Host-parasitoid relationships of *Anagyrus* sp. near *pseudococci* (Girault), (Hymenoptera, Encyrtidae), as a basis to improve biological control of pest mealybugs (Hemiptera, Pseudococcidae)

TESE APRESENTADA PARA OBTENÇÃO DO GRAU DE DOUTOR EM ENGENHARIA AGRONÓMICA

Abdalbaset Abusalah Ali Bugila

Orientador: Professor Doutor José Carlos Franco Santos Silva
Co-orientador: Professora Doutora Manuela Rodrigues Branco Simões

JÚRI:

Presidente: Reitor da Universidade de Lisboa

Vogais:

Doutora Laura Monteiro Torres
Professora Catedrática, Escola de Ciências Agrárias e Veterinárias da Universidade de Trás-os-Montes e Alto Douro

Doutor António Maria Marques Mexia
Professor Catedrático, Instituto Superior de Agronomia da Universidade de Lisboa

Doutor David João Horta Lopes
Professor Auxiliar com agregação, Universidade dos Açores;

Doutor José Carlos Franco Santos Silva
Professor Auxiliar, Instituto Superior de Agronomia da Universidade de Lisboa

Doutora Elisabete Tavares Lacerda de Figueiredo Oliveira
Professora Auxiliar, Instituto Superior de Agronomia da Universidade de Lisboa.

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Abstract

The host-parasitoid relationships of *Anagyrus* sp. nr. *pseudococci* were investigated, including host selection behavior, host defenses, host suitability and parasitoid functional response in relation to five mealybug species with different phylogenetic relationships and geographical origins: i) a Mediterranean native species, *Planococcus ficus*, with a long co-evolutionary history with the parasitoid; ii) three alien species, *Planococcus citri*, *Pseudococcus calceolariae* and *Pseudococcus viburni*, with a more recent co-evolutionary history; and iii) a fourth alien species, *Phenacoccus peruvianus*, with no previous common history with the parasitoid. The parasitoid recognized as potential hosts and complete development in all five mealybug species, but showed a clear preference for *Planococcus* spp. Host suitability of the studied mealybugs seems to fit a phylogenetic/biogeographic trend, showing the highest level in *Pl. ficus* and its closely related congener *Pl. citri*, followed by the Australasian *Ps. calcelolariae*, and the Neotropical *Ps. viburni* and *Ph. peruvianus*. The functional response of the parasitoid varied between host species, with a type II and type III responses observed for *Ps. calceolariae* and *Pl. ficus*, respectively. The results suggest that *A.* sp. nr. *pseudococci* has a broader host range and a more generalist behavior in comparison with other *Anagyrus* species.

Key-words: host selection, host defense, host suitability, functional response, biological control

Resumo

As relações parasitóide-hospedeiro de *Anagyrus* sp. próx. *pseudococci,* nomeadamente o comportamento de selecção do hospedeiro, as defesas do hospedeiro e sua adequação e a resposta funcional do parasitóide, foram estudadas em relação a cinco espécies de cochonilhas-algodão, com diferentes relações filogenéticas e origens geográficas: i) uma espécie nativa do Mediterrâneo, *Planococcus ficus,* com longa história co-evolutiva com o parasitóide; ii) três espécies exóticas, *Planococcus citri, Pseudococcus calceolariae* e *Pseudococcus viburni,* com história co-evolutiva mais recente; e iii) uma quarta espécie exótica, *Phenacoccus peruvianus*, sem relação evolutiva com o parasitóide. O parasitóide reconheceu como hospedeiros potenciais as cinco espécies de cochonilhas e em todas elas completou o desenvolvimento, mas evidenciou clara preferência por *Planococcus* spp. A adequação das espécies estudadas de cochonilhas como hospedeiros de *A.* sp. próx. *pseudococci* parece seguir um padrão fiologenético/biogeográfico, tendo evidenciado o nível mais elevado em *Pl. ficus* e *Pl. citri,* seguido da espécie de origem australiana, *Ps. calceolariae* e das duas espécies neotropicais, *Ps. viburni* e *Ph. peruvianus.* A resposta funcional do parasitóide variou entre hospedeiros, tendo-se observado uma resposta do tipo II e III em *Ps. calceolariae* e *Pl. ficus,* respectivamente. Os resultados sugerem que *A.* sp. próx. *pseudococci* apresenta maior leque de hospedeiros e comportamento mais generalista em comparação com outras espécies de *Anagyrus.*

Palavras-chave: selecção do hospedeiro, defesas do hospedeiro, adequação do hospedeiro, resposta funcional, luta biológica

1. Introduction

1.1. State of the art

Mealybugs (Hemiptera: Pseudococcidae) are soft-bodied piercing-sucking insects constituting the second largest family of scale insects (Hemiptera: Coccoidea), with more than 2000 described species (Ben-Dov, 1994; Downie & Gullan, 2004). About 160 species of mealybugs are recognized as pests worldwide (Miller, Miller, & Watson, 2002). Many of them are cosmopolitan species belonging to the genera *Planococcus*, *Pseudococcus* and *Phenacoccus*, such as the citrus mealybug *Planococcus citri* (Risso), the vine mealybug *Pl. ficus* (Signoret), the citrophilus mealybug *Pseudococcus calceolariae* (Maskell), the obscurus mealybug *Ps. viburni* (Signoret), and the bougainvillea mealybug *Phenacoccus peruvianus* Granara de Willink (Ben-Dov, 1994; Beltrà et al., 2010; Franco, Zada, & Mendel, 2009; Hardy, Gullan, & Hodgson, 2008). Mealybugs are notorious invaders because they are small insects, often live in hidden habitats, and frequently are transported on commodities that are common in international commerce (Miller et al., 2002). Damage originated by mealybugs is often linked to sap feeding, honeydew excretion and associated sooty mold development, toxin injection and virus transmission, including leaf yellowing, defoliation, reduced plant growth, and in some cases death of plants (Franco et al., 2009). For example, in Georgia in 1996, the estimated losses and cost of mealybugs amounted to $98,658,000 (Chong, Oetting, & Iersel, 2003).

Adult females of mealybugs are wingless, often elongate or oval, with about 0.4 to 0.8 mm in body length, resembling immature stages, whereas adult males are winged, short-lived, non-feeding and rarely seen insects (Kosztarab & Kozár, 1988). Females usually lay 100-400 eggs into a white, filamentous ovisac, which they secrete from glands in their cuticle (Cox & Pearce, 1983; Mckenzie, 1967). Typically, mealybugs reproduce sexually, but some species are parthenogenetic (Kosztarab & Kozár, 1988; McKenzie, 1967; Nur, 1977). Their life cycle includes five stages for females (egg - 1th instar - 2th instar - 3th instar - adult) and six stages for males (egg - 1th instar - 2th instar - prepupa - pupa - adult) (Chong et al., 2003; Mckenzie, 1967; Walton & Pringle, 2004). Mealybugs often complete several generations per year, depending on temperature, allowing a quick buildup of their populations (Franco et al., 2009).

Planococcus ficus is a major pest in many grapevine-growing regions in the world (Ben-Dov, 1994; Daane et al., 2006; Walton, Daane, & Pringle, 2004). *Planococcus citri*, *Ps. calceolariae* and *Ps. viburni* are polyphagous mealybugs with pest status on different crops, including citrus and ornamental plants (Ben-Dov, 1994; Franco, Suma, Silva, Blumberg & Mendel, 2004; Franco et al., 2009; Pellizzari & Germain, 2010). *Phenacoccus peruvianu*s is a major pest of *Bougainvillea* spp. (Beltrà et al., 2010). *Planococcus ficus* is considered native to the Mediterranean basin (Cox & Ben-Dov, 1986), whereas *Planococcus citri*, although of uncertain origin, is believed to be Afrotropical (Franco et al., 2008). *Pseudococcus calceolariae* is from Australasia (Pellizzari & Germain, 2010), and *Ps. viburni* and *Ph. peruvianus* are native to South America (Beltrà et al., 2010; Charles, 2011). According to Pellizzari and Germain (2010), *Pl. citri, Ps. calceolariae* and *Ps. viburni* arrived and established in Europe during the 19th century. However, studies on the biogeographic origin and molecular characterization of *Ps. viburni* suggest that its introduction in Europe may have occurred much earlier, in the 16th century (Charles, 2011; Correa, Germain, Malausa, & Zaviezi, 2012). This is possibly also the case for *Pl. citri* and *Ps. calceolariae*. Nevertheless, *Ph. peruvianu*s was only recently introduced into Europe (Beltrà et al., 2010).

Chemical control is still the most common control tactic used against mealybug pests. However, the cryptic behavior of mealybugs, their typical waxy body cover, and clumped spatial distribution pattern render the use of many insecticides ineffective. Repeated insecticide use, especially of broad-spectrum chemicals, also has adverse ecological and environmental impacts (Franco et al., 2009). Therefore, biological control has been considered an environmentally friendly alternative tactic to be used in integrated pest management strategies for the control of pest mealybugs (Franco et al., 2009).

The Encyrtidae are considered one of the six most successful families of Hymenoptera used in biological control programs. Within this family, the tribe Anagyrini consists mainly of mealybug primary endoparasitoids, including several species of the genus *Anagyrus*, which is the most successful Anagyrini genus used in biological control (Noyes & Hayat, 1994). *Anagyrus pseudococci* s.l. (i.e., *sensu latu*, corresponding to the references before Triapitsyn, González, Vickerman, Noyes, & White, 2007) is a koinobiont solitary endoparasitoid of mealybugs (Islam & Copland, 1997; Noyes & Hayat, 1994). It has been used as a biological control agent, especially against *Pl. citri* and *Pl. ficus* (Noyes & Hayat, 1994; Triapitsyn et al., 2007). About 24 mealybug species have been reported as hosts of *A. pseudococci* s.l., representing 11 different genera (Noyes & Hayat, 1994). However, some of these records are possibly erroneous. For example, Noyes and Hayat (1994) refer to records of *Rastrococcus iceryoides* (Green) and *Saccharicoccus sacchari* (Cockerell) as hosts of *A. pseudococci* s.l. are probably misidentifications. Recently, Triapitsyn et al. (2007) showed that *Anagyrus pseudococci* s.l. comprises two sibling, reproductively incompatible and genetically different species: *Anagyrus pseudococci* (Girault) and *Anagyrus* sp. nr. *pseudococci* (Girault). *Anagyrus pseudococci* is apparently restricted to Sicily, Argentina (introduced), and Cyprus, whereas *A.* sp. nr. *pseudococci* seems to be more widely distributed, since it has been recorded from many countries including Portugal, Spain, Italy, Greece, Israel, Turkmenistan, South Africa, Brazil and USA (Franco et al., 2011; Guerrieri & Pellizzari 2009; Karamaouna, Menounou, Stathas, & Avtzis, 2011; Mgocheki & Addison, 2009; Triapitsyn et al., 2007). Therefore, there is a need to study the biology of both parasitoid species, in order to further clarify the taxonomic status of *A.* sp. near *pseudococci,* as well as to support decision making about their use in biological control of pest mealybugs. A further understanding of their host-parasitoid relationships, in particular their host range is needed (Triapitsyn et al., 2007).

1.2. Objectives

In this research work, we aimed at studying the host-parasitoid relationships of *A.* sp. nr. *pseudococci* by investigating host selection behavior, host defenses, host suitability, and functional response in relation to host mealybugs of different geographical and phylogenetic origin, as a basis to further clarify the taxonomic status and biological traits of this parasitoid species, as well as to improve its effective use as a biological control agent of pest mealybugs. With that purpose, we selected five pest mealybug species from three different genera (*Planococcus, Pseudococcus,* and *Phenacoccus*) and two subfamilies (Pseudococcinae and Phenacoccinae) (Downie & Gullan, 2004; Hardy, Gullan, & Hodgson, 2008): i) *Pl. citri* and *Pl. ficus* (Pseudococcinae, Planococcini); ii) *Ps. calceolariae* and *Ps. viburni* (Pseudococcinae, Pseudococcini); and iii) *Ph. peruvianus* (Phenacoccinae). Although in the same subfamily, *Pseudococcus* is a distant genus in respect to *Planococcus,* belonging to a different tribe. *Phenacoccus* is even more phylogenetically distant from *Planococcus,* being part of a different subfamily (Downie & Gullan, 2005; Hardy et al., 2008). Therefore, the selected mealybug species are expected to present different evolutionary relationships with the parasitoid *A.* sp. nr. *pseudococci.* This parasitoid is considered to have a close evolutionary relationship with *Pl. ficus* (Franco et al., 2008, 2011). However, *Pl. citri, Ps. calceolariae* and *Ps. viburni* have been possibly in contact with *A.* sp. nr. *pseudococci* only for the last few centuries, at most. Finally, the lack of previous contact of the parasitoid with *Ph. peruvianu*s excludes any previous adaptation in this host-parasitoid system.

The specific objectives of this research were:

1. Comparing the host selection behavior of *A.* sp. nr. *pseudococci* among the select mealybug species, focusing on close range host location, host recognition, and host acceptance components (Chapter 2);
2. Analyzing differences on the host defense behavior and immune response of the selected mealybug species to the attack of *Anagyrus* sp. nr. *pseudococci* (Chapter 3);

3. Assessing host suitability of the selected mealybug species for the development of *A.* sp. nr. *pseudococci* based on different fitness parameters of the parasitoid, such as body size, development time, emergence rate, and sex ratio (Chapter 4);
4. Investigating the functional response of *A.* sp. nr. *pseudococci* and testing if it could be affected by the host species, depending on its evolutionary history (Chapter 5).

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2. Host selection behavior and specificity of the solitary parasitoid of mealybugs *Anagyrus* sp. nr. *pseudococci* (Girault) (Hymenoptera, Encyrtidae)

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Abstract

The host selection behavior of *Anagyrus* sp. nr. *pseudococci* was compared in no-choice tests among five mealybug species of different geographical and phylogenetic origin, including the Mediterranean native host, *Planococcus ficus*, and four exotic mealybug species, one of the same genus, *Pl. citri*, two *Pseudococcus* species, *Ps. calceolariae* and *Ps. viburni*, and a more distant one, *Phenacoccus peruvinaus*. All five studied mealybug species were recognized by the parasitoid as potential hosts and parasitized, but the behavioral pattern of host recognition, host handling and the level of host acceptance of *Anagyrus* sp. nr. *pseudococci* significantly varied among the five studied species, indicating a clear preference for the two *Planococcus* species, *Pl. ficus* in particular. The results suggest that *A.* sp. nr. *pseudococci* has a broader host range and a more generalist behavior in comparison with other *Anagyrus* species. Practical implications of the findings are discussed.

Key-words: parasitoids, mealybugs, foraging behavior, host range, handling time, biological control

2.1. Introduction

Parasitoids are the most important and successful group of natural enemies used in biological control of insect pests (Mills & Wajnberg, 2008; Noyes & Hayat, 1994). The behavioral ecology of parasitoids is critical for the success of biological control implementation, as the effective suppression of insect pests depends on the parasitoid behavioral decisions during host searching and acceptance (Mills & Wajnberg, 2008). In general, host selection behavior of parasitic Hymenoptera involves a series of steps, including host habitat location, host location, host recognition and host acceptance, each involving different kinds of cues (Vinson, 1998).

Considering that the fundamental host range of a parasitoid is expected to be largely influenced by parasitoid host selection process (Vinson, 1998), the study of behavioral aspects involved in parasitoid-host relationships is important for predicting parasitoid host range. Parasitoid host range has been attracting much attention from researchers and is considered a central question for both theoretical and applied reasons. Knowledge on host range is of critical importance to understanding the functioning and evolution of parasitoid communities (Shaw, 1994; Stireman & Singer, 2003a,b), as well as to assess the risk of non-target impacts of biological control of insect pests (Wajnberg, Scott & Quimby, 2001). Realized host range, i.e., the host species actually used by the parasitoid (Nechols, Kauffman & Schaefer, 1992) is traditionally obtained from literature. However, published host records are often unreliable, especially those from older literature, due to misidentification of parasitoid and/or host (Conti, Salerno, Bin, & Vinson, 2004; Hopper, 2001; Shaw, 1994). The realized host range of a parasitoid integrates its natural host range (in the area of origin of the parasitoid) and novel host range (in areas where it has been introduced) (Barratt et al., 2012), which are delimited by the fundamental host range, i.e. genetically defined (Nechols et al., 1992). The study of parasitoid behavior can also contribute to our knowledge on parasitoid taxonomy and co-evolution between parasitoids, their hosts and the plants the host lives on (van Alphen & Jervis, 1996).

Mealybugs (Hemiptera: Pseudococcidae) constitute the second largest family of scale insects (Hemiptera: Coccoidea), with more than 2000 described species (Ben-Dov, 1994; Downie & Gullan, 2004), of which about 160 species are recognized as pests worldwide (Miller, Miller, & Watson, 2002). Many of them are cosmopolitan species belonging to the genera *Planococcus, Pseudococcus* and *Phenacoccus* (Franco et al., 2009). Damage originated by mealybugs is often linked to sap feeding, honeydew excretion and associated sooty mold development, toxin injection and virus transmission (Franco, Zada, & Mendel, 2009).

The Encyrtidae are considered one of the six most successful families of Hymenoptera used in biological control programs. Within this family, the tribe Anagyrini consists mainly of mealybug primary endoparasitoids, including several species of the genus *Anagyrus*, which is the most successful Anagyrini genus used in biological control (Noyes & Hayat, 1994). *Anagyrus pseudococci* s.l. (i.e., *sensu latu,* corresponding to the references before Triapitsyn, González, Vickerman, Noyes, & White, 2007) is a koinobiont solitary endoparasitoid of mealybugs (Islam & Copland, 1997; Noyes & Hayat, 1994). It has been used as a biological control agent, especially against the citrus mealybug, *Planococcus citri* (Risso) and the vine mealybug, *Planococcus ficus* (Signoret) (Noyes & Hayat, 1994; Triapitsyn et al., 2007). About 24 mealybug species have been reported as hosts of *A. pseudococci* s.l., representing 11 different genera, namely *Antonina* (1 species), *Dysmicoccus* (1), *Maconellicoccus* (1), *Nipaecoccus* (2), *Peliococcus* (1), *Phenacoccus* (5), *Planococcoides* (1), *Planococcus* (3), *Pseudococcus* (7), *Rastrococcus* (1), and *Saccharicoccus* (1) (Noyes & Hayat, 1994). However, some of these records are possibly erroneous. For example, Noyes and Hayat (1994) refer to records of *Rastrococcus iceryoides* (Green) and *Saccharicoccus sacchari* (Cockerell) as hosts of *A. pseudococci* s.l. are probably misidentifications. Recently, Triapitsyn et al. (2007) showed that *Anagyrus pseudococci* s.l. comprises two sibling, reproductively incompatible and genetically different species: *Anagyrus pseudococci* (Girault) and *Anagyrus* sp. nr. *pseudococci* (Girault). *Anagyrus* sp. nr. *pseudococci* is apparently the more common of the two parasitoid species in the Mediterranean basin (Triapitsyn et al., 2007; Guerrieri & Pellizzari, 2009; Franco et al., 2011; Karamaouna, Menounou, Stathas & Avtzis, 2011). According to Triapitsyn et al. (2007), "the host range and host preference of both *A. pseudococci* and *A.* sp. nr. *pseudococci* need to be further investigated (…) in order to provide taxonomists with the information needed for further clarification of the taxonomic status of *A.* sp. nr. *pseudococci*, and also to help biological control practitioners make proper decisions about the use of both forms against the citrus, vine, and possibly other mealybugs (such as some *Pseudococcus* spp.)."

In the present study, we aimed at studying the host range of *A.* sp. nr. *pseudococci* by investigating its relationship with host mealybugs of different geographical and phylogenetic origin. With that purpose, we selected five pest mealybug species from three different genera (*Planococcus, Pseudococcus* and *Phenacoccus*) and two subfamilies (Pseudococcinae and Phenacoccinae) (Downie & Gullan, 2004; Hardy, Gullan, & Hodgson, 2008): i) *Pl. citri* and *Pl. ficus* (Pseudococcinae, Planococcini); ii) the citrophilus mealybug, *Pseudococcus calceolariae* (Maskell) and the obscure mealybug, *Pseudococcus viburni* (Signoret) (Pseudococcinae, Pseudococcini); and iii) the bougainvillea mealybug, *Phenacoccus peruvianus* Granara de

Willink (Phenacoccinae). Although in the same subfamily, *Pseudococcus* is a distant genus in respect to *Planococcus,* belonging to a different tribe. *Phenacoccus* is even more phylogenetically distant from *Planococcus,* being part of a different subfamily (Downie & Gullan, 2005; Hardy et al., 2008).

Except for *Pl. ficus*, which is considered native to the Mediterranean basin (Cox & Ben-Dov, 1986), all other selected mealybug species are alien, having different origin and history of invasion of the Mediterranean basin. *Planococcus citri* is believed to have Afrotropical origin (Franco et al., 2008), *Ps. calceolariae* is from Australasia (Pellizzari & Germain, 2010), and *Ps. viburni* and *Ph. peruvianus* are native to South America (Beltrà et al., 2010, Charles, 2011). *Planococcus ficus* is a major pest in many grapevine-growing regions in the world (Ben-Dov, 1994; Daane et al., 2006; Walton, Daane, & Pringle, 2004). *Planococcus citri, Ps. calceolariae* and *Ps. viburni* are cosmopolitan, polyphagous mealybugs with pest status on different crops, including citrus and ornamental plants (Ben-Dov, 1994; Franco, Suma, Silva, Blumberg, & Mendel, 2004; Franco et al., 2009; Pellizzari & Germain, 2010). *Phenacoccus peruvianu*s is a major pest of *Bougainvillea* spp. (Beltrà et al., 2010).

According to Pellizzari and Germain (2010), *Pl. citri, Ps. calceolariae* and *Ps. viburni* arrived and established in Europe during the 19th century. However, studies on the biogeographic origin and molecular characterization of *Ps. viburni* suggest that its introduction in Europe may have occurred much earlier, in the 16th century (Charles, 2011; Correa, Germain, Malausa, & Zaviezi, 2012). This is possibly also the case for the other two mealybug species. Nevertheless, *Ph. peruvianu*s was only recently introduced into Europe (Beltrà et al., 2010).

Therefore, the selected mealybug species are expected to present different evolutionary relationships with the parasitoid *A.* sp. nr. *pseudococci.* This encyrtid is considered to have a close evolutionary relationship with *Pl. ficus* (Franco et al., 2008, 2011). However, *Pl. citri, Ps. calceolariae* and *Ps. viburni* have been possibly in contact with *A.* sp. nr. *pseudococci* only for the last few centuries, at most. Finally, the lack of previous contact of the parasitoid with *Ph. peruvianu*s excludes any previous adaptation in this host-parasitoid system. As an experimental approach, we compared in no-choice tests the host selection behavior of *A.* sp. nr. *pseudococci* among the selected mealybug species, focusing on close range host location, host recognition, and host acceptance components.

2.2. Material and methods

2.2.1. Mealybug rearing

The origin of the mealybugs used in the study is referred to in Table 2.1. Mealybugs were reared on sprouted potatoes (*Solanum tuberosum* L.) during multiple generations. Third instars of each species were isolated on sprouted potatoes within ventilated plastic boxes seven days before the beginning of the experiments to standardize age, physiological state and obtain pre-reproductive adult females. Isolated mealybugs were kept at controlled conditions (25.0±0.5ºC, 55-65% RH, in the dark).

Table 2.1- Origin of the mealybug populations used in the experiments.

Mealybug species	Region	Host plant
Planococcus citri	Silves (Mainland Portugal)	Sweet orange
Planococcus ficus	Tavira (Mainland Portugal)	Grapevine
Pseudococcus calceolariae	Loulé (Mainland Portugal)	Sweet orange
Pseudococcus viburni	Biscoitos (Azores, Portugal)	Grapevine
Phenococcus peruvianus	Queluz (Mainland Portugal)	*Bougainvillea glabra*

2.2.2. Parasitoid rearing

Anagyrus sp. nr. *pseudococci* was collected in the region of Silves (Portugal) and reared within ventilated plastic boxes on *Pl. citri* for multiple generations under controlled conditions (25.0±0.5ºC, 55-65% RH, 16L:8D photoperiod). To obtain naïve adult female wasps less than 24h old, the rearing plastic boxes were first observed and kept free of parasitoids, and then checked every 24h. Before the experiments, each female wasp was fed and mated by introducing it into a new box containing one drop of honey and two male wasps, in which they were kept for 72h under the same controlled conditions mentioned above, until the beginning of the experiment.

2.2.3. Experiments

The experiments were conducted between 12:00h and 19:00h, under laboratory conditions (19-22°C and 55-65% RH). In each of the 22 replicates, one naïve adult parasitoid female was exposed to 10 pre-reproductive adult mealybug females in a Petri-dish (9cm diameter), and observed during 30 min. The behavior of wasp females was described according to the following five categories (Heidari & Jahan, 2000; Karamaouna & Copland, 2000): i) searching (the parasitoid moved randomly while moving its antennae upward and downward successively); ii) antennation (the female wasp examines the host mealybug, by drumming the antennae); iii) probing (the females inserts the ovipositor to collect information from inside the host); iv) oviposition (the female wasp turns her body clockwise or counterclockwise and flexes the tip of her abdomen to place the ovipositor in position and insert it into the host); and v) grooming and resting (the parasitoid cleans its body involving the mouthparts, antennae, legs and wings, and afterwards eventually remains motionless). For each replicate, the duration of each type of the parasitoid behavior was recorded in seconds, using a chronometer.

2.2.4. Dissection of mealybugs

After the end of each experiment, the mealybugs of each replicate were maintained in the same Petri-dish under laboratory conditions during seven days. After this period, the mealybugs were individually immersed in a clarification solution consisting of 1 part glacial acetic acid and 1 part chloral-phenol and then dissected to determine the number of mealybugs parasitized as well as the total number of oviposited wasp eggs per replicate.

2.2.5. Statistical analysis

The number of host encounters, number of mealybugs parasitized, number of parasitoid eggs oviposited, as well as the number of times each type of parasitoid behavior was observed were analyzed using Generalized Linear Models, by fitting a Poisson distribution.

Univariate General Linear Models (ANOVA) were used for the analysis of time duration of each parasitoid behavior, percentage of total time allocated to host searching and to host handling (antennation + probing + oviposition), and handling time per parasitized host. Normal distribution and homogeneity of variances were tested based on Shapiro-Wilk and Levene´s tests, respectively. When necessary, a square root or angular transformation of data was used for time duration of parasitoid behavior and percentage of total time allocated to host searching and to host handling, respectively. The angular transformation, corresponding to arsin√p where p is a proportion, was used as a tool to stabilize variances and normalize data in percentages or proportions (Sokal & Rohlf, 1981).

Data are presented as mean ± SEM (standard error of the mean). The significance level was set at α=0.05. All statistical tests were carried out using IBM SPSS 20.0 for Windows (IBM Corporation, Armonk, New York, USA).

2.3. Results

2.3.1. Parasitism

The number of observed encounters between *A.* sp. nr. *pseudococci* and the host mealybugs did not significantly vary among host species (Table 2. 2). Yet, the number of mealybugs parasitized by the wasp was significantly higher in *Planococcus* species than in the other mealybug species tested, with the exception of *Pl. ficus* and *Ps. viburni* (Table 2.2). No significant differences were observed between *Pl. citri* and *Pl. ficus,* or among *Pseudococcus* and *Phenacoccus* species. The number of eggs oviposited by *A.* sp. nr. *pseudococci* was significantly higher in *Planococcus* spp. than in all other mealybug species tested (Table 2.2). No significant differences were registered between the two *Planococcus* species, and among *Pseudococcus* and *Phenacoccus* species

Table 2.2 - Mean number of mealybugs parasitized by female of *Anagyrus* sp. nr. *pseudococci* and mean number of wasp eggs oviposited per replicate on the studied five host mealybug species in no-choice test. For each replicate, 10 individuals were exposed to one female parasitoid for 30 min (N=22).

Host species	Number of host encounters*	Number of parasitized mealybugs	Number of wasp eggs
Planococcus citri	8.1±0.6	3.0±0.4a	3.2±0.4a
Planococcus ficus	8.7±0.6	2.2±0.3ab	2.6±0.3a
Pseudococcus calceolariae	7.5±0.6	1.4±0.3c	1.5±0.3b
Pseudococcus viburni	7.6±0.6	1.6±0.3bc	1.7±0.3b
Phenacoccus peruvianus	8.9±0.6	1.1±0.2c	1.3±0.3b
$X^2{}_4$	4.164	25.49	25.79
p	0.384	< 0.001	<0.001

*Within columns, means followed by the same letter are not significantly different (p=0.05)

2.3.2. Host selection behavior

Description of wasp behavior. When encountered, mealybugs were usually examined and eventually accepted or rejected by the wasp based on information collected from the host body surface through antennation. If the host is accepted then the wasp turns her abdominal end towards the host, and repositions to insert her ovipositor into the host and deposit an egg. Sometimes, after probing, the wasp rejects the mealybug and does not oviposit. The frequency of rejection after probing, when a female parasitoid was exposed to 10 mealybugs for 30 min, was on average 1.5±0.3, 1.2±0.2, 0.7±0.2, and 1.2±0.2, for *Pl. citri, Pl. ficus, Ps. calceolariae,* and *Ps. viburni,* respectively. No rejection after probing was observed in the case of *Ph. peruvianus.* Host-feeding was observed in none of the studied mealybug species. Usually, after oviposition the wasp moves away from the host and may spend some time cleaning her antennae, legs and wings and eventually resting. In some cases, in *Planococcus* and *Pseudococcus* species, but especially in *Pl. ficus*, the wasp showed a particular behavior of host acceptance after antennation. She stayed motionless nearby the host with her antennae in upper position for a period of 50 seconds up to about 7.5 minutes, during which the antennae came down gradually. Then the wasp turned back for reexamining the host for no longer than 15 seconds, resuming antennation and ovipositing. In this case oviposition takes more than 50 seconds.

Frequency of each type of behavior. The frequency of host searching behavior of *A.* sp. nr. *pseudococci* females was not significantly different among mealybug species (X^2_4=7.54, P=0.11). However, significant differences were found among host mealybug species (X^2_4=18.32, P=0.001) for the frequency of antennation of female wasps. The higher frequency of antennation was observed on *Pl. ficus* (14.0±0.8) and the lowest on *Ph. peruvianus* (10.4±0.7). No significant differences were detected between species within both *Planococcus* and *Pseudococcus* genera and between *Pseudococcus* species and *Ph. peruvianus*.

The frequency of host probing by wasp females was significantly different among mealybug species (X^2_4=31.433, P<0.001). The highest value was registered in *Pl. ficus* (7.8±0.7). Neverthless, similar values to *Pl. ficus* were found for *Pl. citri* (7.6±0.2), and *Ps. viburni* (7.2±0.9), whereas significantly lower values were found for *Ps. calceolariae* (5.4±0.7) and *Ph. peruvianus* (4.3±0.9).

The frequency of oviposition behavior observed in the females of *A.* sp. nr. *pseudococci* significantly differed among mealybug host species (X^2_4=15.74, P=0.003). However, no significant differences were detected between species within the genus *Planococcus* (6.6±0.5 and 6.2±0.6 for *Pl. ficus* and *Pl. citri*, respectively) and the genus *Pseudococcus* (6.0±0.5 and 4.7 ± 0.5 for *Ps. viburni* and *Ps. calceolariae*, respectively). *Pseudococcus viburni* did not differ from both *Planococcus* species and *Ps. calceolariae* showed no significant differences in relation to *Ph. peruvianus* (4.3±0.4).

Finally, the frequency of wasp grooming and resting also differed significantly among host species (X^2_4=17.56, P=0.002). This parameter was significantly higher on *Pl. ficus* (5.6±0.5), *Ps. viburni* (5.2±0.5), and *Ps. calceolariae* (5.1±0.45), compared to *Pl. citri* (3.7±0.4) and *Ph. peruvianus* (3.5±0.4).

Time duration of each type of behavior. The duration of host searching behavior showed by females of *A.* sp. nr. *pseudococci* was significantly influenced by the host mealybug species (Table 2.3). The time the wasps spent searching was significantly higher in *Ph. peruvianus* than in the other mealybug species. No significant differences were observed among *Pl. citri*, *Ps.*

calceolariae and *Ps. viburni.* The lowest time was observed in *Pl. ficus* but it did not differ significantly from *Pl. citri* and *Ps. viburni.*

Table 2.3 - Mean time duration (±SE) (in minutes) spent by female *Anagyrus* sp. nr. *pseudococci* on host searching, antennation, oviposition and grooming + resting when exposed to each of the studied five host mealybug species in no-choice test. For each replicate, 10 mealybugs were exposed to one female parasitoid for 30 min (N=22).

Host species	Searching*	Antennation	Oviposition	Grooming and resting
Planococcus citri	12.1±1.0bc	5.2±0.5a	3.3±0.4ab	7.2±1.0b
Planococcus ficus	8.6±0.8c	5.2±0.4a	4.7±0.5a	9.6±0.9ab
Pseudococcus calceolariae	13.9±1.0b	4.0±0.6ab	2.2±0.3bc	7.8±1.2b
Pseudococcus viburni	10.0±0.9bc	2.5±0.4b	1.3±0.2cd	14.4±1.1a
Phenacoccus peruvianus	22.5±1.4a	1.1±0.2c	1.0±0.3d	4.2±1.1c
$F_{4, 105}$	28.40	21.02	17.55	14.44
p	<0.001	< 0.001	<0.001	<0.001

* Within columns, pairs of means followed by the same letters are not significantly different (p=0.05)

The amount of time the parasitoid spent examining the host through antennation was significantly dependent on mealybug species (Table 2.3). The lowest value was registered in *Ph. peruvianus* and the highest values were observed in *Planococcus* species.

The amount of time the parasitoid spent ovipositing was also significantly influenced by the host species (Table 2.3). The highest and lowest values were registered in *Pl. ficus* and *Ph. peruvianus,* respectively. No significant differences were observed between species within *Planococcus* and *Pseudococcus* genera*. Planococcus citri* did not significantly differ from *Ps. calceolariae,* and *Ps. viburni* from *Ph. peruvianus.*

The time spent grooming and resting by the parasitoid females significantly varied among mealybug species (Table 2.3). When exposed to *Ps. viburni,* the wasps spent a significantly higher amount of time grooming and resting compared to all other mealybug species except for *Pl. ficus.* No significant differences were observed among *Pl. citri, Pl. ficus* and *Ps. calceolariae. Phenacoccus peruvianus* was significantly different from all other mealybug species.

<u>Percentage of time allocated to host searching and handling</u>. The percentage of time allocated to host searching by the parasitoid was significantly affected by the host mealybug

species (Table 2.4). The highest and lowest values were registered in *Ph. peruvianus* and *Pl. ficus,* respectively. No significant differences were observed between *Ps. calceolariae* and *Pl. citri* and among *Pl. citri, Pl. ficus* and *Ps. viburni.*

Table 2.4 - Percentage (±SD) of time allocated by females *Anagyrus* sp. nr. *pseudococci* for host searching and handling (antennation + probing + oviposition) in each of the studied five host mealybug species (no-choice test). For each replicate, 10 mealybugs were exposed to one female parasitoid for 30 min (N=22).

Host species	Searching*	Handling
Planococcus citri	42.5±3.1bc	32.3±3.1ab
Planococcus ficus	30.1±2.9c	36.7±2.5a
Pseudococcus calceolariae	49.7±3.5b	22.8±2.8bc
Pseudococcus viburni	35.4±3.4c	14.0±1.7c
Phenacoccus peruvianus	78.0±4.5a	7.3±1.4d
$F_{4,105}$	28.02	27.59
p	<0.001	<0.001

* Within columns, pairs of means followed by the same letters are not significantly different (p=0.05).

The percentage of time dedicated to host handling by the wasps, including antennation, probing and oviposition, was significantly dependent on the host mealybug species (Table 2.4). Apparently, it decreased according to the following sequence: *Pl. ficus* > *Pl. citri* > *Ps. calceolariae* > *Ps. viburni* > *Ph. peruvianus* (Fig. 2.1). However, no significant differences were found between *Planococcus* species, as well as between *Pseudococcus* species. *Planococcus citri* did not significantly differ from *Ps. calceolariae* for the same parameter. The percentage of time allocated to host handling by female *A.* sp. nr. *pseudococci* in *Ph. peruvianus* was significantly lower than in all other mealybug species (Table 2.4).

The handling time was significantly influenced by the host species, varying between 2.1 and 5.2 minutes per parasitized mealybug in *Ph. peruvianus* and *Pl. ficus*, respectively (Table 2.5). This parameter was significantly higher in *Pl. ficus* compared to all other mealybug species except for *Pl. citri* and *Ps. calceolariae.* No significant differences were observed among *Pl. citri*, *Ps. calceolariae, Ps. viburni* and *Ph. peruvianus*.

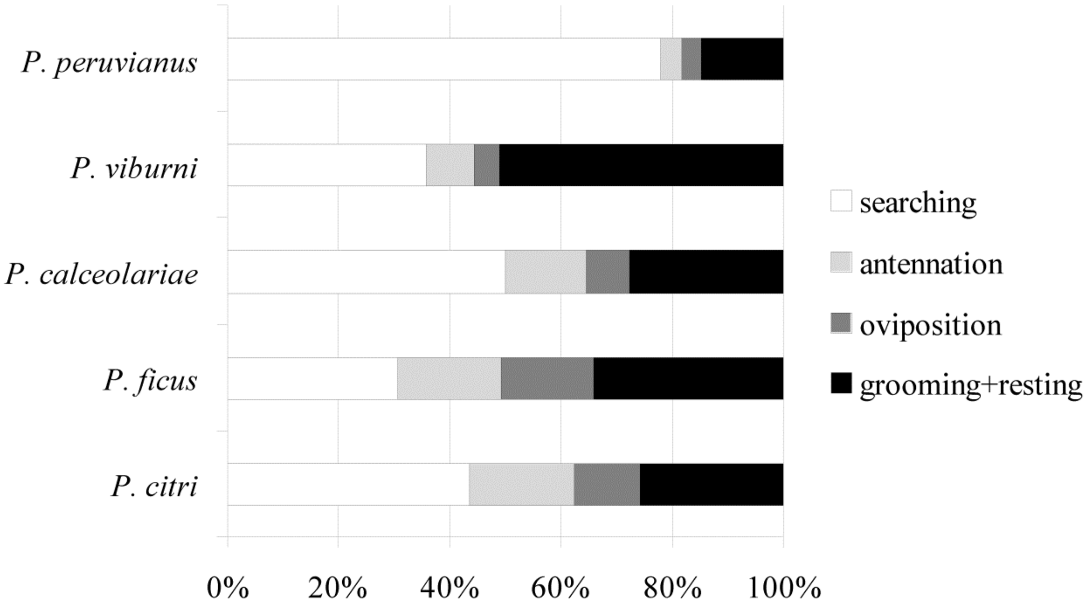

Figure 2.1 - Percentage of time allocated to each behavior of *Anagyrus* sp. nr. *pseudococci* with five mealybug species (*Pl. citri, Pl. ficus, Ps. calceolariae, Ps. viburni, Ph. peruvianus*) in no-choice tests.

Table 2.5 - Mean handling (antennation + probing + oviposition) time (minutes per parasitized mealybug ±SE) of females of *Anagyrus* sp. nr. *pseudococci* for the studied five host mealybug species (no-choice test). For each of the 22 replicates, 10 mealybugs were exposed to one female parasitoid for 30 min.

Host species	N	Handling time*
Planococcus citri	21	3.6±0.7ab
Planococcus ficus	20	5.2±0.6a
Pseudococcus calceolariae	17	4.3±0.7ab
Pseudococcus viburni	18	2.5±0.6b
Phenacoccus peruvianus	11	2.1±0.6b
$F_{4,82}$		3.54
p		0.01

* Within columns, pairs of means followed by the same letters are not significantly different (p=0.05).

2.4. Discussion

The observed host selection behavior of the females of *A.* sp. nr. *pseudococci* was in general similar to that described by Avidov et al. (1967) and Heidari and Jahan (2000) for *A. pseudococci* s.l. No host-feeding was observed in wasp females. However, we cannot exclude the possibility of host-feeding by *A.* sp. nr. *pseudococci* in younger host stages, such as first and second instars, as our observations were carried out only on pre-reproductive adult mealybug females. For example, Karamaouna and Copland (2000) observed that the females of *Leptomastix epona* (Walker) might host feed on second and third instar nymphs of *Ps. viburni* in which they do not oviposit. Host-feeding is used by many synovigenic parasitoids as a source of proteinaceous nutrients for egg production, and can be of biological significance in pest suppression (Karamaouna & Copland, 2000).

Host location by parasitoid females generally involves ambulatory searching behavior for slightly volatile chemical cues, i.e., searching stimulants, such as frass, defensive secretions, pheromones, or feeding secretions, which after encountered will retain the wasp and stimulate the searching for a certain amount of time, depending on experience, host encounter rate, the nature of the substrate, or changes in the concentration of the chemical cues (Vinson, 1998). In previous works, we have shown that the females of *A.* sp. nr. *pseudococci* are attracted to (S)-(+)-lavandulyl senecioate, the sex pheromone of *Pl. ficus* (Franco et al., 2008) and use this kairomonal cue in host location, possibly as an arrestant (Franco et al., 2011). Other mealybug products, such as honeydew, are likely to be used by *A.* sp. nr. *pseudococci* as kairomonal cues in host location (Franco et al., 2008; Islam & Jahan, 1993). Recently, Dhami, Gardner-Gee, Van Houtte, Villas-Bôas, & Beggs (2011) showed that the honeydew excreted by each scale insect species have a distinctive amino acid and carbohydrate signature. This signature may be used as a chemical cue by mealybug parasitoids to distinguish among hosts.

In the present study, host location was limited by the size of Petri dish arena. In such a scenario only short range searching behavior is possible. No significant differences were observed among mealybug species on the searching frequency of *A.* sp. nr. *pseudococci* as well as on the frequency of host encounters. However, the amount of time spent searching by the wasp varied among mealybugs host species, with the shortest time registered in *Pl. ficus* and the longest in *Ph. peruvianus* (Table 2.3). The differences observed among mealybug species on the level of parasitim by *A.* sp. nr. *pseudococci* were not apparently determined by the frequency of host encounters, as no significant differences were found among host species for this parameter (Tables 2.2).

Host recognition by parasitoid females is expected to be based on the external examination of the host using nonvolatile chemicals or physical characteristics as cues (Vinson, 1998). If the host is eventually recognized and considered suitable the parasitoid female might resume antennation and probe the host with the ovipositor (Vinson, 1998). After probing the wasp will eventually accept the host based on the presence of the right cues and the absence of deterrents (Vinson, 1998). In the present study, *A.* sp. nr. *pseudococci* recognized and accepted all five tested mealybug species as potential hosts despite their different geographical origin and phylogenetic relationships. Nevertheless, the behavioral pattern of host recognition and the level of host acceptance significantly varied among host species. The number of parasitized mealybugs in *Pl. citri* and *Pl. ficus* was about twice as higher as in *Pseudococcus* and *Phenacoccus* species. The cues used by female *A.* sp. nr. *pseudococci* in host recognition through antennal examination are probably related to the waxy secretions covering the body of mealybugs. These secretions are produced by epidermal wax glands whose function has been associated with protection against water loss, wet conditions, natural enemies, and contamination with their own honeydew and defensive exudates (Cox & Pearce, 1983; Gulan & Kosztarab, 1997). The chemical composition of these wax secretions differ among mealybug species (Zvi Mendel, pers. communication, 2013). The females of *A.* sp. nr. *pseudococci* present uniporous *chaetica* sensillae in the ventral side of the antennal club which are apparently contact chemoreceptors and may be associated with infochemical detection during external examination of the host through antennation (Fortuna, Franco, & Rebelo, 2013). Mozaddedul and Copland (2003) reported that searching behavior of the parasitoid *Leptomastix* nr. *epona* (Walker) is arrested by the wax secretions of its mealybug host. The ostiolar secretions, which can be produced by the mealybugs when attacked by parasitoids or predators (Gullan & Kosztarab, 1997), may also affect host recognition and acceptance of *A.* sp. nr. *pseudococci.* This reflex bleeding behavior is much more frequent in *Ps. viburni* than in the other mealybug species (Bugila et al., in prep), which may explain the much higher amount of time spent by the parasitoid in grooming and resting when exposed to this mealybug, in comparison with the other studied mealybugs (Table 2.4; Fig. 2.1).

The females of *A.* sp. nr. *pseudococci* rejected some individuals after probing all mealybug species except for *Ph. peruvianus*. Some of the cues detected by probing are possibly related to mealybug resistance. Mealybugs are known to resist the attack of parasitoids through immune defense response by encapsulation of their eggs or larvae (Blumberg, 1997; Blumberg, Klein, & Mendel, 1995). On the other hand, it has been hypothesized that superparasitism might be used by *A.* sp. nr. *pseudococci* and other solitary parasitoids of mealybugs as a strategy for counteracting host immune defenses (Blumberg et al., 2001; Suma et al., 2011). The fact that female parasitoids tend to lay higher number of eggs in more resistant host mealybugs (Blumberg et al., 2001; Suma et al., 2011) suggests that they are able to access the level of host resistance based on the detection of internal chemical cues through ovipositor probing. We hypothesize that eventually female wasps may decide to reject the most resistant hosts after probing. The ability of the five studied mealybugs to encapsulate eggs or larvae of *A.* sp. nr. *pseudococci* will be addressed elsewhere (Bugila et al., in prep).

The duration of host handling may be influenced by host species, as well as by host aggregation, host size, host developmental stage, host state, and parasitoid experience (Segoli, Harari, Bouskila, & Keasar, 2009, and references therein). Our results showed that host handling time by female *A.* sp. nr. *pseudococci* was affected by host species, with the highest value registered in *Pl. ficus*, the host for which the parasitoid showed highest host searching efficiency. A reduction in host handling time is expected to increase reproductive success of parasitoids which require more time for searching suitable hosts than for egg production (Heimpel, Mangel, & Rosenheim, 1998). The observed variation in host handling time among mealybug species may also be related to differences in behavioral defenses among host mealybugs. We would expect a reduction in handling time of female *A.* sp. nr. *pseudococci* with respect to mealybug species reacting more aggressively to parasitoid attack. For example, it is known that the process of ovipositor insertion by female wasps is longer when a sessile host is parasitized and often faster in more mobile and defensive hosts (Vinson, 1998). Our observations on defensive behavior of the five studied mealybugs support this hypothesis, as *Ps. viburni* showed the highest level of defensive behavior and *Planococcus* species the lowest ones (Bugila et al., in prep.). A more rapid host-handling may also reduce the exposure to the predators, such as it seems the case of parasitoids more adapted to successfully attack ant-tended scale insects (Barzman & Daane, 2001). Although mealybugs are known to be commonly ant-tended insects and ants may disrupt the activity of mealybug parasitoids (Daane, Sime, Fallon, & Cooper, 2007; Gullan & Kosztarab, 1997; Way, 1963), it is not likely that the observed differences among host mealybugs on host handling time of female *A.* sp. nr. *pseudococci* are related to ant-tending.

Anagyrus sp. nr. *pseudococci* seems to be much less host specific than its congeners *A.* sp. nr. *sinope* Noyes & Menezes and *A. kamali* Moursi. *Anagyrus kamali* is a solitary endoparasitoid of the pink hibiscus mealybug, *Maconellicoccus hirsutus* Green (Sagarra, Vincent, & Stewart, 2001), whereas *A.* sp. nr. *sinope* is a gregarious endoparasitoid of the Madeira mealybug, *Ph. madeirensis* (Chong & Oetting, 2007). In Table 2.6, we compare the results of the studies by Sagarra et al. (2001) and Chong and Oetting (2007) on the host ranges of these two parasitoids with those obtained by us for *A.* sp. nr. *pseudococci*. *Anagyrus* sp. nr. *sinope* and *A. kamali* were shown to be very selective mealybug parasitoids, only completing development in their principal host species (Table 2.6). In most of the cases, the two parasitoids were able to discriminate among the tested mealybug species and select the most suitable ones.

However, they showed different behavioral response to the non-selected mealybug species. Some mealybug species were almost ignored and did not induce searching behavior by the parasitoid (e.g., *A. kamali*) (Table 2.6). Other mealybugs were rejected by the parasitoids after external antennal examination (e.g., *Ps. longispinus* and *F. virgata* for *A.* sp. nr. *sinope*; *L. neotropicus* and *Pu. barberi* for *A. kamali*) or after being probed with the ovipositor (e.g., *Pl. citri*, *Ps. viburni,* and *Ph. solani* for *A.* sp. nr. *sinope*; *Ps. elisae* for *A. kamali*) (Table 2.6). Finally, a few other mealybug species were accepted by the parasitoid as potential hosts despite being unsuitable hosts (Table 2.6). In contrast, *A.* sp. nr. *pseudococci* accepted and is able to complete development in all tested mealybugs (Bugila et al., in prep.), despite their different geographical origin and phylogenetic relationships. Nevertheless, the behavioral pattern of host recognition, host handling and the level of host acceptance significantly varied among host species, indicating a clear preference for the two *Planococcus* species, *Pl. ficus* in particular. Our results suggest a broader host range and a more generalist behavior for *A.* sp. nr. *pseudococci* in comparison with other *Anagyrus* species, which is in accordance with the hypothesis that this wasp might have evolved by expanding its host range (Franco et al., 2008). In previous studies we found that *A.* sp. nr. *pseudococci* responded to the sex pheromone of *Pl. ficus* (Franco et al., 2008) and use this chemical cue as a kairomone in host location (Franco et al., 2011). This innate kairomonal response of *A.* sp. nr. *pseudococci* females to a chemical cue of a specific host species indicates an intimate evolutionary relationship between the wasp and *Pl. ficus*, suggesting that this mealybug species was its primary host in the region of origin (Franco et al., 2008). However, all the available data, including the innate kairomonal response to the pheromone of *Pl. ficus*, the host selection behavior in comparison to specialist *Anagyrus* species, and an apparent realized host range with several mealybug species from different genera (Guerrieri & Pellizzari, 2009; Triapitsyn et al., 2007), support the hypothesis that *A* sp. nr. *pseudococci* evolved from a specialist to a more generalist strategy (Franco et al., 2008).

Table 2.6 - Specificity of *Anagyrus* sp. nr. *pseudococci* in comparison with two other mealybug parasitoids of the same genus, *A.* sp. nr. *sinope* and *A. kamali*. Elaborated based on data from Chong and Oeting (2007), Sagarra et al. (2001), and the present study, for *A.* sp. nr. *sinope, A. kamali,* and *A.* sp. nr. *pseudococci,* respectively. Mealybug species are organized according to their phylogenetic relationships (Hardy et al., 2008). Legend: **N** (no response) - The host did not induce searching behavior on the parasitoid; **R** - All the available hosts were rejected after antennation or probing; **A** - At least part of the available hosts were accepted and parasitized (% parasitism); **D** - the parasitoid was able to complete development in this host.

Family/Subfamily	Mealybug species	Parasitoid

		A. sp. nr. *sinope*	*A. kamali*	*A.* sp. nr. *pseudococci*
Pseudococcidae				
- Pseudococcinae	*Nipaecoccus nipae*	-	N	-
	Planococcus citri	R	A (11%)	A (30%) D
	Planococcus ficus	-	-	A (22%) D
	Planococcus halli	-	A (8%)	-
	Saccharicoccus sacchari	-	N	-
	Dysmicoccus brevipes	-	N	-
	Leptococcus (=*Plotococcus*) *neotropicus*	-	R	-
	Pseudococcus elisae	-	R	-
	Pseudococcus longispinus	R	-	-
	Pseudococcus calceolariae	-	-	A (14%) D
	Pseudococcus viburni	R	-	A (16%) D
	Ferrisia virgata	R	-	-
	Maconellicoccus hirsutus	-	A (45%) D	-
- Phenacoccinae	*Phenacoccus madeirensis*	A (17%)	-	-
	Phenacoccus peruvianus	-	-	A (11%) D
	Phenacoccus solani	R	-	-
Putoidae	*Puto barberi*	-	R	-

This is in line with the idea that the innate use of semiochemicals by generalist carnivores is the result of evolving from monophagous ancestors (Steidle & van Loon, 2003). Based on the host range information available for about 104 *Anagyrus* species, among the 270 described species, it seems that most of them (ca. 76%) are specialists, with less than five known hosts, and only few species show a more generalist behavior (Noyes, 2012).

The specificity of a parasitoid is considered an important attribute in selected candidates for classical biological control programs aiming to minimize the risks of impacts on non-target native species. In this respect, the use of *A.* sp. nr. *pseudococci* in classical biological control may present risks of impact on native species of mealybugs due to its apparent generalist behavior. Nevertheless, it has been used both in classical biological control and augmentative releases in different areas (Triapitsyn et al., 2007) and there is no evidence of negative impacts on native mealybug species. On the other hand, the existence of alternative hosts is considered important for the success of biological control as it will support parasitoid populations over periods of scarcity of the primary hosts (Chong & Oetting, 2007; DeBach & Bartlett, 1964).

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3. Defense response of native and alien mealybugs (Hemiptera: Pseudococcidae) against the solitary parasitoid *Anagyrus* sp. nr. *pseudococci* (Girault) (Hymenoptera: Encyrtidae)

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Abstract

The host behavioral and immune (encapsulation) defenses against the parasitoid *Anagyrus* sp. nr. *pseudococci* were compared for five mealybug species with different phylogenetic relationships and geographical origins: i) a Mediterranean native mealybug species, *Planococcus ficus,* with a long co-evolutionary history with the parasitoid; ii) three alien mealybugs species, *Planococcus citri, Pseudococcus calceolariae* and *Pseudococcus viburni*, with a more recent co-evolutionary history; and iii) a fourth alien mealybug species, *Phenacoccus peruvianus*, with no previous common history with the parasitoid. Three host defense behaviors were registered: abdominal flipping, reflex bleeding and walking away. The native host *Pl. ficus* and its congener *Pl. citri* exhibited the lowest probability of defense behavior (0.11±0.01 and 0.09±0.01 respectively), whereas the highest value was observed in *P. viburni* (0.31±0.02). Intermediate levels of defense behavior were registered for *Ps. calceolariae,* and *Ph. peruvianus*. The probability of parasitoid encapsulation was lowest and highest for two alien host species, *Ph. peruvianus* (0.20±0.07) and *Ps. viburni* (0.86±0.05), respectively. The native host *Pl. ficus,* its congener *Pl. citri* and *Ps. calceolariae* showed intermediate values (0.43±0.07, 0.52±0.06, and 0.45±0.09, respectively). The results are relevant with respect to biological control and to understand possible evolutionary processes involved in host range of *A.* sp. nr. *pseudococci.*

Key-words: behavioral defense; biological control; encapsulation; host parasitoid co-evolution; host resistance; immune defense

3.1. Introduction

In general, host selection behavior of parasitoid Hymenoptera includes habitat location, host location, host recognition and host acceptance, eventually resulting in oviposition (Vinson, 1998). As an evolutionary response to the selective pressure from parasitoids, insect hosts have developed defensive strategies which may affect parasitoid activity and development along the different steps of host selection. These host defenses can be divided in three major categories (Gross, 1993): i) host characteristics, that reduce the probability of being located by parasitoids (e.g., refugia, elimination of cues); ii) host behavioral and morphological defenses, which may act after host location by reducing the probability of parasitoid oviposition (e.g., evasive behavior, defensive secretions); and iii) host physiological defenses, that if parasitoid oviposition occurs may prevent the successful development of endoparasitoids (e.g., sequestration of allelochemicals, encapsulation). Host defenses can also be designated as (Gentry & Dyer, 2002): i) primary defenses, which prevent enemies from encountering the host/prey; ii) secondary defenses, that are activated once the host/prey has been encountered by a parasitoid/predator; and iii) tertiary defenses, which include host immune responses after an endoparasitoid attack. In the present work we will deal with host defenses of categories ii) and iii), in particular behavioral defenses and encapsulation.

Host behavioral defenses against parasitoids include evasive and aggressive behaviors. Evasive behaviors allow the host to escape from the attacking parasitoid and often involve vigorous wriggling, thrashing, rolling, curling, jumping, walking way, or dropping of the plant, whereas aggressive behaviors are responsible for driving away or disabling adult parasitoids (e.g., defensive secretions), as well as dislodging or killing parasitoid eggs or larvae (Gross, 1993).

Encapsulation is an immune defense mechanism of insect hosts triggered by eggs and larvae of parasitoids which involves the production by hemocytes of a multilayered capsule around the invader, usually associated with melanization (Carton, Poirié, & Nappi, 2008; Schmid-Hempel, 2005; Strand, 2008; Strand & Pech, 1995).

The study of host defenses will contribute to understand the evolution of parasitoid oviposition behavior as well as to understand why some insect species are less susceptible to parasitism than others (Gross, 1993). This knowledge is also of practical importance as it will provide the theoretical background to support decision-making for the selection of best candidates to be used in biological control of insect pests.

Mealybugs (Hemiptera: Pseudococcidae) are the second most diverse family of scale insects (Coccoidea) comprising more than 2000 species, distributed worldwide including many economic important agricultural and ornamental pests (Hardy, Gullan, & Hodgson, 2008), such has the citrus mealybug *Planococcus citri* (Risso), the vine mealybug *Pl. ficus* (Signoret), *Pseudococcus calceolariae* (Maskell), the obscurus mealybug *Ps. viburni* (Signoret), and the bougainvillea mealybug *Phenacoccus peruvianus* Granara de Willink (Ben-Dov, 1994; Beltrà et al., 2010; Franco, Silva, & Carvalho, 2000). Chemical control is still the most common control tactic used against mealybug pests. However, the cryptic behavior of mealybugs, their typical waxy body cover, and clumped spatial distribution pattern render the use of many insecticides ineffective. Repeated insecticide use, especially of broad-spectrum chemicals, also has adverse ecological and environmental impacts (Franco, Zada, & Mendel, 2009). Biological control has been considered an environmentally friendly alternative tactic to be used in integrated pest management strategies for the control of pest mealybugs (Franco et al., 2009). Among mealybug parasitoids, many Encyrtidae wasps (Hymenoptera), such as *Anagyrus* spp., have been used for the biological control of pest mealybugs (Noyes & Hayat, 1994). The efficacy of biological control relies on the host-parasitoid interactions and of their co-evolutionary history. In particular, understanding the ability of the host to escape parasitism by immune response and of the parasitoid to overcome host defense strategies has been considered a most relevant factor for the success of the parasitoid in biological control programs (Blumberg & van Driesche, 2001).

Among parasitoids used for the biological control of mealybugs, the genus *Anagyrus* has been one of the most studied. Recently, Triapitsyn, González, Vickerman, Noyes, & White (2007) have shown that *Anagyrus pseudococci* s.l. (i.e., *sensu latu*, corresponding to the previous references) comprises two sibling species, i.e. *A. pseudococci* (Girault) and *A.* sp. nr. *pseudococci* (Girault), which are reproductively incompatible and genetically different, also differing on their geographical distribution. *Anagyrus* sp. nr. *pseudococci*, apparently the more common species is widely distributed throughout the Mediterranean Basin (Triapitsyn et al., 2007; Franco et al., 2011), whereas *A. pseudococci* seems to be restricted to Sicily and Cyprus, and was apparently introduced in Argentina (Triapitsyn et al., 2007). *Anagyrus* sp. nr. *pseudococci* is a common parasitoid of *Pl. citri* and *Pl. ficus* (Franco et al., 2011; Mgocheki & Addison, 2009). The foraging behavior of female wasps when parasitizing mealybugs has been described as a sequence of events involving searching, antennation, probing, oviposition, and resting (Bugila, Branco, Silva, & Franco, 2014; Heidari & Jahan, 2000). Usually, after antennation, when a suitable host is found, the wasp female turns her body and flexes the tip of her abdomen to oviposit (Bugila et al., 2014; Heidari & Jahan, 2000). During this process mealybugs may escape parasitism by responding with particular defense behaviors. Three types of active defense behaviors have been described for mealybugs against their natural enemies (Gillani & Copland, 1999; Heidari & Jahan, 2000): i) abdominal flipping; ii) escaping; and iii) reflex bleeding. Abdominal flipping refers to repeated up and down movements of the hind half of the mealybug body that may force the attacking parasitoid or predator to leave. By just walking away the mealybug may also escape from the aggressor. Finally, reflex bleeding refers to the secretion of ostiolar fluid. When disturbed mealybugs may segregate one droplet of a waxy fluid, from one or more of the two pairs of dorsal ostioles, which quickly solidifies on contact with air, a reflex bleeding that has been assumed to be a defensive behavior of these insects (Gullan & Kosztarab, 1997). However, only very few studies have been carried out demonstrating this function (e.g., Gillani & Copland, 1999).

Heidari and Jahan (2000) suggested that different species of mealybugs may differ on their behavioral defense reaction against the wasp *A. pseudococci* s.l., in particular when comparing *Pl. citri* and *Ps. viburni*. So far such possible differences of behavior among mealybug species were not studied. After successful parasitoid oviposition, the host mealybugs may still respond with their immune defenses by encapsulating the eggs or larvae of the parasitoid. Well succeed encapsulation will arrest the development of the parasitoid and allow the mealybug to resist the attack and survive (Blumberg & van Driesche, 2001; Güleç, Kilinçer, Kaydan, & Ülgentürk, 2007). Only little information is available on encapsulation of *A.* sp. nr. *pseudococci* by mealybugs (Suma et al., 2012).

In a previous work we compared the foraging behavior of *A.* sp. nr. *pseudococci* among five host mealybugs with different phylogenetic relationships and geographical origins (Bugila et al., 2014). Here we aimed at analyzing differences on the host defense behavior and immune response of the same mealybug species to the attack of *Anagyrus* sp. nr. *pseudococci*. Especially we intend to compare the defense reaction pattern against this wasp among potential hosts, including i) a Mediterranean native mealybug species, *Pl. ficus*, considered to have a long co-evolutionary history with the parasitoid; ii) three alien mealybugs species, *Pl. citri, Ps. calceolariae* and *Ps. viburni*, with a more recent co-evolutionary history; and iii) a fourth alien mealybug species, *Ph. peruvianus*, with no previous common history with the parasitoid, in order to understand host-parasitoid relationships. A complete description of the phylogenetic relationships of the selected mealybug species, as well as their possible regions of origin and history of introduction in the Mediterranean basin was presented in Bugila et al. (2014).

We hypothesize that the defensive behavior and immune response of the five mealybug species selected for our study differ in relation to *A.* sp. nr. *pseudococci* as a consequence of different life traits and evolutionary histories. From a practical point of view, understanding the parasitoid-host relationships between *A.* sp. nr. *pseudococci* and selected mealybugs will allow us to predict its potential as a biological control agent of these and other mealybug species.

3.2. Material and methods

3.2.1. Mealybug rearing

The origin of mealybugs used in the experiments is reported in Table 3.1. All mealybug species were reared on sprouted potatoes (*Solanum tubersum* L.) on laboratory conditions (25.0±0.5°C, 55-65% R.H., in the dark). Before the beginning of the experiments, third instars nymphs of each species were isolated on sprouted potatoes within ventilated plastic boxes (25 x 15 x 12 cm) and kept at the same laboratory conditions as mentioned before for seven days to obtain pre-reproductive females (Bugila et al., 2014).

Table 3.1 - Region and host plant of origin of the studied mealybug species.

Mealybug species	Species origin	Population origin	Host plant
Planococcus citri	Afrotropical	Silves-Algarve	Sweet orange, *Citrus sinensis*
Planococcus ficus	Mediterranean	Tavira-Algarve	Vineyard, *Vitis vinifera*
Pseudococcus calceolariae	Australasian	Loulé-Algarve	Sweet orange, *Citrus sinensis*
Pseudococcus viburni	Neotropical	Biscoitos-Terceira	Vineyard, *Vitis vinifera*
Phenococcus peruvianus	Neotropical	Queluz-Lisboa	*Bouganvillea glabra*

3.2.2. Parasitoid rearing

The parasitoid *Anagyrus* sp. nr. *pseudococci* was obtained from parasitized mealybugs *Pl. citri* collected in citrus orchards in the region of Silves (Portugal). About 30 individuals were used to start a colony in the laboratory. Rearing was done within ventilated plastic boxes on *Pl. citri* under laboratory conditions (25.0±0.5°C, 55-65% R.H., and photoperiod 16L:8D). To obtain naïve adult female wasps, parasitized mealybugs were first isolated on separate boxes. Then the boxes were checked every 24h, in order to collect wasps less than 24h old. Two males and one female were then moved to a new box containing one drop of honey and maintained for 72h until the beginning of the experiments, to allow fertilization and feeding of female wasps (Bugila et al., 2014).

3.2.3. Mealybug defense behavior

The experiments were conducted between 12h and 19h, under laboratory conditions. For each of the five studied mealybug species, 22 replicates were performed. In each replicate, one naïve adult parasitoid female was exposed to 10 pre-reproductive adult mealybug females in a Petri-dish (9cm diameter) and observed during 30 min (Bugila et al., 2014; Sagarra, Vincent, & Stewart, 2001). The defense behavior of the mealybugs was described according to the following three categories: i) abdominal flipping; ii) escaping, by walking away; and iii) reflex bleeding. The frequency of each defensive behavior category, following the parasitoid contact was recorded for each replicate.

3.2.4. Mealybug immune response: encapsulation

After the end of each experiment, mealybugs were maintained for 7 days in the same Petri-dish, under laboratory conditions in order to allow encapsulation to occur before dissection. After this period, the mealybugs were individually immersed in phenol-chloroform (50%) and acetic acid (50%), for 24h for clarification and then dissected in order to count the number of wasp eggs or larvae, as well as the number of encapsulated eggs and larvae. Encapsulation was considered based on the existence of melanin deposition on eggs or larvae (Blumberg, 1997; Blumberg, Klein, & Mendel, 1995). Observations were carried out under magnification (40X) using a stereomicroscope (Leica MZ6).

3.2.5. Statistical methods

Generalized Linear Models (GLM) were used to estimate the probability of a defense behavior pattern of mealybugs to occur after wasp contact, using a Binomial distribution model. Host species was considered a factor explanatory variable. The following types of defensive behavior were considered as dependent variables: i) abdominal flipping; ii) walking away; iii) reflex bleeding; and iv) any type of defense behavior. The same approach was used to model the probability of an egg of the parasitoid to be encapsulated, either at the egg or larval stage (aggregated encapsulation). Behavior patterns and encapsulation were expressed in mean probability of occurrence ± standard error (SE). Least significant differences (LSD) test was used to compare host species (α=0.05). GLM were further used to test differences on the scale dependent variables: i) number of eggs oviposited by *A.* sp. nr. *pseudococci*; ii) number of wasp eggs encapsulated by mealybugs; iii) number of wasp larvae encapsulated by mealybugs; and iv) number of parasitoids escaping encapsulation, in relation to the explanatory variable host species. Poisson distribution, which best fit the data, was used as model function.

Pearson correlations (r) were used to test relationship among behavior patterns and between active defense and encapsulation.

Data are presented as mean ± standard error of the mean (SEM). GLM results are presented in the form of Wald Chi-square X^2 test and P values. The significance level was set at $\alpha=0.05$. All statistical tests were carried out using IBM SPSS 20.0 for Windows (IBM Corporation, Armonk, New York, USA).

3.3. Results

3.3.1. Mealybug defense behavior

Except for reflex bleeding, which was not displayed by *Pl. citri,* all three defensive behaviors were observed in the studied mealybug species. The five mealybug species varied significantly in respect to the frequency of abdominal flipping (Chi-square: $X^2_4=89.89$, $P<0.001$), reflex bleeding ($X^2_4=26.26$, $P<0.001$) and walking away ($X^2_4=81.95$, $P<0.001$) behaviors (Table 3.2). In agreement, differences were found in the display of any type of defense behavior among the five mealybug species ($X^2_4=65.3$, $P<0.001$). For all the variables, *Ps. viburni* had the highest probability of displaying a defensive behavior, except on walking away for which the probability did not differ significantly from *Ph. peruvianus* (Table 3.2). In general, the probability of a mealybug responding with a defensive behavior to the attack of *A.* sp. nr. *pseudococci* decreased according to the following sequence: *Ps. viburni* > *Ph. peruvianus* = *Ps. calceolariae* > *Pl. ficus* = *Pl. citri* (Table 3.2). The probability of showing any type of defense behavior was about three times higher in *Ps. viburni* than in *Pl. citri* and *Pl. ficus*.

The three defense behavior variables were significantly correlated both at individual host-parasitoid contact level (n=1997) and at species level (n=5): abdominal flipping with walking away (individual: r=0.540, $P<0.001$; species: r=0.943, p=0.016); abdominal flipping with reflex bleeding (individual: r=0.351, $P<0.001$; species: r=0.921, p=0.026); and walking away with reflex bleeding (individual: r=0.278, $P<0.001$; species: r=0.967, p=0.007).

Table 3.2 - Mean probability of occurrence (±SE) of different types of defense behavior of five mealybug species belonging to the genera, *Planococcus, Pseudococcus* and *Phenacoccus,* when exposed to the parasitoid *Anagyrus* sp. nr. *pseudococci*.

Host mealybug	Abdominal flipping*	Reflex bleeding	Walking away	Any type of defense behavior
Planococcus citri	$0.07^{c}\pm0.012$	$0.00^{c}\pm0.000$	$0.04^{c}\pm0.010$	$0.09^{c}\pm0.014$
Planococcus ficus	$0.08^{c}\pm0.012$	$0.00^{c}\pm0.002$	$0.05^{c}\pm0.010$	$0.11^{c}\pm0.014$
Pseudococcus calceolariae	$0.18^{b}\pm0.021$	$0.02^{b}\pm0.008$	$0.08^{b}\pm0.014$	$0.21^{b}\pm0.022$
Pseudococcus viburni	$0.27^{a}\pm0.023$	$0.07^{a}\pm0.013$	$0.21^{a}\pm0.021$	$0.32^{a}\pm0.023$
Phenacoccus peruvianus	$0.19^{b}\pm0.022$	$0.06^{b}\pm0.013$	$0.14\ \pm0.019$	$0.21^{b}\pm0.023$

*Within columns, means followed by the same letter are not significantly different (P=0.05)

3.3.2. Mealybug immune response

Significant differences were registered among mealybug species for the total number of eggs oviposited (X^2_4=21.35, *P*=0.001), the number of encapsulated eggs (X^2_4=29.66, *P*=0.001) and the number of encapsulated larvae (X^2_4=13.92, *P*=0.003) (Table 3.3). Significant differences were also found on the number of parasitoid eggs escaping from encapsulation (X^2_4=18.15, *P*=0.001).

Both total oviposited and encapsulated eggs were higher in *Pl. citri* than in the other four mealybug species. Yet, encapsulated larvae were significantly higher in *Ps. viburni* which also showed significantly higher probability of aggregated encapsulation (eggs+larvae) than all other mealybug species (Table 3.4). The probability of encapsulation was similar for *Pl. citri*, *Pl. ficus* and *Ps. calceolariae*, but significantly lower in *Ph. peruvianus* than in all other mealybugs (Table 3.4). The percentage of aggregated encapsulation (number of eggs+larvae encapsulated/total eggs) was 59%, 46%, 45%, 86% and 23% for *Pl. citri*, *Pl. ficus*, *Ps. calceolariae, Ps. viburni* and *Ph. peruvianus*, respectively.

The probability of expression of any defense behavior and of encapsulation were not correlated (r=0.205, n=5, *P*=0.741).

Table 3.3 - Mean number (±SE) of oviposited eggs, encapsulated eggs and larvae of *Anagyrus* sp. nr. *pseudococci*, as well as of eggs escaping from encapsulation by the host in no-choice test with five mealybug species.

Host of mealybugs	Total oviposition*	Encapsulated eggs	Encapsulated larvae	Escaping from encapsulation
Planococcus citri	3.1 ± 0.38^{a}	1.6 ± 0.27^{a}	0.0^{c}	1.5 ± 0.26^{a}
Planococcus ficus	2.1 ± 0.31^{b}	0.7 ± 0.18^{bc}	0.2 ± 0.09^{b}	1.2 ± 0.23^{ab}
Pseudococcus calceolariae	1.5 ± 0.26^{bc}	0.4 ± 0.14^{c}	0.3 ± 0.12^{b}	0.6 ± 0.17^{bc}
Pseudococcus viburni	2.0 ± 0.29^{bc}	0.9 ± 0.21^{ab}	0.8 ± 0.21^{a}	0.3 ± 0.11^{c}
Phenacoccus peruvianus	1.3 ± 0.25^{c}	0.2 ± 0.11^{d}	0.1 ± 0.06^{b}	1.1 ± 0.21^{ab}

*Within columns, means followed by the same letter are not significantly different (P=0.05)

Table 3.4 - Estimated probability (±SE) of aggregated encapsulation of *Anagyrus* sp .nr *pseudococci* by five mealybug species.

Host mealybug	Probability of encapsulation*
Planococcus citri	0.52 ± 0.060^{b}
Planococcus ficus	0.43 ± 0.073^{b}
Pseudococcus calceolariae	0.45 ± 0.087^{b}
Pseudococcus viburni	0.86 ± 0.053^{a}
Phenacoccus peruvianus	0.20 ± 0.073^{c}

*Within columns, means followed by the same letter are not significantly different (P=0.05)

3.4. Discussion

3.4.1. Mealybug defense behavior

Mealybugs may respond to the attack of parasitoids by displaying defense behaviors which eventually may allow them escaping parasitism (Gillani & Copland, 1999; Heidari & Jahan, 2000). Three types of defense behavior, namely walking away, reflex bleeding and abdominal flipping, may be activated by a mealybug when attacked by a parasitoid. These three types of defensive behavior can be further divided into: 1) Evasive behaviors, in the case of walking away; or 2) Aggressive behaviors, in the case of reflex bleeding and abdominal flipping (Firlej, Lucas, Coderre, & Boivin, 2010; Gross, 1993). So far, very few studies have been carried out on mealybug defense behavior (Gillani & Copland, 1999; Heidari & Jahan, 2000). Heidari and Jahan (2000) suggested that mealybug defense behavior could vary among mealybug species, but at the extent of our knowledge this hypothesis was not tested before. In the present study, we comprehensively quantified the mealybug defense behaviors and tested differences among five mealybug species with different phylogenetic relationship, geographical origin and history of host-parasitoid relationship with *A.* sp. nr. *pseudococci*. Our data clearly show that the studied mealybugs respond differently to the attack of *A.* sp. nr. *pseudococci* by combining different levels of evasive and aggressive behavioral responses. A similar trend was observed among mealybug species for both types of aggressive behavior (abdominal flipping and reflex bleeding): the highest level of response was registered in *Ps. viburni*; the lowest was observed in both *Planococcus* species; and an intermediate one in *Ps. calceolariae* and *Ph. peruvianus*. A slightly different pattern was shown in the case of the evasive behavior (walking away). Globally, considering all types of defense behavior, both *Pseudococcus* species, which were probably introduced in the Mediterranean basin over 400 years ago, and the recently introduced *Ph. peruvianu*s exhibited higher active defensive behavior than the two most common host species in the Mediterranean, the native *Pl. ficus* and the phylogenetic related *Pl. citri.*

The impact of host defense behaviors on parasitoid fitness is dependent on their effectiveness in affecting host handling time and host acceptance, and thus reducing parasitism rate, or even in harming or killing parasitoids in some cases (Firlej et al., 2010 and references therein). We can evaluate the effectiveness of defense behaviors of the studied mealybugs by comparing the level of these defenses (Table 3.2) with the parasitism rate of the mealybugs by *A.* sp. nr. *pseudococci* determined by us in a previous study (Bugila et al., 2014): *Pl. citri* (30%); *Pl. ficus* (22%); *Ps. calceolariae* (14%); *Ps. viburni* (16%); and *Ph. peruvianus* (11%). There is an inverse relationship between these two parameters suggesting that mealybug defensive behaviors in the studied species affect host acceptance, and thus parasitism rate by *A.* sp. nr. *pseudococci.* A similar inverse relationship also exists between the level of mealybug defensive behaviors and the parasitoid handling (antennation + probing + oviposition) time (Bugila et al., 2014): *Pl. citri* (3.6 minutes per parasitized mealybug); *Pl. ficus* (5.2); *Ps. calceolariae* (4.3); *Ps. viburni* (2.5); and *Ph. peruvianus* (2.1). The reduction of handling time in those mealybug species with higher level of behavioral defenses indicates that possibly in such hosts the parasitoid strikes back by reducing the time spent in host processing for host acceptance and ovipositing in order to limit the impact of mealybug defenses. This may explains why the impact on the parasitism rate of *Ps. viburni* was lower than expected considering its relatively high level of behavioral defenses.

According to Gross (1993), many host defenses are possibly not an evolutionary response to selective pressure from parasitoids. Instead, they probably evolved for biological functions not related with parasitoids, but which eventually provided some protection against them. For example, aphids (Aphidoidea), present a pair of dorsal glandular cornicles or siphunculi, that similarly to the dorsal ostioles of mealybugs also release waxy droplets (Gullan & Kosztarab, 1997). Although the major function of these sticky secretions is probably to dispense the alarm pheromone, they may also have defensive purposes as they are capable of incapacitating the aggressors (Dill, Fraser, & Roitberg, 1990). The ostiolar secretions of mealybugs which are associated with the defensive behavior of reflex bleeding may have also other functions (Gullan & Kosztarab, 1997). For example, they have been suggested of mediating interactions between ants and obligate ant-attended mealybugs (Williams, 1978).

Therefore, the defense behaviors of mealybugs are likely generalist responses. That is, although behaviors such as walking away, abdominal flipping or reflex bleeding may allow mealybugs to defend themselves against attacks by enemies, they probably did not evolve in response to a specific parasitoid or predator species. This hypothesis is supported by the fact that mealybugs apparently respond with behavioral defenses not only against parasitoid attacks, as evidenced by our results but also against predation, as reported by Gillani and Copland (1999). These authors observed that the longtailed mealybug, *Pseudococcus longispinus* (Targioni Tozzetti) respond with reflex bleeding to the attack of the predatory larvae of *Sympherobius fallax* Navas (Neuroptera: Hemerobiidae), which eventually could die from starvation if their mouthparts were blocked by the ostiolar fluid. However, it is reasonable to expect that the pattern of mealybug behavioral defenses, as well as the way these defenses are combined, may evolve differently depending on the type of selection pressure produced by the corresponding community of enemies. Thus, mealybug species with higher defense behavior, such as *Ps. viburni* might have evolved under higher pressure from natural enemies, in comparison with those species showing lower behavioral defenses, such as *Planococcus* spp. Nevertheless, host insects may also modulate their behavioral defenses depending on the risk of attack and/or the virulence of the parasitoid (Ennis, Dillon, & Griffin, 2010). This hypothesis should be tested in mealybugs by comparing the pattern of behavioral defenses of a particular mealybug species in response to parasitoid species with different levels of virulence.

Trade-offs between defense strategies with variable cost-benefit balances may also be expected. Protection against natural enemies by attending ants as a result of mutualistic interactions with honeydew-excreting hemipterans is considered another category of behavioral defense, i.e. associative (Gross, 1993). Both *Pl. citri* and *Pl. ficus* are known to produce copious honeydew excretion and thus attracting hemipteran-tending ants, which in turn may protect it against predators and parasitoids (Mgocheki & Addison, 2009; Way, 1963; Way & Khoo, 1992). In particular, in Mediterranean fruit crops such as citrus orchards, ant-mealybug interactions are common, involving different ant species (Cerdá, Palacios, & Retana, 2009;

Pekas, Tena, Agular, & Garcia-Marí, 2011; Zina, Soares, Laranjo, & Franco, 2011; V. Zina and J.C. Franco unpublished data). We observed that *Pl. citri* and *Pl. ficus* are slower mealybugs, and its roundish shape may constrain the abdominal movements, when compared with the faster and elongated *Pseudococcus* species, which may reduce their ability of defense by walking away or abdominal flipping, respectively. However, the lower mobility and active defense of *Planococcus* species might be compensated by ant-mediated protection through higher honeydew excretion in comparison with mealybug species presenting more active defensive behaviors, such as the studied *Pseudococcus* species and *Ph. peruvianus.* The hypothesis that the intensity of ant-tending may differ among mealybug species depending on the amount of honeydew they are capable of excreting is supported by the findings recently published by Zhou, Lu, Zeng, Xu, & Liang (2012). These authors showed that the foraging intensity of the ant *Solenopsis invicta* was directly related with the amount of honeydew produced by tended hemipterans. Differences in honeydew sugar composition among ant-tented hemipterans may also influence the response intensity of foraging ants (Völkl, Woodring, Fischer, Lorenz, & Hoffmann, 1999).

The three types of defense behavior showed by mealybugs against the parasitoid were significantly correlated, suggesting possible direct or indirect relationships among them. Naturally, higher movement capacity may reflect both on higher probability of abdominal flipping and walking away. For reflex bleeding a direct relationship with the other variables is not so obvious. Nevertheless, the differences on the probability of occurrence among the three types of defense behavior are likely a reflex of different cost-benefit balances among them. Dill et al. (1990) showed that the likelihood of two alternative defense behaviors of aphids (dropping *versus* walking away) when attacked by a predator could be predicted using a cost-benefit approach. In the case of mealybugs, we would expect that walking away would present higher cost than abdominal flipping. In order to walk away, additionally to the energetic cost of walking, the mealybug should withdraw the stylets from the host plant tissue and thus possible losing a feeding opportunity. Abdominal flipping does not imply stop feeding and is expected to have lower energetic cost than walking away. Reflex bleeding is expected to have higher cost since this defense reaction is dependent on a secretion from hemolymph (Gullan & Kosztarab, 1997) and is source limited (Gillani & Copland, 1997). These authors observed that the capacity of *Ps. longispinus* producing ostiolar secretions against the attack of the predator *S. fallax* was exhausted after the mealybug releasing six or seven waxy droplets. In agreement with these expected costs, our results showed that in all studied mealybug species abdominal flipping had the highest probability of occurrence, followed by walking away and finally by reflex bleeding.

However, a complete analysis should also consider the benefits of each defense behavior, which were not estimated by us, as we do not know if they are equally effective in preventing parasitism. The benefits can be evaluated by determining the effective impact of each behavior on the rate of parasitism.

Here we studied only the individual defense behavior of mealybugs against a parasitoid. However, collective behavioral defenses have recently been shown to exist in hemipteran living in large aggregates of related individuals, such as aphid colonies (Hartbauer, 2010). The hypothesis of collective defensive behaviors also occurring in mealybugs should be investigated as these insects often aggregate in large colonies of related individuals descending from one or few females (Franco et al., 2009; unpublished data; Nestel, Cohen, Saphir, Klein, & Mendel, 1995). Hamilton's theory of kin selection predicts that collective defense is more likely to evolve in groups consisting of highly related individuals (Hamilton, 1964).

3.4.2. Mealybug immune response

Mealybugs respond to parasitism with variable levels of encapsulation of parasitoid eggs or larvae, depending on different factors such as: i) host and parasitoid species; ii) host physiological age and condition; iii) host and parasitoid origins (or strains); iv) temperature; and v) host plant species and stress conditions (Giordanengo & Nenon, 1990; Blumberg, 1997; Blumberg, Franco, Suma, Russo, & Mendel, 2001; Sagarra, Peterkin, Vincent, & Stewart, 2000; Chong & Oetting, 2007). The probability of *A.* sp. nr. *pseudococci* encapsulation varied among the studied mealybug species. The highest value was registered in *Ps. viburni* and the lowest one in *Ph. peruvianus*, whereas intermediate encapsulation probabilities were registered for the native *Pl. ficus*, the congener *Planococcus* species and for *Ps. calceolariae.* Thus, our data do not support the hypothesis suggested by Blumberg et al. (2001), according to which low levels of encapsulation, corresponding to high physiological adaptation of the parasitoid to the host, should occur for co-evolving hosts or closely related ones. Oppositely, high levels of encapsulation were expected to occur when mealybugs are attacked by parasitoids with no co-evolutionary history. However, coevolution in coupled host-parasitoid systems is expected to involve an arms race between host resistance and parasitoid countermeasures (virulence), and thus no-resistance of the host is unlikely unless the costs of resistance are relatively high (Sasaki & Godfary, 1999). Based on this prediction and on our results, we suggest in alternative to the hypothesis proposed by Blumberg et al. (2001) that both low and high levels of encapsulation by mealybugs may be connected with recent host-parasitoid associations, such as between *A.* sp. nr. *pseudococci* and the two alien mealybugs *Ph. peruvianus* and *Ps. viburni,* respectively.

Intermediate levels are expected in associations between a parasitoid and its principal host or closely related ones, such as between *A.* sp. nr. *pseudococci* and the native *Pl. ficus* or with its closely related species *Pl. citri.* Similar levels of encapsulation in closely related mealybug species may further result from cross resistance (Kraaijeveld, van Alphen, & Godfray, 1998). Our previous finds showing that *A.* sp. nr. *pseudococci* responds to the sex pheromone of *Pl. ficus* (Franco et al., 2008) and uses this kairomone in host location (Franco et al., 2011) suggest an intimate evolutionary relationship between the wasp and this mealybug species. Therefore, *Pl. ficus* is likely the primary host of *A.* sp. nr. *pseudococci* in its region of origin (Franco et al., 2008; 2011), which probably evolved by expanding its host range (Bugila et al., 2014; Franco et al., 2008). Further studies comparing the immune defense of a range of mealybugs in response to the attack by parasitoids with different host selectivity are needed in order to test our hypothesis and further clarify this issue.

The outcome of mealybug resistance through encapsulation is usually associated merely with its survival (Blumberg, 1997; Blumberg et al., 2001). However, immune defenses are maintained at some cost. Evolutionary costs may exist owing to pleiotropic effects or genetic covariance, when the selection for a more effective immune defense correlates with a loss in another trait with fitness relevance. The cost of activating immune defense may further include longer development time or decreased fecundity (Schmid-Hempel, 2005). Nevertheless, there is a lack of knowledge on the eventual costs of parasitoid encapsulation for mealybugs, such as about its effects on fecundity, development time or longevity, which is critical to better understand the impact of different parasitoid species as biological control agents.

The aggregate encapsulation of the studied Portuguese population of *A.* sp. nr. *pseudococci* by *Pl. ficus* (46%) was lower than that reported for the Sicilian ecotype of the parasitoid (58%) by Suma et al. (2012), and for the Turkish ecotype of *A. pseudococci* s.l. (60%) by Güleç et al. (2007), and higher than that registered by Blumberg et al. (1995) in the Israeli ecotype of *A. pseudococci* s.l. (20%). In the case of *Pl. citri,* our estimate (59%) was also lower than that reported for the Sicilian ecotype of *A.* sp. nr. *pseudococci* (75%) (Suma et al., 2012) and higher than the values observed for the Israeli ecotype (39%) (Blumberg et al., 1995). Furthermore, the encapsulation level originated by *Ps. calceolariae*, was not significantly different from that registered for *Pl. ficus* and *Pl. citri,* which apparently contradicts the results reported by Suma et al. (2012) for the Sicilian ecotype of *A.* sp. nr. *pseudococci.* These authors observed a significantly higher level of encapsulation of the parasitoid in this mealybug species (94%). These apparent discrepancies might result in part from different experimental procedures (e.g., time of exposure of the parasitoid to the mealybugs; number of mealybugs per replicate) or parasitoid identity (*A.* sp. nr. *pseudococci* versus *A. pseudococci* s.l.). Yet, geographical differences among populations of the parasitoid and the mealybugs are also likely to occur as a consequence of different evolutionary processes (Thompson, 2001). This hypothesis is supported by the work of Blumberg et al. (2001). The authors compared the immune response of *P. citri* among the combination of three allopatric ecotypes of the mealybug (Portuguese, Sicilian, Israeli) and three allopatric ecotypes of *A. pseudococci* s.l. (Portuguese, Sicilian, Israeli) and observed a high variation on the encapsulation levels (58-88%) among the nine studied combinations. Geographic variation in host resistance and parasitoid virulence has been also documented in other insects, and alternative parasitoids and hosts have been suggested to be the most important determinant of that variation (Kraaijeveld et al., 1998).

As hosts may evolve different defense mechanisms against parasitoids, we may hypothesize that an investment on a defense strategy may eventually compensate a lower level of defense from other adaptations to resist parasitism. For example, the lower level of behavioral defense observed in the two *Planococcus* species against *A.* sp. nr. *pseudococci* could be in part compensated by a moderate-high encapsulation. Nevertheless, the probability of expression of any defense behavior by the studied mealybugs did not correlate with the probability of encapsulation of *A.* sp. nr. *pseudococci*, suggesting that behavioral and immune defenses are independent on mealybugs.

3.4.3. General remarks

Here we present a comparison among mealybug species of both behavioral and immune defenses against a parasitoid. A relationship with the host phylogenetic closeness was found. The native *Pl. ficus* and its congener *Pl. citri* presented the lowest and an intermediate level of behavioral and immune defenses, respectively (Table 3.5). Yet, differences on band evolutionary history on diverse interacting communities might account for the divergences on the behavioral patterns observed. The present results together with those obtained in a previous study on host selection behavior of *A.* sp. nr. *pseudococci* (Bugila et al., 2014) will contribute for a better definition of both the ecological and the fundamental (or physiological) host ranges (Strand & Obrycki, 1996) of this parasitoid. Host suitability will be analyzed elsewhere (Bugila et al., submitted). Altogether, these results will have a practical relevance for the biological control of mealybugs.

Table 3.5 - Relative defense level of the five studied mealybug species against the parasitoid *Anagyrus* sp. nr. *pseudococci*: + lowest level; ++ intermediate level; +++ highest level.

Host mealybug	Behavioral defenses	Immune defenses (encapsulation)	Global defense
Planococcus citri	+	++	+/++
Planococcus ficus	+	++	+/++
Pseudococcus calceolariae	++	++	++
Pseudococcus viburni	+++	+++	+++
Phenacoccus peruvianus	++	+	++/+

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4. Suitability of five mealybug species (Hemiptera, Pseudococcidae) as hosts for the solitary parasitoid *Anagyrus* sp. nr. *pseudococci* (Girault) (Hymenoptera: Encyrtidae)

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Abstract

Anagyrus sp. nr. *pseudococci* is an endoparasitoid which has been used as biological control agent of pest mealybugs. In this study, we compared the suitability of five mealybugs species with different phylogenetic relationships and geographical origins as hosts of this parasitoid. The selected mealybugs included: i) a Mediterranean native species, *Planococcus ficus,* sharing a long co-evolutionary history with the parasitoid; ii) three exotic species, the Afrotropical *Pl. citri*, the Australasian *Pseudococcus calceolariae* and the Neotropical *Ps. viburni*, with a more recent co-evolutionary history; and iii) the Neotropical *Phenacoccus peruvianus,* with no previous common history with the parasitoid. Host suitability was assessed based on different fitness parameters, such as body size, development time, emergence rate, and sex ratio. The parasitoid was able to complete development in all mealybug species. Nevertheless, its emergence rate significantly varied among mealybug species, with the highest values observed in *Pl. ficus* and *Pl. citri,* intermediate values in *Ps. calceolariae,* and the lowest ones in *Ps. viburni* and *Ph. peruvianus.* The body size of adult wasp females varied with host suitability and was positively correlated with other measures of parasitoid fitness, including the emergence rate and the sex-ratio. The parasitoid development time differed among mealybug species, but did not correlate with any other measure of fitness. A female biased sex ratio was found in the parasitoid progeny emerged from all mealybug species, except in *Ps. viburni* and *Ph. peruvianus.* There was a direct relationship between the proportion of females in the parasitoid progeny and the emergence rate.

Key-words: host range, host suitability, scale insect, natural enemy, biological control

4.1. Introduction

Mealybugs are the second largest family of scale insects (Hemiptera: Coccoidea), including many economic important species, which are considered pests of agricultural crops and ornamentals, such as the citrus mealybug *Planococcus citri* (Risso), the vine mealybug *Pl. ficus* (Signoret), the citrophilus mealybug *Pseudococcus calceolariae* (Maskell), the obscurus mealybug *Ps. viburni* (Signoret), and the bougainvillea mealybug *Phenacoccus peruvianus* Granara de Willink (Ben-Dov, 1994; Beltrà et al., 2010; Franco, Zada, & Mendel, 2009; Hardy, Gullan, & Hodgson, 2008). Due to the ineffectiveness of many insecticides used to control pest mealybugs, as well as to their adverse health, ecological and environmental impacts, biological control tactics has been suggested as an environmentally friendly alternative to be considered in integrated pest management strategies for the control of these insect pests (Franco et al., 2009).

Encyrtids are amongst the most successful natural enemies used in biological control programs against mealybugs (Noyes & Hayat, 1994). *Anagyrus pseudococci* (Girault) is a well-known solitary encyrtid endoparasitoid which has been commonly used as a biological control agent of mealybugs of the genera *Planococcus* and *Pseudococcus*, specially against *Pl. citri* (Noyes & Hayat, 1994; Triapitsyn, Gonzalez, Vickerman, Noyes, & White, 2007). However, it was recently shown that *A. pseudococci* s.l. (i.e., *sensu latu*, corresponding to the previous references) in fact comprises two sibling species, *Anagyrus pseudococci* (Girault) and *Anagyrus* sp. nr. *pseudococci* (Girault) (Triapitsyn et al., 2007). *Anagyrus pseudococci* is apparently restricted to Sicily, Argentina (introduced), and Cyprus, whereas *A.* sp. nr. *pseudococci* seems to be more widely distributed, since it has been recorded from many countries including Portugal, Spain, Italy, Greece, Israel, Turkmenistan, South Africa, Brazil and USA (Franco et al., 2011; Guerrieri & Pellizzari 2009; Karamaouna, Menounou, Stathas, & Avtzis, 2011; Mgocheki & Addison, 2009; Triapitsyn et al., 2007). Therefore, there is a need to further investigate the host-parasitoid relationship of both *Anagyrus* species in order to further clarify the taxonomic status of *A.* sp. nr. *pseudococci* and improve their effective use in biological control of pest mealybugs (Triapitsyn et al., 2007).

The success of host-parasitoid relationship involves a number of events including host selection, host suitability, and host regulation (Vinson & Iwantsch, 1980). Host selection comprises a series of behaviors, such as host habitat location, host location, host recognition, and host acceptance (Vinson, 1998). In previous works, we studied the host selection behavior of *A.* sp. nr. *pseudococci,* including host location (Franco et al., 2008, 2011), host recognition and host acceptance (Bugila et al., 2014a). More recently, we also investigated the host defense behavior and immune response of different mealybug species to the attack of the parasitoid (Bugila et al., 2014b). In the present work we aimed at studying host suitability by comparing the ability of *A*. sp. nr. *pseudococci* to complete development in five host mealybugs with different phylogenetic relationships and geographical origins. Host suitability was assessed based on different fitness parameters of the parasitoid, such as body size, development time, emergence rate, and sex ratio.

These five mealybug species from three different genera (*Planococcus, Pseudococcus,* and *Phenacoccus*) were the same selected in previous studies (Bugila et al., 2014a, 2014b): i) a Mediterranean native species, *Pl. ficus*, sharing a long co-evolutionary history with the parasitoid; ii) three exotic species, the possibly Afrotropical *Pl. citri*, the Australasian *Ps. calceolariae* and the Neotropical *Ps. viburni*, with a more recent co-evolutionary history; and iii) a fourth one, the Neotropical *Ph. peruvianus*, with no previous common history with the parasitoid. The phylogenetic relationships of these mealybug species, as well as their possible origin and history of introduction in the Mediterranean basin were described in Bugila et al. (2014a).

4.2. Material and Methods

4.2.1. Mealybug rearing

Feral mealybugs were collected from different regions and host plants according to each species habitat in order to start the rearing in laboratory (Table 4.1). All five studied mealybug species were reared on sprouted potatoes (*Solanum tuberosum* L.) under controlled conditions (25.0±0.5ºC, 55-65% R.H., in the dark) in individual climatic chambers (FitoClima, ARALAB) during multiple generations to provide the necessary individuals for the experiments. Mealybug colonies were regularly refreshed by adding new individuals collected from the field. Before the beginning of the experiments, third instars of each mealybug species were isolated on sprouted potatoes within ventilated plastic boxes kept for seven days under the same controlled conditions referred before, to standardize age, physiological state and obtain pre-reproductive adult females (Bugila et al., 2014a).

4.2.2. Parasitoid rearing

Feral individuals of *Anagyrus* sp. nr. *pseudococci* were obtained from parasitized adult females of *Pl. citri* collected in citrus orchards in the region of Silves (Portugal) and reared for multiple generations within ventilated plastic boxes on *Pl. citri* under controlled conditions (25.0±0.5ºC, 55-65% R.H., 16L:8D). To obtain naive adult female wasps, the rearing plastic boxes were first observed and kept free of parasitoids, and then checked every 24h, in order to collect wasps with less than 24h old. Two males and one female were then moved to a new box containing one drop of honey as food in which they were maintained for 72h under laboratory conditions, in order to allow mating and feeding before the experiments (Bugila et al., 2014a).

Table 4.1 - Origin of the studied mealybug populations.

Mealybug species	Region	Host plant
Planococcus citri	Silves (Mainland, Portugal)	Sweet orange
Planococcus ficus	Tavira (Mainland, Portugal)	Vineyard
Pseudococcus calceolariae	Loulé (Mainland, Portugal)	Sweet orange
Pseudococcus viburni	Biscoitos (Azores, Portugal)	Vineyard
Phenacoccus peruvianus	Queluz (Mainland, Portugal)	*Bougainvillea glabra*

4.2.3. Experiments

The experiments were conducted between 12:00h and 19:00h, under laboratory conditions (19-22°C and 55-65% R.H.), using 20 replicates for each of the five studied mealybug species. Each replicate consisted of one naïve adult parasitoid female exposed to 10 pre-reproductive adult mealybug females in a Petri-dish (9cm diameter) during 30 min (Bugila et al., 2014a). During this time observations were carried out and the number of ovipositions by each female wasp was registered. Then the parasitoid female was removed and all Petri-dishes with the exposed mealybugs were maintained under controlled conditions (25.0±0.5ºC, 55-65% R.H., 16L:8D) until wasp progeny emergence. The gender of each emerged wasp was identified and the wasps kept in separate vials for further analysis.

4.2.4. Size of wasp female progeny

The mean size of an adult female of *A.* sp. nr. *pseudococci* progeny was estimated based on the hind tibia length (Chong & Oetting, 2007; Sagarra, Vincent, & Stewart, 2001a; West, Flanagan, & Godfray, 1996). With that purpose, the left hind tibia of the emerged adult females was removed and mounted on microscope slides and then measured under a binocular microscope (100X magnification). Measurements were carried out in at least five specimens per host species.

4.2.5. Host size

The size of the adult mealybug females was estimated based on the projected area (mm^2) of the body, assuming an elliptic shape. This parameter (A) was determined using the following equation, corresponding to the area of an ellipse:

$A = LW\pi/4$

where L and W are the length and width of the female body, respectively.

The measurements of the body length and width of mealybug females were carried out using image capture software (Jenoptik ProgRes CT5, Germany) connected to a stereomicroscope (20X magnification; Meiji Techno EMZ-13TR, Japan). A total of 3-4 specimens were used for each mealybug species.

4.2.6. Data and statistical analysis

The number of parasitized mealybugs, as well as the number of emerged wasps and their gender was recorded per replicate. These data were used for estimating the rate of emergence of the parasitoid (number of emerged wasps/number of parasitized mealybugs) and the parasitism rate (number of emerged wasps per 10 exposed mealybugs). The number of days since oviposition until emergence was recorded for each wasp offspring as a measure of its development time.

The rate of emergence of *A.* sp. nr. *pseudococci* and tibia size of wasp adult females were compared among host species by one-way ANOVA. Development time of emerged individuals was analyzed using full factorial two-way ANOVA, considering the factors gender of the progeny and host species. Differences among host species were subsequently tested by LSD test. Normality assumption was previously tested by Kolmogorov-Smirnoff test. Relationship between variables was tested by Pearson bivariate correlation.

Sex ratio was analyzed by using Generalized Linear Model with Binomial model distribution considering the binary dependent variable (male, female), and host species as predictor variable. A logistic regression was used, to relate the probability of female progeny (dependent variable) with the rate of emergence of the parasitoid (explanatory variable).

Statistical analyses were performed using IBM SPSS 20.0 for Windows (IBM Corporation, Armonk, New York, USA).

4.3. Results

4.3.1. Emergence and parasitism rate

The emergence rate of *A.* sp. nr. *pseudococci* significantly varied among mealybug species ($F_{4, 95}$=16.59, *p*<0.001), with the highest values observed in *Planococcus* spp. (Table 4.2). Intermediate values were found in *Ps. calceolariae,* whereas *Ps. viburni* and *Ph. peruvianus* exhibited the lowest ones.

Table 4.2 - Parasitism rate and emergence rate of *Anagyrus* sp. nr. *pseudococci* for each studied mealybug species.

Mealybug species	Emergence rate (%) *	Parasitism rate (%)
Planococcus citri	65.6±6.23a	22.5±2.28b
Planococcus ficus	67.0±5.97a	31.5±3.42a
Pseudococcus calceolariae	40.6±7.98b	15.0±2.67c
Pseudococcus viburni	14.8±4.44c	4.5±1.35d
Phenacoccus peruvianus	16.8±5.90c	5.5±1.53d

*Within columns, means followed by the same letter are not significantly different (P=0.05)

The parasitism rate originated by the parasitoid was also significantly dependent ($F_{4, 95}$=23.30, *p*<0.001) on the host species (Table 4.2). The highest value was registered in *Pl. ficus* and the lowest ones were observed in *Ps. viburni* and *Ph. peruvianus*. *Planococcus citri* and *Ps. calceolariae* showed intermediate values of parasitism.

4.3.2. Development time

The development time of *A.* sp. nr. *pseudococci* significantly varied with both the progeny gender ($F_{1,4}$=15.86, p<0.001) and the host species ($F_{4,4}$=14.761, p<0.001). No significant interaction was found between the two factors, host species and progeny gender ($F_{4,148}$=1.398, p<0.237). The development time of female wasps in *Pseudococcus* spp. was significantly higher than in the other mealybug species (Table 4.3). Intermediate values were found in *Pl. citri* and *Pl. ficus*. Finally, a significantly lower development time of the parasitoid was observed in *Ph. peruvianus*. This parameter registered higher values in female wasps (20.4±0.23) than in males (18.7±0.36). Mean development time of female wasps was significantly correlated with that of males for all mealybug species (*r*=0.99, n=5, *p*<0.001).

Table 4.3 - Development time, sex ratio, and body size of the progeny of *Anagyrus* sp. nr. *pseudococci* originated from each of the studied mealybug species.

Mealybug species	Development time of progeny females (days)*	Proportion of progeny females	Tibia length of progeny females (x 10^{-3} mm)
Planococcus citri	19.8±0.30b	0.60±0.07b	0.56±0.008b
Planococcus ficus	18.5±0.15c	0.83±0.05a	0.58±0.005a
Pseudococcus calceolariae	23.3±0.51a	0.79±0.07ab	0.53±0.011c
Pseudococcus viburni	22.4±0.84a	0.33±0.10c	-
Phenacoccus peruvianus	17.8±0.48c	0.18±0.08c	0.47±0.015d

*Within columns, means followed by the same letter are not significantly different (P=0.05)

4.3.3. Sex ratio of progeny

The proportion of female progeny of *A.* sp. nr. *pseudococci* significantly differed among mealybug species (Wald χ^2=38.35, p<0.001). More females than males of the parasitoid emerged from all tested mealybugs, except in *Ps. viburni* and *Ph. peruvianus* (Table 4.3). The proportion of parasitoid female progeny was highest in *Pl. ficus*, followed by *Ps. calceolariae* and *Pl. citri*. *Planococcus ficus* showed for the same parameter significantly higher values compared to all other mealybug species, except for *Ps. calceolariae* (p=0.685). On the other hand, *Ps. viburni* and *Ph. peruvianus* showed significantly lower values than other mealybug species. The probability of parasitoid female progeny was significantly explained by the rate of parasitoid emergence through logistic regression (χ^2=24.72, df=1, p<0.001). From the regression parameter estimate (β=0.575±0.116) we predicted that the parasitoid emergence would increase 1.8-fould for females than males.

4.3.4. Host size

The size of the adult female mealybugs significantly differed among species ($F_{4,16}$=25.95, p<0.001) varying according to the following sequence, from the largest to the smallest species: *Ps. calceolariae, Pl. citri*, *Ps. viburni, Pl. ficus,* and *Ph. peruvianus* (Table 4.4).

4.3.5. Size of wasp adult females

The size of the adult females of *A.* sp. nr. *pseudococci,* expressed as hind tibia length, varied significantly with the host species ($F_{3,66}$=18.71, p<0.001; Table 4.3). The size was higher on *Pl. ficus*, followed by *Pl. citri*, then *Ps. calceolariae* and finally *Ph. peruvianus*. Body size could not be determined in the wasps emerged from *Ps. viburni* due to the accidental loss of the low number of wasp females obtained from this mealybug species. A high and significant correlation was found between the size of the tibia of wasp females and the emergence rate of the parasitoid (Figure 4.1). The body size of the adult female of the parasitoid did not correlate with the size of the host species (*r*=0.282, *n*=4, *p*=0.718).

Table 4.4 - Length, width and area of female body of the studied mealybug species.

Mealybug species	Length (mm)*	Width (mm)	Area (mm^2)
Planococcus citri	3.00±0.06b	1.80±0.06b	4.25±0.22b
Planococcus ficus	2.47±0.03d	1.47±0.03c	2.84±0.10cd
Pseudococcus calceolariae	3.20±0.06a	2.00±0.09a	5.03±0.32a
Pseudococcus viburni	2.80±0.06c	1.53±0.03c	3.37±0.14c
Phenacoccus peruvianus	2.17±0.07d	1.33±0.03c	2.27±0.13d

*Within columns, means followed by the same letter are not significantly different (P=0.05)

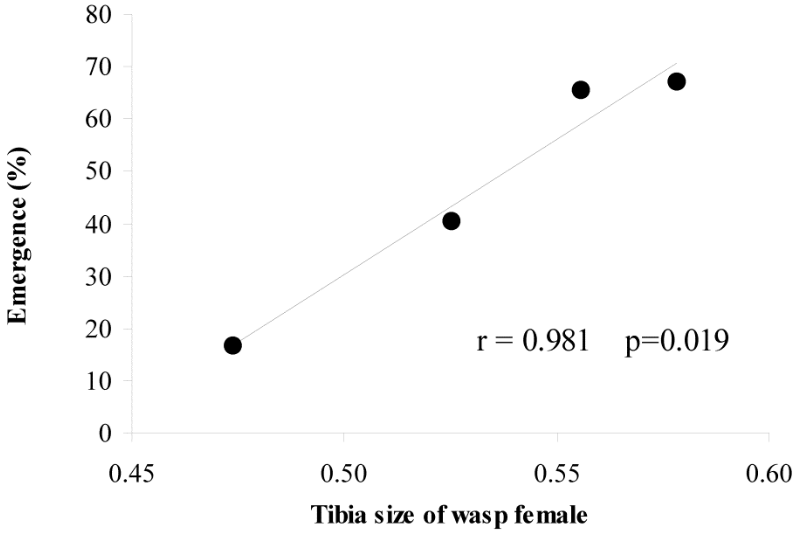

Figure 4.1 - Relationship between the emergence rate of *Anagyrus* sp. nr. *pseudococci* and the size (x 10^{-3} mm) of adult female progeny of the parasitoid according to the host species.

4.4. Discussion

Fitness of endoparasitoid adult females is directly influenced by host characteristics through larval development of its offspring (Firlej, Lucas, Coderre, & Boivin, 2007). Therefore, it is expected that parasitoid females will recognize and accept the hosts that will allow the development of larvae and optimize their fitness, based on external and internal characteristics which are monitored through antennation and ovipositor probing, respectively (Firlej et al., 2007; Vinson, 1998). The ability of a parasitoid completing development is related with the host suitability which in turn is dependent on several factors, such as: i) host immune defenses; ii) host nutritional suitability; iii) presence in the host of substances toxic to the immature parasitoid; and iv) environmental factors (Vinson & Iwantsch, 1980). Therefore, we may divide hosts in three different classes according to their quality: i) suitable hosts, in which most of the parasitoid larvae are allowed to complete development; ii) marginal hosts, in which only a small percentage of parasitoid individuals will develop; and iii) unsuitable hosts, in which no parasitoid development will occur (Firlej et al., 2007). The successful parasitism also depends on the ability of parasitoids manipulating host physiology through gene products (e.g., venom, polydnaviruses, teratocytes), which eventually will benefit the survival and development of the parasitoid, namely by suppressing host immune defenses (e.g., encapsulation), and increasing nutrient availability (Harvey, 2005; Pennacchio & Strand, 2006; Strand & Casas, 2008).

In an earlier study, we observed that the behavioral pattern of host recognition and the level of host acceptance of *A.* sp. nr. *pseudococci* significantly differed among the five mealybug species here studied (Bugila et al., 2014a). More recently, we showed that the rate of host acceptance by *A.* sp. nr. *pseudococci* might be affected by the level of behavioral defenses of each mealybug species (Bugila et al., 2014b). Here we investigated the host suitability of those mealybug species for the development of *A.* sp. nr. *pseudococci* and tested if, as predicted, female wasps really know the best for their progeny. Our results showed that the parasitoid was able to complete development in all five studied mealybug species, despite the fact of these hosts representing three different genera (*Planococcus, Pseudococcus,* and *Phenacoccus*) which are not closely phylogenetically related (Downie & Gullan, 2004; Hardy et al., 2008). Nevertheless, the emergence rate of the parasitoid significantly varied among mealybug species, with the highest values observed in the native *Pl. ficus* and the phylogenetically related *Pl. citri,* intermediate values in *Ps. calceolariae,* and the lowest ones in *Ps. viburni* and *Ph. peruvianus* (Table 4.2). Thus, the observed differences in host suitability apparently reflect the phylogenetic relationships of the studied mealybug species and the differences in their co-evolutionary history with *A.* sp. nr. *pseudococci*.

To test the hypothesis that female wasps are capable of accepting or rejecting a potential host in function of its suitability for their progeny we correlated the emergence rate of *A.* sp. nr. *pseudococci* with the level of host acceptance (i.e. number of parasitized individuals per 10 exposed mealybugs) observed by Bugila et al. (2014a) for the same mealybug species: 3.0±0.4 (*Pl. citri*), 2.2±0.3 (*Pl. ficus*), 1.4±0.3 (*Ps. calceolariae*), 1.6±0.3 (*Ps. viburni*), 1.1± 0.2 (*Ph. peruvianus*). The correlation between these two parameters is not significant (r=0.818, n=5, p =0.091), indicating that in the case of *A.* sp. nr. *pseudococci* not always “mother knows the best” (Henry, Gillespie, & Roitberg, 2005). This output was mainly due to the fact that the parasitoid emergence rate from *Ps. viburni* (14.8%) was lower than expected based on the corresponding level of host acceptance and compared to that of *Ps. calceolariae* (40.6%), for which the parasitoid showed a similar level of host acceptance. Similar results suggesting that the assessment of host quality by female wasps is not perfect have been also reported for other parasitoid species (e.g., Henry et al., 2005; Sagarra, Vincent, & Stewart, 2001b). However, these apparent wrong decisions of the wasp females in host acceptance may favour the recruitment of new host species, as it will be discussed later.

The parameters of *A.* sp. nr. *pseudococci* fitness including the rate of emergence, the body size of adult females, the progeny sex ratio, and the development time significantly differed among host species. As mentioned before, the emergence rate of a parasitoid is dependent on different factors, including host immune defences, host nutritional suitability, presence of toxic substances within the host, and environmental factors. Encapsulation is a common immune defense mechanism of mealybugs against the eggs and larvae of their parasitoids (Blumberg, 1997; Blumberg, Klein, & Mendel, 1995; Blumberg & van Driesche, 2001; Sagarra, Peterkin, Vincent, & Stewart, 2000; Suma et al., 2012b). In a previous work we found that the probability of encapsulation of *A.* sp. nr. *pseudococci* was highest in *Ps. viburni* (0.86), lowest in *Ph. peruvianus* (0.20), and intermediate in *Pl. citri* (0.52), *Ps. calceolariae* (0.45), and *Pl. ficus* (0.43) (Bugila et al., 2014b). Therefore, the observed differences in the emergence rate of the parasitoid are apparently not explained only by the different level of encapsulation in the mealybug species. This suggests that besides encapsulation other factors related with host quality are also involved. Considering that the experimental environmental conditions were the same for all five mealybug species, differences among mealybug species in the presence of toxic substances accumulated from the host plant or in the environmental factors are unlikely. Thus, host nutritional suitability is possibly other factor involved.

Female size is an important measure of parasitoid fitness and is known to influence other fitness parameters, including mating capacity, dispersal, longevity, fecundity and reproductive rate (Godfray, 1994; Harvey, 2005; Jervis & Copland, 1996). In the present work, we found as expected that the body size of adult females of *A.* sp. nr. *pseudococci* varied with host suitability and was positively correlated with other measures of parasitoid fitness, such as the emergence rate and the sex-ratio of the progeny. Similar relationships between female body size and other fitness parameters have been also reported for other *Anagyrus* species, as for example *A. kamali* (Sagarra et al., 2001b).

The effects of development time on parasitoid fitness are still little known. A trade-off between this parameter and parasitoid body size may exist, that is a faster development can occur at the expense of a reduction in body size and *vice versa* (Harvey, 2005; Harvey & Strand, 2002). In our study, the development time of *A.* sp. nr. *pseudococci* varied with host species, but did not correlate with any other measure of parasitoid fitness. Still, development time was similar within each host genus, being highest in *Pseudococcus*, intermediate in *Planococcus* and lowest in *Ph. peruvianus*. In opposition to other fitness parameters, no clear relationship between parasitoid development time and host suitability was found. The development time was longer in females than in males. This result is apparently in contrast with those reported in other studies for *A.* sp. nr. *pseudococci* (Karamaouna et al., 2011; Suma et al., 2012a) and *A. pseudococci* s.l. (Avidov et al., 1967) in which no significant differences on development time were observed between wasp genders. However, as suggested by Gülec, Kilincer, Kaydan, and Ülgentürk (2007) differences in development time between male and female wasps might be related with host stage. These authors observed similar development time on male and female wasps when *A. pseudococci* s.l. developed on third instar mealybugs, whereas a shorter development time was registered for male wasps emerging from adult female mealybugs. Our results are consistent with those obtained by Gülec et al. (2007) for adult female mealybugs.

Haplodiploidy is the sex determination system of most of the hymenopteran parasitoids, in which haploid males originate from unfertilized eggs, whereas diploid females are the result of fertilized eggs (Jervis & Copland, 1986). Therefore, female wasps are able to control the sex of their progeny by regulating the release of sperm from spermatheca during oviposition. Sex allocation in parasitoids is known to be influenced by host quality. Sex ratio theory predicts that female wasps should oviposit female eggs in higher quality hosts and male eggs in lower quality hosts, as females are considered the sex in which the increment gain in fitness per host is higher (Charnov, 1982; Godfray, 1994; King, 1987; West, Reece, & Sheldon, 2002). In the case of *A. pseudococci* s.l., it has been shown that the sex ratio is influenced by the host stage/size within the same mealybug species, with male biased sex ratio observed in third instars or younger host stages, and female biased ones in adult female mealybugs (Suma et al., 2012a and references therein). Here we investigated whether differences in host quality across the five studied mealybug species would affect the sex ratio of *A.* sp. nr. *pseudococci* progeny. We found a significant relationship between this parameter and the emergence rate of the parasitoid. That is, the proportion of females in the parasitoid progeny was highest (female biased sex ratio) in the native mealybug species, *Pl. ficus* and lowest (male biased sex ratio) in the alien Neotropical mealybug species, *Ps. viburni* and *Ph. peruvianus*. Apparently, the observed relationship between the two parameters is not explained by host-size variation among mealybug species, as no significant correlation was found between host size and offspring sex-ratio of *A.* sp. nr. *pseudococci*. Therefore, besides host size other factors of host quality, host immune defenses and host nutritional suitability were possible responsible for the registered differences in sex allocation by the parasitoid females among mealybug species. Nevertheless, we cannot exclude the hypothesis that the observed sex ratios might also have resulted from different survival rate of female and male wasps, depending on host quality, as we did not determine the primary sex ratio in present study. Taken together, the available data suggest that sex ratio of progeny can be used as a predictor of host suitability in *A.* sp. nr. *pseudococci*.

The higher emergence rate, larger tibia length of wasp females, and higher proportion of progeny females clearly indicate that *Pl. ficus* is the most suitable host for *A.* sp. nr. *pseudococci*, closely followed by *Pl. citri*. In contrast, the lower emergence rate, smaller tibia length of wasp females, as well as the male biased sex ratio registered in the parasitoid progeny obtained from *Ps. viburni* and *Ph. peruvianus* suggest that these two mealybug species are poor quality hosts for *A.* sp. nr. *pseudococci*, and thus may be considered marginal hosts. *Pseudococcus calceolariae* seems to be in an intermediate position.

Our results showing that *A.* sp. nr. *pseudococci* is capable of developing in not closely related mealybug species corroborate its generalist behavior suggested in previous studies on host recognition and acceptance (Bugila et al., 2014a), in contrast with other congeneric species which display a much higher degree of specialization. For example, *Anagyrus kamali* Moursi and *Anagyrus amnestos* Rameshkumar, Noyes & Poorani (=*Anagyrus* sp. nr. *sinope* Noyes & Menezes) are only able to complete development in their principal host species, respectively the hibiscus mealybug, *Maconellicoccus hirsutus* and the Madeira mealybug, *Ph. madeirensis*, although the first parasitoid species may accept to oviposit in a few unsuitable hosts (Chong & Oetting, 2007; Rameshkumar, Noyes, Poorani, & Chong, 2013; Sagarra et al., 2001b). On the other hand, *Anagyrus* sp. nr. *pseudococci* has apparently a close evolutionary relationship with *Pl. ficus*, since the parasitoid shows an innate kairomonal response to the sex pheromone of the vine mealybug (Franco et al., 2008) and uses this kairomone in host location (Franco et al., 2011). Overall, the available information, including its host selection behavior and apparent realized host range, indicates that *A.* sp. nr. *pseudococci* evolved from a specialist to a more generalist strategy (Bugila et al., 2014a; Franco et al., 2008). Therefore, we suggest that the host range of *A.* sp. nr. *pseudococci* might have evolved according to the “host-ecology hypothesis” (Shaw, 1994; Tschopp, Riedel, Kropf, Nentwig, & Klopfstein, 2013; Zaldivar-Riverón et al., 2008), which assumes that a parasitoid may expand its host range by recruiting new host species within its searching niche. The data on the host acceptance behavior (Bugila et al., 2014a) and host suitability (here presented), as well as the records of mealybug parasitism from field samples (Beltrà, Tena, & Soto, 2013; Franco et al., 2011; Guerrieri & Pellizzari, 2009; Karamaouna et al., 2011; Mgocheki & Addison, 2009; Triapitsyn et al., 2007) indicate that *A.* sp. nr. *pseudococci* has been expanding the host range from its possible original host, *Pl. ficus* (Franco et al., 2008) by recruiting new host species, specially within the genera *Planococcus* and *Pseudococcus,* but also in *Phenacoccus*. For example, in field surveys carried out in Spain, the parasitoid was found, although in very low numbers, parasitizing *Ph. peruvianus,* which has recently invaded Europe (Beltrà et al., 2010, 2013). It is expected that the recruitment of new hosts will be possible only in parasitoid species presenting a not very selective behavioral pattern of host acceptance, which eventually may oviposite in marginal or even unsuitable hosts. *Anagyrus* sp. nr. *pseudococci* fits this behavioral pattern.

In conclusion, *Pl. ficus* was the most suitable host species for *A.* sp. nr. *pseudococci* corroborating the hypothesis of a close evolutionary history of the parasitoid with this Mediterranean-native mealybug. Host suitability of the studied mealybug species seems to fit a phylogenetic/biogeographic trend, showing the highest level in *Pl. ficus* and its closely related congener *Pl. citri*, followed by the Australasian *Ps. calcelolariae,* and the Neotropical *Ps. viburni* and *Ph. peruvianus*. The results have also implications in the effective use of *A.* sp. nr. *pseudococci* for the biological control of pest mealybugs. By adding data on host suitability, we complemented the information collected in previous studies on host recognition/acceptance behavior (Bugila et al., 2014a), and host defenses (Bugila et al., 2014b), allowing a more clear picture on the host selectivity and host range of the parasitoid.

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5. Functional response of the solitary parasitoid of mealybugs *Anagyrus* sp. nr. *pseudococci* (Hymenoptera, Encyrtidae): comparative analysis between a native and an alien host species

Abstract

Anagyrus sp. nr. *pseudococci* is a solitary parasitoid worldwide used in biological control of pest mealybugs. In this work, we compared the functional response of *A.* sp. nr. *pseudococci* between a Mediterranean native host species, *Planococcus ficus* and an alien mealybug species, *Pseudococcus calceolariae*. Densities of 2, 5, 10, 20, 30, 40, 50 and 60 pre-reproductive adult females of each of the two studied mealybug species were exposed to mated and fed parasitoid females during 24h. The number and gender of the emerged parasitoid offspring was determined. The results showed that the functional response of the parasitoid varied between host species. A type II response was observed for *Ps. calceolariae,* whereas for *Pl. ficus* a type III model better describe the response of the parasitoid. The upper limit of the functional response, i.e. the highest number of progeny produced per wasp, was about three times higher in *Pl. ficus* (18.1±5.40) than in *Ps. calceolariae* (6.31±1.24). The estimated handling time of the parasitoid was longer in *Ps. calceolariae* (0.159 d) than in *Pl. ficus* (0.067 d). The proportion of female progeny was significantly higher in *Pl. ficus* (0.78±0.02) than in *Ps. calceolariae* (0.70 ±0.02) (*P*=0.031). However, the progeny sex ratio was not affected by host density (*P*=0.824). The results are discussed in terms of host traits and practical implications for biological control.

Key-words: host density, handling time, sex ratio, *Planococcus ficus, Pseudococcus calceolariae,* Pseudococcidae, biological control

5.1. Introduction

The efficiency of a biological control organism depends in great part on its response to the variation in population density of its host/prey (Mills & Getz, 1996). Two non-exclusive mechanisms can play a role in this density-dependent relationship: i) a numerical response, in which the predator/parasitoid responds to the increase in prey/host density by increasing its reproductive or migratory rate; and ii) a functional response, where the response is translated by an increase in the number of prey/host consumed per individual and per unit of time (van Alphen & Jervis, 1996). Three main types of functional response have been considered, in function of the curve shape (Holling, 1959; van Alphen & Jervis, 1996). In type I, a positive linear relationship is assumed; type II is described by a decelerated curve, with a constantly decreasing rate; whereas in type III the relationship is sigmoid, initially accelerating and then decelerating. In any case, there is a saturation level, corresponding to a maximum in the number of prey/host attacked per predator/parasitoid, imposed by its behavioral and physiological characteristics. In terms of mortality rate the three types of curves result in a constant (type I), decreasing (type II), or modal variable, with an initial increasing and then decreasing curve (type III).

For population biologists, the density response, linking two trophic levels, greatly explains the fluctuation dynamics of predator/prey or parasitoid/host populations in an interdependent way (Hassell, 2000). Functional and numerical responses can be used for evaluating the potential of a parasitoid to regulate the populations of its host species (Luck, van Lenteren, Twine, Juenen, & Unruh, 1979; Murdoch & Briggs, 1996). The stabilization and destabilization of the population dynamics in host-parasitoid interactions has been associated with type III and type II functional responses, respectively (Chong & Oetting, 2006; Hassell, 1978; Murdoch, & Oaten, 1975). Functional responses may also be used in biological control inundative releases for estimating the optimal dose to obtain a fast decrease in pest numbers (Chong & Oetting, 2006; Mills & Lacan, 2004). Nevertheless, some authors question the relevance of the functional response for the success of biological control (Fernández-Arhex & Corley, 2003; Lester & Harmsen, 2002).

Anagyrus sp. nr. *pseudococci* (Girault) is a solitary koinobiont parasitoid of the vine mealybug, *Planococcus ficus* (Signoret) and the citrus mealybug, *Pl. citri* (Risso) (Hemiptera: Pseudococcidae), among other mealybug species of economic importance, which has been recently separated from its sibling species *A. pseudococci* (Girault) (Triapitsyn, González, Vickerman, Noyes, & White, 2007). Since then, several studies have been conducted in order to clarify its host-parasitoid relationships, including the kairomonal response to host sex pheromone (Franco et al., 2008, 2011), host selection behavior (Bugila, Branco, Silva, & Franco, 2014a), host defenses (Bugila, Franco, Silva, & Branco, 2014b; Suma et al., 2012b), and host suitability (Bugila, Franco, Silva, & Branco, 2014c; Suma et al., 2012a). In the present work, we aimed at investigating the functional response of *A.* sp. nr. *pseudococci* by comparing two host species with different evolutionary relationships with the parasitoid, as well as different geographical origin: the vine mealybug, *Planococcus ficus* (Signoret), a Mediterranean native host species which is considered the primary host of *A.* sp. nr. *pseudococci* in its region of origin (Franco et al., 2008), and the citrophilus mealybug, *Pseudococcus calceolariae* (Maskell), an Australasian alien species (Pellizzari & Germain, 2010). The parasitoid is believed to have a close evolutionary relationship with *Pl. ficus,* whereas its relationship with *Ps. calceolariae* is much more recent, as this mealybug species possibly invaded the Mediterranean basin only few centuries ago (Bugila et al., 2014a and references therein). Our main aim was to test if the functional response of the parasitoid could be affected by the host species, depending on its evolutionary history. Besides the effect on the progeny production by the parasitoid, we also considered the effect on sex allocation as an indicator of fitness. All together, the accumulated knowledge on the host-parasitoid relationships will contribute to further clarify the taxonomic status of *A.* sp. nr. *pseudococci,* as well as to improve its effective use as a biological control agent of pest mealybugs.

5.2. Material and Methods

5.2.1. Mealybug rearing

Specimens of the two mealybugs species *Pl. ficus* and *Ps. calceolariae* were collected in Algarve, Portugal, from vineyards and sweet orange orchards, respectively. The collected individuals were used to start laboratory colonies. The two mealybug species were reared on sprouted potatoes (*Solanum tuberosum* L.) under controlled conditions (25.0±0.5^{o}C, 60-70% r.h., in the dark). Seven days before the experiments, third instars of each species were isolated on sprouted potatoes within ventilated plastic boxes to standardize age, physiological state and obtain pre-reproductive adult females and kept at laboratory conditions as described above.

5.2.2. Parasitoid rearing

Specimens of *A.* sp. nr. *pseudococci* were obtained from parasitized colonies of *Pl. citri* collected in citrus orchards in the region of Silves (Algarve, Portugal) and reared within ventilated plastic boxes on *Pl. citri* under laboratory conditions (25.0±0.5^{o}C, 60.0-70% r.h., and photoperiod 16L:8D). To obtain naive adult female wasps, the rearing boxes were first observed and kept free of parasitoids, and then checked every 24h, in order to collect wasps less than 24h old. For each replicate, two males and one female were introduced into a plastic box containing one drop of honey as food and maintained for 72h under laboratory conditions until the setup of the experiments for allowing mating.

5.2.3. Experiments

Eight densities of each of the two studied mealybug species (2, 5, 10, 20, 30, 40, 50, and 60 adult females) were exposed to the parasitoid. For *Pl. ficus* density 70 was further tested. For each density 20 replicates were performed in separated boxes. For each of the 20 replicates considered in each mealybug density, the mealybugs were exposed inside a plastic box to one mated and fed adult female during 24h under controlled conditions (24°C, 60-70% r.h., and photoperiod 16L:8D). After the exposure period the parasitoid was removed from the box and the mealybugs were kept under the same controlled conditions until the emergence of the parasitoid progeny. The total number of emerged wasps per replicate was recorded, as well as the corresponding gender of each individual.

5.2.4. Model fitting and data analysis

Model fitting was done in two steps. In a first step, we used a logistic regression to model the proportion of parasitized host mealybugs, $p=Na/No$, considering a binomial response. The model was fitted to all data using Generalized Linear Models (GLM) and maximum likelihood estimation techniques. The functional response data satisfy the assumptions of logistic regression analysis and this method is considered more robust than applying least squares techniques (Trexler, Charles, & Joseph, 1988). As dependent variables, we used linear, quadratic and cubic terms of the host density. The sign of the parameter estimates for the polynomial equation allows the differentiation between types of functional response models. A negative estimate for the linear term indicates type II model, whereas a positive estimate for the linear term associated with a negative quadratic term reveals type III model (Griffen & Delaney, 2007; Chong & Oetting, 2007). Plotting the proportion of parasitized mealybugs against mealybug density allowed further confirmation of the type of functional response. A decreasing function reveals type II model, whereas a modal curve confirms Type III model.

In a second step, we fitted by non-linear regression the two types of models, according to the following equations:

Type II $N_a = No(1\text{-}exp(-ab/(b+aNo)))$ (eq. 1)

$N_a = a\,N_o/(1+(a/b)N_o))$ (eq. 2)

Type III $N_a = N_o(1\text{-}exp(-aN_o/(1+cN_o+(a/b)N_o^2)))$ (eq. 3)

$N_a = a\,N_o^2/(b^2+N_o^2)$ (eq. 4)

whereas N_a is the number of parasitized mealybugs, N_o is the total number of available hosts and a, b and c are constant parameters determined by model fitting.

We used the mean estimate of parasitoid progeny for each initial host density to fit equations (1) to (4). Several initial set of values were used for the parameters a, b, and c, to guarantee best and unique parameter estimate and eliminate the possibility of local minima estimates. From Holling type II model, the prey capture rate increases linearly with the prey density. The handling time is thus constant allowing to estimate the handling time of the parasitoid h, i.e. the average time spent in host processing, using the following equation: $h=1/b$ (Holling, 1959).

A univariate ANOVA was used to analyse differences in the progeny sex ratio between host species, considering the initial host density as covariate.

Data are presented as mean ± standard error (SE), unless otherwise referred.

5.3. Results

5.3.1. Functional response

The average maximum number of parasitized mealybugs, indicating the threshold of the functional response, i.e. the highest number of progeny produced by wasp, was 18.4±9.34 wasps for *Pl. ficus* and 6.2±0.02 wasps for *Ps. calceolariae* (Figure 4.1). The proportion of parasitized mealybugs varied between 0.20 and 0.45 for *Pl. ficus* and between 0.04 and 0.58 for *Ps. calceolariae*. The shape of the function relating the proportion of parasitized mealybugs with host density further indicates a modal function for *Pl. ficus,* whereas a monotonous decreasing function is observed for *Ps. calceolariae* (Figure 5.1).

Results from the logistic regression support a type II model for *Ps. calceolariae* with a negative parameter estimate for the linear term (Table 5.1). Yet, for *Pl. ficus* a positive linear trend together with a negative quadratic term suggests a type III model (Table 5.1). Model fitting was better adjusted for *Ps. calceolariae* than for *Pl. ficus* as indicated by the likelihood ratio Chi-Square (Table 5.1).

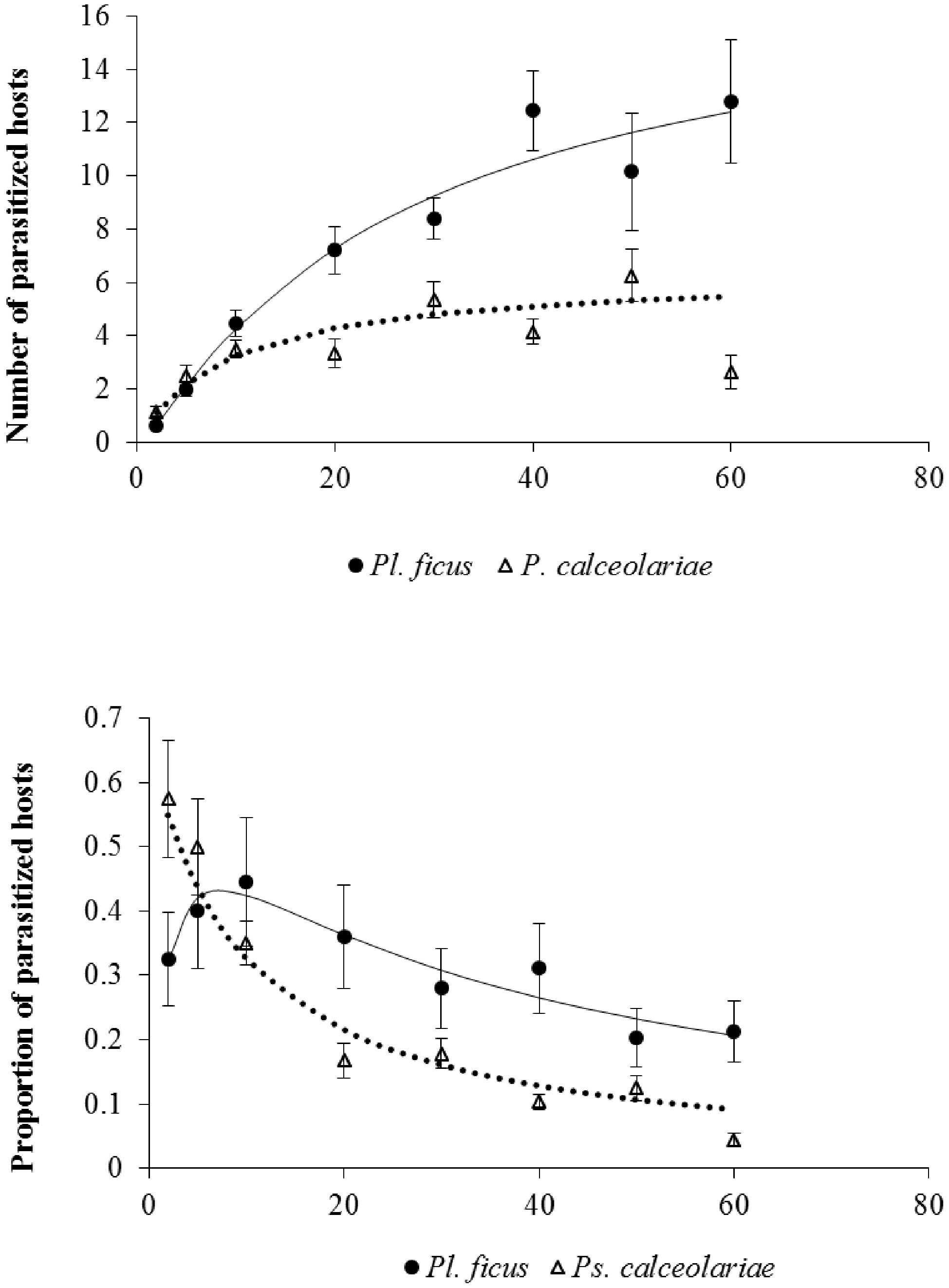

Figure 5.1 - Relationship between mean (±SEM) density (number of exposed adult female mealybugs) of the mealybug species *Planococcus ficus* (●) and *Pseudococcus calceolariae* (△) and the number (top) and proportion (bottom) of parasitized mealybugs by *Anagyrus* sp. nr. *pseudococci*. The solid and dashed lines represent the best-fitted functional response curves for *Pl. ficus* (type III model) and *Ps. calceolariae* (type II model), respectively.

Both Type II and type III models were fitted by non-linear regression. Due to difficulties with model convergence, host density N=70 for *Pl. ficus* was excluded from the non-linear regression analysis. Type III equation (3) provided better fit to *Pl. ficus* but was only slightly better than type II model equation (Table 5.2). Parameter estimates *a*, and *c* were not significantly different from zero. As expected from previous analysis, Type II model provided the best fit to *Ps. calceolariae* (Table 5.2). Estimated curves are indicated in Figure 5.1.

The estimated handling times were 0.067 days for *Pl. ficus* and 0.159 days for *Ps. calceolariae*.

5.3.2. Sex ratio

The sex ratio of the wasp progeny was higher for *Pl. ficus* (0.778±0.024) compared to that obtained for *Ps. calceolariae* (0.703±0.024). A significant effect of host species was observed, ($F_{1,282}$=4.674, *P*=0.031), but not of host density ($F_{1,282}$=0.761, *P*=0.384).

Table 5.1 - Results from logistic regression for the response variable proportion of parasitized mealybugs in relation to the linear, quadratic and cubic terms of the initial density.

Parameter	Estimate	Standard error	Wald Chi-Square	P
Planococcus ficus (LRC*=84.63, df=3, P<0.001)				
Intercept	-0.500	0.2063	5.874	0.015
Linear	0.024	0.0210	1.356	0.244
Quadratic	-0.002	0.0006	6.653	0.010
Cubic	$1.61\ 10^{-5}$	$5.36\ 10^{-6}$	9.005	0.003
Pseudococcus calceolariae (LRC=290.80, df=3, P<0.001)				
Intercept	0.927	0.2413	14.763	<0.001
Linear	-0.219	0.0312	49.131	<0.001
Quadratic	0.006	0.0011	31.222	<0.001
Cubic	$-5.88\ 10^{-5}$	$1.10\ 10^{-6}$	28.836	<0.001

* Likelihood Ratio Chi-Square

Table 5.2 - Results from non-linear regression to relate the number of parasitized mealybugs and the initial host density.

Species / Model	Parameter	Estimate	Standard error	R^2
Planococcus ficus Type II eq. (1)	*a*	0.776	0.269	0.954
	b	20.158	4.528	
Type II eq. (2)	*a*	0.543	0.130	0.954
	b	20.401	4.566	
Type III eq. (3)	*a*	0.338	0.856	0.956
	b	18.143	5.400	
	c	0.326	1.082	
Type III eq. (4)	*a*	12.536	1.130	0.937
	b	15.616	3.356	
*Pseudococcus calceolariae** Type II eq. (1)	*a*	1.064	0.699	0.819
	b	6.280	1.035	
Type II eq. (2)	*a*	0.675	0.272	0.820
	b	6.312	1.024	
Type III eq. (4)	*a*	4.929	0.538	0.731
	b	-5.388	1.907	

* Type III eq. (3) is not displayed as convergence was not achieved.

5.4. Discussion

We investigated the functional response of *A.* sp. nr. *pseudococci* and tested if the parasitoid could respond differently depending on its evolutionary relationship with the host mealybug. The results showed that both the asymptote, as well as the type of functional response of the parasitoid was affected by the host species. A higher asymptote of the curve (upper threshold) was observed in *Pl. ficus* compared with *Ps. calceolariae*. At higher mealybug densities, the progeny produced by *A.* sp. nr. *pseudococci* was about three times higher in *Pl. ficus* (18.4±3.38) than in *Ps. calceolariae* (6.2±0.02). This result may be explained by different behavioral responses exhibited by the parasitoid against the two mealybug species, as well as by their different host suitability. In a previous study, we observed that rate of host acceptance by *A.* sp. nr. *pseudococci* was significantly higher in *Pl. ficus* than in *Ps. calceolariae*, when the parasitoid was exposed to a density of 10 mealybug adult females (Bugila et al., 2014a). Furthermore, *Ps. calceolariae* also showed to be a host with higher level of defenses against *A.* sp. nr. *pseudococci* in comparison with *Pl. ficus* (Bugila et al., 2014b; Suma et al., 2012b). In accordance, we recently observed that the survival rate of *A.* sp. nr. *pseudococci* when developing in *Pl. ficus* was significantly higher than in *Ps. calceolariae* (Bugila et al., 2014c). The results obtained by Chong and Oetting (2007) when comparing between stages of the host mealybug, *Phenacoccus madeirensis* Green, the response of *Anagyrus amnestos* Rameshkumar, Noyes & Poorani (=*Anagyrus* sp. nr. *sinope* Noyes & Menezes) (Rameshkumar, Noyes, Poorani, & Chong, 2013) to increasing host densities also indicate that host suitability can influence parasitoid functional response. These authors reported that although the type of functional response was not affected by the host stage, the asymptote of the curve was highest for the preferred host stage.

Anagyrus sp. nr. *pseudococci* exhibited a type III functional response when foraging in the native mealybug species, *Pl. ficus*, whereas a type II response was observed in the case of the alien mealybug species, *Ps. calceolariae*. Most of the studied parasitoids of homopteran species showed either a type II or type III functional response (Chong & Oetting, 2006, and references therein), but type II has been the most common functional response documented for parasitoids (Chen, Leopold, & Harris, 2006; Chong & Oetting, 2007; Sagarra, Vincent, Peters, & Stewart, 2000), including other *Anagyrus* species, such as *A. amnestos* (Chong & Oetting, 2006, 2007) and *A. ananatis* (González-Hernández, H., Pandey, & Johnson, 2005). However, it has been suggested that this lower frequency of type III functional response might be an experimental artifact (Chong & Oetting, 2006; van Lenteren & Bakker, 1977). For example, Sagarra et al., (2000) reported that *A. kamali* could exhibit either a type II or type III functional response depending on the experimental conditions: a type II response was observed when parasitoids were restricted to the experimental arenas during all the bioassay, where a type III response was shown if the parasitoids were free to decide their residence time within the arenas. Nevertheless, in our study the observed differences in the type of functional response between host species were not due to the experimental conditions as these were the same for both mealybug species studied.

Host specificity may influence the type of functional response of parasitoids (van Lenteren, Cock, Hoffmeister, & Sands, 2006). It is expected that specialists tend to have a type III functional response, whereas generalists tend to show a type II response (Chesson, 1983; Hassell, Lawton, & Beddington, 1978; Jeschke, Kopp, & Tollrian, 2002). Overall, the accumulated data from previous studies on the kairomonal response of *A.* sp. nr. *pseudococci* to the sex pheromone of *Pl. ficus* (Franco et al., 2008, 2011), as well as on the host selection behavior (Bugila et al., 2014a), host defenses (Bugila et al., 2014b), and host suitability (Bugila et al., 2014c) support the hypothesis that the parasitoid evolved from a specialist, with a close relationship with *Pl. ficus,* its possible principal host in the region of origin (Franco et al., 2008), to a more generalist strategy, by recruiting new host species such as *Ps. calceolariae* (Bugila et al., 2014c). Therefore, the observed differences in the type of functional response of *A.* sp. nr. *pseudococci,* between *Pl. ficus* and *Ps. calceolariae,* may reflect this evolutionary trait.

The estimated handling time of *A.* sp. nr. *pseudococci* for *Pl. ficus* (0.067 d) was about half of that for *Ps. calceolariae* (0.159 d). These values, determined based on the parameters of the functional response models, are apparently in contradiction with those estimated in a previous study based on direct observations of the parasitoid behavior (Bugila et al., 2014a), in which the estimated handling time for *Pl. ficus* (5.2±0.6 minutes) was higher but not significantly different from that for *Ps. calceolariae* (4.3±0.7 minutes). This apparent contradiction is at least in part explained by the different criteria for estimating handling time in the two studies, as well as by the different exposure times of the parasitoid to the host mealybugs. The handling time in Bugila et al. (2014a) corresponded to the mean time spent by the parasitoid in antennation, probing and oviposition per parasitized mealybug. Here we used the emergence rate of the parasitoid progeny for determining the parasitism level in the functional response. Therefore, the obtained response reflects not only the host-selection behavioral characteristics of the parasitoid, but also the level of suitability of the host species. As mentioned before, *Ps. calceolariae* exhibits higher level of defenses against *A.* sp. nr. *pseudococci* (Bugila et al., 2014b; Suma et al., 2012b), and is a less suitable host for the parasitoid (Bugila et al., 2014c), in comparison with *Pl. ficus*. In the present study, the exposure time of *A.* sp. nr. *pseudococci* to the host mealybugs was 24h, whereas in Bugila et al. (2014a) the observation of the host selection behavior of the parasitoid was limited to 30 minutes. Furthermore, estimates of the parasitoid's handling times obtained from functional response models tend to be overestimated, and thus direct behavioral observations are essential for more reliable estimates (Chong & Oetting, 2007).

Sex ratio of the parasitoid progeny is an indicator of its fitness (Visser, 1994). We registered a significant higher proportion of *A.* sp. nr. *pseudococci* female progeny when the parasitoid developed on *Pl. ficus* compared to that observed on *Ps. calceolariae,* corroborating the results obtained in a previous work in which *Ps. calceolariae* was shown to be a less suitable host (Bugila et al., 2014b). On the other hand we did not find an effect of host density on the sex ratio of *A.* sp. nr. *pseudococci* progeny for both mealybug species. Similar results were reported for other *Anagyrus* species, such as *A.* (=*Epidinocarsis*) *lopezi* (De Santis) and *A. kamali* Moursi (van Dijken, van Alphen, & van Stratum, 1989; Sagarra et al., 2000). However, Chong and Oetting (2006) observed that *A. amnestos* significantly increased the proportion of female progeny in response to the increase of host density for both studied stages of the host mealybug, *Ph. madeirensis*. The observed differences among parasitoid species might be related with their different reproductive behavior: *A.* sp. nr. *pseudococci*, *A. lopezi*, and *A. kamali* are a solitary parasitoids, whereas *A. amnestos* is gregarious.

Our study was performed in laboratory conditions with a fixed amount of time. This allowed controlling for environmental factors that might influence parasitoid behavior (Sagarra et al., 2000). In field conditions, other factors, such as weather, refuges, competitors, patch size, and host plant, interfere directly or indirectly with predators or parasitoids activity and thus may affect their functional response (Bezemer & Mills, 2001; Farrokhi, Ashouri, Shirazi, Allahvari, & Huigens, 2010; Milonas, Dimitrios, & Angélique, 2011).

Studies comparing the functional response of a parasitoid among different host species are rare. Here, we have shown that the host species may affect the functional response of *A.* sp. nr. *pseudococci*, not only regarding the efficiency of the parasitoid, but also the asymptote of the curve, as well as the type of curve, with type III and type II functional responses exhibited when foraging on a suitable and marginal host, respectively. To our knowledge, this is the first time a parasitoid of mealybugs is shown to exhibit different type of functional response depending on the host species. The results have practical implications for biological control of pest mealybugs. Based on the type III functional response exhibited by *A.* sp. nr. *pseudococci* in the case of *Pl. ficus*, we would expect that the parasitoid is capable of maintaining a stable host–parasitoid dynamics after augmentative releases for controlling this mealybug species (Berryman, 1999). In contrast, the type II functional response observed for *Ps. calceolariae* indicates that in this case the parasitoid may not be able to guaranty a stable dynamics, due to inverse density-dependent host mortality (Chong & Oetting, 2006). Nevertheless, no clear relationship between the type of functional responses and success in biological control was found by Fernández-Arhex and Corley (2003). Further studies are needed, namely by comparing the response of *A.* sp. nr. *pseudococci* to other mealybug species, in order to confirm if the parasitoid exhibit the same type of functional response observed by us in the present study and to assess whether our results reflect the type of response of the parasitoid to varying host densities in field conditions.

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6. Conclusions

- *Anagyrus* sp. nr. *pseudococci* recognized and accepted all five tested mealybug species as potential hosts despite their different geographical origin and phylogenetic relationships. Nevertheless, the behavioral pattern of host recognition and the level of host acceptance exhibited by the parasitoid varied among host species. The parasitism level in *Planococcus* species was about twice as higher as in *Pseudococcus* and *Phenacoccus* species. We suggested that waxy secretions covering the body of mealybugs, as well as their ostiolar secretions may influence host recognition and acceptance by parasitoid females.
- We hypothesized that the females of *Anagyrus* sp. nr. *pseudococci* are capable of assessing the level of host resistance through probing and eventualy use this information for host rejection or acceptance.
- Our results suggest a broader host range and a more generalist behavior for *A.* sp. nr. *pseudococci* in comparison with other *Anagyrus* species, which is in accordance with the hypothesis that this wasp might have evolved by expanding its host range.
- The host handling (antennation + probing + oviposition) time by female *A.* sp. nr. *pseudococci* was affected by host species, with the highest value registered in *Pl. ficus,* the host for which the parasitoid showed highest host searching efficiency.
- The studied mealybugs responded differently to the attack of *A.* sp. nr. *pseudococci* by combining different levels of three types of defensive behavior, which can be classified according to the following two categouries: i) Evasive behavior (walking away); and ii) Aggressive behavior (reflex bleeding and abdominal flipping). Globally, considering all types of defense behavior, both *Pseudococcus* species, which were probably introduced in the Mediterranean basin over 400 years ago, and the recently introduced *Ph. peruvianu*s exhibited higher active defensive behavior than the two most common host species in the Mediterranean, the native *Pl. ficus* and the phylogenetic related *Pl. citri.*

- Our observations lead us to hypothesize that the defense behaviors of mealybugs are likely generalist responses, as they probably did not evolve in response to a specific parasitoid or predator speciesWe observed an inverse relationship between the level of mealybug defense behaviors and the parasitism rate of the mealybugs by *A.* sp. nr. *pseudococci* suggesting that defensive behaviors in the studied mealybug species were effective in affecting host acceptance, and thus parasitism rate by the parasitoid.We hypothesised that the intensity of ant-tending (associative defenses) may differ among mealybug species depending on the amount of honeydew they are capable of excreting and that a trade-off between associative defenses and mealybug behavioral defenses may exist.
- The hypothesis of collective defensive behaviors, which has been recently shown in aphids, also occurring in mealybugs should be investigated as these insects often aggregate in large colonies of related individuals descending from one or few females.
- The probability of *A.* sp. nr. *pseudococci* encapsulation varied among the studied mealybug species, with the highest value registered in *Ps. viburni* and the lowest one in *Ph. peruvianus,* whereas intermediate encapsulation probabilities were registered for the native *Pl. ficus,* the congener *Planococcus* species and for *Ps. calceolariae.*
- Based on our results on the immune defences of the studied mealybug species against *A.* sp. nr. *pseudococci,* we proposed a new hypothesis to explain the differences in the level of encapsulation exhibited by mealybug species against parasitoids, according to which both low and high levels of encapsulation by mealybugs are connected with recent host-parasitoid associations, such as between *A.* sp. nr. *pseudococci* and the two alien mealybugs *Ph. peruvianus* and *Ps. viburni,* respectivelly. Intermediate levels are expected in associations between a parasitoid and its principal host or closely related ones, such as between *A.* sp. nr. *pseudococci* and the native *Pl. ficus* or with its closely

related species *Pl. citri.* Similar levels of encapsulation in closely related mealybug species may further result from cross resistance. Further studies comparing the immune defense of a range of mealybugs in response to the attack by parasitoids with different host selectivity are needed in order to test our hypothesis and further clarify this issue.

- *Anagyrus* sp. nr. *pseudococci* was able to complete development in all five studied mealybug species, but the emergence rate of the parasitoid varied among mealybug species, with the highest values observed in the native *Pl. ficus* and the phylogenetically related *Pl. citri,* intermediate values in *Ps. calceolariae*, and the lowest ones in *Ps. viburni* and *Ph. peruvianus.* The observed differences in host suitability apparently reflect the phylogenetic relationships of the studied mealybug species and the differences in their co-evolutionary history with the parasitoid.
- It is expected that parasitoid females will recognize and accept the hosts that will allow the development of larvae and optimize their fitness. Our results did not support this prediction, suggesting that in the case of *A.* sp. nr. *pseudococci* not always "mother knows the best". However, these apparent wrong decisions of the wasp females in host acceptance may favour the recruitment of new host species.
- We found as expected that the body size of adult females of *A.* sp. nr. *pseudococci* varied with host suitability and was positively correlated with other measures of parasitoid fitness, such as the emergence rate and the sex-ratio of the progeny.
- In opposition to other fitness parameters, no clear relationship between parasitoid development time and host suitability was found. The development time was longer in females than in males.
- We found a significant relationship between sex ratio *A.* sp. nr. *pseudococci* progeny and the emergence rate of the parasitoid. That is, the proportion of females in the parasitoid progeny was highest (female biased sex ratio) in the native mealybug species,

Pl. ficus and lowest (male biased sex ratio) in the alien Neotropical mealybug species, *Ps. viburni* and *Ph. peruvianus*.

- The higher emergence rate, larger tibia length of wasp females, and higher proportion of progeny females clearly indicate that *Pl. ficus* is the most suitable host for *A.* sp. nr. *pseudococci*, closely followed by *Pl. citri.* In contrast, the lower emergence rate, smaller tibia length of wasp females, as well as the male biased sex ratio registered in the parasitoid progeny obtained from *Ps. viburni* and *Ph. peruvianus* suggest that these two mealybug species are poor quality hosts for *A.* sp. nr. *pseudococci*, and thus may be considered marginal hosts. *Pseudococcus calceolariae* seems to be in an intermediate position.
- Host suitability of the studied mealybug species seems to fit a phylogenetic/biogeographic trend, showing the highest level in *Pl. ficus* and its closely related congener *Pl. citri*, followed by the Australasian *Ps. calcelolariae,* and the Neotropical *Ps. viburni* and *Ph. peruvianus*.
- Both the asymptote, as well as the type of functional response of *A.* sp. nr. *pseudococci* was affected by the host species. A higher asymptote of the curve was observed in *Pl. ficus* compared with *Ps. calceolariae.* The parasitoid exhibited a type III functional response when foraging in the native mealybug species, *Pl. ficus*, whereas a type II response was observed in the case of the alien mealybug species, *Ps. calceolariae*.
- We did not find an effect of host density on the sex ratio of *A.* sp. nr. *pseudococci* progeny for both *Pl. citri* and *Ps. calceolariae.*
- All the available data indicate that *A* sp. nr. *pseudococci* evolved from a specialist to a more generalist strategy, expanding the host range from its possible original host, *Pl. ficus* by recruiting new host species, specially within the genera *Planococcus* and *Pseudococcus,* but also in *Phenacoccus*.

- The specificity of a parasitoid is considered an important attribute in selected candidates for classical biological control programs aiming to minimize the risks of impacts on non-target native species. In this respect, the use of *A.* sp. nr. *pseudococci* in classical biological control may present risks of impact on native species of mealybugs due to its apparent generalist behavior. Nevertheless, it has been used both in classical biological control and augmentative releases in different areas and there is no evidence of negative impacts on native mealybug species. On the other hand, the existence of alternative hosts is considered important for the success of biological control as it will support parasitoid populations over periods of scarcity of the primary hosts.
- Based on the type III functional response exhibited by *A.* sp. nr. *pseudococci* in the case of *Pl. ficus,* we would expect that the parasitoid is capable of maintaining a stable host–parasitoid dynamics after augmentative releases for controlling this mealybug species. In contrast, the type II functional response observed for *Ps. calceolariae* indicates that in this case the parasitoid may not be able to guaranty a stable dynamics, due to inverse density-dependent host mortality. Nevertheless, further studies are needed, namely by comparing the response of *A.* sp. nr. *pseudococci* to other mealybug species, in order to confirm if the parasitoid exhibit the same type of functional response observed by us in the present study and to assess whether our results reflect the type of response of the parasitoid to varying host densities in field conditions.

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