

Population Dynamics and Growth Rates of Endosymbionts During *Diaphorina citri* (Hemiptera, Liviidae) Ontogeny

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Abstract The infection density of symbionts is among the major parameters to understand their biological effects in host–endosymbionts interactions. *Diaphorina citri* harbors two bacteriome-associated bacterial endosymbionts (*Candidatus Carsonella ruddii* and *Candidatus Profftella armatura*), besides the intracellular reproductive parasite *Wolbachia*. In this study, the density dynamics of the three endosymbionts associated with the psyllid *D. citri* was investigated by real-time quantitative PCR (qPCR) at different developmental stages. Bacterial density was estimated by assessing the copy number of the 16S rRNA gene for *Carsonella* and *Profftella*, and of the *ftsZ* gene for *Wolbachia*. Analysis revealed a continuous growth of the symbionts during host development. Symbiont growth and rate curves were estimated by the Gompertz equation, which indicated a negative correlation between the degree of symbiont–host specialization and the time to achieve the maximum growth rate (t^*). *Carsonella* densities were significantly lower than those of *Profftella* at all host developmental stages analyzed, even though they both displayed a similar trend. The

growth rates of *Wolbachia* were similar to those of *Carsonella*, but *Wolbachia* was not as abundant. Adult males displayed higher symbiont densities than females. However, females showed a much more pronounced increase in symbiont density as they aged if compared to males, regardless of the incorporation of symbionts into female oocytes and egg laying. The increased density of endosymbionts in aged adults differs from the usual decrease observed during host aging in other insect–symbiont systems.

Introduction

Symbiosis commonly occurs in insects and more than 10 % of insects depend on the interactions with intracellular mutualistic symbionts for their development [1–3]. Symbiosis is based mainly on metabolic innovations related to ecological advantages obtained by the host, such as the supplementation of missing or insufficient nutrients in the host diet, tolerance to stress factors, and increased host plant use due to metabolic compensation [1, 4–6].

Intracellular symbionts can be functionally and phylogenetically distinguished as obligate (primary) or facultative (secondary) based on the degree of co-evolution and co-dependency, specialization of their genome, and location in the host organism [2, 6, 7]. Obligate mutualistic symbionts are essential to the host, with which they have a high degree of co-speciation and dependence as a consequence of a shared evolutionary history. They are vertically transferred to the host progeny through transovarial transmission [2, 6, 8, 9]. On the other hand, facultative symbionts have a more recent history of association, establishing diverse interactions that may or may not contribute to host nutrition and tolerance to stress factors, and affect host fitness in several ways [4, 10, 11]. An example of this is the intracellular facultative symbiont *Wolbachia*, which is widely distributed among arthropods

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and is better known by inducing several reproductive phenotypes (male feminization, parthenogenesis, male killing, and cytoplasmic incompatibility) [12, 13]. But many recent reports have also demonstrated the role this symbiont may have on the host immune responses against microbial pathogens [14], tolerance to stress factors [15], nutritional supplementation [16], and host fecundity and fertility [17, 18]. However, this symbiotic association may also have adaptive costs yielding short-lived and less fecund hosts [19, 20].

Primary symbionts are harbored within specialized host cells (bacteriocytes), which can be organized in more complex tissues, forming the bacteriome [2]. Depending on the host species, the bacteriome may harbor one or more symbionts specifically arranged in the cytoplasm of the bacteriocytes or in the syncytium of the bacteriome [2, 21, 22]. This type of endosymbiotic association is maintained across generations through transovarial transmission, in which part of the free symbionts or intact bacteriocytes present in the maternal bacteriome migrate to the ovaries, where they are deposited at various stages of oogenesis depending on the host [23–26]. As a consequence of this process of vertical transmission, only part of the maternal symbiotic content is allocated to the progeny [9, 24, 26]. Thus, the population of symbionts suffers a bottleneck effect in the process of transmission to the offspring [9, 27], accentuating the processes of genetic deterioration and specialization of the symbiont [28, 29].

Symbiont density may be influenced by factors such as temperature [30, 31], age [32, 33], host gender [13], reproductive cycle [34], polymorphism [35, 36], larval density [37], competition among symbionts [38], location, and host immune response [39, 40], which directly or indirectly affect host fitness. Management of chronic infections with endosymbionts may be achieved either by specific bacterial adaptations [9, 16, 41–45] or by host modulation of the innate defense mechanisms against its microbial partners [39, 45, 46]. Even in those cases in which mutual speciation has led to the integration of endosymbionts as a complementary source of host defense (e.g., against pathogens and predators) [4, 11, 47, 48], insect host still control endosymbiont proliferation in their tissues [39, 46, 49–51]. In most cases, the mechanisms involved in symbiont control are still unknown, but they may involve the synthesis of antimicrobial peptides [46] or molecules involved in controlling symbiont cell division and metabolism [51] or rely on the control of the host immune response elicitation [39]. In addition to the host control on symbiont proliferation, coinfections can also lead to symbiont–symbiont interactions that affect symbiont infection density [38].

The Asian citrus psyllid *Diaphorina citri* is a most widely distributed vector of the *Huanglongbing*-causing bacteria, a major citrus disease. This psyllid has a bilobed, typical bacteriome in which two main bacterial symbionts are harbored either in the syncytium or in the surrounding uninucleate bacteriocytes [2, 21, 24]. The bacteriocytes are inhabited

by *Candidatus Carsonella ruddii*, whereas the syncytium by *Candidatus Proffittella armatura* [2, 47]. Additionally, psyllids may also support a number of secondary symbionts, including the intracellular facultative symbiont *Wolbachia* [52]. We demonstrate here how the population density of the three major symbionts associated with *D. citri* change as the host develops by assessing the number of copies of the 16S ribosomal RNA (rRNA) gene of the symbionts associated with the bacteriome (*Carsonella* and *Proffittella*) and the *ftsZ* gene of the *Wolbachia* using real-time quantitative PCR (qPCR).

Methods

Insect Rearing

The insects used in the experiments were obtained from a stock lab population of *D. citri* kept under controlled conditions (28±2 °C; 60±10 % RH; 14 h photophase) on the orange jasmine *Murraya exotica* (Rutaceae) as the host plant [53].

Samplings

Symbiont density was assessed at different ages of the egg, nymphal, and adult stages of *D. citri*. Eggs were collected at three stages of the embryonic development: early (egg-I=0–10 h old), intermediate (egg-II=48–56 h old), and late stage (egg-III=72–84 h old). The five nymph stadia (N1, N2, N3, N4, and N5) were sampled on their first day of development, i.e., just after hatching (for the first instar) or immediately after ecdysis (for the remaining instars). Male and female adult samplings were also subdivided into three distinct physiological periods: pre-reproductive (0–24 h; adult-I), reproductive (10–25 days; adult-II), and post-reproductive (25–35 days; adult-III) based on the morphology of their spermatheca [54] and on their reproductive behavior [55].

Five-day-old adults at a 1:1 sex ratio were offered to the host plant in cages (65×65×40 cm) and allowed to lay eggs for 4 h under the same rearing conditions earlier mentioned. Adults were then removed and infested plants were maintained under controlled conditions for further sampling of the desired stage of development. The collected samples were fixed in absolute ethanol and stored at 4 °C until genomic DNA (gDNA) extraction.

Genomic DNA Extraction

Total DNA extraction was performed in groups containing the same amount of specimens to reduce possible variations in the efficiency of the extraction method. The extraction of gDNA from eggs ($n=100$ eggs/embryonic stage/biological replicate), nymphs (for N1, N2, and N3, $n=100$ specimens/stage/biological replicate; for N4 and N5, $n=30$ specimens/stage/

biological replicate), and adults ($n=30$ adults/sex/age/biological replicate) of *D. citri* followed by Gilbert et al. [56]. Samples were macerated and incubated in the digestion buffer (3 mM CaCl₂, 2 % SDS, 40 mM dithiothreitol, 20 mg/mL proteinase K, 100 mM Tris buffer, pH 8.0, 100 mM NaCl) for 20 h. After incubation, one volume of phenol (pH 7.8) was added, samples were vortexed, and centrifuged for collection of the gDNA-containing phase. The same procedure was repeated using an equivalent volume of chloroform instead of phenol. The recovered gDNA was precipitated using isopropanol:sodium acetate (volume ratio of 0.7:0.1) added with 1 μ L of glycogen (20 mg/mL; Thermo Scientific #R0561) for 30 min at -80 °C and then centrifuged and washed in 85 % ethanol. The gDNA pellet was dried using a SpeedVac machine for 15 min and resuspended in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) after 30 min at 37 °C. gDNA concentration and quality were assessed on a Nanodrop 2000/2001 spectrophotometer (Thermo Scientific) and by 0.8 % agarose gel electrophoresis using Tris-acetate-EDTA (TAE) (40 mM Tris-acetate, 1 mM EDTA, pH 7.2) buffer at a constant voltage (100 V). Only samples with high-quality gDNA were used in qPCR analysis with a user-designed set of specific primers (Table 1). The specific primers for each symbiont were designed using the OligoPerfect™ Designer software (Invitrogen) (<http://tools.invitrogen.com/content.cfm?pageID=9716>), and quality parameters (dimerization, hairpin, and melting temperature) were checked using the OligoCalc tools (<http://www.basic.northwestern.edu/biotools/oligocalc.html>). The primers were designed to target the 16S rRNA gene sequences of *Carsonella* [57] and *Proffittella* (Salvador and C onsoli, unpublished data—GenBank accession number: EU570830.1), and the *ftsZ* gene of *Wolbachia* [58] (Table 1).

The amount of gDNA per individual (ng/insect- Δ eq) was used to calculate the copy number of each symbiont.

Real-Time Quantitative PCR Analysis

The target genes selected for each symbiont were amplified and the obtained PCR products were purified and inserted into the pGEM®-T Easy Vector System (Promega) and used to transform OneShot® TOP10 (Invitrogen) highly competent cells, which were grown in an Luria-Bertani (LB) culture medium

supplemented with 100 μ g/mL ampicillin and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-GAL), following the manufacturer recommendations. Positive clones were isolated, cultivated in LB liquid medium supplemented with 100 μ g/mL ampicillin, and subjected to plasmid extraction using the alkaline lysis method [59].

The obtained plasmids were subjected to PCR amplification using the specific primer sets developed for each target symbiont in a thermocycler set at 95 °C for 5 min (1 cycle); 95 °C for 45 s, 55 °C for 30 s, 72 °C for 45 s (40 cycles); 72 °C for 5 min (final extension), followed by verification of the insert size on a 1 % agarose gel electrophoresis as earlier described. Plasmids containing the correct insert size were used to produce a dilution standard curve. A series of six dilutions containing 20 ng/ μ L–6.4 pg/ μ L of plasmid + insert was amplified in triplicate using optimized cycling conditions for each primer set on a StepOne (Applied Biosystems) thermocycler as follows: DcMycF/DcMycR (*Carsonella*) and DcftsZF/DcftsZR (*Wolbachia*)—50 °C for 2 min, 95 °C for 10 min, 45 cycles at 95 °C for 15 s, and 58 °C for 30 s, followed by a melting curve at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s; and DcSynF/DcSynR (*Proffittella*)—50 °C for 2 min, 95 °C for 10 min, 45 cycles at 95 °C for 15 s, and 60 °C for 15 s, followed by a melting curve at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s.

The number of copies (N) of the target genes per microliter was determined using the following equation [60]:

$$N = \frac{X \text{ g}/\mu\text{L DNA}}{\text{size of the clone in bp} \times 660} 6.022 \times 10^{23}$$

where

X	Quantity of DNA in g/ μ L
Clone	Plasmid + insert
660 g/mol	Average molecular weight of 1 DNA bp
6.022×10^{23}	Number of molecules in 1 mol (Avogadro constant)

The symbiont density was obtained as a measure of the number of copies of the selected target gene using the Ct values obtained for each sample against the standard curve [61] produced for each target gene using the tools available in the qPCR StepOne system. The qPCR reactions were performed using

Table 1 Primers used in the real-time qPCR analysis of *Diaphorina citri*-associated symbionts

Symbiont	Target gene	Primer sequence	Accession number
<i>Carsonella (myc)</i>	16S rRNA	Myc-F (5'-TGGGAACGCCATATGCTAAT-3') Myc-R (5'-GTCCCAATGGGTGTTTCATC-3')	GenBank: EF450250
<i>Proffittella (syn)</i>	16S rRNA	Syn-F (5'-GCCTTTATGGGTAGGGCTTC-3') Syn-R (5'-CCGGACTACGATGCACCTTT-3')	GenBank: EU570830
<i>Wolbachia (ftsZ)</i>	<i>ftsZ</i> -81	<i>ftsZ</i> -F (5'-AGCAGCCAGAGAAGCAAGAG-3') <i>ftsZ</i> -R (5'-TACGTGCGACACCTTCAAAA-3')	pubMLST: ST 173

12.5 μL of the Maxima SYBR Green/ROX qPCR Master Mix (2 \times) buffer (Fermentas), 0.9 μL of the primer set (concentration 10 μM), 60 ng gDNA (eggs, nymphs, or adults), and 8.7 μL water (nuclease-free), totaling a final volume of 25 μL . Each sample had three biological replicates and each biological replicate was run in technical triplicates.

Statistical Analysis

The qPCR data for each symbiont were subjected to the Levene and Cramér-von Mises tests. The data were transformed into $\ln(x)$ and subjected to ANOVA followed by the Tukey's test ($p \leq 0.05$) using the SAS 9.1 software (SAS Institute, Cary, NC).

Additionally, the Gompertz growth model [62] was used to transform the qPCR data into a growth curve of each symbiont based on the average time required for a full cycle of the host (from egg to adult, 45 days) [53]. The growth and maximum growth rates for each symbiont were also estimated by the Gompertz equation [62] according to the age of the host, as follows:

$$N_c = P_f \times e^{(-e((\ln(-\ln(\frac{P_i}{P_f}))))^{-B \times t})}$$

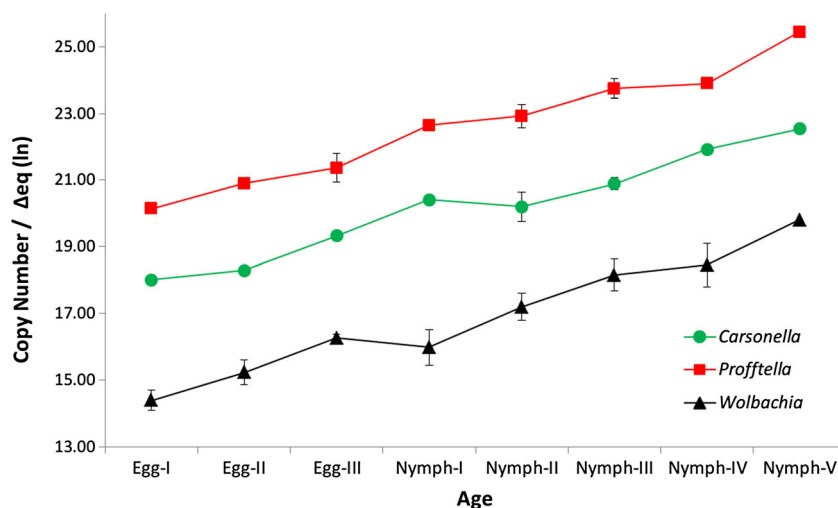
and

$$t^* = \frac{(-1) \times \ln\left(-\ln\left(\frac{P_i}{P_f}\right)\right)}{B}$$

where

- N_c Density of the symbiont, estimated from the number of copies
- P_f Size of the final population or maximum growth of the population
- P_i Size of the population at the beginning or initial growth conditions
- B Relative growth rate

Fig. 1 Average copy numbers per equivalent individual (Δeq) for each endosymbiont during immature *Diaphorina citri* developmental stage. The values are expressed in natural log (\ln)



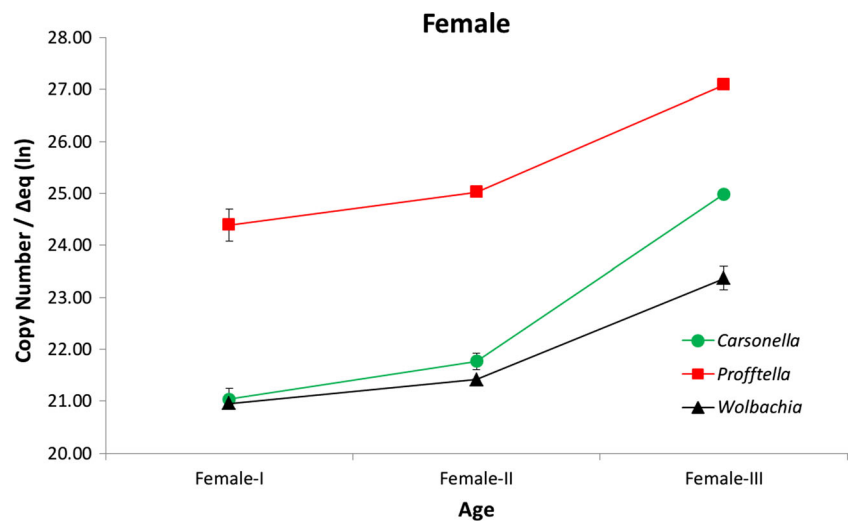
- t Age of the host
- t^* Time in which the growth rate is maximal
- e Euler number
- \ln Natural log

Results

All three investigated symbionts associated with *D. citri* showed exponential growth from the embryonic to the adult stage (Figs. 1, 2, and 3). Despite the expected increase in symbiont density during the initial embryonic stages, significant differences ($p < 0.0001$) were observed only between the early (egg-I) and late (egg-III) embryonic stages. The same was observed for the nymphal stage, with significant differences being detected only between the first and the last nymphs (Fig. 1). Adult females (Fig. 2) showed a progressive increase ($p < 0.0001$) in *Carsonella* and *Profftella* density as they aged, peaking at the post-reproductive period (female-III), while *Wolbachia* density only increased at the post-reproductive period (female-III). In adult males, a significant difference in density was observed for *Carsonella* ($p < 0.0035$) and *Profftella* ($p < 0.0039$) only at the post-reproductive period (male-III) (Fig. 3). No differences were observed in *Wolbachia* density in aging adult males ($p > 0.05$) (Fig. 3).

Estimation of the copy number of the three symbionts indicated a high growth rate during the immature developmental stages of the host, as well as differences between females and males at the initial (P_i) and final (P_f) symbiont densities (Table 2) during the period required for *D. citri* to fully develop from egg to adult (45 days) (for details on biological cycle of *D. citri*, please see [53]). The growth curves estimated by the Gompertz model (Fig. 4) and the average copy numbers showed different rates and times in achieving maximum growth rates (t^*). For example, *Wolbachia* reached the maximum growth rate during the host third instar, and slowed down thereafter. *Profftella* attained its

Fig. 2 Average copy numbers per equivalent individual (Δ_{eq}) for each endosymbiont of adult female *Diaphorina citri* at different reproductive stages. The values are expressed in natural log (ln)



maximum growth rate at the last instar, whereas *Carsonella* reached its maximum growth rate at the adult stage during the reproductive period (female-II) for females and the pre-reproductive period (male-I) for males (Table 2 and Fig. 4).

Discussion

In the *D. citri*–endosymbiont complex, there is a positive correlation between the growth pattern of the symbionts and host development, as indicated by qPCR analyses and estimations for the symbionts growth rates. *D. citri* has an obligate association with *Carsonella* and *Profftella*, as well as with the reproductive parasite *Wolbachia* [47–52]. The growth of symbionts during the embryonic development of *D. citri* is synchronized with the organogenesis of the bacteriome, as previously observed by microscopy [63] and is relatively slow during the initial periods (egg-I and II), then increases its rate during the final third of embryogenesis (egg-III). The

significant increase in symbiont densities at the end of the embryonic development of *D. citri* is different from other species in which symbiont density decrease during this stage, such as *Periplaneta americana* (Blattaria: Blattidae) [64] and *Mastotermes darwiniensis* (Isoptera: Mastotermitidae) [65]. The same has been observed during the later stages of development of *Acyrtosiphon pisum* (Hemiptera: Aphididae) [36] and *Camponotus floridanus* (Hymenoptera: Formicidae) [40], in which part of the symbiont population is destroyed due to the host nutritional demand. Adaptive changes in the regulation of the host immune response to control the density of obligate and facultative symbionts may also explain changes in symbiont density during host development [36, 39, 66–68].

The similar growth trend both primary symbionts associated with the bacteriome of *D. citri* displayed, by continuously increasing in number as the host developed, may serve as a response to the increase in the host metabolic demand [24]. In this case, *Carsonella* and *Profftella* act as syntrophic partners and are in charge of the biosynthesis of compounds required to

Fig. 3 Average copy numbers per equivalent individual (Δ_{eq}) for each endosymbiont of adult male *Diaphorina citri* at different reproductive stages. The values are expressed in natural log (ln)

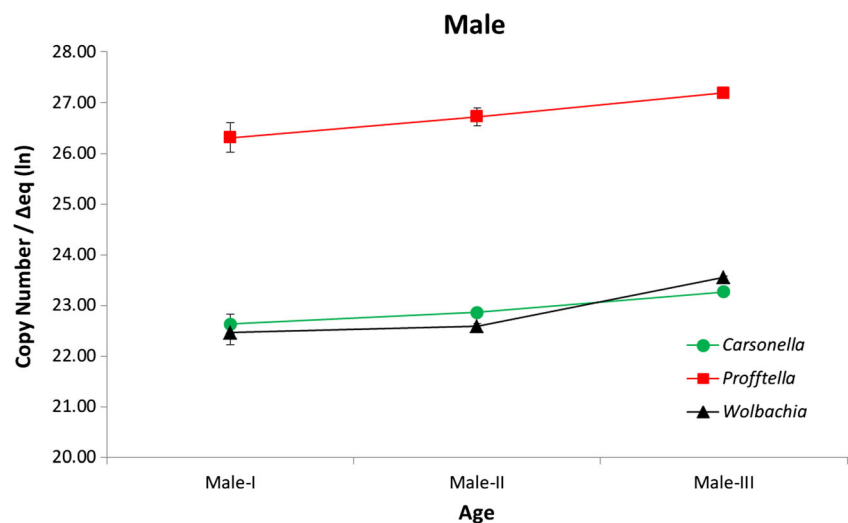


Table 2 Parameters estimated by the Gompertz equation based on the copy number of each endosymbiont during male and female *Diaphorina citri* ontogeny

Parameters	Average copy number per equivalent individual (Δ_{eq}) symbiont					
	<i>Carsonella (myc)</i>		<i>Profftella (syn)</i>		<i>Wolbachia (ftsZ)</i>	
	Male	Female	Male	Female	Male	Female
P_f	26.4471	24.9259	28.0481	26.7086	23.6752	23.4894
P_i	17.9479	18.2546	20.1414	20.1984	14.0304	14.2713
B	0.00204	0.00196	0.00259	0.00309	0.00303	0.00267
t^*	464 h	595 h	426 h	412 h	213 h	260 h
Age (t^*)	Male-I	Female-II	Nymph-V	Nymph-V	Nymph-III	Nymph-III

P_f size of the final population or maximum growth of the population, estimated in natural log (ln) (copy number); P_i size of the population in the beginning or initial growth conditions, estimated in ln (copy number); B relative growth ratio; t^* time (h) in which the growth rate is maximal; Age (t^*) development stage of the host in which t^* is reached

sustain host growth and development [6, 69]. The continuous growth of the symbionts associated with the bacteriome of *D. citri* may also be attributable to their tolerance/escape to/from the hosts' immune system, as suggested for other symbiont–host complexes [39, 46, 70].

The similarity in the growth pattern of *Wolbachia* to that of the symbionts in the bacteriome of *D. citri* is probably due to its capacity to manipulate the hosts' immune response, although this mechanism seems highly specific for the

Wolbachia–host interactions and thus require further studies (see review by Ratzka et al. [39]). *Wolbachia* infects several tissues of this psyllid [FLC unpublished] and infection is fixed in *D. citri* populations in Brazil [58], suggesting this symbiont may act more than a reproductive parasite in this system, as there are examples in which *Wolbachia* is required for host egg development [17] and for metabolic provisioning [71].

Different factors may influence the density of obligate and facultative symbionts during host development, which include

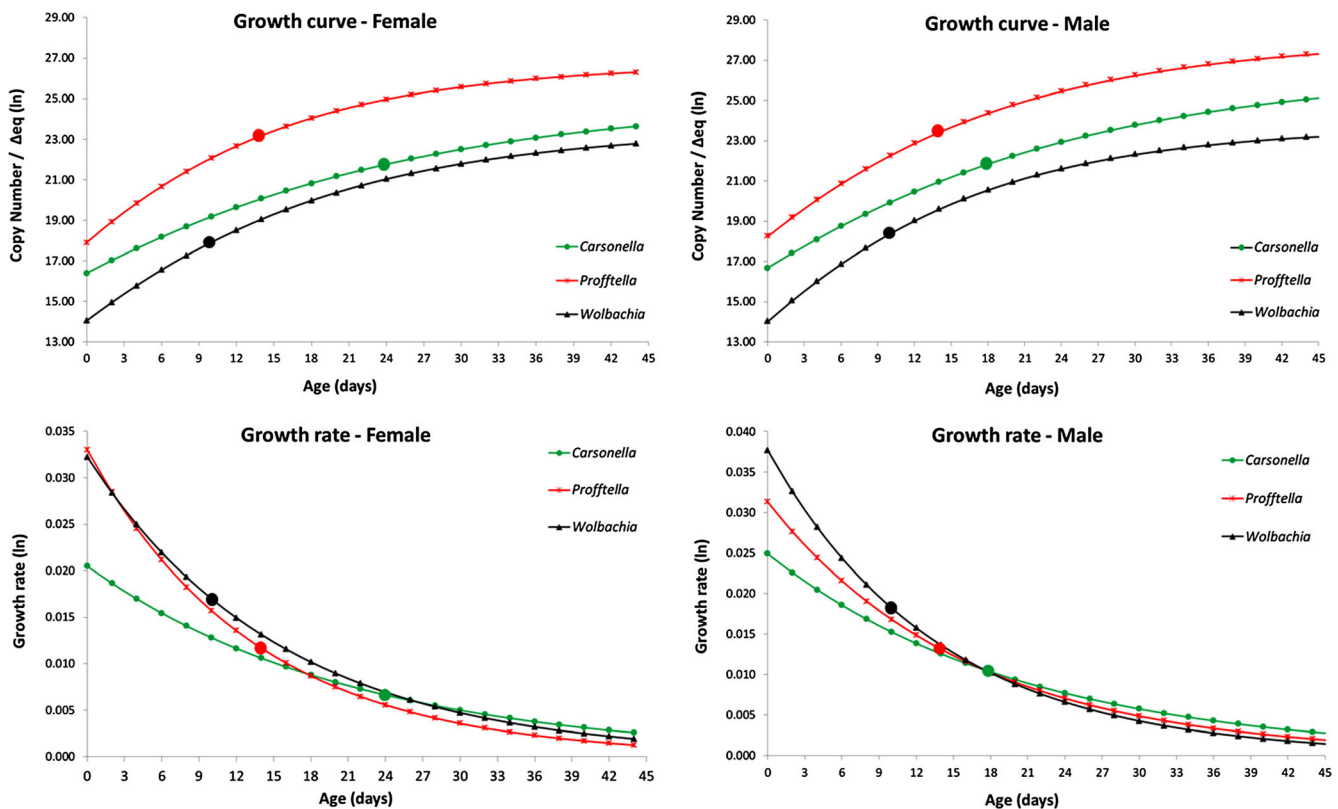


Fig. 4 Curves (ln copy number) (a, b) and rates (ln copy number/h) (c, d) of the growth of symbionts associated with female (a, c) and male (b, d) *Diaphorina citri* (Hemiptera, Liviidae) from egg to adult. The highlighted

points refer to the development period in which the growth rate of each symbiont is maximum (t^*). N3 third instar, N5 fifth instar, PV♂ pre-reproductive male adult, VT♀ female adult of reproductive age

restrictions imposed by the space microbial symbionts have available for their growth in the cell/tissue they are harbored [26, 72], their location inside cells or specialized tissues [2, 21], degradation of symbionts in specific phases of the development of the host [32, 69, 73], fluctuation in symbiont density in response to stress factors and symbiont–symbiont interactions [10, 38, 74, 75], regulation by the host immune system [39], and possibly even competition between/among symbionts [38]. Although all three symbionts investigated displayed similar growth patterns, each one of them reached their maximum growth rate (t^*) at a particular stage of the host development, suggesting they may require specific stimuli and carry different levels of interactions with their host as a result of the evolutionary history of their association with the host and of the interactions among symbionts. *Carsonella*, the older primary symbiont with *D. citri*, showed the lowest growth rate among the studied symbionts, reaching a maximum growth rate (t^*) in early adults, while the more recent primary symbiont *D. citri* acquired, *Proffittella* [6], grew at a faster rate and reached its maximal values in the host fifth instar. But none grew as fast as *Wolbachia*, which attained its t^* in the host third instar.

Although the estimated growth rate decreased during the adult stage of *D. citri*, *Carsonella* and *Proffittella* showed a significant increase in their densities during the hosts' adult life. The growth rate shown during the reproductive period was high enough to overcome the loss of symbionts due to their migration to the oocytes for transmission to the offspring (female-II) because symbiont density was higher than that in the pre-reproductive females (female-I). However, the most surprising fact was the high density of symbionts observed in the post-reproductive period (female-III), both in females and males, in contrast with previous observations in senescent aphids [32, 35, 36].

The density fluctuations of mutualistic obligate symbionts of *D. citri* suggest a parallelism with the host's metabolic demand during the sexual maturation and egg production phases, a condition similar to what was observed during the reproductive period of tsetse flies *Glossina* sp. (Diptera: Glossinidae) and the high densities of the mutualistic symbiont *Wigglesworthia* [70]. The lowest density of *Wolbachia* in females of *D. citri* during the reproductive period as compared to males of *D. citri* is contrary to previous reports on many other species [13, 76, 77]. Little is known on the adaptive cost of infection and the presumable differences in the proliferation rate of *Wolbachia* in the reproductive tissues of males and females [13, 76, 77], but the lower density of *Wolbachia* in older females could be a consequence of the reduced growth rate after t^* (third instar) and/or due to the process of transovarian transmission. Additionally, factors related with the adaptive cost of infection and the presumable differences in the proliferation rate of *Wolbachia* in the reproductive tissues of males and females need further examination [13, 76, 77].

Overall, the growth of the symbionts during the developmental stages of *D. citri* is similar to the patterns observed in other symbiosis systems [32], except for the post-reproductive period. Symbiont density is usually shown to decrease at the post-reproductive period [34, 36], but *D. citri* had a substantial increase in symbiont density. The increased density of symbionts during the post-reproductive period of *D. citri* may be related to the decline in reproductive activities and/or in the regulatory mechanisms controlling symbiont multiplication due to the aging of the host [78].

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