Neotrop Entomol (2018) 47:440–446
DOI 10.1007/s13744-017-0532-0

ECOLOGY, BEHAVIOR AND BIONOMICS

Diversity of the Most Commonly Reported Facultative Symbionts in Two Closely-Related Aphids with Different Host Ranges

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Keywords

Symbiont richness, host plant, oligophagy, polyphagy, *Spiroplasma*

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Edited by Marcelo N Rossi – UNIFESP

Received 9 December 2016 and accepted 2 May 2017

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 aurantii* (Boyer de Fonscolombe) (Hemiptera: Aphididae). *Aphis 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.

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 *et al* 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 *et al* 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), *Spiroplasma* protect against microbials and *R. insecticola* against both biological agents (Ferrari *et al* 2001, Ferrari *et al* 2004, Vorburger *et al* 2010, 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, 2005, 2009, 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 *et al* 2000, Montllor *et al* 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* (Harris) (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, Lagos & Voeftlin 2014).

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.

Material and Methods

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. Samples were collected randomly from 20 trees throughout a year. Aphids were brought to the laboratory and classified following the key to wingless adults provided by Halbert & Brown (1996).

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 × 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 a week to observe aphid development and eliminate parasitized and/or unhealthy aphids. 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 *B. aphidicola* and the APSE-1 bacteriophage (Sandstrom *et al* 2001, Tsuchida *et al* 2002, Oliver *et al* 2005, Oliver *et al* 2006).

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 *et al* (1996). DNA quality was verified by spectrophotometer measurements of the A260/280 ratio and by agarose gel electrophoresis on a 1% 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, 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, 1× 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 1). Amplicons were resolved by agarose gel electrophoresis on a 1.5% gel slab and visualized under the same conditions described above.

Table 1 Primers and cycling conditions used for assessing the association of the most common aphid symbionts infecting *Aphis* (*Toxoptera*) *citricidus* and *Aphis* (*Toxoptera*) *aurantii* by using diagnostic PCR.

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

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 ($p < 0.05$).

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 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 (Fig 1). Overall, *A. aurantii* was more frequently associated with secondary symbionts than *A. citricidus* ($x^2 = 27.0$, $df = 1$, $p < 0.001$), 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* ($x^2 = 7.5$, $df = 1$, $p < 0.001$). In addition, *Spiroplasma* was the only secondary symbiont to be persistently associated with *A. aurantii* in single and multiple infections with other secondary symbionts (Fig 1 and Table 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 APSE-1 (Fig 1). While *Arsenophonus* association with *A. citricidus* was much more common than with *A. aurantii* ($x^2 = 10.2$, $df = 1$, $p < 0.001$), *Rickettsia* showed the opposite trend ($x^2 = 5.2$, $df = 1$, $p < 0.001$). No difference in association with *H. defensa* ($x^2 = 1.5$, $df = 1$, $p = 0.22$), *S. symbiotica* ($x^2 = 0.05$, $df = 1$, $p = 0.83$), or *R. insecticola* ($x^2 = 0.16$, $df = 1$, $p = 0.69$) was detected between the two aphids.

In both species, several isolines were infected with multiple facultative symbionts. Nearly two-thirds of *A. citricidus* isolines

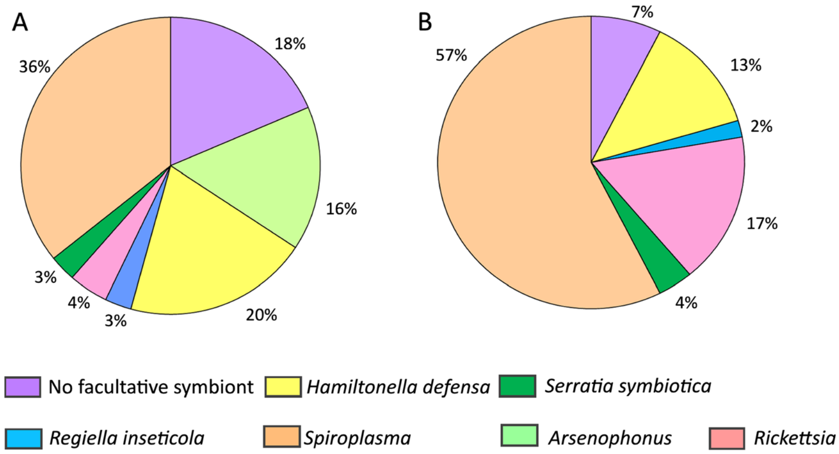

Fig 1 Occurrence and frequency (%) of facultative symbionts in isolines of *Aphis citricidus* (**A**) and *Aphis aurantii* (**B**) collected from the same ecological niche.

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 (Fig 2).

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 2).

Table 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	No. 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

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.

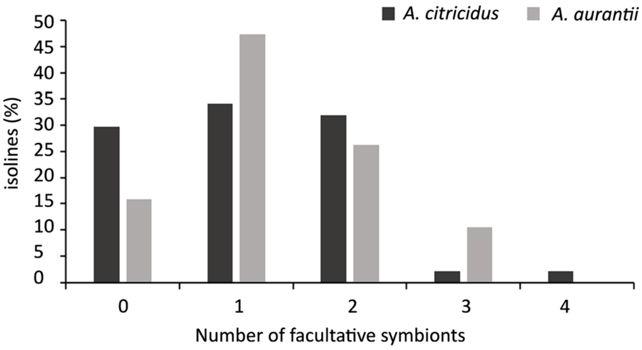

Fig 2 Isolines (%) of *Aphis citricidus* and *Aphis aurantii* from the same ecological niche carrying none, single, or multiple infections of facultative symbionts.

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 *et al* 2014). Associations with defensive facultative symbionts are more prevalent under parasitoid selection pressure (Oliver *et al* 2009, 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, b, c).

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 *et al* 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 *et al* 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 *Aphis* 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 were 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.

Acknowledgments The authors thank FAPESP by providing a PhD fellowship to ASG (Grant No. 2012/04287-0, São Paulo Research Foundation). The authors also thank CNPq (Grant No. 490474/2011-0) and FAPESP (Grant No. 2011/50877-0, São Paulo Research Foundation) for providing research funds to the senior author FLC.

References

Arneodo JD, Ortego J (2014) Exploring the bacterial microbiota associated with native South American species of Aphis (Hemiptera: Aphididae). Environ Entomol 43:589–594

Asplen MK, Bano N, Brady CM, Desneux N, Hopper KR, Malouines C, Oliver KM, White JA, Heimpel GE (2014) Specialisation of bacterial endosymbionts that protect aphids from parasitoids. Ecol Entomol 39:736–739

Augustinos AA, Santos-Garcia D, Dionyssopoulou E, Moreira M, Papapanagiotou A, Scarvelakis M, Doudoumis V, Ramos S, Aguiar AF, Borges PAV, Khadem M, Latorre A, Tsiamis G , Bourtzis K (2011) Detection and characterization of Wolbachia infections in natural populations of aphids: is the hidden diversity fully unraveled? Plos One 6: doi: 10.1371/journal.pone.0028695

Baumann P (2005) Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. Annu Rev Microb 59:155–189

Brady CM, Asplen MK, Desneux N, Heimpel GE, Hopper KR, Linnen CR, Oliver KM, Wulff JA, White JA (2014) Worldwide populations of the aphid *Aphis craccivora* are infected with diverse facultative bacterial symbionts. Microbial Ecol 67:195–204

Buchner P (1965) Endosymbiosis of animals with plant microorganisms. Endosymbiosis of animals with plant microorganisms. Interscience Publishers/John Wiley, New York, p 909

Cayetano L, Rothacher L, Simon JC , Vorburger C (2015) Cheaper is not always worse: strongly protective isolates of a defensive symbiont are less costly to the aphid host. Proc R Soc B 282: doi: 10.1098/rspb.2014.2333

Chandler SM, Wilkinson TL, Douglas AE (2008) Impact of plant nutrients on the relationship between a herbivorous insect and its symbiotic bacteria. Proc R Soc B 275:565–570

Chen DQ, Montllor CB, Purcell AH (2000) Fitness effects of two facultative endosymbiotic bacteria on the pea aphid, *Acyrthosiphon pisum*, and the blue alfalfa aphid, *A. kondoi*. Entomol Exp Appl 95:315–323

Chen DQ, Purcell AH (1997) Occurrence and transmission of facultative endosymbionts in aphids. Curr microbiol 34:220–225

Cockburn SN, Haselkorn TS, Hamilton PT, Landzberg E, Jaenike J, Perlman SJ (2013) Dynamics of the continent-wide spread of a *Drosophila* defensive symbiont. Ecol Lett 16:609–616

Douglas AE (2009) The microbial dimension in insect nutritional ecology. Funct Ecol 23:38–47

Douglas AE, Francois CLMJ, Minto LB (2006) Facultative 'secondary' bacterial symbionts and the nutrition of the pea aphid, *Acyrthosiphon pisum*. Physiol Entomol 31:262–269

Duron O, Hurst GDD (2013) Arthropods and inherited bacteria: from counting the symbionts to understanding how symbionts count. BMC Biology 11: doi: 10.1186/1741-7007-11-45

Ferrari J, Darby AC, Daniell TJ, Godfray HCJ, Douglas AE (2004) Linking the bacterial community in pea aphids with host-plant use and natural enemy resistance. Ecol Entomol 29:60–65

Ferrari J, Godfray HCJ, Faulconbridge AS, Prior K, Via S (2006) Population differentiation and genetic variation in host choice among pea aphids from eight host plant genera. Evolution 60:1574–1584

Ferrari J, Muller CB, Kraaijeveld AR, Godfray HCJ (2001) Clonal variation and covariation in aphid resistance to parasitoids and a pathogen. Evolution 55:1805–1814

Ferrari J, West JA, Via S, Godfray HCJ (2012) Population genetic structure and secondary symbionts in host-associated populations of the pea aphid complex. Evolution 66:375–390

Fukatsu T (1994) Endosymbiosis of aphids with microorganisms: a model case of dynamic endosymbiotic evolution. Plant Spec Biol 9:145–154

Fukatsu T, Tsuchida T, Nikoh N, Koga R (2001) *Spiroplasma* symbiont of the pea aphid, *Acyrthosiphon pisum* (Insecta: Homoptera). Appl Environ Microbiol 67:1284–1291

Gao SX, Liu DG, Chen H, Meng XX (2014) Fitness traits and underlying genetic variation related to host plant specialization in the aphid *Sitobion avenae*. Insect Sci 21:352–362

Halbert SE, Brown LG (1996) *Toxoptera citricida* (Kirkaldy), brown citrus aphid—identification, biology and management strategies. Florida Department of Agriculture and Consumer Services Division of Plant Industry Entomology Circular, 374, 6pp

Halbert SE, Genc H, Cevik B, Brown LG, Rosales IM, Manjunath KL, Pomerinke M, Davison DA, Lee RF, Niblett CL (2004) Distribution and characterization of *Citrus tristeza virus* in South Florida following establishment of *Toxoptera citricida*. Plant Dis 88:935–941

Haselkorn TS, Jaenike J (2015) Macroevolutionary persistence of heritable endosymbionts: acquisition, retention and expression of adaptive phenotypes in *Spiroplasma*. Mol Ecol 24:3752–3765

Haynes S, Darby AC, Daniell TJ, Webster G, van Veen FJF, Godfray HCJ, Prosser JI, Douglas AE (2003) Diversity of bacteria associated with natural aphid populations. Appl Environ Microbiol 69:7216–7223

Henry LM, Maiden MCJ, Ferrari J, Godfray HCJ (2015) Insect life history and the evolution of bacterial mutualism. Ecol Lett 18:516–525

Klepzig KD, Adams AS, Handelsman J, Raffa KF (2009) Symbioses: a key driver of insect physiological processes, ecological interactions, evolutionary diversification, and impacts on humans. Environ Entomol 38:67–77

Lagos DM, Voeftlin DJ, d'acier AC, Giordano R (2014) Aphis (Hemiptera: aphididae) species groups found in the Midwestern United States and their contributon to the phylogenetic knowledge of the genus. Insect Sci 21:374–391

Leonardo TE, Muiru GT (2003) Facultative symbionts are associated with host plant specialization in pea aphid populations. Proc R Soc B 270: 209–212

Lukasik P, Dawid MA, Ferrari J, Godfray HCJ (2013a) The diversity and fitness effects of infection with facultative endosymbionts in the grain aphid, *Sitobion avenae*. Oecologia 173:985–996

Lukasik P, Guo H, Van Asch M, Ferrari J, Godfray HCJ (2013b) 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. J Evol Biol 26:2654–2661

Lukasik P, van Asch M, Guo H, Ferrari J, Godfray HCJ (2013c) Unrelated facultative endosymbionts protect aphids against a fungal pathogen. Ecol Lett 16:214–218

McLean AHC, van Asch M, Ferrari J, Godfray HCJ (2011) Effects of bacterial secondary symbionts on host plant use in pea aphids. Proc R Soc B 278:760–766

Montllor CB, Maxmen A, Purcell AH (2002) Facultative bacterial endosymbionts benefit pea aphids *Acyrthosiphon pisum* under heat stress. Ecol Entomol 27:189–195

Moran NA, Degnan PH, Santos SR, Dunbar HE, Ochman H (2005) The players in a mutualistic symbiosis: insects, bacteria, viruses, and virulence genes. Proc Natl Acad Sci U S A 102:16919–16926

Oliver KM, Degnan PH, Burke GR, Moran NA (2010) Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. Annu Rev Entomol 55:247–266

Oliver KM, Degnan PH, Hunter MS, Moran NA (2009) Bacteriophages encode factors required for protection in a symbiotic mutualism. Science 325:992–994

Oliver KM, Moran NA, Hunter MS (2005) Variation in resistance to parasitism in aphids is due to symbionts not host genotype. Proc Natl Acad Sci U S A 102:12795–12800

Oliver KM, Moran NA, Hunter MS (2006) Costs and benefits of a superinfection of facultative symbionts in aphids. Proc R Soc B 273:1273–1280

Oliver KM, Russell JA, Moran NA, Hunter MS (2003) Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. Proc Natl Acad Sci U S A 100:1803–1807

Oliver KM, Smith AH, Russell JA (2014) Defensive symbiosis in the real world—advancing ecological studies of heritable, protective bacteria in aphids and beyond. Funct Ecol 28:341–355

Russell JA, Moran NA (2006) Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. Proc R Soc B 273:603–610

Russell JA, Weldon S, Smith AH, Kim KL, Hu Y, Lukasik P, Doll S, Anastopoulos I, Novin M, Oliver KM (2013) Uncovering symbiont-driven genetic diversity across North American pea aphids. Mol Ecol 22:2045–2059

Sandstrom JP, Russell JA, White JP, Moran NA (2001) Independent origins and horizontal transfer of bacterial symbionts of aphids. Mol Ecol 10:217–228

Simon JC, Boutin S, Tsuchida T, Koga R, Le Gallic JF, Frantz A, Outreman Y, Fukatsu T (2011) Facultative symbiont infections affect aphid reproduction. Plos One 6: doi: 10.1371/journal.pone.0021831

Smith AH, Lukasik P, O'Connor MP, Lee A, Mayo G, Drott MT, Doll S, Tuttle R, Disciullo RA, Messina A, Oliver KM, Russell JA (2015) Patterns, causes and consequences of defensive microbiome dynamics across multiple scales. Mol Ecol 24:1135–1149

Su Q, Zhou X, Zhang Y (2013) Symbiont-mediated functions in insect hosts. Commun Integr Biol 6: doi: 10.4161/cib.23804

Sunnucks P, De Barro PJ, Lushai G, MacLean N, Hales N (1996) Genetic structure of an aphid studied using microsatellites: cyclic parthenogenesis, differentiated lineages and host specialization. Mol Ecol 6: 1059-1073

Telesnicki MC, Ghersa CM, Martinez-Ghersa MA, Arneodo JD (2012) Molecular identification of the secondary endosymbiont *Hamiltonella defensa* in the rose-grain aphid *Metopolophium dirhodum*. Rev Argent Microbiol 44:255–258

Tsuchida T, Koga R, Horikawa M, Tsunoda T, Maoka T, Matsumoto S, Simon JC, Fukatsu T (2010) Symbiotic bacterium modifies aphid body color. Science 330:1102–1104

Tsuchida T, Koga R, Matsumoto T, Fukatsu T (2004) Secondary endosymbiotic microbiota of the pea aphid, *Acyrthosiphon pisum*, in natural populations. Aphids in a New Millennium 93–96

Tsuchida T, Koga R, Shibao H, Matsumoto T, Fukatsu T (2002) Diversity and geographic distribution of secondary endosymbiotic bacteria in natural populations of the pea aphid, *Acyrthosiphon pisum*. Mol Ecol 11:2123–2135

Vorburger CA, Gehrer L, Rodriguez P (2010) A strain of the bacterial symbiont *Regiella insecticola* protects aphids against parasitoids. Biol Lett 6:109–111

Wagner SM, Martinez AJ, Ruan YM, Kim KL, Lenhart PA, Dehnel AC, Oliver KM, White JA (2015) Facultative endosymbionts mediate dietary breadth in a polyphagous herbivore. Funct Ecol 29:1402–1410

Wuff JA, Buckman KA, Wu K, Heimpel GE, White JA (2013) The endosymbiont *Arsenophonus* is widespread in aphid, *Aphis glycines*, but does not provide protection from parasitoids or fungal pathogen. Plos One 8: doi:10.1371/journal.pone.0062145

Xie J, Butler S, Sanchez G, Mateos M (2014) Male killing *Spiroplasma* protects *Drosophila melanogaster* against two parasitoid wasps. Heredity 112:399–408

Zytynska SE, Weisser WW (2016) The natural occurrence of secondary bacterial symbionts in aphids. Ecol Entomol 41:13–26