University of São Paulo
"Luiz de Queiroz" College of Agriculture

Multipartite interactions of *Aphis (Toxoptera)* and their associated symbionts

Aline Sartori Guidolin

Thesis presented to obtain the degree of Doctor in Sciences. Area: Entomology

Piracicaba
2016

Aline Sartori Guidolin
Bachelor in Biological Sciences

Multipartite interactions of *Aphis (Toxoptera)* and their associated symbionts

versão revisada de acordo com a resolução CoPGr 6018 de 2011

Advisor:
Prof. Dr. **FERNANDO LUIS CÔNSOLI**

Thesis presented to obtain the degree of Doctor in Sciences: Area: Entomology

Piracicaba
2016

Dados Internacionais de Catalogação na Publicação
DIVISÃO DE BIBLIOTECA - DIBD/ESALQ/USP

Guidolin, Aline Sartori
Multipartite interactions of *Aphis* (*Toxoptera*) and their associated symbionts / Aline Sartori Guidolin. - - versão revisada de acordo com a resolução CoPGr 6018 de 2011. - - Piracicaba, 2016.
205 p. : il.

Tese (Doutorado) - - Escola Superior de Agricultura “Luiz de Queiroz”.

1. Fisiologia 2. Interações multitróficas 3. Manejo sustentável de pragas 4. Simbiose I. Título

CDD 632.752
G948m

DEDICATION

This thesis is dedicated to my loving ones that stood by my side all over the way.

ACKNOWLEDGEMENTS

To God that always gave me the answers in the moments of doubts.

To Professor Fernando Luís Cônsoli for all the guidance, scientific discussion and persistence.

To Professor Frederic Francis, University of Liège-Gembloux, for the opportunity and support during my sandwich program.

To FAPESP for the fellowship in Brazil (FAPESP n. 2012/04287-0) and in Belgium (FAPESP-BEPE n. 2014/12067-5).

To the Graduate Program in Entomology of Escola Superior de Agricultura "Luiz de Queiroz" for all the professional support and opportunities.

To all professors that participated of my graduation by teaching in classroom and/or discussing in the hallways.

To my friends from Insect Interaction Lab that listened my complains and happy news in many coffee breaks: Camila de Paiva Gomes, Taisa Pavani Gomes, Mariane Possignolo Gomes, Bianca Soares Carbogin, Fabio Ramon Martins Duarte, Nathalia Cavichiolli de Oliveira, Wanessa Scopel, Bruna Lais Merlin, Luis Gustavo de Almeida, Felipe Antonio Domingues, Fábio Cleisto Alda Dossi, Mirela Batista Coelho e Ana Flávia Canovas Martinez.

To the dearest "sister friends" Aline Bertolosi Bombo, Eveline Calderan Meneguetti, Juliana de Lima Matos, Keini Dressano, Mariana da Silva Azevedo and Thais Degasperi. Always and forever there (many times in the WhatsApp)!

To my parents and grandmothers who support me without a question.

To my best friend, and luckily my boyfriend, Antonio Rogério Bezerra do Nascimento for all the help in the work and in my life, giving me strength and enlightenment.

To all others that passed through my life these years, helping me even when I did not noticed.

My sincere THANK YOU!

EPIGRAPH

I have fought the good fight, I have finished the race, I have kept the faith.

2 Timothy 4:7

SUMMARY

RESUMO

Interações multitróficas de *Aphis (Toxoptera)* e seus simbiontes secundários

A associação insetos - bactérias simbiontes resulta em várias implicações bioecológicas para o hospedeiro e se estende em uma rede de interações que inclue outros microrganismos, o ambiente e outros níveis tróficos. A interação bactérias simbiontes e pulgões tem sido amplamente estudada, especialmente em espécies polífagas do hemisfério norte, tal como o pulgão modelo *Acyrthosiphon pisum* (Harris). Essas pesquisas indicam que simbiontes influenciam vários aspectos ecológicos do hospedeiro, tais como: nutrição, resistência ao estresse térmico e inimigos naturais, capacidade de transmissão de fitovírus, uso de plantas hospedeiras, entre outros. Entretanto, o resultado da interação simbionte-pulgão é contexto dependente, no qual o fenótipo depende da variabilidade intra- e interespecífica, da linhagem do simbionte e de diversos fatores bióticos e abióticos. Assim, este trabalho aborda as interações do pulgão *Aphis* (*Toxoptera*) *citricidus* (Kirkaldy) e seus simbiontes, com o objetivo de contribuir com um sistema ecológico diferente. Foi investigada a influência da estratégia alimentar na diversidade da microbiota, comparando-se uma espécie oligófaga, *A. citricidus*, a outra polífaga, *Aphis* (*Toxoptera*) *aurantii* Boyer de Fonscolombe. Foram utilizadas diversas abordagens experimentais (biológica, metagenômica, genômica, transcritômica e proteômica) para atingir os objetivos de *i)* investigar o impacto das plantas hospedeiras na aptidão biológica de *A. citricidus* e *A. aurantii* e sua consequência ao simbionte primário, *ii)* explorar as diferenças no genoma do simbionte primário de *A. citricidus* e *A. aurantii*, *iii)* investigar a influência da planta hospedeira na distribuição de simbiontes secundários em *A. citricidus*, *iv)* pesquisar a riqueza e abundância de simbiontes secundários nas duas espécies de pulgão e indagar sobre a influência de *Spiroplasma v)* na aptidão biológica, *vi)* no transcritoma e *vii)* na proteômica de *A. citricidus* em duas plantas hospedeiras (laranja vs. murta). Os resultados indicaram ser laranja o melhor hospedeiro para ambos pulgões, sendo *A. citricidus* mais sensível à planta hospedeira que *A. aurantii*. As duas espécies de pulgão utilizam estratégias distintas para controlar o crescimento de *Buchnera*, assim como a utilização do alimento influenciou diferentemente cada fase do desenvolvimento dos pulgões estudados. A planta hospedeira também influenciou a abundância de simbiontes secundários; porém, *Spiroplasma* foi o simbionte secundário mais abundante em ambas espécies. *Spiroplasma* não afetou a biologia de *A. citricidus*, mas causou alterações no transcritoma e no proteoma do hospedeiro. A planta hospedeira também exerceu forte regulação na transcrição gênica de *A. citricidus*, mas esse efeito foi dependente da infecção do pulgão por *Spiroplasma*. Análises de transcritoma em pulgões infectados indicaram a regulação de transcritos relacionados à resposta imunológica quando em laranja, mas de chaperoninas em murta. A regulação gênica de *A. citricidus* foi fortemente influenciada pela planta hospedeira, mas enquanto insetos livres de *Spiroplasma* apresentaram superexpressão gênica em laranja quando comparada a murta, pulgões infectados com esse simbionte apresentaram padrão de expressão oposto para o mesmo conjunto de transcritos. Análises comparativas do proteoma de pulgões infectados ou não por *Spiroplasma* nas plantas hospedeiras estudadas indicaram diminuição dos mecanismos de defesa em favor de um aumento de proteínas ligadas à nutrição em insetos infectados quando se alimentando em murta.

Palavras-chave: Fisiologia; Interações multitróficas; Manejo sustentável de pragas; Simbiose

ABSTRACT

Multipartite interactions of *Aphis (Toxoptera)* and their associated symbionts

Insect-symbiont interactions have many bioecological consequences to the host. Their relationships expand through a complex network that includes other microorganisms, interactions with the environment and other trophic levels. An extensive literature has been produced on bacterial symbionts and aphids, especially for polyphagous aphids from North America and Europe, such as *Acyrthosiphon pisum* (Harris). They indicated symbionts influence host nutrition, heat tolerance, defense against natural enemies, virus transmission, host plant exploitation, among others. However, the outcome from host-symbiont interactions is context-dependent, with the expressed phenotype depending on intra and interspecific variations, symbiont strain, and biotic/abiotic stimuli. We explored the interaction between aphids and associated symbionts, aiming to contribute to this field by exploring new aphid systems, and focused on *Aphis (Toxoptera) citricidus* (Kirkaldy) and associated microbiota. We investigated the influence of the feeding habits on symbiont diversity in an oligophaogus, *A. citricida*, as compared to a polyphagous species, *Aphis (Toxoptera) aurantii* Boyer de Fonscolombe. We employed several approaches (biological, metagenomics, genomic, and proteomic) to investigate *i)* the impact of host plants on fitness traits and primary symbiont abundance in the oligophagous and polyphagous species, *ii)* differences in the draft genome of the primary aphid symbiont between *A. citricidus* and *A. aurantii*, *iii)* the influence of host plant in secondary symbiont distribution in *A. citricidus*, *iv)* secondary symbiont richness and abundance in both aphids, and investigate the effects of *Spiroplasma* infections *v)* in the fitness traits, *vi)* transcriptome and *ii)* proteome of *A. citricidus* when reared on two host plants (sweet orange and orange jasmine). Our data indicated that sweet orange is a better host plant than orange jasmine for both aphids, and that *A. citricidus* was more negatively affected by lower-quality host than *A. aurantii*. *A. citricidus* and *A. aurantii* had different strategies regarding *Buchnera* growth and the use of food in different stages of development. We observed differences in the draft genome of *Buchnera* associated to *A. citricidus* and *A. aurantii*. Host plant affected secondary symbiont abundance, but *Spiroplasma* was the most abundant symbiont in both aphids. *Spiroplasma* had neutral effects on *A. citricidus* biology, but affected host transcriptome and proteome. The host plant affected gene expression of *A. citricidus*, but the effect was dependent on *Spiroplasma* infection. Transcriptome analysis indicated *Spiroplasma* down-regulated aphid immune response genes on sweet orange, while regulating an entire different set of genes on orange jasmine, mainly chaperonins. Gene transcription of *A. citricidus* was strongly influenced by the host plant. But while a large number of transcripts were up-regulated in uninfected aphids in sweet orange as compared to orange jasmine, the same set of genes had an opposite pattern of expression in *Spiroplasma*-infected aphids. Comparative proteomic analysis of *Spiroplasma*-infected and uninfected aphids on sweet orange and orange jasmine demonstrated regulation of a larger number proteins on orange jasmine than on sweet orange. *Spiroplasma* down-regulated the immune response of aphids and up-regulated proteins related to nutritional processes when developing on a low-quality host plant, orange jasmine, while no such trend was observed on sweet orange.

Keywords: Physiology; Multitrophic interactions; Sustainable pest management; Symbiosis

FIGURE LIST

TABLE LIST

SUPLEMENTARY TABLE LIST

1 INTRODUCTION

Plants, herbivorous insects, and associated microorganisms participate of complex multitrophic interactions in which insect-plant relationships are influenced by microbial associates of either plants or insects (SUGIO et al., 2015). This thesis will not cover plant microbial diversity, but instead address how insect-associated bacterial endosymbionts are influenced by host plant use, and how a particular symbiont affects host fitness traits, host transcriptome and host proteome on different host plants.

Aphids are model insects used to study insect-symbiont interactions, and their relationships with host plants and natural enemies. The association between aphids and the obligate symbiont *Buchnera aphidicola* is largely known, and there is a robust body of information for *Acyrthosiphum pisum* (Harris), including its host races and secondary symbionts (MORAN; TELANG, 1998; SABATER et al., 2001; SIMON et al., 2003; TSUCHIDA et al., 2004; LAUGHTON; FAN; GERARDO, 2014).

B. aphidicola is an obligate intracellular bacterium sharing a long history of association with aphids, with differences among strains closely representing the phylogeny of their hosts (NOVAKOVA et al., 2013). This bacterium is harbored in specific cells of the host called bacteriocytes (BUCHNER, 1965), and is maternally transmitted to the progeny (WILKINSON; FUKATSU; ISHIKAWA, 2003). *B. aphidicola* is known to supplement the aphid diet by supplying the host mainly with essential amino acids that are not provided by the host diet, but also vitamins and sterols (DOUGLAS, 2009). In return, the host aphid provides *B. aphidicola* an optimum niche for survival (HANSEN; MORAN, 2014).

The long history of association of this symbiont with host aphids led to the reduction of the *B. aphidicola* genome, as symbionts loose several of their genes in the process of symbiogenesis (WERNEGREEN, 2002). *B. aphidicola* lost critical genes for DNA replication and repair, and genes coding for most enzymes in the biosynthetic pathways for non-essential amino acids (MORAN; DEGNAN, 2006). However, whole genome sequencing of *B. aphidicola* from multiple aphid species demonstrated interspecific variation in the ability to synthesize amino acids (JIANG et al., 2013). Interspecific variation was observed due to the presence of specific mutations in *B. aphidicola* genome that drove host plant adaptation, leading to the development of host-adapted races in *Schizaphis graminum* (Rondani) (VOGEL; MORAN, 2011), and also resulted from the intensity with which *Buchnera* genome reduction occurred in particular host species (KOGA; TSUCHIDA; FUKATSU, 2003; PEREZ-BROCAL et al., 2006). Genome reduction in *B. aphidicola* harbored by *Cinara* sp. was so intense that this

symbiont can no longer provide the host aphid with all of the required nutritional needs, and the host has to rely on an additional secondary symbiont to aid *Buchnera* to fulfill its nutritional role (KOGA; TSUCHIDA; FUKATSU, 2003; SAKURAI et al., 2005; PEREZ-BROCAL et al., 2006).

Several secondary symbionts are known to be associated with aphids. *Hamiltonella defensa*, *Regiella insecticola*, *Serratia symbiotica*, *Rickettsiella viridis*, *Rickettsia*, *Spiroplasma*, X-type, *Arsenophonus* and *Wolbachia* are among the most common aphid-associated secondary symbionts. Although these symbionts are not vital for host survival and/or reproduction, they may influence host fitness traits by altering the host response to a number of abiotic and biotic conditions (OLIVER et al., 2010; SU; ZHOU; ZHANG, 2013; WAGNER et al., 2015).

Several aphid-associated secondary symbionts are considered to aid the aphid defensive response to natural enemies (SU; ZHOU; ZHANG, 2013; OLIVER; SMITH; RUSSELL, 2014). Therefore, increased survival to parasitism was linked to aphids harboring *H. defensa* and *S. symbiotica* (OLIVER et al., 2003), although pea aphid resistance to parasitoids in *H. defensa*-associated aphids was dependent on the symbiont strain and on the presence of the infective bacteriophage APSE (OLIVER et al., 2009). But protection against fungal pathogens was observed in aphids carrying *R. insecticola*, *R. viridis*, *Rickettsia*, *Spiroplasma* and the X-type symbiont (FERRARI et al., 2001; LUKASIK et al., 2013; TSUCHIDA et al., 2014; HEYWORTH; FERRARI, 2015). Additionally, *R. viridis* was reported to indirectly protect the host aphid against predatory insects (TSUCHIDA et al., 2014). *Wolbachia* are known to protect *Drosophila* and mosquitoes against viruses; nonetheless, no protective phenotype was recognize in aphids so far (TEIXEIRA; FERREIRA; ASHBURNER, 2008; MOREIRA et al., 2009; AUGUSTINOS et al., 2011)

Furthermore, secondary symbionts can also lead to other phenotypic responses of aphid to abiotic and/or biotic stressors. *S. symbiotica* and *R. insecticola* were associated with aphid tolerance to heat stress (MONTLLOR; MAXMEN; PURCELL, 2002; RUSSELL; MORAN, 2006), *R. insecticola* and *Arsenophonus* with host plant utilization by aphids (LEONARDO; MUIRU, 2003; FERRARI et al., 2006), and *Spiroplasma* with sex determination in sexual generations and induced fitness costs, as it reduced aphid fecundity and longevity (FUKATSU et al., 2001; SIMON et al., 2011).

Host plant use is the most debatable phenotype in aphid-symbiont interactions. Regardless the unquestionable contribution and importance of *Buchnera* to support aphids in exploiting a nutritionally limited food resource as the phloem (DOUGLAS, 2009), secondary

symbionts place host plant use by aphids into an interesting ecological perspective, since secondary symbionts allow the use of additional plant species and promote dietary specialization by increasing performance on one species while decreasing on another (HANSEN; MORAN, 2014).

Host race adaptation driven by secondary symbionts has been reported in aphids (LEONARDO, 2004; WAGNER et al., 2015). In the aphid *A. pisum* for example, the secondary symbiont *R. insecticola* was associated with a race adapted to white clover (*Trifolium repens*), while *H. defensa* to a race adapted to alfalfa (*Medicago sativa*) (LEONARDO; MUIRU, 2003). But this issue remains controversial due to variable intra and interspecific results (LEONARDO, 2004; TSUCHIDA; KOGA; FUKATSU, 2004; FERRARI; VIA; GODFRAY, 2008 ; PECCOUD et al., 2015). Another issue affecting the proper evaluation of the role of secondary symbionts in aphid host-race adaptation is the biased screening for secondary symbionts on aphids and the limited biogeographic coverage, limiting the proposal of a hypothetical pattern of association with secondary symbionts and host plant specialization (ZYTYNSKA; WEISSER, 2016).

Host plants are also reported to limit the spread of secondary symbionts, as secondary symbiont - host plant interactions are also dependent on plant nutritional quality and plant species diversity in a patch. *S. symbiotica* was demonstrated to be affected by plants exposed to low levels of nitrogen (WILKINSON; KOGA; FUKATSU, 2007), while plant diversity in a patch favored aphid symbiont diversity (HENRY et al., 2015).

The range of host plants exploited depends on the aphid feeding strategy and on the co-evolutionary history with its host plants. Monophagous aphids are specialized in host plant use, and are able to tolerate or even sequestrate host plant defensive compounds. Polyphagous aphids, on the other hand, are commonly spread geographically and are reported to survive periods of environmental instability, even though they are less prone to tolerate secondary defensive compounds from the host plant (ALI; AGRAWAL, 2012). The association of secondary symbiont distribution with aphids-feeding habits has been recently discussed. *S. symbiotica* was commonly associated with aphids with a narrow host-plant range (HENRY et al., 2015). However, few studies that screened for secondary symbionts included oligophagous aphids, and none of them discussed symbiont distribution and feeding habits (HENRY et al., 2015; ZYTYNSKA; WEISSER, 2016).

Additionally, the increasing use of next generation sequencing technologies to elucidate insect-symbiont interactions is shedding some light on key questions. The use of metagenomics based on the 16S rRNA gene revealed a more precise symbiont diversity, as it allowed the

detection of low density or even new symbionts (RUSSELL et al., 2013; BANSAL; MIAN; MICHEL, 2014). Furthermore, transcriptome analyses allowed for investigations on the molecular mechanisms by which phenotypes induced by symbionts act on host plants and vice-versa (LIU et al., 2012; BAUER et al., 2014; UPADHYAY et al., 2015). Moreover, proteomic approaches investigated insect proteins that could influence host phenotype, such as proteins of the aphid saliva that could be involved in virus transmission to plants or act as effector proteins (CILIA et al., 2011; ELZINGA; DE VOS; JANDER, 2014; PINHEIRO et al., 2014).

We investigated host-aphid-plant relationship of an oligophagous aphid inhabiting tropical areas of South America, an area still underrepresented in the growing body of information that accumulates in this field of research. *Aphis (Toxoptera) citricidus* (Kirkaldy) (Hemiptera, Aphididae) is an oligophagous aphid with a diet range restricted to *Citrus* species and a few closely-related genera (HALBERT; BROWN, 1996). This aphid vectors the *citrus tristeza virus* (CTV), a disease that caused severe damage to global citriculture until resistant varieties became available (MORENO et al., 2008). CTV is mainly transmitted by *A. citricidus*, although *Aphis (Toxoptera) aurantii* Boyer de Fonscolombe can also vector this disease, but with low efficiency. *A. aurantii* can co-occur with *A. citricidus* in *Citrus* trees, and in such conditions it is usually misidentified as *A. citricidus*, since they are morphologically similar. Besides, *A. aurantii* differs from *A. citricidus* in many ecological aspects, particular the diet breadth. While *A. citricidus* is reported almost exclusively on citrus (HALBERT; BROWN, 2011), *A. aurantii* hosts includes coffee, tea, cacao, avocado, macadamia, litchi, mango, piper and fig as host plants, and it has been considered an important pest in many of them (CARVER, 1978; CORTEZ-MADRIGAL et al., 2003; SEVIM; CELEBI; SEVIM, 2012; WAHEED et al., 2014). There are few studies on the biology of *A. citricidus* and *A. aurantii* (TSAI, 1998; TSAI; WANG, 1999; WANG; TSAI, 2001), but data are quite variable from one study to another, which has been conferred to the variation in the genetic background of aphids and host plants tested (TANG et al., 1999). Nothing is known on secondary symbiont diversity in these species.

Thus, we provide new data on aphid-symbiont associations for aphids inhabiting tropical areas in the New World. We employed several approaches to investigate the association of secondary symbionts with aphids and their role in host plant use, aphid/primary symbiont gene expression and aphid proteomics. We also investigated the genome of the primary symbiont - *Buchnera* - of two closely related aphid species with different feeding habits – oligophagy vs. polyphagy – to search for differences in their symbionts to explain their range of host plants they use.

References

ALI, J.G.; AGRAWAL, A.A. Specialist versus generalist insect herbivores and plant defense. **Trends in Plant Science**, Oxford, v. 17, n. 5, p. 293-302, 2012.

AUGUSTINOS, A.A.; SANTOS-GARCIA, D.; DIONYSSOPOULOU, E.; MOREIRA, M.; PAPAPANAGIOTOU, A.; SCARVELAKIS, M.; DOUDOUMIS, V.; RAMOS, S.; AGUIAR, A.F.; BORGES, P.A.V.; KHADEM, M.; LATORRE, A.; TSIAMIS, G.; BOURTZIS, K. Detection and characterization of *Wolbachia* infections in natural populations of aphids: is the hidden diversity fully unraveled? **Plos One**, San Francisco, v. 6, n. 12, p. e28695, 2011.

BANSAL, R.; MIAN, M.A.R.; MICHEL, A.P. Microbiome diversity of *Aphis glycines* with extensive superinfection in native and invasive populations. **Environmental Microbiology Reports**, West Sussex, v. 6, n. 1, p. 57-69, 2014.

BAUER, E.; SALEM, H.; MARZ, M.; VOGEL, H.; KALTENPOTH, M. Transcriptomic immune response of the cotton stainer *Dysdercus fasciatus* to experimental elimination of vitamin-supplementing intestinal symbionts. **Plos One**, San Francisco, v. 9, n. 12, p. e114865, 2014.

BUCHNER, P. **Endosymbiosis of animals with plant microorganisms.** New Jersey: Interscience Publ.; John Wiley, 1965. 909 p.

CARVER, M. The black citrus aphids, *Toxoptera citricidus* (Kirkaldy) and *T. aurantii* (Boyer de fonscolombe) (Homoptera: Aphididae). **Australian Journal of Entomology**, Yarra, v. 17, n. 3, p. 263-270, 1978.

CILIA, M.; TAMBORINDEGUY, C.; FISH, T.; HOWE, K.; THANNHAUSER, T.W.; GRAY, S. Genetics coupled to quantitative intact proteomics links heritable aphid and endosymbiont protein expression to circulative polerovirus transmission. **Journal of Virology**, Washington, v. 85, n. 5, p. 2148-2166, 2011.

CORTEZ-MADRIGAL, H.; ALATORRE-ROSAS, R.; MORA-AGUILERA, G.; BRAVO-MOJICA, H.; ORTIZ-GARCIA, C.F.; ACEVES-NAVARRO, L.A. Characterization of multisporic and monosporic isolates of *Lecanicillium* (= *Verticillium*) *lecanii* for the management of *Toxoptera aurantii* in cocoa. **Biocontrol,** Dordrecht, v. 48, n. 3, p. 321-334, 2003.

DOUGLAS, A.E. The microbial dimension in insect nutritional ecology. **Functional Ecology**, Oxford, v. 23, n. 1, p. 38-47, 2009.

ELZINGA, D.A.; DE VOS, M.; JANDER, G. suppression of plant defenses by a *Myzus persicae* (green peach aphid) salivary effector protein. **Molecular Plant-Microbe Interactions**, Saint Paul, v. 27, n. 7, p. 747-756, 2014.

FERRARI, J.H.; GODFRAY, C.J.; FAULCONBRIDGE, A.S.; PRIOR, K.; VIA, S. Population differentiation and genetic variation in host choice among pea aphids from eight host plant genera. **Evolution**, West Sussex, v. 60, n. 8, p. 1574-1584, 2006.

_____. Clonal variation and covariation in aphid resistance to parasitoids and a pathogen. **Evolution**, West Sussex, v. 55, n. 9, p. 1805-1814, 2001.

FERRARI, J.; VIA, S.; GODFRAY, H.C.J. Population differentiation and genetic variation inperformance on eight hosts in the pea aphid complex. **Evolution**, West Sussex, v. 62, n. 10, p. 2508-2524, 2008.

FUKATSU, T.; TSUCHIDA, T.; NIKOH, N.; KOGA, R. *Spiroplasma* symbiont of the pea aphid, *Acyrthosiphon pisum* (Insecta : Homoptera). **Applied and Environmental Microbiology**, Washington, v. 67, n. 3, p. 1284-1291, 2001.

HALBERT, S.E.; BROWN, L.G. ***Toxoptera citricida* (Kirkaldy), brown citrus aphid - identification, biology and management strategies.** Gainesville: Florida Department of Agriculture and Consumer Services Division of Plant Industry, 1996. 6p. (Entomology Circular, 374).

_____. **Brown Citrus Aphid, Toxoptera citricida (Kirkaldy) (Insecta: Hemiptera: Aphididae)**. Gainesville: University of Florida, IFAS Extension,2011. 6p. (EENY,007)..

HANSEN, A.K.; MORAN, N.A. The impact of microbial symbionts on host plant utilization by herbivorous insects. **Molecular Ecology**, Oxford, v. 23, n. 6, p. 1473-1496, 2014.

HENRY, L.M.; MAIDEN, M.C.J.; FERRARI, J.; GODFRAY, H.C.J. Insect life history and the evolution of bacterial mutualism. **Ecology Letters,** Oxford, v. 18, n. 6, p. 516-525, 2015.

HEYWORTH, E.R.; FERRARI, J.A facultative endosymbiont in aphids can provide diverse ecological benefits. **Journal of Evolutionary Biology**, Basel, v. 28, n. 10, p. 1753-1760, 2015.

JIANG, Z.; JONES, D.H.; KHURI, S.; TSINOREMAS, N.F.; WYSS, T.; JANDER, G. WILSON, A.C.C. Comparative analysis of genome sequences from four strains of the *Buchnera aphidicola* Mp endosymbion of the green peach aphid, *Myzus persicae*. **Bmc Genomics**, London, v. 14, 2013. Available at: < DOI: 10.1186/1471-2164-14-917>.

KOGA, R.; TSUCHIDA, T.; FUKATSU, T. Changing partners in an obligate symbiosis: a facultative endosymbiont can compensate for loss of the essential endosymbiont *Buchnera* in an aphid. **Proceedings of the Royal Society B-Biological Sciences**, London, v. 270, n. 1533, p. 2543-2550, 2003.

LAUGHTON, A.M.; FAN, M.H.; GERARDO, N.M. The combined effects of bacterial symbionts and aging on life history traits in the pea aphid, *Acyrthosiphon pisum.* **Applied and Environmental Microbiology**, Washington, v. 80, n. 2, p. 470-477, 2014.

LEONARDO, T.E. Removal of a specialization-associated symbiont does not affect aphid fitness. **Ecology Letters**, Oxford, v. 7, n. 6, p. 461-468, 2004.

LEONARDO, T.E.; MUIRU, G.T. Facultative symbionts are associated with host plant specialization in pea aphid populations. **Proceedings of the Royal Society B-Biological Sciences**, London, v. 270, p. S209-S212, 2003.

LIU, S.; CHOUGULE, N.P.; VIJAYENDRAN, D.; BONNING, B.C. Deep sequencing of the transcriptomes of soybean aphid and associated endosymbionts. **Plos One**, San Francisco, v. 7, n. 9, p. e45161, 2012.

LUKASIK, P.; GUO, H.; VAN ASCH, M.; FERRARI, J.; GODFRAY, H.C.J. Protection against a fungal pathogen conferred by the aphid facultative endosymbionts *Rickettsia* and *Spiroplasma* is expressed in multiple host genotypes and species and is not influenced by co-infection with another symbiont. **Journal of Evolutionary Biology**, Basel, v. 26, n. 12, p. 2654-2661, 2013.

MONTLLOR, C.B.; MAXMEN, A.; PURCELL, A. H. Facultative bacterial endosymbionts benefit pea aphids *Acyrthosiphon pisum* under heat stress. **Ecological Entomology**, London, v. 27, n. 2, p. 189-195, 2002.

MORAN, N.A.; DEGNAN, P.H. Functional genomics of *Buchnera* and the ecology of aphid hosts. **Molecular Ecology**, Oxford, v. 15, n. 5, p. 1251-1261, 2006.

MORAN, N.A.; TELANG, A. Bacteriocyte-associated symbionts of insects - A variety of insect groups harbor ancient prokaryotic endosymbionts. **Bioscience**, Washington, v. 48, n. 4, p. 295-304, 1998.

MOREIRA, L.A.; ITURBE-ORMAETXE, I.; JEFFERY, J.A.; LU, G.; PYKE, A.T.; HEDGES, L.M.; ROCHA, B.C.; HALL-MENDELIN, S.; DAY, A.; RIEGLER, M.H.; UGO, L.E.; JOHNSON, K.N.; KAY, B.H.; MCGRAW, E.A.; VAN DEN HURK, A.F.; RYAN, P.A.; O'NEILL, S.L.A *Wolbachia* Symbiont in *Aedes aegypti* limits infection with Dengue, Chikungunya, and *Plasmodium*. **Cell**, Cambridge, v. 139, n. 7, p. 1268-1278, 2009.

MORENO, P.; AMBROS, S.; ALBIACH-MARTI, M.R.; GUERRI, J.; PENA, L. Plant diseases that changed the world - *Citrus tristeza virus* : a pathogen that changed the course of the citrus industry. **Molecular Plant Pathology**, Maryland Heights, v. 9, n. 2, p. 251-268, 2008.

NOVAKOVA, E.; HYPSA, V.; KLEIN, J.; FOOTTIT, R.G.; VON DOHLEN, C.D.; MORAN, N.A. Reconstructing the phylogeny of aphids (Hemiptera: Aphididae) using DNA of the obligate symbiont *Buchnera aphidicola*. **Molecular Phylogenetics and Evolution,** Maryland Heights, v. 68, n. 1, p. 42-54, 2013.

OLIVER, K.M.; DEGNAN, P.H.; BURKE, G.R.; MORAN, N.A. Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. **Annual Review of Entomology**, Stanford, v.55, n. 3, p.247-266, 2010.

_____. Bacteriophages encode factors required for protection in a symbiotic mutualism. **Science**, Washington,v. 325, n. 5943, p. 992-994, 2009.

_____. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. **Proceedings of the National Academy of Sciences of the United States of America**, Washington, v. 100, n. 4, p. 1803-1807, 2003.

OLIVER, K.M.; SMITH, A.H.; RUSSELL, J.A. Defensive symbiosis in the real world - advancing ecological studies of heritable, protective bacteria in aphids and beyond. **Functional Ecology** , Oxford, v. 28, n. 2, p. 341-355, 2014.

PECCOUD, J.; MAHEO, F.; DE LA HUERTA, M.; LAURENCE, C.; SIMON, J.-C. Genetic characterisation of new host-specialised biotypes and novel associations with bacterial symbionts in the pea aphid complex. **Insect Conservation and Diversity**, West Sussex, v. 8, n. 5, p. 484-492, 2015.

PEREZ-BROCAL, V.; GIL, V.R.; RAMOS, S.; LAMELAS, A.; POSTIGO, M.; MANUEL MICHELENA, J.; SILVA, F.J.; MOYA, A.; LATORRE, A. A small microbial genome: The end of a long symbiotic relationship? **Science**, Washington, v. 314, n. 5797, p. 312-313, 2006.

PINHEIRO, P.; BEREMAN, M.S.; BURD, J.; PALS, M.; ARMSTRONG, S.; HOWE, K.J.; THANNHAUSER, T.W.; MACCOSS, M.J.; GRAY, S.M.; CILIA, M. Evidence of the biochemical basis of host virulence in the greenbug aphid, *Schizaphis graminum* (Homoptera: Aphididae). **Journal of Proteome Research**, Washington, v. 13, n. 4, p. 2094-2108, 2014.

RUSSELL, J.A.; MORAN, N.A. Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. **Proceedings of the Royal Society B-Biological Sciences**, London,v. 273, n. 1586, p. 603-610, 2006.

RUSSELL, J.A.; WELDON, S.; SMITH, A.H.; KIM, K.L.; HU, Y.; LUKASIK, P.; DOLL, S.; ANASTOPOULOS, I.; NOVIN, M.; OLIVER, K.M. Uncovering symbiont-driven genetic diversity across North American pea aphids. **Molecular Ecology**, Oxford, v. 22, n. 7, p. 2045-2059, 2013.

SABATER, B.; VAN HAM, R.; MARTINEZ-TORRES, D.; SILVA, F.; LATORRE, A.; . MOYA, A. Molecular evolution of aphids and their primary (*Buchnera* sp.) and secondary endosymbionts: Implications for the role of symbiosis in insect evolution. **Interciencia**, Caracas, v. 26, n. 10, p. 508-512, 2001.

SAKURAI, M.; KOGA, R.; TSUCHIDA, T.; MENG, X.Y.; FUKATSU, T. *Rickettsia* symbiont in the pea aphid *Acyrthosiphon pisum*: Novel cellular tropism, effect on host fitness, and interaction with the essential symbiont Buchnera. **Applied and Environmental Microbiology**, Washington, v. 71, n. 7, p. 4069-4075, 2005.

SEVIM, E.; CELEBI, O.; SEVIM, A. Determination of the bacterial flora as a microbial control agent of *Toxoptera aurantii* (Homoptera: Aphididae). **Biologia**, Berlin, v. 67, n. 2, p. 397-404, 2012.

SIMON, J.-C.; BOUTIN, S.; TSUCHIDA, T.; KOGA, R.; LE GALLIC, J.-F.; FRANTZ, A.; OUTREMAN, Y.; FUKATSU, T. Facultative symbiont infections affect aphid reproduction. **Plos One**, San Francisco, v. 6, n. 7, p. e21831, 2011.

SIMON, J.C.; CARRE, S.; BOUTIN, M.; PRUNIER-LETERME, N.; SABATER-MUNOZ, B.; LATORRE, A.; BOURNOVILLE, R. Host-based divergence in populations of the pea aphid: insights from nuclear markers and the prevalence of facultative symbionts. **Proceedings of the Royal Society B-Biological Sciences**, London,v. 270, n. 1525, p. 1703-1712, 2003.

SU, Q.; ZHOU, X.; ZHANG, Y. Symbiont-mediated functions in insect hosts. **Communicative & Integrative Biology**, New York, v. 6, n. 3, p. e23804-e23804, 2013.

SUGIO, A.; DUBREUIL, G.; GIRON, D.; SIMON, J.-C. Plant-insect interactions under bacterial influence: ecological implications and underlying mechanisms. **Journal of Experimental Botany**, Oxford, v. 66, n. 2, p. 467-478, 2015.

TANG, Y.Q.; LAPOINTE, S.L.; BROWN, L.G.; HUNTER, W.B. Effects of host plant and temperature on the biology of *Toxoptera citricida* (Homoptera: Aphididae). **Environmental Entomology**, College Park, v. 28, n. 5, p. 895-900, 1999.

TEIXEIRA, L.; FERREIRA, A.; ASHBURNER, M. The bacterial symbiont *Wolbachia* induces resistance to rna viral infections in *Drosophila melanogaster*. **Plos Biology**, San Francisco, v. 6, n. 12, p. 2753-2763, 2008.

TSAI, J. H. Development, survivorship, and reproduction of *Toxoptera citricida* (Kirkaldy) (Homoptera : Aphididae) on eight host plants. **Environmental Entomology,** College Park, v. 27, n. 5, p. 1190-1195, 1998.

TSAI, J.H.; WANG, K.H. Life table study of brown citrus aphid (Homoptera : Aphididae) at different temperatures. **Environmental Entomology**, College Park,v. 28, n. 3, p. 412-419, 1999.

TSUCHIDA, T.; KOGA, R.; FUJIWARA, A.; FUKATSU, T. Phenotypic effect of "*Candidatus* Rickettsiella viridis," a facultative symbiont of the pea aphid (*Acyrthosiphon pisum*), and its interaction with a coexisting symbiont. **Applied and Environmental Microbiology**, Washington, v. 80, n. 2, p. 525-533, 2014.

TSUCHIDA, T.; KOGA, R.; FUKATSU, T. Host plant specialization governed by facultative symbiont. **Science**, Washington, v. 303, n. 5666, p. 1989-1989, 2004.

TSUCHIDA, T.; KOGA, R.; MATSUMOTO, T.; FUKATSU, T. Secondary endosymbiotic microbiota of the pea aphid, *Acyrthosiphon pisum*, in natural populations. In: SYMPOSIUM ON APHIDS, 2001, Rennes. **Aphids in a a new Millennium: proceedings …** 2001. p. 93-96.

UPADHYAY, S.K.; SHARMA, S.; SINGH, H.; DIXIT, S.; KUMAR, J.; VERMA, P.C.; CHANDRASHEKAR, K. Whitefly genome expression reveals host-symbiont interaction in amino acid biosynthesis. **Plos One**, San Francisco, v. 10, n. 5, 2015. Available at: < DOI: 10.1371/journal.pone.0126751>.

VOGEL, K.J.; MORAN, N.A. Effect of host genotype on symbiont titer in the aphid-*Buchnera* symbiosis. **Insects**, Basel, v. 2, n. 3, p. 423-434, 2011.

WAGNER, S.M.; MARTINEZ, A.J.; RUAN, Y.-M.; KIM, K.L.; LENHART, P.A.; DEHNEL, A.C.; OLIVER, K.M.; WHITE, J.A. Facultative endosymbionts mediate dietary breadth in a polyphagous herbivore. **Functional Ecology** , West Sussex, v.29, n.11, p. 1402-1410, 2015.

WAHEED, A.; HAMID, F.S.; ASLAM, S.; ASLAM, N.; AHMED, F.; ZAMAN, Q.; AHMED, N.; ISLAM, S.; KHAN, N., SHAH, B.H. Field evaluation of different chemical pesticides against aphid *Toxoptera aurantii* on different tea (*Camellia sinensis* L.) cuttings under high shade nursery. **Moroccan journal of chemistry**, Oujda, v.33, p. 127-135. 2014.

WANG, J.J.; TSAI, J.H. Development, survival and reproduction of black citrus aphid, *Toxoptera aurantii* (Hemiptera : Aphididae), as a function of temperature. **Bulletin of Entomological Research**, Farnham Royal, v. 91, n. 6, p. 477-487, 2001.

WERNEGREEN, J.J. Genome evolution in bacterial endosymbionts of insects. **Nature Reviews Genetics**, London, v. 3, n. 11, p. 850-861, 2002.

WILKINSON, T.L.; FUKATSU, T.; ISHIKAWA, H. Transmission of symbiotic bacteria *Buchnera* to parthenogenetic embryos in the aphid *Acyrthosiphon pisum* (Hemiptera : Aphidoidea). **Arthropod Structure & Development**, Oxford, v. 32, n. 2-3, p. 241-245, 2003.

WILKINSON, T.L.; KOGA, R.; FUKATSU, T. Role of host nutrition in symbiont regulation: Impact of dietary nitrogen on proliferation of obligate and facultative bacterial endosymbionts of the pea aphid *Acyrthosiphon pisum*. **Applied and Environmental Microbiology**, Washington, v. 73, n. 4, p. 1362-1366, 2007.

ZYTYNSKA, S.E.; WEISSER, W.W. The natural occurrence of secondary bacterial symbionts in aphids. **Ecological Entomology,** London, v. 41, n. 1, p. 13-26, 2016.

2 NUTRITIONAL ECOLOGY OF OLIGOPHAGOUS AND POLYPHAGOUS APHIDS AFFECTED BY HOST SUITABILITY AND ITS EFFECTS ON THE OBLIGATE SYMBIONT GROWTH

Abstract

Nutrition is an important aspect of insect physiology and ecology, and insects may develop different levels of diet specialization leading herbivores to explore from a narrow to a broad range of hosts. Polyphagous insects feed on a broad range of host plants belonging to different botanic families, while oligophagous insects feed on a restrict number of plants, usually within the same family. As a consequence, diet limitation will lead to nutritional specialization in oligophagous. Nutritional specialization may become even more severe in cases in which the diet can pose nutritional quality limitations. Hemipterans are examples of insects that feed on nutritionally poor diets. In such cases, diet use was made possible through the establishment of associations with nutritional symbionts that supply essential nutrients as diet supplements. Here we investigate the effects of host plant suitability on the biology of the oligophagous aphid *Aphis (Toxoptera) citricidus* (Kirkaldy) and the polyphagous *Aphis (Toxoptera) aurantii* Boyer de Fonscolombe, and on their obligate symbiont *B. aphidicola*. The effects of host plant quality on fitness of oligophagous and polyphagous aphids were assessed by investigating their development when restricted to host plants with different nutritional value (sweet orange and orange jasmine) and when alternating host plant quality between immature and adult stages. Sweet orange was the most adequate host for both aphid species, but the poor nutritional quality of orange jasmine was much more severe on the fitness of the oligophagous aphid than the polyphagous aphid. The growth pattern of *Buchnera* differed between aphids and host plants. *Buchnera* increases in density as *A. citricidus* develops on sweet orange, but *Buchnera* density will decrease in aphids feeding on orange jasmine. Yet, *Buchnera* will increase in density in *A. aurantii* regardless the host plant in use, although following a different growth trend. Finally, *Buchnera* density fluctuation reflects the contribution of food source in each developmental stage of both aphids and we argue on the dynamic of the interaction among aphids, *Buchnera* and host plant suitability considering the oligophagous - polyphagous dichotomy.

Keywords: *Buchnera aphidicola*; Comparative biology; Host plant; Nutrition

2.1 Introduction

Insects may develop different strategies for host plant use. Specialist herbivores reduce the range of host plants they can feed on, but do so more efficiently as they develop strategies to benefit themselves from host plants defensive compounds (ALI; AGRAWAL, 2012). On the other hand, generalist herbivores have a large range of host plants available, favoring their distribution and persistence. But their strategies to handle the anti-herbivory arsenal of their host plants are costly and may result in fitness costs (KATSIR et al., 2008; ALI; AGRAWAL, 2012).

Despite the controversial benefits/costs in host plant specialization and the insect´s qualitative nutritional requirements be essentially the same (SCRIBER; SLANSKY, 1981; HOUSE, 1961), differences in the relative abundance of plant nutrients may substantially affect the host plant suitability to herbivores (JOERN; PROVIN; BEHMER, 2012). The nutritional quality of the host plant to herbivores is extremely important as it interferes with a number of biological and ecological features of insects, such as fecundity (AWMACK; LEATHER, 2002), embryo initiation and maturation or resorption (MOORE; ATTISANO, 2011), adult size (COLASURDO et al., 2009), progeny fitness (FUENTEALBA; BAUCE, 2012), mate choice (KNUTTEL; FIEDLER, 2001), and migration (CZERNIEWICZ et al., 2011).

Response of herbivores to host plant quality and within- and between-host plant variation can be species-specific and differ among specialists and generalists (GUTBRODT et al., 2012). Insects can be highly plastic in their responses to changes in host plant quality, and host plant quality can also affect insect reproductive strategies, influencing the decisions on nutrient allocation (soma vs. reproduction) and, consequently, the size and quality of the egg produced (AWMACK; LEATHER, 2002). Yet, diet alternation and/or switching may benefit generalists to regulate the intake of appropriate nutrients or escape the negative effects of host plant defensive secondary metabolites (MODY; UNSICKER; LINSENMAIR, 2007; JOHNS et al., 2015; DU PLESSI; BYRNE; VAN DEN BERG., 2015; MILANOVIC et al., 2016).

Insects adapted to exploit limited food resources, such as plant sap, add another level of complexity to the feeding strategies herbivores use to explore their range of hosts as they can depend on the association with microbial symbionts (HANSEN; MORAN, 2014, SUGIO et al., 2015). Hemipterans are highly specialized to explore unbalanced food sources, such as blood, xylem and phloem (DOUGLAS, 2006). Plant sap is rich in sugars but has a reduced nitrogen content, especially low in essential amino acids. The ratio of essential amino acids to nonessential amino acids in plant sap ranges from 1:4 to 1:20 (DOUGLAS, 2006). Exploration of such unbalanced food source became possible through the establishment of obligate nutritional symbiosis with microbes. Auchenorrhyncha (leafhoppers) and Sternorrhyncha (aphids, mealybugs, psyllids and whiteflies) are associated with endocytobionts harbored in bacteriocytes (DOUGLAS, 1998; THAO et al., 2000; THAO; BAUMANN, 2004; BAUMANN; BAUMANN, 2005; MORAN;MCCUTCHEON; NAKABACHI 2008), while Plataspidae heteropterans are associated with gut bacterial symbionts (HOSOKAWA et al., 2006).

Buchnera aphidicola is certainly one of the most studied nutritional symbiont in sap sucking insects (BUCHNER, 1965; SHIGENOBU et al., 2000; HANSEN; MORAN, 2011;

NOVAKOVA et al., 2013; MORAN; YUN, 2015). The long coevolutionary history of the aphid-*Buchnera* association led to the complementarity of their metabolic pathways. Aphids provide non-essential amino acids acquired from their food sources to *Buchnera*, which in turn will synthesize the required essential amino acids (DOUGLAS, 1998; HANSEN; MORAN, 2011; HANSEN; MORAN, 2014) and vitamins to complement the aphid diet (NAKABACHI et al., 2014). Such dependence on amino acid provision by the aphid host to support growth of this symbiont arises from the erosion of the *Buchnera* genome, which lost several genes required for non-essential amino acid synthesis (SHIGENOBU et al., 2000; VAN HAM, et al., 2001; PEREZ-BROCAL et al., 2006; MORAN; DEGNAN, 2006; MORAN*;* MCLAUGHLIN; SOREK, 2009; MCCUTCHEON; MORAN, 2012)

Therefore, the system host plant - aphid - *Buchnera* establishes an interdependent multitrophic level of interactions, with the host plant quality affecting the nutritional status of the aphid, which will influence the growth and nutritional contribution of the symbiont to the aphid. Moreover, there are aphids that can enhance the nutritional quality of their host plants by inducing systemic alterations in host plant that leads to an elevated level of essential amino acids in the host sap (TELANG et al., 1999; SANDSTRÖM; TELANG; MORAN, 2000). Thus, variation in the aphid´s ability to regulate the nutritional content of their host plants is a strong evidence that aphids interactions with their nutritional symbiont may follow different patterns of regulation.

As plant sap are quite variable among plant species, ranging from 15-48% in amino acid content (SANDSTRÖM; MORAN, 1999) and aphid dietary nitrogen and host plant species can interfere with the proliferation of *Buchnera* (WILKINSON; KOGA; FUKATSU, 2007; CHANDLER; WILKINSON; DOUGLAS, 2008), and host plant quality can affect herbivores with different feeding strategies distinctly, we decided to investigate the effects of host plant and host plant switch on the biology of two closely-related species with different diet breadth and on the growth of the primary symbiont associated.

We evaluated how optimal and suboptimal host plants would affect the aphid biology and growth of *Buchnera* in aphids with a narrow host range, *Aphis (Toxoptera) citricidus* (Kirkaldy), and a broad host range, *Aphis (Toxoptera) aurantii* Boyer de Fonscolombe. *A. citricidus* is mostly restricted to *Citrus* and few other members of Rutaceae (CARVER, 1978), whereas *A. aurantii* also feeds on coffee, tea, cacao, avocado, macadamia, litchi, mango, piper and fig as host plants (CARVER, 1978). Both species have better fitness when feeding on *Citrus* as compared to *Murraya paniculata* (Rutacea), a common alternative host (TSAI, 1998; TANG et al., 1999; WANG; TSAI, 2001). Therefore, we considered *Citrus* spp. as an optimal host and

M. paniculata as a sub-optimal host. We also investigated the impact of host plant switching would have on the reproductive biology of *A. citricidus* and *A. aurantii*.

2.2 Material and methods

2.2.1 Insects

Adult aphids were collected in unmanaged *Citrus* sp. and *Murraya paniculata* trees in the municipality of Piracicaba (22º42´30´´S, 47º38´00´´W), state of São Paulo, and brought to the laboratory for the establishment of isolines. Aphids were classified by using the field key available for the identification of wingless adults (HALBERT; BROWN, 2011).

Adult females of *A. citricidus* and *A. aurantii* were individualized in cages (50 cm high x 15 cm diameter, containing 2 lateral openings closed with cloth for ventilation) containing new shoots of *Citrus sinensis* as the host plant. Cages were maintained under controlled conditions (25±2°C; 60±10% RH; 14h photophase) until aphids completed their life cycle. Isolines that succeeded in laboratory conditions were screened for infections with common secondary symbionts (SANDSTROM et al., 2001; TSUCHIDA et al., 2002; OLIVER, MORAN, HUNTER 2005; OLIVER, MORAN, HUNTER 2006). Isolines exclusively infected with *Buchnera aphidicola* were selected and used for further biological investigations.

The selected isolines of *A. citricidus* (*Ac-iso1*) and *A. aurantii* (*Aa*-iso1) were reared on *Citrus limonia* (Rangpur) seedlings in individual cages maintained under controlled conditions (25±2°C; 60±10% RH; 14h photophase) for at least three generations before the experiments.

2.2.2 Effects of host plant on *Aphis (Toxoptera) citricidus* and *Aphis (Toxoptera) aurantii* biology and *Buchnera* growth

Comparative biological analyses of *Ac-iso1*and *Aa-iso1*developing on two host plants, orange jasmine (*Murraya paniculata*) and sweet orange (*Citrus sinensis* var. Pêra), were carried out under controlled conditions (25±2°C; 60±10% RH; 14 h photophase). Observations were made by using 100 newly-laid nymphs (0-12 h-old) placed in new flushes from each one of the plants tested (orange jasmine or sweet orange). Flushes containing the nymphs were maintained isolated within rearing cages (9.0x7.5 cm transparent plastic cups). Samples were daily observed and the development time to adulthood and the nymph survival were recorded. Experiments were replicated four times.

Twenty newly-emerged females from each line in each host plant were selected and individually caged in a new flush of the host plant they fed as nymphs. Females were observed daily for the assessment of their fecundity and longevity. Fecundity was assessed by counting the number and removing the nymphs laid every day.

Data on immature development time and survival, and female fecundity and longevity were tested for normality and homocedasticity using the Shapiro and the Bartlett tests, respectively. Data were transformed with boxcox algorithm (λ=0.4) whenever required. *Ac-iso1*_nymph development time and female fecundity were analyzed using the Kruskall-Wallis test, while the remaining data were subject to *t* test comparisons using the R Statistical software (v.3.2.1). Data on the number of reproductively active females (females that produced offspring) were compared using the chi-square test. All statistical tests were performed with a significance level of $p \leq 0.05$.

Data obtained for *Aa-iso1*and *Ac-iso1*on sweet orange and orange jasmine were used to calculate life table parameters (IMG: average time between generations; *Ro*: net reproductive rate; *rm*: intrinsic rate of increase; TD: time for duplication; λ: the finite growth rate) following Andrewartha and Birch (1954). Life table parameters of each aphid line obtained for each host plant were subjected to a paired t test ($\alpha = 0.05$) for comparisons based on Jackknife estimates using the SAS® software (SAS INSTITUTE 2015) (MAIA, LUIZ, 2006).

2.2.3 Growth of *Buchnera aphidicola*

As *Buchnera* also goes through genome duplication during growth, assessment of *Buchnera* density reflects the number of copies of the genome instead of the exact number of *Buchnera* cells available (KOMAKI; ISHIKAWA, 1999); nevertheless, we will further refer to density as the number of cells for the easiest of reading. Densities of *B. aphidicola* were measured in nymphs and adults of *Aa-iso1* and *Ac-iso1* developing on sweet orange and orange jasmine. Samples for the immature development were obtained from groups of 50 first instars (0-12h-old), each placed on shoots of sweet orange or orange jasmine. Three nymphs were daily collected and fixed in absolute ethanol until adulthood. Nymphs were then checked under a stereomicroscope, and the first three nymphs from each instar were pooled, yielding three biological replicates (each containing a pool of three nymphs) for each aphid line and host plant.

Samples were subjected to genomic DNA extraction following Sunnucks *et al.* (1997). DNA quality and concentration were verified by spectrophotometry (A260/280 ratio). DNA samples were standardized and used for qPCR using the single-copy gene *dnaK* as the

molecular marker by following the absolute quantification approach. All amplifications were done using the ViiA7 Real-Time PCR System (Thermo Fisher Scientific, USA).

Standard curves for PCR quantification were obtained by cloning a fragment of the *dnaK* gene for later use in the determination of the number of copies available (WHELAN; RUSSELL; WHELAN, 2003). *dnaK* was amplified using 20 ng/µL of aphid gDNA, 1x PCR buffer, 1.5 mM $MgCl_2$, 10 µM from each dNTP, 0.32 µM from each primer (BuchDnaK12F - 5'TAT TGG TAT TGA CTT GGG AA3' and BuchDnaK162R - 5'AGC AGG TTG TCC TAC TAA AAC3') and 0.5 U of Taq DNA polimerase, to a final volume of 25 µL. Cycling condition was set at 94°C for 1 min (1 cycle), 94°C for 30 s, 55°C for 1 min and 72°C for 45 s (35 cycles), and 72°C for 5 min (1 cycle) (KOGA *et al.*, 2003). Amplicons were resolved on a 1.0% agarose gel slab added with 0.5 µg/mL ethidium bromide in Tris-acetate-EDTA buffer (40 mM Tris-acetate, 1 mM EDTA; pH 7.2) at 5 V/cm and visualized on a UV transilluminator (DNR Bio-imaging system). The PCR product was purified using the Montage PCR kit (Millipore) following the manufacturer's instructions. Concentration and purity of the purified amplicon were measured by spectrophotometry at the A260 and 280.

The purified amplicon was then inserted into the pGEM®-T Easy Vector System (Promega) and used to transform DH5-α highly competent cells (Invitrogen). Transformed cells were grown in SOC medium (Invitrogen) for 1h at 37°C under constant agitation (240 rpm). Cells were then plated on LB agar plates supplemented with 100 µg/mL ampicillin and 50 µg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL) for 16h at 37°C. Positive clones were isolated, cultivated in LB liquid medium supplemented with 100 µg/mL ampicillin, and subjected to plasmid extraction by alkaline lysis (SAMBROOK, 2001).

Standard curve for *dnaK* was determined using eight serial dilutions of the transformed plasmid. All biological replicates were ran in technical triplicates using the Platinum® SYBR® Green qPCR SuperMix-UDG kit (Invitrogen). Reactions contained 1x Maxima SYBR Green/ROX qPCR Master Mix, 0.16 µM each primer (BuchDnaK12F 5'TAT TGG TAT TGA CTT GGG AA3' and BuchDnaK162R 5'AGC AGG TTG TCC TAC TAA AAC3'), 10 ng gDNA and sterile water to a final volume of 20 µL. Cycling conditions were set at 50°C for 2 min, 95°C for 10min, followed by 40 cycles of 95°C for 15 s and 60°C for 1min. Final dissociation curves were obtained after 1 cycle at 95°C for 15s, 60°C for 1 min and 95°C for 15 s.

B. aphidicola quantification was obtained by comparing the *Ct* values obtained in each sample against the standard curve generated for DnaK, using tools available in the qPCR ViiA7. Data were analyzed with generalized linear model (GLM) using Poisson distribution with proc

GENMOD and predicted values were obtained with proc LIN on the SAS® software (SAS INSTITUTE 2015).

2.2.4 Effects of host plant switching on *Aphis (Toxoptera) citricidus* and *Aphis (Toxoptera) aurantii* reproduction

In order to evaluate the effects of the host plants studied at the immature and adult stages of both aphid lines, *Ac-iso1* and *Aa-iso1* were switched hosts as they turned into adults. In order to investigate the effects of the nutritional quality of the host during the immature stage, first instars (0-12h) of *Ac-iso1* and *Aa-iso1* were reared on *C. sinensis* or *M. paniculata*, and 20 newly-emerged adult females (0-24h)/host plant were individually transferred to seedlings of *Citrus limonia* (Rangpur) as a host for aphid reproduction. Females were maintained in individual cages as earlier described, and their fecundity and longevity was daily assessed as before. Additionally, three groups of five newly-emerged adult females from each host plant were fixed in absolute ethanol for assessment of the density of *B. aphidicola* via quantitative PCR.

The effect of the nutritional quality of *C. sinensis* and *M. paniculata* on the adult stage of *Ac-iso1* and *Aa-iso1* was evaluated by transferring adult females obtained from nymphs reared on *Citrus limonia* (Rangpur) as a host plant to the test plants (*C. sinensis* and *M. paniculata*). Twenty newly-emerged adult females (0-24h) of *Ac-iso1* and *Aa-iso1* were obtained from *C. limonia* seedlings and individually transferred to either *C. sinensis* or *M. paniculata*. These females were daily observed and their fecundity and longevity assessed as before.

Data were tested for normality and homocedasticity using the Shapiro and the Bartlett tests, respectively. Data on female fecundity and longevity were transformed using boxcox (adults on rangpur - *Tc-iso1*: λ=0.4 for fecundity and λ= 0.28 for longevity; *Ta-iso1*: λ=0.08 for fecundity and λ= 0.01 for longevity; adults on sweet orange or orange jasmine - *Tc-iso1*: λ=0.3 for fecundity and λ= 0.4 for longevity; *Ta-iso1*: λ=0.25 for fecundity and longevity). Data were analyzed using *t* test in R Statistical software (v.3.2.1). The percentage of reproductively active females was verified by chi-square test.

2.3 Results

2.3.1 Effects of host plant on *Aphis (Toxoptera) citricidus* and *Aphis (Toxoptera) aurantii* biology and *Buchnera* growth

Data on immature development and female reproduction demonstrated sweet orange as a better quality host than orange jasmine for both *A. citricidus* (*Ac-iso1*) and *A. aurantii* (*Aa-iso1*) lines studied. *A. citricidus* immature survival was nearly 7-fold higher on sweet orange than on orange jasmine, but no difference in the time of development to the adult stage was observed (Table 2.1). Adult reproductive fitness was also affected by the host plant, as female fecundity on sweet orange was 3-fold that on orange jasmine; but no difference was observed in female longevity between these host plants. Besides the lower fecundity observed in orange jasmine for *Ac-iso1*, only 70% of females reared on this host plant were reproductively active (produced offspring), against 95% among females reared on sweet orange (Table 2.1).

Differences in biological parameters of the polyphagous *A. aurantii* on sweet orange and orange jasmine as host plants were much less conspicuous than those observed for the oligophagous *A. citricidus*. Immature development of *A. aurantii* was shorter and survival was twice as higher on sweet orange than on orange jasmine (Table 2.1). Females reared on sweet orange were more fecund than those on orange jasmine, but no differences in female longevity and in the percentage of reproductively active females were observed (Table 2.1).

Table 2.1 - Nymph development time (days) and survivorship (%), female fecundity (no. nymphs/female) and longevity (days), and percentage of reproductively active females of *Aphis (Toxoptera) citricidus (Ac-iso1)* and *Aphis (Toxoptera) aurantii (Aa-iso1)* reared on sweet orange and orange jasmine under controlled conditions (25±2°C; 60±10% RH; 14h photophase)

Treatment	**Reared from sweet orange**	**Reared from orange jasmine**	**Statistics**
A. citricidus (Ac-iso1)			
Nymph development time (days)	6.7 ± 0.03 a	5.9 ± 2.03 a	χ^2 (1, n = 4) = 1.33, p = 0.24
Nymph survivorship (%)	69.7 ± 4.38 a	7.0 ± 4.84 b	t (3) = 9.6, $p < 0.05$
Reproductively active females (%)	95 a	70 b	χ^2 (1, n = 20) = 6.57, $p < 0.05$
Female longevity (days)	12.1 ± 1.51 a	7.2 ± 1.55 a	χ^2 (1, n = 20) = 1.58, p = 0.2
Female fecundity (Nº nymph/♀)	38.3 ± 6.32 a	12.7 ± 3.38 b	t (19) = 2.6, $p < 0.05$
A. aurantii (Aa-iso1)			
Nymph development time (days)	6.1 ± 0.07 b	7.7 ± 0.16 a	t (3) = -6.09, $p < 0.05$
Nymph survivorship (%)	87.5 ± 9.21 a	40.5 ± 13.95 b	t (3) = 2.81, $p < 0.05$
Reproductively active females (%)	100 a	100 a	χ^2 (1, n = 20) = 0.0, p = 1
Female longevity (days)	11.2 ± 1.11 a	9.8 ± 0.91 a	t (19) = 0.96, p = 0.33
Female fecundity (Nº nymph/♀)	43.0 ± 4.30 a	20.6 ± 2.92 b	t (19) = 4.3, $p < 0.05$

* Means followed by the same letter within a line are not significantly different at $p > 0.05$ using t or χ^2 tests.

The majority of the life table parameters of *A. citricidus* and *A. aurantii* differed between the host plants tested, and corroborated the biological data obtained to demonstrate sweet orange as a much higher nutritional quality host when compared to orange jasmine for both oligophagous and polyphagous aphid species. The time for duplication (TD) for *A. citricidus* and the mean time between generations (IMG) for *A. aurantii* were the only calculated parameters that did not differ between host plants (Table 2.2).

Table 2.2 - Jackkinfe estimates of life table parameters calculated for isolines of *A. citricidus* and *A. aurantii* reared on two host plants, sweet orange and orange jasmine, under controlled conditions (25±2°C; 60±10% RH; 14h photophase)

Treatment	**Reared from sweet orange**	**Reared from orange jasmine**
A. citricidus (Ac-iso1)		
IMG	3.8 ± 0.20 b	7.4 ± 0.65 a
Λ	2.3 ± 0.09 a	0.9 ± 0.03 b
Rm	0.9 ± 0.04 a	-0.009 ± 0.03 b
Ro	26.4 ± 4.35 a	0.8 ± 0.23 b
TD	0.8 ± 0.03 a	164.6 ± 99.74 a
A. aurantii (Aa-iso1)		
IMG	3.6 ± 0.19 a	3.9 ± 0.31 a
Λ	2.6 ± 0.10 a	1.6 ± 0.05 b
Rm	0.9 ± 0.03 a	0.5 ± 0.03 b
Ro	37.4 ± 3.74 a	8.2 ± 1.16 b
TD	0.6 ± 0.02 b	1.3 ± 0.07 a

* Means followed by the same letter within a line are not significantly different at $p > 0.05$, using t test. Life table parameters - IMG: mean time between generations, λ: finite growth rate, *rm:* intrinsic rate of increase, *Ro*: net reproductive rate, TD: time for duplication.

2.3.2 Effects of the host plant on *Buchnera* growth

Density of *Buchnera* in *A. citricidus* and *A. aurantii* followed different trends of growth during the aphid development, but in both cases *Buchnera* density in newly-emerged adults reached the same density (Figure 2.1). *Buchnera* density, assessed by a direct estimation of the number of copies of the *dnaK* gene, increased with aphid development *in A. citricidus* reared on sweet orange but decreased with development on orange jasmine (Figure 2.1 A, B). This

opposite trend was observed mainly because *Buchnera* density in first instars was nearly 90-fold higher in *A. citricidus* on orange jasmine than on sweet orange (Figure 2.1 A, B).

In *A. aurantii*, density of *Buchnera* showed a clear pattern of gradual increment (Figure 2.2 A, C). However, on sweet orange *Buchnera* growth on sweet orange was more stable with a continuous increase in density until adulthood, while on orange jasmine *Buchnera* density decreased after peaking on second instar before a rapid growth from fourth instar to the adult stage (Figure 2.1 A, C). Increase in *Buchnera* density from fourth instar to adult was nearly 1000-fold in aphids developing in orange jasmine, but only 10-fold in aphids developing in sweet orange (Figure 2.1 C, D).

Buchnera growth and final density in newly-emerged adults were much more intense in the polyphagous *A. aurantii* than in the oligophagous *A. citricidus* in both host plants studied (Figure 2.1). In *A. aurantii*, *Buchnera* grew from anywhere near 10^9 in orange jasmine and 10^8 in sweet orange to reach a density of 10^{13} at the adult stage, 100-fold higher than in *Buchnera* in *A. citricidus* (Figures 2.1). Although the density of *Buchnera* was the same early at the adult stage in either host plants for both aphids, it is clear that *Buchnera* growth was affected in orange jasmine, the suboptimal host plant.

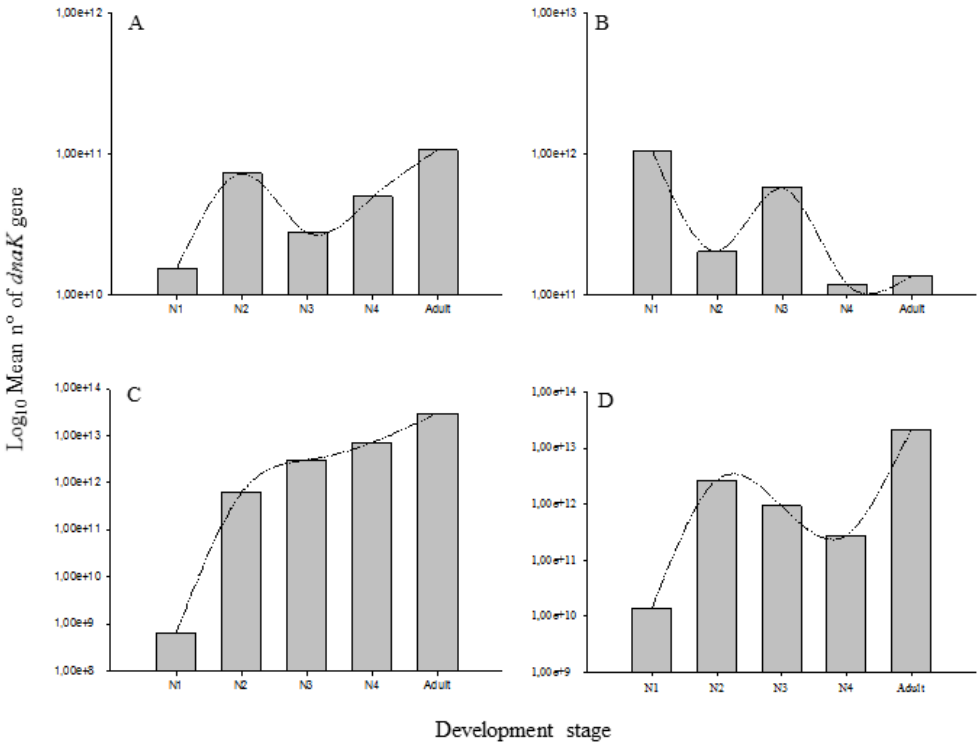


Figure 2.1 - Mean number of copies of dnaK gene for each instar and the adult of *Aphis (Toxoptera) citricidus* reared on sweet orange (A) and on orange jasmine (B), and *Aphis (Toxoptera) aurantii* reared on sweet orange (C) and orange jasmine (D)

2.3.3 Contribution of host nutrition on different stages of development to adult reproductive performance

The effects of the optimal (sweet orange) and sub-optimal host (orange jasmine) on *A. citricidus* and *A. aurantii* was investigated by comparing the contribution of both host plants to their reproductive fitness by allowing their access to these plants during different stages of development. Fecundity of *A. citricidus* females obtained from nymphs reared on sweet orange was much higher than those obtained from orange jasmine, when adults were maintained on rangpur. No differences in longevity and fecundity were observed for *A. aurantii*, regardless the host plant females developed from. But the percentage of reproductively active females on rangpur was always lower for females of *A. citricidus* and *A. aurantii* that developed from nymphs reared on orange jasmine (Table 2.3).

But when nymphs were reared on the same host plant (rangpur), and adult females were transferred to sweet orange or orange jasmine, *A. aurantii* females were the only to show an effect on their longevity and fecundity. In this case, females maintained on sweet orange lived twice as long and produced nearly twice as much nymphs than females reproducing on orange jasmine (Table 2.4). No significant differences were observed in the number of reproductively active females of both aphids on either host plants (sweet orange and orange jasmine) when nymphs were reared on rangpur (Table 2.4).

Table 2.3 - Female longevity (days) and fecundity (no. of nymphs/female) and percentage of reproductive females of *Aphis (Toxoptera) citricidus (Ac-iso1)* and *Aphis (Toxoptera) aurantii (Aa-iso1)* on rangpur, when females were obtained from nymphs reared either on sweet orange or orange jasmine (25±2°C; 60±10% RH; 14h photophase)

Treatment	**Reared from sweet orange**	**Reared from orange jasmine**	**Statistics**
A. citricidus (Ac-iso1)			
Reproductively active females (%)	90 a	50 b	χ^2 (1, 20) = 17.7, $p < 0.05$
Female longevity (days)	5.5 ± 0.92 a	3.4 ± 0.84 a	t (19) = -0.16, $p = 0.86$
Female fecundity (Nº nymph/♀)	19.1 ± 3.73 a	3.1 ± 1.04 b	t (19) =2.32, $p < 0.05$
A. aurantii (Aa-iso1)			
Reproductively active females (%)	95 a	60 b	χ^2 (1, 20) = 12.9, $p < 0.05$
Female longevity (days)	7.6 ± 1.52 a	4.0 ± 1.14 a	t (19) = 0.36, $p = 0.71$
Female fecundity (Nº nymph/♀)	13.0 ± 3.33 a	3.9 ± 1.46 a	t (19)= -1.82, $p = 0.08$

* Means followed by the same letter within a line are not significantly different at $p > 0.05$ using t or χ^2 tests.

Table 2.4 - Female longevity (days) and fecundity (no. of nymphs/female) and percentage of reproductive females of *Aphis (Toxoptera) citricidus (Ac-iso1)* and *Aphis (Toxoptera) aurantii (Aa-iso1)* on sweet orange and orange jasmine, when females were obtained from nymphs reared on rangpur (25±2°C; 60±10% RH; 14h photophase)

Treatment	**Reared from sweet orange**	**Reared from orange jasmine**	**Statistics**
A. citricidus (Ac-iso1)			
Reproductively active females (%)	80 a	70 a	χ^2 (1, 20) = 1.2, p = 0.6
Female longevity (days)	6.9 ± 1.15 a	3.8 ± 0.89 a	t (19) = 1.7, p = 0.09
Female fecundity (Nº nymph/♀)	13.8 ± 3.29 a	7.0 ± 1.99 a	t (19) = 1.3, p = 0.19
A. aurantii (Aa-iso1)			
Reproductively active females (%)	90 a	85 a	χ^2 (1, 20) = 0.3, p = 0.59
Female longevity (days)	9.0 ± 1.40 a	4.5 ± 0.74 b	t (19) = 2.5, p < 0.05
Female fecundity (Nº nymph/♀)	21.2 ± 4.16 a	8.7 ± 1.61 b	t (19) = 2.4, p < 0.05

* Means followed by the same letter within a line are not significantly different at p > 0.05 using t or χ^2 tests.

2.4 Discussion

Biology of *A. citricidus* and *A. aurantii* on sweet orange and orange jasmine demonstrated that sweet orange is a higher-quality host plant for both aphids species. Although orange jasmine negatively impacted fitness of both species, the oligophagous aphid *A. citricidus* was more affected than the polyphagous aphid *A. aurantii*. Yet, aphids showed different strategies regarding the obligate symbiont *Buchnera aphidicola* and host plant influenced the growth trend, but not the density of *Buchnera* attained at the early adult stage. Nevertheless, symbiont increment from first instar to early adult stage in the polyphagous aphid is more intense than on the oligophagous aphid. In both aphids, symbiont growth on the suboptimal host plant was thinned. Additionally, host switch between nymph and adult stages showed that nutrition during the nymph stage was important for *A. citricidus*, whereas adult nutrition influence *A. aurantii* reproduction.

The fecundity and longevity reported for the isolines of *A. citricidus* and *A. aurantii* we investigated on sweet orange and orange jasmine are lower than those reported in the literature (TSAI ,1998; TANG et al., 1999; WANG; TSAI, 2001), but direct comparisons of biological data with those from the literature are limited as fitness traits of aphids may be influenced by intraspecific variability (SANDSTRÖM; PETTERSSON, 1994; TANG et al., 1999; HALBERT; BROWN, 2011).

Host plant quality is crucial for aphid reproduction, but the polyphagous aphid has a better strategy to handle the sub-optimal host plant. We showed that sweet orange is a higher-quality host for both oligophagous and polyphagous aphids, but fitness of the oligophagous aphid *A. citricidus* was more severely affected in orange jasmine. *A. citricidus* reared on orange jasmine resulted in a negative intrinsic rate of increase (*rm*), indicating the population would go to extinction on this host plant. But the polyphagous *A. aurantii* had a positive population growth, even though *rm* when reared in orange jasmine was reduced if compared to that on sweet orange. The higher sensitivity of the oligophagous aphid to a low-quality host plant was also reflected on the net reproductive rate (*Ro*). Both aphid species showed reduced *Ro* on orange jasmine if compared to sweet orange, but *A. citricidus* had a 33-fold difference in *Ro* between sweet orange and orange jasmine, while such difference for the polyphagous *A. aurantii* was less than 5-fold.

Thus, the oligophagous aphid suffered a higher impact on its fitness traits as compared to the polyphagous aphid when exploring a sub-optimal host plant. The better development of

the polyphagous *A. aurantii* over the oligophagous *A. citricidus* when exposed to a suboptimal host plant would be expected as polyphagous herbivores are better adapted to protect themselves from the plant defensive chemical responses (STAM et al., 2014; MULLER et al., 2015; SIMON et al., 2015).

Aphids shared their nutritional ecology with an obligate nutritional symbiont, which is known to respond to the nutritional quality of the host plant the host aphid is feeding on (WILKINSON; KOGA; FUKATSU, 2007; CHANDLER; WILKINSON; DOUGLAS, 2008). Aphids were demonstrated to explore unsuitable host plants due nutrient provisioning by the associated symbiont (AKMAN GÜNDÜZ; GOUGLAS, 2009), but the fact some aphids have strategies to regulate the nutritional quality of their host plant (TELANG et al., 1999; SANDSTRÖM; TELANG; MORAN, 2000) suggests they may share different levels of nutritional dependency of their symbionts and establish different mechanisms of interaction with their symbionts.

We demonstrated that *A. citricidus* and *A. aurantii* have different strategies to regulate the growth of *Buchnera*, and the growth pattern of the symbiont is affected by the quality of the host plant. Recent data on the growth of *Buchnera* in the pea aphid *A. pisum* demonstrated the symbiont will increase in number and size during the immature stage until adulthood, and progressively decrease in number during aphid aging (SIMONET et al., 2016). We did not follow the density of *Buchnera* during the aging of either aphids studied, but the growth pattern of *Buchnera* associated with *A. aurantii* when feeding on sweet orange was as reported in *A. pisum* (SIMONET et al., 2016). The growth pattern of *Buchnera* in *A. aurantii* feeding on orange jasmine and in *A. citricidus* feeding on sweet orange resemble the pattern obtained in *A. pisum*, although presenting a discontinuous instead of a continuous increase in density during the immature stage (SIMONET et al. 2016).

Despite differences in the growth pattern of *Buchnera* in each aphid host, the density of *Buchnera* attained at the early adult stage was similar in each aphid species regardless of the host plant. However, *Buchnera* density in early adult females of the polyphagous *A. aurantii* was much higher than in the oligophagous *A. citricidus*. Variation in *Buchnera* density has been reported to several aphids and such differences have been argued to be influenced by the host age, host generation, amino acids composition of the food source, aphid morphs, temperature and virus infection (HUMPHREYS; DOUGLAS, 1997; WILKINSON et al., 2003; VOGEL; MORAN, 2011; LU et al., 2014; CASSONE et al., 2015).

We hypothesized that the limited changes in density of *Buchnera* during aphid development and the lower abundance of symbiont in the oligophagous aphid *A. citricidus* as

compared to the polyphagous *A. aurantii* may be related with the lower dependence of *A. citricidus* to the nutritional contribution provided by *Buchnera*. The reduction in the dependency of its symbiont would be a consequence of the ability of *A. citricidus* to regulate the composition of its host plant through adaptation to the narrow range of hosts it can successfully explore. Mechanisms of host regulation are common in organisms that share a history of adaptation, as it can be observed in interactions among other groups of organisms (PENNACCHIO; CACCIA; DIGILIO, 2014). Nonetheless, such hypothesis would have to be proven analyzing clonal lines of *A. citricidus* on several host species within the *Citrus* genus to assess the host sap and the symbiont growth.

Food switching indicated the reproductive fitness of *A. citricidus* and *A. aurantii* was differently affected by the quality of the nutrients they received at the immature and adult stages. Exposure to optimal (sweet orange) and suboptimal (orange jasmine) diets during the immature development and then switching the adults to a common optimal host (rangpur) indicated nutrient allocation to reproduction in *A. citricidus* relies on the quality of nutrient uptake during the immature stage, while *A. aurantii* can reduce the effects of the suboptimal diet used during the immature stage by allocating nutrient obtained from the adult stage. Experiments in which the immatures were reared on a similar optimal host plant (rangpur) and adults were transferred to optimal (sweet orange) and suboptimal (orange jasmine) diets to reproduce confirmed that nutrient acquisition during the immature stage is much more critical to the oligophagous than to the polyphagous aphid, demonstrating the use of different reproductive strategies in these species. If such differences would correlate with the variation in the density of *Buchnera* these aphids harbor when developing on the tested plants remains to be investigated.

In conclusion, this research brings new data on how host plant affects oligophagous and polyphagous aphids and its obligate symbiont *Buchnera aphidicola*. Here we demonstrated that sweet orange is higher-quality host plant for both *A. citricidus* and *A. aurantii* when compared to orange jasmine, and that the suboptimal host plant impacts more severely the oligophagous than the polyphagous aphid. The growth pattern of the obligate symbiont *B. aphidicola* also differ between aphids and was influenced by the host plant, although no differences were observed in the final density for each host aphid. *A. citricidus* and *A. aurantii* have different strategies to allocate their resources to reproduction. While *A. citricidus* reproduction is based on nutrient acquisition at the immature stage, in *A. aurantii* nutrient acquisition at the adult stage can be directed to reproduction and overcome nutritional deficiencies at the immature stage.

2.5 Conclusions

- Sweet orange a higher-quality host plant than orange jasmine as host for *Aphis (Toxoptera) citricidus* and *Aphis (Toxoptera) aurantii;*
- The oligophagous aphid *A. citricidus* was more affected by the suboptimal host plant, orange jasmine, than the polyphagous aphid *A. aurantii;*
- *A. citricidus* and *A. aurantii* have different strategies to control *Buchnera* growth, and *Buchnera* is differently affected by the host plant in these aphids;
- *A. citricidus* and *A. aurantii* have different strategies for resource allocation;
- *A. citricidus* and *A. aurantii* have different reproductive strategies;
- Reproduction in *A. citricidus* relies on nutrient acquisition at the immature stage, while in *A. aurantii* reproduction is affected by nutrient acquisition at the adult stage.

References

ALI, J.G.; AGRAWAL, A.A. Specialist versus generalist insect herbivores and plant defense. **Trends in Plant Science**, Oxford, v. 17, n. 5, p. 293-302, 2012.

AKMAN GÜNDÜZ, E.; DOUGLAS, A.E. Symbiotic bacteria enable insect to use a nutritionally inadequate diet. **Proceedings of the Royal Society B-Biological Science**, London, v. 276, p. 987-991, 2009.

ANDREWARTHA, H. G.; BIRCH, L. C. The innate capacity for increase in numbers, p. 31-54. In: H. G. ANDREWARTHA & L. C. BIRCH. (eds.). **The distribution and abundance of animals**. Chicago, University of Chicago Press, 782 p. 1954.

AWMACK, C.S.; LEATHER, S.R. Host plant quality and fecundity in herbivorous insects. **Annual Review of Entomology**, Stanford, v. 47, p. 817-844, 2002

BAUMANN, L.; BAUMANN, P. Growth-Kinetics of the endosymbiont *Buchnera Aphidicola* in the Aphid *Schizaphis Graminum*. **Applied and Environmental Microbiology**, Washington, v. 60, n. 9, p. 3440-3443, 1994.

_____. Cospeciation between the primary endosymbionts of mealybugs and their hosts. **Current Microbiology**, New York, v. 50, n. 2, p. 84-87, 2005.

BUCHNER, P. **Endosymbiosis of animals with plant microorganisms**. New Jersey: Interscience Publ.; John Wiley, 1965. 909 p.

CARVER, M. The black citrus aphids, *Toxoptera citricidus* (Kirkaldy) and *T. Aurantii* (boyer de fonscolombe) (Homoptera: Aphididae). **Australian Journal of Entomology**, Yarra, v. 17, n. 3, p. 263-270, 1978.

CASSONE, B.J.; REDINBAUGH, M.G.; DORRANCE, A.E.; MICHEL, A.P. Shifts in *Buchnera aphidicola* density in soybean aphids (*Aphis glycines*) feeding on virus-infected soybean. **Insect Molecular Biology**, West Sussex, v. 24, n. 4, p. 422-431, 2015.

CHANDLER, S.M.; WILKINSON, T.L.; DOUGLAS, A.E. Impact of plant nutrients on the relationship between a herbivorous insect and its symbiotic bacteria. **Proceeding of the Royal Society B-Biologial Science**, London, v. 275, p.: 565-570, 2008.

COLASURDO, N.; GELINAS, Y.; DESPLAND, E. Larval nutrition affects life history traits in a capital breeding moth. **Journal of Experimental Biology**, Cambridge, v. 212, n. 12, p. 1794-1800, 2009.

CZERNIEWICZ, P.; LESZCZYNSKI, B.; CHRZANOWSKI, G.; SEMPRUCH, C.; SYTYKIEWICZ, H. Effects of host plant phenolics on spring migration of bird cherry-oat aphid (*Rhopalosiphum padi* L.). **Allelopathy Journal**, Haryana, v. 27, n. 2, p. 309-316, 2011.

DOUGLAS, A. E. Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. **Annual Review of Entomology**, Stanford, v. 43, p. 17-37, 1998.

_____. Phloem-sap feeding by animals: problems and solutions. **Journal of Experimental Botany**, Oxford, v. 57, n. 4, p. 747-754, 2006.

DU PLESSI, H.; BYRNE, M.; VAN DEN BERG, J. The effect of different host plants on development and survival fo *Nysius natalensis* (Hemiptera: Orsillidae). **Environmental Entomology**, College Park, v. 44, p. 122-127, 2015.

FUENTEALBA, A.; BAUCE, E. Carry-over effect of host nutritional quality on performance of spruce budworm progeny. **Bulletin of Entomological Research**, Farnham Royal, v. 102, n. 3, p. 275-284, 2012.

GUTBRODT, B.; DORN, S.; UNSICKER, S.B.; MODY,K. Species-specific responses of herbivores to within-plant and environmentally mediated between-plant variability in plant chemistry. **Chemoecology**, Basel, v. 22, p. 101-111, 2012.

HALBERT, S.E.; BROWN, L.G. **Brown Citrus Aphid, Toxoptera citricida (Kirkaldy) (Insecta: Hemiptera: Aphididae)**. Gainesville: University of Florida IFAS Extension. EENY-007**:** 1-6 p. 2011.

HANSEN, A.K.; MORAN, N.A. The impact of microbial symbionts on host plant utilization by herbivorous insects. **Molecular Ecology**, Oxford, v. 23, n. 6, p. 1473-1496, 2014.

_____. Aphid genome expression reveals host–symbiont cooperation in the production of amino acids. **Proceedings of the national academy of science of the united States of America**, v.108, n.7, p. 2849-2854, 2011.

HOSOKAWA, T.; KIKUCHI, Y.; NIKOH, N.; SHIMADA, M.; FUKATSU, T. Strict host-symbiont cospeciation and reductive genome evolution in insect gut bacteria. **Plos Biology**, San Francisco, v. 4, n. 10, p. 1841-1851, 2006.

HOUSE, H.L. Insect Nutrition. **Annual Review of Entomology**, Stanford, v. 6, p. 13-26, 1961.

HUMPHREYS, N.J.; DOUGLAS, A.E. Partitioning of symbiotic bacteria between generations of insect: a quantitative study of a *Buchnera* sp in the pea aphid (*Acyrthosiphon pisum*) reared at different temperatures. **Applied and Environmental Microbiology**, Washington, v. 63, n. 8, p. 3294-3296, 1997.

JOHNS, R.C.; TOBITA, H.; HARA, H.; OZAKI, K. Adaptive advantages of dietary mixing different-aged foliage within conifers for a generalist defoliator. **Ecological Research**, Tokyo, v. 30, p. 793-802, 2015.

JOERN, A.; PROVIN, T.; BEHMER, S.T. Not just the usual suspects: insect herbivore populations and communities are associeted with multiple plant nutrients. **Ecology**, Hoboken, v.93, p. 1002-1015, 2012

KATSIR, L.; CHUNG, H.S.; KOO, A.J.K.; HOWE, G.A. Jasmonate signaling: a conserved mechanism of hormone sensing. **Current Opinion in Plant Biology**, Oxford, v. 11, n. 4, p. 428-435, 2008.

KNUTTEL, H.; FIEDLER, K. Host-plant-derived variation in ultraviolet wing patterns influences mate selection by male butterflies. **Journal of Experimental Biology**, Cambridge, v. 204, n. 14, p. 2447-2459, 2001.

KOGA, R.; TSUCHIDA, T.; FUKATSU, T. Changing partners in an obligate symbiosis: a facultative endosymbiont can compensate for loss of the essential endosymbiont *Buchnera* in an aphid. **Proceedings of the Royal Society B-Biological Sciences**, London, v. 270, n. 1533, p. 2543-2550, 2003.

KOMAKI, K.; ISHIKAWA, H. Genomic copy number of intracellular bacterial symbionts of aphids varies in response to developmental stage and morph of their host. **Insect Biochemistry and Molecular Biology**, Oxford, v. 30, n. 3, p. 253-258, 2000.

LU, W.-N.; CHIU, M.-C.; KUO, M.-H. Host life stage- and temperature-dependent density of the symbiont Buchnera aphidicola in a subtropical pea aphid (*Acyrthosiphon pisum*) population. **Journal of Asia-Pacific Entomology**, Amsterdam, v. 17, n. 3, p. 537-541, 2014.

MAIA, A.D.H.N.; LUIZ, A.J.B. **Programa SAS para análise de tabelas de vida e fertilidade de artrópodes:** o método Jackknife. Jaguariúna: EMBRAPA, 2006.11 p. (Comunicado Técnico, 33).

MCCUTCHEON, J.P.; MORAN, N.A. Extreme genome reduction in symbiotic bacteria. **Nature Reviews Microbiology**, London, v. 10, n. 1, p. 13-26, 2012.

MILANOVIĆ, S.; JANKOVIĆ-TOMANIĆ, M.; KOSTIĆ, I.; KOSTIĆ, M.; MORINA, F.; ŽIVANOVIĆ, B.; LAZAREVIĆ, J. Behavioural and physiological plasticity of gypsy moth larvae to host plant switching. **Entomologia Experimentalis et Applicata**, West Sussex, v.2, p.152-158, 2016.

MODY, K.; UNSICKER, S.B.; LINSENMAIR, K.E. Fitness related dietmixing by intraspecific host-plant-switching of specialist insect herbivores. **Ecology**, Hoboken, v.88, p.1012–1020, 2007.

MOORE, P.J.; ATTISANO, A. Oosorption in response to poor food: complexity in the trade-off between reproduction and survival. **Ecology and Evolution**, New Jersey, v. 1, n. 1, p. 37-45, 2011.

MORAN, N.A.; DEGNAN, P.H. Functional genomics of *Buchnera* and the ecology of aphid hosts. **Molecular Ecology**, Oxford, v. 15, n. 5, p. 1251-1261, 2006.

MORAN, N.A.; YUN, Y. Experimental replacement of an obligate insect symbiont. **Proceedings of the National Academy of Sciences of the United States of America,** Washington, v. 112, n. 7, p. 2093-2096, 2015.

MORAN, N. A.; MCCUTCHEON, J. P.; NAKABACHI, A. Genomics and Evolution of Heritable Bacterial Symbionts. **Annual Review of Genetics**, Palo Alto, v. 42, n. 1, p. 165-190, 2008.

MORAN, N.A.; MCLAUGHLIN, H.J; SOREK, R. The dynamics and time scale of ongoing genomic erosion in symbiotic bacteria. **Science**, Washington, v.323, 2009. Available at:< doi: 10.1126/science.1167140.>.

MULLER, K.; VOGELWEITH, F.; THIERY, D.; MORET, Y.; MOREAU, J. Immune benefits from alternative host plants could maintain polyphagy in a phytophagous insect. **Oecologia,** Heidelberg, v. 177, n. 2, p. 467-475, 2015.

NAKABACHI, A.; ISHIDA, K.; HONGOH, Y.; OHKUMA, M.; MIYAGISHIMA, S-Y. Aphid gene of bacterial origin encodes a protein transported to an obligate endosymbiont. **Current Biology,** Heidelberg, v. 24, n. 14, p. R640-R641, 2014.

NOVAKOVA, E.; HYPŠA, V.; KLEIN, J.; FOOTTIT, R.G.; von DOHLEN, C.D.; MORAN, N.A. Reconstructing the phylogeny of aphids (Hemiptera: Aphididae) using DNA of the obligate symbiont *Buchnera aphidicola*. **Molecular Phylogenetics and Evolution,** Maryland Heights, v. 68, n. 1, p. 42-54, 2013.

OLIVER, K. M.; MORAN, N. A.; HUNTER, M. S. Variation in resistance to parasitism in aphids is due to symbionts not host genotype. **Proceedings of the National Academy of Sciences of the United States of America**, Washington, v. 102, n. 36, p. 12795-800, 2005.

_____. Costs and benefits of a superinfection of facultative symbionts in aphids. **Proceedings of the Royal Society B-Biological Sciences**, London, v. 273, n. 1591, p. 1273-1280, 2006.

PENNACCHIO, F.; CACCIA, S.; DIGILIO, M.C. Host regulation and nutritional exploitation by parasitic wasp. **Current Opinion in Insect Science**, Amsterdam, v.6, p.74-79, 2014.

PEREZ-BROCAL, V.; GIL, V.R.; RAMOS, S.; LAMELAS, A.; POSTIGO, M.; MANUEL MICHELENA, J.; SILVA, F.J.; MOYA, A.; LATORRE, A. A small microbial genome: The end of a long symbiotic relationship? **Science**, Washington, v. 314, n. 5797, p. 312-313, 2006.

SAMBROOK, J.; RUSSELL, D. W. **Molecular cloning:** a laboratory manual. 3rd edition, Cold Spring Harbor: Cold Spring Harbor Laboratoty Press, 2001. v. 1, 999 p.

SANDSTRÖM, J.; MORAN, N.A. How nutritionally imbalanced is phloem sap for aphids? **Series Entomologica,** Oxford, v.56, p. 203-210, 1999. Apresentado no INTERNATIONAL SYMPOSIUM ON INSECT PLANT INTERACTIONS, 10., 1999, Oxford. **Proccedings…**

SANDSTRÖM, J.; PETTERSSON, J. Amino acid composition of phloem sap and the relation to intraspecific variation in pea aphid (Acyrthosiphon pisum) performance. **Journal of Insect Physiology**, Oxford, v. 40, p. 947-955, 1994.

SANDSTROM, J.P.; RUSSELL, J.A.; WHITE, J.P.; MORAN, N.A. Independent origins and horizontal transfer of bacterial symbionts of aphids. **Molecular Ecology**, Oxford, v. 10, n. 1, p. 217-228, 2001.

SANDSTRÖM, J.; TELANG, A.; MORAN, N.A. Nutritional enhancement of host plants by aphids: a comparison of three aphid species on grasses. **Journal of Insect Physiology**, Oxford,v.46, p. 33-40, 2000.

SAS INSTITUTE INC. **SAS language v.9.4.** 4th ed. Cary, SAS Institute, 2015.

SCRIBER, J.M.; SLANSKY JR, F. The nutritional ecology of immature insects. **Annual Review of Entomology**, Palo Alto, v.26, p.183-211, 1981.

SIMONET , P.; DUPORT, G.; GAGET, K.; WEISS-GAYET, M.; COLELLA, S.; FEBVAY, G.; CHARLES, H.; VINUELAS, J.; HEDDI, A.; CALEVRO, F. Direct flow cytometry measurements reveal a fine-tuning of symbiotic cell dynamics according to the host developmental needs in aphid symbiosis. **Scientific Reports**, London, v. 6, 2016. Available < DOI: 10.1038/srep19967>.

SHIGENOBU, S.; WATANABE, H.; HATTORI, M.; SAKAKI, Y.; ISHIKAWA, H. Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp APS. **Nature**, London, v. 407, n. 6800, p. 81-86, 2000.

SIMON, J-C.; D'ALENÇON, E.; GUY, E.; JACQUIN-JOLY, E.; JAQUIÉRY, J.; NOUHAUD, P.; PECCOUD, J.; SUGIO, A.; STREIFF, R. Genomics of adaptation to host-plants in herbivorous insects. **Briefings in Functional Genomics**, Oxford, v. 14, 2015. Available at: <doi: 10.1093/bfgp/elv015 >.

STAM, J.M.; KROES, A.; LI, Y.; GOLS, R.; VAN LOON, J.J.A.; POELMAN, E.H.; DICKE, M. Plant Interactions with Multiple Insect Herbivores: From Community to Genes. **Annual Review of Plant Biology**, Palo Alto, v. 65, p. 689-713, 2014.

SUGIO, A.; DUBREUIL, G.; GIRON, D.; SIMON, J.-C. Plant-insect interactions under bacterial influence: ecological implications and underlying mechanisms. **Journal of Experimental Botany**, Oxford, v. 66, n. 2, p. 467-478, 2015.

SUNNUCKS, P.; DE BARRO, P.J.; LUSHAI, G.; MACLEAN, N.; HALES, D. Genetic structure of an aphid studied using microsatellites: cyclic parthenogenesis, differentiated lineages and host specialization. **Molecular Ecology**, Oxford, v. 6, n. 11, p. 1059-1073, 1997.

TANG, Y.Q.; LAPOINTE, S.L.; BROWN, L.G.; HUNTER, W.B. Effects of host plant and temperature on the biology of *Toxoptera citricida* (Homoptera : Aphididae). **Environmental Entomology**, College Park,v. 28, n. 5, p. 895-900, 1999.

TELANG A.; SNADSTRÖM, J.; DYRESON, E.; MORAN, N.A. Feeding damage by Diuraphis noxia results in a nutritionally enhanced phloem diet. **Entomologia Experimentalis et Applicata**, West Sussex, v. 91, p. 403-412, 1999.

THAO, M.L.; BAUMANN, P. Evolutionary relationships of primary prokaryotic endosymbionts of whiteflies and their hosts. **Applied and Environmental Microbiology**, Washington, v. 70, n. 6, p. 3401-3406, 2004.

THAO, M.L.; MORAN, N.A.; ABBOT, P.; BRENNAN, E.B.; BURCKHARDT, D.H.; BAUMANN, P: Cospeciation of psyllids and their primary prokaryotic endosymbionts. **Applied and Environmental Microbiology**, Washington,v. 66, n. 7, p. 2898-2905, 2000.

TSAI, J.H. Development, survivorship, and reproduction of *Toxoptera citricida* (Kirkaldy) (Homoptera : Aphididae) on eight host plants. **Environmental Entomology,** College Park, v. 27, n. 5, p. 1190-1195, 1998.

TSUCHIDA, T.; KOGA, R.; HORIKAWA, M.; TSUNODA, T.; MAOKA, T.; MATSUMOTO, S.; SIMON, J.C.; FUKATSU, T: Diversity and geographic distribution of secondary endosymbiotic bacteria in natural populations of the pea aphid, *Acyrthosiphon pisum*. **Molecular Ecology**, Oxford, v. 11, n. 10, p. 2123-2135, 2002.

VAN HAM, R. VAN HAM, R.C.H.J.; KAMERBEEK, J.; PALACIOS, C.; RAUSELL, C.; ABASCAL, F.; BASTOLLA, U.; FERNANDEZ, J.M.; JIMENEZ, L.; POSTIGO, M.; SILVA, F.J.; TAMMAMES, J.; VIGUERA, E.; LATORRE, A.; VALENCIA, A.; MORAN, F.; MOYA, A. Reductive genome evolution in *Buchnera aphidicola*. **Proceedings of the National Academy of Sciences of the United States of America**, Washington, v. 100, n. 2, p. 581-586, 2003.

VOGEL, K.J.; MORAN, N.A. Effect of Host Genotype on Symbiont Titer in the Aphid-*Buchnera* Symbiosis. **Insects**, Basel, v. 2, n. 3, p. 423-434, 2011.

WANG, J.J.; TSAI, J.H. Development, survival and reproduction of black citrus aphid, *Toxoptera aurantii* (Hemiptera : Aphididae), as a function of temperature. **Bulletin of Entomological Research**, Farnham Royal, v. 91, n. 6, p. 477-487, 2001.

WHELAN, J. A.; RUSSEL, N.B.; WHELAN, M.A. A method for the absolute quantification of cDNA using real-time PCR. **Journal of Immunological Methods**, Amsterdam, v.278, p. 261-269, 2003.

WHITEHEAD, L.F.; DOUGLAS, A.E. Populations of symbiotic bacteria in the parthenogenetic pea aphid (*Acyrthosiphon-pisum*) symbiosis. **Proceedings of the Royal Society B-Biological Sciences**, London, v. 254, n. 1339, p. 29-32, 1993.

WILKINSON, T.L.; DOUGLAS, A.E. Host cell allometry and regulation of the symbiosis between pea aphids, *Acyrthosiphon pisum*, and bacteria, *Buchnera*. **Journal of Insect Physiology**, Oxford, v. 44, n. 7-8, p. 629-635, 1998.

WILKINSON, T.L.; FUKATSU, T.; ISHIKAWA, H. Transmission of symbiotic bacteria *Buchnera* to parthenogenetic embryos in the aphid *Acyrthosiphon pisum* (Hemiptera : Aphidoidea). **Arthropod Structure & Development**, Oxford, v. 32, n. 2-3, p. 241-245, 2003.

WILKINSON, T.L.; KOGA, R.; FUKATSU, T. Role of host nutrition in symbiont regulation: Impact of dietary nitrogen on proliferation of obligate and facultative bacterial endosymbionts of the pea aphid *Acyrthosiphon pisum*. **Applied and Environmental Microbiology**, Washington, v. 73, n. 4, p. 1362-1366, 2007.

3 DRAFT GENOME OF *Buchnera aphidicola* FROM TWO CLOSELY RELATED APHIDS WITH DIFFERENT HOST RANGES

Abstract

Buchnera aphidicola is the obligate symbiont of aphids. This bacterium provides essential amino acids and vitamins to the host, while host provides a safe intracellular environment. One of the consequences of the specialization to the intracellular mode of life and the establishment of a long co-evolutionary history with aphid hosts is the drastic reduction in the genome of *Buchnera*. Gene losses among different lineages of *Buchnera* were unequal, but all *Buchnera* strains sequenced so far lack genes related to DNA repair and replication and members of pathways for non-essential amino acids. However, there are also cases in which losses of specific sets of genes in *Buchnera* were related to the aphid nutrition ecology. We produced *de novo* assemblies of draft genomes for *Buchnera* strains associated with close-related aphid species, *Aphis (Toxoptera) citricidus* (Kirkaldy) and *Aphis (Toxoptera) aurantii* Boyer de Fonscolombe, which differ in the range of host plants they explore, in order to investigate if differences in the genome of the obligate symbiont would reflect the oligophagy/polyphagy strategies of their host aphids. Draft genomes of *Buchnera* associated with *A. citricidus* (oligophagous) and *A. aurantii* (polyphagous) were assembled following paired-end sequencing (250 bp-pairs) on an Illumina MiSeq platform. *Buchnera* associated with *A. citricidus* (*BAc* strain) and *A. aurantii* (*BAa*) were quite similar, with 570 predicted coding sequences (CDS) in common. Only two CDS and one tRNA were observed to differ between the *BAc* and *BAa* strains: a riboflavin kinase, a DNA helicase and a tRNA-Leu. The riboflavin kinase is a pseudogene in *BAa*, while DNA helicase and a duplication in the tRNA-Leu were only observed in *BAc*. Indeed, the *BAc* strain is the only *Buchnera* strain so far to carry a DNA helicase. Duplications in tRNA-Leu were also observed in the APS strain of *Buchnera*. We compared both draft genomes with those available for other *Buchnera* strains and discuss their differences based on the importance of these gene functions to the host nutritional ecology, considering the oligophagous-polyphagous dichotomy.

Keywords: Comparative genome; Genomics; Host nutrition ecology; Obligatory symbiont

3.1 Introduction

Nutritional symbiotic associations among bacteria and hemipteran hosts are common due to the use of unbalanced diet, such as blood or phloem/xylem hemipterans explore (HANSEN; MORAN, 2014). *Buchnera aphidicola* is the obligate symbiont aphids carry to supplement their nutrient-deficient diet by providing aphids with essential amino acids and vitamins, while harbored in a protected, nutrient-rich environment represented by the cytoplasm of specialized host cells called bacteriocytes (DOUGLAS, 1998; 2009; BAUMANN, 2005). Aphid-*Buchnera* association is a true mutualism, as fitness of *Buchnera*-depleted aphids is severely affected and bacterial growth is dependent on the host intracellular environment (KOGA; TSUCHIDA; FUKATSU, 2003). Moreover, the understanding of their close association has demonstrated bacterial growth is under aphid nutritional regulation (HANSEN;

MORAN, 2011). The intimacy of aphid–*B. aphidicola* association was developed over an ancient relationship established at nearly 80–150 Myr (VON DOHLEN; MORAN, 2000), and has been efficiently maintained by vertical transmission (WILKINSON, FUKATSU, ISHIKAWA, 2003).

The shared life history through many millions of years led to the loss of several genes and to the reduction of the genome of *B. aphidicola*, as typically observed as a consequence of adaptation to endocytobiosis (GOSALBES et al., 2010). Under such conditions many gene products become unnecessary, as they can be acquired directly from the host. Additionally, in the absence of selection pressure and horizontal gene transfer, only essential genes are maintained in the process of genome size reduction (WERNEGREEN, 2002). To date, 19 complete genomes of strains of *B. aphidicola* are available; they are highly syntenic and share extreme genome reduction as compared to their closest free-living relative *Escherichia coli* (SHIGENOBU et al., 2000; VAN HAM et al., 2003; PEREZ-BROCAL et al., 2006; MORAN, MCLAUGHLIN, SOREK, 2009; DEGNAN, OCHMAN, MORAN, 2011; LAMELAS et al., 2011; JIANG et al., 2013; CASSONE et al., 2015).

B. aphidicola lost many genes related to DNA replication, recombination and repair, and synthesis of non-essential amino acids (SHIGENOBU et al., 2000; PEREZ-BROCAL et al., 2006). Even though genome organization was conserved in different strains of *B. aphidicola*, interspecific variation in gene loss occurs among aphid species and host-adapted aphid races (MORAN et al., 2009). The *B. aphidicola* genomes known differ in size, pseudogene content, gene losses and the presence of plasmids. Several of these differences were related to aphid ecology (MORAN; DEGNAN, 2006). For example, the genome of *B. aphidicola* associated with *Schizaphis graminum* (Rondani) lost the capacity to synthesize cysteine and to reduce sulphur due to frameshift and/or point mutations that resulted in stop codons. The functional losses of these genes were correlated with the fact the aphid host plants (grasses) have a high fixed sulphur content (TAMAS et al., 2002).

Associations with symbionts have proven crucial for aphid ecology, since symbionts can narrow or broaden the host range of aphids (LEONARDO; MUIRU, 2003; FERRARI et al., 2006). Nevertheless, there are few cases in which the metabolic capabilities of *B. aphidicola* were linked with host plant (VOGEL; MORAN, 2011; JIANG et al., 2013), despiteless the considerable number of investigations on host-adapted races and aphid symbionts (McLEAN et al., 2011; FERRARI et al., 2012; HENRY et al., 2013; GAUTHIER et al., 2015). Host race adaptation in *Acyrthosiphum pisum* (Harris) was driven by a mutation in the *argC* gene of the associated *B. aphidicola* leading to dietary arginine requirements by *A. pisum* (VOGEL;

MORAN, 2011). On the other hand, no *B. aphidicola* changes were related to host-adapted races in *Myzus persicae* (Sulzer) (JIANG et al., 2013).

Comparative genome analysis of *B. aphidicola* associated with different aphid species and/or races have been carried, but comparisons focusing on oligophagy/polyphagy are still missing. Therefore, we report a comparative analysis of the genome of *B. aphidicola* strains associated with aphid species belonging to *Aphis (Toxoptera)* that differ in the range of host plants they use. *Aphis (Toxoptera) citricidus* (Kirkaldy) is an oligophagous aphid feeding on *Citrus* spp., while *Aphis (Toxoptera) aurantii* Boyer de Fonscolombe is a polyphagous species that feeds on a diversity of plant genera, including *Citrus* (CARVER, 1978; HALBERT; BROWN, 2011). They are both tropical species, with little information on their interactions with symbionts.

3.2 Material and Methods

3.2.1 Samples

A. citricidus and *A. aurantii* were collected in unmanaged *Citrus* sp. and *Murraya paniculata* trees, brought to the laboratory and identified following the key to wingless adults provided by Halbert and Brown (2011). Isolines were established and screened for secondary symbiont infections using diagnostic PCR as earlier described (see Chapter 2 for details). Only isolines exclusively infected with the obligate symbiont *B. aphidicola* were further used. Selected isolines of *A. citricidus* (*Ac-iso1*) and *A. aurantii* (*Aa-iso1*) harboring only *B. aphidicola* as symbiont were maintained in individual cages (50 cm high x 15 cm diameter) containing *Citrus sinensis* var. Pera (sweet orange) or *Murraya paniculata* (orange jasmine) seedlings as host plants, respectively. Cages were maintained under controlled conditions (25±2°C; 60±10% RH; 14 h photophase).

3.2.2 DNA extraction and sequencing

Nearly 100 mg of newly-emerged adults from each *Ac*-iso1 and *Aa*-iso1 isoline were macerated in 1 mL Ringer solution (100 mM NaCl, 4 nM KCl, 1 nM $CaCl_2$, pH 7.3) and used for genomic DNA extraction. Macerated aphids were filtered twice through a series of filters with decreasing mesh sizes (25 µm, 12 µm and 5 µm). The final filtrate obtained was briefly

centrifuged on a tabletop centrifuge to collect the bacterial cells. The supernatant was discarded, and the pellet obtained resuspended in 1 mL of Ringer solution before new centrifugation at 5.939g for 15 min. This procedure was repeated three times. The final pellet was resupended in extraction buffer and subjected to DNA extraction following the manufacturer's instructions (DNeasy Tissue Kit, Qiagen Inc., California). The DNA extracted was recovered in nuclease-free water, and DNA integrity and quantity were verified by spectrophotometry and agarose gel electrophoresis using standard procedures (SAMBROOK; RUSSELL, 2001).

DNA sequencing was performed at the "Centro de Biotecnologia Agricola, Departamento de Zootecnia, ESALQ/USP", following 250bp paired-end strategy on an Illumina MiSeq platform.

3.2.3 Genome assembly and annotation of *Buchnera aphidicola* associated with *Aphis (Toxoptera) citricidus* and *Aphis (Toxoptera) aurantii*

All reads were imported into CLC Genomics Workbench (CLCBio) as paired-reads and trimmed for quality and adapter indexes using the default settings (ambiguous limit = 2, quality limit = 0.05). Reverse reads had five nucleotides of their 3´end removed after trimming. The paired-reads were then subjected to the *de novo* assembly using the Spades (v.3.5) software with default parameters (BANKEVICH et al., 2012), and 500 bp as a cut-off for contig size. Due to remainings of DNA from the host aphids, a large number of contigs were assembled (*A. citricidus* = 80,462; *A. aurantii* = 64,045). Contigs related to *Buchnera* were selected based on their taxonomical identification after heuristic similarity search against the nr/nt database from NCBi, using the Blastn algorithm. Contigs matching to *Buchnera* were selected and the total number of contigs, contigs size and N50 were calculated using Quast v.2.3 (GUREVICH et al., 2013).

Contigs that matched to *Buchnera* were annotated with RAST software (http://rast.nmpdr.org/). The same software was used to performe functional comparisons between draft genomes of *B. aphidicola* associated to *Ac*-iso1 (*BAc* strain) and *Aa*-iso1 (*BAa* strain), and between each *Buchnera* strain and the strains with complete genome available (Table 3.1). Coding regions (CDS) exclusive to *BAc* or *BAa* strain were then subjected to manual annotation.

Table 3.1 - Reference genomes used to order contigs from genome draft of *Buchnera aphidicola* associated to *Aphis (Toxoptera) citricidus* and *Aphis (Toxoptera) aurantii*

Genbank accession number	Strain	Host
NC_017256.1	Ak	*Acyrthosiphon kondoi*
NC_002528.1	APS	*Acyrthosiphon pisum*
NZ_CP009253.1	BAg	*Aphis glycines*
NC_004545	Bp	*Baizongia pistaciae*
NC_008513	BCc	*Cinara cedri*
NC_015662	BCt	*Cinara tujafilina*
NZ_CP002701.1	G002	*Myzus persicae*
NC_00406.1	Sg	*Schizaphis graminum*
NC_017259.1	Ua	*Uroleucon ambrosiae*

3.3 Results

Sequencing yielded 630,904 reads for DNA samples from *A. citricidus* and 625,688 reads from *A. aurantii*. Assemblies resulted in a high number of contigs, but only 0.18-0.35% of them were assigned to *Buchnera*, resulting in nearly 753 and 617 thousand nucleotides assembled for the chromosomes of *Buchnera* associated with *A. citricidus* and *A. aurantii*, respectively (Table 3.2). Assemblies also yielded contigs similar to the pLeu plasmid for both species, but pTrp plasmid was observed only in *A. citricidus*. Genome assembly features were similar in *A. citricidus* and *A. aurantii*, with both presenting nearly 26% of GC content (Table 3.2).

Comparison between the draft genomes of *BAc* and *BAa* showed that 89.4% of the nucleotide sequences representing the 620 CDS predicted in *BAc* have homologue regions in *BAa*, while 84.4% of the nucleotide sequences for the 570 CDS in the *BAa* have homologues in *BAc*. *BAc* and *BAa* shared 527 gene functions for their predicted CDS, and *BAc* included 21 gene functions not represented in *B. aphidicola BAa*, while 46 were represented in *BAa* but not in *BAc*

Table 3.2 - Assembly statistics for the draft genome of *Buchnera aphidicola* associated with *Aphis (Toxoptera) citricidus* and *Aphis (Toxoptera) aurantii*

	A. citricidus	*A. aurantii*
No. of contigs similar to *Buchnera* chromosome	287	113
Total no. of nucleotides	753,486	616,873
N50	5,173	8,722
% GC	26.1	26.6
N° of contigs similar to pLeu plasmid	6	2
Total no. of nucleotides	11,698	6,816
N50	7,653	5,270
%GC	26.2	27.2
N° of contigs similar to pTrp plasmid	1	-
Total no. of nucleotides	3,047	-
%GC	22.7	-

Functional comparison of the draft genomes of *BAa* and *Bac* showed that the number of predicted CDS and tRNAs in *BAc* and *BAa* were very similar to other *Buchnera* genomes available (Table 3.3). The *BAc* strain had one CDS not reported in any other *Buchnera* strain sequenced so far, the CDS that codes for ATP-dependent DNA helicase UvrD/PcrA gene. On the other hand, all CDS of *BAa* were found in at least one *Buchnera* genome. Additionally, only one pseudogene was predicted in *BAa* while other *Buchnera* may carry from 4 (*BCc*) up to 38 (*BSg*) pseudogenes. The limited number of pseudogenes in *BAc* and *BAa* may be due to sequence gaps, as much of the pseudogenes from other *Buchnera* strains are represented in areas of the genome not covered in the draft assemblies obtained for *BAc* and *BAa* strains using the *Sg* genome as a reference (data not shown).

Table 3.3 - Comparison among the chromosome of *Buchnera aphidicola* strains

Host	Strain	Genome size (bp)	N° CDS	N° tRNAs	N° plasmid	Reference
A. aurantii	BAa	616,873	570	32	2	This chapter
A. citricidus	BAc	753,486	620	34	1	This chapter
A. glycines	BAg	638,851	603	35	2	CASSONE et al., 2015
Ac. pisum	APS	640,681	569	32	2	SHIGENOBU et al 2000
Ac. kondoi	BAk	641,794	559	32	2	DEGNAN et al 2011
B. pistaciae	Bp	615,980	504	32	0	VAN HAM et al 2003
C. cedri	BCc	416,380	353	31	1	PÉREZ-BROCAL et al 2006
C. tujafilina	BCt	397,353	367	31	1	LAMELAS et al 2011
M. persicae	G002	643,517	581	32	2	JIANG et al 2013
S. graminum	Sg	641,454	545	32	0	TAMAS et al 2002
U. ambrosiae	Ua	615,380	529	32	2	DEGNAN et al 2011

3.4 Discussion

Draft genomes of *Buchnera aphidicola* associated with *A. citricidus* (*BAc*) and *A. aurantii* (*BAa*) were similar to other *Buchnera* genomes available. They shared a conserved pool of genes, and the number of CDS and tRNAs predicted were similar to other known genomes. The putative size of the *Buchnera* genome associated with the oligophagous *A.*

citricidus is larger than the one associated with the polyphagous *A. aurantii*, and the largest *Buchnera* genome sequenced so far.

A few differences were observed between the genome of *Buchnera* associated with the oligophagous *A. citricidus* and that associated with the polyphagous *A. aurantii*. Only *BAc* carries functional riboflavin kinase, ATP-dependent DNA helicase UvrD/PcrA and duplicated tRNA-Leu codon GAG genes.

The Riboflavin kinase (ribF) gene also occurred in *BAa*, but as a pseudogene. ribF (EC 2.7.1.26) is responsible for a catalytic reaction using ATP that produces ADP and flavin mononucleotide (FNM). FNM acts as a co-factor in several metabolic processes, such as electron transport in the mitochondria, oxidation of fatty acids and metabolism of vitamin B complex. Besides the production of FNM, in bacteria ribF also acts like an adeniltransferase of FNM. In such cases, this enzyme produces ADP and adenine flavin dinucleotide (FAD), another co-factor with similar functions of FNM (KRUPA et al., 2003). Compared with other available *Buchnera* genomes, *Buchnera* associated with *A. pisum* (strain Ap) is the only *Buchnera* to carry the ribF gene. Genes related to riboflavin synthesis (ribB and ribE) are available in the genoma of Bp, Ak and Ua *Buchnera* strains (LAMELAS et al., 2011), but no riboflavin synthases were detected in the draft genomes of BCc and BCt. Here, *BAc* and *BAa* also present the genes ribB and ribE in their genome.

The genome of *Buchnera BAc* genome also presents an ATP-dependent DNA helicase UvrD/PcrA that is not present in the *BAa* strain. Indeed, this gene was not found in the genome of any other *Buchnera* sequenced so far, even though this gene is common to the free-living ancestor of *Buchnera, Escherichia coli* (SHIGENOBU et al., 2000). Helicases in general are enzymes that aid the unwindening of DNA double strands to form single strands DNA, a vital process to cellular function (BHATTACHAYYA; KECK, 2014). The ATP-dependent DNA helicase UvrD/PcrA gene is part of the protein subfamily PcrA/UvrD/Rep. *BAc* and *BAa* carry Rep helicases genes as all other *Buchnera* strains. Rep helicases are widespread in both prokaryotes and eukaryotes, while PcrvA are usually found in Gram-positive bacteria and UvrD in Gram-negative bacteria (PETIT; EHRLICH, 2002). Some homologous sequences of PcvrA helicases were found in *Buchnera* strains Bp, Sg and Ap (CHÈNE, 2008). However, ATP-dependent DNA helicase UvrD/PcrA is only reported in the *Buchnera* ancestor *E. coli*. The effects of the maintainance of this gene in the *BAc* strain remains to be explained.

Theories about *Buchnera* genome reduction usually balance between adaptive and degenerative perspective, althought genomic drift seems more plausive in this case. The degenerative theory predicts that genome reduction was caused by deleterious mutations that

occurred by drift. This hypothesis is supported by the lack of repair genes in *Buchnera* genomes, although no explaination is provided on why repair genes were initially lost (van HAM et al., 2003). On the other hand, the adaptive theory predicts that genes were lost based on their function and on the provisioning of these functions by the host metabolic pathways (MORAN, MCLAUGHLIN, SOREK, 2009).

Finally, a duplication of the tRNa-Leu codon GAG gene was found only in *Buchnera BAc*. Duplication of such gene may one consider the need for higher amounts of leucine production in such aphid-symbiont system. Leucine is an essential amino acid and has been shown to increase the weight and prolong the survival of pea aphids when added to the diet (SRIVASTAVA; AUCLAIR, 1975), but leucine can also participate in several physiological processes, serving as a source of proprionate for juvenile hormone production (BRINDLE et al., 1987), control insect reproduction by affecting vitelogenin gene expression (ATTARDO et al., 2006; SMYKAL; RAIKHEL, 2015), and serve as an important activator of the nutrient sensing TOR pathway (COLOMBANI et al., 2013; MIYAMOTO; WRIGHT; AMREIN, 2013), among others functions. However, understanding the needs of the oligophagous aphid *A. citricidus* for larger supplies of this essential amino acid by the oligophagous aphid *A. citricidus* requires further physiological investigations.

In conclusion, the draft genomes of *B. aphidicola* associated with *A. citricidus* and *A. aurantii* indicated their genome content is highly similar to other *Buchnera* genomes available. As both genomes are incomplete, it is hard to provide any in depth discussion on the functional role of the set of genes that were exclusively predicted in one or other strain of *Buchnera* we senquenced in comparison to the feeding strategies of their host aphids, oligophagy vs. polyphagy.

3.5 Conclusions

- Predicted coding sequences from *Buchnera aphidicola* associated with *Aphis (Toxoptera) citricidus* (strain *BAc*) and *Aphis (Toxoptera) aurantii* (strain *BAa*) are similar to other *Buchnera* genomes;
- *B. aphidicola BAc* genome differs from *Buchnera BAa* by carrying a functional riboflavin kinase gene, a DNA helicase and a duplication in the tRNA-Leu gene;
- The draft genome of *B. aphidicola BAc* is the largest *Buchnera* genome sequenced so far.

References

ATTARDO, G.M.; HANSEN, I.A.; SHIAO, S.H.; RAIKHEL, A.S. Identification of two cationic amino acid transporters required for nutritional signaling during mosquito reproduction. **Journal of Experimental Biology**, Cambridge,v. 209, p. 3071-3078, 2006.

BANKEVICH, A.; NURK, S.; ANTIPOV, D.; GUREVICH, A.A.; DVORKIN, M.; KULIKOV, A.S.; LESIN, V.M.; NIKOLENKO, S.I.; PHAM, S.; PRIJIBELSKI, A.D.; PYSHKIN, A.V.; SIROTKIN, A.V.; VYAHHI, N.; TESLER, G.; ALEKSEYEV, M.A.; PEVZNER, P.A. SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. **Journal of Computational Biology**, New Rochelle, v. 19, n. 5, p. 455-477, 2012.

BAUMANN, P. Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. **Annual Review of Microbiology**, Palo Alto, v. 59, p. 155-189, 2005.

BHATTACHARYYA, B.; KECK, J.L. Grip it and rip it: Structural mechanisms of DNA helicase substrate binding and unwinding. **Protein Science,** Hoboken, v. 23, p. 1498-1507, 2014.

BRINDLE, F.C.B.; TSAI, L.W.; REUTER, C.C.; SCHOOLEY, D.A. Sources of proprionate for the biogenesis of ethyl-branched insect juvenile hormones: role of isoleucine and valine. Proceedings of the National Academy of Sciences of the United States of America, v. 84, p. 7906-7910, 1987.

CARVER, M. The black citrus aphids, *Toxoptera citricidus* (Kirkaldy) and *T. aurantii* (boyer de fonscolombe) (Homoptera:Aaphididae). Australian Journal of Entomology, Yarra, v. 17, n. 3, p. 263-270, 1978.

CASSONE, B.J.; WENGER, J.A.; MICHEL, A.P. Whole Genome Sequence of the Soybean Aphid Endosymbiont *Buchnera aphidicola* and Genetic Differentiation among Biotype-Specific Strains. **Journal of Genomics**, Sydney, v. 3, p. 85-94, 2015.

CHÈNE, P. PcrA/UvrD/Rep DNA helicases in bacterial genomes. Biochemical Systematics and Ecology, Oxford, v. 36, p. 101-109, 2008.

CLCbio. **CLC Genomics Workbench v.8.5.1.** Aarhus, 2015. Available at: www.clcbio.com>.

COLOMBANI, J.; RAISIN, S.; PANTALACCI, S.; RADIMERSKI, T.; MONTAGNE, J.; LÉOPOLD, P. A nutrient sensor mechanism controls *Drosophila* growth. **Cell**, Cambridge, v. 114, p. 739-749, 2013.

DEGNAN, P.H.; OCHMAN, H.; MORAN, N.A. Sequence Conservation and Functional Constraint on Intergenic Spacers in Reduced Genomes of the Obligate Symbiont *Buchnera*. **Plos Genetics**, San Franscisco, v. 7, n. 9, p. e1002252, 2011.

DOUGLAS, A.E. Nutritional interactions in insect-microbial symbioses: Aphids and their symbiotic bacteria *Buchnera*. **Annual Review of Entomology**, Palo Alto, v. 43, p. 17-37, 1998.

_____. The microbial dimension in insect nutritional ecology. **Functional Ecology**, Oxford, v. 23, n. 1, p. 38-47, 2009.

FERRARI, J.H.; GODFRAY, C.J.; FAULCONBRIDGE, A.S.; PRIOR, K.; VIA, S. Population differentiation and genetic variation in host choice among pea aphids from eight host plant genera. **Evolution**, West Sussex, v. 60, n. 8, p. 1574-1584, 2006.

_____. Population genetic structure and secondary symbionts in host-associated populations of the pea aphid complex. **Evolution**, West Sussex, v. 66, n. 2, p. 375-390, 2012.

GAUTHIER, J-P.; OUTREMAN, Y.; MIEUZET, L.; SIMON, J-C. Bacterial Communities Associated with Host-Adapted Populations of Pea Aphids Revealed by Deep Sequencing of 16S Ribosomal DNA. **Plos One**, San Francisco, v. 10, n. 3, p. e0120664, 2015.

GOSALBES, M.J.; LATORRE, A.; LAMELAS, A.; MOYA, A. Genomics of intracellular symbionts in insects. **International Journal of Medical Microbiology**, Muenchen,v. 300, n. 5, p. 271-278, 2010.

GUREVICH, A.; SAVELIEV, V.; VYAHHI, N.; TESLER, G. QUAST: quality assessment tool for genome assemblies. **Bioinformatics**, Oxford, v. 29, n. 8, p. 1072-1075, 2013.

HALBERT, S. E.; BROWN, L. G. **Brown Citrus Aphid, *Toxoptera citricida* (Kirkaldy) (Insecta: Hemiptera: Aphididae)**. Gainesville: University of Florida IFAS Extension, 2011.6 p. (EENY,007).

HANSEN, A.K.; MORAN, N.A. The impact of microbial symbionts on host plant utilization by herbivorous insects. **Molecular Ecology**, Oxford, v. 23, n. 6, p. 1473-1496, 2014.

_____. Aphid genome expression reveals host–symbiont cooperation in the production of amino acids. **Proceedings of the national academy of science of the united States of America**, v.108, n.7, p. 2849-2854, 2011.

HENRY, L.M.; PECCOUD, J.; SIMON, J.C.; HADFIELD, J.D.; MAIDEN, M.J.; FERRARI, J.; GODFRAY, H.C. Horizontally transmitted symbionts and host colonization of ecological niches. **Current biology**, Cambridge, v. 23, n. 17, p. 1713-7, 2013.

JIANG, Z.; JONES, D.H.; KHURI, S.; TSINOREMAS, N.F.; WYSS, T.; JANDER, G.; WILSON, A.C.C. Comparative analysis of genome sequences from four strains of the *Buchnera aphidicola* Mp endosymbion of the green peach aphid, *Myzus persicae*. **Bmc Genomics**, San Francisco, v. 14, 2013. Available at: < DOI: 10.1186/1471-2164-14-917>.

KOGA, R.; TSUCHIDA, T.; FUKATSU, T. Changing partners in an obligate symbiosis: a facultative endosymbiont can compensate for loss of the essential endosymbiont *Buchnera* in an aphid. **Proceedings of the Royal Society B-Biological Sciences**, London, v. 270, n. 1533, p. 2543-2550, 2003.

LAMELAS, A.; JOSE GOSALBES, M.; MOYA, A.; LATORRE, A. New Clues about the Evolutionary History of Metabolic Losses in Bacterial Endosymbionts, Provided by the Genome of *Buchnera aphidicola* from the Aphid *Cinara tujafilina*. **Applied and Environmental Microbiology,** Washington, v. 77, n. 13, p. 4446-4454, 2011.

LEONARDO, T.E.; MUIRU, G.T. Facultative symbionts are associated with host plant specialization in pea aphid populations. **Proceedings of the Royal Society B-Biological Sciences**, London, v. 270, p. S209-S212, 2003.

MCLEAN, A.H.; VAN ASCH, M.; FERRARI, J.; GODFRAY, H.C. Effects of bacterial secondary symbionts on host plant use in pea aphids. **Proceedings of the Royal Society B-Biological Sciences**, London, v. 278, n. 1706, p. 760-766, 2011.

MIYAMOTO, T.; WRIGHT, G.; AMREIN, H. Nutrient sensors. **Current Biology**, Cambridge, v. 9, p.369-373, 2013.

MORAN, N.A.; DEGNAN, P.H. Functional genomics of *Buchnera* and the ecology of aphid hosts. **Molecular Ecology**, Oxford, v. 15, n. 5, p. 1251-1261, 2006.

MORAN, N.A.; MCLAUGHLIN, H.J.; SOREK, R. The Dynamics and Time Scale of Ongoing Genomic Erosion in Symbiotic Bacteria. **Science**, Washington, v. 323, n. 5912, p. 379-382, 2009.

PEREZ-BROCAL, V.; GIL, R.; RAMOS, S.; LAMELAS, A.; POSTIGO, M.; MANUEL MICHELENA, J.; SILVA, F.J.; MOYA, A.; LATORRE, A. A small microbial genome: The end of a long symbiotic relationship? **Science**, Washington, v. 314, n. 5797, p. 312-313, 2006.

PETIT, M.A.; EHRLICH, D. Essential bacterial helicases that counter act the toxicity of recombination proteins. **The EMBO Journal**, West Sussex, v. 21, p. 3137-3147, 2002.

SAMBROOK, J.; RUSSELL, D. W. **Molecular cloning: a laboratory manual.** 3rd edition. Cold Spring Harbor: Cold Spring Harbor Laboratoty Press, 2001. 999p.

SHIGENOBU, S.; WATANABE, H.; HATTORI, M.; SAKAKI, Y.; ISHIKAWA, H. Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp APS. **Nature**, London, v. 407, n. 6800, p. 81-86, 2000.

SMYKAL, V.; RAIKHEL, A.S. Nutritional control of insect reproduction. **Current Opnion in Insect Science,** Amsterdam, v. 11, p. 31-38, 2015.

SRIVASTAVA, P.N.; AUCLAIR, J.L. Rôle of single amino acids in phasgostimulation, growth, and survival of *Acyrthosiphon pisum*. **Journal of Insect Physiology**, Oxford, v. 21, p. 1865-1871, 1975.

TAMAS, I.; KLASSON, L.; CANBACK, B.; NASLUND, A.K.; ERIKSSON, A.S.; WERNEGREEN, J.J.; SANDSTROM, J.P.; MORAN, N.A.; ANDERSSON, S.G.E. 50 million years of genomic stasis in endosymbiotic bacteria. **Science**, Washington, v. 296, n. 5577, p. 2376-2379, 2002.

VAN HAM, R.C.H.J.; KAMERBEEK, J.; PALACIOS, C.; RAUSELL, C.; ABASCAL, F.; BASTOLLA, U.; FERNANDEZ, J.M.; JIMENEZ, L.; POSTIGO, M.; SILVA, F.J.; TAMMAMES, J.; VIGUERA, E.; LATORRE, A.; VALENCIA, A.; MORAN, F.; MOYA, A. Reductive genome evolution in *Buchnera aphidicola*. **Proceedings of the National Academy of Sciences of the United States of America**, Washington, v. 100, n. 2, p. 581-586, 2003.

VOGEL, K.J.; MORAN, N.A. Sources of variation in dietary requirements in an obligate nutritional symbiosis. **Proceedings of the Royal Society B-Biological Sciences**, London, v. 278, n. 1702, p. 115-121, 2011.

VON DOHLEN, C.D.; MORAN, N.A. Molecular data support a rapid radiation of aphids in the Cretaceous and multiple origins of host alternation. **Biological Journal of the Linnean Society**, West Sussex, v. 71, n. 4, p. 689-717, 2000.

WERNEGREEN, J. J. Genome evolution in bacterial endosymbionts of insects. **Nature Reviews Genetics**, Palo Alto, v. 3, n. 11, p. 850-861, 2002.

WILKINSON, T.L.; FUKATSU, T.; ISHIKAWA, H. Transmission of symbiotic bacteria *Buchnera* to parthenogenetic embryos in the aphid *Acyrthosiphon pisum* (Hemiptera : Aphidoidea). **Arthropod Structure & Development**, Oxford, v. 32, n. 2-3, p. 241-245, 2003.

4 SYMBIONT DIVERSITY OF *Aphis (Toxoptera) citricidus* (HEMIPTERA: APHIDIDAE) AS INFLUENCED BY HOST PLANTS

Abstract

Aphids are well known for their association with endosymbiont bacteria. Almost all aphids harbor *Buchnera aphidicola* as an obligate symbiont and several other bacteria as facultative symbionts. Associations of facultative symbionts and aphids are quite variable in terms of diversity and prevalence across aphid species. Facultative symbionts can have a major impact on aphid bioecological traits. A number of factors shape the outcome of the facultative symbiont-aphid association, including aphid clone, bacterial genotype, geography, and host plant association. The effects of host plant on aphid-facultative symbiont associations are the least understood. We performed deep sequencing of the bacterial community associated with field populations of the oligophagous aphid *Aphis (Toxoptera) citricidus* (Kirkaldy) collected from different host plants. We demonstrate that *i) A. citricidus* has low symbiont diversity, *ii)* symbiont diversity is affected by host plant, and *iii)* host plants affect the relative abundance of the obligate symbiont *Buchnera* and an unknown genus of Enterobacteriaceae.

Keywords: Bacterial community; Food source; Illumina; Metagenomics; Symbiont

4.1 Introduction

A number of insect groups are known to associate with endosymbiotic bacteria, with aphids providing some of the best known examples of such interactions. Almost all aphids are infected with the obligate nutritional symbiont *Buchnera aphidicola*, and several aphid species also harbor facultative symbionts in coexistence with *Buchnera* (MORAN; MCCUTCHEON; NAKABACHI, 2008). *Buchnera* is fundamental for aphid development, as it is responsible for biosynthesis of essential amino acids that are not sufficiently abundant in the aphid food source (phloem) (DOUGLAS, 1998). Though quite common among aphids, the role of secondary symbionts in aphid biology remains poorly understood in the majority of cases, particularly as regards their contribution towards aphid nutritional ecology (DOUGLAS, 2009; HANSEN; MORAN, 2014).

Hamiltonella defensa, Serratia symbiotica and *Regiella insecticola* are facultative symbionts commonly associated with aphids. *H. defensa* is known to confer protection against a parasitoid through an associated bacteriophage (OLIVER et al., 2003; OLIVER; MORAN; HUNTER, 2005; OLIVER et al., 2008; OLIVER et al., 2012). *S. symbiotica* increases protection against parasitoids and fungi and heat stress (MONTLLOR; MAXMEN; PURCELL, 2002; OLIVER et al., 2003), while *R. insecticola* protects only against fungal pathogens (SCARBOROUGH; FERRARRI; GODFRAY, 2005). *Spiroplasma, Rickettsia* and

Rickettsiella are not as commonly associated with aphids as the aforementioned species, but they reportedly increase aphid resistance to fungal infections (SU; XHOU; ZHANG, 2013; ŁUKASIK et al., 2013). In addition, *Spiroplasma* has been shown to influence aphid reproduction, and *Rickettsiella* has been shown to regulate aphid body coloration (TSUCHIDA et al., 2010; TSUCHIDA et al., 2014).

Aphid facultative symbionts were identified by amplification of the 16S rRNA gene from bacteria associated with various aphid species (FUKATSU et al., 2001; TSUCHIDA et al., 2002; HAYNES et al., 2003; BURKE et al., 2009; CLARK et al., 2012; SEVIM; CELEBI; SEVIM, 2012; ŁUKASIK et al., 2013; BRADY et al., 2014). The pea aphid *Acyrthosiphon pisum*¸ the most studied aphid, harbors at least eight facultative symbionts, either singly or in multiple infections (FERRARI et al., 2012; RUSSELL et al., 2013).

Diversity and occurrence of facultative bacterial symbionts can differ across aphid species/populations (BRADY et al., 2014). While the frequency of facultative symbiont infection is similar in *Acyrthosiphon pisum* (Harris), *Microlophium carnosum* (Buckton), and *Sitobion avenae* (Fabricius), facultative symbionts are nearly inexistent in other aphid species (BRADY et al., 2014). A number of factors influence the diversity of symbionts associated with a particular host or the infection rate at which they occur; some factors known to influence the aphid-symbiont association include host and symbiont genotypes, host plant, geographical niche and the outcome of the host-symbiont interaction (HENRY et al., 2013).

The role of aphid host plants on facultative symbiont diversity and occurrence remains poorly known. Surveys of facultative symbionts clearly show that particular species are strongly associated with aphids feeding on certain food sources, but it remains unclear whether these patterns reflect the role of these symbionts in host plant use or are a result of simple historical contingency, as studies focusing on these issues have been inconclusive (MCLEAN et al., 2011). To date, the effects of host plant on aphid symbiont diversity have been investigated only for polyphagous aphids, particularly *A. pisum*. However, even for this model aphid species, several experiments that either removed or introduced symbionts have yielded controversial results (VIA, 1991; LEONARDO; MUIRU, 2003; LEONARDO, 2004; TSUCHIDA; KOGA; FUKATSU, 2004; MCLEAN et al., 2011; BRADY; WHITE, 2013; BRADY et al., 2014), leaving the effects of host plant on facultative symbiont diversity open to further investigation.

Thus, we provide additional data on the influence of host plant association on aphid symbiont diversity by screening the bacterial community of *Aphis (Toxoptera) citricidus* (Kirkaldy), an oligophagous aphid species naturally associated with species of *Citrus*. Existing records related to other host plants seem to be incidental, as these plants are not suitable for

aphid development (MICHAUD, 1998). Our data provide a basis for future investigations into the effects of oligophagy/polyphagy on aphid – facultative symbiont associations and into the role of symbionts on aphid multitrophic interactions. We demonstrate that *A. citricidus* harbors very low symbiont diversity as compared to polyphagous aphids, and that symbiont diversity and abundance are affected by host plant.

4.2 Material and Methods

4.2.1 Insect collection

Aphids were sampled from species of *Citrus* (Table 4.1), specimens were screened for parasitization, and only adult specimens of *A. citricidus* were selected after species identification (HALBERT; BROWN, 1996). Specimens were washed twice with absolute ethanol, then fixed in clean absolute ethanol for subsequent extraction of genomic DNA.

4.2.2 DNA extraction

Specimens were removed from ethanol and air-dried at room temperature. Genomic DNA from each individual was extracted using the salt extraction protocol (SUNNUCKS et al., 1997). The DNA pellets were washed with ethanol, air-dried, re-suspended in 20 µL of autoclaved Milli-Q water, and stored at -20°C until further analysis. DNA integrity was assessed by electrophoresis on a 1% agarose gel slab containing 0.5 µg/mL ethidium bromide in Tris-acetate-EDTA buffer (40 mM Tris-acetate, 1 mM EDTA; pH 7.2) at 5 V/cm, and visualized on a UV transilluminator.

Table 4.1 Host plant and localities of *Aphis (Toxoptera) citricidus* populations.

Host plant	Localities	Latitude	Longitude	Collection date
Citrus sinensis	Piracicaba – SP	22 42' 30"S	47 38' 30"W	28.II.2012
Citrus paradisi x Poncirus trifoliata	Cordeirópolis – SP	22°27'39"S	47°24'4"W	10.IV.2012
Poncirus trifoliata Limeira	Cordeirópolis – SP	22°27'39"S	47°24'4"W	10.IV.2012
Citrus latifólia	São José do Cedro - SC	26° 27' 18"S	53° 29' 39"W	16.VIII.2013
Citrus deliciosa Tenore	São Miguel do Oeste - SC	26° 43' 31"S	53° 31' 05"W	06.VIII.2013
Citrus bergamia	São Miguel do Oeste - SC	26° 43' 31"S	53° 31' 05"W	20.VIII.2013
Citrus aurantifolia	São José do Cedro - SC	26° 27' 18"S	53° 29' 39"W	16.VIII.2013

4.2.3 Metagenomic amplification and sequencing

Pools of DNA from 10 specimens from each sampled host plant were subjected to metagenomics analysis through amplification and sequencing of the V4 region of the 16S rDNA. Polymerase chain reactions (PCR) were performed on a total volume of 25 µL containing 20 ng genomic DNA, 1x PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.5 U Taq polymerase, and 0.32 µM of each primer (16SV4F – 3'TAT GGT AAT TGT GTG CCA GCM GCC GCG 5'; 16SV4R – 3'AGT CAG TCA GCC GGA CTA CHV GGG TWT CTA AT 5'). Reactions were run with the following cycling parameters: 98°C for 2 min, 30 cycles at 98°C for 45 s, 56°C for 1 min, 72°C for 1.5 min, and a final extension at 72°C for 10 min.

Amplicons were visualized on a UV transilluminator after electrophoresis on a 1.0% agarose gel slab containing 0.5 µg/mL ethidium bromide in TAE buffer. Samples were purified with the Montage PCR kit (Millipore), following the manufacturer's instructions. The quality and quantity of the purified amplicons were determined by spectrophotometry before further use.

A second PCR reaction was performed on 50 ng of the purified amplicons obtained from each sample, using the tagged V4 16S rDNA primers available with the NEXTflex TM 16SV4 Amplicon-Seq kit (Bioo Scientific). Reactions were performed as described previously. Amplicons were purified by adding 90 µL of Agencourt® AMPure®XP (Beckman Coulter); samples were incubated for 5 min, then placed on a magnetic plate. The magnetic beads were washed twice with 70% ethanol for 30s and subsequently eluted in 40 µL of TE buffer (Tris-EDTA).

Samples were submitted to the Agricultural Biotechnology Center at the Biological Sciences Department – ESALQ/USP for sequencing. Real-time PCR quantification and library normalization were performed before sequencing (250 bp paired-end) in an Illumina MiSeq platform, following the guidelines provided with the NEXTflex TM 16SV4 Amplicon-Seq kit (Bioo Scientific).

4.2.4 Data analysis

Analyses were carried out using QIIME v. 1.8 (CAPORASO et al., 2010). Initially, forward and reverse reads were paired, and a map file with sample information was prepared and validated. Paired reads were demultiplexed and split into libraries corresponding to each

host plant.

Operational taxonomic units (OTUs) were assigned using the open-reference OTU-picking protocol, with a pre-filter of > 60% identity and limit of 97% identity. OTUs were defined at 97% sequence similarity against representative sequences from the Greengenes bacterial 16S rRNA database (v. 13_8), and representative OTUs sequences were classified using the Ribosomal Database Project (RDP) set at a minimum confidence level of 0.5. OTUs were aligned with PyNAST, and a tree was generated using FastTree. OTUs with less than 50 reads were excluded from subsequent analyses.

Alpha-diversity indices (Shannon index, ChaoI, observed OTU, and phylogenetic diversity – PD) were calculated to ensure that enough sequencing coverage had been achieved. The bacterial communities obtained for aphids from each host plant were compared using unweight and weight UniFrac beta-diversity indices. UniFrac indices were visualized with Principal Coordinates Analysis (PCoA) using 1,000 Jackknife replicates.

4.2.5 Phylogenetic analysis

Sequences from OTUs classified as unidentified genera of Anaplasmataceae and Enterobacteriaceae were recovered and subjected to phylogenetic analysis using MEGA 6 (TAMURA et al., 2013). Sequences were aligned with the ClustalW algorithm, phylogenetic analysis was inferred using Neighbor-Joining with p-distance, and bootstrap values were determined after 1000 replications.

4.3 Results

Illumina sequencing of the microbial community associated with seven natural populations of *A. citricidus* from different host plants yielded over 1,600,000 250-base long reads of the V4 region of the 16S rRNA gene. Libraries ranged from 187,542 to 287,481 paired-reads, with a mean length of 187.3 bases. Rarefaction curves indicated that the alpha-diversity indices plateaued at less than 50,000 reads sampled, showing that sequencing was adequate for estimating within-sample diversity (Figure 4.1).

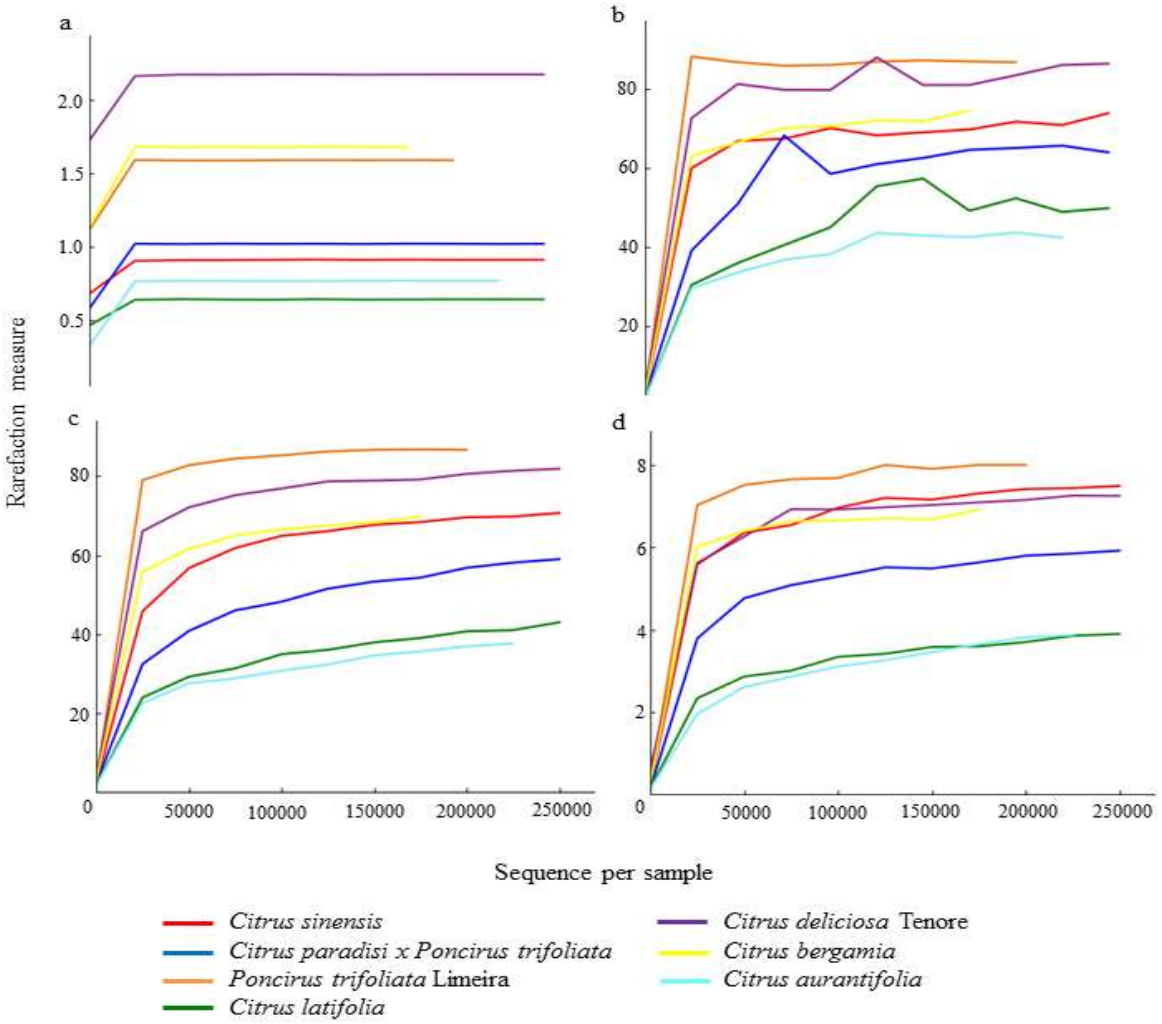


Figure 4.1 - Rarefaction curves form alpha diversity indices of *Aphis (Toxoptera) citricidus* (A) Shannon, (B) Chao I, (C) Observed species, and (D) PD tree.

OTU picking resulted in 168 OTUs that were assigned to 64 genera, distributed in 39 families, 20 orders and eight phyla of Bacteria, but only slightly less than half of the genera and families yielded known matches (Supp. tables S4.1 and S4.2). Most genera (34) were rare members of this aphid microbial community, accounting for less than 0.1% of total abundance (Supp. table S4.1). To allow for a better presentation of the data obtained, we considered further only those taxa that represented 0.1% or more of the microbial community of *A. citricidus* associated with different host plants (Supp. table S4.2).

An overall analysis of the bacterial community associated with *A. citricidus* developing on different *Citrus* species indicated Proteobacteria (99.5%) as the highly dominant phylum, followed by minor occurrences of Firmicutes (0.3%), Actinobacteria (0.1%) and Cyanobacteria (0.1%).

Cyanobacteria were represented only by members of Streptophyta; a higher diversity

was observed for the other bacterial phyla. Actinobacteria were represented by members of Corynebacteriaceae (*Corynebacterium*) and Rubrobacteraceae (*Rubrobacter*), and Firmicutes by Bacillaceae, Planococcaceae, Aerococcaceae and Streptococcaceae. Proteobacteria was the most abundant and diverse phylum. All three classes of Proteobacteria were represented: α-Proteobacteria by Brucellaceae (*Ochrobactrum*), Anaplasmataceae (*Wolbachia*) and Sphingomonadaceae (*Sphingomonas*); β-Proteobacteria were represented by Burkholderiaceae (*Burkholderia*), Comamonadaceae (*Comamonas*) and Hydrogenophilaceae (*Hydrogenophilus*); and γ-Proteobacteria were the most abundant bacteria in the microbial community of *A. citricidus* regardless of host plant, mainly represented by Enterobacteriaceae (Supp. table S4.2). Most of the Enterobacteriaceae OTUs observed belonged to the aphid primary symbiont *Buchnera aphidicola* (Table 4.2). But other genera of Enterobacteriaceae, including *Citrobacter*, *Erwinia*, *Trabulsiella* and *Serratia*, were also observed. γ-Proteobacteria were also represented by Halomonadaceae (*Halomonas*), Moraxellaceae (*Acinetobacter* and *Enhydrobacter*), Pseudomonadaceae (*Pseudomonas*), Sinobacteraceae and Xanthomonadaceae, but at much lower levels (Supp. table S4.2).

Table 4.2 - Bacterial community composition (%) of the phyla Proteobacteria of *Aphis (Toxoptera) citricidus* at different taxonomic level considering all host plant.

Phylum	%	Class	%	Order	%	Family	%	Genus	%
Proteobacteria	99.5	*α-Proteobacteria*	5.6	*Rhizobiales*	0.1				
				Rickettsiales	5.5	*Anaplasmataceae*	5.5	Cluster E	4.7
								Wolbachia	0.8
		β-Proteobacteria	0.1						
		γ-Proteobacteria	93.8	*Enterobacteriales*	93.5	*Enterobacteriaceae*	93.5	Other	0.2
								Cluster C	17.0
								Buchnera	76.2
				Oceanospirillales	0.1	*Halomonadaceae*	0.1	*Halomonas*	0.1
				Pseudomonadales	0.2	*Moraxellaceae*	0.2	*Acinetobacter*	0.2

Phylogenetic analysis of the OTUs belonging to the most abundant families, Enterobacteriaceae and Anaplasmataceae, yielded different clusters for OTUs not assigned to known genera. Rickettsiaceae (α-Proteobacteria) produced two clusters: one represented by *Wolbachia* (Anaplasmataceae), and the other (Cluster E) represented only by unidentified OTUs. Enterobacteriaceae (γ-Proteobacteria) resulted in well-supported clades for *Buchnera*, *Citrobacter*, and most of the OTUs similar to *Erwinia*. Two clades of unidentified OTUs, Clusters A and C, also had high bootstrap support, with Cluster C representing the highly abundant unidentified OTUs in the microbiota of *A. citricidus* (Figure 4.2).

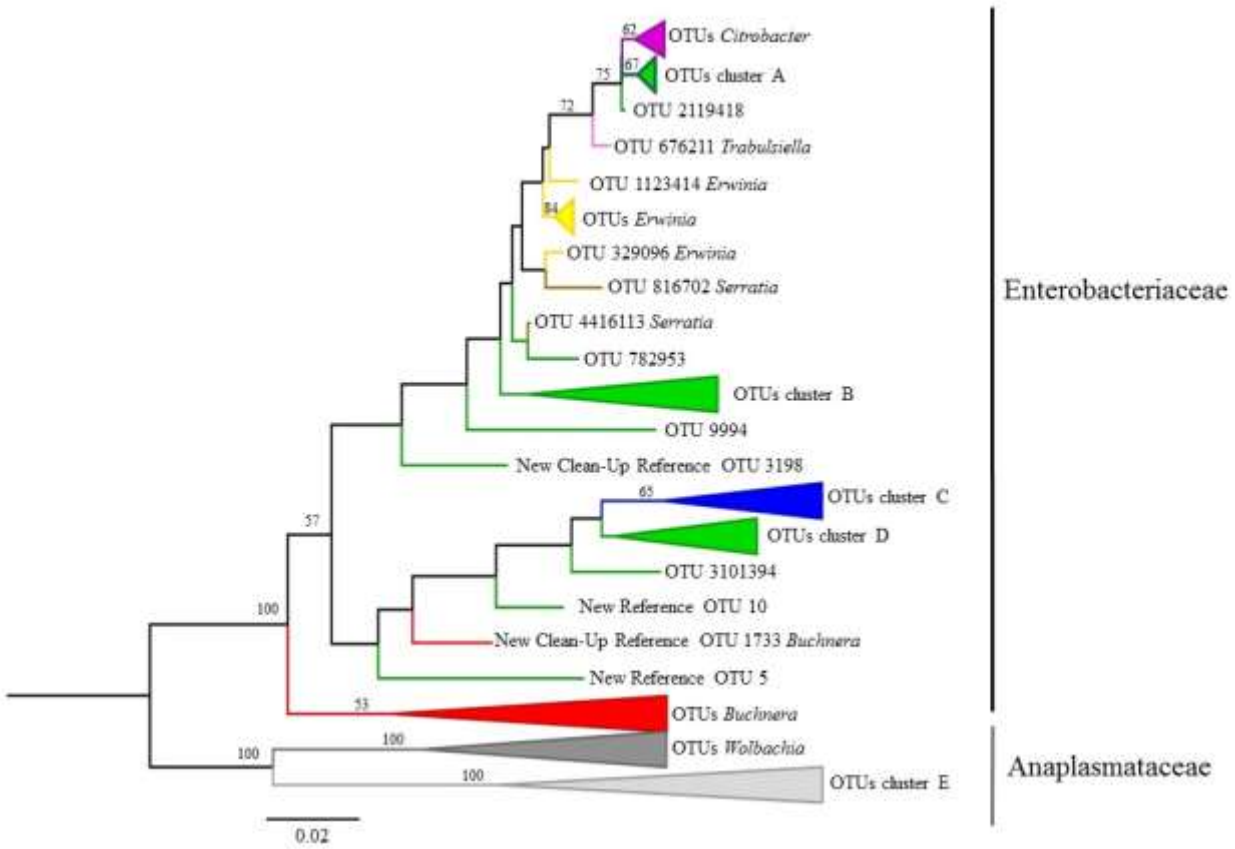


Figure 4.2 - Phylogenetic analysis of Anaplasmataceae and Enterobacteriaceae associated with *Aphis (Toxoptera) citricidus.* The evolutionary history was inferred using the Neighbor-Joining, with evolutionary distances were computed using the *p*-distance method. The percentage of replicate trees was verified with bootstrap of 1,000 replicates

Enterobacteriaceae and Anaplasmataceae were most strongly affected by host plant (Table 4.3). OTUs related to *Buchnera* and Cluster C were the only ones present in 100% of

the samples, and the relative abundance of these OTUs within the microbial community of *A. citricidus* varied according to host plant. *Buchnera* abundance was highest on *Citrus latifolia* (94.4%) and *Citrus aurantifolia* (91.7%), on which Cluster C represented less than 1%. But Cluster C abundance ranged from 10.4 to 17.2% of the microbial communities of aphids associated with *Citrus sinensis*, *Citrus paradisi x Poncirus trifoliata* and *Poncirus trifoliata*, peaking at 32-36% in aphids from *Citrus deliciosa* and *Citrus bergamia* (Table 4.3). The abundance of Cluster C was always inversely related to that of *Buchnera*. But in the microbial community of aphids from *Citrus deliciosa,* Cluster E (Anaplasmataceae) was as abundant as Cluster C (Table 4.3). Cluster E only occurred in aphids associated with *C. deliciosa,* but other Anaplasmataceae (*Wolbachia)* were detected in aphids from *C. aurantifolia* (0.1%) and *P. trifoliata* (0.2%) (Table 4.3).

Unweighted and weighted beta-diversity indices produced similar topologies for the bacterial communities associated with populations of *A. citricidus* developing on different host plants, all of them with high bootstrap values (Figure 4.3). However, the UPGMA when bacterial densities were considered (weighted UniFrac analysis) resulted in clusters that better represented the differences we observed in the microbial communities of *A. citricidus* from different host plants (Figure 4.3b, Table 4.2).

Table 4.3 - Bacterial community composition (%) of *Aphis (Toxoptera) citricidus* among different host plant

	Enterobacteriaceae			Anaplasmataceae	
Host plant	*Buchnera*	Other	Cluster C	*Wolbachia*	Cluster E
Citrus sinensis (sweet orange)	88.4	0.1	10.4	0.0	0.0
Citrus paradisi x Poncirus trifoliata (Citrange)	88.7	0.0	10.7	0.0	0.0
Poncirus trifoliata Limeira (Pomeroy)	77.6	0.6	17.2	0.2	0.0
Citrus latifolia	94.4	0.0	0.5	0.0	0.0
Citrus deliciosa Tenore (Willowleaf mandarin)	32.5	0.1	32.2	0.0	33.0
Citrus bergamia	60.4	0.1	36.6	0.0	0.0
Citrus aurantifolia (Mexican lime)	91.7	0.0	0.7	0.1	0.0

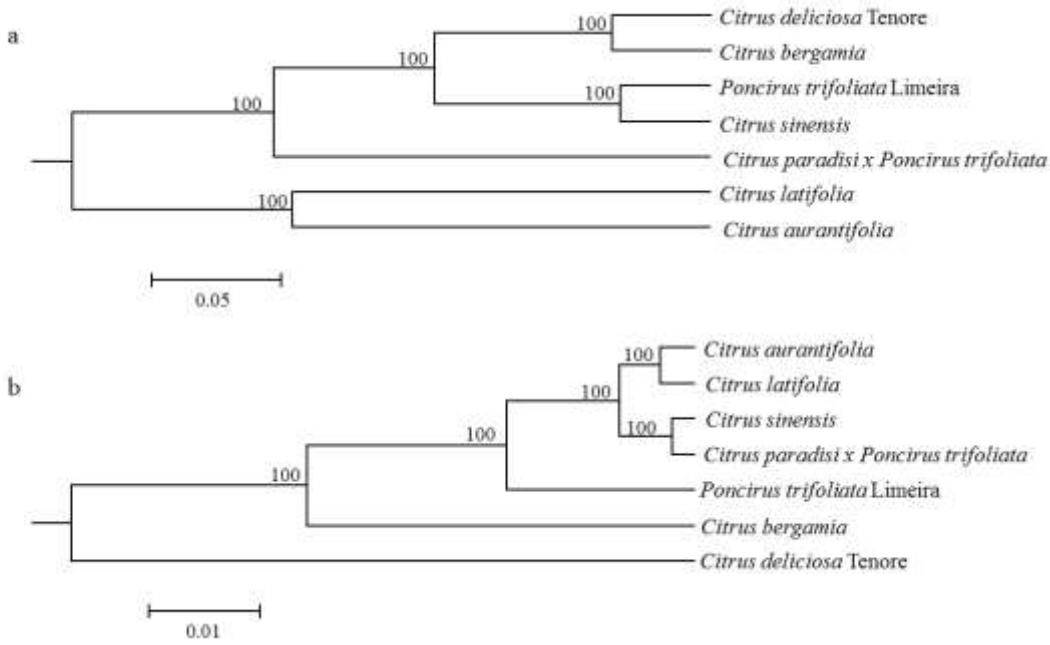


Figure 4.3 - UPGMA analysis of Unweight (a) and weight (b) UniFrac beta-diversity indices, using 1.000 Jackknife replicates, for the secondary symbionts associated with *Aphis (Toxoptera) citricidus* exploiting different host species

4.4 Discussion

We showed that host plants influence the diversity and abundance of primary and secondary symbionts of *A. citricidus*. The bacterial community associated with *A. citricidus* consisted of 168 OTUs, although most of these OTUs represented less than 0.1% of total observed diversity. Enterobacteriaceae and Anaplasmataceae are the main representatives of the bacterial community of *A. citricidus*. OTUs similar to *Buchnera* and to Enterobacteriaceae Cluster C were the most abundant in almost all samples. In aphids from *C. deliciosa*, Cluster C shared the bacterial community with *Buchnera* and with Cluster E of Anaplasmataceae in equal amounts. Apart from *Buchnera*, Cluster C was the only secondary symbiont associated with aphids from all samples to be affected by host plant use.

Occurrence of intra- and extracellular bacterial symbionts can vary considerably within and between aphid species (TSUCHIDA et al., 2002; HAYNES et al., 2003; RUSSELL et al., 2003; FERRARI et al., 2004; JONES et al., 2011), but the low diversity of symbionts associated with *A. citricidus* is particularly interesting due to the lack of association with the most common aphid-associated secondary symbionts, namely *H. defensa* and *R. insecticola* (RUSSELL; MORAN, 2006).

Secondary symbionts influence a number of bioecological traits of aphids, as they provide protection against natural enemies (OLIVER et al., 2003; OLIVER; MORAN; HUNTER, 2005; GUAY et al., 2009; NYABUGA et al., 2010; ŁUKASIK et al., 2013) and heat shock (MONTLLOR; MAXMEN; PURCELL, 2002; RUSSELL; MORAN, 2006), in addition to their effect on aphid nutrition (TSUCHIDA; KOGA; FUKATSU, 2004; MCLEAN et al., 2011) and reproduction (LEONARDO; MONDOR, 2006). These associations directly affect the trophic interactions of aphids and their overall fitness. One may wonder whether the lack of such secondary interactions in *A. citricidus* might be due to the restricted number of host plants associated with this species, and/or whether the usual contribution of secondary symbionts to aphid phenotypic variability could be provided by mechanisms associated with the primary aphid symbiont.

Notwithstanding, some secondary symbionts were identified in the analyzed populations of *A. citricidus*, among them the α-proteobacterium *Wolbachia*. Symbiotic *Wolbachia* are widely found among insects (WERREN; BALDO; CLARCK, 2008), but seldom reported in aphids, including *A. citricidus* (JEYAPRAKASH; HOY, 2000; GOMEZ-VALERO et al., 2004; WANG et al., 2009). But the low association of *Wolbachia* with natural populations of aphids is argued to be resultant of the low titers of this bacterium in aphids (AUGUSTINOS et al., 2011). In addition to its best-known effects on host reproduction, *Wolbachia* has been reported to influence several aspects of host physiology (WERREN; BLADO; CLARCK, 2008). However, no reports are available on the role of *Wolbachia* in known aphid hosts (AUGUSTINOS et al., 2011).

Our results also showed that the microbial community associated with *A. citricidus* is affected differently according to host plant. Host plant effects on microbial communities of aphids have already been reported, and some studies have indicated a role of secondary symbionts in widening the diet breadth of aphids (LEONARDO; MUIRU, 2003; MCLEAN et al., 2011; BRADY; WHITE, 2013), although some of the reported data is controversial (LEONARDO, 2004). However, secondary symbionts may reduce or widen the diet breadth of their host aphids depending on host genotype, as recently demonstrated for *Arsenophonus* and *Aphis craccivora* (WAGNER et al., 2015). The microbial community of aphids can also be shaped by and related with host-race specialization, as indicated by a broad analysis of host-adapted pea aphid lines (GAUTHIER et al., 2015). Yet the effects of host plants on aphid microbial communities also depend on bioecological factors (NAJAR-RODRIGUEZ et al., 2009; TOJU; FUKATSU, 2011; JONES et al., 2011; ZYTYNSKA; WEISSER, 2016). Our data suggest that differences in the microbial community of *A. citricidus* may occur even in the

absence of host plant-adapted races.

The growing body of studies on the role played by bacterial endosymbionts in host plant use by insects basically focuses on *Acyrthosiphon pisum* and other polyphagous aphids (SU et al., 2013), and little information is available for monophagous and/or oligophagous aphid species (ZYTYNSKA; WEISSER, 2016). *A. citricidus* is primarily associated with plants in the *Citrus* genus (HALBERT; BROWN, 1996; MICHAUD, 1998), although some other Rutaceae and Malvaceae (cotton) host plants have been reported, always under laboratory conditions (TSAI, 1998; MICHAUD, 2004). Nevertheless, *A. citricidus* survival and/or fitness on these host plants is severely reduced when compared to its usual *Citrus* hosts (TSAI, 1998; MICHAUD, 2004).

Citrus taxonomy is quite complex, and species definition has been problematic due to the close interaction of humans with these plants, which led to the production of various hybrids. But a number of studies have used different molecular approaches in the attempt to elucidate the phylogenetic relationships among commonly grown commercial species and other closely-related genera (FEDERICI et al., 1998; NICOLOSI et al., 2000; BARKLEY et al., 2006; PANG; HU; DENG, 2007).

Recent data, including complete chloroplast genome information, have yielded a very comprehensive phylogenetic analysis of wild and commercial *Citrus* and related species, with well-defined clusters based on chloroplast type (Pummelo, Micrantha, Mangshan, Mandarin, apeda, *Fortunella*, *Poncirus*, citron, *Microcitrus*, *Eremocitrus*, and *Severinia*) (CARBONELL-CABALLERO et al., 2015). Comparing the phylogenetic information available for *Citrus* with the data on *A. citricidus* fitness on different host plants, we can easily observe that the reduction in intrinsic rate of natural increase (*rm*) reported by Tsai (1998) for different host plants reflects the phylogenetic position of these plants. The best *Citrus* hosts belong to the clade represented by the Pummelo type of chloroplast (e.g., *Citrus paradisi* and *C. aurantium*), followed by the Micrantha (*Citrus aurantifolia*) and Mandarin (*Citrus jambhiri*) chloroplast types. Citrus with *Fortunella* (e.g., *Citrofortunella microcarpa*) and *Severinia* (e.g., *Severinia buxifolia*) type chloroplasts were among the least suitable for *A. citricidus*, as reported by Tsai (1998).

Comparing this information with our present data on the microbial community of *A. citricidus*, we observed a microbial community consisting almost exclusively of *Buchnera* in aphids developing on *C. aurantifolia* and *C. latifolia*, both limes and both carrying the Pummelo chloroplast type, the most suitable host plants as tested by Tsai (1998). *Citrus aurantifolia* was included in the study by Carbonell-Caballero (2015), but assignment of *C. latifolia* to the Pummelo chloroplast group was based on the phylogeny reported by Froelicher et al. (2011),

which resolved *C. sinensis* (a Pummelo chloroplast type in Carbonell-Caballero (2015) in the same subclade with *C. latifolia*. But in *C. sinensis*, nearly 10% of the aphid microbiota was already represented by Cluster C of Enterobacteriaceae, similarly to the microbiota of *A. citricidus* from the hybrid *Citrus paradisi x Poncirus trifoliata,* whose source plants carry two distinct chloroplast types, Pummelo and *Poncirus*.

On the other hand, *C. bergamia,* which clustered with *C. sinensis, C. paradisi* and *C. aurantium* (FROELICHER et al., 2011), all of them reported to carry the Pummelo chloroplast type (CARBONELL-CABALLERO et al., 2015), was the host plant with the second highest abundance of Cluster C. The cluster formed by species assigned to the Pummelo chloroplast type by Carbonell-Caballero et al. (2015) consists of three distinct subclades. One includes species of limes (*C. aurantium* and *Citrus limon*), one consists of sweet orange only (*C. sinensis*), and one includes five different cultivars of pummelos, including *C. paradisi* (CARBONELL-CABALLERO et al., 2015). These results indicate the existence of some variability within the Pummelo chloroplast type, and although RFLP data indicated *C. bergamia* as a hybrid of *Citrus limetta* and *C. aurantium* (FEDERICI et al., 2000), we do not believe the available information ensures a correct placement of *C. bergamia* within this clade.

It was clear to us that the greater the phylogenetic distance between a host plant and the lime subclade of the Pummelo chloroplast type, the lower would be the relative abundance of *Buchnera* and the more abundant the Cluster C bacteria. Cluster C abundance was higher in aphids from a *Poncirus* chloroplast type plant (*Poncirus trifoliata*, 17.6%), and peak abundance of Cluster C was observed in aphids reared from a Mandarin type host plant, *Citrus deliciosa*, although in this case the microbial community also showed a high abundance of an unknown Anaplasmataceae (Cluster E). Citrus with Mandarin type chloroplasts were among the host plants that yielded intermediate results for *A. citricidus* (TSAI, 1998).

Therefore, a comparison of our microbial diversity data with the existing data on *A. citricidus* fitness based on *Citrus* chloroplast type strongly suggests that the microbial community of *A. citricidus* may respond to host plant suitability, with a decrease in relative abundance of *Buchnera* as host suitability decreases. The association of Enterobacteriaceae Cluster C with all natural populations sampled in our study and its changing abundance in response to changes in host plant – with abundance increasing as *A. citricidus* moves from more suitable (Pummelo and Micrantha chloroplast types) to less suitable host plants (Mandarin and *Poncirus* chloroplast types) – suggest that the Cluster C symbiont may play key roles in helping *A. citricidus* exploit less suitable host plants by complementing the contribution provided by *Buchnera*. Moreover, the frequency of occurrence of Cluster C and *Buchnera* and their

interdependent responses to host plant may suggest Cluster C as an additional primary symbiont of *A. citricidus.*

Symbiont complementarity or even replacement have already been reported for a number of species, as the process of genome reduction faced by all bacterial symbionts as they established ancient interactions may lead to a complete loss of several important pathways (GOSALBES et al., 2010). In aphids, *S. symbiotica* and *Rickettsia* are known to suppress *Buchnera* titers, negatively affecting host fitness (KOGA; TSUCHIDA; FUKATSU, 2003; SAKURAI et al., 2005). *S. symbiotica* has also been shown to provide positive effects by compensating for the removal of the essential symbiont *Buchnera* (KOGA; TSUCHIDA, FUKATSU, 2003).

In the microbial community of *A. citricidus*, we observed a process of dysbiosis associated with most of the host plants assessed in the present study. Dysbiosis, represented by changes in microbial balance not related to its diversity, has been deeply investigated in human and mice gut microbiota in response to stress conditions, commonly associated with illnesses (MYERS, 2004). Recently, there have also been increasing reports of dysbiosis in microbial communities associated with insects under stressful conditions, including nutritional stress (HAMDI et al., 2011; MINARD; MAVINGUÉ; MORO, 2013; MA et al., 2015). Such changes have also been observed in aphids, including alterations in *Buchnera* densities in *Aphis glycines* as a consequence of feeding on diseased host plants, for example (CASSONE et al., 2015).

Nevertheless, a broad screening of *A. citricidus* symbionts across *Citrus* host plants, coupled with a chemical analysis of the phloem nutrients of *Citrus* species/varieties, would aid in better understanding the role of host plants in shaping the microbial symbiont community of an aphid with a narrow host range. Likewise, further research into the full identification of Enterobacteriaceae Cluster C associated with *A. citricidus*, natural infection rates within and among aphid populations, and the effects of nutritional sources on abundance of Cluster C under controlled conditions would be required for a deeper understanding of the role played by this symbiont in *A. citricidus.*

4.5 Conclusions

- *Aphis (Toxoptera) citricidus* has low symbiont diversity, as bacterial community associated with *A. citricidus* was represented mainly represented for Enterobacteriaceae and Anaplasmataceae;

- Symbiont diversity is affected by host plant;
- Host plants affect the relative abundance of the obligatory symbiont *Buchnera* and an unknown genus of Enterobacteriaceae.

References

AUGUSTINOS, A.A.; SANTOS-GARCIA, D.; DIONYSSOPOULOU, E.; MOREIRA, M.; PAPAPANAGIOTOU, A.; SCARVELAKIS, M.; DOUDOUMIS, V.; RAMOS, S.; AGUIAR, A.F.; BORGES, P.A.V.; KHADEM, M.; LATORRE, A.; TSIAMIS, G.; BOURTZIS, K. Detection and characterization of *Wolbachia* infections in natural populations of aphids: is the hidden diversity fully unraveled? **Plos One**, San Francisco, v. 6, n. 12, p. e28695, 2011.

BARKLEY, N.A.; ROOSE, M.L.; KRUEGER, R.R.; FEDERICI, C.T. Assessing genetic diversity and population structure in a citrus germplasm collection utilizing simple sequence repeat markers (SSRs). **Theoretical and Applied Genetics**, Heidelberg, v. 112, n. 8, p. 1519-1531, 2006.

BRADY, C.M.; ASPLEN, M.K.; DESNEUX, N.; HEIMPEL, G.E.; HOPPER, K.R.; LINNEN, C.R.; OLIVER, K.M.; WULFF, J.A.; WHITE, J.A. Worldwide populations of the aphid *Aphis craccivora* are infected with diverse facultative bacterial symbionts. **Microbial Ecology**, New York, v. 67, n. 1, p. 195-204, 2014.

BRADY, C. M.; WHITE, J. A. Cowpea aphid (*Aphis craccivora*) associated with different host plants has different facultative endosymbionts. **Ecological Entomology,** London, v. 38, n. 4, p. 433-437, 2013.

BURKE, G.R.; NORMARK, B.B.; FAVRET, C.; MORAN, N.A. Evolution and diversity of facultative symbionts from the aphid subfamily Lachninae. **Applied and environmental microbiology**, Washington, v. 75, n. 16, p. 5328-5335, 2009.

CAPORASO, J. G.; KUCZYNSKI, J.; STOMBAUGH, J.; BITTINGER, K.; BUSHMAN, F.D.; COSTELLO, E.K.; FIERER, N.; PENA, A.G.; GOODRICH, J.K.; GORDON, J.I.; HUTTLEY, G.A.; KELLEY, S.K.; KNIGHTS, D.; KOENIG, J.E.; LEY, R.E.; LOZUPONE, C.A.; MCDONALD, D.; MUEGGE, B.D.; PIRRUNG, M.; REEDER, J.; SEVINSKY, J.R; TURNBAUGH, P.J.; WALTERS, W.A.; WIDMANN, J.; YATSUNENKO, T.; ZANEVELD, J.; KNIGHT, R. QIIME allows analysis of high-throughput community sequencing data. **Nature Methods**, London, v. 7, n. 5, p. 335-336, 2010.

CARBONELL-CABALLERO, J.; ALONSO, R.; IBANEZ, V.; TEROL, J.; TALON, M.; DOPAZO, J. A Phylogenetic Analysis of 34 Chloroplast Genomes Elucidates the Relationships between Wild and Domestic Species within the Genus Citrus. **Molecular Biology and Evolution**, Cary, v. 32, n. 8, p. 2015-2035, 2015.

CASSONE, B.J.; REDINBAUGH, M.G.; DORRANCE, A.E.; MICHEL, A.P. Shifts in *Buchnera aphidicola* density in soybean aphids (*Aphis glycines*) feeding on virus-infected soybean. **Insect Molecular Biology**, West Sussex, v. 24, n. 4, p. 422-431, 2015.

CLARK, E.L.; DANIELL, T.J.; WISHART, J.; HUBBARD, S.F.; KARLEY, A.J. How conserved are the bacterial communities associated with aphids? A detailed assessment of the *Brevicoryne brassicae* (Hemiptera: Aphididae) using 16S rDNA. **Environmental Entomology,** College Park, v. 41, n. 6, p. 1386-97, 2012.

DOUGLAS, A. E. Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. **Annual Review of Entomology**, Stanford, v. 43, p. 17-37, 1998.

_____. The microbial dimension in insect nutritional ecology. **Functional Ecology**, Oxford, v. 23, n. 1, p. 38-47, 2009.

FEDERICI, C.T.; FANG, D.Q.; SCORA, R.W.; ROOSE, M.L. Phylogenetic relationships within the genus *Citrus* (Rutaceae) and related genera as revealed by RFLP and RAPD analysis. **Theoretical and Applied Genetics**, Heidelberg, v. 96, n. 6-7, p. 812-822, 1998.

FEDERICI, C. T.; ROOSE, M. L.; SCORA, R. W. RFLP analysis of the origin of *Citrus bergamia*, *Citrus jambhiri*, and *Citrus limonia*. **Proceedings of the First International Symposium on Citrus Biotechnology**, Eilat, n. 535, p. 55-62, 2000.

FERRARI, J.; DARBY, A.C.; DANIELL, T.J.; GODFRAY, H.C.J.; DOUGLAS, A.E. Linking the bacterial community in pea aphids with host-plant use and natural enemy resistance. **Ecological Entomology** , London, v. 29, n. 1, p. 60-65, 2004.

FERRARI, J.; WEST, J.A.; VIA, S.; GODFRAY, H.C.J. Population genetic structure and secondary symbionts in host-associated populations of the pea aphid complex. **Evolution**, West Sussex, v. 66, n. 2, p. 375-390, 2012.

FROELICHER, Y.; MOUHAYA, W.; BASSENE, J-B.; COSTANTINO, G.; KAMIRI, M.; LURO, F.; MORILLON, R.; OLLITRAULT, P. New universal mitochondrial PCR markers reveal new information on maternal citrus phylogeny. **Tree Genetics & Genomes,** Heidelberg, v. 7, n. 1, p. 49-61, 2011.

FUKATSU, T.; TSUCHIDA, T.; NIKOH, N.; KOGA, R. Spiroplasma symbiont of the pea aphid, *Acyrthosiphon pisum* (Insecta : Homoptera). **Applied and Environmental Microbiology**, Washington, v. 67, n. 3, p. 1284-1291, 2001.

GAUTHIER, J-P.; OUTREMAN, Y.; MIEUZET, L.; SIMON, J-C. Bacterial Communities Associated with Host-Adapted Populations of Pea Aphids Revealed by Deep Sequencing of 16S Ribosomal DNA. **Plos One**, San Francisco, v. 10, n. 3, p. e0120664, 2015.

GOMEZ-VALERO, L.; SORIANO-NAVARRO, M.; PEREZ-BROCAL, V.; HEDDI, A.; MOYA, A.; GARCIA-VERDUGO, J.M.; LATORRE, A. Coexistence of *Wolbachia* with *Buchnera aphidicola* and a secondary symbiont in the aphid *Cinara cedri*. **Journal of bacteriology**, Washington, v. 186, n. 19, p. 6626-6633, 2004.

GOSALBES, M.J.; LATORRE, A.; LAMELAS, A.; MOYA, A. Genomics of intracellular symbionts in insects. **International Journal of Medical Microbiology**, Muenchen, v. 300, n. 5, p. 271-278, 2010.

GUAY, J.F.; BOUDREAULT, S.; MICHAUD, D.; CLOUTIER, C. Impact of environmental stress on aphid clonal resistance to parasitoids: Role of *Hamiltonella* defensa bacterial symbiosis in association with a new facultative symbiont of the pea aphid. **Journal of insect physiology**, Oxford, v. 55, n. 10, p. 919-26, 2009.

HALBERT, S.E.; BROWN, L.G. ***Toxoptera citricida*** **(Kirkaldy), brown citrus aphid** - identification, biology and management strategies. Gainesville, Florida Department of Agriculture and Consumer Services, Division of Plant Industry, 1996. 6p. (Entomology Circular, v. 374).

HAMDI, C.; BALLOI, A.; ESSANAA, J.; CROTTI, E.; GONELLA, E.; RADDADI, N.; RICCI, I.; BOUDABOUS, A.; BORIN, S.; MANINO, A.; BANDI, C.; ALMA, A.; DAFFONCHI, D.; CHERIF, A. Gut microbiome dysbiosis and honeybee health. **Journal of Applied Entomology**, Berlin, v. 135, n. 7, p. 524-533, 2011.

HANSEN, A.K.; MORAN, N.A. The impact of microbial symbionts on host plant utilization by herbivorous insects. **Molecular Ecology**, Oxford, v. 23, n. 6, p. 1473-1496, 2014.

HAYNES, S.; DARBY, A.C.; DANIELL, T.J.; WEBSTER, G.; VAN VEEN, F.J.; GODFRAY, H.C.; PROSSER, J.I.; DOUGLAS, A.E. Diversity of bacteria associated with natural aphid populations. **Appl Environ Microbiol**, Washington, v. 69, n. 12, p. 7216-7223, 2003.

HENRY, L.M.; PECCOUD, J.; SIMON, J.C.; HADFIELD, J.D.; MAIDEN, M.J.; FERRARI, J.; GODFRAY, H.C. Horizontally transmitted symbionts and host colonization of ecological niches. **Current biology**, Cambridge, v. 23, n. 17, p. 1713-7, 2013.

JEYAPRAKASH, A.; HOY, M. A. Long PCR improves *Wolbachia* DNA amplification: wsp sequences found in 76% of sixty-three arthropod species. **Insect Molecular Biology**, West Sussex, v. 9, n. 4, p. 393-405, 2000.

JONES, R.T.; BRESSAN, A.; GREENWELL, A.M.; FIERER, N. Bacterial communities of two parthenogenetic aphid species cocolonizing two host plants across the Hawaiian Islands. **Applied and environmental microbiology**, Washington, v. 77, n. 23, p. 8345-8349, 2011.

KOGA, R.; TSUCHIDA, T.; FUKATSU, T. Changing partners in an obligate symbiosis: a facultative endosymbiont can compensate for loss of the essential endosymbiont *Buchnera* in an aphid. **Proceedings of the Royal Society B-Biological Sciences**, London, v. 270, n. 1533, p. 2543-2550, 2003.

LEONARDO, T.E. Removal of a specialization-associated symbiont does not affect aphid fitness. **Ecology Letters**, Oxford, v. 7, n. 6, p. 461-468, 2004.

LEONARDO, T. E.; MONDOR, E. B. Symbiont modifies host life-history traits that affect gene flow. **Proceedings of the Royal Society B-Biological Sciences**, London, v. 273, n. 1590, p. 1079-84, 2006.

LEONARDO, T.E.; MUIRU, G.T. Facultative symbionts are associated with host plant specialization in pea aphid populations. **Proceedings of the Royal Society B-Biological Sciences**, London, v. 270, p. S209-S212, 2003.

ŁUKASIK, P.; VAN ASCH, M.; GUO, H.; FERRARI, J.; CHARLES, J.; GODFRAY, H. Unrelated facultative endosymbionts protect aphids against a fungal pathogen. **Ecology Letters**, West Sussex, v. 16, n. 2, p. 214-218, 2013.

MA, D.; STORELLI, G.; MITCHELL, M.; LEULIER, F. Studying host-microbiota mutualism in *Drosophila*: Harnessing the power of gnotobiotic flies. **Biomedical journal**, Munich, v. 38, n.4, p.285-293, 2015.

MCLEAN, A.H.; VAN ASCH, M.; FERRARI, J.; GODFRAY, H.C. Effects of bacterial secondary symbionts on host plant use in pea aphids. **Proceedings of the Royal Society B-Biological Sciences**, London, v. 278, n. 1706, p. 760-766, 2011.

MICHAUD, J. P. Assessment of cotton as an alternative host plant for the brown citrus aphid, *Toxoptera cytricida* (Homoptera : Aphididae). **Florida Entomologist**, Lutz, v. 87, n. 2, p. 105-111, 2004.

_____. A review of the literature on *Toxoptera citricida* (Kirkaldy) (Homoptera: Aphididae). **Florida Entomologist,** Lutz, v.3, p. 37-61, 1998.

MINARD, G.; MAVINGUI, P.; MORO, C. V. Diversity and function of bacterial microbiota in the mosquito holobiont. **Parasites & Vectors**, London, v. 6, n. 146, p. 1-12, 2013.

MONTLLOR, C.B.; MAXMEN, A.; PURCELL, A. H. Facultative bacterial endosymbionts benefit pea aphids *Acyrthosiphon pisum* under heat stress. **Ecological Entomology**, London, v. 27, n. 2, p. 189-195, 2002.

MORAN, N. A.; MCCUTCHEON, J. P.; NAKABACHI, A. Genomics and Evolution of Heritable Bacterial Symbionts. **Annual Review of Genetics**, Palo Alto, v. 42, n. 1, p. 165-190, 2008.

MYERS, S. P. The causes of intestinal dysbiosis: a review. **Alternative Medicine Review**, Napa, v. 9, n. 2, p. 180-197, 2004.

NAJAR-RODRIGUEZ, A.J.; MCGRAW, E.A.; MENSAH, R.K.; PITTMAN, G.W.; WALTER, G.H. The microbial flora of *Aphis gossypii*: Patterns across host plants and geographical space. **Journal of Invertebrate Pahtology,** Maryland Heights, v.100, p.123-126, 2009.

NICOLOSI, E.; DENG, Z.N.; GENTILE, A.; LA MALFA, S.; CONTINELLA, G.; TRIBULATO, E. Citrus phylogeny and genetic origin of important species as investigated by molecular markers. **Theoretical and Applied Genetics,** Heidelberg, v. 100, n. 8, p. 1155-1166, 2000.

NYABUGA, F.N.; OUTREMAN, Y.; SIMON, J-C.; HECKEL, D.G.; WEISSER, W.W. Effects of pea aphid secondary endosymbionts on aphid resistance and development of the aphid parasitoid *Aphidius ervi*: a correlative study. **Entomologia Experimentalis et Applicata,** West Sussex, v. 136, n. 3, p. 243-253, 2010.

OLIVER, K.M.; CAMPOS, J. MORAN, N.A.; HUNTER, M.S. Population dynamics of defensive symbionts in aphids. **Proceedings of the Royal Society B-Biological Sciences**, London, v. 275, n. 1632, p. 293-299, 2008.

OLIVER, K. M.; MORAN, N. A.; HUNTER, M. S. Variation in resistance to parasitism in aphids is due to symbionts not host genotype. **Proceedings of the National Academy of Sciences of the United States of America**, Washington, v. 102, n. 36, p. 12795-800, 2005.

OLIVER, K.M.; NOGE, K.; HUANG, E.M.; CAMPOS, J.M.; BECERRA, J.X.; HUNTER, M.S. Parasitic wasp responses to symbiont-based defense in aphids. **BMC Biology**, San Francisco, v. 10, p. 11, 2012. Available at: < DOI: 10.1186/1741-7007-10-11>.

_____. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. **Proceedings of the National Academy of Sciences of the United States of America**, Washington, v. 100, n. 4, p. 1803-1807, 2003.

PANG, X.-M.; HU, C.-G.; DENG, X.-X. Phylogenetic relationships within Citrus and its related genera as inferred from AFLP markers. **Genetic Resources and Crop Evolution**, Dordrecht, v. 54, n. 2, p. 429-436, 2007.

RUSSELL, J.A.; LATORRE, A.; SABATER-MUNOZ, B.; MOYA, A.; MORAN, N.A: Side-stepping secondary symbionts: widespread horizontal transfer across and beyond the Aphidoidea. **Molecular Ecology**, Oxford, v. 12, n. 4, p. 1061-1075, 2003.

RUSSELL, J.A.; MORAN, N.A. Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. **Proceedings of the Royal Society B-Biological Sciences**, London, v. 273, n. 1586, p. 603-610, 2006.

RUSSELL, J.A.; WELDON, S.; SMITH, A.H.; KIM, K.L.; HU, Y.; LUKASIK, P.; DOLL, S.; ANASTOPOULOS, I.; NOVIN, M.; OLIVER, K.M. Uncovering symbiont-driven genetic diversity across North American pea aphids. **Molecular Ecology**, Oxford, v. 22, n. 7, p. 2045-2059, 2013.

SAKURAI, M.; KOGA, R.; TSUCHIDA, T.; MENG, X.Y.; FUKATSU, T. Rickettsia symbiont in the pea aphid Acyrthosiphon pisum: Novel cellular tropism, effect on host fitness, and interaction with the essential symbiont Buchnera. **Applied and Environmental Microbiology**, Washington, v. 71, n. 7, p. 4069-4075, 2005.

SCARBOROUGH, C. L.; FERRARI, J.; GODFRAY, H. C. Aphid protected from pathogen by endosymbiont. **Science**, Washington, v. 310, n. 5755, p. 1781, 2005.

SEVIM, E.; CELEBI, O.; SEVIM, A. Determination of the bacterial flora as a microbial control agent of Toxoptera aurantii (Homoptera: Aphididae). **Biologia**, Berlin, v. 67, n. 2, p. 397-404, 2012.

SU, Q.; ZHOU, X.; ZHANG, Y. Symbiont-mediated functions in insect hosts. **Communicative & Integrative Biology**, New York, v. 6, n. 3, p. e23804-e23804, 2013.

SUNNUCKS, P.; DE BARRO, P.J.; LUSHAI, G.; MACLEAN, N.; HALES, D. Genetic structure of an aphid studied using microsatellites: cyclic parthenogenesis, differentiated lineages and host specialization. **Molecular Ecology**, Oxford, v. 6, n. 11, p. 1059-1073, 1997.

TAMURA, K.; STECHER, G.; PETERSON, D.; FILIPSKI, A.; KUMAR, S: MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. **Molecular Biology and Evolution**, Cary, v. 30, n. 12, p. 2725-2729, 2013.

TOJU, H.; FUKATSU, T. Diversity and infection prevalence of endosymbionts in natural populations of the chestnut weevil: relevance of local climate and host plants. **Molecular Ecology,** West Sussex, v.20, p.853-868, 2011.

TSAI, J. H. Development, survivorship, and reproduction of *Toxoptera citricida* (Kirkaldy) (Homoptera : Aphididae) on eight host plants. **Environmental Entomology,** College Park, v. 27, n. 5, p. 1190-1195, 1998.

TSUCHIDA, T.; KOGA, R.; FUJIWARA, A.; FUKATSU, T. Phenotypic Effect of "Candidatus Rickettsiella viridis," a Facultative Symbiont of the Pea Aphid (*Acyrthosiphon pisum*), and Its Interaction with a Coexisting Symbiont. **Applied and Environmental Microbiology**, Washington, v. 80, n. 2, p. 525-533, 2014.

TSUCHIDA, T.; KOGA, R.; FUKATSU, T. Host plant specialization governed by facultative symbiont. **Science**, Washington, v. 303, n. 5666, p. 1989-1989, 2004.

TSUCHIDA, T.; KOGA, R.; HORIKAWA, M.; TSUNODA, T.; MAOKA, T.; MATSUMOTO, S.; SIMON, J.C.; FUKATSU, T: Symbiotic bacterium modifies aphid body color. **Science**, Washington, v. 330, n. 6007, p. 1102-1104, 2010.

TSUCHIDA, T.; KOGA, R.; HORIKAWA, M.; TSUNODA, T.; MAOKA, T.; MATSUMOTO, S.; SIMON, J.C.; FUKATSU, T: Diversity and geographic distribution of secondary endosymbiotic bacteria in natural populations of the pea aphid, *Acyrthosiphon pisum*. **Molecular Ecology**, Oxford, v. 11, n. 10, p. 2123-2135, 2002.

VIA, S. Specialized Host Plant Performance of Pea Aphid Clones is not Altered by Experience. **Ecology**, Hoboken, v. 72, n. 4, p. 1420-1427, 1991.

WAGNER, S.M.; MARTINEZ, A.J.; RUAN, Y.-M.; KIM, K.L.; LENHART, P.A.; DEHNEL, A.C.; OLIVER, K.M.; WHITE, J.A. Facultative endosymbionts mediate dietary breadth in a polyphagous herbivore. **Functional Ecology** , West Sussex, v.29, n.11, p. 1402-1410, 2015.

WANG, Z.; SHEN, Z-R.; SONG, Y.; LIU, H-Y.; LI, Z-X: Distribution and diversity of *Wolbachia* in different populations of the wheat aphid *Sitobion miscanthi* (Hemiptera: Aphididae) in China. **European Journal of Entomology**, Ceske Budejovice, v. 106, n. 1, p. 49-55, 2009

WERREN, J. H.; BALDO, L.; CLARK, M. E. *Wolbachia*: master manipulators of invertebrate biology. **Nature Reviews Microbiology**, London, v. 6, n. 10, p. 741-51, 2008.

ZYTYNSKA, S.E.; WEISSER, W.W. The natural occurrence of secondary bacterial symbionts in aphids. **Ecological Entomology,** London, v. 41, n. 1, p. 13-26, 2016.

Supplementary table S 4.1 - Taxonomy taxa obtained from *Aphis (Toxoptera) citricidus* developing on different host plant that showed less than 0.1% of representatively.

(to be continued)

Kingdom	Phylum	Class	Order	Family	Genus
Archaea	*Euryarchaeota*	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	undetermined
Bacteria	*Actinobacteria*	*Actinobacteria*	*Actinobacteriales*	*Intrasporangiaceae*	undetermined
				Streptomycetaceae	*Streptomyces*
	Bacteriodetes	*Flavobacteriia*	*Flavobacteriales*	*Weeksellaceae*	*Chryseobacterium* *Cloacibacterium* *Wautersiella*
		Sphingobacteriia	Sphingobacteriales	undetermined	undetermined
				Sphingobacteriaceae	Undetermined *Sphingobacterium*
	Cyanobacteria	*4COd-2*	*MLE1-12*	undetermined	Undetermined
	Elusimicrobia	*Elusimicrobia*	*Elusimicrobiales*	undetermined	Undetermined
	Firmicutes	*Bacilli*	*Bacillales*	undetermined	Undetermined
				Undetermined	Undetermined
				Alicyclobacillaceae	*Alicyclobacillus*
				Bacillaceae	*Bacillus* *Geobacillus*
				Staphylococcaceae	*Staphylococcus*
			Lactobacillales	*Enterococcaceae*	*Enterococcus*
	Proteobacteria	*Alpha-proteobacteria*	*Caulobacteriales*	*Caulobacteraceae*	Undetermined
			Rhizobiales	undetermined	Undetermined
				Bradyrhizobiaceae	Undetermined
				Hyohomicrobiaceae	*Pedomicrobium*
				Methylobacteriaceae	undetermined *Methylobacterium*
				Phyllobacteriaceae	*Aminobacter*
			Sphingomonadales	*Sphingomonadaceae*	undetermined *Novosphigobium*

Supplementary table S 4.1 - Taxonomy taxa obtained from *Aphis (Toxoptera) citricidus* developing on different host plant that showed less than 0.1% of representatively

(conclusion)

Kingdom	Phylum	Class	Order	Family	Genus
		Beta-proteobacteria	*Burholderiales*	*Alcaligenaceae*	*Achromobacter*
					undetermined
				Comamodaceae	undetermined
					Delftia
				Oxalobacteraceae	*Ralstonia*
		Gamma-proteobacteria	*Enterobacteriales*	*Enterobacteriaceae*	*Serratia*
			Pseudomonales	*Pseudomonadaceae*	Undetermined
	Verrucomicrobia	*Opitutae*	*Opitutales*	*Opitutaceae*	Undetermined

Supplementary table S4.2 - Percentage of taxonomy taxa obtained from *Aphis (Toxoptera) citricidus* developing on different host plant that showed 0.1% or more of representatively in at least one host plant

(to be continued)

Phylum	**Class**	**Order**	**Family**	**Genus**	**A1***	**A3**	**A5**	**A6**	**A7**	**A8**	**A9**
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Corynebacteriaceae*	*Corynebacterium*	0.0	0.0	0.0	0.0	0.0	0.1	0.0
	Rubrobacteria	*Rubrobacteriales*	*Rubrobacteriaceae*	*Rubrobacter*	0.0	0.0	0.0	0.0	0.1	0.2	0.0
Cyanobacteria	*Chloroplast*	*Streptophyta*	undetermined	undetermined	0.0	0.0	0.1	0.0	0.1	0.4	0.0
Firmicutes	*Bacilli*	*Bacillales*	*Bacillaceae*	undetermined	0.0	0.0	0.0	0.0	0.1	0.1	0.0
				Anoxybacillus	0.0	0.0	0.0	0.0	0.0	0.1	0.0
			Planococcaceae	undetermined	0.0	0.0	0.2	0.0	0.4	0.9	0.0
			Aerococcaceae	undetermined	0.0	0.0	0.1	0.0	0.0	0.0	0.0
			Streptococcaceae	*Lactococcus*	0.0	0.0	0.1	0.0	0.0	0.0	0.0
				Streptococcus	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Proteobacteria	*Alpha-proteobacteria*	*Rhizobiales*	*Brucellaceae*	undetermined	0.0	0.0	0.0	0.0	0.1	0.0	0.0
				Ochrobactrum	0.0	0.0	0.2	0.0	0.0	0.0	0.0
			Rickettsiaceae	undetermined	0.0	0.0	0.0	0.0	33.0	0.0	0.0
				Wolbachia	0.8	0.0	2.5	0.9	0.1	0.1	1.1
		Sphingomonadales	*Sphingomonadaceae*	*Sphingomonas*	0.0	0.1	0.1	0.0	0.0	0.0	0.0
	Beta-proteobacteria	*Burkholderiales*	*Burkholderiaceae*	*Burkholderia*	0.0	0.0	0.0	0.0	0.0	0.1	0.0
			Comamonadaceae	*Comamonas*	0.1	0.1	0.1	0.0	0.0	0.0	0.0
		Rhodocyclades	*Rhodocyclaceae*	*Hydrogenophilus*	0.0	0.0	0.1	0.0	0.0	0.0	0.0
	Gamma-proteobacteria	*Enterobacteriales*	*Enterobacteriaceae*	*X1*	0.2	0.3	0.4	0.1	0.3	0.5	0.4
				X2	10.3	10.5	17.5	4.6	31.9	36.2	6.8
				Buchnera	88.3	88.6	77.4	94.4	32.4	60.3	91.7
				Citrobacter	0.0	0.2	0.0	0.0	0.0	0.0	0.0
				Erwinia	0.0	0.0	0.0	0.0	0.0	0.0	0.0
				Trabulsiella	0.0	0.0	0.1	0.0	0.0	0.0	0.0

Supplementary table S4.2 - Percentage of taxonomy taxa obtained from *Aphis (Toxoptera) citricidus* developing on different host plant that showed 0.1% or more of representatively in at least one host plant

(conclusion)

Phylum	Class	Order	Family	Genus	A1*	A3	A5	A6	A7	A8	A9
		Oceanospirillales	*Halomonadaceae*	*Halomonas*	0.0	0.0	0.1	0.0	0.1	0.2	0.0
		Pseudomonadales	*Moraxellaceae*	*Acinetobacter*	0.0	0.0	0.1	0.0	0.7	0.3	0.0
				Enhydrobacter	0.0	0.0	0.0	0.0	0.2	0.0	0.0
			Pseudomonadaceae	*Pseudomonas*	0.0	0.0	0.1	0.0	0.0	0.1	0.0
		Xanthomonadales	*Sinobacteraceae*	undetermined	0.0	0.0	0.1	0.0	0.0	0.0	0.0
			Xanthomonadaceae	undetermined	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Thermi	*Deinococci*	*Thermales*	*Thermaceae*	*Thermus*	0.0	0.0	0.0	0.0	0.0	0.1	0.0

* A1= *Citrus sinensis*, A3= *Citrus paradisi x Poncirus trifoliata*, A5= *Poncirus trifoliata* Limeira,A6= *Citrus latifolia*,A7= *Citrus deliciosa* Tenore,A8= *Citrus bergamia*,A9= *Citrus aurantifolia*.

5 FACULTATIVE SYMBIONT DIVERSITY AT THE SAME ECOLOGICAL NICHE IN TWO CLOSELY-RELATED APHIDS WITH DIFFERENT HOST RANGES

Abstract

Richness and abundance of facultative symbionts vary strongly with aphid species and genotype, symbiont strain, host plant, biogeography and a number of abiotic factors. Despite indications that aphids in the same ecological niche show similar levels of facultative symbiont richness, existing reports do not consider the potential role of host plants on aphid microbial community. Little is known about how oligophagy and polyphagy may be influenced by secondary symbiont distribution, mainly because studies on secondary symbiont diversity are biased towards polyphagous aphids from the Northern Hemisphere. Here, we demonstrate the richness and abundance of the most common aphid-associated facultative symbionts in two tropical aphid species, the oligophagous *Aphis (Toxoptera) citricidus* (Kirkaldy) (Hemiptera: Aphididae) and the polyphagous *Aphis (Toxoptera) aurantii* Boyer de Fonscolombe (Hemiptera: Aphididae). *A. citricidus* is restricted to *Citrus* sp. host plants and closely-related genera, whereas *A. aurantii* successfully exploits a wide variety of host plants from different families. Both were collected in the same ecological niche and our data basically indicated the same richness of secondary symbionts, but the abundance at which secondary symbionts occurred was very distinct between the two species. *Spiroplasma* was the most abundant facultative symbiont associated with *A. citricidus* and *A. aurantii* in the ecological niche studied. Single and multiple secondary symbiont infections were observed, but diversity of multiple infections was particularly high in *A. citricidus*. We discuss our findings and suggest hypotheses to explain causes and consequences of the differences in secondary symbiont diversity observed between these two aphid species.

Keywords: Host plant; Microbial community; Oligophagy; Polyphagy; Symbiont richness

5.1 Introduction

Bacterial symbionts are widespread among invertebrates and are quite common in insects. Their associations with host insects can lead to interactions ranging from mutualism to parasitism (BAUMANN, 2005; DURON; HURST, 2013; KLEPZIG et al., 2009). In insects, endosymbiotic bacteria are categorized into two groups: obligate/primary and facultative/secondary symbionts (BUCHNER, 1965; BAUMANN, 2005). Obligate symbionts establish mutualistic interactions with their hosts, often contributing to insect nutrition by providing essential nutrients that are limited or completely absent from the diet of the host (MORAN et al., 2005; DOUGLAS, 2009). Facultative symbionts are not vital for survival and/or reproduction of their hosts, but may influence their fitness traits by altering host response to a number of abiotic and biotic factors (OLIVER et al., 2003; OLIVER et al., 2010; SU; ZHOU; ZHANG, 2013; ASPLEN et al., 2014; GAO et al., 2014; WAGNER et al., 2015).

Several aspects of aphid-symbiont interactions have been studied. The genomic composition and physiological contributions of the aphid obligate symbiont *Buchnera aphidicola* have been determined for a number of aphid species, and the effects of several commonly reported secondary symbionts of aphids (*Hamiltonella defensa*, *Serratia symbiotica*, *Regiella insecticola*, *Spiroplasma* sp., *Arsenophonus* sp. and *Rickettsia* sp.) have been widely investigated (FUKATSU, 1994; CHEN; PURCELL, 1997; FUKATSU et al., 2001; LEONARDO; MUIRU, 2003; OLIVER et al., 2003; DOUGLAS; FRANCOIS; MINTO, 2006; SIMON et al., 2011; LUKASIK et al., 2013b; TSUCHIDA et al., 2004; WAGNER et al., 2015).

Aphid secondary symbionts influence the fitness traits of their hosts in different ways. *Arsenophonus*, *H. defensa*, *S. symbiotica*, *Spiroplasma* and *R. insecticola* have been reported to protect their host aphids from natural enemies. *Hamiltonella defensa* and *S. symbiotica* provide protection against macrobials (parasitoids) (OLIVER et al., 2003), *R. insecticola* and *Spiroplasma* protect against microbials (FERRARI et al., 2001; FERRARI et al., 2004; LUKASIK et al., 2013a) , and *Arsenophonus* have no protective role (WULFF et al., 2013). Pea aphid resistance to parasitoids was much higher when associated with *H. defensa*, and resistance was dependent on symbiont strain and presence of the infective bacteriophage APSE (OLIVER et al., 2003; OLIVER; MORAN; HUNTER, 2005; OLIVER et al., 2009; OLIVER; SMITH; RUSSELL, 2014).

Aphid secondary symbionts have also been shown to affect other host traits. Besides influencing host resistance to natural enemies, *S. symbiotica* and *R. insecticola* have also been linked to aphid heat tolerance (CHEN; MONTLLOR; PURCELL, 2000; MONTLLOR; MAXMEN; PURCELL, 2002; RUSSELL; MORAN, 2006). *Regiella insecticola* and *Arsenophonus* may also interfere in host plant use, as aphid fitness on host plants is different in symbiont-free and symbiont-associated aphids (LEONARDO; MUIRU, 2003; FERRARI et al., 2006). *Spiroplasma* has been shown to affect sex determination in sexual generations of aphids by eliminating male progeny (SIMON et al., 2011) and to induce fitness costs by reducing aphid fecundity and longevity (FUKATSU et al., 2001; SIMON et al., 2011).

Secondary symbiont diversity can vary greatly within and among aphid species (TSUCHIDA et al., 2002; HAYNES et al., 2003; BRADY et al., 2014). Within-species variation may occur due to ecological factors related to aphid geographical distribution and/or host plant use (FERRARI et al., 2004; MCLEAN et al., 2011), but little information is available on among-species variation (BRADY et al., 2014). Although a fair amount of information has been published on the diversity and interactions of aphid symbionts, most of the available data focuses on polyphagous aphids, primarily the pea aphid *Acyrthosiphon pisum* (TSUCHIDA et

al., 2004; FERRARI et al., 2012; RUSSELL et al., 2013). Very few studies have focused on aphid species that have a narrow host range or are found in tropical areas (HENRY et al., 2015; ZYTYNSKA; WEISSER, 2016).

Aphis (Toxoptera) citricidus (Kirkaldy) is an oligophagous aphid commonly found in tropical areas feeding on *Citrus* plants and a few relative species. It is also an efficient vector of *citrus tristeza virus* (CTV) to citrus plants. Another aphid species, *Aphis (Toxoptera) aurantii* Boyer de Fonscolombe, usually co-occurs with *A. citricidus* in *Citrus* groves. However, *A. aurantii* is polyphagous and a poor vector of CTV (HALBERT; BROWN, 1996; HALBERT et al., 2004).

As aphid-symbiont associations in aphids with restricted diet breadth still remain to be investigated, we screened for the most commonly reported aphid-associated facultative symbionts in an oligophagous (*A. citricidus*) as compared to a polyphagous (*A. aurantii*) aphid species within the same genus and in the same ecological niche.

5.2 Material and methods

5.2.1 Aphid isolines

Adult aphids were collected from an ecological niche on unmanaged *Citrus* sp. and *Murraya paniculata* trees in the municipality of Piracicaba, SP, Brazil Aphids were brought to the laboratory and classified following the key to wingless adults provided by Halbert and Brown (2011).

After species identification, specimens of *A. citricidus* and *A. aurantii* were individually placed on new shoots of *Citrus sinensis* seedlings in a rearing cage (50 cm high x 15 cm diameter, with two lateral openings covered with cloth for ventilation). Cages were kept under controlled conditions (25±2°C; 60±10% RH; 14 h photophase) for aphid development and reproduction. After two weeks, aphid lines that had successfully reproduced and established themselves were used to assess the presence of known aphid-associated symbionts. Symbiont assessments were carried out by diagnostic PCR to detect the six most common secondary symbionts associated with aphids, as well as the primary symbiont *Buchnera aphidicola* and the APSE-1 bacteriophage (SANDSTROM et al., 2001; TSUCHIDA et al., 2002; OLIVER; MORAN; HUNTER, 2005; OLIVER et al., 2006).

5.2.2 DNA extraction and symbiont screening

Three adult aphids from each isoline were randomly selected, placed into 1.5 mL plastic tubes and subjected to genomic DNA (gDNA) extraction following Sunnucks and Hales (1996). DNA quality was verified by spectrophotometer measurements of the A260/280 ratio, and by agarose gel electrophoresis on a 1% gel stab containing 0.5 µg/mL ethidium bromide in Tris-acetate-EDTA buffer (40 mM Tris-acetate, 1 mM EDTA; pH 7.2) at 5 V/cm, followed by visualization on a UV transilluminator (DNR Bio-imaging system).

Diagnostic PCR reactions were set to a final volume of 25 µL and contained 20 ng/µL of gDNA, 1x PCR buffer, 1.5 mM $MgCl_2$, 10 µM dNTP, 0.32 µM of each primer and 0.5 U of Taq polymerase. Cycling conditions and primer sets depended on the symbiont screened (Table 5.1). Amplicons were resolved by agarose gel electrophoresis on a 1.5% gel stab and visualized under the same conditions described above.

5.2.3 Statistical analysis

Differences in frequency of association of secondary symbionts with the oligophagous *A. citricidus* and the polyphagous *A. aurantii* were tested using a contingency table with a chi-square test ($p < 0.05$).

Table 5.1 - Primers and cycling conditions used for assessing the association of the most common aphid symbionts with *Aphis (Toxoptera) citricidus* and *Aphis (Toxoptera) aurantii* by using diagnostic PCRs

Symbionts	Primers (5'-3')	Cycling conditions	Reference
Buchnera aphidicola	Buch16S1F - GAGCTTGCTCTCTTTGTCGGCAA Buch16S1R - CTTCTGCGGGTAACGTCACGAA	1x: 2 min at 95ºC; 35x: 30 s at 95ºC, 1 min at 53ºC, 2 min at 72ºC; 1x: 5 min at 72ºC; 1x: hold at 10ºC.	TSUCHIDA et al., 2002
Serratia symbiotica	16SA1 - AGAGTTTGATCMTGGCTCAG PASScmp - GCAATGTCTTATTAACACAT	Same as for *Buchnera*	TSUCHIDA et al., 2002
Regiella insecticola	U1279F - CGAACGTAAGCGAACCTCAT 35R - CCTTCATCGCCTCTGACTGC	1x: 1min at 94ºC; 35x: 1min at 94ºC, 1min at 58ºC, 2 min at 72ºC; 1x: 6 min at 72ºC; 1x: hold at 10ºC.	OLIVER, 2006
Rickettsia	PAR4F - GGCTCAGAACGAACGCTATC PAR1213R - CACCGTCTTGCTTCCCTCTG	1x: 1min at 94ºC; 35x: 1min at 94ºC, 1min at 55ºC, 2min 72ºC; 1x: 6 min at 72ºC; 1x: hold at 10ºC.	OLIVER, 2006
Spiroplasma	16SA1 - AGAGTTTGATCMTGGCTCAG TKSSspR - TAGCCGTGGCTTTCTGGTAA	Same as *Buchnera*	TSUCHIDA et al., 2002
Hamiltonella defensa	T1279F - CGAGGGAAAGCGGAACTCAG 35R - CTTCATCGCCTCTGACTGC	1x: 1min at 94ºC; 30x: 1min at 94ºC, 1 min at 54ºC, 1.5 min at 72ºC; 1x: 5min at 72ºC; 1x: hold at 10ºC.	OLIVER, 2005
Arsenophonus	16SA1 - AGAGTTTGATCMTGGCTCAG Ars16SR - TTAGCTCCGGAGGCCACAGT	1x: 4min at 95ºC 40x: 30s at 95 ºC, 30s at 55 ºC, 30s at 72ºC; 1x: 5min at 72ºC 1x: hold at 10ºC.	TSUCHIDA et al., 2002
APSE-I Bacteriophage	APSE-1F - GCAACGCTTTTTACTCCTTT APSE-1R - TCGATAAAAACGGAGAATGC	1x: 2 min at 94ºC; 35x: 1min at 94ºC, 1min at 50ºC, 1min at 72ºC; 1x: 5min at 72ºC; 1x: hold at 10ºC.	SANDSTROM, 2001

5.3 Results

Establishing isolines from field-collected adults was quite difficult, ultimately limiting the number of isolines screened for symbionts. Even so, 47 isolines of *A. citricidus* and 37 of *A. aurantii* originating from different field collections were subjected to diagnostic PCRs to determine their association with the most commonly reported aphid symbionts.

Aphis (Toxoptera) citricidus and *A. aurantii* showed high rates of infection by secondary symbionts, as exclusive infections by the primary symbiont *B. aphidicola* comprised only 18% of all tested isolines of *A. citricidus* and 7% of *A. aurantii* isolines (Figure 5.1). Overall, *A. aurantii* was more frequently associated with secondary symbionts than *A. citricidus* (χ^2= 27.0, *df*=1, p<0.001), and the frequency of occurrence of each facultative symbiont was different between aphid species. *Spiroplasma* was the most frequent secondary symbiont in both *A. citricidus* (25/47) and *A. aurantii* (31/37), but occurred at a higher frequency in *A. aurantii* (χ^2= 7.5, *df* =1, p<0.001). In addition, *Spiroplasma* was the only secondary symbiont to be persistently associated with *A. aurantii* in single and multiple infections with other secondary symbionts (Figure 5.1 and Table 5.2). Many isolines of *A. citricidus* harbored *H. defensa* (14/47) or *Arsenophonus* (11/47), whereas few isolines carried *Rickettsia* (3/47), *R. insecticola* (2/47) or *S. symbiotica* (2/47). On the other hand, several isolines of *A. aurantii* were infected with *Rickettsia* (9/37) or *H. defensa* (7/37), a few with *S. symbiotica* (2/37) or *R. insecticola* (1/37) and none with *Arsenophonus*. In addition, even though many isolines of *A. citricidus* and *A. aurantii* were infected with *H. defensa*, we were unable to detect infections with the bacteriophage APSE1 (Figure 5.1). While *Arsenophonus* association with *A. citricidus* was much more common than with *A. aurantii* (χ^2=10.2, *df*=1, p<0.001), *Rickettsia* showed the opposite trend (χ^2=5.2, *df*=1, p<0.001). No difference in association with *H. defensa* (χ^2=1.5, *df*=1, p=0.22), *S. symbiotica* (χ^2=0.05, *df*=1, p=0.83) or *R. insecticola* (χ^2=0.16, *df*=1, p=0.69) was detected between the two aphids.

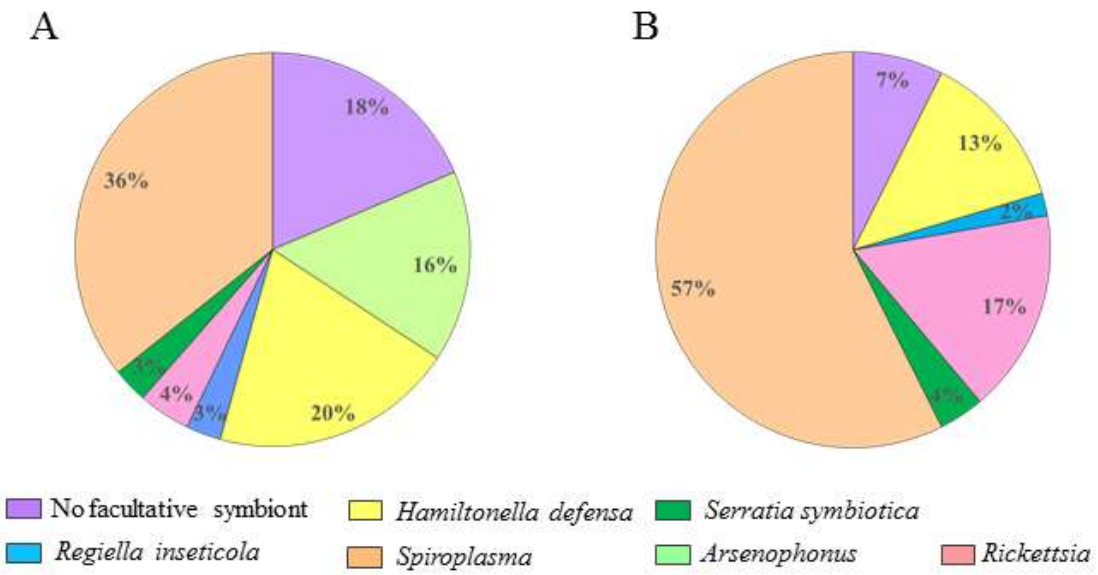


Figure 5.1 - Occurrence and frequency (%) of facultative symbionts in isolines of *Aphis (Toxoptera) citricidus* (A) and *Aphis (Toxoptera) aurantii* (B) collected from the same ecological niche

In both species, several isolines were infected with multiple facultative symbionts. Nearly two-thirds of *A. citricidus* isolines were infected with one (34% - 16/47) or two (34% - 16/47) secondary symbionts, 2% carried three and 2% carried four facultative symbionts. Almost half of the isolines of *A. aurantii* (47%) harbored only one secondary symbiont, 26% carried two, 11% carried three facultative symbionts, and none were infected with four facultative symbionts (Figure 5.2).

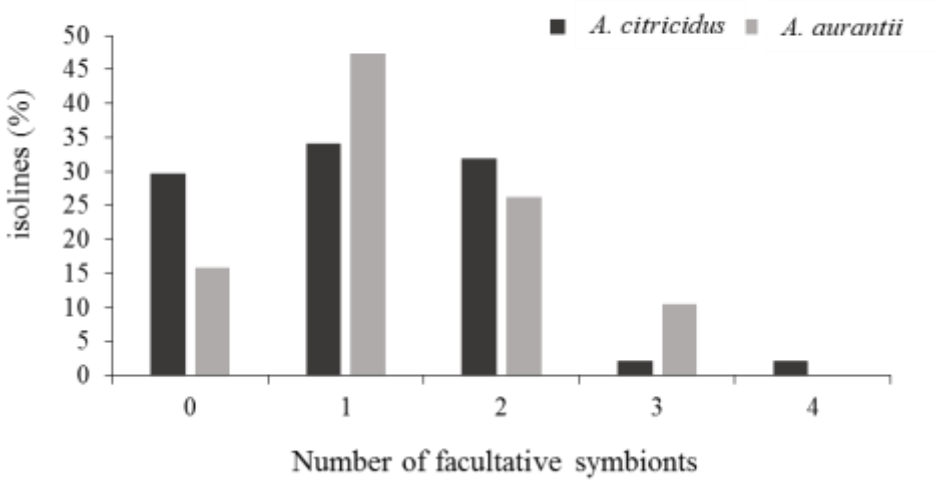


Figure 5.2 - Isolines (%) of *Aphis (Toxoptera) citricidus* and *Aphis (Toxoptera) aurantii* from the same ecological niche carrying none, single or multiple infections of facultative symbionts

An analysis of associations among facultative symbionts in multiple-infected aphids indicated that some multiple infections were more common in *A. citricidus* than in *A. aurantii*. In *A. citricidus, Spiroplasma* and *H. defensa* were observed in single infection events, followed by several combinations of double, triple and quadruple secondary symbiont infections, with *Spiroplasma* as the most common symbiont in aphids with multiple infections. *Spiroplasma* was present in all multiple infections in *A. aurantii* and in five out of eight combinations of multiple infections in *A. citricidus* (Table 5.2).

Table 5.2 - Occurrence of facultative symbionts in singly and multiply infected isolines of *Aphis (toxoptera) citricidus* and *Aphis (Toxoptera) aurantii* inhabiting the same ecological niche

Facultative symbionts	N° of isolines
A. citricidus	
Spiroplasma	13
H. defensa	3
H. defensa + Arsenophonus	5
Spiroplasma + Arsenophonus	3
Spiroplasma + H. defensa	3
Spiroplasma + S. symbiotica	2
R. insecticola + Arsenophonus	2
Spiroplasma + Rickettsia	1
R. insecticola + Arsenophonus+ H. defensa	1
Spiroplasma + H. defensa + Arsenophonus + Rickettsia	1
A. aurantii	
Spiroplasma	18
Spiroplasma + Rickettsia	8
Spiroplasma + H. defensa	3
Spiroplasma +Rickettsia + S. symbiotica	2
Spiroplasma +Rickettsia + R. insecticola	1

5.4 Discussion

Richness and abundance of facultative symbionts commonly associated with aphids differed between the closely related aphid species *A. citricidus* and *A. aurantii* exploiting the

same ecological niche, with the polyphagous species *A. aurantii* showing higher frequencies of infection by secondary symbionts. *Spiroplasma* was the most abundant facultative symbiont associated with these aphid species in single or multiple infections. *Rickettsia* was the second most abundant facultative symbiont associated with *A. aurantii,* but was seldom associated with *A. citricidus*; on the other hand, *Arsenophonus* was common in *A. citricidus*, but was not associated with *A. aurantii*. Multiple infections were common. Among these, double infections prevailed, although we observed infections by up to four facultative symbionts.

Hamiltonella defensa, *R. insecticola*, *S. symbiotica*, *Rickettsia*, *Spiroplasma*, X-type, *Arsenophonus* and *Wolbachia* are regularly reported as facultative symbionts of aphids, although their richness and abundance can differ from one host aphid to another (SANDSTROM et al., 2001; TSUCHIDA et al., 2002; AUGUSTINOS et al., 2011; ZYTYNSKA; WEISSER, 2016). The diversity of aphid-associated facultative symbionts has been intensively investigated in polyphagous aphids, and the factors influencing these associations are mostly known for *Aphis craccivora*, *Aphis fabae*, *A. pisum* and *Sitobion avenae* (FERRARI et al., 2012; LUKASIK et al., 2013a; BRADY et al., 2014; CAYETANO et al., 2015). Aphid genotype, symbiont strain, host plant and biogeography are among the factors affecting the occurrence of facultative symbionts in aphids (MCLEAN et al., 2011; FERRARI et al., 2012). Extensive data on the influence of biogeography is still lacking, as most of the available information on facultative symbiont richness and abundance focuses on natural populations of aphids in the Northern Hemisphere (ZYTYNSKA; WEISSER, 2016) and very little is known about aphid species in the Southern Hemisphere, particularly in the Neotropics (TELESNICKI et al., 2012; ARNEODO; ORTEGO, 2014). Therefore, our data on *A. citricidus* and *A. aurantii* add to current knowledge by providing additional information about tropical areas in the southern part of the Neotropical region, also contributing towards an elucidation of how polyphagy/oligophagy may interfere with aphid-facultative symbiont associations.

Recent research on aphid-symbiont associations has focused on the importance of ecological niche on symbiont distribution, as aphids with similar feeding habits are more likely to share similar symbiont communities (HENRY et al., 2015). These authors also showed that the mean proportion of aphids carrying *S. symbiotica*, but not *H. defensa* or *R. insecticola*, increased with aphid specialization in host plant use (HENRY et al., 2015). These data show that certain associations are affected by the life history traits of the host aphid. We did not find such a correlation with *S. symbiotica* in the polyphagous/oligophagous aphids we tested, as the abundance of infection by *S. symbiotica* ranged from 4.2% (2/47) in *A. citricidus* (oligophagous) to 5.4% (2/37) in *A. aurantii* (polyphagous).

But we did find differences in the frequency at which certain facultative symbionts were associated with *A. citricidus* and *A. aurantii* aphids inhabiting the same ecological niche. *Arsenophonus* was exclusively associated with *A. citricidus*; frequency of infection by *Rickettsia* differed between the two aphids, occurring in nearly 24.3% (9/37) of *A. aurantii* isolines, but only 6.4% (3/47) of *A. citricidus* isolines tested. *Arsenophonus* has been associated with host plant specialization in *A. craccivora* (WAGNER et al., 2015), and its high abundance in *A. citricidus* could be associated with the narrow host range of this aphid. Differences in *Rickettsia* abundance between *A. citricidus* and *A. aurantii* could be related to the role this facultative symbiont may play on host defense against microbial infections (LUKASIK et al., 2013b). This would indicate that *A. aurantii* may be under higher selection pressure due to pathogen infection than *A. citricidus*, as maintaining such associations involves fitness costs to the host (OLIVER; SMITH; RUSSELL, 2014). Associations with defensive facultative symbionts are more prevalent under parasitoid selection pressure (OLIVER et al., 2008; OLIVER; SMITH; RUSSELL, 2014) and are more often observed in aphids that are not tended by ants that provide the same defensive services (HENRY et al., 2015).

Two other facultative symbionts that reportedly enhance host defense against natural enemies, namely *Spiroplasma* and *H. defensa* (LUKASIK et al., 2013b; OLIVER et al., 2003), were very frequent in single- or multiple-infected *A. citricidus* and *A. aurantii*. This also supports the hypothesis that these aphid species are kept under strong selective pressure by natural enemies in field conditions. Although we found no difference in frequency of infection by *H. defensa* between the two aphids, which indicates that they may be under the same selective pressure by parasitoids, our data clearly suggest that *A. aurantii* may be more subject to infection by entomopathogenic fungi than *A. citricidus*, as *Spiroplasma* and *Rickettsia* were detected more frequently in *A. aurantii*. *Spiroplasma* is by far the most frequently-associated facultative symbiont of *A. citricidus* and *A. aurantii*, and although a fair amount of information on the role of this symbiont in fruit flies is known (COCKBURN et al., 2013; XIE et al., 2014; HASELKORN; JAENIKE, 2015), investigations into its role in aphid associations began only recently (LUKASIK et al., 2013a; LUKASIK et al., 2013b; LUKASIK et al., 2013c).

Multiple infections by more than two facultative symbionts were also detected in *A. citricidus* and *A. aurantii*, but at a lower frequency than single or double infections, as expected. Multiple-infected hosts are subject to a bottleneck as a result of the associated fitness costs (OLIVER et al., 2006). Most field trials conducted thus far have been carried out with *A. pisum*, which usually harbors one or two facultative symbionts (FERRARI et al., 2012; RUSSELL et al., 2013). Data on the dynamics of symbionts associated with *A. pisum* have suggested that

multiple infections may not be efficiently maintained in the population, due to inefficient transmission. In addition, temporal changes in frequency have been reported for all symbionts associated with *A. pisum*, and increases in the frequency of certain symbionts in response to bioecological stressors have indicated that symbionts participate in seasonal adaptation of aphids (SMITH et al., 2015). However, initial studies on other species of aphids did not follow this pattern (CHANDLER; WILKINSON; DOUGLAS, 2008; HENRY et al., 2015). Laboratory surveys identified up to four facultative symbionts in a single clone, providing enhanced resistance to parasitoids at the expense of decreased fecundity (OLIVER et al., 2006). Differences between field and laboratory data can be attributed to the transmission of facultative symbionts, as multiple infections are more prone to transmission failures, which can be more severe under variable environmental field conditions (OLIVER; SMITH; RUSSELL, 2014).

Secondary symbionts occurred in different combinations in multiple-infected *A. citricidus* and *A. aurantii*. Surveys of natural populations have rarely reported the abundance at which facultative symbionts co-occur. Co-infections by *H. defensa* and *S. symbiotica* have been associated with increased defense against parasitoids, but the cost of this association would decrease its abundance in the field, particularly under low selection pressure (OLIVER et al., 2006). *Hamiltonella* and *Serratia* have also been shown to co-infect *A. pisum* with *Rickettsiella*, a facultative symbiont that alters the body color of the aphid due to accumulation of blue-green polycyclic quinones (TSUCHIDA et al., 2010). The accumulation of these compounds and co-occurrence of *Rickettsiella* with *Hamiltonella* and *Serratia* led to the hypothesis that *Rickettsiella* may also benefit the aphid by improving its immune response against natural enemies (TSUCHIDA et al., 2010). Co-infections involving *R. insecticola* and *H. defensa* or the X-type, or *Rickettsiella* and *Spiroplasma*, are less frequent in field conditions, probably due to a fitness cost component (FERRARI et al., 2012) or the incompatible nature of *R. insecticola* (SMITH et al., 2015). *Regiella insecticola* was very rare, infecting a single line of each species of *Toxoptera* studied. In both cases, *R. insecticola* was detected in triple infections, co-occurring with *H. defensa* and *Arsenophonus* in *A. citricidus*, but with *Spiroplasma* and *Rickettsia* in *A. aurantii*.

We showed that facultative symbiont richness and abundance was different between two aphids with different host ranges in the same ecological niche, with the polyphagous species being more frequently infected with secondary symbionts than the oligophagous one. We also reported *Spiroplasma* as the most common facultative symbiont of *A. citricidus* and *A. aurantii,* but the diversity of associations with other facultative symbionts was quite high between the two aphids, with *A. citricidus* carrying a considerably higher number of facultative symbiont

combinations. However, the relevance of such multiple infections for the host aphid should not be exclusively viewed from the perspective of richness/abundance of associated microbiota, but rather their role and functional complementarity (ZYTYNSKA; WEISSER, 2016). Therefore, the nature of the facultative symbiont associations we detected in natural populations of *A. citricidus* and *A. aurantii*, as relates to host range use and role in ecological and fitness traits of host aphids, remains to be investigated.

5.5 Conclusions

- *Aphis (Toxoptera) citricidus* and *Aphis (Toxoptera) aurantii* harbour the most common facultative symbionts associated with aphids, *Hamiltomella defensa, Regiella insecticola, Serratia symbiotica, Spiroplasma,* and *Rickettsia;*
- Frequency of infection with secondary symbionts in higher in the polyphagous *A. aurantii;*
- *Arsenophonus* was found infecting only *A. citricidus;*
- *Spiroplasma* was the most frequent facultative symbiont in *A. aurantii* and *A. citricidus*, and was the only one to occur in single infections in *A. aurantii*;
- *Rickettsia* was very common in *A. aurantii*, but rarely detected in *A. citricidus;*
- Single and double infections by facultative symbionts prevailed over triple and quadruple infections with facultative symbionts.

References

ARNEODO, J. D.; ORTEGO, J. Exploring the bacterial microbiota associated with native south american species of aphis (Hemiptera: Aphididae). **Environmental Entomology,** College Park, v. 43, n. 3, p. 589-594, 2014.

ASPLEN, M. K.; BANO, N.; BRADY, C. M.; DESNEUX, N.; HOPPER, K. R.; MALOUINES, C.; OLIVER, K. M.; WHITE, J. A.; HEIMPEL, G. E. Specialisation of bacterial endosymbionts that protect aphids from parasitoids. **Ecological Entomology,** London, v. 39, n. 6, p. 736-739, 2014.

AUGUSTINOS, A. A.; SANTOS-GARCIA, D.; DIONYSSOPOULOU, E.; MOREIRA, M.; PAPAPANAGIOTOU, A.; SCARVELAKIS, M.; DOUDOUMIS, V.; RAMOS, S.; AGUIAR, A. F.; BORGES, P. A. V.; KHADEM, M.; LATORRE, A.; TSIAMIS, G.; BOURTZIS, K. Detection and characterization of *Wolbachia* infections in natural populations of aphids: is the hidden diversity fully unraveled? **Plos One,** San Francisco, v. 6, n. 12, p. e28695, 2011.

BAUMANN, P. Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. **Annual Review of Microbiology,** Palo Alto, v. 59, p. 155-189, 2005.

BRADY, C. M.; ASPLEN, M. K.; DESNEUX, N.; HEIMPEL, G. E.; HOPPER, K. R.; LINNEN, C. R.; OLIVER, K. M.; WULFF, J. A.; WHITE, J. A. Worldwide populations of the aphid *Aphis craccivora* are infected with diverse facultative bacterial symbionts. **Microbial Ecology,** New York, v. 67, n. 1, p. 195-204, 2014.

BUCHNER, P. **Endosymbiosis of animals with plant microorganisms.** New Jersey: Interscience Publishers / John Wiley, 1965. 909p.

CAYETANO, L.; ROTHACHER, L.; SIMON, J. C.; VORBURGER, C. Cheaper is not always worse: strongly protective isolates of a defensive symbiont are less costly to the aphid host. **Proceedings of the Royal Society B-Biological Sciences,** London, v. 282, n. 1799, 2015.

CHANDLER, S. M.; WILKINSON, T. L.; DOUGLAS, A. E. Impact of plant nutrients on the relationship between a herbivorous insect and its symbiotic bacteria. **Proceedings of the Royal Society B-Biological Sciences,** London, v. 275, n. 1634, p. 565-570, 2008.

CHEN, D. Q.; MONTLLOR, C. B.; PURCELL, A. H. Fitness effects of two facultative endosymbiotic bacteria on the pea aphid, *Acyrthosiphon pisum*, and the blue alfalfa aphid, *A. kondoi*. **Entomologia Experimentalis Et Applicata,** West Sussex, v. 95, n. 3, p. 315-323, 2000.

CHEN, D. Q.; PURCELL, A. H. Occurrence and transmission of facultative endosymbionts in aphids. **Current Microbiology,** New York, v. 34, n. 4, p. 220-225, 1997.

COCKBURN, S. N.; HASELKORN, T. S.; HAMILTON, P. T.; LANDZBERG, E.; JAENIKE, J.; PERLMAN, S. J. Dynamics of the continent-wide spread of a *Drosophila* defensive symbiont. **Ecology Letters,** Oxford, v. 16, n. 5, p. 609-616, 2013.

DOUGLAS, A. E. The microbial dimension in insect nutritional ecology. **Functional Ecology,** West Sussex, v. 23, n. 1, p. 38-47, 2009.

DOUGLAS, A. E.; FRANCOIS, C. L. M. J.; MINTO, L. B. Facultative 'secondary' bacterial symbionts and the nutrition of the pea aphid, *Acyrthosiphon pisum*. **Physiological Entomology,** West Sussex, v. 31, n. 3, p. 262-269, 2006.

DURON, O.; HURST, G. D. D. Arthropods and inherited bacteria: from counting the symbionts to understanding how symbionts count. **Bmc Biology,** London, v. 11, n.45, 2013. Available at: < DOI: 10.1186/1741-7007-11-45>.

FERRARI, J.; DARBY, A. C.; DANIELL, T. J.; GODFRAY, H. C. J.; DOUGLAS, A. E. Linking the bacterial community in pea aphids with host-plant use and natural enemy resistance. **Ecological Entomology,** London, v. 29, n. 1, p. 60-65, 2004.

FERRARI, J.; GODFRAY, H. C. J.; FAULCONBRIDGE, A. S.; PRIOR, K.; VIA, S. Population differentiation and genetic variation in host choice among pea aphids from eight host plant genera. **Evolution,** West Sussex, v. 60, n. 8, p. 1574-1584, 2006.

FERRARI, J.; MULLER, C. B.; KRAAIJEVELD, A. R.; GODFRAY, H. C. J. Clonal variation and covariation in aphid resistance to parasitoids and a pathogen. **Evolution,** West Sussex, v. 55, n. 9, p. 1805-1814, 2001.

FERRARI, J.; WEST, J. A.; VIA, S.; GODFRAY, H. C. J. Population genetic structure and secondary symbionts in host-associated populations of the pea aphid complex. **Evolution,** West Sussex, v. 66, n. 2, p. 375-390, 2012.

FUKATSU, T. Endosymbiosis of aphids with microorganisms: a model case of dynamic endosymbiotic evolution. **Plant Species Biology,** Richmond, v. 9, n. 3, p. 145-154, 1994.

FUKATSU, T.; TSUCHIDA, T.; NIKOH, N.; KOGA, R. *Spiroplasma* symbiont of the pea aphid, *Acyrthosiphon pisum* (Insecta : Homoptera). **Applied and Environmental Microbiology,** Washington, v. 67, n. 3, p. 1284-1291, 2001.

GAO, S.-X.; LIU, D-G.; CHEN, H.; MENG, X-X. Fitness traits and underlying genetic variation related to host plant specialization in the aphid Sitobion avenae. **Insect Science,** Richmond, v. 21, n. 3, p. 352-362, 2014.

HALBERT, S.E.; BROWN, L.G. ***Toxoptera citricida* (Kirkaldy), brown citrus aphid:** identification, biology and management strategies. Gainesville: Florida Department of Agriculture and Consumer Services, Division of Plant Industry, 1996. 6 p. (Entomology Circular, 374).

HALBERT, S. E.; GENC, H.; CEVIK, B.; BROWN, L. G.; ROSALES, I. M.; MANJUNATH, K. L.; POMERINKE, M.; DAVISON, D. A.; LEE, R. F.; NIBLETT, C. L. Distribution and characterization of *Citrus tristeza virus* in South Florida following establishment of *Toxoptera citricida*. **Plant Disease,** San Paul, v. 88, n. 9, p. 935-941, 2004.

HASELKORN, T. S.; JAENIKE, J. Macroevolutionary persistence of heritable endosymbionts: acquisition, retention and expression of adaptive phenotypes in Spiroplasma. **Molecular Ecology,** New York, v. 24, n. 14, p. 3752-3765, 2015.

HAYNES, S.; DARBY, A. C.; DANIELL, T. J.; WEBSTER, G.; VAN VEEN, F. J. F.; GODFRAY, H. C. J.; PROSSER, J. I.; DOUGLAS, A. E. Diversity of bacteria associated with natural aphid populations. **Applied and Environmental Microbiology,** Washington, v. 69, n. 12, p. 7216-7223, 2003.

HENRY, L. M.; MAIDEN, M. C. J.; FERRARI, J.; GODFRAY, H. C. J. Insect life history and the evolution of bacterial mutualism. **Ecology Letters,** Oxford, v. 18, n. 6, p. 516-525, 2015.

KLEPZIG, K. D.; ADAMS, A. S.; HANDELSMAN, J.; RAFFA, K. F. Symbioses: a key driver of insect physiological processes, ecological interactions, evolutionary diversification, and impacts on humans. **Environmental Entomology,** College Park, v. 38, n. 1, p. 67-77, 2009.

LEONARDO, T. E.; MUIRU, G. T. Facultative symbionts are associated with host plant specialization in pea aphid populations. **Proceedings of the Royal Society B-Biological Sciences,** London, v. 270, p. 209-212, 2003.

LUKASIK, P.; DAWID, M. A.; FERRARI, J.; GODFRAY, H. C. J. The diversity and fitness effects of infection with facultative endosymbionts in the grain aphid, *Sitobion avenae*. **Oecologia,** Heidelberg, v. 173, n. 3, p. 985-996, 2013a.

LUKASIK, P.; GUO, H.;, VAN ASCH, M.; FERRARI, J.; GODFRAY, H. C. J. Protection against a fungal pathogen conferred by the aphid facultative endosymbionts *Rickettsia* and *Spiroplasma* is expressed in multiple host genotypes and species and is not influenced by co-infection with another symbiont. **Journal of Evolutionary Biology,** Basel, v. 26, n. 12, p. 2654-2661, 2013b.

LUKASIK, P.; VAN ASCH, M.; GUO, H.; FERRARI, J.; GODFRAY, H. C. J. Unrelated facultative endosymbionts protect aphids against a fungal pathogen. **Ecology Letters,** Oxford, v. 16, n. 2, p. 214-218, 2013c.

MCLEAN, A. H. C.; VAN ASCH, M.; FERRARI, J.; GODFRAY, H. C. J. Effects of bacterial secondary symbionts on host plant use in pea aphids. **Proceedings of the Royal Society B-Biological Sciences,** London, v. 278, n. 1706, p. 760-766, 2011.

MONTLLOR, C. B.; MAXMEN, A.; PURCELL, A. H. Facultative bacterial endosymbionts benefit pea aphids Acyrthosiphon pisum under heat stress. **Ecological Entomology,** London, v. 27, n. 2, p. 189-195, 2002.

MORAN, N. A.; DEGNAN, P. H.; SANTOS, S. R.; DUNBAR, H. E.; OCHMAN, H. The players in a mutualistic symbiosis: Insects, bacteria, viruses, and virulence genes. **Proceedings of the National Academy of Sciences of the United States of America,** Washington, v. 102, n. 47, p. 16919-16926, 2005.

OLIVER, K. M.; DEGNAN, P. H.; BURKE, G. R.; MORAN, N. A. Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. **Annual Review of Entomology**, Standford, v.55, p.247-266, 2010.

OLIVER, K. M.; DEGNAN, P. H.; HUNTER, M. S.; MORAN, N. A. Bacteriophages encode factors required for protection in a symbiotic mutualism. **Science,** Oxford, v. 325, n. 5943, p. 992-994, 2009.

OLIVER, K. M.; MORAN, N. A.; HUNTER, M. S. Variation in resistance to parasitism in aphids is due to symbionts not host genotype. **Proceedings of the National Academy of Sciences of the United States of America,** Washington, v. 102, n. 36, p. 12795-12800, 2005.

_____. Costs and benefits of a superinfection of facultative symbionts in aphids. **Proceedings of the Royal Society B-Biological Sciences,** London, v. 273, n. 1591, p. 1273-1280, 2006.

OLIVER, K. M.; OLIVER, K. M.; RUSSELL, J. A.; MORAN, N. A.; HUNTER, M. S. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. **Proceedings of the National Academy of Sciences of the United States of America,** Washington, v. 100, n. 4, p. 1803-1807, 2003.

OLIVER, K. M.; SMITH, A. H.; RUSSELL, J. A. Defensive symbiosis in the real world - advancing ecological studies of heritable, protective bacteria in aphids and beyond. **Functional Ecology,** West Sussex, v. 28, n. 2, p. 341-355, 2014.

RUSSELL, J. A.; MORAN, N. A. Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. **Proceedings of the Royal Society B-Biological Sciences,** London, v. 273, n. 1586, p. 603-610, 2006.

RUSSELL, J. A.; WELDON, S.; SMITH, A. H.; KIM, K. L.; HU, Y.; LUKASIK, P.; DOLL, S.; ANASTOPOULOS, I.; NOVIN, M.; OLIVER, K. M. Uncovering symbiont-driven genetic diversity across North American pea aphids. **Molecular Ecology,** New York, v. 22, n. 7, p. 2045-2059, 2013.

SANDSTROM, J. P.; RUSSELL, J. A.; WHITE, J. P.; MORAN, N. A. Independent origins and horizontal transfer of bacterial symbionts of aphids. **Molecular Ecology,** New York, v. 10, n. 1, p. 217-228, 2001.

SIMON, J.-C.; BOUTIN, S.; TSUCHIDA, T.; KOGA, R.; LE GALLIC, J. F.; FRANTZ, A.; OUTREMAN, Y.; FUKATSU, T. Facultative Symbiont Infections Affect Aphid Reproduction. **Plos One,** San Francisco, v. 6, n. 7, 2011. Available at: < DOI: 10.1371/journal.pone.0021831>.

SMITH, A. H.; LUKASIK, P.; O'CONNOR, M. P.; LEE, A.; MAYO, G.; DROTT, M. T.; DOLL, S.; TUTTLE, R.; DISCIULLO, R. A.; MESSINA, A.; OLIVER, K. M.; RUSSELL, J. A. Patterns, causes and consequences of defensive microbiome dynamics across multiple scales. **Molecular Ecology,** New York, v. 24, n. 5, p. 1135-1149, 2015.

SU, Q.; ZHOU, X.; ZHANG, Y. Symbiont-mediated functions in insect hosts. **Communicative & Integrative Biology, New York,** v. 6, n. 3, p. e23804-e23804, 2013.

TELESNICKI, M. C.; GHERSA, C. M.; MARTINEZ-GHERSA, M. A.; ARNEODO, J. D. Molecular identification of the secondary endosymbiont *Hamiltonella defensa* in the rose-grain aphid Metopolophium dirhodum. **Revista Argentina De Microbiologia,** Buenos Aires, v. 44, n. 4, p. 255-258, 2012.

TSUCHIDA, T.; KOGA, R.; HORIKAWA, M.; TSUNODA, T.; MAOKA, T.; MATSUMOTO, S.; SIMON, J. C.; FUKATSU, T. Symbiotic bacterium modifies aphid body color. **Science,** Oxford, v. 330, n. 6007, p. 1102-1104, 2010.

TSUCHIDA, T.; KOGA, R.; MATSUMOTO, T.; FUKATSU, T. Secondary endosymbiotic microbiota of the pea aphid, *Acyrthosiphon pisum,* in natural populations. In: SYMPOSIUM ON APHIDS , 2001, Rennes. **Aphids in a a new Millennium: Proceedings of the Sixth International Symposium on Aphids**, 2001, p. 93-96.

TSUCHIDA, T.; KOGA, R.; SHIBAO, H.; MATSUMOTO, T.; FUKATSU, T. Diversity and geographic distribution of secondary endosymbiotic bacteria in natural populations of the pea aphid, *Acyrthosiphon pisum*. **Molecular Ecology,** New York, v. 11, n. 10, p. 2123-2135, 2002.

WAGNER, S. M.; MARTINEZ, A.J.; RUAN, Y.; KIM, K.L.; LENHART, A.; DEHNEL, A.C.; OLIVER, K.M.; WHITE, J.A. Facultative endosymbionts mediate dietary breadth in a polyphagous herbivore. **Functional Ecology**, West Sussex, v.29, n.11, p. 1402-1410, 2015.

WULFF, J.A.; BUCKMAN, K.A.; WU, K.; HEIMPEL, G.E.; WHITE, J.A. The endosymbiont arsenophonus is widespread in soybean aphid, *Aphis glycines*, but does not provide protection from parasitoids or a fungal pathogen. **PLOS One,** San Franscico, v.8, p. e62145, 2013.

XIE, J.; BUTLER, S.; SANCHEZ, G.; MATEOS, M. Male killing *Spiroplasma* protects *Drosophila melanogaster* against two parasitoid wasps. **Heredity,** London, v. 112, n. 4, p. 399-408, 2014.

ZYTYNSKA, S.E.; WEISSER, W.W. The natural occurrence of secondary bacterial symbionts in aphids. **Ecological Entomology,** London, v. 41, n. 1, p. 13-26, 2016.

6 NO FITNESS COSTS ASSOCIATED WITH INFECTIONS OF *Aphis (Toxoptera) citricidus* BY THE SECONDARY SYMBIONT *Spiroplasma* ON TWO HOST PLANTS

Abstract

Aphids are associated with many bacterial symbionts, with the obligate symbiont *Buchnera aphidicola* supplementing the host diet with essential amino acids and vitamins, while secondary symbionts inducing different host phenotypes. Aphids can harbor several secondary symbionts that alter important aphid-related ecological traits, such as defense against natural enemies, heat tolerance and host plant utilization. One of these secondary symbionts, *Spiroplasma*, is well known in *Drosophila* as a sex modulator and by interacting with the host immune system. However, little is known on the effects of *Spiroplasma* on aphids, such as its influence on the host immune defense against fungi and on host plant utilization. Aphid infections by *Spiroplasma* are low and few aphid species were reported to be infected with this secondary symbiont. Thus, we investigated the association of *Spiroplasma* with the tropical aphid *Aphis (Toxoptera) citricidus* (Kirkaldy) through comparative biology experiments on two host plants with different nutritional value to the aphid. We demonstrate *Spiroplasma* induced no significant fitness costs to *A. citricidus* on either host plant.as no changes in the fitness traits we assessed were observed. *Spiroplasma* infection only induced sutle changes on host longevity and fecundity. Therefore, we concluded *Spiroplasma* established a neutral interaction with *A. citricidus* under the selection pressure we tested, and argue on stress conditions that could better demonstrate the role of *Spiroplasma* in *A. citricidus* bioecology and associated costs involved.

Keywords: Aphid-symbionts interactions; Comparative biology; Host plant suitability; Nutritional ecology

6.1 Introduction

Aphids harbor many bacterial symbionts. *Buchnera aphidicola* is the obligate symbiont of aphids, supplementing the host diet with essential amino acids and vitamins (DOUGLAS, 1998; HANSEN; MORAN, 2014). Aphid - *B. aphidicola* association has been under investigation for a long period of time (BUCHNER, 1965; MORAN; DEGNAN, 2006; DOUGLAS, 2009; VOGEL; MORAN, 2011; MORAN; YUN, 2015), but is the diversity of secondary symbionts and the ecological traits they influence that holds the most surprising discoveries (RUSSELL; MORAN, 2006; MORAN, 2007; OLIVER et al., 2008; BURKE; MORAN, 2011; FERRARI; VAVRE, 2011; McLEAN et al., 2011; SIMON et al., 2011; FERRARI et al., 2012; LUKASIK et al., 2013b; PECCOUD et al., 2014; SUGIO et al., 2015).

The secondary symbionts so far identified infecting aphids are *Arsenophonus, Hamiltonella defensa, Regiella insecticola, Rickettsia, Rickettisiella viridis, Serratia*

symbiotica, *Spiroplasma*, *Wolbachia* and the X-type symbiont. These bacteria may produce phenotypic responses ranging from defense against natural enemies to host plant use (SU; ZHOU; ZHANG, 2013). Most of the aphid-associated secondary symbionts were partially or exclusively associated with host aphid defensive behavior. *H. defensa* and *S. symbiotica* are linked to resistance to parasitoids (OLIVER et al., 2003), *R. insecticola*, *R. viridis*, *Rickettsia*, *Spiroplasma* and X-type are associated with protection against fungal pathogens (FERRARI et al., 2001; LUKASIK et al., 2013a,b; TSUCHIDA et al., 2014; HEYWORTH; FERRARI, 2015), and *R. viridis* indirectly protects the host against predatory insects (TSUCHIDA et al., 2014). In addition, *Wolbachia* is known to confer protection against viruses in *Drosophila* and mosquito, but the role of this bacterium in aphids remains to be explored (TEIXEIRA; FERREIRA; ASHHBURNER, 2008; MOREIRA et al., 2009; AUGUSTINOS et al., 2011).

Besides their defensive role in associated aphids, *R. insecticola*, *S. symbiotica* and X-type were also linked with tolerance to heat stress (MONTLLOR; MAXMEN; PURCELL; 2002; RUSSELL; MORAN, 2006; HEYWORTH; FERRARI, 2015), *Spiroplasma*, *Arsenophonus* and *Wolbachia* with reproductive alterations (BOURTZIS; MILLER, 2006), and *R. insecticola* and *Arsenophonus* with host plant use (LEONARDO; MUIRU, 2003; FERRARI et al., 2006).

Spiroplasma is a particular interesting genus of bacteria since its members may be associated as ecto and endocytobionts with a variety of plants and arthropods (ANBUTSU; FUKATSU, 2011). Remarkably, all member of the genus *Spiroplasma* are obligatory associated with insects in some part of their life, either as commensals, pathogens or mutualists (GASPARICH, 2002). *Spiroplasma* are long known as sex ratio distorters in *Drosophila* species (EBBERT, 1991), and to participate in host defense against nematode and parasitic wasps in *Drosophila* (JAENIKE et al., 2010; XIE; VILCHEZ; MATEOS, 2010).

In aphids, *Spiroplasma* infections were first reported in *A. pisum* in 2001 (FUKATSU et al., 2001), and a diversity of aphid phenotypes have been described since then in association with this symbiont. *Spiroplasma* were reported to reduce fecundity and longevity (FUKATSU et al., 2001; SIMON et al., 2011), induce ‘male-killing’ in the sexual generation (SIMON et al., 2011), confer protection against fungi in *A. pisum* (LUKASIK et al., 2013), and to broaden host plant range in aphids (TSUCHIDA; KOGA; FUKATSU, 2004). However, symbiont-induced phenotypes are usually context-dependent, being affected by the symbiont strain, aphid species/clone, host plant, biogeography and abiotic factors (MONTLLOR; MAXMEN; PURCELL; 2002; LEONARDO; MUIRU, 2003; BRADY; WHITE, 2013; RUSSELL et al., 2013).

Host plant and biogeography are the two most inconclusive factors that influence symbiont diversity and distribution (McLEAN et al., 2011), and data on biogeography been inconsistent, mainly due to unbalanced research efforts focusing in few aphids species constrained to a narrow global range (TSUCHIDA et al., 2002; BRADY et al., 2014; FORISTER et al., 2015; ZYTYNSKA; WEISSER, 2016). *Spiroplasma* has been rarely investigate in South America, although there are indications it may be more prevalent in tropical areas (ZYTYNSKA; WEISSER, 2016). Indeed, there are very few studies on aphid species with a narrow host range and/or on tropical areas.

Aphis (Toxoptera) citricidus (Kirkaldy) is a common aphid in tropical areas, following its preferred host plant, *Citrus*. Its diet breath is restricted to *Citrus* plants and a few relative species, and acquired increased economic pest status by vectoring the *citrus tristeza virus* (CTV) to citrus plants (HALBERT; BROWN, 1996; HALBERT et al., 2004). Although there are few studies on secondary symbiont diversity associated with aphids in tropical areas, our previous investigations on *A. citricidus* association with microbial secondary symbionts indicated *Spiroplasma* as the most common symbiont associated with this aphid (see Chapter 5). Thus, considering the possible aphid host phenotypes that may arise from interactions with *Spiroplasma*, we wondered if *Spiroplasma* infection would result in any fitness costs to the aphid *A. citricidus*, and if it would influence host plant use by assessing several aphid fitness traits on a highly suitable host plant (*Citrus sinensis*) as compared to a less suitable host plant (*Murraya paniculata*) (see Chapter 2).

6.2 Material and methods

6.2.1 Aphid isolines

Adult aphids were collected on unmanaged *Citrus* sp. groves and on *Murraya paniculata* in different localities within the municipality of Piracicaba, SP, Brazil. Aphids were brought to the laboratory and classified following the key for wingless adults provided by Halbert and Brown (2011).

After species identification, specimens of *A. citricidus* were individually placed on *Citrus sinensis* seedlings inside rearing cages (50 cm high x 15 cm diameter) containing two lateral openings closed with cloth for ventilation. Cages were maintained under controlled conditions (25±2°C; 60±10% UR; 14 h photophase) for aphid reproduction and development. Isolines that

produced second generation were used for symbiont screening, as previously described (Chapter 5). However, all isolines established carrying *Spiroplasma* were also infected by another secondary symbiont. Therefore, an isoline carrying only *Buchnera* was selected and infected with *Spiroplasma* donated by a laboratory-established isoline of *A. aurantii*.

6.2.2 Artificial infection of *Aphis (Toxoptera) citricidus* with *Spiroplasma* by microinjection

An isoline of *A. citricidus* carrying only the obligate symbiont *Buchnera aphidicola* was selected for the establishment of a sister isoline infected with *Spiroplasma* for comparative analysis of fitness traits between *Spiroplasma*-infected (Ac-BS) and *Spiroplasma*-free (Ac-B) sister lines and assessment of fitness costs. Assessments of fitness traits were done on two host plants. *Spiroplasma* was obtained from a donor isoline of *A. aurantii*, exclusively infected with this secondary symbiont.

The *Spiroplasma*-free isoline of *A. citricidus* was divided into two groups, and third instars of one of these groups were microinjected with hemolymph collected from the *Spiroplasma*-infected *A. aurantii* isoline to originate the *Spiroplasma*-infected sister isoline (Ac-BS). Third instars of the remaining group of *A. citricidus* were microinjected with PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) as control, for the establishment of the so-called *Spiroplasma*-free isoline (Ac-B).

Microinjections were performed with ultra-thin needles made from borosilicate glass capillary tubes (1 mm e.d. x 0.78 i.d. - cat# BF100-78-10, Sutter Instrument Company). The borosilicate glass capillary tubes were shaped into glass needles using a P-1000 Flaming/Brow Micropipette Puller (Sutter Instruments Inc.) with heat set to 550°C, pull at 60 U, velocity at 60s, pressure at 500 U, and time at 250 U. Needles were sharpened using a 1300M Beveler (World Precision Instruments Inc.) for 30s under 45°. Injections were carried by coupling the needles to a PV830 Pneumatic Pico Pump (World Precision Instruments Inc.) connected to a nitrogen cylinder. Hemolymph was collected using a glass needle after removal of a leg of *Spiroplasma*-infected *A. aurantii* adults. The same needle was immediately used to microinject a third instar *Spiroplasma*-free *A. citricidus*. Injections were made on the lateral side of the abdomen of *A. citricidus*. Hemolymph collected from one *Aphis (Toxoptera) aurantii* Boyer de Fonscolombe adult was injected into a single third instar of *A. citricidus*. The needle was replaced after every three injections.

Newly-injected third instars of *A. citricidus* (Ac-BS and Ac-B) were maintained under controlled conditions (25±2°C; 60±10% UR; 14:10 h) on *Citrus sinensis* var. Pêra (sweet orange) as a host plant until they completed their development. Adults obtained from these plants were transferred to *Citrus limonia* (rangpur) for several generations before their use in further experiments. Nymphs from Ac-B and Ac-BS isolines on rangpur were sampled, subjected to DNA extraction and diagnostic PCR for confirmation of the infection status by *Spiroplasma*. DNA extraction and diagnostic PCR confirmation of *Spiroplasma* infection were performed as earlier described (Chapter 5).

6.2.3 Biology of *Aphis* (*Toxoptera)citricidus* Ac-BS and Ac-B isolines on sweet orange and orange jasmine

Spiroplasma-infected (Ac-BS) and *Spiroplasma*-free (Ac-B) isolines of *A. citricidus* maintained on rangpur were transferred to orange jasmine (*M. paniculata*) and sweet orange (*C. sinensis* var. Pêra). They were reared on these host plants for six generations before biological observations were made.

Plastic cups (9.5 x 7.5cm) containing a ventilation hole at the bottom closed with cloth were used as rearing cages to isolate aphids in new flushes of each host plant to allow for the observation of their development. One-hundred nymphs (0-24h-old) were placed in a new flush within each rearing cage in each one of the host plants studied (orange jasmine or sweet orange), and their time to adult development and survivorship were recorded. Four replicates were made for each isoline in each host plant, with each 100 nymphs as an experimental unit.

For observation of adult reproduction and longevity, 20 newly-emerged adults from each isoline in each one of the host plants were individually caged on a new host plant similar to the host plant they fed as nymphs. Female fecundity and longevity were recorded daily by counting and removing new nymphs until the adult female died.

All colony maintenance and experiments were conducted under controlled conditions (25±2°C; 60±10% RH; 14 h photophase).

6.2.4 Statistical analysis

Data normal distribution was checked with Shapiro test and homocedasticity with Bartlett test. To attend normal distribution, data on adult fecundity and longevity on sweet orange were transformed with boxcox algorithm $\lambda = 0.4$. The effect of *Spiroplasma* infection on

the biological parameters observed for *A. citricidus* within each host plant was compared using *t* test ($p < 0.05$). All statistical analyses were carried using the R Statistical software (v.3.2.1) (R CORE TEAM, 2015).

The biological data obtained for Ac-BS and Ac-B isolines of *A. citricidus* in each one of the host plants were also used to estimate life table parameters, such as the mean time between generations (IMG), the net reproductive rate (*Ro*), the intrinsic rate of increase (*rm*), the time for duplication (TD) and the finite growth rate (λ). Life table parameters were compared by using a paired *t* test ($p < 0.05$) based on Jackknife estimates using SAS® (SAS INSTITUTE 2015) (MAIA; LUIZ, 2006).

6.3 Results

Spiroplasma had no effect on most of the biological parameters of *A. citricidus* studied regardless the host plant (sweet orange or orange jasmine) (Table 6.1). Nymph development time was the only parameter affected by *Spiroplasma* infection, leading to a small delay in immature development in infected aphids when reared on sweet orange (Table 6.1).

Likewise, life table parameters between Ac-BS and Ac-B isolines of *A. citricidus* were similar within each host plant (Table 6.2). However, values obtained for *A. citricidus* on sweet orange, regardless of *Spiroplasma* infection, indicated sweet orange as a much better host to *A. citricidus* than orange jasmine, as aphids took a much longer period of time to double their population and had much lower finite growth rates in orange jasmine if compared to sweet orange (Table 6.2).

Table 6.1 - Nymph development time and survivorship, percentage of reproductive females and adult longevity and fecundity of *Spiroplasma*–free (Ac-B) and *Spiroplasma*–infected *A. citricidus* (Ac-BS) isolines reared on sweet orange and orange jasmine as host plants (25±2°C; 60±10% RH; 14 h photophase)

Treatment	**Ac-B**	**Ac-BS**	**Statistical test**	***df***	***p***
Sweet Orange					
Nymph development time (days)	6.1 ± 0.06 b	6.5 ± 0.09 a	*t*= -3.2	5, 5	<0.05
Nymph survivorship (%)	83.7 ± 2.59 a	88.2 ± 4.97 a	*t*= -0.8	4, 5	0.46
Reproductive female (%)	95 a	95 a	χ^2= 0	1	1
Adult longevity (days)	8.8 ± 1.16 a	7.2 ± 1.39 a	*t*= 1.1	34, 7	0.26
Adult fecundity (Nº nymph/♀)	20.1 ± 2.57 a	23.6 ± 4.97 a	*t*= -0.07	30, 5	0.94
Orange jasmine					
Nymph development time (days)	6.5 ± 0.23 a	6.5 ± 0.18 a	*t*= -0.2	5, 6	0.86
Nymph survivorship (%)	69.5 ± 3.66 a	72.7 ± 3.06 a	*t*= -0.68	5, 8	0.52
Reproductive female (%)	90 a	85 a	χ^2= 0.29	1	0.58
Adult longevity (days)	10.0 ± 1.83 a	8.7 ± 1.7 a	*t*= 0.2	32, 9	0.79
Adult fecundity (Nº nymph/♀)	23.1 ± 4.34 a	20.9 ± 4.63 a	*t*= 0.15	32, 9	0.87

* Means with the same letter in line are not significantly different at $p > 0.05$ using t-test.

Table 6.2 - Mean time between generations (IMG), the finite growth rate (λ), the intrinsic rate of increase (*rm*), the net reproductive rate (*Ro*) and the time for duplication (TD) of *Spiroplasma*-free (Ac-B) and *Spiroplasma*-infected (Ac-BS) isolines of *Aphis (Toxoptera) citricidus* reared on sweet orange and orange jasmine as host plants (25±2°C; 60±10% RH; 14 h photophase)

Treatment	**Ac-B**	**Ac-BS**
Sweet Orange		
IMG	3.1 ± 0.25 a	2.9 ± 0.52 a
λ	2.4 ± 0.16 a	2.6 ± 0.49 a
rm	0.8 ± 0.06 a	0.9 ± 0.16 a
Ro	16.6 ± 2.13 a	20.8 ± 4.37 a
TD	0.7 ± 0.05 a	0.6 ± 0.09 a
Orange jasmine		
IMG	4.4 ± 0.33 a	4.1 ± 0.68 a
λ	1.9 ± 0.07 a	1.9 ± 0.19 a
rm	0.6 ± 0.04 a	0.6 ± 0.09 a
Ro	15.9 ± 2.99 a	15.0 ± 3.33 a
TD	1.1 ± 0.07 a	1.0 ± 0.14 a

* Means with the same letter in line are not significantly different at $p > 0.05$ using t-test with Jacknife replicates.

6.4 Discussion

In here we demonstrated *Spiroplasma* induced subtle changes on the fitness traits assessed for *A. citricidus*, independently of the host plant tested. Nymph development time of Ac-BS on sweet orange was the only trait to be marginally affected by *Spiroplasma* infection, with aphids showing a slower growth. The lack of any significant effects on the host aphid fitness traits under the ecological conditions tested and the frequency of occurrence we detected this symbiont in natural populations of *A. citricidus* and *A. aurantii* at the same ecological niche (see Chapter 5), suggest an efficient mechanism for vertical transmission of *Spiroplasma* and its adaptation to *A. citricidus* as a host.

Vertical transmission occurs through maternal germlines, and usually maternally-inherited symbionts must benefit their host fitness to guarantee their maintenance and spread in the host population. Reproductive parasites usually benefit the reproductive fitness of infected

females by either rendering uninfected females infertile when mating infecting males (cytoplasmic incompatibility), favoring female-biased sex ratio in infected females, providing increased resistance against diseases, and adding little fitness cost to the associated host (STOUTHAMER; BREEWER; HURST, 1999; DURON et al., 2008; ENGELSTAEDTER; HURST, 2009; JAENIKE et al., 2010). However, secondary symbionts other than reproductive parasites that are also vertically transmitted must establish relationships in which infected hosts will gain fitness benefits over uninfected hosts to guarantee their persistence in the host population (OLIVER; MORAN; HUNTER, 2006; RUSSELL; MORAN, 2006; OLIVER et al., 2008). It is very unlikely that *Spiroplasma* assumes a role of a reproductive parasite in *A. citricidus*, since reproduction of this species in tropical areas is exclusively by telytokous parthenogenesis.

Moreover, benefits to the host and persistence of the interaction will depend on the evolutionary history of the host-symbiont. In the case of aphids, most of the facultative associated symbionts share recent evolutionary histories with their hosts as the benefits symbionts provide to their hosts are highly influenced by several biotic and abiotic conditions (FERRARI; SCARBOROUGH; GODFRAY; 2007; VORBURGER; GOUSKOV, 2011; LUKASIK et al., 2013a; OLIVER et al., 2008; CASS et al., 2016). Yet, there is a cost associated to harbor one or more secondary symbiont, and persistence and spread of a new infection in a host population depends on the trade-offs between the costs of the maintenance of the symbiont and the benefits the symbiont provide to the host (DYKSTRA et al., 2014; OLIVER; SMITH; RUSSELL, 2014; CAYETANO et al., 2015).

The lack of fitness costs in the association of *A. citricidus* – *Spiroplasma* demonstrates that the transfection of a *Spiroplasma* strain originally infecting *A. aurantii* into *A. citricidus* indicates the horizontal transmission of this symbiont may be a natural event in the field where these populations co-occur. Such natural transmission of *Spiroplasma* in between both aphid species would be possible as these species can co-occur in the same host plants and are attacked by the same natural enemies, some of the conditions required for natural horizontal transmission to occur (DURON et al. 2010). The absence of fitness costs in transfected *A. citricidus* and the fast establishment and maintenance of the infection status in laboratory conditions are also indicatives the *Spiroplasma* line transferred to *A. citricidus* was well-adapted to the host immune system (SCHMID-HEMPEL, 2005; BRIGHT; BULGHERESI, 2010).

The successful establishment of an infection by *Spiroplasma* in *A. citricidus* and the persistent association through vertical transmission could be associated with the fact that *A. citricidus* and *A. aurantii* share similar ecology and provide a similar physiological environment

to *Spiroplasma*. Interspecific transfections were highly successful when species shared similar physiological enviroments and ecology (PERLMAN; JAENIKE, 2003; LONGDON et al. 2011; LUKASIK et al., 2015).

We have to consider that the neutral outcome of the interaction between *Spiroplasma* and *A. citricidus* may be due to the fact infected aphids were not exposed to a selection pressure, such as a fungal pathogen and/or an abiotic stress condition, in which the costs of infection to the host could be perceived, as often observed for infected aphids under some stress conditions (OLIVER et al. 2008).

As a conclusion, *Spiroplasma* infection did not yield any fitness cost to *A. citricidus* as no changes in the assessed fitness traits were observed in response to the nutritional quality of the host plant. However, comparative proteomic analysis of *Spiroplasma*-infected and healthy aphids on host plants with different nutritional value to the aphid indicated *Spiroplasma* may have a strong influence on the host nutritional immunity (see Chapter 8). Therefore, the phenotype induced by *Spiroplasma* infection of *A. citricidus* remains to be determined and will require further investigations under different selection pressures, such as those that challenge the host immune system.

6.5 Conclusions

- *Spiroplasma* delays the immature development of *Aphis (Toxoptera) citricidus* in sweet orange as compared to orange jasmine;
- *Spiroplasma* does not affect adult fecundity or longevity of *Aphis (Toxoptera) citricidus*, regardless the host plant used;
- Life table parameters of *Aphis (Toxoptera) citricidus* on sweet orange and orange jasmine are not affected by *Spiroplasma* infection;
- No significant fitness costs were induced in *Aphis (Toxoptera) citricidus* by *Spiroplasma* infection under nutritional stress.

Reference

ANBUTSU, H.; FUKATSU, T. *Spiroplasma* as a model insect endosymbiont. **Environmental Microbiology Reports,** West Sussex, v. 3, n. 2, p. 144-153, 2011.

AUGUSTINOS, A.A.; SANTOS-GARCIA, D.; DIONYSSOPOULOU, E.; MOREIRA, M.; PAPAPANAGIOTOU, A.; SCARVELAKIS, M.; DOUDOUMIS, V.; RAMOS, S.; AGUIAR, A.F.; BORGES, P.A.V.; KHADEM, M.; LATORRE, A.; TSIAMIS, G.; BOURTZIS, K. Detection and characterization of *Wolbachia* infections in natural populations of aphids: is the hidden diversity fully unraveled? **Plos One**, San Francisco, v. 6, n. 12, p. 2011. Available at: < doi: 10.1371/journal.pone.0028695>.

BOURTZIS, K.; MILLER, T. A. **Insect symbiosis.** Volume 2. Boca Raton: CRC Press, p. 304, 2006.

BRADY, C.M.; ASPLEN, M.K.; DESNEUX, N.; HEIMPEL, G.E.; HOPPER, K.R.; LINNEN, C.R.; OLIVER, K.M.; WULFF, J.A.; WHITE, J.A. Worldwide populations of the aphid *Aphis craccivora* are infected with diverse facultative bacterial symbionts. **Microbial Ecology,** New York, v. 67, n. 1, p. 195-204, 2014.

BRADY, C. M.; WHITE, J. A. Cowpea aphid (*Aphis craccivora*) associated with different host plants has different facultative endosymbionts. **Ecological Entomology,** West Sussex, v. 38, n. 4, p. 433-437, 2013.

BRIGHT, M.; S. BULGHERESI. A complex journey: transmission of microbial symbionts. **Nature Review Microbiology**, London, v.8, n.3, p.218–230, 2010.

BUCHNER, P. **Endosymbiosis of animals with plant microorganisms**. New Jersey: Interscience Publishers/John Wiley, p. 909, 1965.

BURKE, G. R.; MORAN, N. A. Responses of the pea aphid transcriptome to infection by facultative symbionts. **Insect Molecular Biology,** West Sussex, v. 20, n. 3, p. 357-365, 2011.

CASS, B.N.; HIMLER, A.G.; BONDY, E.C.; BERGEN, J.E.; FUNG, S.K.; KELLY, S.E.; HUNTER, M.S. Conditional fitness benefits of the *Rickettsia* bacterial symbiont in an insect pest. **Oecologia,** Heidelberg, v. 180, n. 1, p. 169-179, 2016.

CAYETANO, L.; ROTHACHER, L.; SIMON, J.C.; VORBURGER, C. Cheaper is not always worse: strongly protective isolates of a defensive symbiont are less costly to the aphid host. **Proceedings of the Royal Society B-Biological Sciences,** Edinburgh, v. 282, n. 1799, 2015. Available at: < DOI: 10.1098/rspb.2014.2333>

DOUGLAS, A. E. Nutritional interactions in insect-microbial symbioses: Aphids and their symbiotic bacteria *Buchnera*. **Annual Review of Entomology,** Stanford, v. 43, p. 17-37, 1998.

_____. The microbial dimension in insect nutritional ecology. **Functional Ecology**, Oxford, v. 23, n. 1, p. 38-47, 2009.

DURON, O.; BOUCHON, D.; BOUTIN, S.; BELLAMY, L.; ZHOU, L.; ENGELSTÄDTER, J.; HURST, G.D. The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. **BMC Biology**, London, v.6, n.27, 2008. Available at: <DOI: 10.1186/1741-7007-6-27>.

DYKSTRA, H.R.; WELDON, S.R.; MARTINEZ, A.J.; WHITE, J.A.; HOPPER, K.R.; HEIMPEL, G.E.; ASPLEN, M.K.; OLIVER, K.M. Factors Limiting the Spread of the Protective Symbiont *Hamiltonella defensa* in *Aphis craccivora* Aphids. **Applied and Environmental Microbiology,** Washington, v. 80, n. 18, p. 5818-5827, 2014.

EBBERT, M. A. The interaction phenotype in the *Drosophia willistoni- Spiroplasma* symbiosis. **Evolution,** West Sussex, v. 45, n. 4, p. 971-988, 1991.

ENGELSTAEDTER, J.; HURST, G. D. D. The ecology and evolution of microbes that manipulate host reproduction. **Annual Review of Ecology Evolution and Systematics,** Palo alto, v. 40, p. 127-149, 2009.

FERRARI, J.; GODFRAY, H.C.; FAULCONBRIDGE, A.S.; PRIOR, K.; VIA, S. Population differentiation and genetic variation in host choice among pea aphids from eight host plant genera. **Evolution,** West Sussex, v. 60, n. 8, p. 1574-1584, 2006.

_____. Clonal variation and covariation in aphid resistance to parasitoids and a pathogen. **Evolution,** West Sussex, v. 55, n. 9, p. 1805-1814, 2001.

FERRARI, J.; SCARBOROUGH, C. L.; GODFRAY, H. C. J. Genetic variation in the effect of a facultative symbiont on host-plant use by pea aphids. **Oecologia,** Heidelberg, v. 153, n. 2, p. 323-329, 2007.

FERRARI, J.; VAVRE, F. Bacterial symbionts in insects or the story of communities affecting communities. **Philosophical Transactions of the Royal Society B-Biological Sciences,** London,v. 366, n. 1569, p. 1389-1400, 2011.

FERRARI, J.; WEST, J.A.; VIA, S.; GODFRAY, H.C.J. Population genetic structure and secondary symbionts in host-associated populations of the pea aphid complex. **Evolution,** West Sussex, v. 66, n. 2, p. 375-390, 2012.

FORISTER, M.L.; NOVOTNY, V.; PANORSKA, A.K.; BAJE, L.; BASSET, Y.; BUTTERILL, P.T.; CIZEK, L.; COLEY, P.D.; DEM, F.; DINIZ, I.R.; DROZD, P.; FOX, M.; GLASSMIRE, A.E.; HAZEN, R.; HRCEK, J.; JAHNER, J.P.; KAMAN, O.; KOZUBOWSKI, T.J.; KURSAR, T.A.; LEWIS, O.T.; LILL, J.; MARQUIS, R.J.; MILLER, S.E.; MORAIS, H.C.; MURAKAMI, M.; NICKEL, H.; PARDIKES, N.A.; RICKLEFS, R.E.; SINGER, M.S.; SMILANICH, A.M.; STIREMAN, J.O.; VILLAMARÍN-CORTEZ, S.; VODKA, S.; VOLF, M.; WAGNER, D.L.; WALLA, T.; WEIBLEN, G.D.; DYER, L.A.The global distribution of diet breadth in insect herbivores. **Proceedings of the National Academy of Sciences of the United States of America,** Washington, v. 112, n. 2, p. 442-447, 2015.

FUKATSU, T.; TSUCHIDA, T.; NIKOH, N.; KOGA, R. *Spiroplasma* symbiont of the pea aphid, *Acyrthosiphon pisum* (Insecta : Homoptera). **Applied and Environmental Microbiology,** Washington, v. 67, n. 3, p. 1284-1291, 2001.

GASPARICH, G. E. *Spiroplasmas*: Evolution, adaptation and diversity. **Frontiers in Bioscience,** New York, v. 7, p. 619-640, 2002.

HALBERT, S.E.; BROWN, L.G. *Toxoptera citricida* (Kirkaldy), brown citrus aphid - identification, biology and management strategies. **Florida Department of Agriculture and Consumer Services Division of Plant Industry Entomology Circular,** Gainesville, v. 374, p. 6pp.-6pp., 1996.

HALBERT, S.E.; GENC, H.; CEVIK, B.; BROWN, L.G.; ROSALES, I.M.; MANJUNATH, K.L.; POMERINKE, M.; DAVISON, D.A.; LEE, R.F.; NIBLETT, C.L. Distribution and characterization of *Citrus tristeza virus* in South Florida following establishment of *Toxoptera citricida*. **Plant Disease,** St. Paul, v. 88, n. 9, p. 935-941, 2004.

HANSEN, A. K.; MORAN, N. A. The impact of microbial symbionts on host plant utilization by herbivorous insects. **Molecular Ecology,** Oxford, v. 23, n. 6, p. 1473-1496, 2014.

HEYWORTH, E.R.; FERRARI, J.A facultative endosymbiont in aphids can provide diverse ecological benefits. **Journal of Evolutionary Biology**, Basel, v. 28, n. 10, p. 1753-1760, 2015.

JAENIKE, J.; UNCKLESS, R.; COCKBURN, S.N.; BOELIO, L.M.; PERLMAN, S.J. Adaptation via symbiosis: recent spread of a *drosophila* defensive symbiont. **Science,** Washington, v. 329, n. 5988, p. 212-215, 2010.

LEONARDO, T. E.; MUIRU, G. T. Facultative symbionts are associated with host plant specialization in pea aphid populations. **Proceedings of the Royal Society B-Biological Sciences,** Edinburgh, v. 270, p. 209-212, 2003.

LONGDON, B.; HADFIELD, J.D.; WEBSTER, C.L.; OBBARD, D.J.; JIGGINS, F.M. Host phylogeny determines viral persistence and replication in novel hosts**. Plos Pathogens**, San Francisco, v.7, n. 9, p. e1002260, 2011. Available at: < DOI: 10.1371/journal.ppat.1002260>.

ŁUKASIK, P.; DAWID, M.A.; FERRARI, J.; GODFRAY, H.C. The diversity and fitness effects of infection with facultative endosymbionts in the grain aphid, *Sitobion avenae*. **Oecologia,** Heidelberg, v. 173, n. 3, p. 985-996, 2013.

LUKASIK, P.; GUO, H.; van ASCH, M.; FERRARI, J.; GODFRAY, H.C. Protection against a fungal pathogen conferred by the aphid facultative endosymbionts *Rickettsia* and *Spiroplasma* is expressed in multiple host genotypes and species and is not influenced by co-infection with another symbiont. **Journal of Evolutionary Biology,** West Sussex, v. 26, n. 12, p. 2654-2661, 2013a.

_____. Unrelated facultative endosymbionts protect aphids against a fungal pathogen. **Ecology Letters,** West Sussex, v. 16, n. 2, p. 214-218, 2013b.

_____. Horizontal transfer of facultative endosymbionts is limited by host relatedness. **Evolution**, West Sussex, v. 69, n.10, p. 2757–2766, 2015.

MAIA, A. D. H. N.; LUIZ, A. J. B. **Programa SAS para Análise de Tabelas de Vida e Fertilidade de Artrópodes: o Método Jackknife**. Jaguariúna- SP: EMBRAPA, 2006. 11 p. (Circular Tecnica, 33).

McLEAN, A.H.; van ASCH, M.; FERRARI, J.; GODFRAY, H.C. Effects of bacterial secondary symbionts on host plant use in pea aphids. **Proceedings of the Royal Society B-Biological Sciences,** Edinburgh, v. 278, n. 1706, p. 760-766, 2011.

MONTLLOR, C. B.; MAXMEN, A.; PURCELL, A. H. Facultative bacterial endosymbionts benefit pea aphids *Acyrthosiphon pisum* under heat stress. **Ecological Entomology,** London, v. 27, n. 2, p. 189-195, 2002.

MORAN, N. A. Symbiosis as an adaptive process and source of phenotypic complexity. **Proceedings of the National Academy of Sciences of the United States of America,** Washington, v. 104, p. 8627-8633, 2007.

MORAN, N.A.; DEGNAN, P.H. Functional genomics of Buchnera and the ecology of aphid hosts. **Molecular Ecology**, Oxford, v. 15, n. 5, p. 1251-1261, 2006.

MORAN, N. A.; YUN, Y. Experimental replacement of an obligate insect symbiont. **Proceedings of the National Academy of Sciences of the United States of America,** Washington, v. 112, n. 7, p. 2093-2096, 2015.

MOREIRA, L.A.; ITURBE-ORMAETXE, I.; JEFFERY, J.A.; LU, G.; PYKE, A.T.; HEDGES, L.M.; ROCHA, B.C.; HALL-MENDELIN, S.; DAY, A.; RIEGLER, M.H.; UGO, L.E.; JOHNSON, K.N.; KAY, B.H.; MCGRAW, E.A.; VAN DEN HURK, A.F.; RYAN, P.A.; O'NEILL, S.L.A Wolbachia Symbiont in *Aedes aegypti* Limits Infection with Dengue, *Chikungunya*, and *Plasmodium*. **Cell**, Cambridge, v. 139, n. 7, p. 1268-1278, 2009.

OLIVER, K. M.; CAMPOS, J.; MORAN, N.A.; HUNTER, M.S. Population dynamics of defensive symbionts in aphids. **Proceedings of the Royal Society B-Biological Sciences,** Edinburgh, v. 275, n. 1632, p. 293-299, 2008.

OLIVER, K.M.; RUSSELL, J.A.; MORAN, N.A.; HUNTER, M.S. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. **Proceedings of the National Academy of Sciences of the United States of America,** Washington, v. 100, n. 4, p. 1803-1807, 2003.

OLIVER, K.M.; SMITH, A.H.; RUSSELL, J.A. Defensive symbiosis in the real world - advancing ecological studies of heritable, protective bacteria in aphids and beyond. **Functional Ecology**, Oxford, v. 28, n. 2, p. 341-355, 2014.

OLIVER, K.M.; MORAN, N.A.; HUNTER, M.S. Costs and benefits of a superinfection of facultative symbionts in aphids. **Proceedings of the Royal Society B-Biological Sciences**, London, v. 273, n. 1591, p. 1273-1280, 2006.

PECCOUD, J.; BONHOMME, J.; MAHÉO, F.; DE LA HUERTA, M.; COSSON, O.; SIMON, J.C. Inheritance patterns of secondary symbionts during sexual reproduction of pea aphid biotypes. **Insect Science,** Richmond, v. 21, n. 3, p. 291-300, 2014.

PERLMAN, S. J., AND J. JAENIKE. Infection success in novel hosts: an experimental and phylogenetic study of *Drosophila*-parasitic nematodes. **Evolution**, West Sussex, v. 57, p. 544–557, 2003.

R CORE TEAM. R: A language and environment for statistical computing. **R Forundata on for Statistical Computing**, Vienna, 2015. Available at:<https://www.R-project.org/>.Acessed in: 04/2016.

RUSSELL, J. A.; MORAN, N. A. Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. **Proceedings of the Royal Society B-Biological Sciences,** Edinburgh, v. 273, n. 1586, p. 603-610, 2006.

_____. Horizontal transfer of bacterial symbionts: Heritability and fitness effects in a novel aphid host. **Applied and Environmental Microbiology,** v. 71, n. 12, p. 7987-7994, 2005.

RUSSELL, J.A.; WELDON, S.; SMITH, A.H.; KIM, K.L.; HU, Y.; LUKASIK, P.; DOLL, S.; ANASTOPOULOS, I.; NOVIN, M.; OLIVER, K.M. Uncovering symbiont driven genetic diversity across North American pea aphids. **Molecular Ecology**, Oxford, v. 22, n. 7, p. 2045-2059, 2013.

SAS INSTITUTE INC. **SAS language v.9.4.** 4th ed. Cary, SAS Institute, 2015.

SCHMID-HEMPEL, P. Evolutionary ecology of insect immune defenses. **Annual Review of Entomology**, Palo Alto, v. 50, p 529-551, 2005

SIMON, J.C.; BOUTIN, S.; TSUCHIDA, T.; KOGA, R.; LE GALLIC, J.; FRANTZ, A.; OUTREMAN, Y.; FUKATSU, T. Facultative Symbiont Infections Affect Aphid Reproduction. **Plos One,** San Franscico, v. 6, n. 7, 2011. Available at: <DOI: 10.1371/journal.pone.0021831 >.

STOUTHAMER, R.; BREEUWER, J. A. J.; HURST, G. D. D. *Wolbachia pipientis*: Microbial manipulator of arthropod reproduction. **Annual Review of Microbiology,** Palo Alto, v. 53, p. 71-102, 1999.

SU, Q.; ZHOU, X.; ZHANG, Y. Symbiont-mediated functions in insect hosts. **Communicative & Integrative Biology,** New York, v. 6, n. 3, 2013. Available at: < doi: 10.4161/cib.23804>.

SUGIO, A.; DUBREUIL, G.; GIRON, D.; SIMON, J.C. Plant-insect interactions underbacterial influence: ecological implications and underlying mechanisms. **Journal of Experimental Botany**, Oxford, v. 66, n. 2, p. 467-478, 2015.

TEIXEIRA, L.; FERREIRA, A.; ASHBURNER, M. The Bacterial Symbiont *Wolbachia* Induces Resistance to RNA Viral Infections in *Drosophila melanogaster*. **Plos Biology,** San Francisco, v. 6, n. 12, p. 2753-2763, 2008.

TSUCHIDA, T.; KOGA, R.; FUJIWARA, A.; FUKATSU, T. Phenotypic Effect of "*Candidatus* Rickettsiella viridis," a Facultative Symbiont of the Pea Aphid (*Acyrthosiphon pisum*), and Its Interaction with a Coexisting Symbiont. Applied and **Environmental Microbiology**, Washington, v. 80, n. 2, p. 525-533, 2014.

TSUCHIDA, T.; KOGA, R.; FUKATSU, T. Host plant specialization governed by facultative symbiont. **Science,** Washington, v. 303, n. 5666, p. 1989-1989, 2004.

_____. Diversity and geographic distribution of secondary endosymbiotic bacteria in natural populations of the pea aphid, *Acyrthosiphon pisum.* **Molecular Ecology,** Oxford, v. 11, n. 10, p. 2123-2135, 2002.

VOGEL, K.J.; MORAN, N.A. Effect of Host Genotype on Symbiont Titer in the Aphid *Buchnera* Symbiosis. **Insects**, Basel, v. 2, n. 3, p. 423-434, 2011.

VORBURGER, C.; GOUSKOV, A. Only helpful when required: a longevity cost of harbouring defensive symbionts. **Journal of Evolutionary Biology,** West Sussex, v. 24, n. 7, p. 1611-1617, 2011.

WAGNER, S. M.; MARTINEZ, A.J.; RUAN, Y.; KIM, K.L.; LENHART, .A.; DEHNEL, A.C.; OLIVER, K.M.; WHITE, J.A. Facultative endosymbionts mediate dietary breadth in a polyphagous herbivore. **Functional Ecology**, West Sussex, v.29, n.11, p. 1402-1410, 2015.

XIE, J.; VILCHEZ, I.; MATEOS, M. *Spiroplasma* Bacteria Enhance Survival of *Drosophila hydei* Attacked by the Parasitic Wasp *Leptopilina heterotoma*. **Plos One,** San Francisco, v. 5, n. 8, 2010. p. e12149. Available at: < doi:10.1371/journal.pone.0012149>.

ZYTYNSKA, S.E.; WEISSER, W.W. The natural occurrence of secondary bacterial symbionts in aphids. **Ecological Entomology**, London, v. 41, n. 1, p. 13-26, 2016.

7 TRANSCRIPTOME ANALYSIS OF *Spiroplasma*-INFECTED *Aphis (Toxoptera) citricidus* ON TWO HOST PLANTS

Abstract

Aphids are associated with several bacterial symbionts. The obligate symbiont *Buchnera aphidicola* is known for supplying the host with essential amino acids and vitamins, while the host provides a safe environment for *Buchnera* growth. This relationship is ancient and led to the establishment of a complementary metabolism between host and *Buchnera*. However, aphids may be associated with a variety of secondary symbionts that alter host ecology and interacts with host immune system. Secondary symbionts may affect host plant use and promote dietary specialization, leading to the establishment of host-adapted races. Moreover, host plant, aphids, and primary and secondary symbionts establish an interesting multipartite interaction. *Spiroplasma* is the least studied secondary symbiont due to low occurrence on aphids, but we observed high abundance of this symbiont on *Aphis (Toxoptera) citricidus* (Kirkaldy). Biology of *A. citricidus* infected with *Spiroplasma* showed a neutral relationship. However, identification of host-induced phenotypes by secondary symbionts may appear only under selection pressure. Therefore, we used transcriptome analysis to investigate the influence of *Spiroplasma* on *A. citricidus* at the molecular level. We also explored the effects of host suitability on the interaction *A. citricidus*-*Spiroplasma*. We found that *Spiroplasma* affected *A. citricidus* transcriptome, and the intensity of the effects on gene expression depends on host plant quality. *Spiroplasma* infections induced up-regulation of transcripts related to immune defense in aphids feeding on sweet orange, while on orange jasmine most of the up-regulated transcripts were linked to nutrition (digestive enzymes and detoxification pathways). Thus, we argue that *Spiroplasma* affects the transcriptome of *A. citricidus,* and regulation of gene expression differs in response to the suitability of the host plant to the host aphid.

Keywords: Gene expression; Host plant suitability; Immune defense; Nutrition; RNA-Seq

7.1 Introduction

Insect symbiosis is a growing research field and a lot of the research in this field is concentrated in the relationship among aphids and their bacterial symbionts. The association of aphids with their obligate symbiont *Buchnera aphidicola* is largely known, with host providing a protected environment, while the symbiont supplies the host with essential amino acids unavailable in phloem diet and vitamins (DOUGLAS, 1998; BAUMANN, 2005; NAKABACHI et al., 2014). Aphids and *B. aphidicola* share a congruent phylogeny due to their co-evolution through millions of years (NOVAKOVA et al., 2013), which led to the establishment of complementary metabolism (RAMSEY et al., 2010; HANSEN; MORAN 2011; RUSSELL et al., 2013). *B. aphidicola* is vertically transmitted, as bacteriocytes-containing *Buchnera* cells are transmitted to germlines during embryogenesis (WILKINSON; FUKATSU; ISHIKAWA, 2003).

However, aphids may also harbor many secondary symbionts that are not required for aphid survival and reproduction, but can alter many of aphid´s ecological traits (OLIVER et al., 2010; SU; ZHOU; ZHANG, 2013; WAGNER et al., 2015). Aphid-secondary symbiont relationship is dynamic and can fluctuate over time depending on the cost-benefit ratio of the association to the host (OLIVER et al., 2008). Aphid secondary symbionts are vertically transmitted, but horizontal acquisition is also a route of transmission to most of them (HENRY et al., 2013; LUKASIK et., 2015). In both cases transmission is always defective, and failures in secondary symbiont transmission are partially responsible for density fluctuations in their hosts (NISHIKORI et al., 2009). Thus, secondary symbionts are much more challenging for the host, as immune system regulation of secondary symbiont density will depend on the phenotype they induce in their hosts and the existing selection pressure the aphid-symbiont system will be exposed to (VIGNERON et al., 2014).

Moreover, host plant, aphids, and primary and secondary symbionts establish an interesting multipartite interaction. Secondary symbionts may allow the use of additional plant species and promote dietary specialization, leading to the establishment of host-adapted races (HANSEN; MORAN, 2014). Yet, plants are reported to limit secondary symbionts distribution, as secondary symbiont - aphid interactions are dependent on host plant nutritional quality and plant species diversity in a patch (HENRY et al., 2015).

Spiroplasma is the least studied aphid secondary symbiont, as aphid infections by this symbiont are generally low (ZYTYNSKA; WEISSER, 2016). However, *Spiroplasma* was demonstrated to affect the fecundity and longevity (FUKATSU et al., 2001), induce 'male-killing' in the sexual generation (SIMON et al., 2011) and confer protection against entomopathogenic fungi in the pea aphid *Acyrthosiphon pisum* (Harris) (LUKASIK et al., 2013), and broaden host range in aphids (TSUCHIDA; KOGA; FUKATSU, 2004). Remarkably, *Spiroplasma* was found to infect two tropical aphids *Aphis (Toxoptera) citricidus* (Kirkaldy) and *Aphis* (*Toxoptera*) *aurantii* Boyer de Fonscolombe in high frequencies in Brazil (see Chapter 5), without inducing any detectable fitness costs to *A. citricidus* (see Chapter 6). However, identification of host-induced phenotypes by secondary symbionts is challenging as phenotypes may appear only under the correct selection pressure (OLIVER, 2008). Thus, neutral fitness effects do not necessarily indicates neutral interactions.

Transcriptomic analysis is an interesting approach to study host-secondary symbiont interactions, as it allows the identification of molecular processes that are affected by symbiont infection (LIU et al., 2012). Thus, considering host plant suitability affects symbiont interactions, *Spiroplasma* interference with host plant use by aphids and the overall impact host

plant suitability may have on the aphid-symbiont system, we used differential gene expression to investigate the effects of *Spiroplasma* infection on the transcriptomics of *A. citricidus* on optimal and suboptimal host plants. We also compared the effects of host plant suitability on the aphid transcriptomics.

7.2 Material and Methods

7.2.1 Insect rearing

Two sister isolines of *A. citricidus*, Ac-B and Ac-BS, obtained as earlier described (see Chapter 6) were used in the experiments. Briefly, the Ac-B isoline of *A. citricidus*, exclusively infected with the obligate symbiont *B. aphidicola* was microinjected with hemolymph from *Spiroplasma*-infected *A. aurantii* for the establishment of a *Spiroplasma*-infected sister line. A subgroup of the *Spiroplasma*-free isoline of *A. citricidus* was microinjected with PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) to produce the control isoline (Ac-B).

Once established, subgroups of each Ac-B and Ac-BS isoline were maintained under controlled conditions (25±2°C; 60±10% UR; 14:10 h) on optimal, *Citrus sinensis* var. Pêra (sweet orange), and suboptimal, *Murraya paniculata* (orange jasmine), host plants for at least six generations before samples for RNA extraction were collected.

7.2.2 RNA isolation and transcriptome sequencing

Samples for RNA-seq analysis consisted of three replicates of 50 newly-emerged adult females (0-24h) for each isoline Ac-B and Ac-BS in each host plant.

Whole body aphids were subjected to RNA extraction using the Direct-zol™ RNA MiniPrep kit (Zymo Research), following the manufacturer´s instruction. After extraction, the precipitated RNA was re-suspended in nuclease-free water and RNA quality and quantity was measured using a Picodrop spectrophotometer 4.0.4. Each RNA sample was treated for the enrichment of mRNA by removing rRNA using the RiboMinus Eukaryote Kit (Invitrogen), following the manufacturer´s instructions. Samples were kept under -80°C and submitted to the "Laboratório de Biotecnologia Animal, Departamento de Zootecnia, Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo", for cDNA library preparation and sequencing in the HiSeq 2500 (Illumina) platform.

Briefly, for cDNA library construction mRNA samples were cleaved in 200 nucleotide fragments, which were tagged with random primers for transcriptase reverse reaction using TruSeq RNASample Prep kit v2 (Illumina). The cDNA obtained was purified with magnetic beads and washed with absolute ethanol. Afterwards, adenosine was added to the 3´-end of each fragment to guide the ligation of specific adaptors. Illumina specific adaptors allow each sample to be distinguished at the end of the run. cDNA libraries linked with specific adaptors were submitted to sequencing using HiSeq SBS kit V4 (Illumina) and HiSeq PE Cluster kit V4 cBot (Illumina) with 125bp *paired-end* protocol.

7.2.3 *De novo* assembly and transcriptome annotation

Read quality was verified using FastQC software v.0.11.3 (ANDREWS, 2010). All nucleotides with Phred quality score lower than Q=20 were trimmed with *seqtk* algorithm (available in: https://github.com/lh3/seqtk/blob/master/seqtk.c). Subsequently, reads were normalized using the *in silico normalization* algorithm and obtained reads were subjected to a *de novo* assembly with default parameters with a fixed *k*-mer size of 25 bp and minimum transcript size of 200bp using the Trinity package (GRABHERR et al., 2011). Reads from all libraries were used to perform one *de novo* reference assembly. Transcripts were verified for redundancy, using 95% similarity threshold to cluster similar contigs by running CD-Hit v.4.6 (LI; GODZIK, 2006).

Finally, selected contigs were used for annotation by similarity search against the GenBank non-redundant database using the BLASTX algorithm with minimum *e*-value of $1.0e^{-3}$ (ALTSCHUL et al., 1997). Gene ontology (GO) annotation was conducted using the Swiss-Prot database (http://www.uniprot.org/) and protein signatures were annotated using InterProScan with a cut-off value of $1.0e^{-5}$ (ZDOBNOV; APWEILER, 2001). Enzymatic code and annotation of metabolic pathways (KEGG – Kyoto Genes and Genomes) were derived from GO mapping (KANEHISA; GOTO, 2000). Annotation was performed using the BLAST2GO platform (CONESA et al., 2005).

7.2.4 RNA-seq experiments

The aphid transcriptome obtained from the *de novo* assembly was used as a reference transcriptome for RNA-seq experiments in which the effects of *Spiroplasma* infection was compared within each host plant (Ac-B vs. Ac-BS in orange jasmine; Ac-B vs Ac-BS in sweet

orange). RNA-seq experiments on the effects of the host plant on uninfected (Ac-B in orange jasmine vs. Ac-B in sweet orange) and infected (Ac-BS in orange jasmine vs. AcBS in sweet orange) aphids were also conducted. The effects of *Spiroplasma* on aphid gene expression feeding on orange jasmine and sweet orange was observed by analyzing the expression profile of genes that were differentially expressed in both conditions (Ac-B in orange jasmine vs. sweet orange, and Ac-BS in orange jasmine vs. sweet orange).

RNA-Seq experiments were obtained by counting the reads mapped against *de novo* assembly using Bowtie2 v.2.2.6 (LANGMEAD; SALZBERG, 2012), and the relative abundance of the transcripts was estimated by FPKM (Fragments Per Kilobase of transcript per Million mapped reads). FPKM was computed by RSEM script (LI; DEWEY, 2011). The RSEM matrix generated was exported to R Statistics software v.3.2.2 and subjected to analysis using a generalized linear model with negative binomial distribution on DESeq package (ANDERS; HUBER, 2010) in order to identify the differentially expressed transcripts. Selection of differentially expressed transcripts was based on a fold change difference > 2 and a p value < 0.05 using t-test.

7.3 Results

7.3.1 *Aphis (Toxoptera) citricidus* reference transcriptome

Sequencing of cDNA libraries from *Spiroplasma*-free and *Spiroplasma*-infected sister lines of *A. citricidus* reared on orange jasmine and sweet orange yielded approximately 191.7 million reads. Reads per libraries ranged from nearly 14.1 million for *Spiroplasma*-free isoline on sweet orange to 18.5 million for *Spiroplasma*-infected isoline on sweet orange. On average, we obtained 15.9 million of reads. After filtering the reads for quality and read normalization, reads ranged from 3.3 million for *Spiroplasma*-infected isoline on sweet orange to 5.1 million for *Spiroplasma*-free isoline on orange jasmine, with an an average of 4.2 million reads/library. Thus, nearly 50.9 million reads were used for the *de novo* assembly of the reference transcriptome of *A. citricidus*.

The *de novo* assembly of the transcriptome yielded 646,253 contigs, representing nearly 462 million nucleotides with a GC content of 30.4% (Table 7.1). Assembled transcriptome produced transcripts as large as 20,140 bp, with a N50 of 990 bp and the majority of the transcripts being distributed within the size range of 2000 bp (Table 7.1, Figure 7.1).

Table 7.1 - Summary statistics of *Aphis citricidus* reference transcriptome

Total number of reads	191,683,996
Total number of reads after quality filtering and read normalization	50,910,936
Total number of transcripts assembled	646,253
Total number of nucleotides in assembled transcripts	461,116,549
Average transcripts size	713
Size of the largest transcript	20,140
%GC	30.4
N50	990

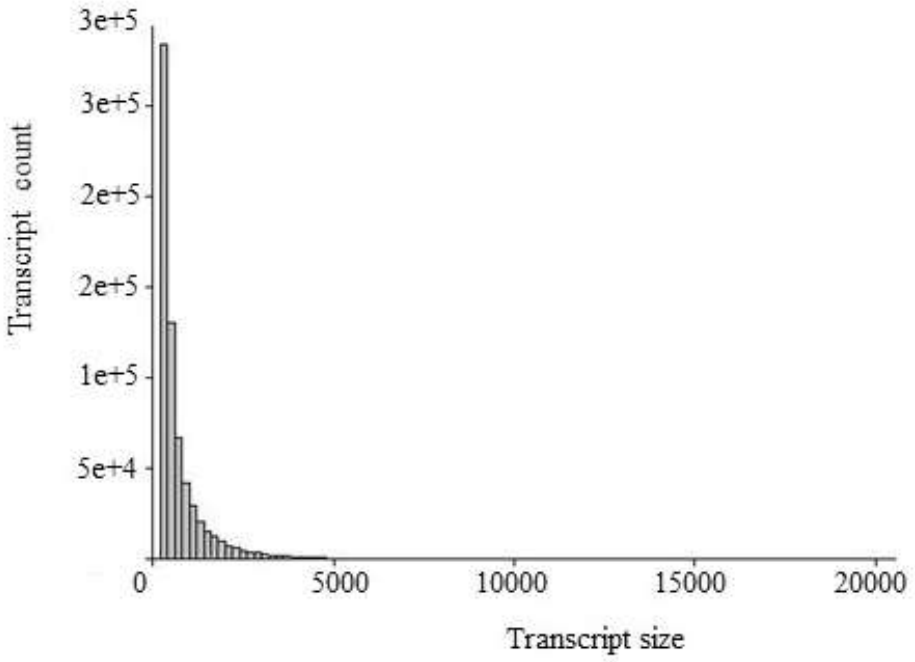


Figure 7.1 - Size distribution of transcripts obtained from the *de novo* assembly of the reference transcriptome of *Aphis (Toxoptera) citricidus*

Transcriptome analysis indicated 191,573 transcripts were common to all four libraries, *Spiroplasma*-infected aphids from sweet orange and orange jasmine shared 249,513 transcripts, and *Spiroplasma*-free aphids from sweet orange and orange jasmine shared 186,671 transcripts

(Figure 7.2). Analysis also demonstrated a large number of transcripts were unique to each one of the libraries analyzed. 5,724 transcripts were unique to the *Spiroplasma*-free isoline on sweet orange, but six-fold more unique transcripts (30,888) were observed in aphids on orange jasmine. In the case of *Spiroplasma*-infected aphids, 17,183 transcripts were detected only on orange jasmine, against 21,235 on sweet orange (Figure 7.2).

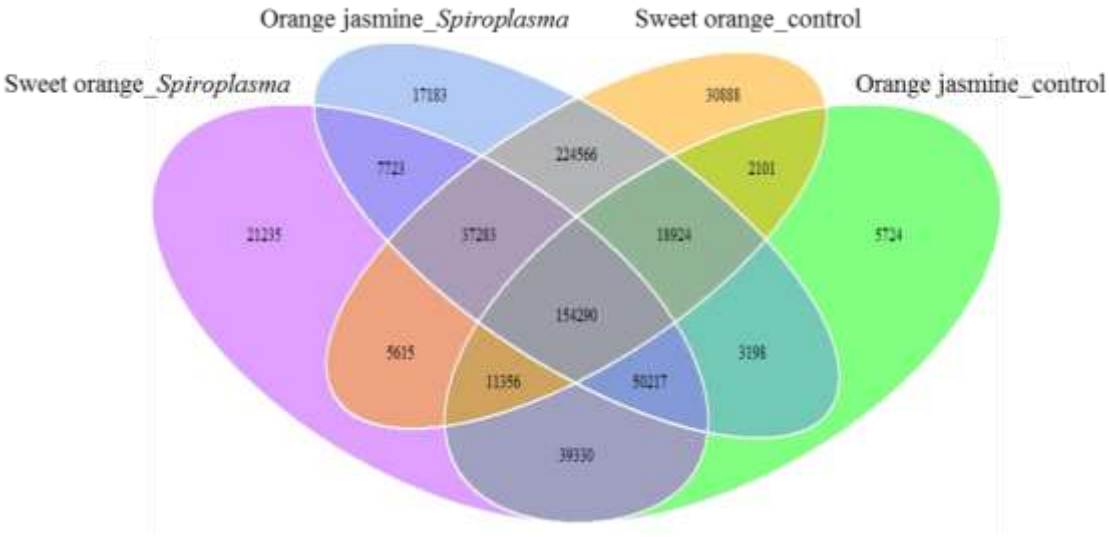


Figure 7.2 - Number of unique and shared transcripts from the *de novo* transcriptome of *Aphis (Toxoptera) citricidus* among *Spiroplasma*-free and *Spiroplasma*-infected aphids on sweet orange and orange jasmine as host plants

7.3.2 Effects of *Spiroplasma* infection on gene expression of *Aphis (Toxoptera) citricidus* on sweet orange and orange jasmine

A. citricidus gene expression when developing either on sweet orange or orange jasmine was affected by *Spiroplasma* infection. When aphids were reared on sweet orange, 270 differentially expressed transcripts between *Spiroplasma*-infected and *Spiroplasma*-free isolines were detected, from which 60 returned significant matches from BLASTX searches against the NCBI non-redundant database (Table 7.2). Identified transcripts were assigned to Gene Ontology (GO) annotations at level 2 and GO terms yielded 15 functional groups, which covered the biological process (7 GO), molecular function (3 GO) and cellular component (5 GO) categories (Figure 7.3 A).

Table 7.2 - Differentially expressed transcripts between *Spiroplasma*-free and *Spiroplasma*-infected isolines of *Aphis (Toxoptera) citricidus* reared on sweet orange

(to be continued)

Transcript	Fold change*	*p-value*	BLAST ID	Organism	*e-value*
TR109124|c0_g2_i1	+2.80	0.048	reverse transcriptase	*Acyrthosiphon pisum*	6.50E-23
TR98673|c0_g1_i1	+2.87	0.044	e3 sumo-protein ligase pias2-like	*Acyrthosiphon pisum*	1.50E-37
TR115352|c0_g2_i1	+2.88	0.044	uncharacterized protein LOC103310444	*Acyrthosiphon pisum*	3.40E-32
TR126789|c2_g6_i1	+2.93	0.037	hypothetical protein G7K_1410-t1	***Penicillium nordicum***	3.30E-25
TR133534|c2_g6_i14	+2.96	0.047	histone-lysine n-methyltransferase partial	*Solenopsis invicta*	1.70E-22
TR134570|c11_g2_i10	+2.97	0.050	cold shock protein 1-like	*Athalia rosae*	1.60E-05
TR130832|c0_g2_i1	+2.99	0.047	uncharacterized protein LOC100570869	*Acyrthosiphon pisum*	2.60E-28
TR100433|c0_g1_i1	+3.00	0.046	enzymatic polyprotein endonuclease reverse	*Lasius niger*	1.90E-25
TR134024|c3_g2_i1	+3.01	0.045	uncharacterized protein LOC103308714	*Acyrthosiphon pisum*	2.50E-23
TR127185|c2_g9_i1	+3.02	0.043	uncharacterized protein LOC103310669	*Acyrthosiphon pisum*	7.80E-19
TR104996|c0_g1_i2	+3.04	0.047	histone-lysine n-methyltransferase partial	*Stegodyphus mimosarum*	7.80E-31
TR124000|c0_g3_i1	+3.08	0.046	uncharacterized protein LOC103309275	*Acyrthosiphon pisum*	9.20E-14
TR126861|c2_g10_i2	+3.10	0.047	uncharacterized protein LOC100572042	*Acyrthosiphon pisum*	7.90E-33
TR104996|c0_g1_i1	+3.32	0.034	histone-lysine n-methyltransferase partial	*Stegodyphus mimosarum*	9.90E-34
TR134569|c17_g1_i1	+3.34	0.047	uncharacterized protein LOC103578886	*Microplitis demolitor*	3.40E-05
TR113381|c1_g2_i2	+3.35	0.042	probable RNA-directed DNA polymerase from transposon bs ame	*Acyrthosiphon pisum*	1.20E-18

Table 7.2 - Differentially expressed transcripts between *Spiroplasma*-free and *Spiroplasma*-infected isolines of *Aphis (Toxoptera) citricidus* reared on sweet orange

(to be continued)

Transcript	Fold change*	*p-value*	BLAST ID	Organism	*e-value*
TR131149|c1_g9_i5	+3.40	0.043	uncharacterized protein LOC103309757	*Acyrthosiphon pisum*	7.00E-43
TR134484|c2_g5_i1	+3.41	0.038	uncharacterized protein LOC100569355	*Acyrthosiphon pisum*	7.00E-28
TR133434|c2_g8_i1	+3.43	0.040	uncharacterized protein LOC103309275	*Acyrthosiphon pisum*	1.80E-45
TR129700|c1_g3_i2	+3.44	0.047	uncharacterized protein LOC103307897	*Acyrthosiphon pisum*	3.10E-17
TR129573|c1_g3_i1	+3.46	0.035	e3 sumo-protein ligase pias2-like	*Acyrthosiphon pisum*	4.10E-04
TR134551|c9_g8_i5	+3.49	0.049	uncharacterized protein LOC100569006	*Acyrthosiphon pisum*	3.10E-43
TR102906|c0_g3_i1	+3.65	0.048	conserved protein	***Listeria monocytogenes*** **	3.30E-30
TR133614|c5_g3_i5	+3.67	0.045	histone-lysine n-methyltransferase setmar-like	*Lasius niger*	2.20E-54
TR126278|c0_g7_i1	+3.72	0.041	reverse transcriptase	*Acyrthosiphon pisum*	1.40E-15
TR133835|c1_g3_i1	+3.76	0.035	uncharacterized protein LOC103308992	*Acyrthosiphon pisum*	3.10E-42
TR133783|c2_g3_i1	+4.01	0.035	RNA-binding protein squid-like	*Acyrthosiphon pisum*	6.10E-27
TR103764|c0_g1_i1	+4.13	0.022	reverse transcriptase	*Acyrthosiphon pisum*	1.90E-54
TR134174|c3_g5_i13	+4.14	0.046	sentrin-specific protease 7-like	*Acyrthosiphon pisum*	4.70E-20
TR89348|c0_g1_i1	+4.27	0.044	uncharacterized protein LOC103308711	*Acyrthosiphon pisum*	9.10E-38
TR122784|c0_g7_i4	+4.38	0.044	hypothetical protein TcasGA2_TC001491	*Tribolium castaneum*	9.70E-31
TR128027|c1_g5_i1	+4.43	0.038	hypothetical protein X777_05351, partial	*Cerapachys biroi*	1.40E-27
TR97409|c0_g1_i1	+4.50	0.030	RNA-directed DNA polymerase from mobile element jockey-like	*Acyrthosiphon pisum*	3.50E-10
TR126789|c2_g10_i4	+4.75	0.008	hypothetical protein ACN38_g13205	***Saitoella complicata***	3.10E-34

Table 7.2 - Differentially expressed transcripts between *Spiroplasma*-free and *Spiroplasma*-infected isolines of *Aphis (Toxoptera) citricidus* reared on sweet orange

(to be continued)

Transcript	Fold change*	*p-value*	BLAST ID	Organism	*e-value*
TR125916|c0_g8_i1	+4.79	0.041	hypothetical ctc00065-like protein	***Clostridium botulinum***	3.90E-45
TR78812|c0_g1_i1	+4.79	0.026	uncharacterized protein LOC100571052	*Acyrthosiphon pisum*	1.60E-32
TR113066|c0_g1_i1	+4.85	0.021	reverse transcriptase	*Acyrthosiphon pisum*	1.10E-73
TR132077|c0_g2_i2	+5.04	0.019	52 kda repressor of the inhibitor of the protein kinase-like	*Acyrthosiphon pisum*	9.40E-36
TR117621|c0_g4_i2	+5.07	0.006	hypothetical protein PSTG_16994	***Puccinia striiformis***	2.00E-64
TR129702|c0_g2_i1	+5.44	<0.001	RNA-dependent RNA polymerase	**Newfield virus**	1.90E-43
TR108471|c0_g2_i1	+5.52	0.003	hypothetical protein PSTG_16993	***Puccinia striiformis***	9.10E-31
TR129702|c0_g1_i1	+5.63	<0.001	RNA-dependent RNA polymerase	***Drosophila* A virus**	5.30E-35
TR254480|c0_g1_i1	+5.64	0.034	alpha-l1 nicotinic acetyl choline receptor	*Diaphorina citri*	2.60E-18
TR130412|c0_g2_i1	+5.76	<0.001	capsid protein	**Bat Soemovirus**	1.20E-17
TR101838|c0_g1_i1	+Inf	0.047	ribosomal protein s6 kinase alpha-2-like	*Acyrthosiphon pisum*	1.60E-08
TR127018|c0_g6_i1	+Inf	0.043	maverick integrase	***Cotesia congregata* Bracovirus**	5.70E-32
TR127287|c0_g1_i1	+Inf	0.022	glutathione synthetase	***Buchnera aphidicola***	1.40E-23
TR128718|c0_g7_i3	+Inf	0.018	e3 sumo-protein ligase pias2-like	*Acyrthosiphon pisum*	1.90E-10
TR131795|c1_g4_i3	+Inf	0.042	histone-like isoform x2	*Haplochromis burtoni*	7.50E-52
TR134155|c3_g1_i1	+Inf	0.043	uncharacterized protein LOC103308063	*Acyrthosiphon pisum*	2.30E-39
TR134562|c10_g1_i6	+Inf	0.014	ryanodine receptor	*Aphis citricidus*	3.60E-45
TR134570|c12_g4_i1	+Inf	0.020	enzymatic polyprotein endonuclease reverse	*Acyrthosiphon pisum*	2.90E-06

Table 7.2 - Differentially expressed transcripts between *Spiroplasma*-free and *Spiroplasma*-infected isolines of *Aphis (Toxoptera) citricidus* reared on sweet orange

(conclusion)

Transcript	Fold change*	*p-value*	BLAST ID	Organism	*e-value*
TR164493|c0_g1_i1	+Inf	0.043	reverse transcriptase	*Euscorpius carpathicus*	2.60E-10
TR224400|c0_g1_i1	+Inf	0.035	hypothetical protein TSTA_040370	***Talaromyces stipitatus***	3.70E-30
TR255326|c0_g1_i1	+Inf	0.030	murein lipoprotein	***Arsenophonus*** *(Nilaparvata lugens)*	2.10E-44
TR283402|c0_g1_i1	+Inf	0.004	avidin family protein	***Burkholderia pseudomallei***	1.20E-27
TR42666|c0_g1_i1	+Inf	0.029	uncharacterized protein LOC100572427	*Acyrthosiphon pisum*	1.20E-22
TR81544|c0_g4_i1	+Inf	<0.001	hypothetical protein EPIR_3224	***Erwinia piriflorinigrans***	2.70E-51
TR36791|c0_g1_i1	+Inf	0.001	uncharacterized protein LOC103516268	*Diaphorina citri*	1.30E-78
TR99343|c0_g2_i1	+Inf	0.024	orf b (bases 1850-5560) first start codon at 2306	*Lasius niger*	3.00E-10

* (+) = up-regulated in *Spiroplasma*-infected *A. citricidus*
** bold letters represent transcripts from prokaryotes or viruses

On orange jasmine, the influence of *Spiroplasma* infection on aphid gene transcription was less intense. Only 31 transcripts were differentially expressed, from which 26 yielded BLASTX results (Table 7.3). These transcripts were divided in 11 functional groups, with gene ontologies distributed in cellular component (4), biological process (5) and molecular function (2) categories (Figure 7.3 B). Although a lower number of differentially expressed transcripts were detected in *A. citricidus* on orange jasmine, changes in gene expression induced by *Spiroplasma* infection in *A. citricidus* led to the up-regulation of genes in infected aphids regardless of the host plant used (Figure 7.4).

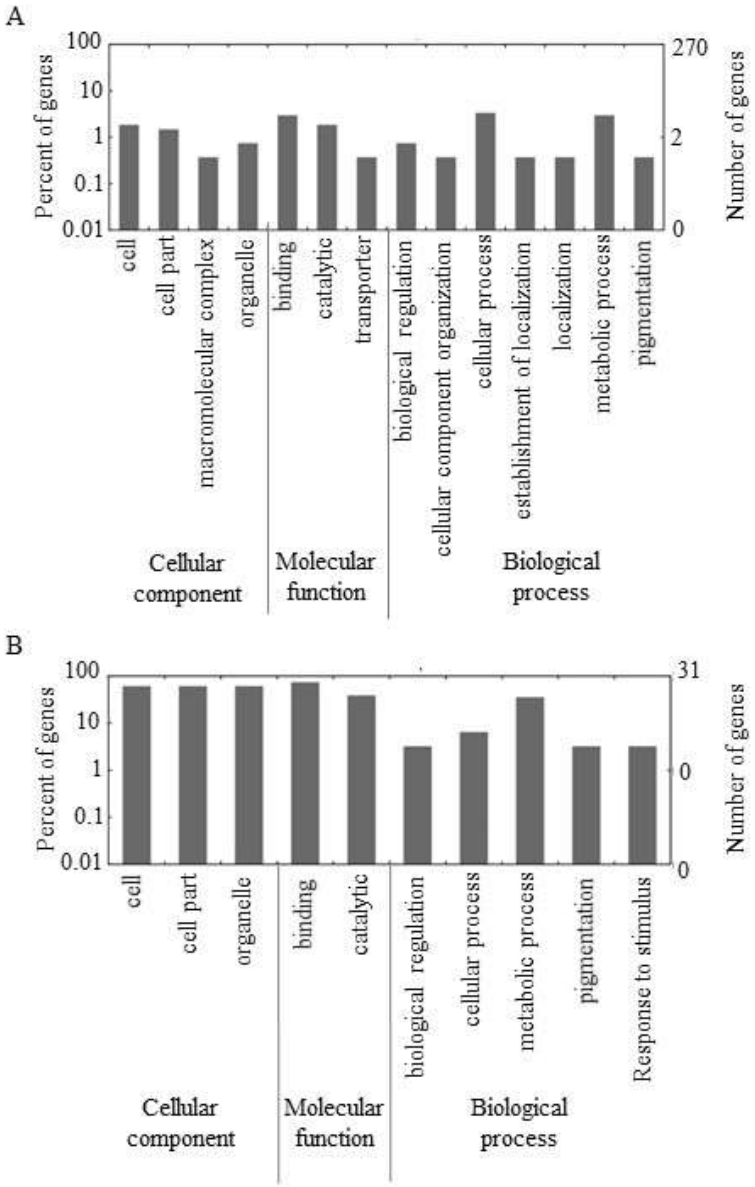


Figure 7.3 - Gene Ontology (GO) annotation terms at level 2 for cellular component, molecular function and biological process categories of differentially expressed transcripts between *Spiroplasma*-free and *Spiroplasma*-infected *Aphis (Toxoptera) citricidus* when reared on (A) sweet orange or (B) orange jasmine

Table 7.3 - Differentially expressed transcripts between *Spiroplasma*-free and *Spiroplasma*-infected isolines of *Aphis (Toxoptera) citricidus* reared on orange jasmine

(to be continued)

Transcript	Fold change*	*p-value*	BLAST ID	Organism	*e-value*
TR131985|c10_g6_i1	-Inf	0.030	nad-dependent dna ligase	***Buchnera aphidicola***	2.60E-42
TR134461|c3_g3_i1	-Inf	0.025	trigger factor	***Buchnera aphidicola***	4.70E-46
TR120399|c0_g1_i1	- Inf	0.023	eukaryotic translation initiation factor 4b	*Acyrthosiphon pisum*	8.30E-17
TR131589|c3_g6_i1	+ 3.47	0.047	heat shock protein 68- partial	*Acyrthosiphon pisum*	1.50E-40
TR131589|c3_g4_i5	+ 3.61	0.039	heat shock protein 68-like	*Aphis glycines*	2.90E-69
TR128956|c0_g2_i8	+ 3.63	0.040	heat shock protein 68-like	*Aphis glycines*	0.00E+00
TR131589|c3_g4_i6	+ 3.63	0.040	heat shock protein 68-like	*Acyrthosiphon pisum*	5.40E-117
TR131589|c3_g4_i1	+ 3.66	0.044	heat shock protein 68- partial	*Acyrthosiphon pisum*	2.60E-147
TR46288|c0_g1_i1	+ 3.66	0.042	heat shock protein 70 a1-like	*Acyrthosiphon pisum*	4.80E-56
TR88293|c0_g1_i1	+ 3.72	0.039	heat shock protein 68-like	*Acyrthosiphon pisum*	7.70E-125
TR121824|c0_g2_i4	+ 3.73	0.036	heat shock protein 68- partial	*Aphis glycines*	2.90E-132
TR128956|c0_g2_i13	+ 3.75	0.037	heat shock protein 68-like	*Aphis glycines*	2.20E-145
TR27885|c0_g1_i1	+ 3.75	0.034	heat shock protein 70 a1-like	*Acyrthosiphon pisum*	2.20E-48
TR116616|c0_g4_i1	+ 3.76	0.035	heat shock protein 68-like	*Acyrthosiphon pisum*	2.20E-108
TR121824|c1_g1_i1	+ 3.76	0.038	heat shock protein 68-like	*Aphis glycines*	1.00E-45
TR131589|c3_g3_i1	+ 3.79	0.034	heat shock protein 68- partial	*Aphis glycines*	0.00E+00
TR128956|c0_g2_i17	+ 3.84	0.033	heat shock protein 68-like	*Aphis glycines*	4.90E-132
TR116616|c0_g2_i2	+ 3.87	0.032	heat shock protein 68-like	*Acyrthosiphon pisum*	6.90E-105

Table 7.3 - Differentially expressed transcripts between *Spiroplasma*-free and *Spiroplasma*-infected isolines of *Aphis (Toxoptera) citricidus* reared on orange jasmine

(conclusion)

Transcript	Fold change*	*p-value*	BLAST ID	Organism	*e-value*
TR128956|c0_g2_i3	+ 4.03	0.031	heat shock protein 68-like	*Acyrthosiphon pisum*	0.00E+00
TR131589|c3_g5_i1	+ 4.07	0.025	heat shock protein 68- partial	*Acyrthosiphon pisum*	8.40E-37
TR131589|c1_g1_i1	+ 4.16	0.026	heat shock protein 68- partial	*Bemisia tabaci*	1.30E-37
TR196958|c0_g1_i1	+ 4.20	0.022	heat shock protein 68-like	*Acyrthosiphon pisum*	4.10E-65
TR128956|c0_g2_i7	+ 4.21	0.026	heat shock protein 70 a1-like	*Acyrthosiphon pisum*	3.40E-79
TR62539|c0_g1_i1	+ 4.53	0.019	heat shock protein 68- partial	*Acyrthosiphon pisum*	2.90E-45
TR121824|c0_g2_i2	+ 4.78	0.014	heat shock protein 68- partial	*Acyrthosiphon pisum*	5.10E-92
TR128956|c0_g2_i12	+ 4.78	0.021	heat shock protein 68-like	*Acyrthosiphon pisum*	2.70E-151

* (+) = up-regulated and (-) = down-regulated in *Spiroplasma*-infected *A. citricidus.*

** bold letters represent transcript of prokaryotes or viruses

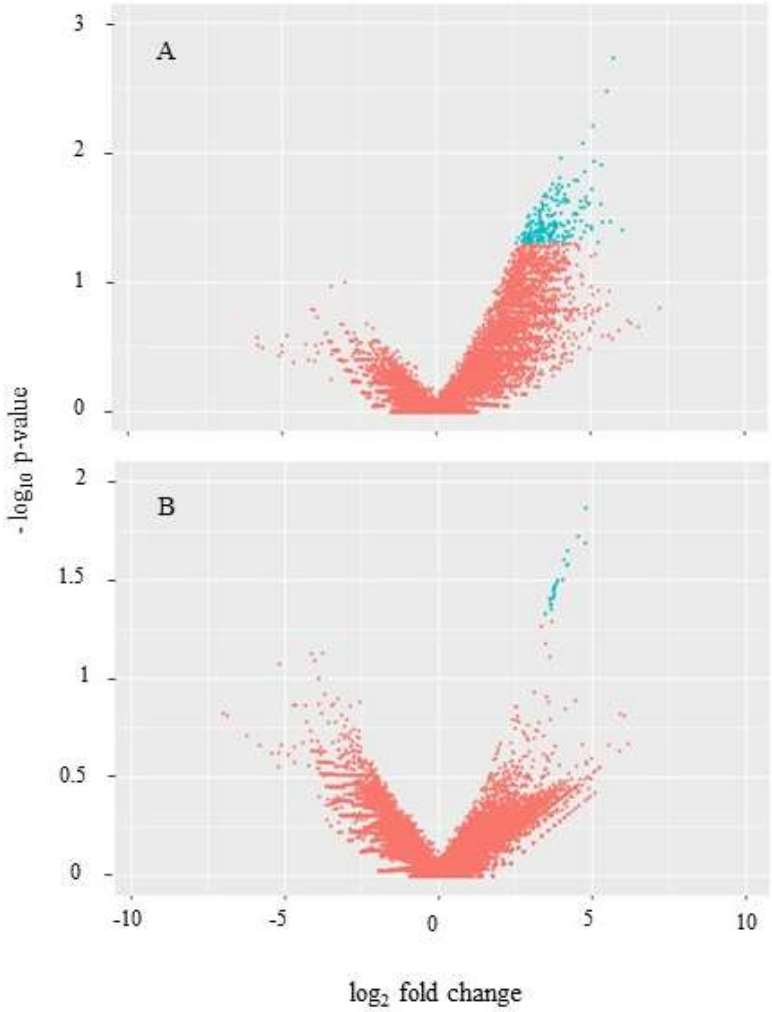


Figure 7.4 - Gene expression changes due to *Spiroplasma* infection in *Aphis (Toxoptera) citricidus* feeding on sweet orange (A) and orange jasmine (B). Transcripts on positive x-axis are up-regulated, while transcripts on negative x-axis are down-regulated. Blue dots indicate differentially expressed transcripts (fold change > 2 and $p < 0.05$)

Analysis only of the transcripts with hits in the BLASTX indicated that the 60 transcripts on sweet orange with BLAST hits were up-regulated by *Spiroplasma* infection, while 21 out of the 23 transcripts on orange jasmine with BLAST hits were up-regulated in *Spiroplasma*-infected aphids. Only three transcripts with BLAST hits were down-regulated in *Spiroplasma*-infected *A. citricidus*: NAD-dependent DNA ligase, eukayotic translation initiation factor 4b and trigger factor (Tables 7.3 and 7.4, Figure 7.5).

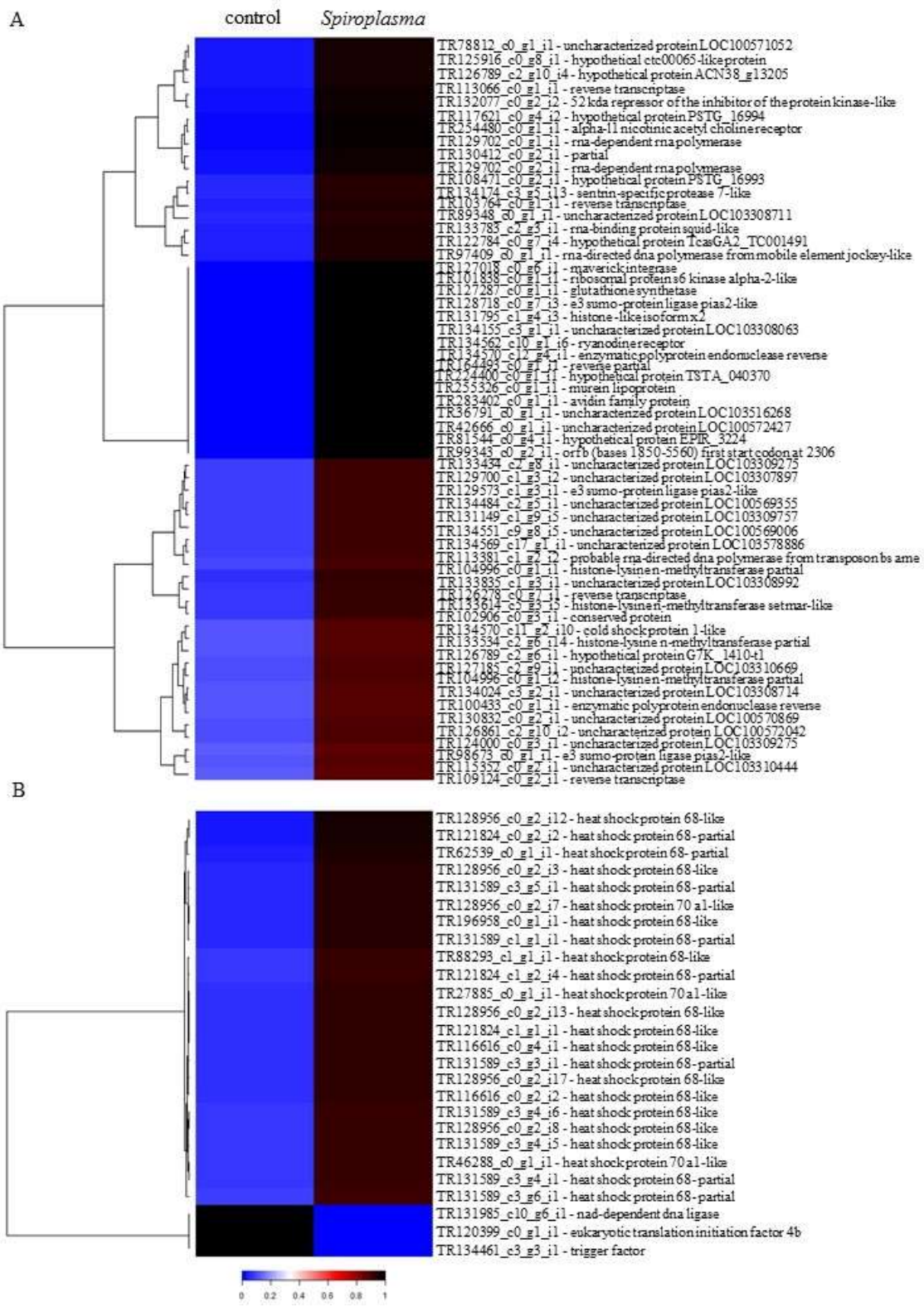


Figure 7.5 - Heatmap of differentially expressed transcripts between *Spiroplasma*-infected and *Spiroplasma*-free isolines of *Aphis (Toxoptera) citricidus* reared on sweet orange (A) and orange jasmine (B). Fold change was transformed in a proportional scale in which blue represents the smallest fold change and black represent the largest fold change

7.3.3 Effects of host plant on gene expression of *Spiroplasma*-free and *Spiroplasma*-infected *Aphis (Toxoptera) citricidus*

A. citricidus gene expression was affected by host plant regardelles *Spiroplasma* infection. 29,193 transcripts were differentially expressed in *Spiroplasma*-free *A. citricidus* when feeding on orange jasmine as compared to sweet orange, from which 16,730 transcripts returned BLASTX hits. Changes in gene expression in *Spiroplasma*-infected aphids due to host plant utilization were less intense than those observed in *Spiroplasma*-free aphids. 10,570 differentially expressed transcripts were detected between infected aphids when feeding on orange jasmine or sweet orange, and nearly 6,113 rendered BLASTX hits.

Differentially expressed transcripts were assigned to Gene Ontology (GO) annotations at level 2. *Spiroplasma*-free isolines yielded 54 GOs distributed in 18 GOs from cellular process, 24 GOs from biological process and 12 GOs from molecular function (Figure 7.6 A), while *Spiroplasma*-infected isolines yielded 31 GOs distribuited in 12 GOs from cellular process, 19 GOs from biological process and 7 GOs from molecular function (Figure 7.6 B).

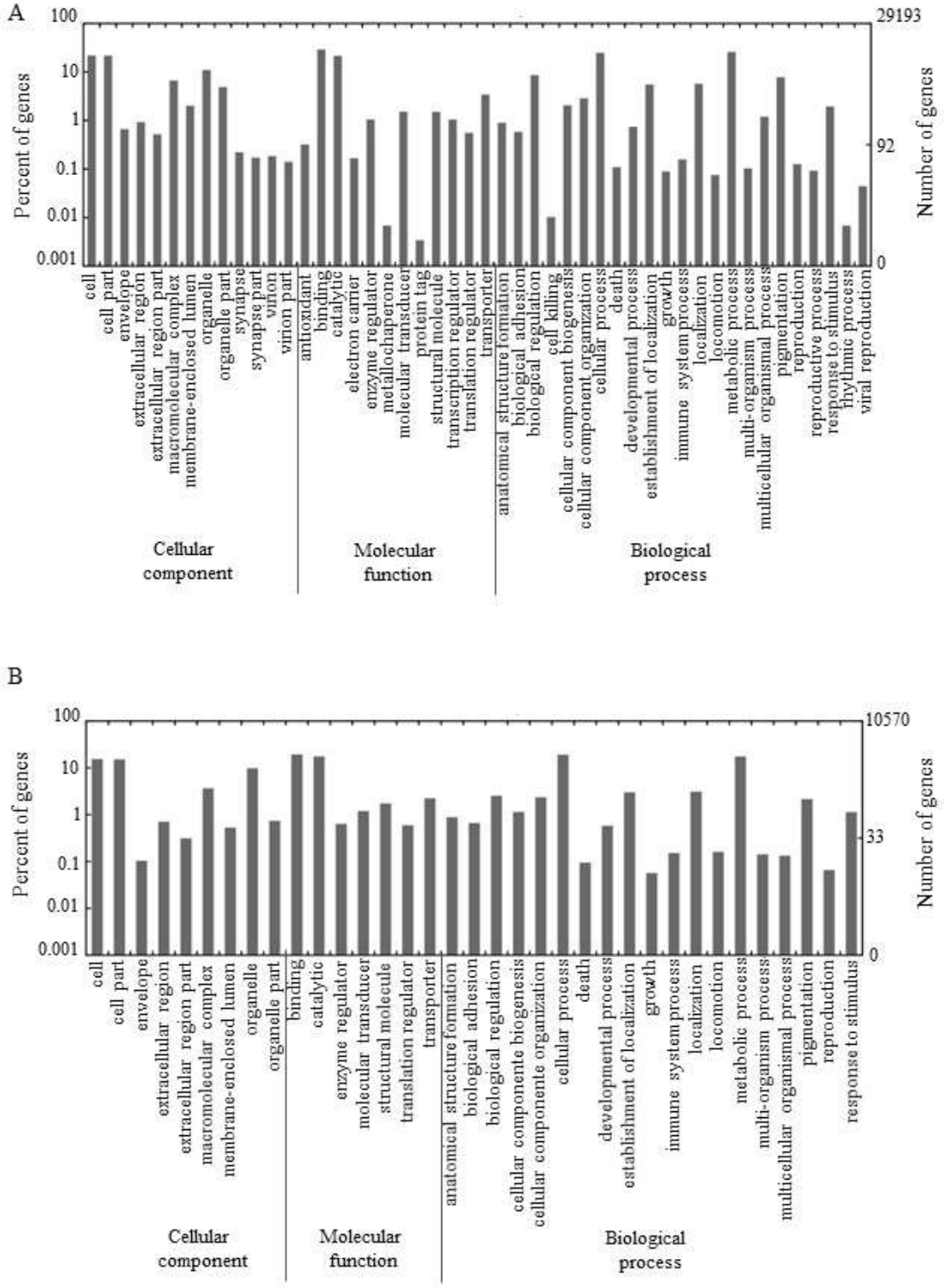


Figure 7.6 - Gene Ontology (GO) annotation terms at level 2 for cellular component, molecular function and biological process categories of differentially expressed transcripts between sweet orange and orange jasmine on *Aphis (Toxoptera) citricidus* from *Spiroplasma*-free isolines (A) and *Spiroplasma*-infected isolines (B)

Besides the larged number of differentially expressed transcripts in *Spiroplasma*-free *A. citricidus* in response to host plants as compared to *Spiroplasma*-infected *A. citricidus* isolines,

the pattern of differential expression in *Spiroplasma*-infected aphis was slightly skewed to the down-regulation of transcripts in aphids feeding on sweet orange as compared to orange jasmine (Figure 7.7).

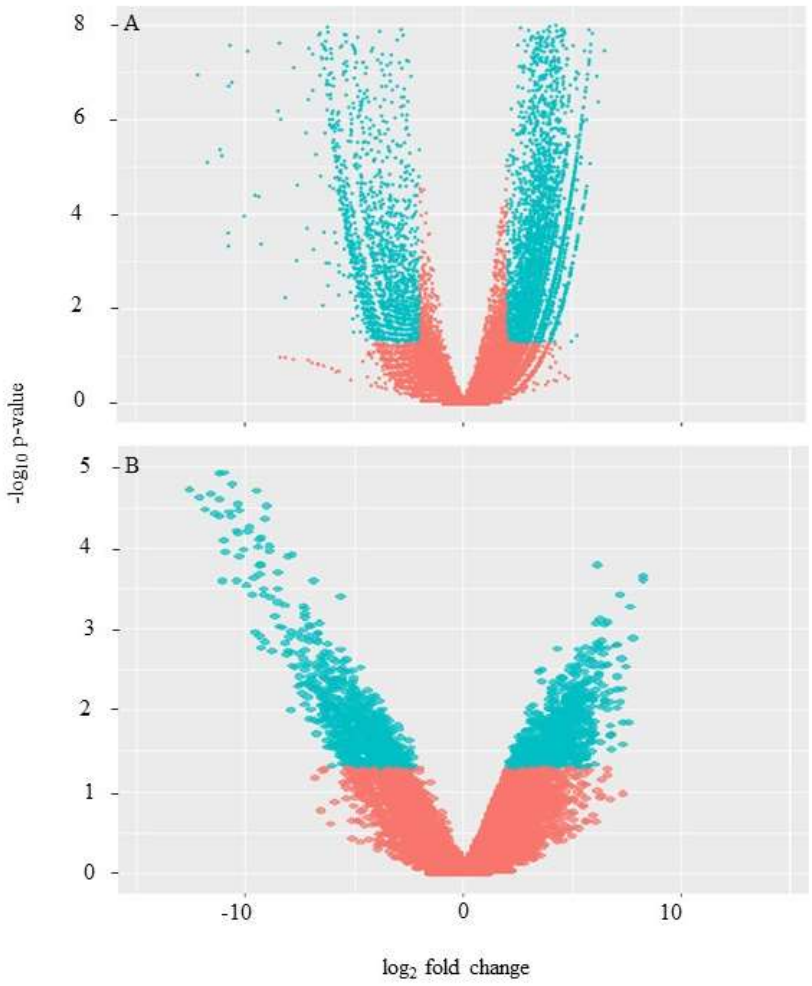


Figure 7.7 - Gene expression changes due to host plant of *Aphis (Toxoptera) citricidus* on *Spiroplasma*-free (A) and *Spiroplasma*-infected isolines (B). Transcripts on positive x-axis are up-regulated, while transcripts on negative x-axis are down-regulated. Blue dots indicate differentially expressed transcripts (fold change > 2 and $p < 0.05$)

Infection and host plant altered gene expression of *A. citricidus*. RNA-Seq experiments with *Spiroplasma*-free and *Spiroplasma*-infected isolines showed 9,170 differentially expressed transcripts in common when comparing host plants, from which 5,701 yielded BLASTX hits. The remaining differentially expressed transcripts were present only in *Spiroplasma*-free (20,023 transcripts) or *Spiroplasma*-infected isolines (1,400 transcripts). Differentially

expressed transcripts common to both isolines followed different patterns of expression and/or intensity of regulation (fold change variation). Therefore, 6,245 transcripts were down-regulated and 89 transcripts were up-regulated in both isolines, but 2,682 transcripts were up-regulated in *Spiroplasma*-free while being down-regulated in *Spiroplasma*-infected isolines. On the contrary, 154 transcripts were up-regulated in *Spiroplasma*-infected while being down-regulated in *Spiroplasma*-free isolines (Table 7.4).

Table 7.4 – Shared differentially expressed transcript between *Spiroplasma*-free and *Spiroplasma*- infected isolines when comparing host plant that inverted fold change

(to be continued)

Transcript	BLAST ID	Fold change S^-	Fold change S^+
TR128068|c1_g1_i9	trifunctional enzyme subunit mitochondrial	-Inf	Inf
TR127615|c7_g1_i3	probable basic-leucine zipper transcription factor q	-Inf	Inf
TR134075|c2_g2_i1	RNA-directed DNA polymerase from mobile element jockey-like	-Inf	5.21
TR123141|c4_g2_i1	reverse transcriptase	-Inf	5.14
TR130902|c0_g3_i1	RNA-directed DNA polymerase from mobile element jockey-like	-Inf	5.03
TR109124|c0_g2_i1	reverse transcriptase	-Inf	4.71
TR129517|c2_g4_i5	ribonuclease h1-like	-Inf	4.69
TR122344|c3_g2_i1	probable aconitate hydrolase mitochondrial	-Inf	4.34
TR134161|c3_g1_i6	low quality protein: protocadherin-15-like	-Inf	4.21
TR111611|c0_g5_i1	enzymatic polyprotein endonuclease reverse	-Inf	4.12
TR134198|c1_g1_i1	steroid dehydrogenase-like	-Inf	4.09
TR121961|c1_g4_i2	reverse transcriptase	-Inf	3.66
TR132075|c5_g1_i7	GTP cyclohydrolase 1 isoform x1	-Inf	3.65
TR129308|c3_g1_i2	acetyl- CoA acetyltransferase B107mitochondrial	-Inf	3.59
TR79389|c0_g1_i1	fatty acid synthase-like	-Inf	3.35
TR86814|c1_g2_i1	condensin complex subunit 2	-Inf	3.26
TR129608|c1_g6_i1	reverse transcriptase	-Inf	3.25

Table 7.4 – Shared differentially expressed transcript between *Spiroplasma*-free and *Spiroplasma*- infected isolines when comparing host plant that inverted fold change

(to be continued)

Transcript	BLAST ID	Fold change S^-	Fold change S^+
TR128606|c1_g1_i7	PREDICTED: fumarylacetoacetase	-Inf	3.11
TR211256|c0_g1_i1	cytochrome p450 6a2-like	-Inf	3.08
TR131320|c4_g21_i1	UDP pyrophosphate phosphatase	-Inf	3.03
TR129306|c1_g2_i2	cytochrome p450 6a2-like	-3.42	3.61
TR128248|c5_g1_i1	ribose-5-phosphate isomerase a	-5.14	3.54
TR9866|c0_g2_i1	cytochrome p450 6k1-like	-3.37	3.44
TR130274|c5_g1_i5	mfs-type transporter slc18b1-like	-5.47	3.14
TR132013|c6_g1_i3	bifunctional aspartokinase i homoserine dehydrogenase i	-3.45	3.13
TR130459|c6_g6_i3	26s proteasome non-atpase regulatory subunit 1	Inf	-Inf
TR133125|c5_g2_i1	2-oxoglutarate dehydrogenase e1 component	Inf	-Inf
TR124951|c0_g1_i8	60 kda heat shock mitochondrial-like	Inf	-Inf
TR125587|c1_g1_i1	6-phosphofructokinase isoform x4	Inf	-Inf
TR132890|c1_g3_i18	adenylate cyclase type	Inf	-Inf
TR107258|c0_g2_i1	adenylate kinase	Inf	-Inf
TR126195|c0_g1_i10	adipocyte plasma membrane-associated	Inf	-Inf
TR126183|c1_g2_i6	alanine aminotransferase 2 isoform x2	Inf	-Inf
TR57343|c1_g1_i1	alanine-tRNA ligase	Inf	-Inf

Table 7.4 – Shared differentially expressed transcript between *Spiroplasma*-free and *Spiroplasma*- infected isolines when comparing host plant that inverted fold change

(conclusion)

Transcript	BLAST ID	Fold change S^-	Fold change S^+
TR126493|c3_g1_i23	amino acid transporter	Inf	-Inf
TR123710|c3_g1_i4	asparagine synthetase	Inf	-Inf
TR128808|c4_g3_i5	ATP-binding cassette sub-family a member 3-like	Inf	-Inf
TR127717|c3_g6_i4	cell-death-related nuclease 7	Inf	-Inf
TR47482|c0_g1_i1	cysteinyl-tRNA synthetase	Inf	-Inf
TR129481|c3_g1_i5	cytochrome p450 18a1	Inf	-Inf
TR128419|c2_g4_i2	cytokine receptor isoform x1	Inf	-Inf
TR133249|c10_g1_i5	death associated protein	Inf	-Inf
TR133245|c0_g2_i1	e3 ubiquitin-protein ligase hecw2-like	Inf	-Inf
TR132152|c9_g2_i11	e3 ubiquitin-protein ligase mylip	Inf	-Inf
TR130669|c7_g6_i7	e3 ubiquitin-protein ligase synoviolin a	Inf	-Inf
TR128773|c3_g2_i1	electron transport complex subunit	Inf	-Inf
TR126066|c7_g1_i10	facilitated trehalose transporter tret1-2 homolog	Inf	-Inf
TR124676|c1_g1_i16	fatty acid synthase-like isoform x3	Inf	-Inf
TR123890|c2_g1_i8	glucose transporter type 1 isoform x5	Inf	-Inf
TR122855|c4_g1_i1	insulin receptor tyrosine kinase substrate-like partial	Inf	-Inf

* (+) = up-regulated and (-) = down-regulated in *A. citricidus* reared on sweet orange

7.4 Discussion

Comparative transcriptome analysis between *Spiroplasma*-free (Ac-B) and *Spiroplasma*-infected (Ac-BS) isolines of *A. citricidus* on two host plants indicated *Spiroplasma* infection interferes with the host transcriptome, especially affecting the expression of genes belonging to the immune system and to stress response. However, host plants induced more conspicuous changes in the host transcriptome regardless of *Spiroplasma* infection. Host plant affected expression of genes related to several gene functions and metabolic pathways, such as digestive enzymes, amino acids synthesis, energy metabolism, detoxification and transcriptional and translational regulatory factors. But gene expression of *A. citricidus* in response to host plant quality (sweet orange and orange jasmine) was influenced by *Spiroplasma* infection.

Changes in gene expression in response to *Spiroplasma* for aphids reared on sweet orange indicated *Spiroplasma* suppresses immune response and cellular gowth in infected as compared to uninfected aphids through the up-regulation of e3 sumo-protein ligase pias2-like and the 52 kDa repressor of the inhibitor of the protein kinase. E3 sumo-protein ligase pias2-like are also known as Protein Inhibitor of Activated STAT (PIAS), which are transcriptional co-regulators that can activate or repress expression by interacting with a large set of proteins involved in transcription. PIAS are also described as important co-regulators of the JAK/STAT signal transduction pathway. In insects, the JAK/STAT pathway leads to the expression of genes coding for cytokines, antimicrobial peptides and proteins involved in the melanization and phagocytosis, acting on both humoral and cellular immune responses (MYLLUMÄKI; RÄMET, 2014). PIAS are reported to negatively regulate transcription by blocking DNA-binding, recruiting other co-regulators, sequestering transcription factors in certain subnuclear structures, or by repressing transcription by sumoylation. PIAS proteins that repress transcription by sumoylation have SUMO-E3-ligase activity and act mainly regulating cellular immune processes (SHUAI; LIU, 2005). Therefore, we believe the up-regulation of the co-activator E3 sumo-protein ligase pias2-like in *Spiroplasma*-infected aphids leads to the repression of transcription of signals of the JAK/STAT signal transduction pathway, ultimately affecting the humoral and/or cellular immune response of *Spiroplasma*-infected *A. citricidus* on sweet orange. Such hypothesis is supported by the high number of up-regulated viral transcripts in *Spiroplasma*-infected *A. citricidus* on sweet orange, as there is evidence of the involvement of Janus kinases in the JAK/STAT signaling pathway in the immune response against viral infection (DOSTERT et al., 2005; SONG et al., 2015).

52 kDa repressor of the inhibitor of the protein kinase ($p52^{rIPK}$) acts upstream of the regulator of interferon-induced serine/threonine protein kinase R (PKR), the cellular protein $p58^{IPK}$. PKRs are activated by a number of environmental stimuli and mediate the phosphorylation of the eukaryotic initiation factor 2-alpha subunit (eIF-2a), blocking mRNA translation and cell growth (GALE et al., 1998). Up-regulation of $p52^{rIPK}$ may lead to the blockage of PKRs-inhibition by $p58^{IPK}$, restoring kinase activity, mRNA translation and cell growth (GALE et al., 1998). In mammalian cells, PKRs are important in the regulation of virus multiplication as phosphorylated eIF-2alpha kinases results in the inhibition of protein translation. $p58^{IPK}$-deficiency resulted in increased eIF-2alpha phosphorylation and decreased virus mRNA translation in mice fibroblasts (GOODMAN et al., 2007; LUIG et al., 2010). $p58^{IPK}$ has also been demonstrated to function as a co-chaperone and play a protective role in response to cytoplasmic stress (GALE et al., 2002) and stressed endoplasmic reticulum, including in insects (RUTKOWSKI et al., 2007; CHOW et al., 2013). Thus, the up-regulation of $p52^{rIPK}$ may be associated with the increased response of *Spiroplasma*-infected aphids to viral protein translation, even though *Spiroplasma*-infected aphids had the JAK/STAT signal transduction pathway inhibited by e3 sumo-protein ligase pias2-like as earlier discussed. Otherwise, overproduction of $p52^{rIPK}$ may be involved with $p58^{IPK}$ inhibition to maintain eIF-2a phosphorylation to regulate cell response to stress and apoptosis (CLEMENS, 2001; GALE et al., 2002;).

Regulation of glutathione synthetase transcription in *Buchnera* associated with *Spiroplasma*-infected aphids on sweet orange may be related with the participation of *Buchnera* in the regulation of reactive oxygen species (ROS) produced due to cellular stress or to the metabolism of xenobiotics. Glutathione synthetase participates in glutathione biosynthesis (LAW; HALLIWELL, 1986), and glutathione can be used to neutralize ROS by glutathione peroxidase or participate in the detoxification of xenobiotics by glutathione-S-transferases. Therefore, regulation in the biosynthesis of glutathione synthetase is crucial in the maintenance of cellular defense against environmental stressors (ORTIZ et al., 2009; NAIR et al., 2013; LIU et al., 2015). Although enhanced glutathione synthesis has been suggested in *Spiroplasma*-infected aphids in sweet orange, no differential transcription was observed for glutathione peroxidases or glutathione-S-transferases transcripts, limiting our hypothesis (but see below our discussion on host plant effects on aphid transcription as affected by *Spiroplasma*).

Regulation of gene expression of *A. citricidus* in response to *Spiroplasma* infection when aphids were reared on orange jasmine indicated *Spiroplasma* interfered with *Buchnera*

growth and activity due to the down-regulation of NAD-dependent DNA ligase and trigger factor, respectively. NAD-dependent DNA ligase fixes DNA brakes during replication, repair and recombination, utilizing NAD+ as cofactor (LEE et al., 2000; SHIGENOBU et al., 2000). Regulation of *Buchnera* genome replication is usually more intense during embryogenesis, as mothers have to pass bacteriocytes with *Buchnera* to their progenies (WILKINSON; FUKATSU; ISHIKAWA, 2003). Trigger factor acts like a chaperonin aiding in the exportation of newly unfolded proteins (MORAN; MCLAUGHLIN; SOREK, 2009). Thus, we believe *Spiroplasma* down-regulates *Buchnera* growth and activity in orange jasmine due to the lower suitability of this host plant to the aphid.

The regulation of heat shock proteins (HSP) in *Spiroplasma*-infected aphids on orange jasmine also indicates the up-regulation of HSPs aids infected aphids to handle stressors associated with the aphid host plant. Heat shock 68 and heat shock 70 are well known in *Drosophila* as they share high similarity in their structure and amino acid composition (KELLETT; MCKECHNIE, 2004; VELIKODVORSKAIA et al., 2005), and are thought to have evolved from a single ancestor by gene duplication, in which *hsp68* locus may have arisen via duplication of the *hsp70* locus (KELLETT; MCKECHNIE, 2005). HSP70 family of proteins are the most temperature sensitive and highly conserved of the HSPs among mammals, insects, yeast and bacteria (FEDER, HOFMANN, 1999). HSP70 are related to responses to cellular stress caused by hypothermia, energy depletion, hypoxia, acidosis, reactive oxygen species (ROS), reactive nitrogen species such as nitric oxide, and viral infection. HSP70 acts on cellular processes, such as activation of proteases, release of lysosomal and proteolytic enzymes, alterations of the cytoskeleton (KREGEL, 2002) and programmed cell death (BEERE et al., 2000; GARRIDO et al., 2001).

Host plant had a strong effect in gene expression of *A. citricidus*, influencing transcription of genes involved in several pathways such as nutrient sensing, energy metabolism, nutrient metabolism, nutrient transport, urea cycle, digestion, transport and inactivation of xenobiotics, immune response and programmed cell death. However, host plant effect in *A. citricidus* gene expression differed in the presence of *Spiroplasma* infection, indicating *Spiroplasma* does influence the aphid - host plant interaction.

The effects of host plant in the nutrient sensing pathway [target of rapamycin (TOR) pathway] and regulation of cell growth was observed by the differential regulation of genes like Rheb-like proteins (TR132940), several insulin-like growth factors (TR122855, TR130160, TR134022, TR204305), RAS-related proteins (transcripts TR115471, TR122892, TR126966, TR124550, TR131340, TR132045, TR133035), MAP kinases (TR121016, TR129411,

TR129682, TR133499), and ribosomal protein S6 kinase (S6K) (TR117520) (JEWELL; GUAN, 2013). Although the signaling pathway is not entirely represented, several of the intermediates of this pathway were identified as differentially expressed, including one of the substrates of the mTORC1, the S6K (JEWELL; GUAN, 2013). The eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1), which is also a substrate of mTORC1 and a repressor of translation initiation by preventing the assembly of eiF4E into the eiF4F complex through competition with eiF4G1/eiG4G3, was not detected (JEWELL; GUAN, 2013). But several eukaryotic translation initiation factors (eiF) were differentially expressed, including subunits of eiF3 (TR117300, TR128965, TR131905) and isoforms of eiF4G (TR128256, TR130620, TR132982), indicating strong translation activity. However, differential expression of these genes in response to host plant (orange jasmine and sweet orange) were affected by *Spiroplasma* infection. While all these genes were up-regulated in *Spiroplasma*-free aphids feeding on sweet orange as compared to orange jasmine, they suffered down-regulation in *Spiroplasma*-infected aphids. Up-regulation of these genes in uninfected aphids correlates with the higher nutrient availability in aphids due to the exploration of a higher quality host, supporting our comparative analysis on the host plant effect on aphid biology (see Chapter 2). However, *Spiroplasma* alters the aphid response to the nutritional quality of the host plant and down-regulates the nutrient sensing pathway and the regulation of cell growth in sweet orange as compared to orange jasmine. However, such strategy does not influence aphid fitness traits either on sweet orange or orange jasmine (see Chapter 6), demonstrating *Spiroplasma* can use such strategies to benefit its own maintenance while still providing adequate stimuli for aphid growth on both host plants.

Host plant also influenced the transport and metabolism of sugars and lipid biosynthesis. Transport and metabolism of sugars was evident by the differential expression of several facilitated trehalose transporter TRET transcripts (TR121366, TR126066, TR127850, TR128134, TR130085, TR131747, TR131926), which are required for trehalose transport from the fat body to tissues requiring a carbon source of energy, and ultimately regulating the levels of glucose in the hemolymph (KANAMORI et al., 2010). High trehalose transport activity is also supported by glycogen conversion into trehalose in the aphid´s fat bodies, as indicated by the differential transcription of glycogen phosphorylases (TR131029 and TR13135), which metabolizes glycogen into glucose-1-phosphate, phosphoglucomutase (TR132675), which converts glucose-1-phosphate into glucose-6-phosphate, uridine diphosphate glucose pyrophosphatase (TR127609), which converts glucose-1-phosphate into trehalose-6-phosphate, and trehalose-6-phosphate synthase (TR121083), which converts glucose-6-phosphate into

trehalose-6-phosphate, being trehalose-6-phosphate the last step for trehalose synthesis in insect fat body cells (BECKER et al., 1996). Acetyl-coenzyme A synthetase (TR127602), an enzyme involved in the production of acetyl-CoA from acetate is also an indicative of regulation of lipogenesis in response to the food sources. Regulation of lipogenesis is corroborated as the activity of acetyl-carboxylase (TR130683, TR130480, TR13083), which converts acetyl-CoA into malonyl-CoA that serves as substrate for fatty acid synthase (TR120208, TR124676, TR130133) to produce fatty acids, and transcripts of elongation fatty acid proteins (TR119308, TR126666, TR128391), which participate in the elongation cycle of fatty acids, follow the expression of acetyl-coenzyme A synthetase. Once again, both glycogenolysis and lipogenesis are positively affected in uninfected aphids feeding on sweet orange as compared to orange jasmine. But both pathways follow a different trend in *Spiroplasma*-infected aphids, with a down-regulation in infected aphids feeding on sweet orange as compared to orange jasmine. These facts demonstrate that *Spiroplasma* also manipulates the glycogenolysis and fatty acid biosynthesis pathways in *A. citricidus* in order to promote a balanced energy metabolism in the host aphid without causing fitness effects.

Substrate degradation by the proteasome was another process affected by the host plant quality, but differently influenced by *Spiroplasma* infection. There were 18 differentially expressed E3-ubiquitin protein ligases in aphids fed on sweet orange as compared to orange jasmine. Gene expression was higher in *Spiroplasma*-uninfected aphids feeding on sweet orange as compared to orange jasmine, but the opposite was observed in *Spiroplasma*-infected aphids. E3-ubiquitin protein ligases recruits E2 ubiquitin-conjugating enzymes loaded with ubiquitin to facilitate the transfer of ubiquitin from the E2 to the protein substrate, a common process to target the ubiquinated substrate to destruction by the proteasome. Ubiquination is associated with a number of processes including apoptosis, cell cycle, immune response, modulation of cell surface receptors, ion channels and secretory pathways, and response to stress (PICAKART, 2001), all of them influenced by host plant utilization in *A. citricidus*. Ubiquitin-tagged proteins are degraded by proteolysis in proteasomes located in the nucleus and cytoplasm to regulate the concentrations of proteins and degrade misfolded proteins. Products of the degradation process can be reutilized for the synthesis of new proteins. Proteosomal degradation is a key process in cell cycle, regulation of gene expression and responses to oxidative stress. Several proteasome transcripts (TR116483, TR118021, TR121879, TR124406, TR131366) were affected by host plant utilization, and their transcription level followed that of the E3-ubiquitin protein ligases.

Proteasomal degradation also occurs in an ubiquitin-independent manner. An important substrate targeted to ubiquitin-independent proteosomal degradation is ornithine decarboxylase. Ornithine decarboxylase (TR126538) (ODC) transcription level was also influenced by the use of host plants, but the antizyme factor required for ODC degradation by the proteasome was not detected. Therefore, increased ODC transcription is not targeted to the increased activity of the proteasome. ODC participates of the first steps involved in the synthesis of polyamines, which are important as antioxidants and for cell growth through stabilization of the DNA structure and repair (PEGG, 1988). Polyamine synthesis is supported as spermidine synthase (TR125061) and a polyamine-binding protein (TR132652) transcription levels followed that of ODC. Thus, increased expression level of such genes indicates uninfected-*A. citricidus* have a higher cellular activity and cell growth when feeding on sweet orange as compared to orange jasmine. Interestingly, *Spiroplasma* clearly up-regulated genes involved with the host cellular metabolism and cell growth when developing on low quality (orange jasmine) as compared to a high quality (sweet orange) hosts.

Ubiquination is also involved in responses to stress conditions, and the increased ubiquination detected in uninfected aphids when feeding on sweet orange may correlate with the increased response to oxidative stress. Increased production of free radicals, mainly reactive oxygen species in uninfected-aphids feeding on sweet orange was suggested by the high number of transcripts involved in the bioactivation of xenobiotics, such as cytochrome P450 (10 transcripts) and prostaglandin synthase (TR120492). Increased ROS inactivation is suggested by the elevated transcription of superoxide dismutase (TR119656), which use reactive oxygen for the production of H_2O_2, and catalase (TR128594), involved in the convection of H_2O_2 in water. Increased activity of ROS inactivation in uninfected-aphid on sweet orange as compared to orange jasmine follows the increased cellular and metabolic activities observed in such conditions. But, as expected due to the regulation of the cellular and metabolic pathways in *Spiroplasma*-infected aphids, ROS inactivation in infected aphids has been reduced in sweet orange as compared to orange jasmine.

Host plants also influence the detoxification machinery of aphids in response to host plant utilization, and all three steps of the detoxification process were represented, with the differential expression of enzymes – P450 monooxygenases (10 transcripts), gluthatione-S-transferases (TR96761, TR120897, TR126759) – and transporters – ATP-binding cassette (ABC) (10 transcripts). Once again, gene regulation of *A. citricidus* in response to the host plant was affected by *Spiroplasma* infection. All of these transcripts were up-regulated in uninfected aphids on sweet orange as compared to orange jasmine, but in *Spiroplasma*-infected aphids the

expression of these genes was increased in orange jasmine when compared to sweet orange instead.

P450s are a diverse class of enzymes that interacts with endogenous (ecdysteroids and juvenile hormone), as with exogenous compounds such as plant allelochemicals and insecticides (FEYEREISEN, 1999). All P450s differentially expressed in aphids feeding on sweet orange as compared to orange jasmine play a role in the detoxification of xenobiotics (WAN et al., 2014; PENG et al., 2016, WEN; SCOTT, 2001; PRIDGEON; ZHANG; LIU, 2003). ABC transporters are involved in the transport of molecules and play an important role in extruding drugs and toxins out of the cell, helping in the detoxification process (HIGGINS, 1995). It is clear that the metabolic cost in host utilization between infected and uninfected aphids is quite different depending on their association with *Spiroplasma*. Thus, the process of detoxification through enzymatic activity and transport of toxins is also influenced by *Spiroplasma* infection and is dependent on the quality of the host plant. Such mechanisms are only influenced by *Spiroplasma* if the host aphid is subjected to nutritional stress.

Spiroplasma induced the up-regulation of several transcripts related to nutrition on orange jasmine. tRNA synthetase and/or tRNA ligases were observed for the non-essential amino acids alanine (TR57343), asparagine (TR123710, TR123330, TR123744), cysteine (TR47482, TR134350), glutamine (TR130602), glycine (TR122402), proline (TR130546) and tyrosine (TR128943), and for the essential amino acids histidine (TR133527), isoleucine (TR1219567, TR167922), leucine (TR127652, TR133930), lysine (TR132064), threonine (TR172342, TR180769) and valine (TR50487, TR132872). Besides, transcripts related to amino acid transport, amino acid synthesis and amino acid release, such as amino acid transporter (TR121633, TR126493), citrate synthase (TR132492), anthranilate synthase (TR125155), and methionine aminopeptidase (TR134113). Aphid nutrition is a balance between host and *Buchnera* amino acid pathways. *Buchnera* has biosynthesis pathways for the non-essential amino acids cysteine, glycine and tyrosine, and for most of the essential amino acids except leucine, isoleucine, valine, methionine, and phenylalanine (HANSEN; MORAN, 2011). Aphid and *Buchnera* shared amino acids pathways were elucidated after *A. pisum* and *Buchnera* complete sequencing (WILSON et al., 2010; HANSEN; MORAN, 2011), and although *Spiroplasma* induces an increase in tRNA synthetases and in enzymes involved with amino acid synthesis and aminoacid transporters, it does not seem to regulate the *Buchnera* amino acid contribution to the host.

In conclusion, *Spiroplasma* affects *A. citricidus* regardless of the host plant use, and the effect of the host plant on aphid gene expression is severely affected by *Spiroplasma* infection.

Uninfected aphids showed high levels of cellular activity and cell growth when feeding on sweet orange, but such trend was just observed for *Spiroplasma*-infected aphids feeding on orange jasmine. In addition, *Spiroplasma* influenced detoxification mechanisms if *A. citricidus* was subjected to nutritional stress. Thus, *Spiroplasma* manipulates several biosynthesis and degradation pathways in *A. citricidus* in order to improve energy metabolism in the host aphid under nutrition stress caused by low quality host plant.

7.5 Conclusions

- *Aphis citricidus* transcriptome was affected by *Spiroplasma* infection on both sweet orange and orange jasmine;
- Gene expression regulation of *Spiroplasma*-infected aphids was more intense on sweet orange than orange jasmine;
- *Spiroplasma* induced the up-regulation of all of the differentially expressed transcripts in infected *Aphis citricidus* on sweet orange and as most of them on orange jasmine;
- Host plant also affected *A. citricidus* transcriptome regardless of *Spiroplasma* infection;
- *Spiroplasma*-free and *Spiroplasma*-infected *Aphis citricidus* shared more than 9,000 differentially expressed transcripts when feeding on sweet orange as compared to orange jasmine;
- Shared differentially expressed transcripts between *Aphis citricidus* on sweet orange and orange jasmine followed a different trend in *Spiroplasma*-infected as compared to uninfected aphids;
- *Spiroplasma* infection and host plant quality affected mainly the expression of digestive enzymes, amino acids synthesis, energy metabolism, and detoxification transcripts and transcriptional and translational regulation factors;
- *Spiroplasma* alters aphid energy allocation by inducing the up-regulation of immune response genes on an optimal host plant, while inducing up-regulation of genes related to nutrient uptake and detoxification of plant secondary metabolites on a suboptimal host plant.

References

ALTSCHUL, S. F.; MADDEN, T.L.; SCHÄFFER, A.A.; ZHANG, J.; ZHANG, Z.; MILLER, W.; LIPMAN, D.J. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. **Nucleic Acids Research,** Oxford, v. 25, n. 17, p. 3389-3402, 1997.

ANDERS, S.; HUBER, W. Differential expression analysis for sequence count data. **Genome Biology,** London, v. 11, n. 10, 2010. Available at: < doi: 10.1186/gb-2010-11-10-r106>.

ANDREWS, S. FastQC A Quality Control tool for High Throughput Sequence Data. 2010. Available at: < http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ >.

AU, K.G.; CLARK, S.; MILLER, J.H.; MODRICH, P. *Escherichia coli* mutY gene encodes an adenine glycosylase active on G-A mispairs. **Proceedings of the National Academy of Sciences of the United States of America**, Washington, v.86, p.8877-8881, 1989.

BAUMANN, P. Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. **Annual Review of Microbiology,** Palo Alto, v. 59, p. 155-189, 2005.

BECKER, A.; SCHLDER, P.; STEELE, J.E.; WEGENER, G. The regulation of trehalose metabolism in insects. Experientia, **Basel**, v.52, p. 433-439, 1996.

BEERE, L.M.; WOLF, B.B.; CAIN, K.; MOSSER, D.D.; MAHBOUBI, A.; KUWANA, T.; TAILOR, P.; MORIMOTO, R.I.; COHEN, G.D.; GREEN, D.R. Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. **Nature Cell Biology**, London, v.2, p. 469-475, 2000.

CHOW, C.Y.; WOLFNER, M.F.; CLARK, A.G. Using natural variation in Drosophila to discover previously unknown endoplasmic reticulum stress genes. **Proceedings of the National Academy of Sciences,** Washington, v.110, p. 9013-9018, 2013.

CIOCCA, D.R.; CAPPELLO, F.; CUELLO-CARRIÓN, F.D.; ARRIGO, A.P. Molecular Approaches to Target Heat Shock Proteins for Cancer Treatment. IN: **Frontiers in Clinical Drug Research - Anti-Cancer Agents,** v.2, p. 3-47, 2015.

CLEMENS, M.J .Initiation factor eIF2a phosphorylation in stress responses and apoptosis. **Progress in Molecular and Subcellular Biology**, New York, v.27, p. 57-89, 2001.

CONESA, A.; GÖTZ, S.; GARCÍA-GÓMEZ, J.M.; TEROL, J.; TALÓN, M.; ROBLES, M. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. **Bioinformatics,** Oxford, v. 21, n. 18, p. 3674-3676, 2005.

DOUGLAS, A. E. Nutritional interactions in insect-microbial symbioses: Aphids and their symbiotic bacteria *Buchnera*. **Annual Review of Entomology,** Stanford, v. 43, p. 17-37, 1998.

DOSTERT, C.; JOUANGUY, E.; IRVING, P.; TROXIER, L.; GALLANA-ARNOUX, D.; HETRU, C.; HOFFMAN, J.A.; IMLER, J.L.The Jak-Stat signaling pathway is required but not sufficient for the antiviral response of *Drosophila*. **Nature Immunology**, New York, v.6, p. 946-953, 2005.

FEDER, M. E.; HOFMANN, G. E. Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. **Annual Review of Physiology,** Palo Alto, v. 61, p. 243-282, 1999.

FEYEREISEN, R. Insect P450 enzymes. **Annual Review of Entomology**, Palo Alto, v.44, p.507-533, 1999.

FUKATSU, T.; TSUCHIDA, T.; NIKOH, N.; KOGA, R. *Spiroplasma* symbiont of the pea aphid, *Acyrthosiphon pisum* (Insecta : Homoptera). **Applied and Environmental Microbiology,** Washington, v. 67, n. 3, p. 1284-1291, 2001.

GALES JR, M.; BLAKELY, C.M.; HOPKINS, D.H.; MELVILLE, M.W.; WAMBACH, M.; ROMANO, P.R.; KATZE, M.G. Regulation of interferon-induced protein kinase PKR: Modulation of P58IPK inhibitory function by a novel protein, P52rIPK . **Molecular and Cellular Biology**, Washington, v. 18, p. 859-871, 1998.

GALE JR, M.; BLAKELY, C.M; DARVEAU, A.; ROMANO, P.R.; KORTH, M.J.; KATZE, M.G. $P52^{rIPK}$ regulates the molecular cochaperone $P58^{IPK}$ to mediate control of the RNA-dependent protein kinase in response to cytoplasmic stress. **Biochemistry,** Washington, v. 41, p.11878-11887, 2002..

GARRIDO, C.; GURBUXANI, S.; RAVAGNAN, L.; KROEMER, G. Heat shock proteins: endogenous modulators of apoptotic cell death. **Biochemical and Biophysical Research Communications**, Philadelphia, v. 286, p. 433-442, 2001.

GOODMAN, A.G.; SMITH, J.A.; BALACHANDRAN, S.; PERWITASARI, O.; PROLL, S.C.; THOMAS, M.J.; KORTH, M.J.; BARBER, G.N.; SCHIFF, L.A.; KATZE, M.G. The cellular protein P58IPK regulates influenza virus mRNA translation and replication through a PKR-mediated mechanism. **Journal of Virology**, Washington, v.81, p.2221–2230, 2007.

GRABHERR, M. G.; HAAS, B.J.; YASSOUR, M.; LEVIN, J.Z.; THOMPSON, D.A.; AMIT, I.; ADICONIS, X.; FAN, L.; RAYCHOWDHURY, R.; ZENG, Q.; CHEN, Z.; MAUCELI, E.; HACOHEN, N.; GNIRKE, A.; RHIND, N.; DI PALMA, F.; BIRREN, B.W.; NUSBAUM, C.; LINDBLAD-TOH, K.; FRIEDMAN, N.; REGEV, A. Full-length transcriptome assembly from RNA-Seq data without a reference genome. **Nature Biotechnology,** New York, v. 29, n. 7, p. 644-652, 2011.

HANSEN, A. K.; MORAN, N. A. The impact of microbial symbionts on host plant utilization by herbivorous insects. **Molecular Ecology,** Oxford, v. 23, n. 6, p. 1473-1496, 2014.

_____. Aphid genome expression reveals host-symbiont cooperation in the production of amino acids. **Proceedings of the National Academy of Sciences USA**, Washington, v.108, p. 2849-2854, 2011.

HENRY, L. M.; MAIDEN, M.C.; FERRARI, J.; GODFRAY, H.C. Insect life history and the evolution of bacterial mutualism. **Ecology Letters,** Oxford, v. 18, n. 6, p. 516-525, 2015.

HENRY, L. M.; PECCOUD, J.; SIMON, J.C.; HADFIELD, J.D.; MAIDEN, M.J.; FERRARI, J.; GODFRAY, H.C. Horizontally Transmitted Symbionts and Host Colonization of Ecological Niches. **Current Biology,** Cambridge, v. 23, n. 17, p. 1713-1717, 2013.

HIGGINS, C.F. The ABC of channel regulation. **Cell**, Cambridge, v.82, p. 693-696, 1995.

JEWELL, J.L.; GUAN, K.L. Nutrient signaling to mTOR and cell growth. **Trends in Biochemical Science**, Oxford, v.38, p.233-242, 2013.

KANAMORI, Y.; SAITOA, A.; HAGIWARA-KOMODA, Y.; TANAKA, D.; MITSUMASU, K.; KIKUTA, S.; WATANABE, M.; CORNETTE, R.; KIKAWASA, T.; OKUDA, T. The trehalose transporter 1 gene sequence is conserved in insects and encodes proteins with different kinetic properties involved in trehalose import into peripheral tissues. **Insect Biochemistry and Molecular Biology**, Oxford, v.40, p. 30-37, 2010.

KANEHISA, M.; GOTO, S. KEGG: Kyoto Encyclopedia of Genes and Genomes. **Nucleic Acids Research,** Oxford, v. 28, n. 1, p. 27-30, 2000.

KELLETT, M.; MCKECHNIE, S.W. A cluster of diagnostic Hsp68 amino acid sites that are identified in *Drosophila* from the melanogaster species group are concentrated around -sheet residues involved with substrate binding. **Genome**, Ottawa, v. 48, p. 226–233, 2005.

KREGEL, K.C. Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. **Journal of Applied Physiology**, Bethesda, v.92, p.2177-2186, 2002.

LANGMEAD, B.; SALZBERG, S. L. Fast gapped-read alignment with Bowtie 2. **Nature Methods,** London, v. 9, n. 4, p. 357-359, 2012.

LAW, M.Y.; HALLIWELL, B. Purification and properties of glutathione synthetase from (*Spinacia oleracea*) leaves. **Plant Science**, Shannon, v.43, p. 185-191, 1986.

LEE, J.Y.; CAHNG, C.; SONG, H.K.; MOON, J.; YANG, J.K.; KIM, H.; KWON, S.; SUH, S.W. Crystal structure of NAD+-dependent DNA ligase: modular architecture and functional implications. **The EMBO Journal**, West Sussex, v.19, p.1119-1129, 2000.

LI, B.; DEWEY, C. N. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. **Bmc Bioinformatics,** London, v. 12, 2011. Available at: < doi: 10.1186/1471-2105-12-323>.

LI, W.; GODZIK, A. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. **Bioinformatics,** Oxford, v. 22, n. 13, p. 1658-1659, 2006.

LIU, S.; CHOUGULE, N.P; VIJAYENDRAN, D.; BONNING, B.C. Deep Sequencing of the Transcriptomes of Soybean Aphid and Associated Endosymbionts. **Plos One,** San Franscico, v. 7, n. 9, 2012. Available at: <DOI: 10.1371/journal.pone.0045161>.

LIU, X.; ZHANG, S.; WHITWORTH, R.J.; STUART, J.J.; CHEN, M.S. Unbalanced activation of glutathione metabolic pathways suggests potential involvement in plant defense against the gall midge *Mayetiola destructor* in wheat. **Scientifc Reports**, London, v.5, 2015. Available at: < doi: 10.1038/srep08092>.

LUIG, C.; KOTHER, K.; DUDEK, S.E.; GAESTEL, M.; HISCOTT, J.; WIXLER, V.; LUDWIG, S .MAP kinase-activated protein kinases 2 and 3 are required for influenza A virus

propagation and act via inhibition of PKR. **The FASEB Journal,** Bethesda, v.24, p. 4068-4077, 2010.

LUKASIK, P.; GUO, H.; van ASCH, M.; FERRARI, J.; GODFRAY, H.C. Protection against a fungal pathogen conferred by the aphid facultative endosymbionts *Rickettsia* and *Spiroplasma* is expressed in multiple host genotypes and species and is not influenced by co-infection with another symbiont. **Journal of Evolutionary Biology,** West Sussex, v. 26, n. 12, p. 2654-2661, 2013.

LUKASIK, P.; GUO, H.; VAN ASCH, M.; HENRY, L.M.; GODFRAY, C.J.; FERRARI, J. Horizontal transfer of facultative endosymbionts is limited by host relatedness. **Evolution**, West Sussex, v.69, p.2757-2766, 2015.

MORAN, N.A.; MCLAUGHLIN, H.J; SOREK, R. The Dynamics and Time Scale of Ongoing Genomic Erosion in Symbiotic Bacteria. **Science**, Washington, v.323, 2009.

MYLLYMÄKI, H.; RÄMET, M. JAK/STAT pathway in *Drosophila* immunity. **Scandinavian Journal of Immunology**, West Sussex, v.79, p. 377–385, 2014.

NAIR, P.M.; PARK, S.Y.; CHUNG, J.W.; CHOI, J. Transcriptional regulation of glutathione biosynthesis genes, γ-glutamyl-cysteine ligase and glutathione synthetase in response to cadmium and nonylphenol in *Chironomus riparius*. **Environmental Toxicology and Pharmacology**, Amsterdam, v.36, p. 265-273, 2013.

NAKABACHI, A.; ISHIDA, K.; HONGOH, Y.; OHKUMA, M.; MIYAGISHIMA, S-Y. Aphid gene of bacterial origin encodes a protein transported to an obligate endosymbiont. **Current Biology,** Heidelberg, v. 24, n. 14, p. R640-R641, 2014.

NISHIKORI, K.; MORIOKA, K.; KUBO, T.; MORIOKA, M. Age- and morph-dependent activation of the lysosomal system and *Buchnera* degradation in aphid endosymbiosis. **Journal of Insect Physiology,** Oxford, v. 55, n. 4, p. 351-357, 2009.

NOVAKOVA, E.; HYPŠA, V.; KLEIN, J.; FOOTTIT, R.G.; von DOHLEN, C.D.; MORAN, N.A. Reconstructing the phylogeny of aphids (Hemiptera: Aphididae) using DNA of the obligate symbiont *Buchnera aphidicola*. **Molecular Phylogenetics and Evolution,** Maryland Heights, v. 68, n. 1, p. 42-54, 2013.

OLIVER, K. M. ; CAMPOS, J.; MORAN, N.A.; HUNTER, M.S. Population dynamics of defensive symbionts in aphids. **Proceedings of the Royal Society B-Biological Sciences,** Edinburgh, v. 275, n. 1632, p. 293-299, 2008.

_____. Facultative Symbionts in Aphids and the Horizontal Transfer of Ecologically Important Traits. **Annual Review of Entomology**, Stanford, v.55, p.247-266, 2010.

OLIVER, K.M.; RUSSELL, J.A.; MORAN, N.A.; HUNTER, M.S. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. **Proceedings of the National Academy of Sciences of the United States of America,** Washington, v. 100, n. 4, p. 1803-1807, 2003.

ORTIZ, J.G.; OPOKA, R.; KANE, D.; CARTWRIGHT, I.L. Investigating arsenic susceptibility from a genetic perspective in *Drosophila* reveals a key role for glutathione synthetase. **Toxicological Sciences**, Oxford, v.107, p.416-426, 2009.

PEGG, A.E. Polyamine metabolism and its importance in neoplastic growth and a target for chemotherapy. **Cancer Research**, Philadelphia, v.48, p. 759-774, 1988.

PENG, T.; PANA, Y.; YANGE, C.; GAOB, X.; XIA, J.; WUA, Y.; HUANGA, X.; ZHUA, E.; XINA, X.; ZHANA, C.; SHANGA, Q. Over-expression of CYP6A2 is associated with spirotetramat resistance and cross-resistance in the resistant strain of *Aphis gossypii* Glover. **Pesticide Biochemistry and Physiology**, Maryland Heights, v.126, p. 64-69, 2016.

PICAKART, C.M. Mechanisms underlying ubitination. **Annual Review of Biochemistry**, Palo Alto, v.70, p.503-533, 2001.

PRIDGEON, J.W.; ZHANG, L.; LIU, N. Overexpression of CYP4G19 associated with a pyrethroid-resistant strain of the German cockroach, *Blattella germanica* (L.). **Gene**, Amsterdam, v.314, p. 157-163, 2003.

RAMSEY, J.S.; MACDONALD, S.J.; JANDER, G.; NAKABACHI, A.; THOMAS, G.H.; DOUGLAS, A.E Genomic evidence for complementary purine metabolism in the pea aphid, *Acyrthosiphon pisum*, and its symbiotic bacterium *Buchnera aphidicola*. **Insect Molecular Biology**, West Sussex, v.19, p. 241-248, 2010.

RUSSELL, C.W.; BOUVAINE, S.; NEWELL, P.D.; DOUGLAS, A.E. Shared metabolic pathways in a coevolved insect-bacterial symbiosis. **Applied and Environmental Microbiology**, Washington, v.79, p. 6117-6123, 2013.

RUSSELL, J. A.; MORAN, N. A. Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. **Proceedings of the Royal Society B-Biological Sciences,** Edinburgh, v. 273, n. 1586, p. 603-610, 2006.

RUTKOWSKI, D,T.; KANG, S.W.; GOODMAN, A.G.; GARRISON, J.L.; TAUNTON, J.; KATZE, M.G.; KAUFMAN, R.J.; HEDGE, R.S. The role of $p58^{IPK}$ in protecting the stressed endoplasmic reticulum. **Molecular Biology of the Cell**, Bethesda, v.18,p. 3681-3691, 2007.

SHIGENOBU, S.; WATANABE, H.; HATTORI, M.; SAKAKI, Y.; ISHIKAWA, H. Genome sequence of the endocellular bacterial symbiont of aphids Buchnera sp APS. **Nature**, London, v. 407, n. 6800, p. 81-86, 2000.

SHUAI, K.; LIU, B. Regulation of gene activation pathways by pias proteins in the immune system. **Nature Reviews: immunology**, London, v.5, p. 593-605, 20

SIMON, J.C.; BOUTIN, S.; TSUCHIDA, T.; KOGA, R.; LE GALLIC, J.; FRANTZ, A.; OUTREMAN, Y.; FUKATSU, T. Facultative Symbiont Infections Affect Aphid Reproduction. **Plos One,** San Franscico, v. 6, n. 7, 2011. Available at: <DOI: 10.1371/journal.pone.0021831 >.

SOGA, T.; BARAN, R. SUEMATSU, M.; UENO, Y.; IKEDA, S.; SAKURAKAWA, T.; KAKAZU, Y.; ISHIKAWA, T.; ROBERT, M.; NISHIOKA, T.; TOMITA, M. Differential

Metabolomics Reveals Ophthalmic Acid as an Oxidative Stress Biomarker Indicating Hepatic Glutathione Consumption. **The Journal of Biological Chemistry**, New York, v.281, p. 16768-16776, 2006.

SONG, X.; ZHANG, Z.; WANG, S.; LI, H.; ZUO, H.; XU, X.; WENG, S.; HE, J.; LI, C. A Janus kinase in the JAK/STAT signaling pathway from *Litopenaeus vannamei* is involded in antiviral immune response. **Fish & Shellfish Immunology,** London, v.44, p. 662-673, 2015.

SU, Q.; ZHOU, X.; ZHANG, Y. Symbiont-mediated functions in insect hosts. **Communicative & Integrative Biology,** New York, v. 6, n. 3, 2013. Available at: < doi: 10.4161/cib.23804>.

TSUCHIDA, T.; KOGA, R.; FUKATSU, T. Host plant specialization governed by facultative symbiont. **Science,** Washington, v. 303, n. 5666, p. 1989-1989, 2004.

VELIKODVORSKAIA, V. V.; LYOZIN, G.T.; FEDER, M.E.; EVGEN'EV, M.B. Unusual arrangement of the hsp68 locus in the virilis species group of *Drosophila* implicates evolutionary loss of an hsp68 gene. **Genome**, Ottawa, v. 48, p. 234–240, 2005.

VIGNERON, A.; MASSON, F.; VALLIER, A.; BALMAND, S.; REY, M.; VINVENT-MONEGAT, C.; AKSOY, E.; AUBAILLY-GIRAUD, E.; ZAIDMAN-REMY, A.; HEDDI, A. Insects Recycle Endosymbionts when the Benefit Is Over. **Current Biology**, Cambridge, v. 24, p.2267-2273, 2014.

WAGNER, S. M.; MARTINEZ, A.J.; RUAN, Y.; KIM, K.L.; LENHART, .A.; DEHNEL, A.C.; OLIVER, K.M.; WHITE, J.A. Facultative endosymbionts mediate dietary breadth in a polyphagous herbivore. **Functional Ecology**, West Sussex, v.29, n.11, p. 1402-1410, 2015.

WAN, H.; LIU, Y.; LI, M.; ZHU, S.; LI, X.; PITTENDRIGH, B.R.; QIU, X. Nrf2/Maf-binding-site-containing functional Cyp6a2 allele is associated with DDT resistance in *Drosophila melanogaster.* **Pest Management Science**, West Sussex, v.70, p. 1048-1058, 2014.

WEN, Z.; SCOTT, J.G. Cloning of two novel P450 cDNAs from German cockroaches, *Blattella germanica.* **Insect Molecular Biology**, West sussex,v.10, p.131-137, 2001.

WILKINSON, T. L.; FUKATSU, T.; ISHIKAWA, H. Transmission of symbiotic bacteria *Buchnera* to parthenogenetic embryos in the aphid *Acyrthosiphon pisum* (Hemiptera : Aphidoidea). **Arthropod Structure & Development,** Oxford, v. 32, n. 2-3, p. 241-245, 2003.

WILSON, A.C.; ASHTON, P.D.; CALEVRO, F.; CHARLES, H.; CALELLA, S.; FEBVAY, G.; JANDER, G.; KUSHLAN, P.F.; MADONALD, S.J.; SCHWARTZ, J.F.; THOMAS, G.H.; DOUGLAS, A.E. Genomic insight into the amino acid relations of the pea aphid, *Acyrthosiphon pisum*, with its symbiotic bacterium *Buchnera aphidicola*. **Insect Molecular Biology**, West Sussex, v.3, 2010, Available at:< doi: 10.1111/j.1365-2583.2009.00942.x.>.

WU, Q.; BROWN, M.R. Signaling and function of insulin-like peptides in insects. **Annual Review of Entomology**, Palo Alto, v.51, p.1-24, 2006.

ZDOBNOV, E. M.; APWEILER, R. InterProScan - an integration platform for the signature-recognition methods in InterPro. **Bioinformatics,** Oxford, v. 17, n. 9, p. 847-848, 2001.

ZYTYNSKA, S.E.; WEISSER, W.W. The natural occurrence of secondary bacterial symbionts in aphids. **Ecological Entomology**, London, v. 41, n. 1, p. 13-26, 2016.

8 PROTEOMICS OF THE INTERACTION *Aphis (Toxoptera) citricidus- Spiroplasma* ON TWO HOST PLANTS

Abstract

Bacterial symbionts are broadly distributed among insects, influencing insect biology and ecology to different degrees. Aphids are commonly associated with primary and secondary symbionts. *Buchnera aphidicola* is the primary symbiont that provides essential amino acids the host is unable to produce or acquire from the host plant. Aphids also carry a number of secondary symbionts that can influence aphid physiology and fitness attributes, including changes in host metabolites and immune defense. *Spiroplasma* is one of such secondary symbionts associated with aphids, but very little is known about such association and its effects on the aphid trophic interactions. *Spiroplasma* is not usually reported as a common aphid secondary symbiont, but a high level of infection has been demonstrated in one population of *Aphis (Toxoptera) citricidus*. We used sister isolines of *Spiroplasma*-infected (Ac-BS) and *Spiroplasma*-free (Ac-B) aphids reared on sweet orange and orange jasmine as host plants to demonstrate the effects of *Spiroplasma* infection on the proteomics of *A. citricidus*. *Spiroplasma* infection affected the aphid proteome in both host plants. A higher number of proteins were found to be affected in aphids feeding on orange jasmine, indicating the host plant quality influence the outcome of the aphid-*Spiroplasma* interactions. In both host plants, the majority of proteins affected by *Spiroplasma* were heat shock proteins, proteins linked to cell function and structure, and energy metabolism. However, *Spiroplasma* also induced changes in proteins involved in antimicrobial activity, carbohydrate processing and metabolism, amino acid synthesis and metabolism in aphids feeding on orange jasmine. We also provide a discussion on how the host proteome is differentially affected by *Spiroplasma* infection when the host is exploiting different host plants.

Keywords: Aphid-symbiont interaction; Host plant use; Mass spectrometry; Metabolism alterations

8.1 Introduction

All multicellular animals have symbiotic microbes that establish biochemical and physiological interactions with their hosts. In insects, microorganisms are known to participate in many of the host physiological processes, e.g. by influencing the host capacity to use host plants or to vector a disease (MORAN, 2007; CILIA et al., 2011; HANSEN; MORAN, 2014). The combination of two metabolic systems (symbiont + host) has a variety of potential consequences to the host, and aphids may carry a great diversity of them. Aphids are known to harbor their obligatory symbiont *Buchnera aphidicola* (BUCHNER, 1965; DOUGLAS, 1998), and yet be associated with several facultative symbionts (CHEN; PURCELL, 1997; TSUCHIDA et al., 2004; MORAN et al., 2005; DOUGLAS, 2014; ZYTYNSKA; WEISSER, 2016). *Buchnera aphidicola* and aphids have a long co-evolutionary history. They share several metabolic pathways related to amino acids synthesis, and *B. aphidicola* provides the aphid with

essential amino acids that are not produced by the host nor are acquired from its natural diet. In many instances, *Buchnera* uses precursors and enzymes from aphid metabolic pathways to contribute to aphid nutrition (CHANDLER et al., 2008; HANSEN; MORAN, 2014). Facultative symbionts are diverse and the outcome of their interactions with their hosts are quite variable as they induce a plethora of phenotypes in host aphids (SU; ZHOU; ZHANG, 2013).

Secondary symbionts have also been related to host plant use in aphids, either by broadening or narrowing the insect host range (McLEAN et al., 2011; FERRARI et al., 2012). Nevertheless, the molecular and physiological mechanisms secondary symbionts interfere with host plant utilization and host range in aphids remain to be understood. Proteomics is an interesting approach for the investigation and identification of metabolic processes and pathways differently expressed in an organism subjected to particular challenges. In fact, proteomics of aphids saliva have been shown successful to investigate the aphid interactions with host plants and viruses they vector, as well as aphids response to stress conditions (FRANCIS et al., 2006; NGUYEN et al., 2007; FRANCIS et al., 2010; ELZINGA et al., 2014; VANDERMOTEN et al., 2014). Aphid proteomics is also a growing area of research to investigate the effects secondary symbionts may have in the association with their hosts.

Spiroplasma is a common facultative symbiont reported in insects, but their role in host biology is still being questioned and explored (GASPARICH, 2010). *Spiroplasma* are known to be associated with at least 16 species of *Drosophila*, but the relationship of this symbiont with their *Drosophila* hosts may be quite variable (HASELKORN, 2010). There are associations between *Drosophila* and *Spiroplasma* in which this symbiont acts as a male killing (VENTURA et al., 2012), but the fitness effects in others are not clear. There are associations with *Spiroplasma*, including those that induce male killing, in which *Drosophila* is benefited by increased immune response to nematodes (JAENIKE et al., 2010; COCKBURN et al., 2013) and parasitoids (XIE et al., 2010; 2014). In aphids, this symbiont was first shown in *Acyrthosiphon pisum* (Harris), and injections into "bacteria-free aphid lines" demonstrated *Spiroplasma* negatively affected host biology (FUKATSU et al., 2001). Later, *Spiroplasma* was discovered to confer protection against a fungal entomopathogen due to aphid behavior manipulation. Aphids carrying *Spiroplasma* would drop off the plant and sporulate away from siblings, limiting the spread of the disease (LUKASIK et al., 2013).

However, *Spiroplasma* is not so frequently found in aphids as other secondary symbionts (RUSSELL et al., 2013; BRADY et al., 2014). Nevertheless, previous work reported high infections rates of *Aphis (Toxoptera) citricidus* (Kirkaldy) and *Aphis (Toxoptera) aurantii* Boyer de Fonscolombe (chapter 5 of this thesis). *A. citricidus* is an oligophagous species,

restricted to *Citrus* as suitable host plants, although it may survive and reproduce poorly the sub-optimum host *Murraya paniculata* (orange jasmine) (HALBERT; BROWN, 2011).

Thus, we aimed to investigate the induced changes in the proteome of *A. citricidus* by the secondary symbiont *Spiroplasma* on two host plants with different suitability to this aphid. We report the overall effects of *Spiroplasma* infection on the proteomics of *A. citricidus* and argue on its implications for host fitness and host plant use in this aphid.

8.2 Material and methods

8.2.1 Insect isolines

Sister isolines of *A. citricidus* infected (Ac-BS) or not (Ac-B) with *Spiroplasma* were established as described in Chapter 6. Briefly, part of an isoline of *A. citricidus* free of secondary symbiont was microinjected with hemolymph from *Spiroplasma*-infected *A. aurantii*, while the other part was microinjected with PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). Thus, infected and control lines of *A. citricidus* have the same genetic background (check sections 6.2.1, 6.2.2 and 6.2.3 in Chapter 6 for additional information).

Ac-B and Ac-BS lines were each maintained on seedlings of *Citrus sinensis* var. Pera (sweet orange) and *Murraya paniculata* (orange jasmine) inside rearing cages (50 cm high x 15 cm diameter), containing two lateral openings closed with cloth for ventilation), and maintained under controlled condition (25±2°C; 60±10% UR; 14:10 h).

8.2.2 Protein extraction

Adults from Ac-B and Ac-BS were collected from their host plants and subjected to protein extraction. Two-hundred and fifty aphids/host plant/isoline were macerated in 1 mL of extraction buffer (8 M Urea, 4% DTT, 4% CHAPS) and incubated in ice for 40 min. The material was centrifuged (12,000 g x 15 min x 4°C) and the supernatant was transferred to a new tube for purification with the commercial system 2D - Clean up (GE Healthcare®). Briefly, purification was made by adding 300 µL of the precipitant buffer followed by incubation on ice for 15 min. Afterwards, 300 µL of co-precipitant was added and the sample centrifuged (12,000 g x 5 min x 4°C) once again. The supernatant was discarded and 40 µL of co-precipitant was layered on top of the pellet. The pellet was allowed to sit on ice for 5 min. The sample was centrifuged (12,000 g x 5 min x 4°C) and the pellet recovered in 25 µL of Milli-Q, 1mL of wash buffer and 5 µL of wash additive. The purification procedure was finalized after centrifugation

(12,000g x 5 min x 4°C) and resuspension of the pellet in UT buffer (7 M Urea, 2 M Thiourea, 20 mM Tris, 2% CHAPS). The pH was measured using Neutralit pH 5.5-9 (Merck) strips and adjusted to pH=8.5 with 1 M NaOH. Total protein concentration was verified by using the commercial system RC DC Protein Assay (Bio-Rad®), following the manufacturer instructions.

8.2.3 2D-gel electrophoresis

Proteomic analyses were made separately in protein samples collected from aphids from different host plants; hence, the effect of *Spiroplasma* infection on the aphid proteome is individually analyzed within each host plant.

To perform analytic 2D gels, protein extracts were labeled with one of the three CyeDye (GE healthcare) in a total of 25 µg of protein by IPG strip (pH3-10NL, 24 cm, GE Healthcare), following the manufacturer instructions. Therefore, each Ac-B and Ac-BS sample from both experimental host plants was labeled in duplicates with Cye3 and Cye5. All IPG strips also carried the internal standard labeled with Cye2. The internal standard was composed of an equimolar mixture of Ac-B and Ac-BS.

The solution with labeled proteins was adjusted to a final volume of 450 µL of UT buffer, 2 M dithiothreitol (DTT) and 0.8% v/v IPG ampholyte (SERVA). IPG strips were set on a PROTEAN i12 IEF (Bio-Rad) for active rehydration for 11h at 50V, followed by isoelectric focalization (IEF) at 200V for 200Vh, 500V for 500Vh, 100V for 100Vh, and 8000V for 60000Vh. Both steps were conducted at 15°C and 50 µA maximum/IPG strip.

After IEF, IPG strips were prepared to second dimension electrophoresis. First, IPG strips were equilibrated for 15 min in 375 mM Tris (pH=8.8), 6 M Urea, 20% glycerol (v/v), 2% SDS and 130 mM dithiothreitol (DTT), followed by an extra 15 min in a similar buffer, in which DTT was replaced by 135 mM iodoacetamide (IAA).

Once treated, IPG strips were positioned onto a 2D 12.5% HPE large gel (SERVA®). Second dimension electrophoresis used the HPE FlatTop tower (GelCompany) under the following conditions: phase I - 100V, 7 mA, 1W for 30 min, 200V, 13 mA, 3W for 30 min, and 300V, 40 mA, 5W for 10 min; phase II - 1500V, 40 mA, 30W for 3h50min, and 1500V, 45 mA, 40W for 50 min. IPG strips were removed after phase I, and only 2D gel was used in phase II.

8.2.4 Image analysis and spot selection

2D gel images were captured with Typhoon Fluorescence Imager (Amersham) at wavelengths corresponding to each dye. A total of 12 images were obtained, three for each gel. Images were analyzed with the software Progenesis SameSpots (Nonlinear Dynamics). Images were aligned based on the internal standard in the semi-automatic mode, in which 90 vectors were manually determined before automatic alignment. Spots significantly different between treatments (ANOVA, $p \leq 0.05$) were selected. For the orange jasmine experiment, an additional filter (fold change ≥ 2) was included, once spots with a fold change lower than 2 were considered dubious due to gel bias.

After the list of spots was defined, a preparative gel was prepared, and another IPG strip loaded with internal standard labeled with Cye2 and 500 µg of unlabeled proteins (250 µg Ac-B and 250 µg Ac-BS) was subjected to electrofocusing. This IPG strip was later used for a second dimension electrophoresis as earlier mentioned. The image of this preparative gel was aligned with the previously obtained gels to locate the spots of interest. Spot picking was made in duplicate using an Ettan Spot Picker Robot (GE Healthcare).

Two equipments were used for spot identification; thus, each spot was subjected to spectrum acquisition on an Ultraflex II MALDI Mass Spectrometer (Bruker) and a *Synapt* G2 HDMS (Waters).

8.2.5 Mass spectrometry using an Ultraflex II MALDI Mass Spectrometer

Excised spots were sent in MilliQ water (Millipore) to the "Laboratoire de Spectrométrie de Masse" (University of Liège), where selected gel pieces were designated for the Proteineer dp automated Digester (Bruker, Bremen,Germany). Briefly, gel pieces were washed with three incubations in 100% of 50 mM ammonium bicarbonate, followed by incubations in a 1:1 mixture of acetonitrile (ACN) and 50 mM ammonium bicarbonate. Two additional washes were performed with 100% acetonitrile to dehydrate the gel. Gel pieces were first soaked in freshly activated trypsin (Porcine, Proteomics Grade, Roche) at 8°C for 30 min, and later subjected to protein trypsinization for 3 h at 30°C. Peptide extractions were performed with 10 µL of 1% formic acid (FA) for 30 min at 20°C.

Protein digests (3 µL) were adsorbed for 3 min on pre-spotted Anchorchips (R) using the Proteineer dp automaton. Spots were washed on-target using 10 mM ammonium dihydrogen phosphate in 0.1% trifluoroacetic acid (TFA) and desalted after washing with MilliQ water (Millipore). High throughput spectra were acquired using an Ultraflex II MALDI Mass Spectrometer (Bruker) in positive reflectron mode with close calibration enabled.

Successful spectra were summed, treated, and de-isotoped in line with an automated SNAP algorithm using Flex Analysis 2.4 software (Bruker), and subsequently submitted in batch mode to the Biotools 3.0 software suite (Bruker) with an in-house hosted MASCOT search engine (www.MatrixScience.com). Three databases were used: (1) the public National Center for Biotechnology Information (NCBI) non-redundant database, (2) the public National Center for Biotechnology Information (NCBI) non-redundant database with parameters set for Arthropoda, and (3) a homemade database containing all available aphid and aphid-symbiont protein sequences. A mass tolerance of 100 ppm with close calibration and one missing cleavage site were allowed. Partial oxidation of methionine residues and complete carbamylation of cystein residues were considered. The probability score calculated by the software was used as a criterion for correct putative identification.

In order to confirm identifications, experimental molecular weight (MW) and pI were compared to the predicted values resulting from the MASCOT analysis.

8.2.6 Mass spectrometry using *Synapt* G2 HDMS

Excised spots were dehydrated with 100% ACN, followed by reduction and alkylation. Reduction was performed in 20 mM DTT and 50 mM ammonium bicarbonate for 40 min at 55°C. The supernatant was removed and alkylation performed in 55 mM IAA and 50 mM ammonium bicarbonate for 30 min in the dark at room temperature. The supernatant was removed and gel fragments were washed in 25 mM ammonium bicarbonate.

Gel fragments were dehydrated with 100% ACN and rehydrated with 20 ng/µL trypsin (Promega V5111) in 50 mM ammonium bicarbonate. Protein digestion with trypsin was carried at 37 °C for 16h; after this period, enzymatic activity was blocked by a solution of 50% ACN and 5% FA in ultrapure water. Peptides were first washed in 60% methanol (MeOH) and 1% FA in ultrapure water, followed by a wash in 50% ACN and 1% FA in ultrapure water, with a final wash in 100% ACN. In each wash, peptides were sonicated for 20 min at 45°C (Ultra Som, Thronton). The supernatant was transferred to a new vial, and dried at room temperature in a vacuum centrifuge (Eppendorf 5301).

Dried samples were rehydrated in 10 µL 0.1% TFA in water and purified using a reverse phase column (*Reverse phase Zip-Tip C18, Millipore).* For this purpose, the column was first equilibrated by sequential washings with *i)* 100% ACN, 0.1% TFA, *ii)* 50% ACN, 0.1% TFA in water, and *iii)* 100% H_2O, 0.1% TFA. Afterwards, protein samples were adsorbed to the column, and washed with 95% H_2O, 5% MeOH, 0.1% TFA solution (v/v). Peptides were eluted

in 10 µL of an acidified (0.1% TFA), 50% ACN solution, dried in a vacuum centrifuge (Eppendorf 5301), and kept at -80°C until analysis.

Samples were eluted in 15 µL of 0.1% TFA acidified water and 8 µL were injected in a NanoAcquity UPLC (Waters, Manchester, UK) connected to a *Synapt* G2 HDMS. Peptides were passed through a C18 Symmetry capture column (5 µm, 180 µm x 20 mm) and separated on a silica-based, reverse phase C18 HSS T3 column (1.8 µm, 75 µm x 100 mm) (Waters, Manchester, UK). Mobile phase was composed of solution A (100% H_2O + 0.1% FA) and solution B (100% ACN + 0.1% FA) starting with a gradient of 99% A and 1% B for 90 min, and then 60% A and 40% B for 2 min and 15% A and 85% B for 3 min. The original condition of the column was reestablished for 25 min, totalling 120 min/run at 300 µL/min of flux.

Acquisition of MS data used a Q-TOF in the *Synapt* G2 HDMS equipped with an ion mobility cell and a nano lock spray source in the positive ion and 'V' mode with typical resolving power of at least 12 500 full-width half-maximum. The human (Glu1)-fibrin-peptide (GFP) (1 *p*mol.µL^{-1}) was used to calibrate the Q-TOF analyzer with data post-acquisition lock mass using the GFP double charged precursor ion, with sampling of the reference spray at a frequency of 30s. MS experiments were performed by switching between low (3eV) and high collision energies (15–50eV) applied to the T-wave cell trap filled with argon. The low and high energy scans from m/z 50 to 2000 used a scan time of 0.8s.

Analysis of MS data was performed using ProteinLynx GlobalServer version 3.1 (Waters) with reverse Uniprot databases: *Acyrtosiphum pisum*, *Myzus persicae*, *Toxoptera* sp., *Buchnera aphidicola* and *Spiroplasma* sp., accessed in August 14th, 2015. Uniprot database of aphids included their secondary symbionts. Analysis followed parameters automatic tolerance for precursor and ion product, minimum of three consecutive fragment-ions for peptide, minimum of seven consecutive fragments-ions for protein, minimum of two peptides for protein, and only one missed cleavage for trypsin, carbamylation of cysteine with fixed modification and methionine oxidation with variable modification; and false positive taxa (FDR) maximum of 4%.

Spots that yield good spectra in both methods adopted not always rendered identical putative protein identification due to differences in databases. Spots that rendered diverse hits on both mass spectrometry systems were assigned to the best hit by considering coverage (%), expected mass in both analysis, and the estimated error (ppm) obtained with the Ultraflex II-MALDI System.

All identified proteins were classified into functional groups. Groups were set based on ontology, protein domain and homology of each searched in the Uniprot, String, InterPro and

Pfam databases. Functional groups establish were: *protein-protein interactions, energy metabolism, antimicrobial peptides, cell function and structure, carbohydrate processing and metabolism, amino acid synthesis and metabolism, ion transport, processing and degradation, anti-oxidants, biosynthesis of bactericides,* and unknown.

8.3 Results

The *Spiroplasma*-injected *A. citricidus* sister line was successfully infected as confirmed by diagnostic PCR (data not shown). Protein extraction and purification also yielded high quality protein samples with enough concentration for proteomic analyses.

Gel images passed to all quality filters, and spots were well distributed (Figure 8.1). Initially, 174 spots were differentially expressed (Suppl. Table S8.1 and S8.2), but only 92 had minimum sample volume for further analysis. Out of the 92 spots analyzed, 80 of them were identified. 53 out of these 80 spots were differentially expressed between Ac-B and Ac-BS using orange jasmine as a host plant, while 27 differentially expressed spots were identified in aphids reared on sweet orange as a host (Tables 8.1 and 8.2).

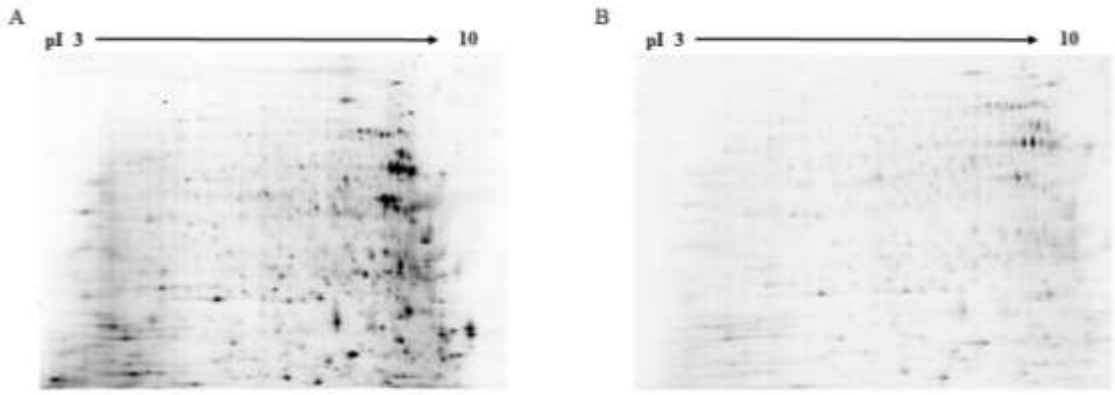


Figure 8.1 - Representative 2D 12.5% gel slab of protein samples labeled with Cye3 from the Ac-B isoline reared on orange jasmine (A) or sweet orange (B)

Proteomic analysis of *A. citricidus* using the Ultraflex II MALDI Mass Spectrometer and the *Synapt* G2 HDMS systems rendered the identification of several putative proteins, which are associated with *stress response, energy metabolism, structural protein* and other functional categories, although a large number of peptides yielded no identification (16 out of 53 in aphids reared on orange jasmine; and 17 out of 27 from aphids reared on sweet orange) (Tables 8.1 and 8.2).

Several of the proteins identified in the proteome of *A. citricidus* were present as isoforms. In aphid samples from orange jasmine, we identified two differentially expressed

isoforms of the transitional endoplasmic reticulum ATPase-TER94 (spots 388 and 389), six of the repetitive proline-rich cell wall protein 2-like isoform X2 (spots 581, 587, 588, 589, 590 and 660), two of the heat shock protein cognate 3 precursor (spots 663 and 699b), two of the uncharacterized protein J9JJY7 (spots 687 and 693), five of the 60 kDa chaperone (spots 776, 832, 841, 855 and 937), and two for the ATP synthase subunit alpha (spots 959 and 1019) (Table 8.2). The overall number of differentially expressed proteins in the Ac-BS as compared to the Ac-B sister isoline was much reduced in sweet orange as a host plant if compared to orange jasmine. In this case, only two isoforms each of the uncharacterized protein J9JWP2 (spots 193 and 194) and the uncharacterized protein J9JJY7 (spots 215 and 217) were found (Table 8.1). We also had two spots rendering more than one protein hit each. Two different heat shock proteins were identified from spot 699 from aphids reared on orange jasmine (Table 8.2); and a heat shock protein (262a) and an unchacterized protein (262b) were obtained from mass data analysis of spot 262 from aphids reared on sweet orange (Table 8.1).

The overall analyses of the differentially expressed proteins indicated *Spiroplasma* regulated the levels of more proteins from the aphid host than from the associated microbiota, mainly from *B. aphidicola* (Tables 8.1 and 8.2). However, the proteomic profile obtained from aphids from the two host plant was quite different, indicating *Spiroplasma* affected *A. citricidus* proteomics differently depending on the host plant.

Table 8.1 - Identification of different expressed spot from *Spiroplasma*-free (Ac-B) and *Spiroplasma* infected (Ac-BS) isolines of *Aphis (Toxoptera) citricidus* feeding on sweet orange.

(to be continued)

Spot	Fold change	Protein	*Species*	NCBInr gi or Uniprot entry name	Score	Mass	pI	N. peptides matched/searched	% coverage	Error (ppm)
Protein-Protein interactions										
Heat shock 60										
531	1.3 (+)*	60 kDa chaperonin[2**]	*Buchnera aphidicola Sg*	CH60_BUCAP	3615	57965		19/45	32.6	
Heat shock 70										
193	1.3 (+)	uncharacterized protein[2]	*Acyrthosiphon pisum*	J9JWP2_ACYPI	4044	72182		26/48	39.3	
194	1.1 (+)	uncharacterized protein[2]	*Acyrthosiphon pisum*	J9JWP2_ACYPI	3291	72182		27/48	40.4	
203	1.2 (+)	uncharacterized protein[2]	*Acyrthosiphon pisum*	J9JWD2_ACYPI	940	73920		13/65	24.1	
204	1.2 (+)	chaperone protein DnaK[2]	*Buchnera aphidicola Sg*	DNAK_BUCAP	1285	70401		14/68	32.4	
214	1.1 (+)	heat shock protein cognate 3 precursor[1]	*Acyrthosiphon pisum*	gi|193716022	259	72993	5.2	28/45	47	20
215	1.3 (+)	uncharacterized protein[2]	*Acyrthosiphon pisum*	J9JJY7_ACYPI	1653	71670		15/49	22.6	

Table 8.1 - Identification of different expressed spot from *Spiroplasma*-free (Ac-B) and *Spiroplasma* infected (Ac-BS) isolines of *Aphis (Toxoptera) citricidus* feeding on sweet orange

(to be continued)

Spot	Fold change	Protein	*Species*	NCBInr gi or Uniprot entry name	Score	Mass	pI	N. peptides matched/searched	% coverage	Error (ppm)
217	1.2 (+)	uncharacterized protein[2]	*Acyrthosiphon pisum*	J9JJY7_ACYPI	2166	71670		17/49	31.9	
262b	1.7 (-)	heat shock protein cognate 3 precursor[1]	*Acyrthosiphon pisum*	gi|193716022	95	72993	5.2	15/42	28	32
274	1.1 (-)	heat shock 70 kDa protein cognate 4[1]	*Acyrthosiphon pisum*	gi|193688192	169	72138	5.3	17/32	31	10
324	1.2 (+)	heat shock 70 kDa protein cognate 4-like[1]	*Acyrthosiphon pisum*	gi|193603578	150	71626	5.3	15/25	33	65
Heat shock 90										
131	1.2 (+)	uncharacterized protein[2]	*Acyrthosiphon pisum*	J9JP34_ACYPI	748	83759		10/69	16.2	
Calcium binding										
883	1.3 (-)	ACYPI007505 protein[2]	*Acyrthosiphon pisum*	C4WYG9_ACYPI	747	17432		5/22	33.7	
Genetic transcription control										
608	1.2 (-)	uncharacterized protein[2]	*Acyrthosiphon pisum*	J9JV14_ACYPI	2984	36544		17/28	55.8	

Table 8.1 - Identification of different expressed spot from *Spiroplasma*-free (Ac-B) and *Spiroplasma* infected (Ac-BS) isolines of *Aphis (Toxoptera) citricidus* feeding on sweet orange

(to be continued)

Spot	Fold change	Protein	*Species*	NCBInr gi or Uniprot entry name	Score	Mass	pI	N. peptides matched/searched	% coverage	Error (ppm)
				Energy metabolism						
440	1.1 (+)	malyl-CoA lyase[1]	*Granulibacter bethesdensis*	gi|499950029	72	36167	5.8	6/14	21	34
788	1.2 (-)	uncharacterized protein[2]	*Acyrthosiphon pisum*	J9JQL8_ACYPI	1316	21268		4/17	23.0	
801	1.2 (+)	glyceraldehyde-3-phosphate dehydrogenase[2]	*Acyrthosiphon pisum*	C4WYF8_ACYPI	521	35735		6/24	24.4	
				Cell function and structure						
363	1.2 (+)	tubulin beta-1 chain[2]	*Acyrthosiphon pisum*	D7RA98_ACYPI	4347	50558		28/29	54.4	
610	1.1 (-)	uncharacterized protein[2]	*Acyrthosiphon pisum*	J9JLA2_ACYPI	3288	32571		9/27	25	
637	1.2 (-)	ACYPI003527 protein[2]	*Acyrthosiphon pisum*	C4WTM0_ACYPI	215	22715		3/11	19.3	
665	1.6 (-)	uncharacterized protein[2]	*Acyrthosiphon pisum*	X1XTT0_ACYPI	151	20386		2/8	12.8	
893	1.2 (+)	ACYPI005394 protein[2]	*Acyrthosiphon pisum*	C4WUV1_ACYPI	1374	20659		7/22	22.3	

Table 8.1 - Identification of different expressed spot from *Spiroplasma*-free (Ac-B) and *Spiroplasma* infected (Ac-BS) isolines of *Aphis (Toxoptera) citricidus* feeding on sweet orange

(conclusion)

Spot	Fold change	Protein	*Species*	NCBInr gi or Uniprot entry name	Score	Mass	pI	N. peptides matched/searched	% coverage	Error (ppm)
962	1.3 (-)	ACYPI008348 protein[2]	*Acyrthosiphon pisum*	C4WTD6_ACYPI	2421	11575		5/9	45.5	
1038	1.1 (-)	putative cofilin/actin depolymerizing factor-like protein[2]	*Acyrthosiphon pisum*	Q1ZZP9_ACYPI	1411	17364		2/16	16.9	
				Ion transport						
262a	1.7 (-)	V-type proton ATPase catalytic subunit A[1]	*Acyrthosiphon pisum*	gi|193690627	116	68057	5	15/42	28	30
				Processing and degradation						
713	1.2 (+)	ACYPI004563 protein[2]	*Acyrthosiphon pisum*	C4WYA2_ACYPI	413	24716		3/14	22.9	
				Unknown						
197	2.0 (-)	uncharacterized protein[2]	*Acyrthosiphon pisum*	J9K6K0_ACYPI	392	72647		9/33	14.6	
825	1.1 (+)	ACYPI003440 protein[2]	*Acyrthosiphon pisum*	C4WUX5_ACYPI	1099	20238		3/20	18.6	

* Protein expression pattern in response to *Spiroplasma* infection. (+) = up-regulated protein, (-) = down-regulated protein

**number indicates the mass spectrometer used in the spot identification, 1= Ultraflex II MALDI and 2 = *Synapt* G2 HDMS

Table 8.2 - Identification of different expressed spot from *Spiroplasma*-free (Ac-B) and *Spiroplasma* infected (Ac-BS) isolines of *Aphis (Toxoptera) citricidus* feeding on orange jasmine

(to be continued)

Spot	Fold change	Protein	Species	NCBInr gi or Uniprot entry name	Score	Mass	pI	N. peptides matched/searched	% coverage	Error (ppm)
Protein-Protein Interactions										
Heat shock 10										
2318	3.3 (+)*	10 kDa chaperonin[2**]	*Buchnera aphidicola* 5[a]	CH10_BUCA5	1703	10243		2/6	28.1	
Heat shock 60										
776	2.0 (-)	60 kDa chaperonin[2]	*Buchnera aphidicola Sg*	CH60_BUCAP	4001	57965		29/45	46.7	776
832	4.6 (-)	60 kDa chaperonin[2]	*Buchnera aphidicola Sg*	CH60_BUCAP	5376	57965		24/45	35.8	832
841	3.1 (-)	60 kDa chaperonin[2]	*Buchnera aphidicola Sg*	CH60_BUCAP	3742	57965		21/45	35.8	841
855	4.3 (-)	60 kDa chaperonin[2]	*Buchnera aphidicola Sg*	CH60_BUCAP	3200	57965		20/45	33.6	855
937	4.3 (-)	60 kDa chaperonin[2]	*Buchnera aphidicola Sg*	CH60_BUCAP	463	57965		11/45	29.1	937
Heat shock 70										
417	2.8 (-)	heat shock 70 kDa protein 4[1]	*Acyrthosiphon pisum*	gi|193596448	59	89426	5.5	6/11	9	69

Table 8.2 - Identification of different expressed spot from *Spiroplasma*-free (Ac-B) and *Spiroplasma* infected (Ac-BS) isolines of *Aphis (Toxoptera) citricidus* feeding on orange jasmine

(to be continued)

Spot	Fold change	Protein	Species	NCBInr gi or Uniprot entry name	Score	Mass	pI	N. peptides matched/searched	% coverage	Error (ppm)
663	2.4 (-)	heat shock protein cognate 3 precursor[1]	*Acyrthosiphon pisum*	gi|193716022	257	72993	5.2	32/66	51	11
685	2.3 (-)	chaperone protein DnaK[2]	*Buchnera aphidicola Sg*	DNAK_BUCAP	1404	70401		9/68	22.4	
687	2.2 (-)	uncharacterized protein[2]	*Acyrthosiphon pisum*	J9JJY7_ACYPI	2919	71671		19/49	33.8	
693	2.7 (-)	uncharacterized protein[2]	*Acyrthosiphon pisum*	J9JJY7_ACYPI	1246	71671		13/49	20.9	
699a	2.4 (-)	heat shock 70 kDa protein cognate 4-like[1]	*Acyrthosiphon pisum*	gi|193603578	112	71626	5.3	16/45	31	43
699b	2.4 (-)	heat shock protein cognate 3 precursor[1]	*Acyrthosiphon pisum*	gi|193716022	112	72993	5.2	17/45	29	42
Heat shock 90										
397	2.7 (-)	endoplasmin[1]	*Acyrthosiphon pisum*	gi|193643557	89	89250	4.8	14/35	16	39
524	3.7 (+)	uncharacterized protein[2]	*Acyrthosiphon pisum*	J9JP34_ACYPI	2221	83759		19/69	26.4	

Table 8.2 - Identification of different expressed spot from *Spiroplasma*-free (Ac-B) and *Spiroplasma* infected (Ac-BS) isolines of *Aphis (Toxoptera) citricidus* feeding on orange jasmine

(to be continued)

Spot	Fold change	Protein	Species	NCBInr gi or Uniprot entry name	Score	Mass	pI	N. peptides matched/searched	% coverage	Error (ppm)
Calcium binding										
2128	2.2 (+)	ACYPI000027 protein[2]	*Acyrthosiphon pisum*	Q1ZZQ3_ACYPI	1093	17649		3/13	22.1	
				Energy metabolism						
951	2.3 (-)	ATP synthase subunit alpha mitochondrial[1]	*Acyrthosiphon pisum*	gi|193666827	84	59986	9.1	12/39	22	49
971	2.6 (-)	uncharacterized protein[2]	*Acyrthosiphon pisum*	J9JNB2_ACYPI	199	48183		8/40	19.6	
959	2.2 (-)	ATP synthase subunit alpha[2]	*Acyrthosiphon pisum*	J9JYX3_ACYPI	1469	60023		11/50	21.6	
1019	4.4 (+)	ATP synthase subunit alpha[2]	*Acyrthosiphon pisum*	J9JYX3_ACYPI	472	60023		7/50	13.2	
1331	2.4 (-)	malate dehydrogenase[2]	*Acyrthosiphon pisum*	Q201V2_ACYPI	1007	36103		9/23	28.9	
1353	2.4 (-)	glyceraldehyde-3-phosphate dehydrogenase[2]	*Acyrthosiphon pisum*	J9K9Z1_ACYPI	2993	35645		9/24	31.0	
1760	2.3 (+)	triosephosphate isomerase[2]	*Acyrthosiphon pisum*	C4WT45_ACYPI	293	27468		3/21	15.4	

Table 8.2 - Identification of different expressed spot from *Spiroplasma*-free (Ac-B) and *Spiroplasma* infected (Ac-BS) isolines of *Aphis (Toxoptera) citricidus* feeding on orange jasmine

(to be continued)

Spot	Fold change	Protein	Species	NCBInr gi or Uniprot entry name	Score	Mass	pI	N. peptides matched/searched	% coverage	Error (ppm)
Anti-microbial peptides										
581	3.2 (-)	repetitive proline-rich cell wall protein 2-like isoform X2[1]	*Acyrthosiphon pisum*	gi|641669847	111	72602	7.7	15/47	22	24
587	4.5 (-)	repetitive proline-rich cell wall protein 2-like isoform X2[1]	*Acyrthosiphon pisum*	gi|641669847	82	72602	7.7	10/35	16	56
588	3.0 (-)	repetitive proline-rich cell wall protein 2-like isoform X2[1]	*Acyrthosiphon pisum*	gi|641669847	94	72602	7.7	15/60	21	23
589	2.9 (-)	repetitive proline-rich cell wall protein 2-like isoform X2[1]	*Acyrthosiphon pisum*	gi|641669847	115	72602	7.7	16/52	21	15
590	3.0 (-)	repetitive proline-rich cell wall protein 2-like isoform X2[1]	*Acyrthosiphon pisum*	gi|641669847	113	72602	7.7	17/70	21	11
Cell function and structure										
1264	2.3 (+)	actin related protein 1[2]	*Acyrthosiphon pisum*	gi|217330650	141	42158	5.3	12/32	40	49
1265	4.6 (+)	putative actin[2]	*Acyrthosiphon pisum*	Q201U9_ACYPI	175	42221		4/34	14.6	
1356	3.2 (+)	uncharacterized protein[2]	*Acyrthosiphon pisum*	C4WTU7_ACYPI	1192	32818		8/26	30.7	

Table 8.2 - Identification of different expressed spot from *Spiroplasma*-free (Ac-B) and *Spiroplasma* infected (Ac-BS) isolines of *Aphis (Toxoptera) citricidus* feeding on orange jasmine

(to be continued)

Spot	Fold change	Protein	Species	NCBInr gi or Uniprot entry name	Score	Mass	pI	N. peptides matched/searched	% coverage	Error (ppm)
660	2.4 (-)	repetitive proline-rich cell wall protein 2-like isoform X2[1]	*Acyrthosiphon pisum*	gi|641669847	79	72602	7.7	8/20	13	50
1541	2.6 (+)	ACYPI003527 protein[2]	*Acyrthosiphon pisum*	C4WTM0_ACYPI	911	22715		6/11	40.4	
1761	2.3 (+)	ACYPI005885 protein[2]	*Acyrthosiphon pisum*	C4WYE4_ACYPI	810	24745		7/24	28.0	
1781	4.0 (+)	ACYPI001342 protein[2]	*Acyrthosiphon pisum*	C4WRW5_ACYPI	355	19792		3/11	18.4	
			Carbohydrate processing and metabolism							
734	2.0 (-)	polysaccharide deacetylase[1]	*Fusobacterium nucleatum*	gi|495968271	66	69834	9.1	15/62	24	50
1744	2.2 (-)	putative diacetyl/L-xylulose reductase[2]	*Acyrthosiphon pisum*	Q201Y9_ACYPI	207	26334		2/17	10.4	
1787	4.3 (+)	ACYPI000057 protein[2]	*Acyrthosiphon pisum*	Q1ZZP5_ACYPI	188	25816		4/18	21.2	
1308	2.1 (+)	ACYPI002851 protein[2]	*Acyrthosiphon pisum*	C4WTT0_ACYPI	818	39779		8/23	28.9	

Table 8.2 - Identification of different expressed spot from *Spiroplasma*-free (Ac-B) and *Spiroplasma* infected (Ac-BS) isolines of *Aphis (Toxoptera) citricidus* feeding on orange jasmine

(to be continued)

Spot	Fold change	Protein	Species	NCBInr gi or Uniprot entry name	Score	Mass	pI	N. peptides matched/searched	% coverage	Error (ppm)
				Amino acid synthesis and metabolism						
913	3.5 (+)	glutamate dehydrogenase[2]	*Acyrthosiphon pisum*	J9KB74_ACYPI	285	59967		9/44	20.7	
975	2.3 (-)	glutamyl-tRNA synthase[1]	*Kyrpidia tusciae*	gi|502839225	78	55629	6.1	14/76	31	50
1215	2.4 (+)	glutamine synthetase[2]	*Acyrthosiphon pisum*	J9JML2_ACYPI	1783	41935		7/25	22.2	
2302	2.1 (+)	aspartate kinase[1]	*Nitrolancea hollandica*	gi|495756139	58	8291	6.6	4/29	38	44
				Ion transport						
1607	4.7 (+)	ACYPI006090 protein[2]	*Acyrthosiphon pisum*	C4WY80_ACYPI	40	26228		2/21	11.9	
1872	2.1 (-)	ATP synthase oligomycin sensitivity conferral protein[2]	*Aphis (Toxoptera) citricidus*	Q5XUB9_TOXCI	157	22719		2/20	9.6	
2293	3.7 (+)	V-type proton ATPase subunit F[2]	*Acyrthosiphon pisum*	Q201X8_ACYPI	1251	13663		5/13	39.3	

Table 8.2 - Identification of different expressed spot from *Spiroplasma*-free (Ac-B) and *Spiroplasma* infected (Ac-BS) isolines of *Aphis (Toxoptera) citricidus* feeding on orange jasmine

(to be continued)

Spot	Fold change	Protein	Species	NCBInr gi or Uniprot entry name	Score	Mass	pI	N. peptides matched/searched	% coverage	Error (ppm)
				Processing and degradation						
388	3.3 (-)	transitional endoplasmic reticulum ATPase TER94[1]	*Acyrthosiphon pisum*	gi|193617621	258	89914	5.1	26/36	32	55
389	2.3 (-)	transitional endoplasmic reticulum ATPase TER94[1]	*Acyrthosiphon pisum*	gi|193617621	187	89914	5.1	27/68	32	17
1704	2.1 (+)	proteasome subunit alpha type[2]	*Acyrthosiphon pisum*	C4WUA6_ACYPI	57	27982		2/22	8.3	
				Antioxidant						
1890	4.5 (+)	uncharacterized protein[2]	*Acyrthosiphon pisum*	J9JUC5_ACYPI	205	27120		2/21	10.5	
1908	2.2 (+)	ACYPI002506 protein[2]	*Acyrthosiphon pisum*	C4WSM1_ACYPI	2239	21794		9/15	48.7	
				Biosynthesis of bactericides						
934	3.4 (-)	radical SAM additional 4Fe4S-binding domain protein[1]	*Acholeplasma sp.*	gi|547179168	73	43424	6.1	8/20	18	34

Table 8.2 - Identification of different expressed spot from *Spiroplasma*-free (Ac-B) and *Spiroplasma* infected (Ac-BS) isolines of *Aphis (Toxoptera) citricidus* feeding on orange jasmine

(conclusion)

Spot	Fold change	Protein	Species	NCBInr gi or Uniprot entry name	Score	Mass	pI	N. peptides matched/searched	% coverage	Error (ppm)
Unknown										
729	3.3 (-)	uncharacterized protein[2]	*Acyrthosiphon pisum*	J9JTV1_ACYPI	379	63934		8/29	12.2	
731	2.6 (-)	uncharacterized protein DDB_G0274915-like[1]	*Acyrthosiphon pisum*	gi|193716064	91	67060	7.7	12/61	16	12

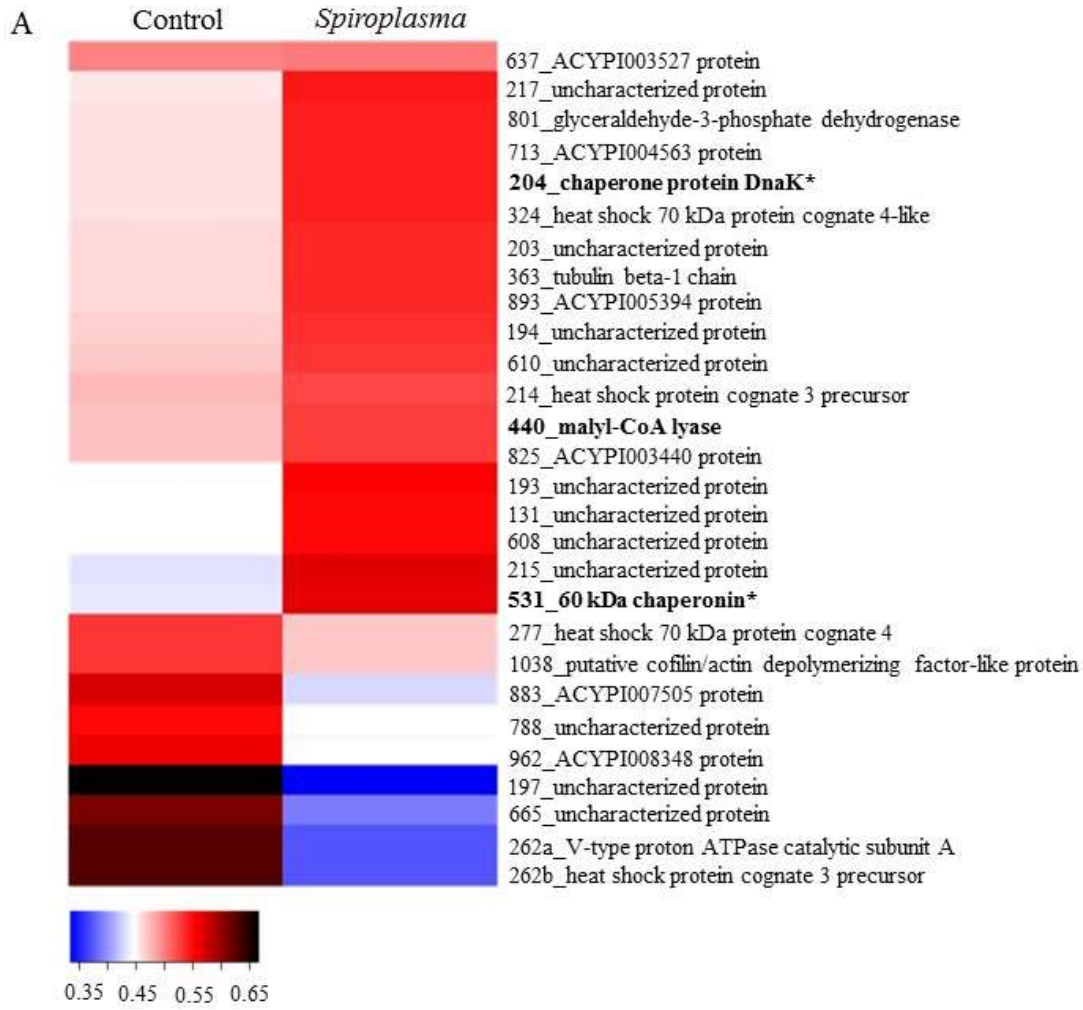
* Protein expression pattern in response to *Spiroplasma* infection. (+) = up-regulated protein, (-) = down-regulated protein
**number indicates the mass spectrometer used in the spot identification, 1= Ultraflex II MALDI and 2 = *Synapt* G2 HDMS

Changes in the proteome of *A. citricidus* induced by *Spiroplasma* infection was less evident in aphids reared in sweet orange, with 19 up-regulated and 9 down-regulated proteins belonging to *protein-protein interaction*, *energy metabolism*, *cell function and structure*, *ion transport* and *processing and degradation* functional groups (Table 8.1 and Figure. 8.2 A). Most of the proteins in aphids reared on sweet orange were uncharacterized, but analyses were made based on their domain. Up and down-regulated proteins were from several functional groups, without a clear division. Most of the eukaryote heat shock proteins and chaperonins from *Buchnera* were up-regulated (Figure 8.2 A), and three eukaryote heat shock proteins (spots 277 and 262b), three proteins from *cell function and structure*, one protein from *ion transport*, one from *energy metabolism* and one from unknown functional group were down-regulated. Moreover, down-regulated protein were all from eukaryote origin, while up-regulated proteins were from both eukaryote and prokaryote (Figure 8.2 A).

On the other hand, *Spiroplasma* infection of *A. citricidus* (Ac-BS) in orange jasmine yielded broader changes in the host proteins belonging to all functional groups established. Differential expression of *antimicrobial peptides*, *carbohydrate processing and metabolism*, *amino acid synthesis and metabolism*, *anti-oxidants* and *biosynthesis of bactericides* was observed only when comparing infected and non-infected aphids fed on orange jasmine. *Spiroplasma* induced the up-regulation of 21 up-regulated and the down-regulation of 33 proteins (Figure 8.2B). All of the differentially expressed proteins of *cell function and structure* and *anti-oxidants*, most of the *amino acid synthesis and metabolism* (except spot 975) and *ion transport* (except spot 1872), and several belonging to other functional groups were up-regulated in *Spiroplasma*-infected hosts (Figure 8.2 B). Down-regulated proteins included mainly those in *protein-protein interaction* and *antimicrobial peptides* functional groups, with three 60 kDa chaperonins (spots 832, 855 and 937) and one repetitive proline-rich cell wall protein 2-like isoform X2 (spot 587) with strong down-regulation ratio (Figure 8.2B). The fucntional groups *energy metabolism*, *carbohydrate processing and metabolism*, *processing and degradation* and *biosynthesis of bactericides* also include proteins that were either up or down regulated in *Spiroplasma*-infected aphids in orange jasmine. Futher, we detected no pattern of protein regulation in regards to protein origin, if eukaryote or prokaryote (Figure 8.2B).

A comparison of the proteome of *A. citricidus* infected by *Spiroplasma* when exploiting sweet orange and orange jasmine indicated a number of differentially expressed proteins common to aphids reared on both host plants, as the uncharacterized protein J9JP34 (spot 524 in orange jasmine and spot 131 in sweet orange), chaperone protein DnaK (spot 685 in orange

jasmine and 204 in sweet orange), uncharacterized protein J9JJY7 (spots 687 and 693 in orange jasmine and spots 215 and 217 in sweet orange), 60 kDa chaperonin (spots 776, 832, 841, 855 and 937 in orange jasmine and spot 531 in sweet orange) and ACYPI003527 protein (spot 1541 in orange jasmine and spot 637 in sweet orange) (Table 8.1 and 8.2). But although sharing similar differentially expressed proteins in two different host plants, only the uncharacterized protein J9JP34 and ACYPI003527 protein followed the same pattern of expression in both plants (up-regulation), with the remaining proteins being up-regulated in sweet orange but down regulated in *Spiroplasma*-infected aphids feeding on orange jasmine (Figure 8.2 A and B). Differentially expressed proteins common to both host plants, but with different pattern of expression were represented by heat shock proteins (*protein-protein interaction*) and the ACYPI003527 protein (*cell function and structure*).



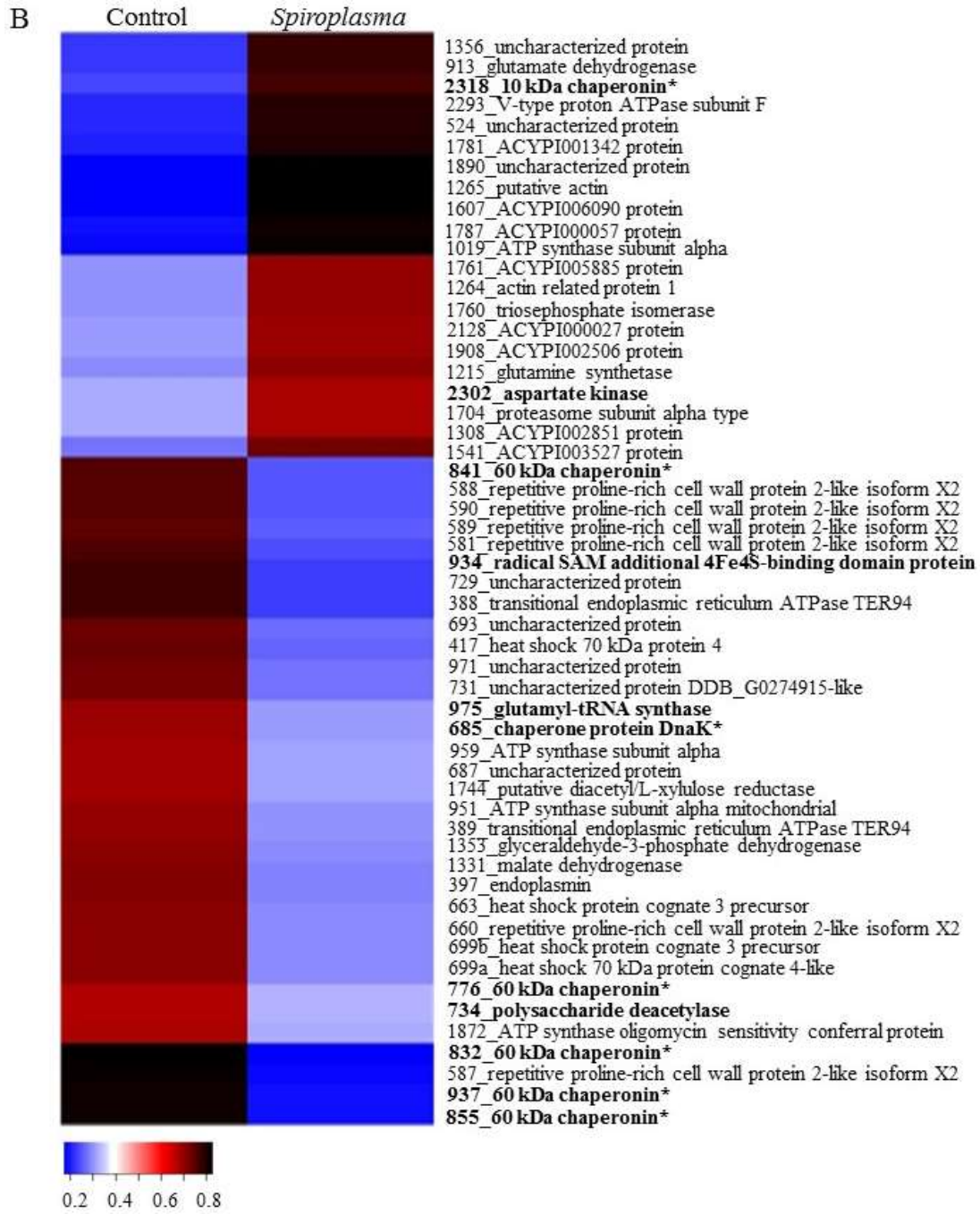


Figure 8.2 - Heatmap of identified proteins in *C. sinensis* (A) and *M. paniculata* (B). Proteins are identified by their spot number followed by Blast2Go or Uniprot identification. Regular letters represents proteins from eukaryote, bold letters represents proteins from prokaryote origin, and bold letters with asteristic (*) represents protein from *Buchnera aphidicola*. Fold change was transformed in a proportional scale in which blue represents the smallest fold change and black represent the largest fold change

8.4 Discussion

Comparative proteomic analysis between *Spiroplasma*-free (Ac-B) and *Spiroplasma*-infected (Ac-BS) sister isolines of *A. citricidus* on two host plants indicated *Spiroplasma* infection affects the host proteome, especially affecting the expression of proteins belonging to *protein-protein interaction, cell function and energy metabolism*. Changes in host proteome due to *Spiroplasma* infection were quite different in aphids feeding on different host plants - orange jasmine or sweet orange, indicating proteome-induced alterations by *Spiroplasma* infection are dependent on the nutritional suitability of the aphid host plant.

One of the major groups of proteins of the holobiome represented by *A. citricidus-Buchnera* that was affected by *Spiroplasma* infection was the heat shock proteins (HSPs). HSPs are ubiquitous proteins common to prokaryotes and eukaryotes and are expressed either under stress or no-stress conditions (FEDER; HOFMANN, 1999). HSPs are reported to respond to a variety of stress abiotic elicitors other than heat, such as ultraviolet radiation, chemical pesticides, but also to biotic sources of stress, such as infection by viruses, bacteria, and fungi, and parasitization by other insects (ZHAO; JONES, 2012). But HSPs are also commonly produced under no stress conditions as they play key roles for the successful folding/unfolding, assembly, intracellular localization, secretion, regulation, and degradation of proteins (KAUFMANN, 1990; FEDER; HOFMANN, 1999). HSPs are divided into families based on molecular weight and sequence homology (KAUFMANN, 1990; FEDER; HOFMANN, 1999), and function of proteins belonging to different families may depend on the development stage, subcellular location, and environmental conditions (KING; MACRAE, 2015). Therefore, the function of HSPs has to be interpreted taking into account changes in proteins they may interact with, considering the physiological state of the aphid as a whole.

Spiroplasma affected the expression of several HSPs (HSP10, HSP60, HSP70 and HSP90) from *A. citricidus-Buchnera.* All of the identified HSP10 and HSP60 and one HSP70 (DnaK) were similar to those from *B. aphidicola,* and the remaining HSP70 and the HSP90 were similar to those from *A. pisum.* But the outcome of the regulation of HSPs expression by *Spiroplasma* was quite influenced by the host plant the aphid was developing on. While the majority of the differentially expressed HSPs were up-regulated in aphis developing on sweet orange, HSPs of aphids on orange jasmine were down-regulated.

GroEL (HSP60) family, GroES (HSP10) and DnaK (HSP70) are commonly expressed in *Buchnera* as they are involved with molecule exchange between host and symbiont (BAUMANN et al., 1997), particularly for Hsp60/Hsp10 which are known to participate in the folding and importing of host proteins to the symbiotic bacterium (RYAN et al., 1997). The down-regulation of GroEl (HSP60) and DnaK (HSP70) proteins in infected aphids in a less

suitable host (orange jasmine) demonstrate *Spiroplasma* can negatively interfere, directly or indirectly, with the exchange of molecules in between the primary symbiont *B. aphidicola* and the aphid, even though a GroES was up-regulated. The regulation of different groups of HSPs by *Spiroplasma* under different nutritional conditions – sweet orange and orange jasmine – demonstrates the intensity of regulation of the interaction host aphid – primary symbiont by *Spiroplasma* can differ depending on the nutritional quality of the host food source.

Two other abundant groups of proteins, *energy metabolism* and *cell function and structure*, were differentially expressed in response to *Spiroplasma* infection, regardless the host plant the aphid was feeding on. HSPs are ATP dependent since association and dissociation of HSPs to/from interacting proteins are facilitated by ATP hydrolysis, and therefore closely linked to ATP availability (KAUFMANN, 1990; KING; MACRAE, 2015). Thus, down-regulation of HSPs in aphids feeding on orange jasmine were followed by down regulation of most of the ATP synthases produced, but no such correlation was observed in infected aphids feeding on sweet orange. Additionally, HSP are also reported to interact with cytoskeletal proteins, as one of the first functions reported for HSP90 family was the protection and recovery of actin and tubulin filaments from prolonged and severe heat stress exposure (SCHLESINGER, 1990). We did observed up-regulation of proteins included in *cell function and structure*, including putative actins and actin-related proteins. But over-expression of these proteins in *Spiroplasma*-infected aphids was observed on both host plants, even under conditions in which HSP expression was reduced.

HSPs are also reported to play a role in immunity by interacting with antimicrobial peptides (OTVOS et al., 2000; POCKLEY, 2003). Proline-rich peptides are common antimicrobial peptides reported to produce pores mainly in the cells of Gram- bacteria to penetrate into susceptible cells and act intracellularly (LI et al., 2012). Such proline-rich peptides are also reported to play key roles in the immune response against microbials by interacting with HSP70 (DnaK), interfering with protein folding and protein expression (KRAGOL et al., 2004; ROY et al., 2015; TANIGUCHI et al., 2016). A strong down-regulation of HSP70 and antimicrobial peptides was observed in *Spiroplasma*-infected aphids developing on orange jasmine. Differentially expressed *antimicrobial peptides* were all isoforms of repetitive proline-rich cell wall protein 2-like isoform X2. Down-regulation of the host immune system in *Spiroplasma*-infected aphids was also observed by the down-regulation of SAM binding domain–containing proteins, which are linked to various bacteriocin biosynthesis proteins (HAFT; BASU, 2011).

Regulation of DnaK of *Buchnera* and/or of proline-rich antimicrobial proteins may indicate *Spiroplasma* interferes with growth of the primary symbiont by reducing the production of antimicrobials, even though aphids are known to use a nutrition-based mechanism to by controlling amino acid provisioning to regulate the growth of *Buchnera* (HANSEN; MORAN, 2011). No differential expression was observed for antimicrobial peptides in infected aphids developing on sweet orange, but antimicrobial peptides were strongly repressed in aphids developing on orange jasmine. If antimicrobial suppression in a poor quality host was a mechanism of regulation exclusively oriented to alleviate the control of primary symbiont growth, on such condition will need to be further investigated. Several other antimicrobial peptides and HSP70s were also affected by *Spiroplasma* infection in aphids reared on orange jasmine, and the impairment of the immune system under nutritional deficiency as a trade-off mechanism is vastly reported in insects (KARIMZADEH; WRIGHT, 2007; LEE et al., 2006; 2008; VOGELWEITH et al., 2011), and should not be discarded as an effect of *Spiroplasma* infections in *A. citricidus*.

The proposed reduced regulation in *Buchnera* growth due the down regulation of DnaK/proline-rich antimicrobial peptides in *Spiroplasma*–infected aphids developing on orange jasmine is supported by the up-regulation of proteins involved in nutrient provisioning to *Buchnera*. Aphids control the primary symbiont growth by regulating the availability of glutamine to the symbiont (HANSEN; MORAN, 2011; 2014). In orange jasmine, we observed an increase in glutamine synthase and glutamate dehydrogenase proteins, which are enzymes involved in the recycling of ammonia. Glutamate dehydrogenase is involved in the interconversion of glutamate to α-ketoglutarate, and glutamine synthase in the production of glutamine through the catalysis of glutamate and ammonia. Glutamine is a host-provided, non-essential amino acid required for *Buchnera* growth (HANSEN; MORAN, 2011). Glutamine and glutamate are amino acids required for the synthesis of amino acids and for purine metabolism in *Buchnera*-carrying bacteriocytes (HANSEN; MORAN, 2011). Regulation of these amino acids has been proposed as key in maintaining amino acids availability balanced for both the host and the symbiont (HANSEN; MORAN, 2011).

Aspartate is also a non-essential amino acid that is not produced by *Buchnera*, and has to be transported into *Buchnera* cells. Aspartate is an important source of nitrogen for the synthesis of essential amino acids (HANSEN; MORAN, 2011). Additionally, we also observed an increase in a prokaryote aspartate kinase, an enzyme involved in the synthesis of essential amino acids (lysine and threonine) through aspartate phosphorylation (VIOLA, 2001; HANSEN; MORAN, 2011). Up-regulation of aspartate kinase is suggestive of increased

synthesis of essential amino acids from aspartate, but malate dehydrogenase was down-regulated in *Spiroplasma*-infected aphids in orange jasmine. Malate dehydrogenase is the enzyme involved in the transamination of oxaloacetate to produce aspartate, but in aphid bacteriocytes synthesis of aspartate is based on the precursor glutamine. Down-regulation of the aphid malate dehydrogenase enzyme may be correlated with the observed reduction in aphid energy metabolism, as oxaloacetate is also an intermediate of the citric acid cycle.

We demonstrated increased glutamine synthesis by the up-regulation of glutamine synthetase, but we did not detected up-regulation of glutamine transporters in the protein spots we able to analyze to demonstrate enhanced transport of glutamine from the hemolymph to the bacteriocyte (PRICE et al., 2014; RUSSELL et al., 2014). Thus, infected aphids feeding on orange jasmine needs to provide a higher nutritional investment to *Buchnera* in order to have the needed synthesis of essential amino acids by the obligate symbiont, and reduce any fitness cost *Spiroplasma* infection could induce under such nutritional stress condition.

Moreover, up-regulation of *antioxidants* and proteasome subunit alpha type proteins in *Spiroplasma*-infected aphids feeding on orange jasmine may also indicate increased cell damage and requirement for cell protection (antioxidants) and degradation of unneeded or degraded proteins. Antioxidants are important to reduce the negative effects of the accumulation of reactive oxygen species, which are involved in the reduction of the reproductive fitness and in the trade-offs between immunity and reproductive traits (DOWLING; SIMMONS, 2009). Proteasome subunit alpha type proteins belong to a selective proteolytic protein machinery (proteasome) acting on ubiquitin-tagged proteins, serving as an important mechanism of regulation of cell cycle, gene transcription, signal transduction and apoptosis (WOLF; HILT, 2004; KLEIGER; MAYOR, 2014), even though proteins from the ubiquitinylation pathway were not identified among the analyzed proteins. Up-regulation of proteasome subunit alpha type proteins indicates the increased need of protein degradation in infected aphids in orange jasmine once proteasome deficiency could lead to the accumulation of damaged and misfolded proteins and result in a number of pathologies (WOLF; HILT, 2004; KLEIGER; MAYOR, 2014). Therefore, the requirement of high levels of proteasome activity may be associated with the reduced availability of HSPs, which in turn may have led to an accumulation of damaged and/or misfolded proteins, as several HSPs are down-regulated in *Spiroplasma*-infected hosts feeding on orange jasmine.

In conclusion, *Spiroplasma* infection affects the aphid-primary symbiont proteome mainly by regulating the activity of heat shock proteins. Regulation of heat shock proteins (HSPs) is dependent on the nutritional quality of the host plant, as HSPs are up-regulated in

infected aphids feeding on a highly suitable host plant, while suffering down-regulation in aphids feeding on a less suitable host plant. Down-regulation of HSPs in *Spiroplasma*-infected aphids in a low quality host plant also resulted in the regulation of proteins involved in processes in which HSPs participate, including the immune response. Thus, the effects of *Spiroplasma* on aphid proteome is highly dependent on the nutritional quality of the host plant, and down regulation of proteins involved in immune response are suggestive of developmental traits trade-offs due to nutritional deficiency. Further studies to investigate the fitness costs of *A. citricidus* association with *Spiroplasma* in response to immune challenges under different nutritional stress conditions will help to shed light on the role this secondary symbiont may play in aphid biology and on the costs involved in such association.

8.5 Conclusions

- *Spiroplasma* alters the proteome of *Aphis (Toxoptera) citricidus* regardless the host plant used;
- Proteome regulation is more conspicuous in *Spiroplasma*-infected aphids feeding on a lower quality (orange jasmine) as compared to the high quality host (sweet orange);
- Heat shock proteins were the common targeted proteins for regulation by *Spiroplasma* infection, regardless the host plant tested;
- Regulation of heat shock proteins in *Spiroplasma*-infected aphids was dependent on the quality of the host plant used, suffering up-regulation in aphids feeding on a high quality host plant (sweet orange) and down–regulation in aphids feeding on a low quality host plant (orange jasmine);
- Down-regulation of proteins involved in immune response in the low quality host orange jasmine is suggestive of trade-offs in developmental traits due to nutritional deficiency.

References

BAUMANN, P.; MORAN, N. A.; BAUMANN, L. The evolution and genetics of aphid endosymbionts. **Bioscience**, Washington, v. 47, n. 1, p. 12-20, 1997.

BRADY, C. M.; ASPLEN, M. K.; DESNEUX, N.; HEIMPEL, G. E.; HOPPER, K. R.; LINNEN, C. R.; OLIVER, K. M.; WULFF, J. A.; WHITE, J. A. Worldwide populations of

the aphid *Aphis craccivora* are infected with diverse facultative bacterial symbionts. **Microbial Ecology**, New York, v. 67, n. 1, p. 195-204, 2014.

BUCHNER, P. **Endosymbiosis of animals with plant microorganisms**. New Jersey: Interscience Publishers/John Wiley, p. 909, 1965.

CHANDLER, S. M.; WILKINSON, T. L.; DOUGLAS, A. E. Impact of plant nutrients on the relationship between a herbivorous insect and its symbiotic bacteria. **Proceedings of the Royal Society B-Biological Sciences**, London, v. 275, n. 1634, p. 565-570, 2008.

CHEN, D. Q.; PURCELL, A. H. Occurrence and transmission of facultative endosymbionts in aphids. **Current Microbiology**, New York, v. 34, n. 4, p. 220-225, 1997.

CILIA, M.; TAMBORINDEGUY, C.; FISH, T.; HOWE, K.; THANNHAUSER, T.W.; GRAY, S. Genetics coupled to quantitative intact proteomics links heritable aphid and endosymbiont protein expression to circulative polerovirus transmission. **Journal of Virology**, Washington, v. 85, n. 5, p. 2148-2166, 2011.

COCKBURN, S. N.; HASELKORN, T. S.; HAMILTON, P. T.; LANDZBERG, E.; JAENIKE, J.; PERLMAN, S. J. Dynamics of the continent-wide spread of a *Drosophila* defensive symbiont. **Ecology Letters**, Oxford, v. 16, n. 5, p. 609-616, 2013.

DOUGLAS, A. E. Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. **Annual Review of Entomology**, Standford, v. 43, p. 17-37, 1998.

_____. Multiorganismal Insects: Diversity and Function of Resident Microorganisms. **Annual Review of Entomology**, Standford, v. 60, p. 17-34, 2014.

DOWLING, D. K.; SIMMONS, L. W. Reactive oxygen species as universal constraints in life-history evolution. **Proceedings of the Royal Society B: Biological science,** London, v. 276, p. 1737-1745, 2009.

ELZINGA, D. A.; DE VOS, M.; JANDER, G. Suppression of plant defenses by a *Myzus persicae* (Green Peach Aphid) salivary effector protein. **Molecular Plant-Microbe Interactions**, Saint Paul, v. 27, n. 7, p. 747-756, 2014.

FEDER, M. E.; HOFMANN, G. E. Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. **Annual Review of Physiology**, Palo Alto, v. 61, p. 243-282, 1999.

FERRARI, J.; WEST, J.A.; VIA, S.; GODFRAY, H.C.J. Population genetic structure and secondary symbionts in host-associated populations of the pea aphid complex. **Evolution**, West Sussex, v. 66, n. 2, p. 375-390, 2012.

FRANCIS, F.; GERKENS, P.; HARMEL, N.; MAZZUCCHELLI, G.; DE PAUW, E.; HAUBRUGE, E. Proteomics in *Myzus persicae*: Effect of aphid host plant switch. **Insect Biochemistry and Molecular Biology**, Oxford, v. 36, n. 3, p. 219-227, 2006.

FRANCIS, F.; GUILLONNEAU, F.; LEPRINCE, P.; DE PAUW, E.; HAUBRUGE, E.; Tritrophic interactions among *Macrosiphum euphorbiae* aphids, their host plants and endosymbionts: Investigation by a proteomic approach. **Journal of Insect Physiology**, Oxford, v. 56, n. 6, p. 575-585, 2010.

FUKATSU, T.; TSUCHIDA, T.; NIKOH, N.; KOGA, R. *Spiroplasma* symbiont of the pea aphid, *Acyrthosiphon pisum* (Insecta: Homoptera). **Applied and Environmental Microbiology**, Washington, v. 67, n. 3, p. 1284-1291, 2001.

GASPARICH, G. E. *Spiroplasmas* and *Phytoplasmas*: Microbes associated with plant hosts. **Biologicals**, London, v. 38, n. 2, p. 193-203, 2010.

HAFT, D. H.; BASU, M. K. Biological systems discovery in silico: radical S-Adenosylmethionine protein families and their target peptides for posttranslational modification. **Journal of Bacteriology**, Washington, v. 193, n. 11, p. 2745-2755, 2011.

HALBERT, S.E.; BROWN, L.G. **Brown Citrus Aphid, Toxoptera citricida (Kirkaldy) (Insecta: Hemiptera: Aphididae)**. Gainesville: University of Florida IFAS Extension. EENY-007**:** 1-6 p. 2011.

HANSEN, A. K.; MORAN, N. A. The impact of microbial symbionts on host plant utilization by herbivorous insects. **Molecular Ecology**, Oxford, v. 23, n. 6, p. 1473-1496, 2014.

_____. Aphid genome expression reveals host–symbiont cooperation in the production of amino acids. **Proceedings of the national academy of science of the united States of America**, Washington, v.108, n.7, p. 2849-2854, 2011.

HASELKORN, T.S. The *Spiroplasma* heritable bacterial endosymbiont of *Drosophila*. **Fly**, New York, v. 4, n. 1, p. 80-87, 2010.

JAENIKE, J.; UNCKLESS, R.L.; COCKBURN, S.N.; BOELIO, L.M.; PERLMAN, S.J. Adaptation via symbiosis: recent spread of a *Drosophila* defensive symbiont. **Science**, Washington, v. 329, p. 212–215, 2010.

KARIMZADEH, J.; WRIGHT, D. J. Bottom-up cascading effects in a tritrophic system: interactions between plant quality and host-parasitoid immune responses. **Ecological Entomology**, London, v. 33, p. 45-52, 2007.

KAUFMANN, S. H. E. Heat-shock proteins and the immune-respose. **Immunology Today**, Oxford, v. 11, n. 4, p. 129-136, 1990.

KING, A. M.; MACRAE, T. H. Insect heat shock proteins during stress and diapause. **Annual Review of Entomology**, Standford, v. 60, p. 59-75, 2015 2015.

KLEIGER, G.; MAYOR, T. Perilous journey: a tour of the ubiquitin-proteasome system. **Trends in Cell Biology**, Oxford, v. 24, p. 352-359, 2014.

KRAGOL, G.; LOVAS, S.; VARADI, G.; CONDIE, B. A.; HOFFMANN, R.; OTVOS-JR, L. The antibacterial peptide pyrrhocoricin inhibits the ATPase action of DnaK and prevents chaperone-assisted protein folding. **Biochemistry**, Washignton, v. 40, p. 3016-3026, 2004.

LEE, K.P.; CORY, J.S.; WILSON, K.; RAUBENHEIMER, D.; SIMPSON, S.J. Flexible diet choice offsets protein costs of pathogen resistance in a caterpillar. **Proceedings of the Royal Society B Biological Sciences**, London, v. 272, p. 823-829, 2006.

LEE, K.P; SIMPSON, S.J.; WILSON, K. Dietary protein-quality influences melanization and immune function in an insect. **Functional Ecology**, Oxford, v. 22, p.1052-1061, 2008.

LI, Y.; XIANG, Q.; ZHANG, Q.; HUANG, Y.; SU, Z. Overview on the recent study of antimicrobial peptides: Origins, functions, relative mechanisms and application. **Peptides**, Philadelphia, v.37, p.207-215, 2012.

LUKASIK, P.; GUO, H.; VAN ASCH, M.; FERRARI, J.; GODFRAY, H.C.J. Protection against a fungal pathogen conferred by the aphid facultative endosymbionts *Rickettsia* and *Spiroplasma* is expressed in multiple host genotypes and species and is not influenced by co-infection with another symbiont. **Journal of Evolutionary Biology**, Basel, v. 26, n. 12, p. 2654-2661, 2013.

MCLEAN, A.H.; VAN ASCH, M.; FERRARI, J.; GODFRAY, H.C. Effects of bacterial secondary symbionts on host plant use in pea aphids. **Proceedings of the Royal Society B-Biological Sciences**, London, v. 278, n. 1706, p. 760-766, 2011.

MORAN, N. A. Symbiosis as an adaptive process and source of phenotypic complexity. **Proceedings of the National Academy of Sciences of the United States of America**, Washington, v. 104, p. 8627-8633, 2007.

MORAN, N. A.; DEGNAN, P. H.; SANTOS, S. R.; DUNBAR, H. E.; OCHMAN, H. The players in a mutualistic symbiosis: Insects, bacteria, viruses, and virulence genes. **Proceedings of the National Academy of Sciences of the United States of America**, Washington, v. 102, n. 47, p. 16919-16926, 2005.

NGUYEN, T. T. A.; MICHAUD, D.; CLOUTIER, C. Proteomic profiling of aphid *Macrosiphum euphorbiae* responses to host-plant-mediated stress induced by defoliation and water deficit. **Journal of Insect Physiology**, Oxford, v. 53, n. 6, p. 601-611, 2007.

OTVOS JR, L.; O, I.; ROGERS, M.E.; CONSOLVO, P.J.; CONDIE, B.A.; LOVAS, S.; BULET, P.; BLASZCZYK-THURIN, M. Interaction between heat shock proteins and antimicrobial peptides. **Biochemistry**, Washington, v. 39, p. 14150-14159, 2000.

POCKLEY, A.G. Heat shock proteins as regulators of the immune response. **Lancet**, London, v. 362, p. 469-476, 2003.

PRICE, D. R. G.; FENG, H.; BAKER, J.D.; BAVAN, S.; LUETJE, C. W.; WILSON, A. C. C. Aphid amino acid transporter regulates glutamine supply to intracellular bacterial symbionts. **Proceedings of the National Academy of science of the United States of America**, Washington, v.111, n.1, p.320-325, 2014.

ROY, R.N.; LOMAKIN, I.B.; GAGNON, M.G.; STEITZ, T.A. The mechanism of inhibition of protein synthesis by the proline-rich peptide oncocin. **Nature Structural & Molecular Biology**, New York, v. 22, p. 466-469, 2015.

RUSSELL, J.A.; WELDON, S.; SMITH, A.H.; KIM, K.L.; HU, Y.; LUKASIK, P.; DOLL, S.; ANASTOPOULOS, I.; NOVIN, M.; OLIVER, K.M. Uncovering symbiont-driven genetic diversity across North American pea aphids. **Molecular Ecology**, Oxford, v. 22, n. 7, p. 2045-2059, 2013.

RUSSELL, C. W.; POLIAKOV, A.; HARIBAL, M.; JANDER, G.; VAN WIJK, K. J.; DOUGLAS, A. E. Matching the supply of bacterial nutrients to the nutritional demand of the animal host. **Proceedings of the royal society B: Biological science**, London, v. 281, n. 1791, 2014. Available at: < DOI: 10.1098/rspb.2014.1163>.

RYAN, M. T.; NAYLOR, D.J.; HØJ, P.B.; CLARK, M.S.; HOOGENRAAD, N. J. The role of molecular chaperones in mitochondrial protein import and folding. **International Review of Cytology - a Survey of Cell Biology**, Maryland Heights, v. 174, p. 127-193, 1997.

SCHLESINGER, M. J. Heat-shock proteins. **Journal of Biological Chemistry**, Bethesda, v. 265, n. 21, p. 12111-12114, 1990.

SU, Q.; ZHOU, X.; ZHANG, Y. Symbiont-mediated functions in insect hosts. **Communicative & Integrative Biology**, New York, v. 6, n. 3, p. e23804-e23804, 2013.

TANIGUCHI, M.; OCHIAI, A.; KONDO, H.; FUKUDA, S.; ISHIYAMA, Y.; SAITOH, E.; KATO, T.; TANAKA, T. Pyrrhocoricin, a proline-rich antimicrobial peptide derived from insect, inhibits the translation process in the cell-free Escherichia coli protein synthesis system. **Journal of Bioscience and Bioengineering**, Suita, v.121, p. 591-598, 2016.

TSUCHIDA, T.; KOGA, R.; MATSUMOTO, T.; FUKATSU, T. Secondary endosymbiotic microbiota of the pea aphid, *Acyrthosiphon pisum*, in natural populations. In: SYMPOSIUM ON APHIDS , 2001, Rennes. **Aphids in a a new Millennium: Proceedings of the Sixth International Symposium on Aphids**, 2001, p. 93-96.

VANDERMOTEN, S.; HARMEL, N.; MAZZUCCHELLI, G.; DE PAUW, E.; HAUBRUGE, E.; FRANCIS, F. Comparative analyses of salivary proteins from three aphid species. **Insect Molecular Biology**, West sussex, v. 23, n. 1, p. 67-77, 2014.

VENTURA, I. M.; MARTINS, A.B.; LYRA, M.L.; ANDRADE, C.A.; CARVALHO, K.A.; KLACZKO, L.B. et al. *Spiroplasma* in *Drosophila melanogaster* populations: prevalence, male-killing, molecular identification, and no association with *Wolbachia*. **Microbial Ecology**, Oxford, v. 64, n. 3, p. 794-801, 2012.

VIOLA, R. E. The central enzymes of the aspartate family of amino acid biosynthesis. **Accounts of Chemical Research**, Washington, v. 34, n.5, p. 339-349, 2001.

VOGELWEITH, F.; THIÉRY, D.; QUAGLIETTI, B.; MORET, Y.; MOREAU, J. Host plant variation plastically impacts different traits of the immune system of a phytophagous insect. **Functional Ecology**, Oxford, v. 25, p. 1241-1247, 2011.

XIE, J.; VILCHEZ, I.; MATEOS, M. *Spiroplasma* Bacteria Enhance Survival of *Drosophila hydei* Attacked by the Parasitic Wasp *Leptopilina heterotoma*. **Plos One,** San Francisco, v. 5, n. 8, 2010. Available at: < doi:10.1371/journal.pone.0012149>.

XIE, J.; BUTLER, S.; SANCHEZ, G.; MATEOS, M. Male killing *Spiroplasma* protects Drosophila melanogaster against two parasitoid wasps. **Heredity**, London, v. 112, p. 399-408, 2014.

WOLF, D. H.; HILT, W. The proteasome: a proteolytic nanomachine of cell regulation and waste disposal. **Biochimica et Piophysica Acta Molecular Cell Research**, Amsterdam, v. 1695, p. 19-31, 2004.

ZHAO, L.; JONES, W. A. Expression of heat shock protein genes in insect stress responses. **Isj-Invertebrate Survival Journal**, Modena, v. 9, n. 1, p. 93-101, 2012.

ZYTYNSKA, S. E.; WEISSER, W. W. The natural occurrence of secondary bacterial symbionts in aphids. **Ecological Entomology**, London, v. 41, n. 1, p. 13-26, 2016.

9 FINAL CONCLUSIONS

We were able to demonstrate the effects of host plant suitability (optimal vs. suboptimal host plants) on the development and reproduction of the aphid *Aphis (Toxoptera) citricidus* (Kirkaldy) and on the growth pattern of the aphid-associated primary symbiont, *Buchnera aphidicola*. We also demonstrated that aphids reproductive strategies and regulation of the growth pattern of the primary symbiont differ between the oligophagous aphid *A. citricidus* and the closely-related polyphagous *Aphis* (*Toxoptera*) *aurantii* Boyer de Fonscolombe. Our investigation on the genomic composition of the primary symbiont associated with these oligophagous and polyphagous aphid species indicated differences in their strategies to explore optimal and suboptimal hosts were not sustained by differences in the genomic composition of their primary symbionts.

We also demonstrated that host plants can affect the diversity of secondary symbionts associated with *A. citricidus*, even if restricted to *Citrus* and *Citrus*-related genera, and that the effects on the aphid associated microbiota followed the phylogeny of the host plants and their suitability to *A. citricidus*. Our investigations also allowed us to explore the diversity of secondary symbionts associated with *A. citricidus* and *A. aurantii* and demonstrate they share the same secondary symbiont richness, but not the secondary symbiont abundance when living in the same ecological niche. Nevertheless, *Spiroplasma* was the most common secondary symbiont infecting both aphid species. We then demonstrated associations with *Spirolasma* induced no fitness costs to *A. citricidus*, but *Spiroplasma* did affect the proteome of aphid host. The effects of *Spiroplasma* infection on aphid proteome were influenced by the quality of the host plant. Transcriptomic analysis between *Spiroplasma*-infected and *Spiroplasma*-free *A. citricidus* reared on optimal and suboptimal hosts indicated *Spiroplasma* interferes with gene expression of *A. citricidus* and its associated primary symbiont, *B. aphidicola*. Once again, the effects of *Spiroplasma* on gene expression profile of infected aphids were influenced by the quality of the host plant (optimal or suboptimal) the aphid was developing on.

In conclusion, we demonstrated that the association of aphid-primary symbiont is influenced by secondary symbionts and that the nutritional quality of the aphid host plant interferes in the multipartite interactions aphids establish with their associated symbionts. We found no clear role of symbiotic associations on the feeding strategies of *A. citricidus* (oligophagy) and *A. aurantii* (polyphagy).