Relative Abundance of *Carsonella ruddii* (Gamma Proteobacterium) in Females and Males of *Cacopsylla pyricola* (Hemiptera: Psyllidae) and *Bactericera cockerelli* (Hemiptera: Triozidae)

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**ABSTRACT.** *Carsonella ruddii* (Gamma Proteobacterium) is an obligate bacterial endosymbiont of psyllids that produces essential amino acids that are lacking in the insect's diet. Accurate estimations of *Carsonella* populations are important to studies of *Carsonella*-psyllid interactions and to developing ways to target *Carsonella* for control of psyllid pests including pear psylla, *Cacopsylla pyricola* (Forster) (Hemiptera: Psyllidae) and potato psyllid, *Bactericera cockerelli* (Sulc) (Hemiptera: Triozidae). We used two methods, namely fluorescence in situ hybridization and quantitative polymerase chain reaction (qPCR), to estimate relative abundance of *Carsonella* in bacteriocytes and whole bodies of psyllids, respectively. Using these two methods, we compared *Carsonella* populations between female and male insects. Estimations using fluorescence in situ hybridization indicated that *Carsonella* was more abundant in bacteriocytes of female *C. pyricola* than in those of males, but *Carsonella* abundance in bacteriocytes did not differ between sexes of *B. cockerelli*. Analyses by qPCR using whole-body specimens indicated *Carsonella* was more abundant in females than in males of both psyllids. Neither fluorescence in situ hybridization nor qPCR indicated that *Carsonella* populations differed in abundance among adults of different ages (0–3 wk after adult eclosion). Using fluorescence in situ hybridization, *Carsonella* was observed in ovarioles of newly emerged females and formed an aggregation in the posterior end of mature oocytes. Results of our study indicate that female psyllids harbor greater populations of *Carsonella* than do males and that sex should be controlled for in studies which require estimations of *Carsonella* populations.

**Key Words:** bacteriome, mycetome, mycetocyte, Psylloidea, jumping plant lice

Psyllids (Hemiptera: Psylloidea) are phloem-feeding insects comprising eight Hemipteran families (*Burckhardt and Ouvrard 2012*). Several psyllid species are major agricultural pests, including pear psylla, *Cacopsylla pyricola* (Forster) (Hemiptera: Psyllidae), and potato psyllid, *Bactericera cockerelli* (Sulc) (Hemiptera: Triozidae). *C. pyricola* is a pest of cultivated pear, *Pyrus communis* L. (Rosaceae) because it produces abundant honeydew which causes fruit russetting, provides medium for the growth of sooty mold and increases labor costs during harvest (*Westigard and Zwick 1972*). *B. cockerelli* is a pest of Solanaceous crops including potato and tomato and is the vector of "*Candidatus Liberibacter solanacearum,*" the pathogen associated with zebra chip disease of potato (*Munyanzeza 2012*). Both psyllids are primarily controlled using insecticides, but current research efforts seek to develop ecologically based strategies to manage these pests.

All psyllids (Hemiptera: Psylloidea) have an obligate relationship with the bacterial endosymbiont, *Carsonella ruddii* (Gamma Proteobacterium) (*Thao et al. 2000*). *Carsonella* is transovarially transmitted and is housed within specialized host cells called bacteriocytes which form aggregates called bacteriomes (*Chang and Musgrave 1969, Thao et al. 2000*). This endosymbiont produces essential amino acids that are lacking in the insect’s diet, and elimination of this endosymbiont using antibiotics leads to death of the insect (*Baumann 2005, Nakabachi et al. 2006*). Because *Carsonella* is vital to the survival of psyllids, novel control strategies for psyllid pests including *C. pyricola* and *B. cockerelli* could be developed by targeting this endosymbiont (*Rio et al. 2004, Douglas 2007, Crotti et al. 2012*).

Paramount to discovering ways to manipulate *Carsonella* is the ability to obtain accurate estimations of *Carsonella* populations. A recent study by *Cooper and Horton* (2014) indicated that bacteriomes of female psyllids were larger than those of males, suggesting that females may harbor more *Carsonella* or other bacteriome-associated endosymbionts than do males. If so, then insect sex represents an important source of biological variation that must be accounted for when estimating *Carsonella* populations in psyllids. The objectives of our study were to 1) develop methods using fluorescence in situ hybridization and quantitative polymerase chain reaction (qPCR) to estimate *Carsonella* populations in *C. pyricola* and *B. cockerelli*, 2) compare *Carsonella* populations between female and male *C. pyricola* of varying ages (fifth-instar nymph to 21-d-old adult), and 3) document in both psyllid species the presence of *Carsonella* in varying ages of oocytes.

**Materials and Methods**

*Insects.* *C. pyricola* adults (150–200) were collected from pear trees located at the USDA experimental farm near Moxee, WA. To facilitate oviposition, psyllids were confined to six potted pear trees using a mesh cage for 36 h at 25°C with a photoperiod of 16:8 (L:D) h. The development of the resulting eggs and nymphs was closely monitored. Twelve-fifth instars (6 per sex) were collected and frozen for qPCR analysis of *Carsonella*. Adults (n = 150) that had emerged within a 24-h period were transferred to new pear trees and used for experiments. Each week for 4 wk (week 0, week 1, week 2, and week 3), 10 adults (five per sex) were collected for fluorescence in situ hybridization and 6 adults (three per sex) were collected for qPCR analysis.

Newly molted *B. cockerelli* adults were obtained from a laboratory colony and were maintained on potato “Russet Burbank” for 5 d. The colony was...
originally established from psyllids of the western haplotype collected near Prosser, WA, in summer 2012. These assays were limited to 5-d-old adults to simplify the experimental design. Female *B. cockerelli* of this age contain mature oocytes. The 5-d-old adults were collected for fluorescence in situ hybridization (5 per sex) and qPCR (10 per sex). The experiment on *B. cockerelli* was conducted twice (repetition) with different cohorts of insects.

For both psyllids, adults collected for fluorescence in situ hybridization were immediately dissected and processed as described in “*Carsonella* Densities in Individual Bacteriocytes” subsection. Adults collected for qPCR were stored in −20°C until they were processed as described in the “*Carsonella* titers in whole insects” subsection.

**Carsonella Densities Within Individual Bacteriocytes.** Fluorescence in situ hybridization was performed using methods similar to those described by Cooper et al. (2014). Each psyllid was mounted ventral side facing up on a glass microscope slide using double-sided tape. A drop of phosphate-buffered saline was placed over the insect and held in place by cohesion. Using two number 5 forceps (D’Outils Dumont SA, Montignez Switzerland), bacteriomes and fat body were removed from each psyllid and were transferred to a positively charged microscope slide (Tissue Tack, Polysciences Inc., Warrington, PA). After the dissected tissues air dried, the slides were maintained for 3 min on a slide warmer set at 50°C to adhere the tissues to the slides. Samples were fixed in Carnoy’s solution for 1 h, briefly rinsed in 100% ethanol, and washed three times for 20 min in hybridization buffer consisting of 20 mM Tris–HCL (pH 8.0), 0.9 M NaCl, 0.01% sodium dodecyl sulfate, and 30% formamide. Samples were hybridized overnight with 250 nM specific probe labeled with Alexa Fluor 488 on the 5'-end and 30% formamide. Samples were washed three times for 20 min in hybridization buffer, followed by two washes for 20 min in hybridization buffer, and one 20-min wash in Tris-buffered saline.

*Carsonella* was observed at 400 x using a fluorescence microscope (Zeiss Axioskop 40 FL, Carl Zeiss USA, Thornwood, NY) with Zeiss filter-set 10 (excitation wavelength = 450–490 nm, beam splitter = 510 nm, and emission wavelength = 515–565 nm). Each sample was photographed using a DP25 camera mounted to the microscope and operated using the CellSens software (Olympus America Inc., Central Valley, PA). All photographs were captured with an ISO of 200 and an exposure time of 80 ms. The mean intensity of fluorescence was estimated from four to nine bacteriocytes from each insect using ImageJ software (http://imagej.nih.gov/ij, last accessed 2 March 2015) (Burgess et al. 2010, Schneider et al. 2012). Fluorescence intensity values associated with *Carsonella* densities within individual bacteriocytes were analyzed using the GLIMMIX procedure of SAS 9.3 (SAS Institute 2012; Cary, NC). For analysis of *Carsonella* densities in *C. pyricola*, sex, age, and the sex by age interaction were included as the fixed effects, and insect (sex age) was included as the random variable. Data were excluded for the following reasons: 1) not enough 72°C cycles for qPCR amplification; 2) less than 25% of 16S of *Escherichia coli* chemically competent cells were included in the qPCR reaction; 3) less than 20 ng of bacteria DNA for qPCR amplification. For each analysis, the log of the estimated number of copies of 16S of *Carsonella* was included as the dependent variable. Sex, age, and the sex by age interactions were included as the fixed effects in analysis of qPCR reactions. For each analysis, the log of the estimated number of copies of 16S of *Carsonella* was included as the dependent variable. Sex, age, and the sex by age interactions were included as the fixed effects in analysis of qPCR reactions.
Carsonella in C. pyricola. For analysis of Carsonella titers in B. cockerelli, sex was included as the fixed effect, and repetition was included as a random variable. For each analysis, data were examined for heterogeneity of variance and nonnormality of errors by inspecting residual and normal quantile-quantile plots, respectively. Based on these plots, data were modeled assuming a Gaussian distribution using the DIST=G option of the MODEL statement.

Carsonella in Developing Oocytes. We used fluorescence in situ hybridization to observe Carsonella in the oocytes of female C. pyricola and B. cockerelli. Five females of each psyllid species were dissected, and the ovaries and developing oocytes were transferred to a microscope slide. Because of difficulties washing probe from psyllid sected, and the ovaries and developing oocytes were transferred to a microscope slide. At least two females of each species were newly reproductive organs of mated females before transferring the tissues to microscope slides. At least two females of each species were newly emerged (un sclerotized) and unmated. Fluorescence in situ hybridization was performed as described in “Carsonella Densities in Individual Bacteriocytes” subsection. Each sample was photographed at 200 or 400× with an ISO of 200 and an exposure time between 80 and 100 ms.

Results

Carsonella Densities Within Individual Bacteriocytes. Fluorescence was not associated with fat body cells hybridized with the 16S probe designed to bind to Carsonella suggesting the probe did not bind to non-target DNA (data not shown because photographs taken with the standardized settings of an ISO of 200 and exposure time of 80 ms appeared black). In addition, fluorescence was not associated with fat body cells or bacteriocytes hybridized without the probe indicating a lack of interfering autofluorescence. However, the cytoplasm of bacteriocytes of both C. pyricola and B. cockerelli fluoresced bright-green indicating the presence of Carsonella (Fig. 1A inset).

In most cases, the bacteriocytes of female C. pyricola fluoresced visibly brighter than those of males suggesting a greater concentration of Carsonella (Fig. 1A inset). Analysis of the fluorescence-intensity values obtained from ImageJ confirmed these visual observations. Fluorescence intensity for bacteriocytes of females was significantly greater than that of males (Fig. 1A; \( F = 19.2; \text{df} = 1, 22; P < 0.001 \)). Fluorescence intensity did not change with insect age (\( F = 2.4; \text{df} = 3, 22; P = 0.095 \)), and the lack of a significant sex \times\ week interaction (\( F = 1.1; \text{df} = 3, 22; P = 0.391 \)) indicated that the differences between sexes in fluorescence intensity were consistent among the different insect ages (newly emerged adults, 1-wk-old adults, 2-wk-old adults, and 3-wk-old adults).

In contrast with our findings on C. pyricola, we did not observe visible differences in fluorescence of bacteriocytes of female and male B. cockerelli (Fig. 2A; \( F = 0.4; \text{df} = 1, 17; P = 0.549 \)).

Fig. 1. (A) Comparison of estimated intensity of fluorescence associated with Carsonella in bacteriocytes of female and male C. pyricola. Inset shows observed Carsonella (green fluorescence) in bacteriocytes of insects. (B) Comparison of the number of copies of 16S from Carsonella in whole bodies of female and male C. pyricola. Error bars represent standard errors.

Carsonella Titers in Whole Psyllids. Linear regression analysis revealed a strong relationship between DNA dilutions and \( C_7 \) values associated with the amplification of 16S of Carsonella of C. pyricola (\( R^2 = 0.998 \)) and B. cockerelli (\( R^2 = 0.993 \)). The estimated efficiency of qPCR amplification for 16S of Carsonella was 71% for C. pyricola and 83% for B. cockerelli. The coefficient of variation was 0.014 for both species, providing evidence that the qPCR assay was highly repeatable.

Analysis of the estimated number of 16S copies of Carsonella of C. pyricola indicated that Carsonella was more abundant in females than in males (Fig. 1B; \( F = 9.3; \text{df} = 1, 22; P = 0.006 \)). We did not observe a significant effect for age (\( F = 1.0; \text{df} = 4, 22; P = 0.421 \)) and the lack of a significant sex \times\ age interaction (\( F = 1.1; \text{df} = 4, 22; P = 0.400 \)) indicated that the observed effects of sex on Carsonella populations were consistent among the different insect ages (fifth instars, newly emerged adults, 1-wk-old adults, 2-wk-old adults, and 3-wk-old adults).

Analysis of Carsonella titers in 1-wk-old B. cockerelli also indicated that females harbored significantly more Carsonella than did males (Fig. 2B; \( F = 10.6; \text{df} = 1, 35.1; P = 0.003 \)).

Carsonella in Developing Oocytes. Carsonella was present in both previtellogenic and vitellogenic ovarioles of C. pyricola and B. cockerelli (Fig. 3), including ovarioles of newly emerged (un sclerotized) females (Fig. 3A). Carsonella was distributed throughout the ovarioles but formed a honeycomb pattern around previtellogenic oocytes and appeared more aggregated near the posterior end of the mature oocyte (Fig. 3B and C).

Discussion

We previously reported that the bacteriomes of female psyllids were larger than those of males (Cooper and Horton 2014). Although
inconclusive, staining of bacteriocytes with eosin/hematoxylin in that study suggested that bacteria densities were also higher in bacteriocytes of female psyllids. We developed two new methods to estimate and compare abundance of *Carsonella* in *C. pyricola* and *B. cockerelli* using fluorescence in situ hybridization and qPCR. Results of fluorescence in situ hybridization indicated that the bacteriocytes of female *C. pyricola* harbor greater densities of *Carsonella* than do those of males. Because female psyllids have larger bacteriomes that harbor more *Carsonella* than those of males, it is not surprising that *Carsonella* was also more abundant in whole bodies of female *C. pyricola*. We also compared the abundance of *Carsonella* between 5-d-old (reproductively mature) females and 5-d-old males of *B. cockerelli* using both techniques. We did not observe differences between *B. cockerelli* sexes in *Carsonella* densities within bacteriocytes, but we did observe significantly higher whole-body *Carsonella* titers in female *B. cockerelli* than in males using qPCR. Dossi et al. (2014) reported that compared with female *Diaphorina citri* (Hemiptera: Psyllidae), males have larger populations of endosymbionts including *Carsonella*. Therefore, our results indicating that females of *C. pyricola* and *B. cockerelli* harbor more *Carsonella* than do males may not be true for all psyllids.

The biological relevance of observed gender-specific variations in bacteriomes of psyllids (Cooper and Horton 2014) and the gender-specific variations in *Carsonella* populations is not known but may be associated with egg production. *Carsonella* produces essential amino acids that are lacking in the psyllid’s diet (Nakabachi et al. 2006), so larger populations of *Carsonella* may be necessary to satisfy the nutritional demands associated with metabolically expensive egg production. This explanation is consistent with previous reports that *Carsonella* proliferation corresponds with insect growth and ovarian development (Waku and Endo 1987, Dossi et al. 2014). Differences in *Carsonella* titers between female and male psyllids may also be due in part to the distribution of *Carsonella* outside the bacteriomes of females. *Carsonella* is vertically transmitted (Thao et al. 2001), and *Carsonella* appeared to be abundant in both previtellogenic and vitellogenic ovarioles of females using fluorescence in situ hybridization. Our observation of *Carsonella* in previtellogenic ovarioles of newly emerged females suggests that undeveloped ovarioles already harbor *Carsonella* prior to adult eclosion, which has been observed in the whitefly, *Aleuorchiton aceris* Modeer (Hemiptera: Aleyrodiniae) (Szklarzewicz and Moskal 2001).

Our observations of *Carsonella* in psyllid ovarioles revealed structural patterns that changed as ovarioles matured. *Carsonella* aggregations formed a honeycomb pattern in previtellogenic ovarioles of both psyllids species, but the cause of the pattern is unknown. Previous researchers showed that endosymbionts of psyllids colonize the hemocoel surrounding the ovarioles, the space between the follicular epithelium, and follicular cells, and form aggregations (peripheral maculae) near the posterior end of mature oocytes (Chang and Musgrave 1969, Waku and Endo 1987). Our observations using fluorescence in situ hybridization showed that *Carsonella* occurred throughout or surrounding follicular cells and developing oocytes consistent with the previous reports. Our observations also confirm the presence of peripheral maculae as previously documented using electron micrographs, but the role of these bacterial aggregations are not known (Chang and Musgrave 1969, Waku and Endo 1987).

Long-term goals of our research include exploring interactions among bacterial endosymbionts, psyllids, and their host plants. These
long-term research goals require control of biological variation that may impede accurate measurements of *Carsonella* abundance. Previous studies that assessed *Carsonella* populations among psyllids did not control for insect sex and age (Nachappa et al. 2011, Alvarado et al. 2012, Arp et al. 2014). The combined results of our previously published study (Cooper and Horton 2014) and our current study clearly indicate that insect sex represents an important source of variability that must be considered in studies which rely on estimates of *Carsonella* populations. In addition, this report describes two new methods to estimate *Carsonella* abundance in psyllids by measuring *Carsonella* densities in individual bacteriocytes (fluorescence in situ hybridization) or *Caronella* titers in whole insects (qPCR). Although fluorescence in situ hybridization has been used previously to observe unculturable bacterial endosymbionts (Fukatsu and Nikoh 1998, Cooper et al. 2014), our study is the first to use relative intensity of fluorescence to estimate relative abundance of endosymbionts in insects.

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