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Symbionts and hosts behavioral interactions: a study from the perspective of host – parasitoid interactions

Caio Henrique Lopes Zitelli

Dissertation presented to obtain the degree of Master in Science. Area: Entomology

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DEDICATION

To Dad, who have never measured any efforts to provide me the best opportunities.
To Mom who have never stopped believing in me and in my potential in the pursuit of my dreams.
To Carlinha, who constantly remember me that the Force will be with us. Always.
To Eloísa, who have never stopped supporting me, even in the most difficult times.
To Our Lady of Aparecida, for empowering me in each and everyday.
I dedicate this work…

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“If I have seen further it is by standing on the shoulders of Giants”

(Sir Isaac Newton, 1675)

“The Force is with me, and I am with the Force.
And I fear nothing.
For all is, as the Force wills it.”

Chirrut Îmwe

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RESUMO

Simbiontes e interações comportamentais de hospedeiros: um estudo da perspectiva das interações hospedeiro-parasitoide

A simbiose é um dos principais agentes na evolução e ecologia de organismos. Tais interações são muito íntimas, podendo ser muito diversas e ter grandes impactos na diversidade biológica. Uma das principais associações que ocorrem na natureza é aquela entre insetos e microrganismos. Microrganismos associados a insetos são capazes de alterar uma gama de eventos fisiológicos, comportamentais, ecológicos e evolutivos em seus hospedeiros. Dois simbiontes de insetos muito comuns são *Wolbachia* e *Spiroplasma*. *Wolbachia* é também muito comum a outros artrópodes e nematoides. Para melhor compreender como essas relações podem influenciar o comportamento de insetos, dois sistemas biológicos foram selecionados para investigar como esses simbiontes podem interferir nas interações hospedeiro-parasitoide. O comportamento de duas espécies de parasitoides, *Aphelinus asychis* (Hymenoptera: Aphididae) e *Trichogramma pretiosum* (Hymenoptera: Trichogrammatidae) foi investigado quando explorando *patches* com seus respectivos hospedeiros, *Aphis citricidus* (Hemiptera: Aphididae) e *Anagasta kuehniella* (Lepidoptera: Pyralidae). No primeiro caso foi investigado como a infecção do hospedeiro por *Spiroplasma*, e no segundo caso como a infecção do parasitoide por *Wolbachia*, afetariam a exploração da *patch* pelos parasitoides. O comportamento dos parasitoides ao explorarem suas *patches* foi registrado, assim como os comportamentos de defesa dos pulgões em resposta ao ataque do parasitoide. Os dados obtidos demonstraram que *Spiroplasma* afetaram o comportamento de defesa e a agressividade de *A. citricidus* em resposta aos ataques de *A. asychis*. *Wolbachia* aumentou o tempo de residência e reduziu o sucesso de parasitismo de ovos do hospedeiro por *T. pretiosum*. A compreensão de tais efeitos certamente contribuirá para o melhor entendimento dos efeitos da associação de insetos a simbiontes, fornecendo bases sólidas para a melhor exploração de tais interações para propósitos de controle biológico de pragas.

Palavras-chave: Comportamento de insetos; Inimigos naturais; Parasitismo; Simbiontes

ABSTRACT

Symbionts and hosts behavioral interactions: a study from the perspective of host – parasitoid interactions

Symbiosis is one of the main players in evolution and ecology of organisms. Such intimate interactions may be diverse and have a great impact in biological diversification. One of the main associations that occur in nature is that of insects and microbes. Insect associated microbes are, capable of altering a wide range of physiological, behavioral, ecological and evolutionary events for their hosts. Two very common insect microbial symbionts are *Wolbachia* and *Spiroplasma*. *Wolbachia* is also common to other arthropods and nematodes. To better understand how these relations could influence the behavior of insects, we selected two biological systems to investigate how these symbionts can interfere in the host – parasitoid interactions. We investigated the behavior of two species of parasitoids, *Aphelinus asychis* (Hymenoptera: Aphididae) and *Trichogramma pretiosum* (Hymenoptera: Trichogrammatidae) when exploting patches with their respective hosts, *Aphis citricidus* (Hemiptera: Aphididae) and *Anagasta kuehniella* (Lepidoptera: Pyralidae). In the first case we looked into how *Spiroplasma* infecting hosts, and in the second case how *Wolbachia* infecting the parasitoid may affect parasitoid patch exploitation. We recorded the wasp's behaviors when exploiting their patches, as well as the aphid defensive behavior in response to parasitoid attack. Our data demonstrate *Spiroplasma* and *Wolbachia* influence the parasitoid patch exploitation decisions. *Spiroplasma* also affected the defense behavior and aggressiveness of *A. citricidus* in response to *A. asychis* attack. *Wolbachia* increased the patch residence time and reduced the successful parasitization of host eggs in *T. pretiosum.* The understanding of such effects will certainly contribute to provide a better knowledge of the outcome of the associations of insects with microbial symbionts, providing ground base for the proper exploitation of such interactions for biological control purposes.

Keywords: Insect behavior; Natural enemies; Parasitism; Symbionts

1. INTRODUCTION

Symbiosis is a common process in nature and it is distributed in many hosts ranging from aquatic to terrestrial habitats, playing an important part in the evolution and ecology of organisms (Oliver et al. 2003; Wernegreen 2004). Symbiosis is so diverse and involves the living together of a range of organisms that the outcome of such associations can be extremely diverse (Nikoh et al. 2014; Lewis and Lizé 2015). The age of the association is one of the major factors affecting the type of relationship the associated organisms will establish. Longer the history of association more likely is the reduction of pathogenesis and high is the development of coadaptive processes due to the coevolutionary history (Hentschel et al. 2000; Steinert et al. 2000).

Symbiosis has a great impact in biological diversification (Zabalou et al. 2004). In the case of insects, microorganisms that established mutualistic associations can be fundamental in providing essential nutrients to insect hosts (Hansen and Moran 2011; McCutcheon and Moran 2012), recycling nitrogen (Fox-Dobbs et al 2010), assisting with food digestion (Engel and Moran 2013) and in supporting host reproduction (Dedeine et al. 2001). These associations most involve endosymbionts or endocytobionts, but there are cases in which the coevolutionary history of the association led to the establishment of external mutualistic associations. One of such example is the ant – fungus association, in which fungus-growing ants rely on the cultivation and use of a mutualistic fungus as food resource (Weber 1966). From the many associations organisms present in the animal kingdom, the ones that are maternally transmitted, like bacteria in insects, are often obligate, reaching such an intimate relationship that one could not survive without the other (Douglas 1998; Ferrari and Vavre 2011).

Facultative symbionts in the other hand are not required for the completion of their hosts life cycle but usually establish beneficial symbiotic interactions (Oliver et al. 2010). Although not always required for their hosts survival, the facultative symbionts may contribute positively to several host fitness traits, such as resistance against natural enemies and xenobiotics, protection against heat stress, and expansion of food sources (Chen and Purcell 1997; Oliver et al. 2003; Ferrari et al. 2007; Kontsedalov et al. 2008; Burke et al. 2010; Xie et al. 2010; Simon et al. 2011). As shown by Oliver et al. (2003), the facultative symbiont *Serratia symbiotica*

improved aphid resistance against its natural enemy, causing high mortality of the developing parasitoid larvae. Other researchers have even found evidence that the presence of facultative symbionts could increase their host fitness, sometimes doubling their offspring (Leonardo and Muiru 2003). Moreover, non-parasitic microbial symbionts can also alter the behavior of their hosts in order to optimize their reproductive fitness or to protect them against pathogens (Brownlie and Johnson 2009).

Facultative symbionts can be acquired from the environment or be horizontally transmitted, but vertical transmission is not uncommon even if not leading to infection fixation (Werren et al. 2008). The most spread and common group of non-obligate symbiont associated with arthropods are the sex-determinant bacteria, particularly. *Wolbachia* is the most common representative microorganism associated with the reproductive system of arthropods, influencing progenie sex determination or even host reproductive fitness *Wolbachia* (Ma et al. 2013; Newton et al. 2016). Other known bacteria that also establish such type of relationship with their arthropod hosts are *Arsenophonus*, *Cardinium*, *Rickettsia* and *Spiroplasma* (Enigl and Schausberger 2007; Duron et al. 2008; Shropshire and Bordenstein 2016; Zhang et al. 2016).

The discoveries in the last decades of the importance of the microbiota in phenotype definition (Sonnenburg et al. 2005; Lyte et al. 2016) and the increased understanding of the role of obligate and non-obligate bacteria in insects, made clear the associated microbiota acts as manipulators of their hosts. In manipulating the host, the microbiome acts as one of the determinants of evolutionary, physiological and behavioral processes driving adaptation, diversification and speciation (Forsythe and Kunze 2013; Rohrscheib and Brownlie 2013; Rohrscheib et al. 2015).

Manipulators of behavioral responses of insect hosts are seen among symbiotic bacteria (Lewis and Lize 2015), fungi (Roy et al. 2006) and virus (Burand et al. 2005), but in many of these cases microorganisms established a pathogenic relationship with their host insects (Evans 1982; van Houte; Ros; van Oers 2013). The entomopathogenic fungus *Ophiocordyceps unilateralis* induces the infected host ant *Camponotus leonardi* (Hymenoptera: Formicidae) to wander towards the north/northwest direction as infected ants die around noon in a position that enhances fungus transmission and spread of infection to other workers of the colony (Holldobler and Wilson 1990; Hughes, et al. 2011). Pathogenic viruses are also reported to affect the behavior of infected hosts, inducing the precocius onset of the

courthip behavior in *Gryllus texensis* (Orthoptera: Gryllidae) male crickets to assure female infection during mating (Knell and Webberley 2004; Adamo et al. 2014).

There are also a few cases of non-pathogenic symbionts that influence the host behavior. Viral particles infecting *Leptopilina boulardi* (Hymenoptera: Figitidae) alter the wasp parasitization behavior inducing a higher tendency to host superparasitization, increasing the chances of horizontal transmission within the parasitized host larva *Drosophila* (Varaldi et al. 2006). Reproductive parasites usually manipulate their hosts' reproduction by increasing the production or survival of female hosts at some cost to the males (Montenegro et al 2006). *Wolbachia*-infected males and females of *Drosophila melanogaster* (Diptera: Drosophilidae) had higher locomotor activity and foraging behavior than uninfected adults probably due to an increase in their metabolic rates (Evans et al. 2009; Caragata et al. 2011). *Wolbachia* was also shown to reduce *D. melanogaster* male aggressiveness by lowering males octopamine levels (Rohrscheib et al. 2015). Additionally, *Wolbachia* was demonstrated to influence *Trichogramma brassicae* (Hymenoptera: Trichogrammatidae) oviposition behavior by affecting the capacity of females to discriminate previously parasitized hosts. In this case, infected females were less effective in host evaluation, leading female to select low quality hosts more frequently (Farahani et al. 2015). The manipulation of the reproductive fitness or traits of the host are very important for determination of the efficiency of symbiont infections. Nonetheless, such manipulations can directly affect host fitness and influence symbiont ability to persist in natural populations (Montenegro et al., 2005b).

Spiroplasma is another important symbiont present in nearly 5% of insects (Duron et al. 2008). *Spiroplasma* is better known as a male-killing symbiont (Harumoto et al. 2014), but that can also stimulate the host immune response capacity (Herren and Lemaitre 2011) and protect the host against natural enemies (Jaenike et al. 2010; Xie et al. 2010).

Thus, microbial manipulators can play important roles in the evolution and ecology of their hosts, and the understanding of these effects may have important applied implications (Lewis and Lizé 2015). Parasitoids are the most common biological control agents in applied biological control of a great number of insect pests, constituting one of the most important strategies in integrated pest management programs (Waage and Hassell 1982; Fernández-Arhex and Corley 2003; Yazdani and Keller 2016). As parasitoids usually have a very short lifespan,

they maximize the deposition of their eggs early in their adult life to avoid dying before females are able to lay their full complement of eggs (Rosenheim 1999; Wajnberg et al. 2006; 2016). Thus, female parasitoids evolved foraging strategies to avoid time limitation by optimization of patch use (Outreman et al. 2005; Wajnberg 2006). A patch is defined as a spatial subunit of the foraging area where host aggregations are available (Hassell and Southwood 1978; Wajnberg 2006). Hosts occurring in discrete and individual patches in the environment lead parasitoids to optimize the time they invest in host exploitation, actively contributing to the costs of reproduction (Charnov and Skinner 1984; Godfray 1994; Rosenheim 1999). A series of theoretical models was proposed to explain the decisions parasitoids make to allocate time for patch exploitation and to maximize the individual capacity of exploitation (Outreman et al. 2005). To enhance egg production, parasitoids need to balance their investments in longevity and host searching in an attempt to lay they full egg complement (Rosenheim et al. 2008).

Female parasitoids may locate patches ranging in quality within their foraging area. Differences in patch quality require females to decide the best time allocation to each particular patch in the process of host selection and exploitation. There are a number of factors that can affect the female´s decision to allocate time to exploit a patch. The identification of such factors and the understanding of how they influence female´s decision to select and exploit a particular patch are not only of relevance for comprehending the behavioral ecology of parasitoids (Outreman et al. 2001, 2005; Desneux et al. 2004; Tentelier et al. 2005; Wajnberg 2006), but also for predicting the efficacy of parasitoids selected for use as biocontrol agents (Waage 1990; Wajnberg et al. 2016).

Microbial associations are quite spread in insects and several insect-associated microorganisms are reported to affect the host insect behavioral decisions. In this dissertation we focused in evaluating two host-parasitoid systems to investigate the impact microbial associations would have on host-parasitoid interactions, particularly in parasitoid patch exploitation. In one of the systems used, the aphid *Aphis citricidus* (Hemiptera: Aphididae) – the wasp *Aphelinus asychis* (Hymenoptera: Aphelinidae), we used sister isolines of *Spiroplasma*-infected and uninfected aphids *A. citricidus* to investigate the role of host infection in patch time use by the parasitic wasp. In the second system, the host *Anagasta kuehniella* (Lepidoptera: Pyralidae) – the wasp *Trichogramma pretiosum* (Hymenoptera:

Trichogrammatidae), we investigated how *Wolbachia* could affect patch exploitation by using sister isolines of *Wolbachia*-infected and uninfected *T. pretiosum*.

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2. *SPIROPLASMA*-INFECTING APHIDS INTERFERES WITH THE *Aphis citricidus* (HEMIPTERA: APHIDIDAE) – *Aphelinus asychis* (HYMENOPTERA: APHELINIDAE) INTERACTIONS

Abstract

There are a wide range of studies focusing on the benefits of secondary associations of insects and microorganisms at the molecular, physiological and behavioral levels. Secondary symbionts have been demonstrated to influence several insect host responses to a number of stressors, including the third trophic level. Insects associated with many secondary symbionts were shown to better survive to the attacks of their natural enemies. Understanding the effects associations with secondary symbionts may have on the output of host-parasitoid interactions are important in the implementation of biological control strategies in the field. Thus, we investigated the effects of *Aphis citricidus* infection with *Spiroplasma* in the patch-time exploitation by the aphid parasitoid *Aphelinus asychis*. We also investigated if *Spiroplasma* infection could affect the host defensive behavior. Investigations were done by recording the host defensive behaviors and the parasitoid patch use in a patch comprised of five-host aphids under controlled conditions (25±2^{o} C; 70±10% RH; 14:10 h). We did not detect overall differences in the exploitation of *Spiroplasma* infected and uninfected hosts by *A. asychis*. The only factors leading to an effect on the patch leaving decisions of *A. asychis* were the time spent in grooming by the parasitic wasp and the host agitated behavior. The aphid´s agitated behavior was positively influenced by *Spiroplasma* infection. *Spiroplasma* also influenced the aphid kicking and whipping behaviors. Kicking was reduced and antennal whipping was increased by *Spiroplama* infection in response to parasitoid attack. *Spiroplasma*-infected hosts were less accepted than uninfected aphids for egg laying by *A. asychis*, demonstrating *Spiroplasma* directly affects *A. asychis* parasitization efficiency by reducing host acceptance and indirectly by enhancing host defensive behaviors that limits parasitoid successful attacks.

Keywords: Host selection; Integrated pest management; Natural enemies; Parasitoid efficacy; Symbiosis

2.1. Introduction

A wide range of studies are focused on the diverse interactions insects have with non-pathogenic microorganisms, with some of which dedicated to understand and explain how these microorganisms interact with their hosts at the molecular, physiological and behavioral levels (Goodacre and Martin 2012). Bacteria that are obligately associated with insects are often involved with host-nutrition supplementation, while the non-obligate, secondary symbionts have been reported to affect a variety of host traits, influencing the host phenotype and the host interactions

with other trophic levels (Oliver et al. 2003; Vásquez et al. 2012; Guidolin et al. 2018).

Non-obligate symbionts can affect host fitness traits by confering resistance against natural enemies and xenobiotics, protection against heat stress, and the host diet breadth (Leonardo and Muiru 2003; Ferrari et al. 2004; Oliver et al. 2003; Kontsedalov et al. 2008; Burke et al. 2010; Xie et al. 2010), and even increase their response to selection pressures (Oliver et al. 2003). There are symbionts able to induce behavioral changes in the host and improve the host´s reproductive capacity (Brownlie and Johnson 2009) while others are capable to confer protection to hosts against entomophagous or entomopathogens (Oliver et al. 2003).

Spiroplasma is better known as a male-killer in a range of host associations (Ebbert 1991; Montenegro et al. 2000; 2005). Male-killing is due the production of high levels of a protein that contains ankyrin repeats and a deubiquitinase domain, which is designated Spaid. Male mortality was suggested to result from the effects of Spaid on the dosage compensation machinery on the male X-chromosome (Harumoto and Lemaitre 2018). Nevertheless, *Spiroplasma* can establish associations with insect hosts playing a range of roles (Jaenike et al. 2010; Xie et al 2010; Herren and Lemaitre 2011; Guidolin et al. 2018). In some of these associations, *Spiroplasma* infection can result in increased protection of the host against bacterial pathogens (Herren and Lemaitre 2011) and parasitoids (Jaenike et al. 2010; Xie et al. 2010). *Spiroplasma* is not commonly reported associated to aphids, but this secondary symbiont prevailed in field collected Brazilian populations of *Aphis citricidus* (Hemiptera: Aphididae) and *Aphis aurantii* (Hemiptera: Aphididadae) (Guidolin and Cônsoli 2018). *Spiroplasma* infections of *A. citricidus* were also shown to affect the aphid proteome. Moreover, the aphid proteome was differently affected depending on the host plant quality, altering the abundance of proteins involved in protein-protein interactions, cell functioning and energy metabolism (Guidolin et al. 2018). Metabolomics analysis also indicated *Spiroplasma* infections affected a large variety of metabolites of *A. citricidus*, including metabolites involved in host selection by aphid's parasitoids (Duarte 2017).

Thus, based on the fact secondary symbionts can alter host phenotypic traits, affect the interactions of the host insect with other trophic levels and that *Spiroplasma* did alter the proteome and metabolome of the host aphid *A. citricidus,* we hypothesize *Spiroplasma* infection affects *A. citricidus* interactions with the

parasitoid *Aphelinus asychis* (Hymenoptera: Aphelinidae), and tested two predictions to prove our hypothesis: 1) *Spiroplasma*-infected aphids will display more efficient defensive behavior against parasitoid attack, and 2) patch time allocation of female parasitoids will be affected when exploting *Spiroplasma*-infected aphids.

2.2. Material and Methods

2.2.1. Insects rearing

Spiroplasma-infected and *Spiroplasma*-free sister lines established from a field population of *A. citricidus* collected at the city of Piracicaba, state of São Paulo, Brazil in 2014 were reared on *Citrus limonia* seedlings under laboratory-controlled conditions (26±1°C, 60±10% RH, 14 h photophase) (Guidolin 2016).

Females of *A. asychis* were obtained from field collected parasitized *A. citricidus. Aphelinus asychis* was reared under laboratory-controlled conditions (25±1°C, 60±10% RH, 14 h photophase) using 2^{nd}-3^{rd} instars of *A. citricidus* as hosts. Nymphs were daily collected from the aphid's stock colony and offered to female wasps for parasitization for at least 6 hours. After parasitization, female wasps were collected and kept in 5 mL glass vials with a drop of pure honey. Parasitized nymphs were transferred to new buds of *C. limonia* seedlings kept inside 500 mL plastic cages containing lateral openings covered with an anti-aphid mesh. Aphid mummies were daily collected from day 7 to day 10 after parasitization and individually placed in 5 mL glass tubes containing a drop of honey as a food source for the emerging wasp. Upon emergence, wasps were either used in colony maintenance or in bioassays. Rearing of *A. asychis* yielded only females, and additional investigation using diagnostic PCR detected females were infected with *Wolbachia*.

2.2.2. Multi-locus sequence typing (MLST) of *Wolbachia* infecting *Aphelinus asychis*

Females of *A. asychis* were subjected to genomic DNA extraction (gDNA) as described by Gilbert et al. (2007). Briefly, females were macerated and incubated at 55^{o} C for 16 h in digestion buffer [3 mM $CaCl_2$; 100 mM Tris-HCl, pH 8; 100 mM NaCl; 400 mM dithiothreitol (DTT); 2% dodecyl sodium sulphate (SDS); 1.5%

proteinase K (20 mg/mL)]. After digestion, 400 µL of phenol:chloroform (25:24) was added, samples were vortexed and centrifuged (14.000*g* × 5 min). The organic phase was transferred to a new vial and the process of extraction with phenol:chloroform was repeated. Samples were then added to 400 µL of chloroform, vigorously homogenized and centrifuged as before. The supernatant was transferred to a new vial and 1 µL of glycogen (20 mg/mL), 100 µL of 3 M sodium acetate (pH 5.2) and 400 µL of isopropanol were added. Samples were incubated at – 80^{o}C for 40 min and centrifuged (16.000 *g* × 30 min × 4^{o}C). The pellet obtained was washed twice with 85% cold ethanol and centrifuged (16.000 g × 10 min × 4^{o} C). The pelleted gDNA obtained was dried in a speedvac centrifuge (15 min x 60^{o} C), recovered in TE buffer (100 mM Tris-HCl; 1 mM EDTA, pH 8), incubated at 37^{o} C for 30 min, quantified using the Epoch Microplate Spectrophotometer (BioTech®) and kept at -20^{o} C until further use.

The strain of *Wolbachia* infecting *A. asychis* was characterized by analyzing the hypervariable regions of the *wsp* gene using the primer set *wsp*81F (5'-TGGTCCAATAAGTGATGAAGAAAC-3') and wsp691R (5'-AAAAATTAAA CGCTACTCCA-3') (Braig et al., 1998), and the multi-locus sequence typing (MLST) (Table 2.1) (Baldo et al. 2006). The amplification reactions were performed in a total volume of 25 µL containing 20 ηg of gDNA, 1× PCR buffer (5×), 1.5 to 2 mM of $MgCl_2$, 200 µM of each dNTP, 0.32 µM of each primer and 0.5 U of the Taq polymerase enzyme, using the following thermocycling conditions: 94^{o}C for 2 minutes (1×); followed by 35 cycles of 94^{o}C for 30 s, annealing temperature in accordance to the target gene for 45 s (Table 2.1), 72^{o}C for 90 s; and 1 cycle for final extension at 70^{o}C for 10 min.

The obtained amplicons were resolved using 1.5% agarose gel electrophoresis in TAE buffer (40 mM Tris-Acetate, 1 mM EDTA, pH 7.2) added of 0.5 µg/mL ethyl bromide and subjected to image capture in an ultraviolet transluminator coupled to a photo documentation system (DNR Bioimaging Systems®). Residual primers, single strand DNA and nucleotides were eliminated by adding 10 U exonuclease (Exo) and 0.5 U alkaline phosphatase (Sap) to 8 µL of the amplification reaction, followed by incubation at 37^{o}C for 30 min. Afterwards, samples were exposed at 80^{o} C for 15 min for enzyme inactivation. ExoSap-treated samples were subjected to bidirectional sequencing using the BigDye® Terminator v3.1 Cycle

Sequencing kit in the ABI-3730 system available at the Laboratory of Agriculture Biotechnology (ESALQ-USP).

Sequences obtained were visualized and edited using the FinchTV v.1.4.0 software (Geospiza inc.). Edited sequences for each MLST locus were then used for heuristic search against reference sequences available in the MLST database (http://pubmlst.org/wolbachia/) for allele identification, determination of the allele combination and identification of the sequence type (ST) of the *Wolbachia* strain infecting *A. asychis* (Baldo et al. 2006). The *wsp* amplicon was used in a batch sequence query against the MLST *Wolbachia* public database (http://pubmlst.org/wolbachia/wsp/) for *wsp* allele identification and HVR peptide assignment (Baldo et al. 2005).

Concatenated sequences from MLST database were selected to analyze the proximity of the sequence-type defined for *Wolbachia* associated to *A. asychis* to other closely related STs already deposited at the database. HVRs peptide sequences were also concatenated and used for phylogenetic analysis.

Both data set had their sequences aligned in the software MEGA 7.0.26 (Kumar et al. 2016) with the tool ClustalW. The phylogenetic analysis of MLST genes used the Maximum Likelihood method available in the software MEGA 7.0.26 based on the Tamura 3-parameter model (T92), with G = 0.05, following the lower value of BIC (Bayesian Information Criterion) while the phylogenetic analysis of HVRs peptide sequences was performed using the Whelan and Goldan model (WAG), with G = 0.05. Phylogenetic trees were visualized and edited in the software FigTree 1.4.3 (Rambaut 2016).

Table 2.1. Loci, primers and annealing temperatures used for the molecular characterization of the *Wolbachia* strain infecting *Aphelinus asychis*

Gene	Primer name	Sequence	Anelling (^{o}C)
coxA	coxA_F1	TTGGRGCRATYAACTTTATAG	55
	coxA_R1	CTAAAGACTTTKACRCCAGT	
fbpA	fbpA_F1	GCTGCTCCRCTTGGYWTGAT	59
	fbpA_R1	CCRCCAGARAAAAYYACTATTC	
ftsZ	ftsZ_F1	ATYATGGARCATATAAARGATAG	54
	ftsZ_R1	TCRAGYAATGATTRGATAT	
gatB	gatB_F1	GAKTTAAAYCGYGCAGGBGTT	54
	gatB_R1	TGGYAAYTCRGGYAAAGATGA	
hcpA	hcpA_F1	GAAATARCAGTTGCTGCAAA	53
	hcpA_R1	GAAAGTYRAGCAAGYTCTG	
wsp	wsp_81F	TGGTCCAATAAGTGATGAAGAAAC	55
	wsp_691R	AAAAATTAAACGCTACTCCA	

2.2.3. Behavioral assays

Second and third instars of *Spiroplasma*-infected and uninfected sister lines of *A. citricidus* were offered to 24-48 h-old, fed, naive *A. asychis* females in order to evaluate the patch time allocation and behavioral responses *of A.* asychis when exploiting patches with infected or uninfected hosts.

Females of *A. asychis* were individually released in glass Petri dishes (9 cm in diameter) containing one leaf of *C. limonia* laid within an area of 4 cm in diameter (patch) delimited on the Petri dish. Each leaf was infested with four aphids. The parasitoid behavior in the patch was analyzed following parameters available in the literature (Outreman et al., 2001, 2005; Desneux et al. 2004; Tentelier; et al. 2005; Wajnberg 2006) and parameters selected from preliminary observations of the insect behavior. The following behaviors of *A. asychis* females were observed: i) patch residence time; ii) patch leaving time; iii) grooming; iv) resting; v) host drumming; vi) host searching; vii) host finding; viii) host feeding; ix) body wiggling; x) turn around (the parasitoid turns around to probe the host); xi) host probing.

The aphid behavior was also observed. The same set up used to study the parasitoid patch exploitation was used to observe the behavioral responses of *A. citricidus* to the wasp attack. The following aphid behaviors were observed: i) agitation (the aphid moves legs, antennae and the body while still feeding); ii) antennal whip; iii) kicking; iv) walking; v) leaving/retuning to the patch; vi) confronting (the aphid confronts and/or chases the wasp); vii) death (number of aphids that died).

Aphids in the patch were digitally numbered and their positions followed during the experiment. Aphids were collected and individually transferred to 250 mg gelatin capsules at the end of each experiment. Aphids remained in the capsules for 3-4 h before dissection under a stereomicroscope for confirmation of parasitization (adapted from Outreman et al. 2001). Hosts that were probed but no parasitoid egg was detected were considered as rejected hosts.

Parasitoid patch exploitation and aphid defense behavior were observed under a stereomicroscope, and the behavioral data observed collected using the JWatcher 1.0 program (Dan Blumstein's Lab University of California Los Angeles & The Animal Behaviour Lab, Macquarie University, Sydney). Behavioral assays were performed from 1 to 7 pm in a quiet room under controlled conditions (25±1°C, 70±10% RH, 14h photophase). Behavioral data collection started once parasitoid females entered the patch and ended either when the parasitoid female rested for more than 5 min or left the patch for more than 1 min. A total of 25 replicates/treatment, *Spiroplasma*-infected and *Spiroplasma*-free aphids, were used. Each patch with one female wasp was considered as one replicate.

2.2.8. Data analysis

The effects of the host infection by *Spiroplasma* on the patch exploitation by *A. asychis* and on the host defensive behavior were tested by analyzing the i) attack on hosts that were not attacked before, ii) re-attack of previously attacked hosts, iii) rejection of host that have not been previously attacked, iv) rejection of hosts that were attacked before, v) wasp grooming behavior, vi) host feeding, vii) host death, viii) aphid agitation, ix) aphid antennal whipping, x) aphid kicking behavior using Cox regression model as implemented in the R statistical software (R Core Team 2018).

2.3. Results

2.3.1. Multi-locus sequence typing (MLST) of *Wolbachia* infecting *Aphelinus asychis*

Aphelinus asychis was infected with an undescribed sequence-type (ST) of a telytoky-inducing *Wolbachia*. The new ST of *Wolbachia* associated to *A. asychis* was characterized by the identification of four new alleles (*coxA*, *fbpA*, *ftsZ* and *gatB*) defined as described in Table 2.2. The *coxA* gene shared 99% similarity with allele 15, with 4 nucleotide substitutions; *fbpA* shared 99.5% similarity with allele 58, with 2 nucleotide substitutions; *ftsZ* shared 99.3% similarity with allele 56, with 3 nucleotide substitutions; and *gatB* allele shared 99.4% similarity with allele 14, with 2 nucleotide substitutions.

Table 2.2. MLST alleles defining the new sequence-type of *Wolbachia* infecting the lab population of *Aphelinus asychis*

	Genes			
ST	*coxA*	*ftsZ*	*fbpA*	*gatB*
	279	239	451	281

Molecular analysis based on the concatenated sequence of all of the MLST alleles identified placed the new ST of *Wolbachia* infecting *A. asychis* in a clade with ST-79 and ST-230 (Figure 2.1). The closest sequence-type of the ST of *Wolbachia* associated to *A. asychis* is ST-79, but information on the associated host remains unavailable in the MLST database. However, the other two closely related STs, ST-34 and ST-230, are sequence-types associated with *Nasonia vitripennis* (Hymenoptera: Pteromalidae) and *Ixias pyrene* (L.) (Lepidoptera: Pieridae) (Figure 2.1).

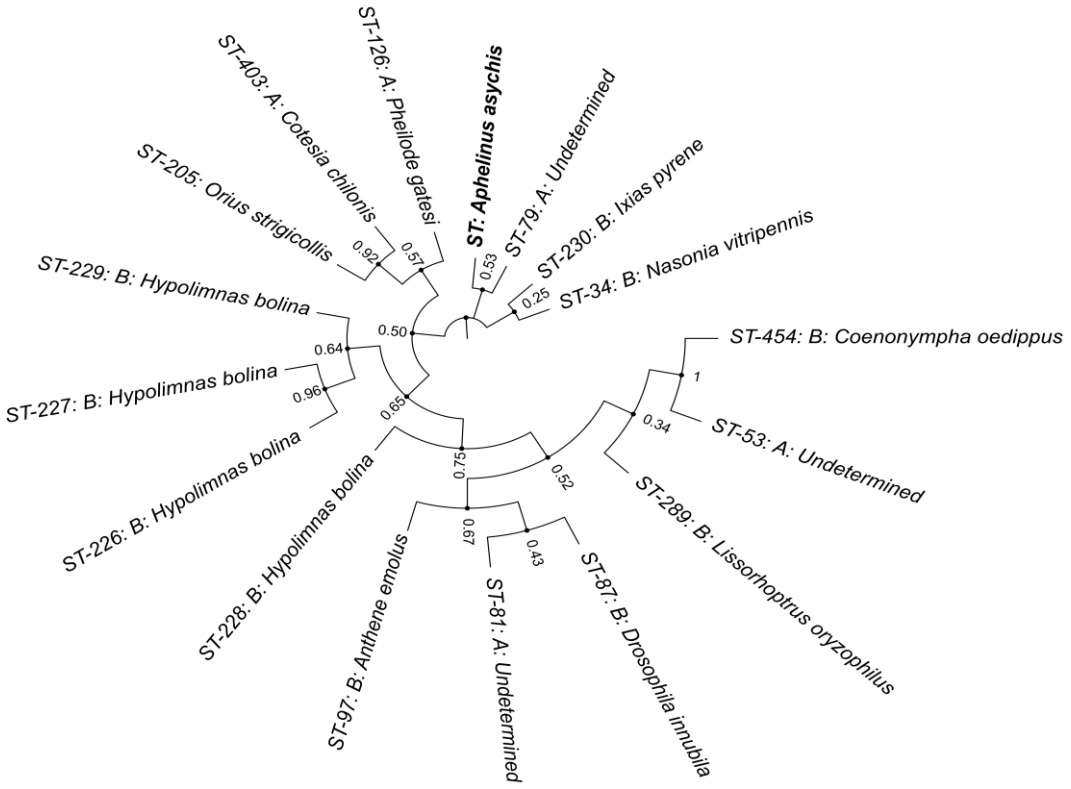

Figure 2.1. Phylogenetic relations of the sequence-type (ST) of *Wolbachia* associated to *A. asychis* when comparing it to the STs of *Wolbachia* associated with other insects already defined in the database, using the concatenated sequences of the molecular markers used in the MLST analysis.

The analysis of the HVRs regions of the *wsp* sequence resulted in the characterization of a new allele for *Wolbachia* infecting *A. asychis*, defined as wsp-726 and characterized by the identification of four new HVR peptide sequences, which were defined as alleles 258 (HVR-1), 292 (HVR-2), 289 (HVR-3) e 321 (HVR-4). There is a change of 3 amino acids in the HVR-1 258 sequence when compared to the closest available allele 17; five amino acid modifications in HVR-2 allele 292 as compared to allele 242; two amino acids modifications in HVR-3 allele 289 as compared to allele12; and seven amino acid modifications in HVR-4 allele 321 as compared to allele 255.

The phylogenetic analysis of the concatenated HVRs peptide regions of the allele *wsp-726* identified in the strain of *Wolbachia* infecting *A. asychis* and the other alleles available in wsp indicated this *Wolbachia* belongs to the supergroup A (Figure 2.2).

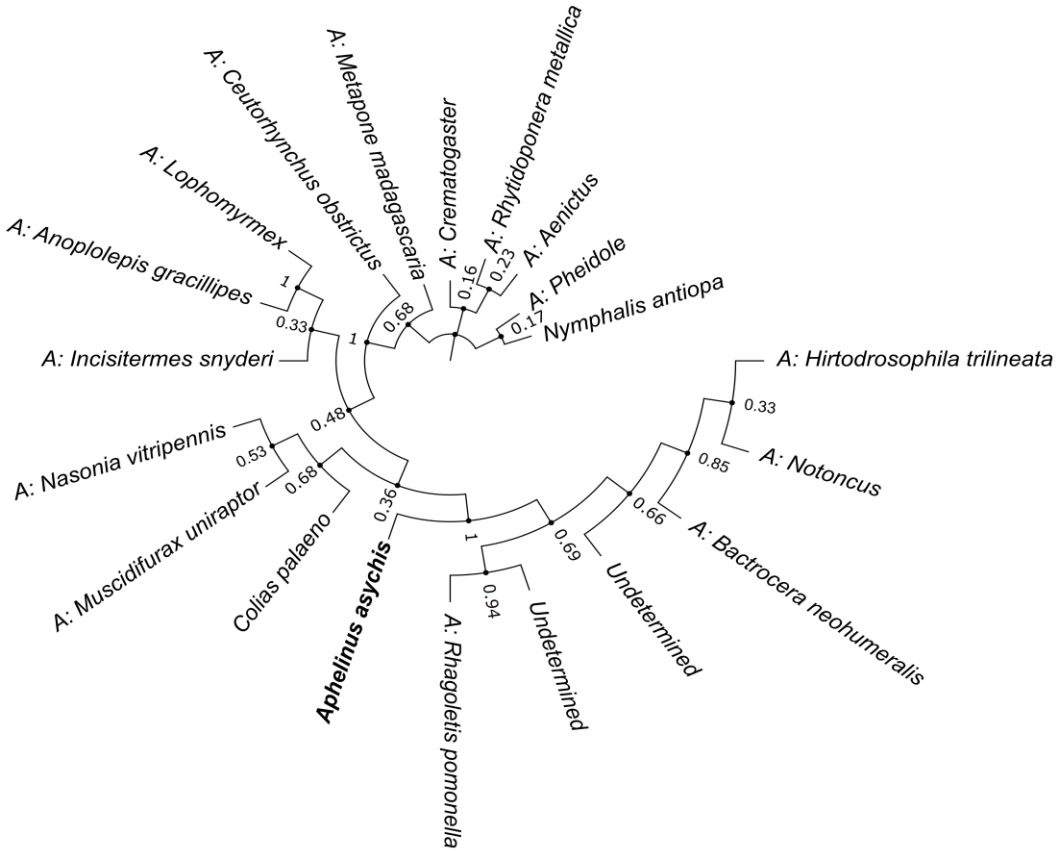

Figure 2.2. Phylogenetic relations of the HVRs of *Wolbachia* associated with *Aphelinus asychis* when compared to HVRs of *Wolbachia* availaible in the *wsp* database

2.3.2. Aphid and wasp behavior

No overall differences were observed in the total patch time allocation of females of *A. asychis* exploiting patches with *Spiroplasma*-free or *Spiroplasma*-infected aphids (χ^2=1,218; *df* = 1; p=0.22698) (Figure 2.3). The total time females of *A. asychis* spent from host contact to host parasitization was not affected by *Spiroplasma* infection (X^2= 1.427; *df*=1; *p*=0.2323) (Figure 2.4).

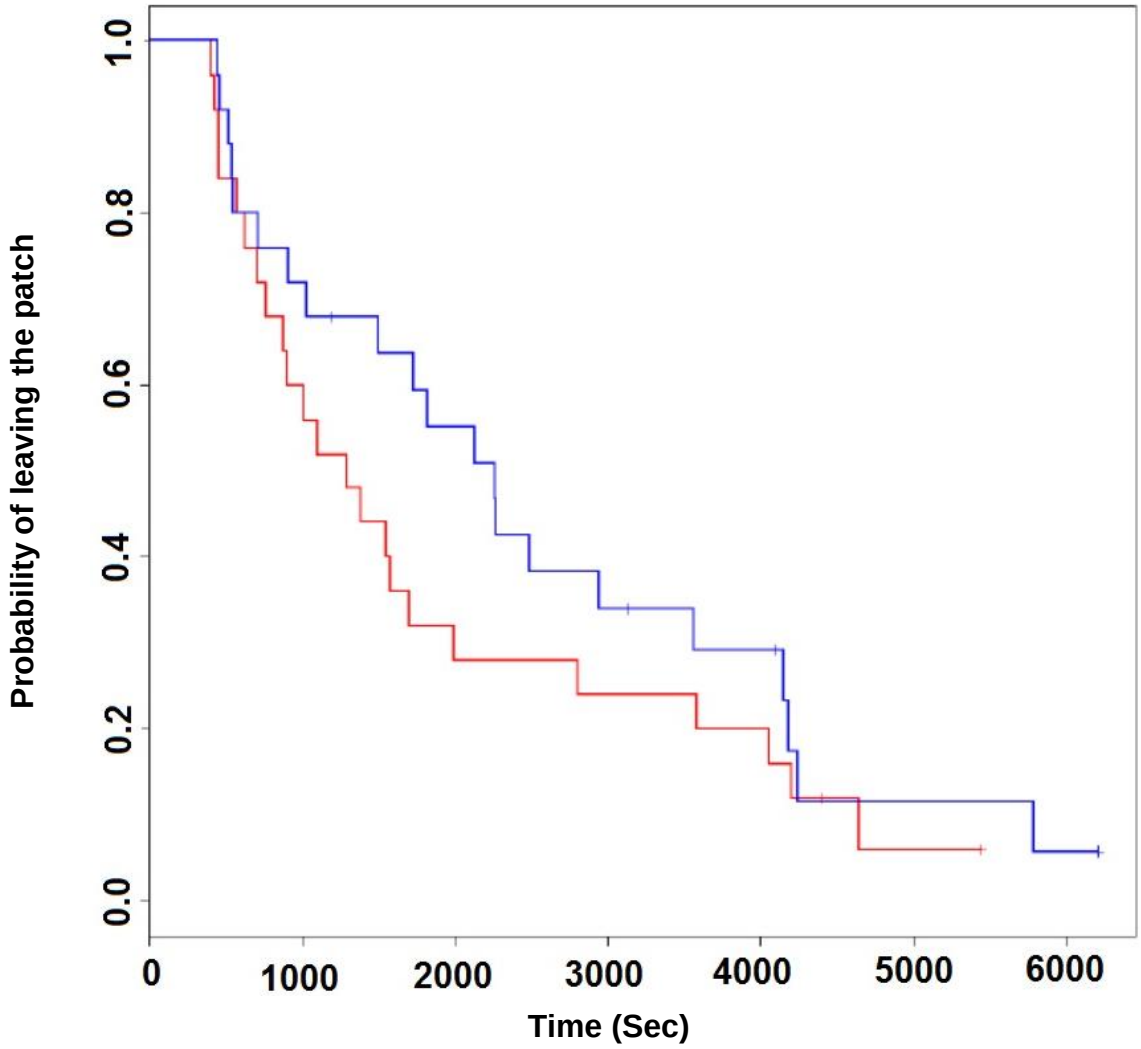

Figure 2.3. Probability of *Aphelinus asychis* females to leave patches containing *Spiroplasma*-free (blue line) or *Spiroplasma*-infected *Aphis citricidus* (red line) hosts over time.

We observed the time allocated in attacking unprobed hosts (χ^2=0.1084; *df*=1; *p*=0.7419), re-attacking hosts (χ^2=1.5623; *df*=1; *p*=0.2113), rejecting unprobed hosts (χ^2=3.0683; *df*=1; *p*=0.0798) and rejecting probed hosts (χ^2=0.0143; *df*=1; *p*=0.9046) did not alter the pacth leaving decisions of A. asychis, nor the number of hosts deaths (χ^2=3.1864; *df*=1; *p*=0.0743). However, patch-leaving decisions were influenced by the wasp grooming behavior. Females that spent less of their time grooming decided to leave the patch earlier (χ^2=7.177; *df*=1; *p*=0.0074). Another factor that induced the patch leaving decisions of *A. asychis* was the agitated behavior of the host (X^2=5.983; *df*=1; *p*=0.0144) (Figure 2.4).

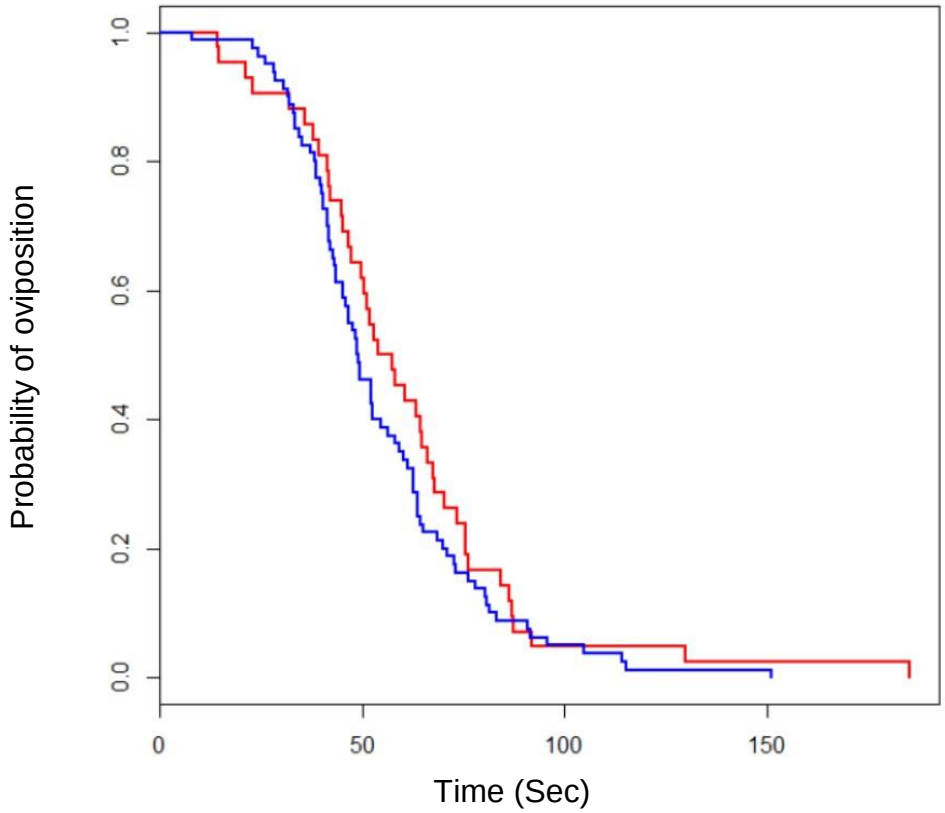

Figure 2.4. Survival density comparing the time used for the wasp to analyze and oviposit in the *Spiroplasma*-infected aphids (blue) and in the *Spiroplasma*-not-infected aphids (red) (X^2= 1.427; *df*=1; *p*=0.2323).

The female's decision to lay eggs was influenced by the host infection status. Successful oviposition was higher in uninfected than in *Spiroplasma*-infected aphids (χ^2=6.55961; *df*=1; *p*=0.01043) (Figure 2.5).

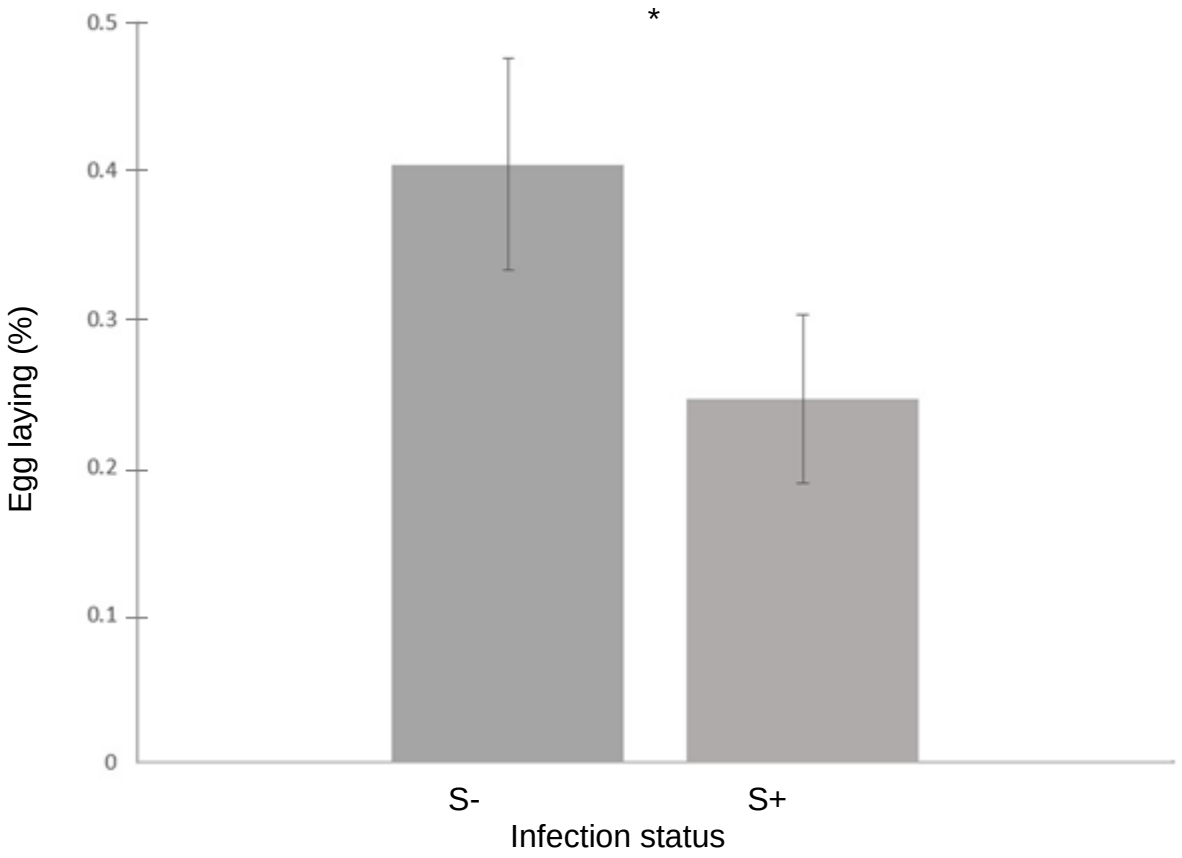

Figure 2.5. Probability of *Aphelinus asychis* to lay an egg in uninfected (S-) and *Spiroplasma*-infected (S+) *Aphis citricidus*.

However, when we compared the presence of eggs inside the hosts to the number of host contacts and probes, we found no overall difference between infected and uninfected aphids (χ^2=2.0066; *df*=1; *p*=0.1566).

But *Spiroplasma* directly interfered in the aphid defense behavior when exposed to *A. asychis* females. *Spiroplasma*-infected aphids moved more actively than uninfected aphids when attacked by *A. asychis* (X^2=5.9754; *df*=1; *p*=0.0145) (Figure 2.6), but uninfected responded more aggressively to parasitoid contact (X^2=17.072; *df*=1; *p*<0.0001) (Figure 2.6). Analysis of the individual aggressive behaviors indicated uninfected aphids threw more kicks (X^2=10.118; *df*=1; *p*=0.0014) (Figure 2.7a) and gave a larger number of antennal whips towards the wasps (X^2=7.3314; *df*=1; *p*<0.0060) than the *Spiroplasma*-infected *A. citricidus* (Figure 2.7b).

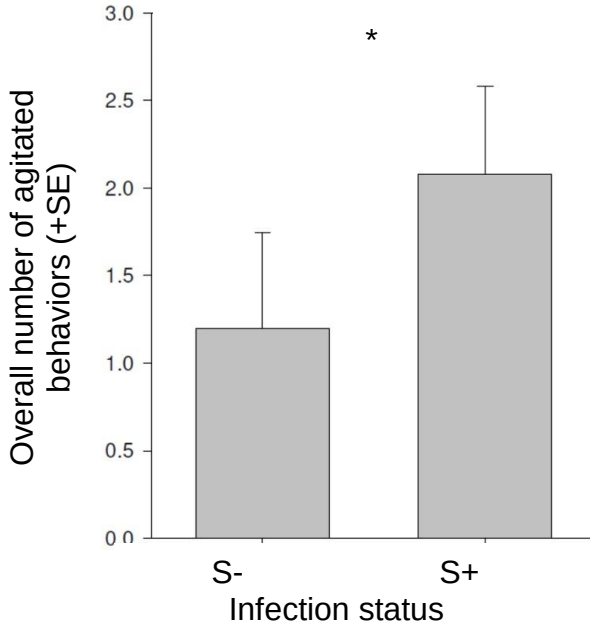

Figure 2.6. Overall number of agitated behaviors from the *Spiroplasma*-infected (S+) and *Spiroplasma*-not-infected (S-), showing a significant difference between the two situations (X^2=5.9754; *df*=1, *p*=0.0145).

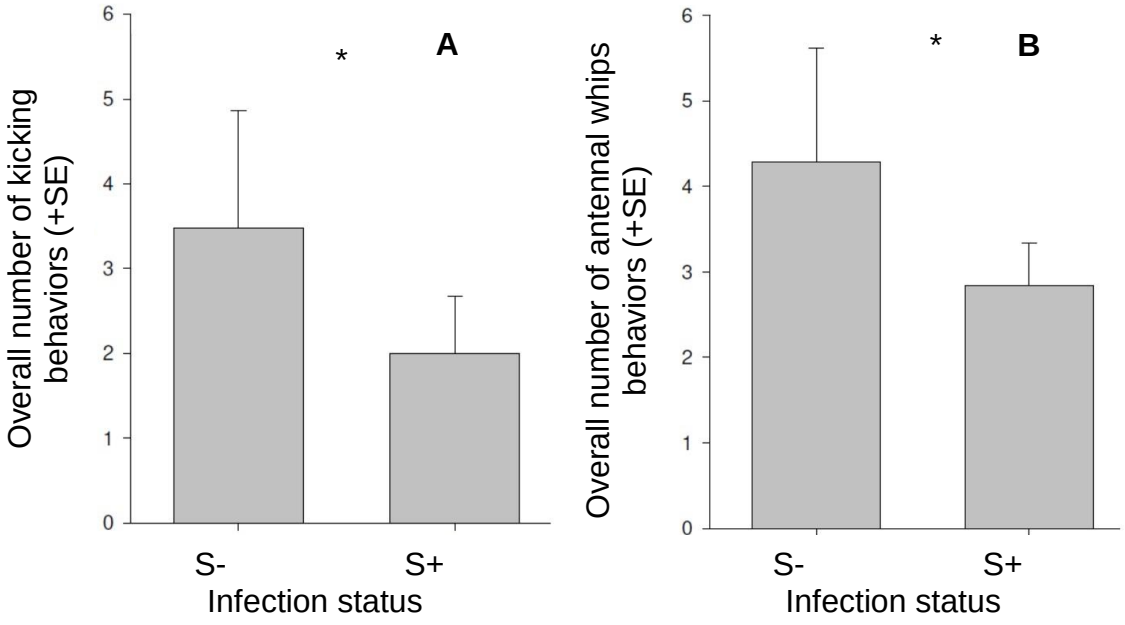

Figure 2.7. Kicking behavior (A) and antennal whips (B) of *Spiroplasma*-infected (S+) and *Spiroplasma*-free (S-) *Aphis citricidus* when attacked by females of *Aphelinus asychis*.

2.4. Discussion

We demonstrated that *Spiroplasma*-host infection can influence directly and indirectly the patch exploitation by *A. asychis*. Successful parasitization (given by egg laying) of *A. citricidus* by *A. asychis* was higher in uninfected than in *Spiroplasma*-infected hosts. Although one would argue that the reduced success should be a differential response of the immune system of infected hosts, once sensitized hosts would have a higher level of expression of immune related genes before parasitization (Oliver et al. 2003; Degnan and Moran 2008), we did not observe any cellular immune activity as we did not record any reaction of encapsulation of eggs of *A. asychis*. The cells involved in immune response are still poorly described in aphids, although the only cell type identified was demonstrated to adhere to the chorion surface of laid parasitoid eggs (Kapranas et al. 2008). It is possible though that the reduced oviposition of *A. asychis* in infected aphids would correlate with increased levels of melanin. Overproduction of prophenoloxidase in *Aphidius ervi* (Hymenoptera: Braconidae) and activation of phenoloxidase (PO) cascade in response to parasitism were suggested as indicators that high levels of melanization activity would be one of the antiparasitic factors affecting host acceptance by aphid parasitoids (Pennacchio et al. 1995; Shelby et al 2000). In this case, microbial infections are known to activate the humoral machinery that includes the production of antimicrobials and the activation of the PO cascade to increase melanin production (Wojda and Jakubowicz 2007). However, other could argue that the reduced number of eggs laid in *Spiroplasma*-infected hosts is more related to changes in the chemical and/or physical stimuli wasp females must perceive to make their decisions to lay eggs (Vinson 1976). There is very little information on the chemical stimuli parasitic wasps rely on to accept a host when evaluating the suitability of a host for the investment of an egg (Vinson and Iwantsh 1980a). Most of the information available is for egg parasitoids, which will accept their hosts as an oviposition substrate in response to the availability and concentration of particular amino acids and/or ions (Vinson and Iwantsh 1980b). *Spiroplasma*-infected *A. citricidus* was shown to suffer alterations in the proteome profile as compared to uninfected aphids, particularly in the abundance of proteins involved in protein-protein interactions, cell functioning and energy metabolism (Guidolin et al. 2018). Moreover, metabolomic analysis also demonstrated *Spiroplasma* infections affected a large number of metabolites of *A.*

citricidus, including nutrients and cuticular hydrocarbons that are used as chemical cues for host location and selection by aphid´s parasitoids (Vinson 1976). Thus, the reduced acceptance of *Spiroplasma* infected aphids by *A. asychis* could result from *Spiroplasma*-induced changes in the host immune system and/or in host metabolites that would indicate female parasitoids a host of a reduced quality. Female wasps evaluate the suitability of hosts by using external chemical and physical cues during host handling but also rely their final decision to accept a host evaluating the internal chemical cues with sensilla located in the ovipositor (Salt 1934; Vinson and Iwantsh 1980a; van Lenteren et al. 2007).

The lack of differences in the time females spent in host handling until host probing when exploiting infected and uninfected aphids and the higher number of successful egg laying in uninfected as compared to infected hosts, support the hypothesis that wasp females were only able to recognize differences in between hosts after probing them with the ovipositor. Females invested the same time and displayed the same number of host contacts and probing when laying an egg in an infected as compared to an uninfected host. Thus, we find no indication that the reported differences in the composition of cuticular hydrocarbons in *Spiroplasma*-infected *A. citricidus* (Duarte 2017) would lead to changes in host recognition by *A. asychis*. Both hypotheses require additional experimentation to substantiate a final conclusion. A higher quality patch could represent a better source for the female to invest in, what could affect the decision to leave it (McNair 1982; Wajnberg et al. 2016). If the hypotheses provided above are true, we should argue that the lower host quality represented by infected aphids did not affect *A. asychis* patch leaving decisions. However, only two parameters affected the patch leaving decisions of *A. asychis* females. One was the amount of time females remained grooming themselves. The other parameter was the host agitated behavior.

Aphids defend themselves from aggressors by displaying behaviors, such as walking away, kicking and even dropping themselves from the host plant (Roitberg and Myers 1978), but no information is available regarding the role of symbionts in stimulating such behaviors. The aphid agitated behavior was higher in infected than in uninfected hosts. Although agitation is clearly a behavior in response to a stress condition (Bateson et al. 2011), the other aggressive related behaviors (kicking and antennal whip) observed for *A. citricidus* did not affect the patch leaving decisions by *A. asychis*. These behaviors were not affected by infection at the same way. While

kicking was inhibited in infected females, antennal whipping of the wasp was stimulated when compared to uninfected aphids. Insect aggressiveness also corresponds to the levels of biogenic amines, particularly octopamine (Davenport and Evans 1984; Stevenson and Rilich 2016) suggesting *Spiroplasma* induces octopamine production in infected hosts.

Wolbachia strains can be important defensive players increasing host aggressiveness (Rohrscheib et al. 2015) and competitiveness (Alexandrov et al. 2007); however, there are few cases in which protective symbionts may negatively affect the defense of their hosts (Dion et al. 2011; Polin et al. 2014), highlighting symbionts can alter their hosts' behaviors differently.

Symbiont induced alterations in the host and in the interactions of the host with the third trophic level can potentially affect the host selection processes and time allocation, which are important parameters affecting parasitoid efficiency in host exploitation. Understanding the many aspects that affects patch exploitation and the behavioral rules involved are important in the decision making process for selecting biological control agents (Wajnberg et al. 2016). Parasitoids that stay longer and better exploit a patch should be preferred for use in biological control programs.

2.5. Conclusions

- *Spiroplasma* infections affect the defensive behavior of *Aphis citricidus;*
- *Spiroplasma*-infected aphids kick and whip females of *Aphelinus asychis* with their antennae more often that uninfected aphids;
- The oviposition decisions of female *A. asychis* are negatively impacted in patches of *Spiroplasm*a-infected *A. citricidus.*

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3. *Wolbachia* INFECTION INTERFERES WITH THE PATCH EXPLOITATION DECISIONS OF THE EGG PARASITOID *Trichogramma pretiosum* (HYMENOPTERA: TRICHOGRAMMATIDAE)

Abstract

Several factors influence the host selection process and the behavioral ecology of parasitic wasps. The assessment and understanding of the factors that result in successful host selection and optimal patch time allocation by parasitic wasps are required for the implementation of successful applied biological control and promotion of conservation biological control. Insect microbial symbionts are diverse and are reported to influence several aspects of the physiology and behavior of their hosts, including *Wolbachia,* the most common non-obligate symbiont associated with arthropods. We investigated the effects of *Wolbachia* infection on patch time allocation and host selection behavior of *Trichogramma pretiosum* (Hymenoptera: Trichogrammatidae) using infected and cured sister lines, when exploiting patches with host eggs of *Anagasta küehniella* (Lepidoptera: Pyralidae). A new sequence type (ST-493) of *Wolbachia* was identified infecting *T. pretiosum* using the multi-locus sequencing typing approach. We recorded the behaviors of infected and cured wasps when exploiting the patch. We found infected females remain longer in the patch when compared to cured females. We also detected mated females remained longer than virgin females regardless of their infection status. The patch leaving decisions of *T. pretiosum* females increased with rate of contact with previously parasitized eggs regardless the mating and infection status. In conclusion, the ST-493 of *Wolbachia* affects the patch leaving decisions and the efficiency of parasitization of *T. pretiosum,* influencing the field efficiency of this parasitoid if used as a biological control agent.

Keywords: Natural enemies; Parasitism; Quality control; Risk assessment; Symbiosis

3.1. Introduction

There is a wide range of symbiont bacteria directly related to their hosts attributes, which can alter the hosts physiology, phenotype expression and behavior. Some bacterial symbionts are key to their hosts as they provide essential nutrients to complement the host´s nutritional requirements, while other can contribute to host defense and host utilization, for example (Steinhaus 1960; Dillon and Dillon 2004).

Wolbachia is by far the most common and widely distributed symbiont in arthropods and nematodes. *Wolbachia* is better known by affecting sex host determination using several different processes, but a number of different interactions can also be established (Werren et al. 2008). There associations in which *Wolbachia* stablishes highly pathogenic interactions with their hosts (Min and Benzer 1997; Woolfit et al. 2013), while in others they can be fundamental in

providing nutrients to the host (Brownlie et al. 2009) or in inducing oogenesis in others (Dedeine et al. 2001; Dedeine et al. 2005). In associations in which *Wolbachia* infection has low adaptive costs, infection by this bacterium enhances the host immune system and contributes to the host immune defense against pathogenic infections (Teixeira et al. 2008).

Several insect associated symbionts are also known to affect the host behavior even when leading of pathogenic interactions (Dion et al. 2011; Ferrari and Vavre 2011). *Wolbachia* is also one of such symbionts, and can induce high levels of octopamine synthesis in their hosts, resulting in increased host aggressiveness (Rohrscheib et al. 2015). *Wolbachia* has also been demonstrated to affect the host selection behavior of natural enemies, reducing the capacity of female parasitic wasps to discriminate parasitized from health hosts (Farahani et al. 2015).

There are also *Wolbachia* strains that adds high fitness costs to their associate hosts negativelly affecting fitness traits that are of particular interest when hosts are important as biocontrol agents in applied or conservative biological control (Mochiah et al. 2002). Biological Control is one of the most important strategies available for sustainable pest control (Jonsson et al. 2014). Implementation of biological control strategies require the understanding of natural enemies' behavior and ecology and their interactions with the hosts for their successful exploitation (Wajnberg et al. 2015).

Egg parasitoids of the genus *Trichogramma* (Hymenoptera: Trichogrammatidae) are the most common and important parasitoids used as biocontrol agents worldwide (Cônsoli et al. 2010). *Trichogramma pretiosum* is polyphagous and widely distributed in this genus, and it has been mass reared and released in millions of hectares for the control of pest species in a number of agroecosystems, including Brazil (Cônsoli et al. 2010; Parra and Zucchi 2004; Parra 2014). *Trichogramma* is also a common host to *Wolbachia*, with several species been associated with this bacterium (Pintureaeu et al. 2002; Werren et al. 2008; Almeida and Stouthamer 2017). Thelytokous parthenogenesis is the most common phenotype induced by *Wolbachia* infections in *Trichogramma* (Werren et al. 2008), and the fact that *Wolbachia*-infected *Trichogramma* would only produce females as progenies has been argued to favor the mass production and use of this natural enemy in applied biological control programs (Stouthamer et al. 1990; 1999; Stouthamer 1993;). But little is known on the effects of *Wolbachia* infections on

behavioral and fitness traits that would interfere with the optimal strategies for host use and successful parasitization (Farahani et al. 2015).

There are a number of factors capable of altering the host selection and behavior ecology of parasitoids. Over recent years, the role of the host microbiome in host phenotype expression and fitness traits attributes has been discovered and increased concern to understand how associates microbes, particularly bacteria, can interfere in host biology, phsiology and ecology has fostered investigations on a wide range of topics, from their role in speciation processes, host selection, host adaptation, among others (Brucker and Bordenstein 2012; Lewis and Lizé 2015; Shropshire and Bordenstein 2016).

In order to better understand the effects of *Wolbachia* in infected wasps target to use as biocontrol agents, we tested the hypothesis that *Wolbachia* affects patch utilization and host parasitization of *T. pretiosum* by analyzing the patch leaving decisions and the successful host paratization of *Wolbachia*-infected and *Wolbachia*-cured sister isolines when exploiting patches with eggs of *Anagasta kuehniella* (Lepidoptera: Pyrallidae). We expect our data will contribute to the understanding of *Wolbachia* – *Trichogramma* associations, and the possible use of *Wolbachia*-infected strains in applied biological control programs.

3.2. Material and Methods

3.2.1. Insects rearing

A *Wolbachia*-infected strain of *T. pretiosum* collected on eggs of *Helicoverpa zea* (Lepidoptera: Noctuidae) in corn fields in the region of Piracicaba, state of São Paulo) was donated by Prof. Sérgio de Bortoli (UNESP – Jaboticabal). This strain was reared on eggs of the factitious host *A. kuehniella* under controlled laboratory rearing conditions (25±1°C, 60±10% RH, 14h photophase). *Anagasta kuehniella* was reared following Parra et al. (2014) and eggs were kindly provided by the Insect Biology Laboratory, Department of Entomology and Acarology, USP/ESALQ.

Thirty-six females of *T. pretiosum* were individualized in glass vials, fed with a drop of pure honey and offered eggs of *A. kuehniella* glued on a 10 x 4 mm cardboard for parasitization. Only the isoline that parasitized most of the eggs was selected and used for further experiments. Emerging females of the selected isoline were individualized and offered eggs for parasitization as before. The cardboard

containing parasitized eggs was split and one half was offered drops of honey added with 0.1% tetracycline, while the other half received drops of pure honey to feed the emerging females. Tetracycline was used to cure females from *Wolbachia* infection allowing male production and sexual reproduction (Stouthamer et al. 1990; 1999).

Males emerging from tetracycline-treated females were used to mate newly-emerged females that were treated with tetracycline for 24 h in advance. Tetracycline-fed (Wolbachia-cured: W^-) and honey-fed females (*Wolbachia*-infected: W^+) were offered new eggs for parasitization. This rearing procedure was repeated for 12 generations, when adult samples from each sister isolines were subjected to genomic DNA extraction and diagnostic-PCR for *Wolbachia* detection using the *wsp* gene as a marker, following Werren et al. (1995). After confirmation that tetracycline-fed females had been cured of their *Wolbachia* infection, the assays to investigate the effects of *Wolbachia* infection in the patch exploitation of *T. pretiosum* females were initiated by comparing female behavior of *Wolbachia*-infected (W^+) and *Wolbachia*-cured (W^-) sister isolines. The W^+ sister line was continually fed with pure honey during the rearing procedure. Females that were used in experiments were not fed with tetracycline.

3.2.3. Multi-locus sequence typing (MLST) of Wolbachia infecting Trichogramma pretiosum

W^+ females of the sister isoline of *T. pretiosum* were collected and subjected to genomic DNA (gDNA) extraction followed the same procedure as in Chapter 2. The multi-locus sequencing typing and the characterization of the hypervariable regions (HVRs) of the *wsp* were conducted following Baldo et al. (2005) as earlier described in Chapter 2.

3.2.4. Phylogenetic analyses

Allele sequences obtained from the MLST database for Wolbachia (http://pubmlst.org/wolbachia/)were recovered and concatenated for proximity sequence analysis with the concatenated sequences of the MLST alleles obtained for the strain of Wolbachia infecting *T. pretiosum.* Similar procedure was followed to recover sequences for HVRs from different *Wolbachia* strains from the *Wolbachia*

wsp database (http://pubmlst.org/wolbachia/wsp/) and used to run proximity analysis with HVRs sequences obtained from the *Wolbachia* associated with *T. pretiosum*. Sequences from each data sets were subjected to sequence alignment using *ClustalW* as implemented in MEGA 7.0.26 (Kumar et al. 2016). The phylogenetic analysis of MLST genes used the maximum likelihood (ML) method available in MEGA 7.0.26 based on the Hasegawa-Kishino-Yano model (HKY) with G = 0.05, following the lower Bayesian Information Criterion value estimated. Phylogenetic analysis using the peptide sequences for the HVRs of the *wsp* gene was performed using the Whelan and Goldan model (WAG) with G = 0.05. The consensus trees were visualized and edited using FigTree 1.4.3 (Rambaut 2016).

3.2.5. Behavioral assays of *Trichogramma pretiosum*

Females of the W^+ and W^- sister isolines were offered UV-treated *A. kuehniella* eggs for parasitization following Parra et al. (2014) and reared under controlled conditions as described before. Parasitized eggs (black eggs) were individualized in 250 mg gelatin capsules containing a drop of honey inside and maintained under controlled conditions until adult emergence. Upon emergence, adults had their sex determined and virgin W^+ and W^- females were either used in the experiments or allowed to mate W^- males in the gelatin capsules. Only females in which male mounting was observed were further used. The behavioral experiment followed a two factor design (*Wolbachia* infection and mating status) with two levels each: i) W^+-virgin females; ii) W^--virgin females; iii) W^+-mated females; iv) W^--mated females.

A patch defined by a 20 x 20 mm square was drawn on the back of a 6-cm Petri dish and nine newly laid eggs of *A. kuehniella* were placed 5 mm apart from each other following a 3 by 3 design. Eggs were numbered depending on their position in the patch.

The following behaviors of parasitoid females were assessed in the patch: i) host encounter, ii) host drumming, iii) host drilling, iv) host oviposition, v) host feeding, vi) walking, vii) grooming, viii) resting, and ix) patch leaving, as defined by others (Salt, 1935; Schimidt and Smith, 1985). Female behavior was observed under a stereomicroscope and data were collected using the JWatcher 1.0 program (Dan Blumstein's Lab University of California Los Angeles & The Animal Behaviour Lab,

Macquarie University, Sydney). Assays were performed in a quiet, controlled environment (25±1°C, 70±10% RH) from 1 p.m. to 7 p.m. with the lights on.

Females were individually released into the arena and time started counting once females encountered the first host. Females were excluded if no host had been encountered within 10 min after their release in the arena. The arena was cleaned with ethanol and the eggs were replaced with fresh ones every time a female entered the arena. The assay ended either when female wasps rested for more than 5 min, remained out of the patch for more than 1 min, or when the assay reached 40 min. Each treatment was replicated 25 times (1 female = 1 replicate).

At the end of each assay, females were collected in 70% ethanol and stored at -20^{o}C for further gDNA extraction and diagnostic PCR for detection of *Wolbachia*-infection as earlier described. Host eggs used in each replicate were collected at the end of the experiments, placed individually in 250 mg clear gelatine capsules and kept under controlled conditions for parasitoid/host development (25±1°C; 60±10% RH; 14 h photophase). After 10 days, eggs were checked for successful parasitization (eggs that turned black) and the sex of the emerged adult(s).

3.2.6. Data analysis

Data were analyzed in a factorial design with two factors (infection and mating status) and their interactions by fitting the data to a Cox regression model using the tools implemented in the R statistical software. Data were analyzed by computing the overall patch residence time taking into account time coordinates of each one of the behaviors described before: i) oviposition in unattacked healthy hosts; ii) oviposition in previously attacked hosts; iii) rejection of healthy hosts; iv) rejection of a host already attacked. We used the number of successful ovipositions and the number of hosts that were rejected as parameters that influence patch leaving decisions (Wajnberg et al. 1999; 2000; 2003). As the hosts were composed of eggs (i.e. stationary), we were able to identify attacks, re-attacks and rejections, which leads us to time-dependent covariates (as performed by Wajnberg et al. 2003), during the replicates. We have also compared a few different parameters between infected and cured females: i) the total number of hosts attacked; ii) the rate (per time unit) of the total number of hosts attacked; iii) the number and percentage of hosts that turn black; iv) the total number of hosts rejected; v) the rate (per time unit) of the total

number of host rejected; vi) the total number of hosts revisited; vii) the rate (per time unit) of the total number of hosts revisited; viii) the percentage of adult emergence, per host attacked. The total number of hosts attacked, rejected or revisited was analyzed using a Poisson regression. ANOVA was used to compare the rate (per time unit) of the total number of hosts attacked, rejected or revisited. Logistic regression were used to compare the number and percentage of host eggs that turned black or developed a wasp, to the number of host eggs that remained white or ecloded a larva. Differences in the percentage of adults that emerged per attacked host were tested using ANOVA. All analyses were performed in R software.

3.3. Results

3.3.1. Multi-locus sequence typing (MLST) of *Wolbachia* infecting *Trichogramma pretiosum*

The MLST analysis of the *Wolbachia* strain associated with *T. pretiosum* resulted in the identification of a new sequence-type (ST) present in this population (Table 3.1). From the studied genes – *coxA*, *fbpA*, *ftsZ*, *gatB* and *hcpA* – only two of them had identical alleles to those available in the wsp database: *ftsZ* allele 19 and *gatB* allele 21, with the three remaining allele being identified as new ones. Alleles *ftsZ*-19 and *gatB*-21 belong to ST-31, which is associated with *A. kuehniella*.

Table 3.1. Alleles of five molecular markers used for MLST of the strain of *Wolbachia* associated with *Tricogramma pretiosum*

	Genes				
ST	*coxA*	*ftsZ*	*fbpA*	*gatB*	*hcpA*
493	276	19	452	21	307

Allele *coxA-276* from the *Wolbachia* ST-493 associated with *T. pretiosum* is 402 nucleotides long and differs from allele *coxA*-22 by one nucleotide substitution; allele *fbpA-452* is 423 bp-long, shares 99.8% similarity with allele *fbpA*-21 and one single substitution; at last, allele *hcpA*-307 is 443 bp-long and have 5-bp substitutions with allele *hcpA*-25.

Phylogenetic analysis of the MLST alleles obtained for the *Wolbachia* strain infecting *T. pretiosum* demonstrated ST-31 is the ST closest related with the new ST-

493 infecting *T. pretiosum*. Both STs resolved in a well-defined internal clade close to ST-486, which infects *Hyponephele lycaon* (Lepidoptera: Nymphalidae) (Figure 3.1).

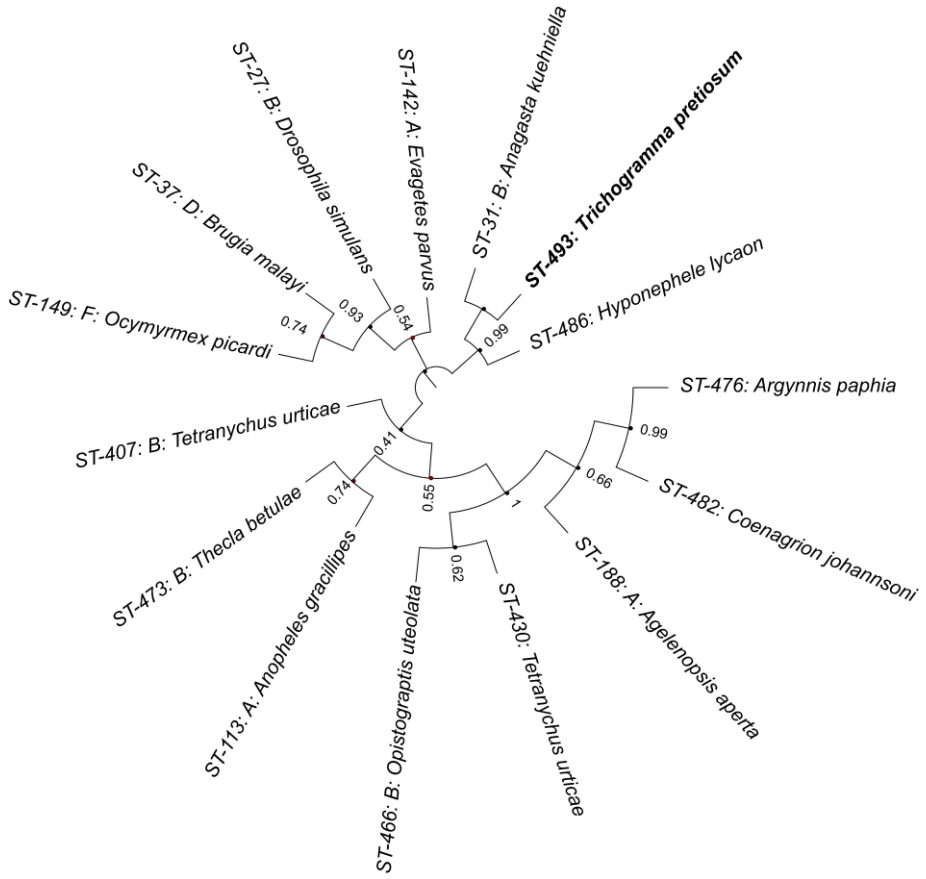

Figure 3.1. Phylogenetic relations of the sequence-type (ST-493) of *Wolbachia* associated with *T. pretiosum* and STs of *Wolbachia* associated with other insects using the concatenated sequences of the molecular markers used in MLST analysis.

Analysis of the nucleotide sequence of the *wsp* obtained for the strain of *Wolbachia* associated with *T.* pretiosum resulted in the identification of a new allele given by four new hypervariable regions (Table 3.2).

Table 3.2. Hypervariable regions of the *wsp* peptide sequence observed in *Wolbachia* population associated to *T. pretiosum* and defined in database

wsp allele	HVR-1	HVR-2	HVR-3	HVR-4
727	259	293	290	322

HVR-1 259 of *Wolbachia* associated with *T. pretiosum* was closest to HVR-1 181, carrying three amino acids changes. The new HVR-2 293 was carried three amino acids changes when compared to the closest HVR-2 149. The peptide sequence of HVR-3 290 presented five amino acids changes in relation to the closest match HVR-3 136, and HVR-4 322 carried four amino acids substitutions and one amino acid addition when compared to the closest match HVR-4 228. The phylogenetic analysis of the concatenated hypervariable regions placed the new allele *wsp*-727 in a highly supported clade with a *wsp* alleles from *Wolbachia* supergroup B. In the most internal clade wsp-727 resolved with a strain associated with an undetermined host, having *Wolbachia* associated with *Gryllus firmus* (Orthoptera: Gryllidae) as the closest related (Figure 3.2).

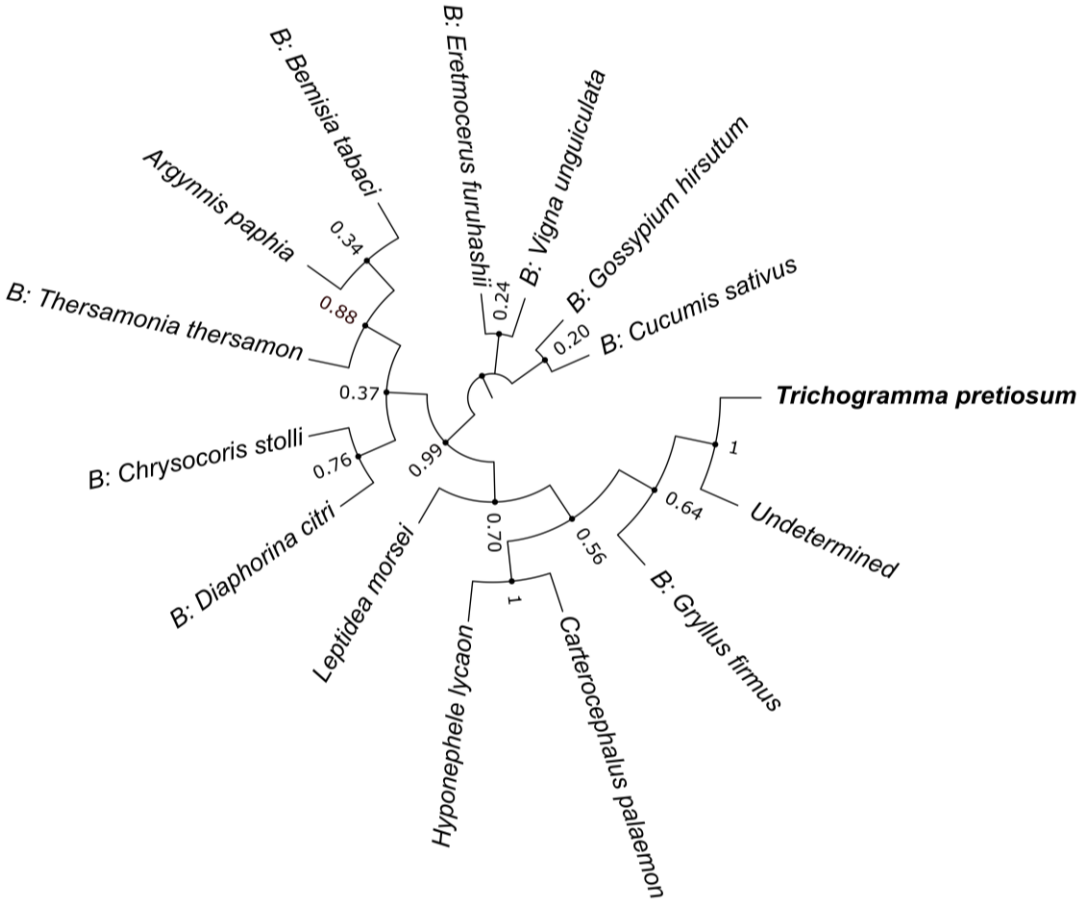

Figure 3.2. Phylogenetic relations of the HVRs of the *wsp* gene of *Wolbachia* associated with *Trichogramma pretiosum* and of *Wolbachia* associated with other insects using the concatenated peptide sequences of the four HVRs.

3.3.2. Behavioral data analysis

Diagnostic PCRs used to verify the presence/abscense of Wolbachia in *T. pretiosum* females used in the behavioral assays conducted indicated tetracycline-treated females were cured of the *Wolbachia* infection, while those fed exclusively with honey were all infected with *Wolbachia.*

Wolbachia infection clearly affected the patch residence time of *T. pretiosum*. *Wolbachia*-infected females remained longer in the patch than cured females (χ^2= 8.653; *df*=1; *p*=0.003) (Figure 3.3). The total patch residence time was also influenced by the mating status. Virgin females left the patch earlier than mated females (χ^2=7.401; *df*=1; *p*=0.006) (Figure 3.3). The effects of *Wolbachia* on the total patch residence time did not interact with the female mating status (χ^2=1.431; *df*=1; p=0.232).

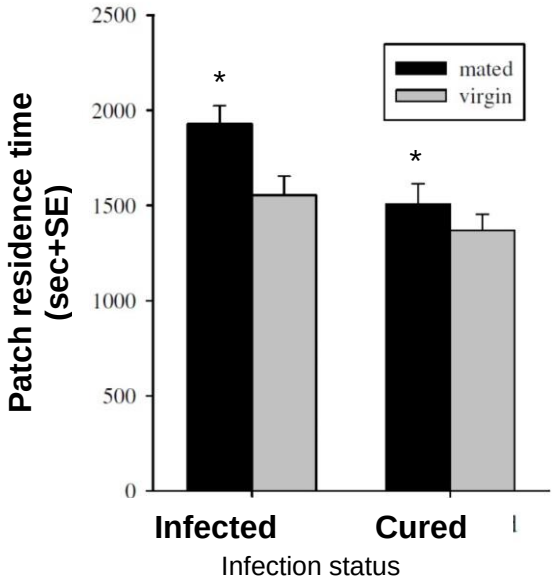

Figure 3.3. Patch residence time of *Wolbachia*-infected and *Wolbachia*-cured virgin or mated females of *Trichogramma pretiosum.* The figure shows a strong difference between the two female wasps (infected and cured), indicating that infected females remained longer in the patch than the cured females (χ2= 8.653, df=1, p=0.003). The figure also shows that the total patch residence time was influenced by the mating status. Virgin females left the patch earlier than mated females (χ2=7.401, df=1, p=0.006). Assays were performed at 25±1°C; 60±10% RH; 14 h photophase.

Females increased their tendency to leave the patch by a factor of 1.71 as affected by each oviposition in an unattacked host (χ^2=41.568; *df*=1; *p*<0.001), resulting in a decremental mechanism. On the other hand, an incremental mechanism was noticed with each oviposition in a previously attacked host

(χ^2=4.416; *df*=1; *p*=0.036), which decreased the female tendency to leave the patch by a factor of 0.771. These patch leaving mechanisms had no interactions with the infection (unattacked host - χ^2=0.539; *df*=1; *p*=0.463; previously attacked host - χ^2=0.958; *df*=1; *p*=0.328) or the mating status (unattacked host - χ^2=0.047; *df*=1; *p*=0.828; previously attacked host - χ^2=0.145; *df*=1; *p*=0.704). Females patch leaving decisions were not affected by the rejection of unattacked hosts (χ^2=3.771; *df*=1; *p*=0.052) or of hosts that were previously attacked (χ^2=0.949; *df*=1; *p*=0.330).

Wolbachia infection did not affect the total number of host eggs that were attacked by female wasps (χ^2=1.059; *df*=1; *p*=0.3034), but infected females had a much lower rate of attack per unit of time when compared to cured females (F=15.828; *df*=1, 98; *p*<0.001) (Figure 3.4). Additionally, analysis of the observed successful parasitization, given by the full larval development of *Trichogramma* as indicated by the blackening of the host chorion (Cônsoli et al. 2000), clearly demonstrated *Wolbachia* infection reduces the successful observed parasitization (χ^2=15.576; *df*=1; *p*<0.001) (Figure 3.5). Nonetheless, no effects of *Wolbachia* infection were detected on the emergence rate of observed successfully parasitized hosts (*F*=0.489; *df*=1, 98; *p*=0.486).

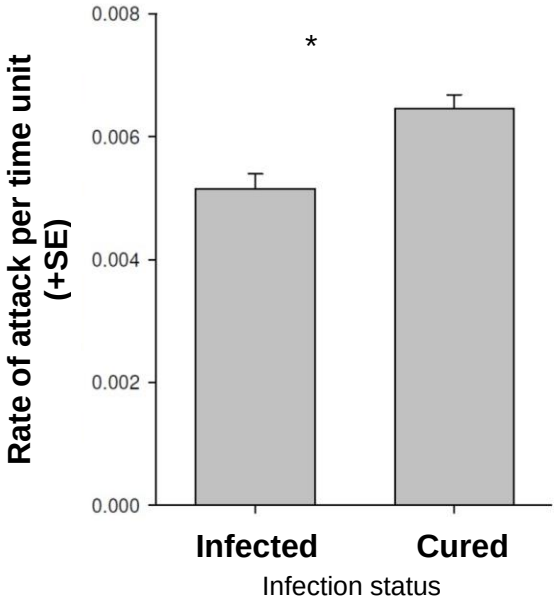

Figure 3.4. Rate of attack per time unit (+SE) of *Wolbachia*-infected and *Wolbachia*-cured females of *Trichogramma pretiosum* when exploiting a patch with eggs of *Anagasta kueniella*. The figure shows a strong difference between the two female wasps (infected and cured), indicating that cured females had a higher rate of attack per time unit than the infected females (F=15.828; *df*=1, 98; *p*<0.001). Assays were performed at 25±1°C; 60±10% RH; 14 h photophase.

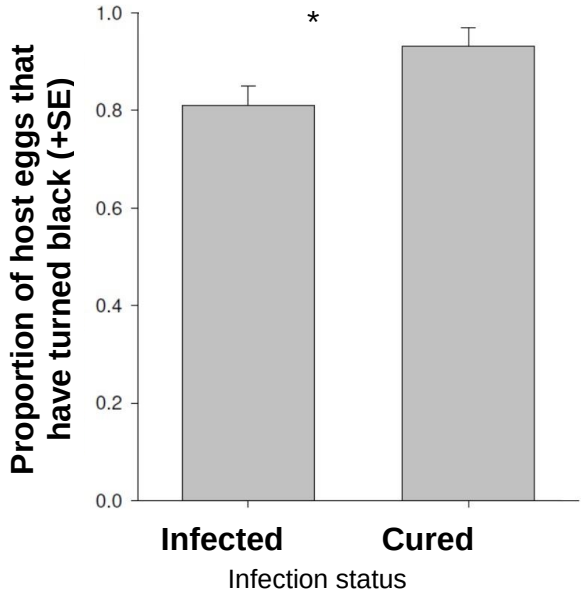

Figure 3.5. Proportion of host eggs that have turned black (+SE). The figure shows a strong difference between the two female wasps (infected and not-infected), indicating an ability of the cured female of producing a higher proportion of hosts into black eggs (χ2=15.576, *df*=1, p=0.001). Assays were performed at 25±1°C; 60±10% RH; 14 h photophase.

Infection of *T. pretiosum* with *Wolbachia* also resulted in changes in the number and in the rate host eggs rejected by female wasps. The number of hosts rejected by female wasps is reduced in *Wolbachia*-infected females (χ^2=6.747; *df*=1; *p*=0.009), while the rate of host rejection by unit time is increased when compared to *Wolbachia*-cured females (F=4.240; *df*=1, 98; *p*=0.042) (Figure 3.6).

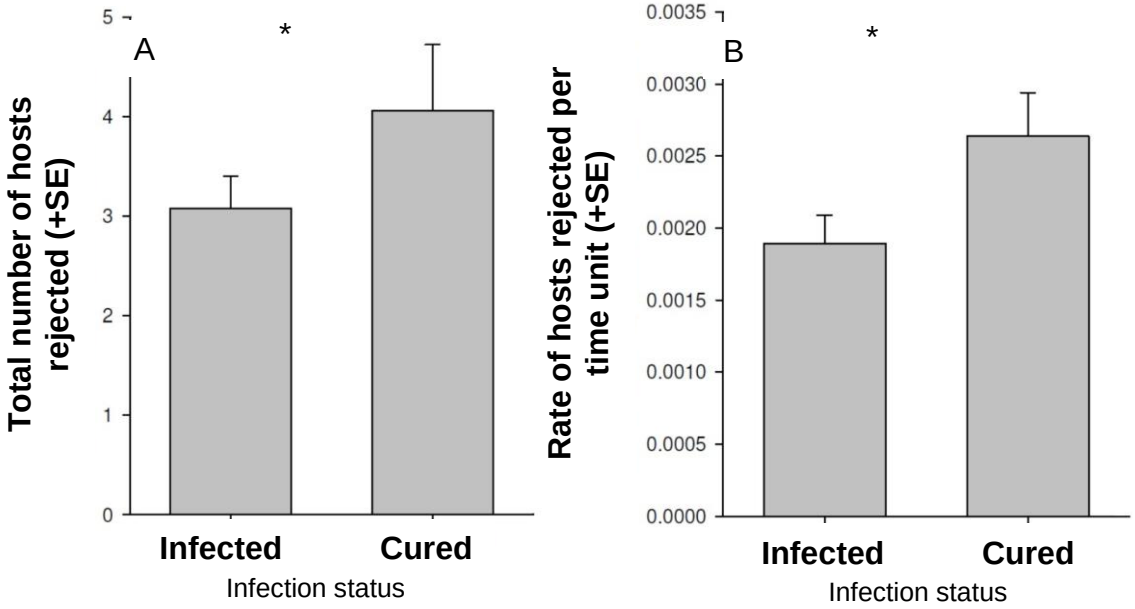

Figure 3.6a. Total number of hosts rejected (+SE) for infected and cured female wasps. The figure shows a a higher rate of rejection by the cured wasps (χ2=6.747; *df*=1; *p*=0.009).
Figure 3.6b. Rate of hosts rejected per time unit (+SE). The figure shows a slight difference between the infected and cured females (F-value= 4.2405; *df*=1, 98; *p*=0.042).
Assays were performed at 25±1°C; 60±10% RH; 14 h photophase.

Wolbachia infection did not affect the number of hosts revisited (χ^2=3.579; *df*=1; *p*=0.051) nor the rate per time unit of the total number of hosts revisited (*F*=1.576; *df*=1, 98; *p*=0.212) by *T. pretiosum* females.

3.4. Discussion

As parasitoids are very short-lived organisms, factors that affect their decisions to remain or leave a patch influencing their patch time allocation play important roles in optimal foraging strategies adopted by parasitoids (Wajnberg et al. 2015). Our data demonstrated *Wolbachia* infection induced *T. pretiosum* females to remain longer in the patch and search for hosts less intensively, as the parasitoid rate of host encounter was severely reduced in infected as compared to cured females. Morever, *Wolbachia* infection made females less efficient to successfully parasize health host eggs. While longer patch residence time can be beneficial to the wasp as it can lead to higher rates of host encounter and consequently host parasitization, residence times longer the required to optimal parasitization of the host are detrimental to the parasitic wasps as it will largely affect the wasp life time fecundity (Wajnberg et al. 2015; Wajnberg 2006). The fact that *Wolbachia* infection did not affect the total number of hosts attacked by *T. pretiosum* suggests *Wolbachia*

may interfere with host acceptance and/or egg quality that would lead to incomplete embryo development or to precocius embryo mortality. *Wolbachia* can affect the proteome of the host ovary by increasing or decreasing the availability of a number of proteins (Christensen et al. 2016). Although the control of the host physiological processes that lead to ovary development and reproduction are expected to optimize female reproduction to improve the spread of infection in the population, the outcome of the interaction will dependent on the virulence of the infecting *Wolbachia* strain. Virulent strains of *Wolbachia* can severely reduce host survival and reproduction (Min and Benzer 1997; Fleury et al. 2000; Vala et al.; Fry et al. 2004). Wolbachia ST-493 also affected host acceptance by *T. pretiosum*. Infected females were less selective than cured females by rejecting a lower number of hosts, although with an increased rate. In a similar study, Wolbachia was demonstrated to reduce the abilities of the female wasp to recognize low quality hosts, interfering with the process of host acceptance of infected *T.brassicae* (Hymenoptera: Trichogrammatidae) (Farahani et al. 2015), but the mechanisms involved in the manipulation of the host physiology were not identified.

Additionally, the reduced rate of host encounter per unit of time infected females exploited the patch suggests *Wolbachia ST-493* could be affecting the locomotary activity of *T. pretiosum* females. Although *Wolbachia* was shown to increase the locomotory activity and consequently the metabolism of *Aedes aegyptii* (Evans et al. 2009), *Wolbachia* was also shown to affect the locomotory activity by interfering with olfactory system of *Drosophila* (Peng et al. 2008). Host searching relies on a number of chemical stimuli acting at long and short ranges or upon contact (Vinson 1998; Collaza et al. 1999). In *Trichogramma* the perception of contact chemical cues induces the parasitoid arrestant behavior and stimulates the wasp to remain searching for hosts where they are available (Jones et al. 1973; Gardner and van Lenteren 1986; Fatouros et al. 2005).

Another possible hypothesis that led infected females of *T. pretiosum* to remain in the patch, display reduced rate of host encounter and successfully parasitize a lower number of host eggs would be the effect of *Wolbachia* in the energy metabolism of *T. pretiosum*, reducing the availability of resources for the wasp. *Wolbachia* compete for amino acids with the host (Caragata et al. 2014), and modulates lipid metabolism in mosquito cells (Molloy et al. 2016). Lipid metabolism is of particular importance to parasitic hymenopterans once they lack lipogenesis at the

adult stage (Giron and Casa 2003; Visser et al. 2010). In the association with *Brugia malayi*, *Wolbachia* depends on nutrients provided by the host, and *Wolbachia* elimination has shown increase in levels of glucose and glycogen in the host eggs (Voronin et al. 2016). Differences in glycogen availability and hexokinase actinity during the embryonic development were also observed between *Aedes fluviatus* infected or not with *Wolbachia.* The knocking down of the enzyme involved in glycogen synthesis led to alterations of in the levels of glycogen and proteins to the embryo, but of most relevance embryo mortality and reduction of *Wolbachia* (Fernandes et al. 2014). These metabolic changes induced by *Wolbachia* in the host and the requirement of *Wolbachia* for glycogen from the embryo of the host could explain the negative effects (reduced rate of host encounter, lower successful parasitization) observed in *T. pretiosum* infected with *Wolbachia* ST-493. The use of glycogen by *Wolbachia* in competition with the developing embryos of *Trichogramma* has the potential to negatively impact embryo successful development. *Trichogramma* lays hydropic eggs that are poor in vitellogenin content, relying on the absorption of nutrients from the host for their full embryonic development. The embryonic development in *T. pretiosum* is quite short (Cônsoli and Parra 1995), and the negative effects of *Wolbachia* utilization of the glycogen available would depend on the host quality and on the initial density of *Wolbachia* infecting the eggs

Our data on the effects of the mating status on patch exploitation by *T. pretiosum* followed the expected, with virgin females abandoning the patch earlier to search for mates to improve their reproductive fitness (Godfray 1994; Abe et al. 2010). However, an interesting fact was the observation that the patch leaving decisions of virgin infected females was similar to uninfected virgin females, which suggests that the alterations *Wolbachia* provokes in the host to produce a female-based progenie does not interfere with the physiological processes induced by mating on female behavior. Males can transfer a set of molecules associated with their seminal fluid that can modulate a number of behaviors and physiological processes in the female, including ovary development and egg deposition (Chen 1996; Wolfner 1997; Tram and Wolfner 1999; Wolfner 2002; Sirot et al. 2014). Thus, these data indicate the patch exploitation behaviors of *Trichogramma* females are affected by molecules males transfer in the seminal fluids during mating.

We successfully characterized the new ST-493 of *Wolbachia* infecting females of *T. pretiosum* and demonstrated this strain affects the patch leaving decisions of

the host, the rate of host encounter per unit of time and the successful parasitization of eggs of *A. kuehniella,* demonstrating *Wolbachia* has the potential to negativelly interfere with the successful use of hosts by *Trichogramma* wasps. Our data then points that the use of *Wolbachia*-infected lines in mass rearing systems for biological control application should be adopted only after the nature of the interactions of the infecting strain of Wolbachia with the particular host genotype are established to allow for a correct risk assessment of the benefits of using *Wolbachia*-infected lines in applied and/or conservation biological control programs.

3.5. Conclusions

- *Trichogramma pretiosum* is infected by the new ST-493 of *Wolbachia;*
- ST-493 of *Wolbachia* affects the patch leaving decisions and the patch residence time of *Trichogramma pretiosum*;
- ST-493 of *Wolbachia* decreases the rate of host encounter per unit time of *Trichogramma pretiosum*;
- ST-493 of *Wolbachia* decreases the number of eggs successfully parasitized by *Trichogramma pretiosum*;
- ST-493 of *Wolbachia* decreases the number of rejected hosts and increases the rate of rejection of eggs per unit time by *Trichogramma pretiosum*;
- Virgin females remain short in the patch as compared to mated females regardless of their infection status.

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