting of shoots are some of the damages caused by aphids. The growth of fungi on the leaf surface occurs directly and indirectly when the injection of toxic salivary secretion is done during feeding and molasses secretion.

Integrated Pest Management (IPM) is a practice found for the maximum use of biological control agents and cultural practices for handling pests and diseases with the appropriate use of selective pesticides (Horne and Page, 2008). The need to develop and implement new ways of handling is urgent since alternatives to insecticide applications are needed (Cameron and Fletcher, 2005).

This need arises from the constant use of insecticides that have already provoked the development of resistance to phytopharmaceutical products by aphids (Martin, 2005).

Silva *et al.* (2012) and Earlham Institute, (2017) prove that *M. persicae* already resists about 70 types of synthetic chemical pesticides.

1.2 Biological control of aphids

Several predators and parasitoids eat aphids (Hosseinzadeh *et al.* 2017). An example of this are spiders (Sunderland et al. 1986), coccinellids (Honěk and Hodek, 1996), crisopids (Principi and Canard, 1984), hoverflies (Gilbert, 1986), cecidomyiid mosquitoes (Madahi *et al.* 2013), and parasitoids (Starý, 1970).

Predators, parasitoids and entomopathogens are fundamental natural enemies for the control of aphids (Hajek and Leger, 1994; Völkl *et al.* 2007). When the success rates of parasitoids (21.8% n = 193) and predators (4, 1%, n = 221) were compared, parasitoids were more effective (Hirose, 2006). Some genera of parasitoids of the order Hymenoptera and the Braconidae and Aphelinidae families are the most used in biological control programs (Boivin *et al.* 2012). *Aphidius*, *Praon*, *Diaeretiella*, *Trioxys* and *Ephedrus* (Wei *et al.* 2005) are some examples, respectively. Jones *et al.* (2003) states that the use of parasitoid wasps for IPM programs has requested greater attention.

The use of pesticides has a negative effect on the environment, reducing the potential for biological control, but in Portugal there is an Integrated Pest Management Directive (Directive nº. 256/2009) that requires extensive knowledge of culture and enemies, as well as the various factors that contribute to their harmfulness (biotic, abiotic, cultural and economic) in order to properly estimate the risk arising from the presence of these enemies. For biodiversity to be restored in Europe and opportunities for crop production to be created using biodiversity-based ecosystem services, such as biological pest control, there must be a shift across Europe so that agriculture uses minimal pesticides in large areas (Geiger et al. 2010). Thus, biological control through natural enemies becomes an important component in Integrated Pest Management (Honěk and Hodek, 1996; Atlihan and Kaydan, 2010).

Normally, biological control programs use only one control agent, but some studies have shown that combining different functional groups helps in cumulative control (Snyder et al. 2003).

Rabb (1974) claims that it is necessary to understand the biological interactions between natural enemies that intervene to increase the effect of natural enemies on a population of prey.

Before being used in biological control programs, Hosseinzadeh *et al.* (2017) states that it is necessary that the effectiveness of natural enemies be measured.

Studies by Straub *et al*. (2008) indicate that the conservation of the wealth of natural enemy species sometimes weakens or has no effect on biological control. The idiosyncratic mix of positive, negative and neutral effects of enemy diversity is caused by niche complementarity, intraguild predation and functional redundancy, respectively.

According to Hall *et al*. (1980) and Phillips *et al*. (2008), approximately 20% of new biological control projects are able to provide partial or complete biological control of invasive pests when classic biological control programs are conducted on the introduction of new enemy species in regions where they are not native.

For Snyder et al. (2003) the characteristics that define a Biological Control Agent (BCA) are its specificity in relation to the prey, its short development time in relation to the prey and its high reproductive capacity.

Previous research projects results have shown that *Scymnus nubilus* Mulsant is a potential biological control agent of aphids (Borges *et al*. 2011, 2013, Meseguer *et al*. 2019). *S.*

nubilus is a small coccinellid that is abundant and widespread across the Azorean archipelago. Biological and behavioral characteristics of ladybirds such as polyphagia, high voracity and numerical and aggregative responses allow them to be considered an important biological control agent (Obrycki and Kring, 1998; Dixon, 2000 and Hodek *et al*. 2012). However, its reduced food requirements allow the ladybird to arrive sooner to aphid colonies in earlier developmental stages, as well as keep on exploiting them at the colonies declining phase contributing to controlling aphis population densities. *A. colemani* has already been found in the nurseries of this Forest park.

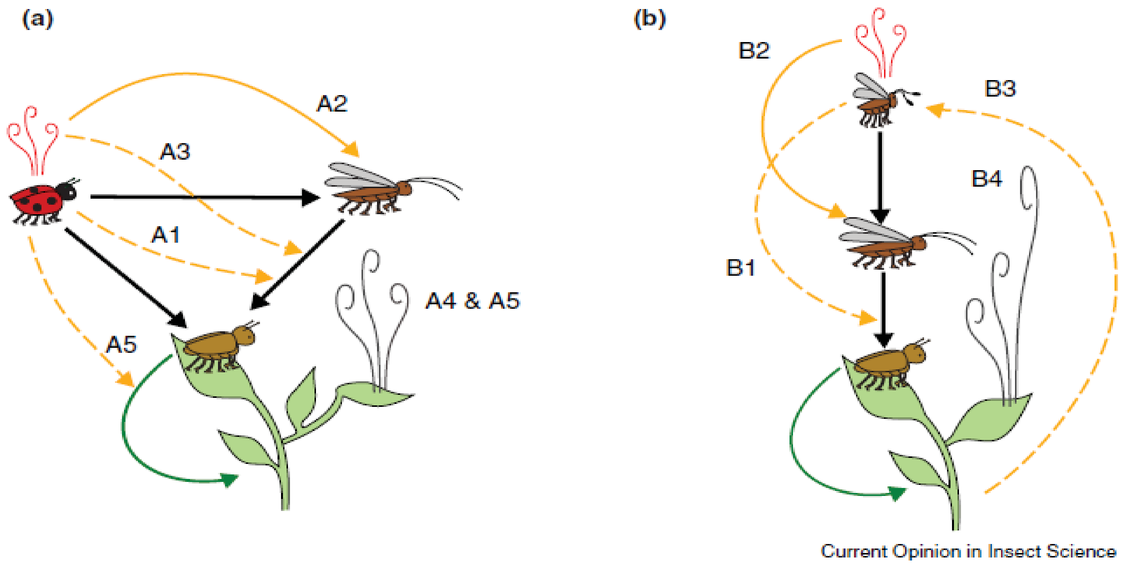


Figure 1. Parasitoids often engage in antagonistic interactions with higher order natural enemies like (a) intraguild predators (b) hyperparasitoids.

1.3 Biotic interactions among Biological Control Agents

Different species of aphids can be found in different densities, providing different types of effects to their biological control agents. These can be intraguild predation, competition, mutualism, and commensalism. Intraguild predation occurs when the predatory species has benefits over the other species while it suffers losses.

Competition occurs whenever both species involved suffer losses. Mutualism arises when both species benefit from interaction and commensalism occurs when one species has benefits, and the other does not suffer any kind of reaction. However, the type of response of predators, when confronted with different relative abundances of non-parasitized and parasitized prey, is not usually known.

1.4 Objective

Thus, the use of *S. nubilus* will be continued, with the objective of using *A. colemani* simultaneously as a biological control agent. To this end, intra-specific treatments with *S. nubilus*, intra-specific treatments with *A. colemani* and inter-specific treatments between *S. nubilus* and *A. colemani* will be carried out.

According to Soares *et al*. (2004), predatory males were able to demonstrate a constant feeding preference for *M. persicae* since predatory females showed no preference. This type of response is called "null switching". With increasing *M. persicae* density, and under a mixed regime, adult voracity gradually increased, thus not affecting the biomass consumed nor the relative growth rate. The parameters of fertility and fertility have increased. The predators are also able to effect antiswitching, that is, before two different prey, they consume the prey that is in lower densities.

1.5 Biological models

1.5.1 *Aphidius colemani* Viereck (Hymenoptera: Braconidae)

A. colemani (Hymenoptera, Braconidae, Aphidiinae) described for the first time in 1912, is one parasitoid important in the control of *M. persicae* (Mani and Krishnamoorthy, 1994;

Kos *et al.* 2008; Acheampong *et al.* 2012). Able to parasitize other aphid species economically important (Starý, 2002), this parasitoid is a solitary and koinobiont endoparasitoid (Khatri, 2017). The idiobiont/koinobiont spectrum is often used as a surrogate for whether a parasitoid of unknown biology is a generalist or specialist (Quicke, 2014). It is currently commercially produced for the control of aphids in Europe, North America (Fernandez and Nentwig, 1997) and New Zealand (Teulon *et al*. 2008; Bioforce, 2017) because of its great parasitism capacity (van Steenis, 1995).

A. colemani is a 2-3 mm long, slender wasp with a black thorax and head and yellowish-brown legs and abdomen. A female has pointed abdomen with an ovipositor (Figure 2).

The Figure 3 and 4 are two *M. persicae* parasitized with *A. colemani* with different ages (3 and 4 days, respectily.

Figure 2. Female adult of *A. Colemani*

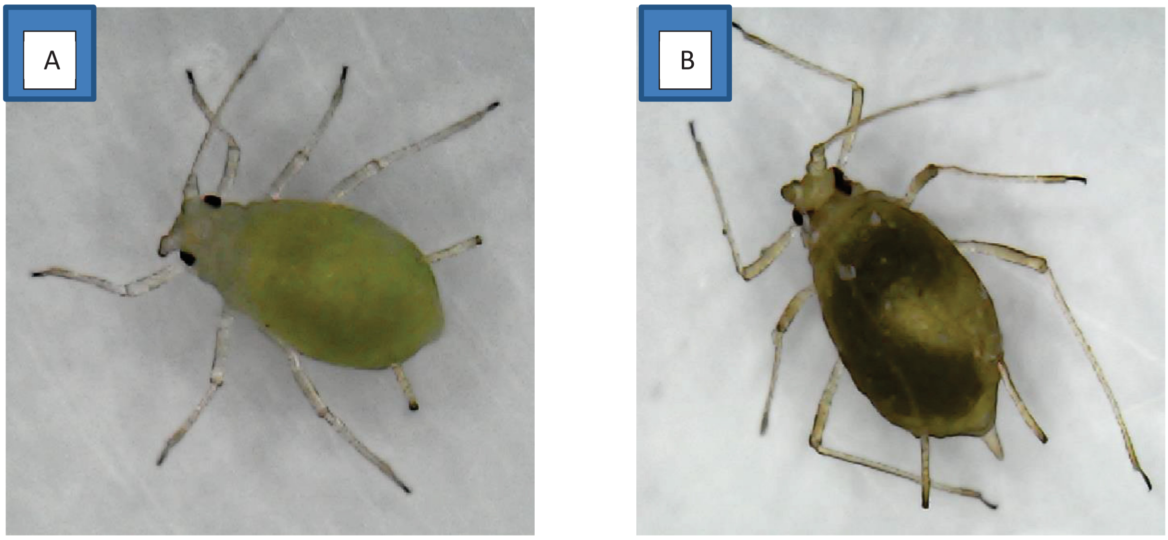


Figure 3. ***M. persicae*** **parasitized with** ***A. colemani*** **with 3 days (A) and with 4 days (B).**

1.5.2 *Scymnus nubilus* Mulsant (Coleoptera: Coccinellidae)

According to Pacheco, (2011), this ladybug is found on the islands of the Azores Archipelago. It is also found in southern Europe, Africa, in the Atlantic islands east of Japan and Micronesia (Canepari, 2011) and in Iran and Yemen (Yazdani, 1990, Raimundo and van Harten, 2000, Raimundo et al. 2006, 2008). *S. nubilus* is a small brown aphidophagous ladybug. *Scymnus* are able to explore aphid colonies at an earlier stage than larger ladybugs (Agarwala and Yasuda, 2001) because larvae of this species are covered by a thick layer of wax produced by dorsal epidermal cells (Agarwala and Yasuda, 2001) providing a defensive morphological mechanism.

Figure 4. Female of ***S. nubilus***

2. Materials and methods

2.1 Laboratory insect population

All insect species used in these experiments were mass reared in the laboratory under optimal conditions, 25 ± 1 ^{o}C, 75 ± 5% RH and a 16L: 8D light regime under fluorescent lamps.

Scymnus nubilus adults were collected in the field. In the laboratory, a stock population was established in rearing cages covered with mousseline fabric. The ladybird predator was fed a mixed diet of *Aphis fabae* Scopoli (Hemiptera: Aphididae) and *M. persicae* infesting *Vicia faba* L. (Fabales: Fabaceae) plants. The diet was complemented with honey diluted in water and pollen. When necessary new infested plants were provided.

APHIPAR was purchased to Koppert. Emerged parasitoid adults were transferred to BugDorm rearing cages with a potted *V. faba* plant infested with *M. persicae*. Diet was complemented with honey diluted in water provided as little droplets in the top lid. As new generations emerged, new infested plants were provided.

2.2 Biotic interaction in intraspecific and interspecific treatments with *S. nubilus* and *A. colemani*

The methodology used in this task will be adapted from Northfield *et al*., 2010. For this purpose, three 24-hours laboratory single species and multi-species treatments will be performed: (i) tests with *A. colemani* adult females (parasitoid);(ii) tests with *S. nubilus* 4th larval stage (predator) and (iii) test with *A. colemani* adult females (parasitoid) and *S. nubilus* 4th larval stage (predator) simultaneously, each with 4 parasitoid/predator densities levels: 1, 2, 4 and 6 in a total of 12 experimental treatments. Eight treatments will allow assessing the impact of intraspecific interactions and the remaining four will allow assessing the impact of interspecific interactions of biological control agents on aphid consumption or parasitism (Table 1). The number of aphids provided in the treatments corresponds to enough prey/host to meet the daily consumption/parasitism rates for the highest density treatment (Meseguer *et al.* 2019; Khatri, 2017). Each treatment will be replicated 15 times.

Table 1. Density of parasitoid *A. colemani* (adult females) and predator *S. nubilus* (4th larval stage) in the 12 experimental treatments designed to assess biotic interactions.

Single-species tests (parasitoid)	Single-species tests (predator)	Multi-species tests (parasitoid+predator)
1	1	1+1= 2
2	2	2+2= 4
4	4	4+4=8
6	6	6+6= 12

It was decided to use *M. persicae* 2nd instar as prey/host because it is the preferred development stage for *A. colemani* (Katri, 2017) whereas the predator does not have preference for any development stage. To obtain the second instar of *M. persicae*, approximately 200 adult apterous females were placed on potted *V. faba* plants for 24 hours and removed in the next day. After 3 days, the aphid nymphs molt into the 2nd instar (Katri, 2017) and were collected to be used in the experiments.

To carry out these tests, a single plant of *V. faba* with approximately 15 cm, seeded inside a vase (8 cm Ø X 11cm height) was placed inside an acrylic box (25 X 18 X 9,5 cm) bearing a hole covered with fine metallic mesh to allow ventilation. One hundred and sixty 2nd instar *M. persicae* were transferred to the plant and to this experimental device it was added the predator 4th instar larvae, adult female parasitoid or both species in the 4 density levels pre-defined according to the treatment to be performed. After 24 hours, the predators and/or parasitoids were removed, and the number of aphids consumed were determined. Preyed aphids are readily recognized because *S. nubilus* larvae feeds by piercing the aphid tegument and sucking up the body fluids leaving the prey deformed. It is noteworthy, however, that a preyed aphid may not be dead. To determine the number of parasitized aphids, the experimental device was maintained until the formation of aphid mummies. The number of emerged parasitoids was also registered.

2.3 Feeding preference of parasitized and non-parasitized aphids by 4th larval instar *S. nubilus*

To assess the effect of aphid parasitism on *S. nubilus* 4th larval instar consumption, preference tests were performed. *Scymnus nubilus* adults were paired in plastic boxes (5 cm Ø x 3 cm height) to obtain eggs. Newly hatched larvae were transferred individually into new boxes and fed a single diet of *M. persicae* until reaching the 4th larval stage. To register the development stage, larvae were observed twice a day, at 9:00 and 17:00 with fresh food being provided daily. Newly molted 4th larval stage predators were allowed to feed for 12 hours and starved for the next 12 hours prior to the experiments. The experimental devices were mounted as previously described. One hundred and sixty aphids with a varying proportion of parasitized prey was put on a *V. faba* plant, according to table 1 (adapted from Soares *et al*. 2004). One *S. nubilus* 4th larval stage was added to the system for 24 hours. The number of preyed aphids was determined, and the remnant aphids were kept for 3 days to easily identify the parasitized and non-parasitized aphids. Non-parasitized 2nd instars of *M. persicae* were obtained as previously mentioned. To obtain parasitized aphids, they were exposed to *A. colemani* females for 24 hours in rearing cages. In the next day, the parasitoids were removed, and the experimental device was kept for 3 days to be possible to identify the parasitized aphids which become turgid, shiny and more immobile.

A preliminary test was conducted to assess the efficiency of identifying parasitized aphids. Ten replicates with 10 aphids identified as parasitized after 3 days of exposure to *A. colemani* female were kept over one *V. faba* leaf in a plastic box (10 cm Ø x 3 cm height) with a hole covered with fine metallic mesh for ventilation. After approximately 3 days, the number of aphid mummies was registered to calculate the success rate in identifying parasitized hosts.

While searching for prey, ladybird predators may find aphid colonies in which a proportion of aphids are parasitized. In this case, the success of predators in a multi-specific biological control program will depend on preference by non- parasitized aphids and by this way keeping the parasitoid fitness intact. Predators often show a preference for one of them and the predator's response is strongly influenced by the relative abundance of the two prey species offered (Murdoch 1969; Cock 1978; Sherratt and Harvey 1993). For this reason, it will be recommendable to test feeding preference with different relative abundance of prey. A predator can show one of the four types of responses; (i) a constant preference for one prey species, (ii) no preference, when the ratio of consumed prey is equal to the ratio of prey individuals in the environment (i.e., null switching) (Chesson

1984), (iii) a switching behaviour, when the predator eats disproportionably more of the more abundant prey (Murdoch 1969) and (iv) an anti-switching behaviour, when the predator eats disproportionably more of the less abundant prey (Chesson, 1984). To do this, three different ratios of parasitized aphids and non-parasitized aphids were offered to S*. nubilus* 4th larval stage (Table 2.), in an experimental set up as previously described. The number aphids eaten in 24 h was recorded. A minimum of 10 replicates was conducted per treatment.

Table 2. Percentage of parasitized and non-parasitized aphids to be provided to S*. nubilus* 4th larval stage in the preference tests.

Number of predators	% of parasitized aphid (nº of aphids)	% of non-parasitized aphid (nº of aphids)
1	0%	100% (160)
1	30% (48)	70% (112)
1	50% (80)	50% (80)
1	70% (112)	30% (48)
1	100% (160)	0%

2.4 Statistical analysis

We assessed the potential effect of biotic interactions on the effectiveness of biological control by quantifying the total and per capita aphid consumption/parasitism under the following factors (independent variables): i) biocontrol agent treatments [S. nubilus (Sn), A. colemani (Ac), S. nubilus (Sn) + A. colemani (Ac)] and ii) prey density (from low - to high prey/host density). Total and per capita aphid consumption / parasitism (dependent variables) were analysed using generalized linear models (GLMZ). Normal distributions of data were assessed by the Kolmogorov-Smirnov test. When error distribution of the dependent variables was confirmed, GLMZ analyses considered the normal error distribution and the identity link function. In the absence normal error distribution, we used Poisson error distribution and the log link function. Factor and interactions were analysed using the Wald Chi-Square test for a confidence level of 95%. Pairwise multi comparisons were performed and P values corrected using Bonferroni test. All means in text and figures were calculated with untransformed data and followed by 1 standard error.

In the heterospecific treatments [*S. nubilus* (Sn) + *A. colemani* (Ac)], we access the relative contribution of predation and parasitism on the effectiveness of biological control of aphid populations. We quantify and compare the ratio between the proportion of aphids consumed by *S. nubilus* and the proportion of mummified aphids (dependent variables), in function of prey density (independent variable: 1 Sn + 1 AC, 2 Sn + 2 AC, 4 Sn + 4 AC, 6 Sn + 6 AC. The ratios were analysed using general linear models (GLMZ). We applied a non-parametric test to independent samples, contrasted by a Kruskal-Wallis test. Pairwise multi comparisons were performed and P values corrected using Bonferroni test.

We evaluated the potential effect of biotic interactions on the fitness of parasitoids. We quantified the total and per capita number of parasitoids emerged and the rate of emergence (dependent variables) under the following factors (independent variables): i) biocontrol agent treatments [*A. colemani* (Ac), *S. nubilus* (Sn) + *A. colemani* (Ac)] and ii) prey density (from low - to high prey/host density) using generalized linear models (GLMZ). Normal distributions of data were assessed by a Kolmogorov-Smirnov test. Only two set of data were not normal and for this reason were normalized using a log function. The GLMZ analyses considered the normal error distribution and the identity link function. Factor and interactions were analysed using the Wald Chi-Square test for a confidence level of 95%. Pairwise multi comparisons were performed and P values corrected using Bonferroni test. All means in text and figures were calculated with untransformed data, and followed by 1 standard error.

Feeding preference was assessed using Chesson's preference Index (Chesson, 1978):

$$\alpha 1 = \frac{Ln\frac{(n1 - r1)}{n1}}{Ln\frac{(n1 - r1)}{n1} + Ln\frac{(n2 - r2)}{n2}}$$

where n_1 and n_2 = number of *M. persicae* non-parasitized and parasitized and r_1 and r_2 = number of *M. persicae* non-parasitized and parasitized eaten in 24 h by the predators. Wilcoxon's matches-pairs signed rank tests (WMPSR) was used to compare feeding preference of S. *nubilus* 4th larval stage on the ratio of parasitized aphid/non-parasitized aphid.

All statistical treatments were performed in SPSS 27.

3. Results

Total aphid consumption/parasitism

When analysing the total number of aphids consumed in 24h, we found a significant effect of both factors; biocontrol agent treatments (Wald Chi-Square = 1120.1, df = 2, P ≤ 0.0001) and prey/host density (Wald Chi-Square = 606.6, df = 3, P ≤ 0.0001). Moreover, there was a significant effect between the independent variables (Wald Chi-Square = 343.3, df = 6, P ≤ 0.0001) (Table 3). Concerning biocontrol agents, there is no significant differences between total aphid consumption by S. nubilus (75.4 ± 1.1) and A. colemani (74.9 ± 1.1), but the total aphid consumed by S. nubilus + A. colemani (127.6 ± 1.4) is significantly higher (Figure 5; Table 3).

In relation to prey/host density, the total aphids consumed/parasitized differs significantly. Average consumption in the lowest density (D1: one single natural enemy in the conspecific treatments or two individuals in the heterospecific S. nubilus + A. colemani treatment) (60.1 ± 1.2) differs significantly from the other higher densities (D2: 101 ± 1.5, D3: 105.6 ± 1.5 and D4: 100.9 ± 1.5). No significant differences were found between the three remaining aphid densities (Figure 5; Table 3).

On the conspecific treatments with A. colemani and heterospecific treatments, we found a significant trend towards an increase in the number of aphid consumed as the number of natural enemies increased. In the conspecific treatments with S. nubilus, on the other hand, the trend is reversed, that is, the number of aphid consumed decreases as the number of natural enemies increases, especially after a density of two individuals (Figure 5; Table 3).

Table 3. GLMZ results on the total aphid consumption/parasitism in the biocontrol agent treatments ([*S. nubilus* (Sn), *A. colemani* (Ac), *S. nubilus* (Sn) + *A. colemani* (Ac)] and prey density (from low - to high prey/host density).

	Wald Chi-Square	df	Sig.
Biocontrol agent treatments	1120.1	2	≤0.0001
Prey density	606.6	3	≤0.0001
Biocontrol agent treatments * Prey density	343.3	6	≤0.0001

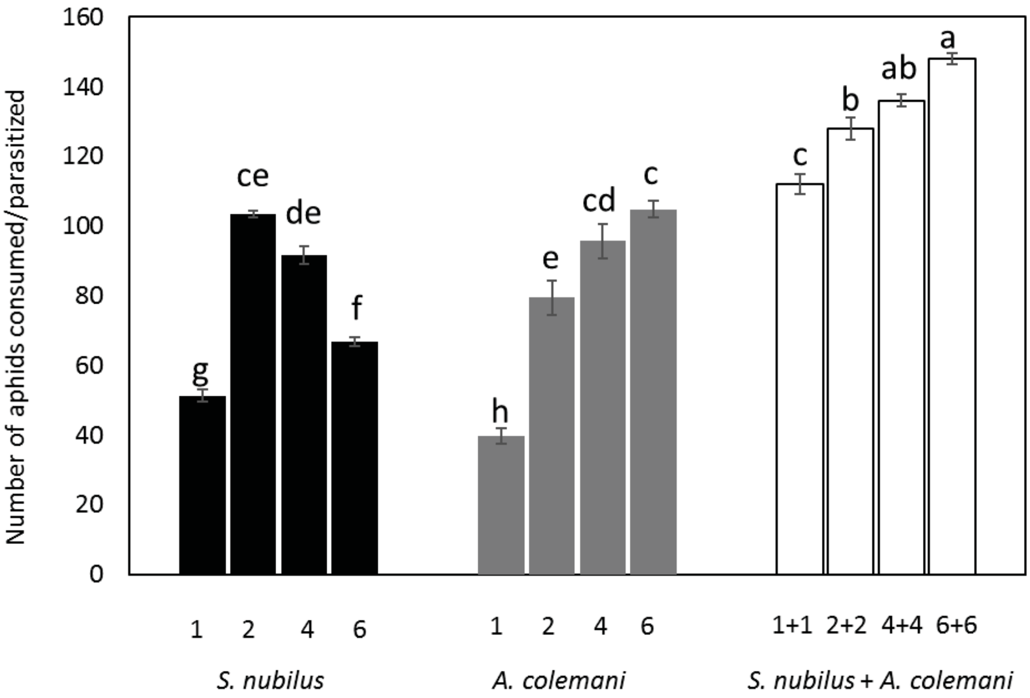


Figure 5. Number aphids consumed/parasitized in 24h (mean number of aphids eaten in 24h ± SE) by S. nubilus, A. colemani and S. nubilus +A. colemani. The numbers 1, 2, 4 and 6 means number of biological control agents used in each conspecific experiments and 1+1, 2+2, 4+4 and 6+6 means number of S. nubilus + A. colemani used in each heterospecific experiments.

Per capita aphid consumption/parasitism

When analysing the per capita aphid consumption/parasitism, we found a significant effect of the both factors; biocontrol agent treatments (Wald Chi-Square = 25.4, df = 2, P ≤ 0.0001) and prey/host density (Wald Chi-Square = 1019.1, df = 3, P ≤ 0.0001). Moreover, there was a significant effect between the independent variables (Wald Chi-Square = 123.6, df = 6, P ≤ 0.0001) (Table 4). Concerning biocontrol agents, there is a significant difference between all treatments, being higher in the S. nubilus (28.9 ± 0.8), followed by A. colemani (28.6 ± 0.7) and S. nubilus + A. colemani treatment (24.3 ± 0.6) (Figure 6; Table 4).

In relation to prey/host density, the per capita aphid consumed/parasitized differs significantly between all treatments, being higher in D1 (47.7 ± 1.0), followed by D2 (40.2

± 0.9), D3 (21.1 ± 0.7) and D4 (13.5 ± 0.6) (Figure 6, Table 4).

For all biocontrol agents treatments, the number aphid consumed/parasitized per capita in 24h decreased significantly with prey density (Figure 6; Table 4).

Table 4. GLMZ results on the aphid consumption/parasitism per capita of biocontrol agent treatments ([S. nubilus (Sn), A. colemani (Ac), S. nubilus (Sn) + A. colemani (Ac)] and prey density (from low - to high prey density).

	Wald Chi-Square	df	Sig.
Biocontrol agent treatments	25.4	2	≤0.0001
Prey density	1019.1	3	≤0.0001
Biocontrol agent treatments * Prey density	123.6	6	≤0.0001

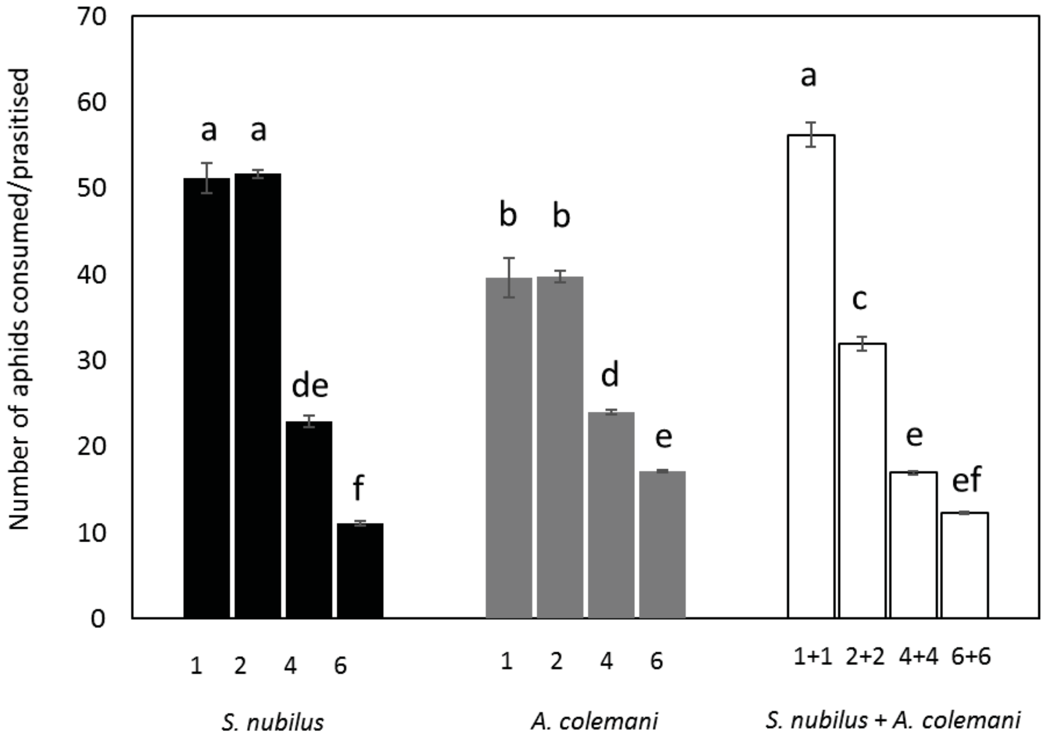


Figure 6. Number of aphids consumed/parasitized per capita in 24h (mean number of aphids eaten in 24h ± SE) by S. nubilus, A. colemani and S. nubilus +A. colemani. The number 1, 2, 4 and 6 means number of biological control agents used in each conspecific experiments and 1+1, 2+2, 4+4 and 6+6 means number of S. nubilus +A. colemani used in each heterospecific experiments.

Proportion of aphids consumed by *S. nubilus* to the proportion aphids mummified

We found that, as the prey density increases, the difference between the proportions of prey consumed in relation to the proportion of mummified aphids becomes greater (Table 5). The ratio between the previous proportion differs significantly (Kruskal-Wallis, P ≤ 0.0001) (Figure 7). These results suggest that the contribution of *S. nubilus* is increasing by increasing predation on parasitized aphids and/or by decreasing the parasitism capacity of *A. colemani* through interference competition (Kruskal-Wallis, P ≤ 0.0001).

Table 5. Proportion of aphids consumed vs aphids mummified in the heterospecific treatments, in function of the prey density.

Treatments	Proportion of aphids consumed	Proportion of aphids mummified
1 Sn + 1 AC	57.1 ± 1.6	42.9 ± 1.6
2 Sn + 2 AC	68.1 ± 0.7	31.9 ± 0.7
4 Sn + 4 AC	79.9 ± 0.9	29.1 ± 0.9
6 Sn + 6 AC	87.4 ± 0.6	12.6 ± 0.6

Legend: Sn = *S. nubilus* and Ac = *A. colemani*

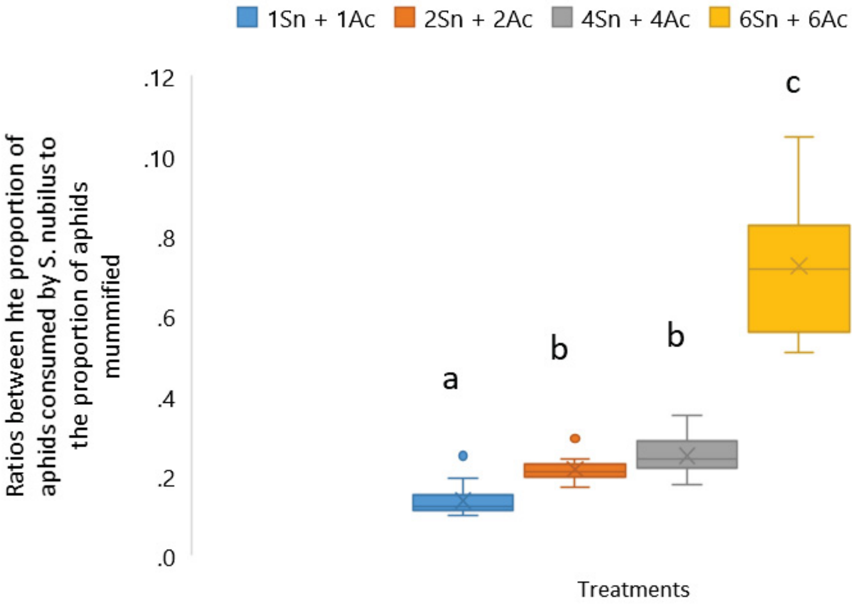


Figure 7. Ratio between the proportion of aphids consumed by S. nubilus to the proportion aphids mummified, in function of prey density (Treatments: 1 Sn + 1 AC, 2 Sn + 2 AC, 4 Sn + 4 AC, 6 Sn + 6 AC).

Effect of biotic interactions on the fitness of *A. colemani*

Total number of parasitoid emerged. When analysing the total number of parasitoid emerged, we found a significant effect of both factors; biocontrol agent treatments (Wald Chi-Square = 1458.9, df = 1, P ≤ 0.0001) and prey/host density (Wald Chi-Square = 156.2, df = 3, P ≤ 0.0001). Moreover, there was a significant effect between the independent variables (Wald Chi-Square = 212.2, df = 3, P ≤ 0.0001) (Table 6). Concerning biocontrol agents, there is a significant difference between total number of parasitoids emerged in the *A. colemani* (60.4 ± 3.8) and S. nubilus + A. colemani (18.6 ± 1.1) treatments (Figure 8A; Table 6). In relation to prey/host density as a whole, the number of parasitoids emerged significantly differs. It was lower in the D1 density (one single natural enemy in the conspecific treatments or two individuals in the heterospecific *S. nubilus* + *A. colemani* treatment) (18.3 ± 0.7) and no significant differences were found between the remaining aphid densities (D2: 43.3 ± 4.8, D3: 46.9 ± 1.2 and D4: 49.6 ± 0.6) (Figure 8A; Table 6). On the conspecific treatments with *A. colemani*, we found a significant trend towards an increase in the number of parasitoids emerged as the number of natural enemies increase. In the heterospecific treatments, the trend is similar except in the last biological control agent densities (Figure 8A).

Number of parasitoids emerged per capita. When analysing the total number of parasitoids emerged per capita, we found a significant effect of both factors; biocontrol agent treatments (Wald Chi-Square = 1212.3, df = 1, P ≤ 0.0001) and prey/host density

(Wald Chi-Square = 440.3, df = 3, P ≤ 0.0001). Moreover, there was a significant effect between the independent variables (Wald Chi-Square = 212.2, df = 3, P ≤ 0.0001) (Table 7). Concerning biocontrol agents, there is a significant difference in the number of parasitoids emerged per capita the between *A. colemani* (21.1 ± 1.3) and S. nubilus + *A. colemani* (4.5 ± 0.4) treatments (Figure 8B; Table 7). In relation to prey/host density as a whole, the number of parasitoids emerged significantly differs. It was significantly higher in the D2 density (two single natural enemy in the conspecific treatments or four individuals in the heterospecific *S. nubilus* + *A. colemani* treatment) (18.8 ± 2.8) and significant differences were found with the remaining aphid densities (D1: 14.3 ± 0.2, D3: 10.1 ± 0.2 and D4: 7.8 ± 0.05). No significant differences were found between D3 and D4 densities (Figure 8B; Table 7). On the conspecific treatments with *A. colemani*, we found an initial and significant increase in the number of parasitoids emerged, followed by a decrease in the two remaining densities, among which there are no significant differences. (Figure 8B). In the heterospecific treatments, we found a significant trend towards a decrease in the number of parasitoids emerged as the number of natural enemies increase. (Figure 8B).

Rate of emergence of the parasitoid. When analysing the rate of emergence of the parasitoid, we found a significant effect of both factors; biocontrol agent treatments (Wald Chi-Square = 1335.3, df = 1, P ≤ 0.0001) and prey/host density (Wald Chi-Square = 61.9, df = 3, P ≤ 0.0001). Moreover, there was a significant effect between the independent variables (Wald Chi-Square = 103.7, df = 3, P ≤ 0.0001) (Table 8). Concerning biocontrol agents, there is a significant difference between total of parasitoids emerged in the *A. colemani* (0.71 ± 0.022) and *S. nubilus* + *A. colemani* (0.15 ± 0.008) treatments (Figure 8C;

Table 8). In relation to prey/host density as a whole, the rate of emergence of parasitoids significantly differs. It was significantly lower in the D1 density (one single natural enemy in the conspecific treatments or two individuals in the heterospecific *S. nubilus* + *A. colemani* treatment) (0.33 ± 0.006) and significant differences were found between the emergency rates between the remaining aphid densities (D2: 0.49 ± 0.008, D3: 0.44 ± 0.008 and D4: 0.46 ± 0.004) (Figure 8C; Table 8). On the conspecific treatments with *A. colemani*, we found an initial and significant increase in the number of parasitoid emerged, followed by a stabilization in the remaining densities, among which there are no significant differences. (Figure 8C). In the heterospecific treatments, the trend is similar except in the last biological control agent densities (Figure 8C).

Table 6. GLMZ results on total number of parasitoids emerged in the biocontrol agent treatments ([A. colemani (Ac), S. nubilus (Sn) + A. colemani (Ac)] and prey density (from low - to high prey density).

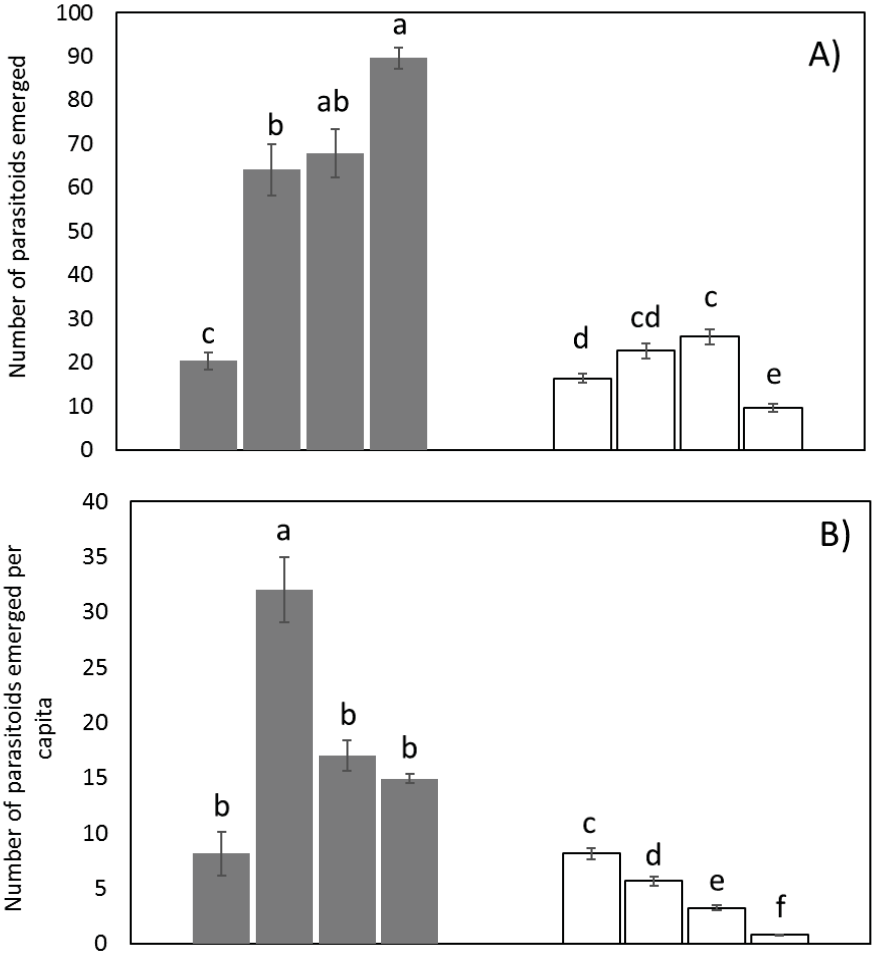
	Wald Chi-Square	df	Sig.
Biocontrol agent treatments	458.9	1	≤0.0001
Prey density	156.2	3	≤0.0001
Biocontrol agent treatments * Prey density	212.2	3	≤0.0001

Table 7. GLMZ results on the per capita number of parasitoids emerged of biocontrol agent treatments ([A. colemani (Ac), S. nubilus (Sn) + A. colemani (Ac)] and prey density (from low - to high prey/host density).

	Wald Chi-Square	df	Sig.
Biocontrol agent treatments	1212.3	1	≤0.0001
Prey density	440.3	3	≤0.0001
Biocontrol agent treatments * Prey density	212.2	3	≤0.0001

Table 8. GLMZ results on the effects of rate of emergence of parasitoids of biocontrol agent treatments ([A. colemani (Ac), S. nubilus (Sn) + A. colemani (Ac)] and prey density (from low - to high prey/host density).

	Wald Chi-Square	df	Sig.
Biocontrol agent treatments	1335.3	1	≤0.0001
Prey density	61.9	3	≤0.0001
Biocontrol agent treatments * Prey density	103.7	3	≤0.0001



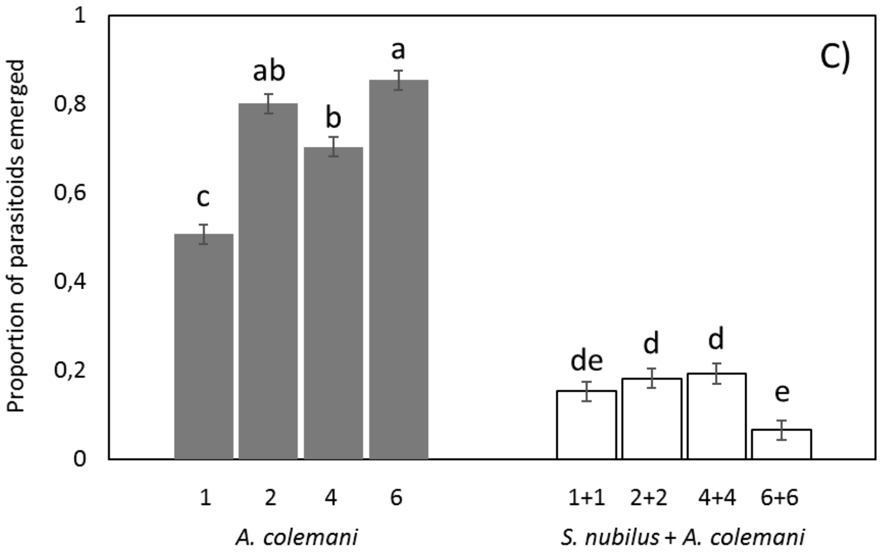


Figure 8. Total number of parasitoid emerged (A), number of parasitoids emerged per capita (B) and rate of emergence (C). The number 1, 2, 4 and 6 means number of biological control agents used in the conspecific experiments with A. colemani and 1+1, 2+2, 4+4 and 6+6 means number of S. nubilus +A. colemani used in each heterospecific experiments.

Feeding preference

4th larval stage of *S. nubilus* show a feeding preference for parasitized aphids on the 70 non-parasitized M. persicae/30 parasitized M. persicae (WMPSR; Z = 2.497, P = 0.013), and 50 non-parasitized M. persicae/50 parasitized M. persicae (WMPSR; Z = 2.803, P = 0.005) ratios. The preference was not statistically significant in the treatment of 30 non-parasitized M. persicae/70 parasitized M. persicae 70 (WMPSR; Z = 1.376, P = 0.169) (Figure 9).

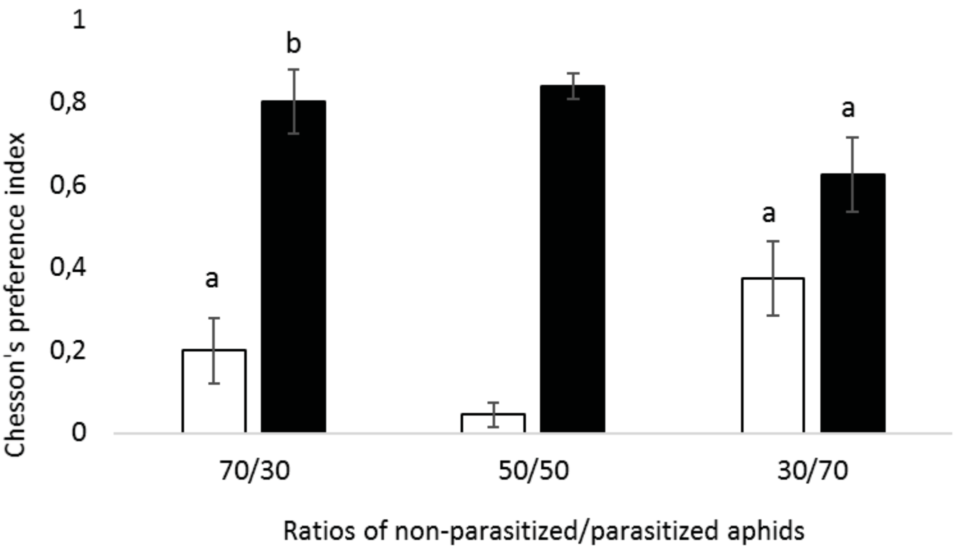


Figure 9. Chesson's preference index (mean ± se) 4th larval stage of S. nubilus fed on three different ratios of non-parasitized and parasitized M. persicae by A. colemani (70 non-parasitized M. persicae/30 parasitized M. persicae, 50 non-parasitized M. persicae/50 parasitized M. persicae and 30 non-parasitized M. persicae/70 parasitized M. persicae). Different letters indicate significant differences (Wilcoxon WMPSR test; $p < 0.05$).

4. Discussion

Forestry nurseries produces Macaronesian endemic plants face major problems with plant aphid infestation. These phytophagous insects cause great damage because the use of pesticides is limited by the certification requirements.

Individually, the aphid control potential is larger for the predator, however at higher densities the aphid consumption drops significantly. For the parasitoid, there is an increasing trend in the number of aphids parasitized as the density of *A. colemani* increases. Our results indicate that when using simultaneously predators and parasitoids, the level of overall aphid control is higher although the per capita aphid consumption/parasitism decreases as the natural enemy density increases because intraguild predation occurs, and predators can feed other predators and parasitoids

(Snyder *et al*. 2003).

The parasitism of *M. persicae* by *A. colemani* did not render the prey unpalatable for the predator. Moreover, the ladybird showed a tendency to prefer parasitized aphids. This may have occurred because the parasitized aphids have less mobility, facilitating their consumption by S. nubilus. When we analyze the total number of emerged parasitoids obtained during biotic interactions in the fitness of A. colemani, we find that there are significant differences. ([*A. colemani* (60.4 ± 3.8)]) and ([*S. nubilus* + *A. colemani* (18.6 ± 1.1)]). The number of emerged parasitoids differed significantly when it comes to the prey/host density as a whole.

Its lowest density was found in the cospecific treatments or two in the heterospecific treatments of *S. nulibus* + *A.colemani*, with no further significant difference between the remaining aphid densities.

There was an increase in the number of parasitoids that emerged with the increase in the number of natural enemies in the co-specific treatment of *A. colemani*. In heterospecific treatments, the trend is similar, except in the densities of the last biological control agent. When the number of emerged parasitoids per capita is evaluated, a significant difference in the number of emerged parasitoids per capita is observed between the treatments of *A. colemani* (21.1 ± 1.3) and *S. nubilus* + *A. colemani* (4.5 ± 0.4). Regarding the density of prey/hosts as a whole, the number of emerged parasitoids differs significantly.

The density where there was a significantly greater difference was in the D2 density (two unique natural enemies in the cospecific treatments or four individuals in the heterospecific treatment of *S. nubilus* + *A. colemani* (18.8 ± 2.8).

There were no significant differences in the remaining aphid densities (D1: 14.3 ± 0.2, D3: 10.1 ± 0.2 and D4: 7.8 ± 0.05). There were also no significant differences between D3 and

D4 densities.

When it comes to co-specific treatments with *A. colemani*, there is also an initial and significant increase in the number of emerged parasitoids following a decrease in the following two densities, with no significant differences between them. In heterospecific treatments, the opposite was verified, that is, there was a significant decrease in the number of parasitoids that arose with the increase in the number of natural enemies.

Comparing the treatments of *A. colemani* (0.71 ± 0.022) and *S. nubilus* + *A. colemani* (0.15 ± 0.008), there is a significant difference between the total of emerged parasitoids.

There was an initial and significant increase in the number of emerged parasitoids, followed by a stabilization in the remaining densities between which there are no significant differences when observing the results obtained in the co-specific treatments with *A. colemani*. The same happens in heterospecific treatments, where there is an increase in the first three densities, with the exception of the last density (6 + 6).

When testing the feeding preference of *S. nubilus* for parasitized or non-parasitized aphids, it was found that the larvae of the 4 instar of this ladybug had a preference for parasitized aphids. This is possibly due to the fact that parasitized aphids exhibit greater immobility when compared to non-parasitized aphids. This preference was verified in the proportions of 70% of non-parasitized *M. persicae* / 30% of parasitized *M. persicae* and 50% of non-parasitized *M. persicae* / 50% of parasitized *M. persicae*, with no significant differences. The same was not verified in the proportions of 30% of non-parasitized *M. persicae* / 70% of parasitic *M. persicae*, where there were no significant differences.

These generalist predators are not restricted to feeding on pests but can also consume parasitoids, reducing the overall effect of pest elimination. (Traugott *et al*. 2012).

According to Brodeur and Rosenheim (2000), ingesting and killing another member of the guild provides energy to the intraguild predator. This act may have an influence on the reduction of potential competition for food and the risk of predation in cases of mutual IGP.

Polis and Holt (1992), Moran *et al*. (1996) and Holt and Polis (1997) claim that theoretical models and empirical evidence suggest that the PGI can lead to the spatial and temporal exclusion of intraguild predators, competitive coexistence or alternative stable states.

For the near future, it will be important to verify the interactions between these two biological control agents under field conditions.

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