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ORIGINAL ARTICLE

Ecological factors influencing the beneficial endosymbionts of the hemlock woolly adelgid (Hemiptera: Adelgidae)

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Abstract Bacterial endosymbionts of sap-sucking insects provide their host with a number of beneficial qualities, including the supply of nutrition, defense against parasitoids, and protection from heat stress. Damage to these bacterial associates can therefore have a negative impact on the fitness of their insect host. We evaluated observational and experimental factors regarding the nonnative hemlock woolly adelgid (*Adelges tsugae* Annand) (Hemiptera: Adelgidae) to help understand the roles of its three recently identified symbionts, including under heat stress conditions. The prevalence of *A. tsugae*'s facultative symbiont (*Serratia symbiotica*) was examined at different spatial scales to determine how variable infection rates are for this symbiont. There was no significant difference found in infection rates between adelgids on a tree, within a plot, or within a state. However, significantly more adelgids in Georgia (95%) had *S. symbiotica* compared to those in New York (68%). Microsatellite genotyping of the adelgids found that this difference was most likely not the result of a second introduction of *A. tsugae* into eastern North America. Comparison of *S. symbiotica* proportions between first and fourth instars showed that symbiont absence did not affect the ability of *A. tsugae* to survive aestivation. Evaluations of symbiont densities within each adelgid found that when *S. symbiotica* was absent, the density of obligate symbionts was significantly higher. Exposure to heat stress (32.5 °C) was not consistently correlated with changes in symbiont densities over a 4-d period. Overall, we have shown that symbiont prevalence and densities vary within the broad population of *A. tsugae* in eastern North America, with potentially significant effects upon the ecology of this important pest.

Key words *Adelges tsugae*; *Annandia adelgestsuga*; bacterial endosymbionts; *Serratia symbiotica*; *Pseudomonas adelgestsugas*

Introduction

Symbiosis, the association between different species living together, is a common occurrence between insects and intracellular bacteria. Many of these bacterial symbionts are maternally transmitted and play diverse beneficial roles that increase the fitness of their insect hosts (Douglas, 1998; Moran *et al.*, 2008; Burke *et al.*, 2010a; Oliver *et al.*, 2010). Obligate (primary) symbionts are those that are essential for the host and usually serve a nutritional role, producing nutrients that are limited in the diet of the insect host (Baumann, 2005; Moran, 2006; Hosokawa *et al.*, 2010; Hansen & Moran, 2014). Conversely, facultative (secondary) symbionts have variable presence in hosts and are considered nonessential, but

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can play ecologically important roles including defense against parasitoids and heat stress (Baumann, 2005; Oliver *et al.*, 2010).

Due to their nutrient-poor diet, sap-sucking insects in the suborder Sternorrhyncha, particularly aphids, have historically been a focus of research on insect bacterial endosymbionts. Besides expanding our knowledge about symbiotic relationships and host-symbiont biology, research regarding bacterial endosymbionts may spur development of novel pest management options, such as targeting the symbiont as a means of reducing host fitness. One of the most ecologically damaging sap-sucking insects in North America is the nonnative hemlock woolly adelgid, *Adelges tsugae* Annand. Native to Japan, *A. tsugae* was accidentally introduced into the eastern United States prior to the 1950s and is currently causing widespread mortality of eastern and Carolina hemlocks (*Tsuga canadensis* (L.) Carrière and *T. caroliniana* Engelmann respectively) (Orwig & Foster, 1998; Havill *et al.*, 2016). The loss of eastern hemlock is expected to negatively affect a number of ecosystem variables, including carbon uptake, vegetative biodiversity, soil characteristics, and stand water yields (Cobb *et al.*, 2006; Eschtruth *et al.*, 2006; Daley *et al.*, 2007; Albani *et al.*, 2010; Lustenhouwer *et al.*, 2012). In North America, due to the absence of reproduction on its primary host (*Picea torano* [Siebold ex. K. Koch] Koehne), *A. tsugae* reproduces parthenogenetically. As a result, all adelgids in eastern North America are clones of the original introduced individuals (McClure & Cheah, 1999; Havill & Foottit, 2007; Havill *et al.*, 2016).

Although bacterial endosymbionts were briefly discussed as potential targets for *A. tsugae* biological control (Shields & Hirth, 2005), there has been little to no investigation into this possibility. Instead, control efforts have focused on the release of predatory insects and the use of insecticides (Webb *et al.*, 2003; Cowles *et al.*, 2006; Havill *et al.*, 2014). One factor that has been shown to have a negative effect on *A. tsugae* populations is temperature. In the northern end of the distribution, cold threshold temperatures are expected to keep adelgid populations from continued range expansion (Parker *et al.*, 1998, 1999). In addition, supraoptimal summer temperatures at the southernmost edge of eastern hemlock's range in Georgia were correlated with increased summer mortality of *A. tsugae* (Mech *et al.*, 2017). During the summer months, *A. tsugae*'s generation of sistens individuals undergoes aestivation (summer diapause) which they enter as first instars shortly after permanently settling at a needle node (McClure *et al.*, 2003). One of the hypothesized reasons for the observed mortality below a critical thermal limit is that bacterial endosymbionts may be sensitive to heat stress, thereby influencing *A. tsugae* fitness (Mech *et al.*, 2017).

Some of the most foundational work on the roles and evolution of bacterial symbionts of insects has been completed using pea aphids (*Acyrthosiphon pisum* Harris) (Hemiptera: Aphididae) and their bacterial symbionts as a model system. '*Candidatus* Buchnera aphidicola' (hereafter *Buchnera*), the obligate nutritional symbiont of aphids, produces amino acids from nutrients present in the aphids' nitrogen-poor phloem sap diet (Douglas, 1998; Sandstrom & Moran, 1999; Shigenobu *et al.*, 2000). Like *A. tsugae*, pea aphids are sensitive to heat, which is in part influenced by the heat sensitivity of the aphids' *Buchnera* symbiont. The symbiotic lifestyle and association of *Buchnera* with aphids for more than 160 million years has resulted in bacterial genome degradation and the loss of many genes, including those involved in responding to thermal stress (Wilcox *et al.*, 2003; Dunbar *et al.*, 2007; Burke *et al.*, 2010b). A short burst of heat (heat shock) can decrease *Buchnera* densities by >90%, with drastic effects upon the fitness of the aphids themselves (Dunbar *et al.*, 2007; Burke *et al.*, 2010a). The detrimental effects of heat stress upon bacterial symbionts are also known to have negative impacts upon host fitness in other insect species (Dunbar *et al.*, 2007; Kikuchi *et al.*, 2016; Moran, 2016).

Pea aphids can also possess facultative symbionts (e.g., "*Ca.* Serratia symbiotica" or "*Ca.* Hamiltonella defensa," hereafter *S. symbiotica* or *H. defensa*) that help to maintain aphid fitness during heat stress to varying degrees (Chen *et al.*, 2000; Montllor *et al.*, 2002; Russell & Moran, 2006; Harmon *et al.*, 2009; Martinez-Diaz *et al.*, 2016). While the molecular mechanisms imparting protection are unknown, the presence of facultative symbionts like *S. symbiotica* may protect the obligate symbiont *Buchnera*. This may be achieved via lysis of the facultative symbiont (resulting in a decrease in density) and the release of protective metabolites (Burke *et al.*, 2010a).

To date, the diversity and roles of microbial symbionts in adelgids is mostly unknown. A recently published survey of symbionts present in *A. tsugae* revealed that there are two symbionts present in all *A. tsugae* populations, named "*Ca.* Annandia adelgestsuga" and "*Ca.* Pseudomonas adelgestsugas" (hereafter *Annandia* and *Pseudomonas*; von Dohlen *et al.*, 2013). One additional symbiont type, "*Ca.* Serratia symbiotica" (hereafter *S. symbiotica*) was identified in *A. tsugae* populations in eastern North America and in the source lineage of adelgids found on southern Japanese hemlock trees (*T. sieboldii* Carrière).

The roles these symbionts play in *A. tsugae* biology is currently unknown. However, we can form predictions

based upon the known functions of aphid *Buchnera*, a relative of *Annandia*, and pea aphid *S. symbiotica*, which is very closely related to adelgid *S. symbiotica* (von Dohlen *et al.*, 2013). While the patterns of symbiont presence in *A. tsugae* populations are suggestive of obligate nutritional roles for *Annandia* and *Pseudomonas*, and a facultative role for *S. symbiotica*, no survey has yet been performed to assess variation in symbiont presence and prevalence in individual adelgids. We performed sampling at the level of trees, sites, or latitudinal extremes of the clonal host, and experiments to test whether *Annandia*, *S. symbiotica*, and *Pseudomonas* are present in all *A. tsugae* individuals and whether they are sensitive to heat stress. Furthermore, we evaluated how the absence of *S. symbiotica* may affect *Annandia* and *Pseudomonas* as well as the adelgid host.

Materials and methods

Sampling design

Aestivating first instar *A. tsugae* were collected in September 2015 from three sites in the Chattahoochee National Forest, Georgia (GA), which varied in elevation—Soque River (457 msl; 34.71590°N, 83.57632°W), Dicks Creek (523 msl; 34.68093°N, 83.93885°W), and Coopers Creek (904 msl; 34.73868°N, 83.99465°W). Samples from New York (NY) were collected from Glenwood Creek (182 msl; 42.49424°N, 75.542838°W). At each site, four eastern hemlock trees were randomly selected and four branches per tree were clipped for the symbiont presence–absence analysis. Six *A. tsugae* were removed from each branch within 24 h of the collection and frozen (−20 °C) until DNA extraction could occur. Collections were repeated in February 2016 at the NY site ($n = 16$ branches; 96 *A. tsugae*) to collect fourth instar individuals and evaluate whether the absence of the facultative symbiont influenced *A. tsugae*'s ability to come out of aestivation.

An additional hemlock tree was randomly selected in September 2015 at both Soque River (GA) and Glenwood creek (NY), and nine branches per tree were collected for the heat stress experiment. Each of the nine branches per geographic location (state) were randomly designated both a treatment (heat or control) and duration (0, 24, 48, 72, or 96 h), and placed in wrapped wet foam to prevent desiccation. The four heat-treatment branches were placed in a growth chamber held at 32.5 °C with 60%–62% relative humidity while the five control branches per geographic location were left at room temperature (~22.5 °C; 60%–65% humidity). Immediately following each time point, six *A. tsugae* per branch deemed to still be alive (based on convexity of body) were removed from their stylet insertion site and stored at −20 °C.

Detection of bacterial presence or absence using symbiont-specific PCR

DNA was extracted from individual aestivating nymphs by crushing them in 3 μL of 20 mg/mL proteinase K, adding 50 μL of 10% w/v Chelex (100 sodium form; 50–100 mesh dry) (Sigma-Aldrich, St. Louis, MO, USA), mixing, and then incubating for 1 h at 36 °C followed by 8 min at 96 °C. The solution was then centrifuged for 5 s to settle insect debris and Chelex beads, and 48–50 μL of the supernatant was transferred to a fresh vial for storage (−20 °C). Polymerase chain reaction (PCR) was used for amplification of *Pseudomonas*, *S. symbiotica*, and *Annandia* 16S small-subunit ribosomal DNA (16S rDNA) genes. We designed primers to amplify 16S rDNA using sequences previously published in von Dohlen *et al.* (2013). These sequences were aligned with MAFFT version 7 and examined manually for regions of divergence that would be appropriate for the design of symbiont-specific primers. To design primers for the amplification of the HWA elongation factor 1α gene (EF1-α), the orthologous sequence from the *A. pisum* genome (Acyr_2.0, LOC100574365) was used to identify EF1-α in the *A. tsugae* transcriptome dataset available in GenBank (accession number GBJX01005462.1). EF1-α primers were designed with Primer3 v0.4.0. We used these primers (Table 1) to amplify an approximately 200 bp product for each symbiont and *A. tsugae* EF1-α. The 10 μL reactions contained 4.3 μL of distilled water, 1 μL 10 × buffer, 1 μL 10 mmol/L dNTPs, 0.8 μL of both 2.5 μmol/L forward and reverse primers, 0.08 μL *Taq* polymerase (5PRIME), and 2 μL of *A. tsugae* DNA. PCR was performed with initial denaturation of 95 °C for 2 min, 35 cycles at 95 °C for 20 s, 55 °C for 20 s, and 65 °C for 45 s, followed by final extension at 65 °C for 7 min on a ProFlex instrument (Applied Biosystems, Grand Island, NY, USA). PCR products were visualized using gel electrophoresis with all negative results being repeated to avoid false negatives.

PCR-based quantification of bacterial density

Quantitative PCR products for each symbiont and *A. tsugae* EF1-α were cloned using the StrataClone PCR cloning kit (Agilent, Santa Clara, CA, USA), and plasmids were isolated with the GeneJET plasmid miniprep kit (Thermo Fisher Scientific, Waltham, MA, USA). Each plasmid insert was sequenced to confirm the correct

Table 1 Primers used for *Adelges tsugae* bacterial symbiont infection rates and densities.

Specificity	Primer	Sequence (5′–3′)	Amplicon size (bp)
Pseudomonas adelgestsugas	GSAf II	AAGCAACGCGAAGAACCTTA	187
	GANA 1118r†	GAGTGCCCACCATGATGT	
Serratia symbiotica	GSBf II	GGTAATGTGTTAATAAGACATTGC	214
	GSBr III	CCTCTACAAGACTCTAGCTTGCC	
Annandia adelgestsuga	GamC 476f†	ATTTTGTTATCAGACGTTAGCTAT	202
	GSCr III	CTCCCTCTACGAAACTCTAGTCTATT	
Adelges tsugae EF1-α	AtEF1F	AAATACGCGTGGGTATTGGA	202
	AtEF1R	TCACCAGTACCAGCAGCAAC	

†von Dohlen *et al.* (2013).

amplicon was cloned. A standard curve was made using diluted plasmids (10^7 down to 10^2 copies in 10 fold increments) as a template for qPCR with putatively symbiont-specific primers on a Rotor-Gene Q machine (Qiagen, Germantown, MD, USA). Quantitative PCR (qPCR) was performed using the Rotor-Gene SYBR Green PCR Kit with 1 μmol/L primers and 1 μL of undiluted DNA per 10 μL reaction. After 5 min of denaturation at 95 °C, a two-step amplification cycle with 95 °C for 5 s denaturation and 60 °C for 20 s of annealing and extension was used for 45 cycles. Each sample was replicated 4 times. An absolute standard curve and the melting temperature of each amplicon were calculated using the Rotor-Gene Q software. To test whether primers were specific for each symbiont type, we examined melt curves for a representative *A. tsugae* sample (possessing all three symbiont types) to ensure they matched melt curves obtained from amplicons cloned into sequence-verified plasmids. qPCR was used to determine the number of copies of each symbiont in *A. tsugae* following each time treatment (0, 24, 48, 72, or 96 h). For each sample and primer set, melt curve analysis was performed to ensure that amplification of the correct product occurred. The relative density of each symbiont in *A. tsugae* was calculated as a ratio to the number of host EF1-α amplicons.

Genotype analysis of HWA

Adelges tsugae DNA samples were genotyped with 14 microsatellite loci using the methods described in Abdoullaye *et al.* (2009). PCR products were analyzed on an ABI 3730 sequencer (Life Technologies, Carlsbad, CA, USA) at the DNA Analysis Facility on Science Hill at Yale University with a Rox 500 internal size standard (Applied Biosystems, Foster City, CA, USA). Alleles were scored using the microsatellite plugin in the software Geneious v. 8.1.6 (Kearse *et al.*, 2012).

Statistical analyses

All analyses were conducted using R statistical software (R Core Team, 2015). Data were first evaluated to determine if the assumptions for analyses were met. Comparisons of *S. symbiotica* infection rates (average proportion of adelgids with *S. symbiotica* present) between trees within a site, between sites within a state, and between states representing the latitudinal extremes within the range (NY and GA) were conducted through nonparametric Kruskal–Wallis tests. *S. symbiotica* infection rates between adelgids that were aestivating versus those that had come out of aestivation were analyzed through a nonparametric Wilcoxon rank-sum test.

The effect of heat was assessed through linear models (R Core Team, 2015) where the relative density over time for each symbiont was tested to determine if symbiont levels varied as a function of the treatment used. Differences between *A. tsugae* exposed to heat and those left at room temperature were compared by evaluating the treatment–time interaction term. To ensure homoscedasticity and normality of residuals, *Annandia* densities were square root transformed and *S. symbiotica* densities were inverse transformed. In addition, trends over time were compared between the latitudinal extremes (NY vs. GA) by evaluating the time–location interaction term for each symbiont and both temperature treatments.

Symbiont densities from the NY adelgids were also used to evaluate how the lack of *S. symbiotica* may affect densities of the other symbionts in the host. The relative densities of both *Annandia* and *Pseudomonas* were compared between adelgids with and without *S. symbiotica* present through a mixed effects model where time nested within treatment (heat or control) was included as a random effect (lme4 package; Bates *et al.*, 2015). *Annandia* and *Pseudomonas* densities were also evaluated to determine if *S. symbiotica* presence influences the effect of

heat upon densities of the other symbionts. Mixed effects models (lme4 package; Bates *et al.*, 2015) were run, with exposure time included as a random effect, to compare obligate symbiont densities between treatments (heat or control) for each *S. symbiotica* level (present or absent) ($n = 4$ models).

Results

Symbiont infection rates

Overall, 378 aestivating first instar *A. tsugae* ($n = 2$ geographic locations, 4 sites, 16 trees, 64 branches) were examined for the presence of *Pseudomonas*, *S. symbiotica*, and *Annandia*. Two of the symbionts, *Pseudomonas* and *Annandia*, were found in almost all of the samples (100% and 99.7%, respectively) supporting their obligate role in *A. tsugae*. *Serratia symbiotica*, on the other hand, was not as consistent (88.3%) indicating that its role is facultative in the adelgid host. When the average proportion of *A. tsugae* with *S. symbiotica* present was calculated for the four collected branches per tree and compared between the four sampled trees within each site, no differences were observed at any of the four site locations: Soque River ($\chi^2 = 2.22$, df = 3, $P = 0.53$), Dicks Creek ($\chi^2 = 3.39$, df = 3, $P = 0.35$), Coopers Creek ($\chi^2 = 2.15$, df = 3, $P = 0.54$), and Glenwood Creek ($\chi^2 = 4.66$, df = 3, $P = 0.20$). There was also no significant difference in the average infection rate of *S. symbiotica* when comparing the three sites within the state of Georgia ($\chi^2 = 1.27$, df = 2, $P = 0.53$). However, the proportion of *A. tsugae* with *S. symbiotica* present per tree was significantly different when comparing populations at the latitudinal extremes of the host range ($\chi^2 = 23.03$, df = 1, $P < 0.001$). NY adelgid samples had a significantly lower proportion with the symbiont ($\bar{x} = 0.68 \pm 0.05$ SEM) versus GA ($\bar{x} = 0.95 \pm 0.02$ SEM) (Fig. 1). Because the NY *A. tsugae* population had lower *S. symbiotica* prevalence, we compared the average proportion of *S. symbiotica*-infected first instars per tree ($n = 93$ *A. tsugae*) with the average proportion of infected fourth instars per tree ($n = 95$ *A. tsugae*) to determine if the lack of *S. symbiotica* was correlated with aestivation survival in NY. The proportion of *A. tsugae* with *S. symbiotica* was not significantly different between the two life stages (Wilcoxon test statistic = 7, $P = 0.88$), indicating that the lack of *S. symbiotica* in *A. tsugae* may not influence their ability to survive aestivation (Fig. 2).

In addition, *A. tsugae* from NY were evaluated to determine whether: (1) *S. symbiotica* is absent in some individuals due to low fidelity of maternal transmission or a rare

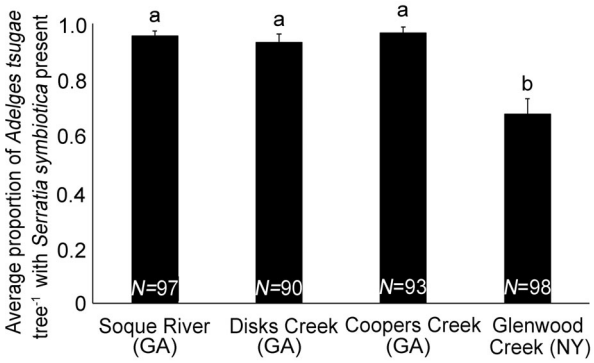

Fig. 1 Average infection rates of *Adelges tsugae* with *Serratia symbiotica* from sites in Georgia (GA) and New York (NY). Numbers within columns represent the number of *A. tsugae* sampled per location. Different letters indicate significant differences ($P < 0.05$) between the sites/states.

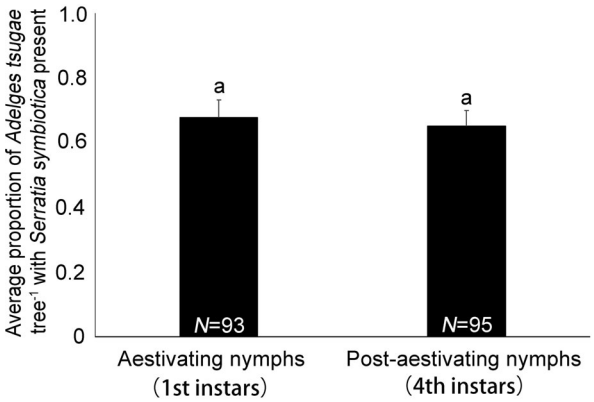

Fig. 2 Average infection rates of *Adelges tsugae* with *Serratia symbiotica* from individuals in aestivation phase and those that came out of aestivation phase. Numbers within columns represent the number of *A. tsugae* sampled. Same letters indicate lack of significant differences ($P = 0.88$) between the life stages.

symbiont loss event followed by spread of that lineage, or (2) differences in *S. symbiotica* infection is evidence for two different introductions of *A. tsugae* into the eastern United States. A random subset of DNA samples was analyzed; 12 first instar and 12 fourth instar for a total of 24 *A. tsugae* samples. Within life stages, samples were chosen so that half were infected with *S. symbiotica* and the remainder were not. All NY samples analyzed had identical microsatellite genotypes, which were also the same as the clonal genotype found throughout eastern United States (Havill *et al.*, 2016). This indicated that the absence of *S. symbiotica* in some *A. tsugae* individuals is likely not the result of two separate introductions of *A. tsugae*.

Effect of heat stress

In total, the symbiont densities from 95 adelgids (47 from GA and 48 from NY) were determined for the evaluation of potential heat-stress effects. For each time interval, the DNA from five or six *A. tsugae* ($\bar{x} = 5.22 \pm 0.10$ SEM) was extracted and the average density of the three symbionts was estimated for each time point. The number of adelgids varied due to the accidental loss of individuals that occurred when opening PCR tube strips used to store frozen adelgids prior to DNA extraction. The number of replicates per time interval was further reduced, to as low as three, for *S. symbiotica* density evaluations based on the discovery that a portion of the population, particularly those in NY, naturally lacked *S. symbiotica*. *Post hoc* evaluations found that 35 of the 48 heat-stress-tested adelgids from NY had *S. symbiotica* present (73%), which fell within the percentage range calculated from the field population ($\bar{x} = 0.68 \pm 0.05$ SEM). Likewise, only one GA adelgid from the heat-stress experiment lacked *S. symbiotica* indicating that the infection rate (98%) also fell within the percentage range calculated from the Soque River, GA population ($\bar{x} = 0.96 \pm 0.02$ SEM). Any density level of zero for this symbiont was therefore removed from analyses since we were unable to ascertain whether the zero value was the result of the experimental treatment or that the adelgid did not have the symbiont present when the experiment began. However, zero density levels were retained for the obligate symbionts *Annandia* and *Pseudomonas*. All *A. tsugae* were positive for EF1-α, demonstrating that DNA extractions were successful and ensuring that any zero symbiont density was not the result of host mortality.

The results of the heat stress experiment were variable. For the GA *A. tsugae*, there were no significant differences between symbiont density trends over time when compared between the heat and control treatments for any of the three symbionts (Figs. 3A, C, E). However, for NY

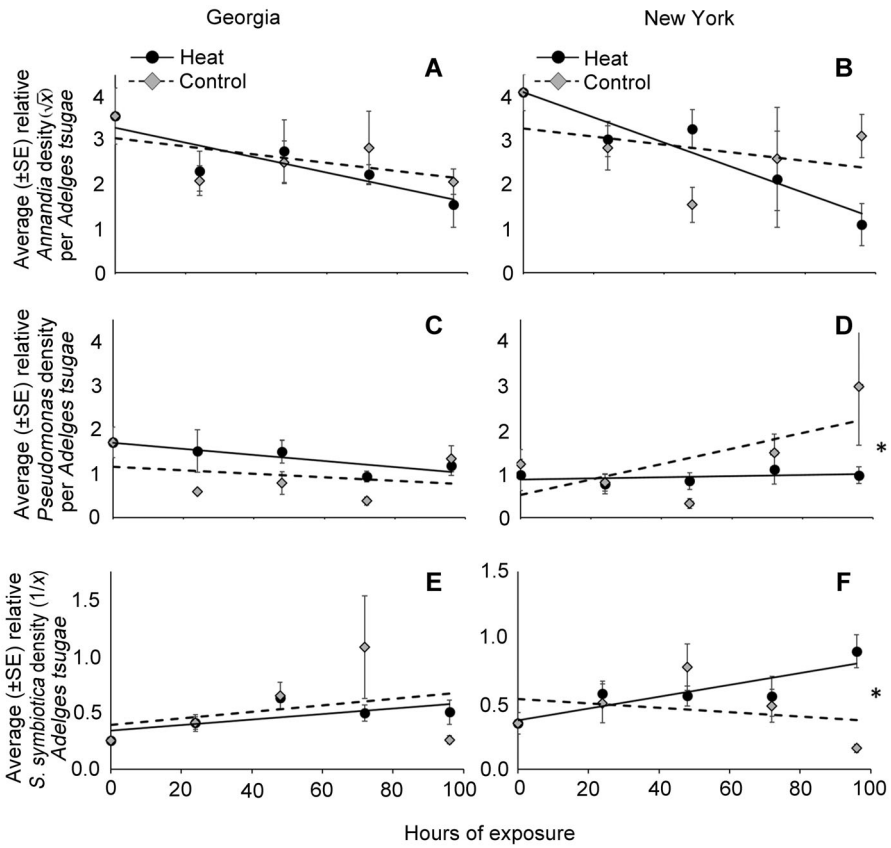

Fig. 3 Average relative densities of bacterial endosymbionts in *A. tsugae* from New York and Georgia over time compared between adelgids exposed to a heat treatment (Heat; 32.5 °C) and those that were maintained at room temperature (Control; 22.5 °C) ($n = 3$–6 *A. tsugae* per treatment-time interval); *Annandia* (square root transformed; A–B), *Pseudomonas* (C–D), and *Serratia symbiotica* (inverse transformed; E–F). Note that inverse transformation makes *S. symbiotica* negative trends appear positive in the figure. Lines represent treatment trends over time (*represents a significant difference in trends between treatments; $P < 0.05$).

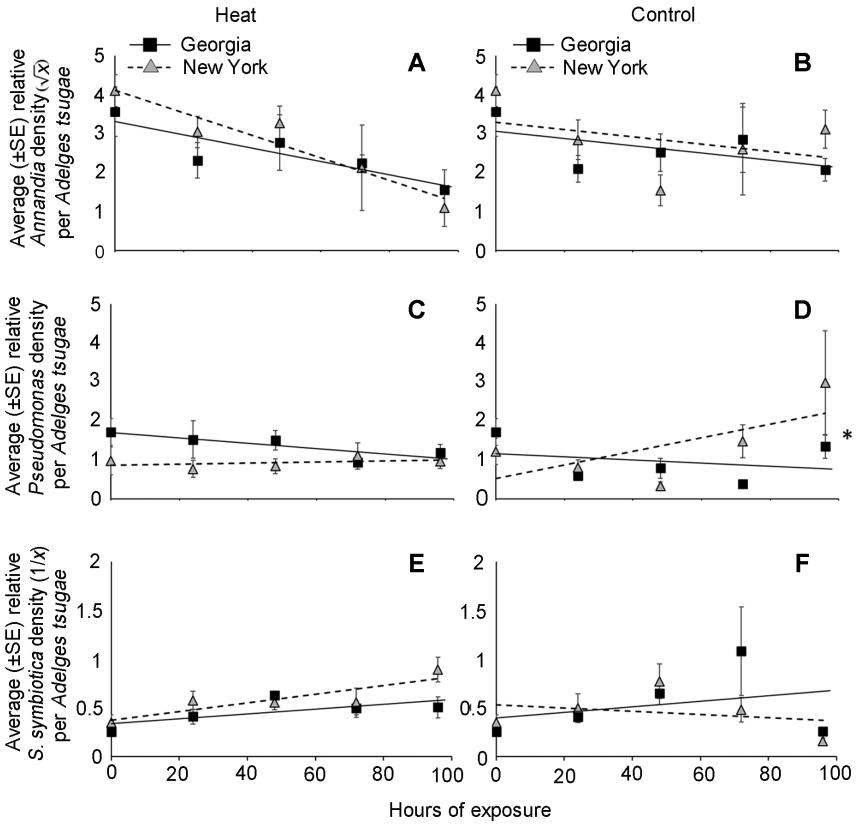

Fig. 4 Average relative density of bacterial endosymbionts in *A. tsugae* over time compared between adelgids from Georgia (squares, solid line) and New York (triangles, dashed line) for both the heat (32.5 °C) and control (22.5 °C) temperature treatments; *Annandia adelgestsuga* (square root transformed; A–B), *Pseudomonas adelgestsugas* (C–D), and *Serratia symbiotica* (inverse transformed; E–F). Note that inverse transformation makes *S. symbiotica* negative trends appear positive in the figure. Lines represent location trends over time (*represents a significant difference in trends between states; $P < 0.05$).

A. tsugae, significantly different trends were found between the heat and control treatments for *Pseudomonas* ($t = -2.64$, $P = 0.01$) and *S. symbiotica* ($t = 2.26$, $P = 0.03$). The NY *Pseudomonas* difference does not appear to be due to an effect of heat, but rather to an unexpected positive trend over time, as indicated by the control adelgids (Fig. 3D). However, there was no significant trend over time for NY *Pseudomonas* densities when evaluated individually for the heat ($t = 1.21$, $P = 0.22$) or control ($t = 1.76$, $P = 0.08$) treatment (i.e. slopes were not significantly different from zero). This indicates that the significantly different treatment slopes for NY *Pseudomonas* are most likely due to natural biological variation rather than a treatment effect. Conversely, NY *S. symbiotica* densities significantly decreased over time when exposed to the heat ($t = 2.32$, $P = 0.02$), but not control ($t = 1.28$, $P = 0.20$), treatment (Fig. 3F). Although this indicates that *S. symbiotica* may be sensitive to heat stress, the sample size was low for NY samples and the same pattern was not observed in GA adelgids (Fig. 3E). Comparisons between NY and GA densities over time found that most of the trends, regardless of treatment, were not significantly different (Fig. 4A–F) except for control *Pseudomonas* levels; the increase in NY control *Pseudomonas* densities over time was different from the decreasing GA control *Pseudomonas* levels ($t = 3.05$, $P = 0.004$) (Fig. 4D).

Analyses found that *A. tsugae* lacking *S. symbiotica* had significantly higher densities of both *Annandia* ($t = -2.14$, $P = 0.03$) and *Pseudomonas* ($t = -3.22$, $P = 0.001$) compared to *A. tsugae* with *S. symbiotica* present (Fig. 5). On average, *A. tsugae* lacking *S. symbiotica* had 66.5% higher *Annandia* densities, and 85.9% higher *Pseudomonas* densities. Results also found that, although *Pseudomonas* levels in adelgids lacking *S. symbiotica* were reduced by ~50% when exposed to heat as compared to those in the control treatment, neither of the obligate symbionts was significantly affected by the

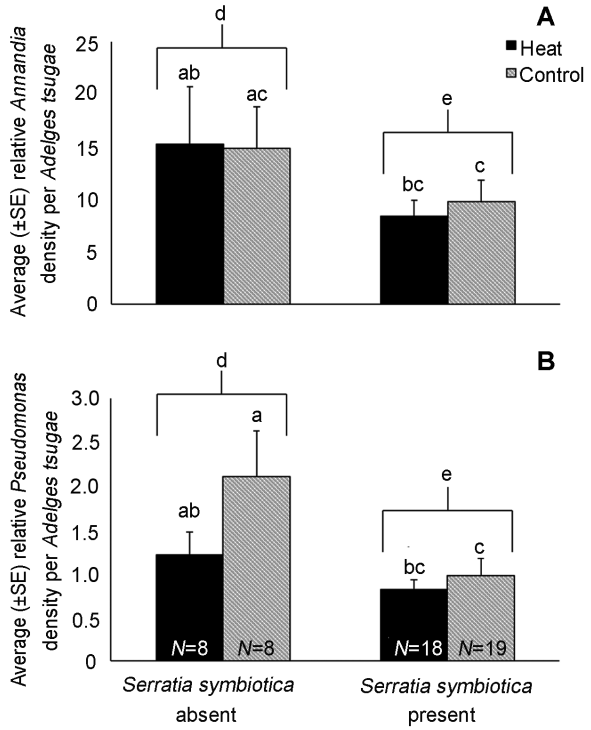

Fig. 5 Average relative density of *Annandia* (A) and *Pseudomonas* (B) in *Adelges tsugae* collected from New York that do and do not have the facultative symbiont, *Serratia symbiotica*, present. Density comparisons were made between adelgids that possessed or lacked *S. symbiotica* (regardless of temperature treatment), between temperature treatments for each *S. symbiotica* level, and between *S. symbiotica* levels for each temperature treatment. Numbers within columns represent the number of *A. tsugae* sampled per category. Different letters indicate significant differences ($P < 0.05$) in symbiont densities.

temperature treatment based on the presence or absence of *S. symbiotica* (all $P > 0.10$; Fig. 5).

Discussion

This study has focused upon the role of bacterial symbionts, particularly *S. symbiotica*, found in the nonnative, invasive *A. tsugae* in the eastern United States. One of the principal findings of this research is that *S. symbiotica* infection rates vary based on location within eastern North America (Fig. 1). Accidental introductions of insect species into new environments often occurs with a very small number of individuals. This founder effect is expected to result in the colonization of a genetically homogenous population, particularly for asexually reproducing species such as *A. tsugae.* Given that *A. tsugae* is invasive in eastern North America, and the presence of *S. symbiotica* was previously reported in this region (von Dohlen *et al.*, 2013), we expected that all adelgids would possess this symbiont. The variability of *S. symbiotica* presence within the invaded range could have originated via one of two likely scenarios. First, the pool of *A. tsugae* individuals introduced into eastern North America could have been relatively large, and varying in *S. symbiotica* infection status. This could have occurred via introduction of *A. tsugae* differing in *S. symbiotica* at a single point in time, or by multiple introductions of *A. tsugae* lineages, some infected with *S. symbiotica* and some uninfected. In this case, we would most likely expect to detect multiple *A. tsugae* genotypes present in eastern North America. In the second scenario, all introduced *A. tsugae* individuals would have contained *S. symbiotica*, but over time, some lineages lost *S. symbiotica* (perhaps due to imperfect fidelity of vertical transmission; Russell & Moran, 2005) and could have become abundant in the *A. tsugae* population at large. Under this scenario, we would expect all *A. tsugae* in eastern North America to have the same genotype regardless of *S. symbiotica* infection status. Because our microsatellite data showed no differences in genotype among adelgid individuals, consistent with a population genetic study across the range of *A. tsugae* (Havill *et al.*, 2016), we suggest that the second scenario is more likely.

Facultative symbionts, although typically not required for host reproduction or development, can be beneficial for the insect by improving fitness and/or protection (Oliver *et al.*, 2010). In aphids, *S. symbiotica* has been found to increase resistance to parasitism (Oliver *et al.*, 2003), approach co-obligate status by sharing in the biosynthesis of amino acids (Lamelas *et al.*, 2011), and protect their hosts against heat stress (Chen *et al.*, 2000; Montllor *et al.*, 2002; Russell & Moran, 2006). In pea aphids, *S. symbiotica* protects obligate symbionts from the effects of heat stress by preventing declines in *Buchnera* density (Burke *et al.*, 2010a), while in other aphids, it may not (Martinez-Diaz *et al.*, 2016). Because there would be no need for facultative parasitoid resistance in *A. tsugae* (there are no known parasitoids for *A. tsugae* or any other adelgid species; Havill & Foottit, 2007), we hypothesized that *S. symbiotica* plays a role in heat tolerance. This might also explain why *A. tsugae* from the hotter regions in Georgia would have higher infection rates of *S. symbiotica* compared to adelgids from the northern, cooler regions in New York (Fig. 1); this trend has also been observed for *S. symbiotica* in aphids (Montllor *et al.*, 2002). Further sampling of *A. tsugae* infection rates from locations along a latitudinal transect could reveal whether this association with temperature is a generalizable pattern as well as determine how variable *S. symbiotica* infection rates are in eastern North America.

If *S. symbiotica* were able to protect the obligate symbionts *Annandia* or *Pseudomonas* during heat stress, we would have expected to see a dramatic shift in the densities of the latter symbionts, such as those observed in aphids (Burke *et al.*, 2010a). However, we did not detect a conclusive effect of heat on any of the three symbionts (Figs. 3, 4). It is possible that *S. symbiotica* alters *A. tsugae* heat tolerance via a mechanism unrelated to the protection of obligate symbionts (Burke *et al.*, 2010a; Martinez-Diaz *et al.*, 2016). Nonetheless, we recognize potential issues with the experiment and emphasize that this conclusion is appropriate for the parameters we tested only.

Given the previously reported presence of *S. symbiotica* in eastern North America and the primarily vertical transmission of endosymbionts, we expected that all *A. tsugae* would be infected with *S. symbiotica*. Because of this, our heat stress experiments were conducted concurrently with the evaluation of symbiont presence or absence in the over 350 field-collected adelgids. The added variable of *S. symbiotica* absence in a number of NY individuals affected our experimental design and reduced the number of replicates we had for each type of temperature–time association. We now know that nearly a third of the NY adelgids lack *S. symbiotica* (Fig. 1), and that the lack of this symbiont affects the densities of the obligate symbionts (Fig. 5). In the future, it may be possible to leverage the natural variation in *S. symbiotica* frequencies to assess the role of heat stress upon symbiont densities and adelgid fitness from both a *S. symbiotica* present and *S. symbiotica* absent *A. tsugae* population. By comparing *A. tsugae* with and without *S. symbiotica*, the role of this symbiont in heat tolerance (either directly imparted upon the adelgids or indirectly by effects upon their obligate symbionts) could be assessed.

It is possible that the temperature tested in our experiment was too low for a heat-induced response. We selected 32.5 °C as our heat treatment to avoid host mortality, which can occur in less than 96 h when exposed to temperatures above 35 °C (Mech *et al.*, 2017), but survival of *A. tsugae* and their bacterial symbionts reported here indicates that a higher range of temperatures could be tested, especially if field populations have not been exposed to long periods of naturally high temperatures. Experiments using these recommendations could determine whether any of the three symbionts are sensitive to heat stress and if *S. symbiotica* protects adelgids or obligate symbionts from heat-induced damage. However, the development of methods to establish clonal lineages of adelgids in laboratory culture is a necessary first step.

One conclusive outcome of this study is that the presence of *S. symbiotica* is negatively correlated with the abundance of both symbionts that putatively have a nutritional role in *A. tsugae*. Changes in the sensitivities of symbionts in response to the presence of other symbionts (coinfections) can influence the fitness of host insects (Oliver *et al.*, 2006). The presence of a facultative symbiont (*Hamiltonella defensa*) in pea aphids has been reported to influence the abundance of its nutritional symbiont *Buchnera*, but whether this has an impact on aphid fitness is unknown (Martinez *et al.*, 2014). Because *S. symbiotica* and *Annandia* share space within the bacteriocyte (von Dohlen *et al.*, 2013), it was not surprising to find higher *Annandia* densities when *S. symbiotica* was absent. However, with *Pseudomonas* only found extracellularly in the haemocoel (von Dohlen *et al.*, 2013), it was unexpected to find lower levels of this symbiont when *S. symbiotica* was present in the bacteriocyte. The reduced obligate symbiont densities associated with *S. symbiotica* infection may have an impact upon *A. tsugae* fitness due to a reduced capacity for the production of essential nutrients. This could explain why *A. tsugae* not infected with *S. symbiotica* have reached a higher prevalence in at least one geographic location in eastern North America.

Bacterial endosymbionts have also been shown to suppress induced plant defenses to benefit the insect host. When tomato (*Solanum lycopersicum* L.) was exposed to orally secreted bacteria of the Colorado potato beetle (*Leptinotarsa decemlineata* Say), it incited a regulated defense that differed from, and suppressed, an herbivory-induced defense (Chung *et al.*, 2013). It has been hypothesized that *A. tsugae* saliva may be toxic to *T. canadensis*, thereby allowing adelgids to successfully populate and utilize the tree (McClure & Cheah, 1999; Radville *et al.*, 2011). When first instar *A. tsugae* were examined, the only bacterial endosymbiont found in their salivary glands was *Annandia* (von Dohlen *et al.*, 2013). The lack of *S. symbiotica* in the salivary glands suggest it is unlikely to play a role in the dysbiosis between *A. tsugae* and *T. canadensis*. Future interpretations of the genome sequences of *A. tsugae*'s bacterial symbionts should shed light on their roles within adelgids and broaden our knowledge of this symbiotic system.

Overall, this study provides an effective methodology for extracting DNA from individual, minute first instar *A. tsugae*, establishes specific quantitative PCR primer sequences for *Annandia*, *Pseudomonas*, and *S. symbiotica* (Table 1), and builds knowledge about the density dynamics, distribution, and function of heritable symbionts. This knowledge is important for understanding *A. tsugae* ecology and is one of the first steps in developing projects that could evaluate the effects of symbionts upon *A. tsugae* fitness.

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Disclosure

All authors of this manuscript declare no conflicts of interest.

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