Heat shock alters pea aphid-*Buchnera* interactions: negative allometry of gene densities

Nousheen Parven[1,2], Izumi Yao[1], Takashi Kanbe[1] & Shin-ichi Akimoto[1]*

[1]Department of Ecology and Systematics, Graduate School of Agriculture, Hokkaido University, Sapporo Japan, and [2]Department of Zoology, University of Dhaka, Dhaka Bangladesh

***Correspondence:** E-mail: akimoto@res.agr.hokudai.ac.jp

Running title: *Heat shock alters aphid*-Buchnera *interactions*

Key words: Acyrthosiphon pisum, bacteriocyte, elongation factor 1-alpha, 16S-rRNA gene, sterility, symbiont, real-time PCR, transgenerational effect, temperature, Hemiptera, Aphididae

Abstract

In the face of global climate change, the understanding of how aphid-symbiont relationships are affected by heat shock is critical. We evaluated the effects of heat shock on the pea aphid, *Acyrthosiphon pisum* Harris (Hemiptera: Aphididae), and its obligate endosymbiont *Buchnera aphidicola* Munson et al. by means of quantitative PCR in treated aphids and their offspring. First-instar aphids received a single heat shock (35 °C for 6 h), repetitive heat shocks (repeat of the single heat shock for 3 days), or a control treatment (constant 20 °C). We evaluated the impacts on aphid body length and *Buchnera* and aphid gene densities, estimated from the number of copies of bacterial 16S-rRNA and nuclear elongation factor 1-alpha (EF1α) genes, respectively. Heat shock negatively affected aphid body length and *Buchnera* and EF1α gene densities. Heat-shocked aphids contained lower densities of *Buchnera* and EF1α genes than control aphids when body length was kept constant. When *Buchnera* and EF1α gene densities were represented on a log-log scale, *Buchnera* densities increased with EF1α densities in all treatments, but *Buchnera* densities showed negative allometry with EF1α densities. Compared to control aphids, heat-shocked aphids contained lower *Buchnera* densities relative to EF1α densities. Some heat-shocked aphids became sterile if their *Buchnera* gene density was lower than a threshold (ca. 42 000 copies). The offspring of aphids subjected to a single heat shock recovered the number of *Buchnera*, but the offspring of aphids subjected to repetitive heat shocks exhibited markedly lower *Buchnera* and EF1α densities. Thus, heat shock negatively affects both aphid and *Buchnera* cell proliferation, in the heat shock-treated generation as well as in their offspring, but the impact is more severe on *Buchnera*. Because the symbiont supplies essential amino acids, vitamins, and an essential protein, this could reduce aphid development and reproduction and possibly leads to extinction of local populations.

Introduction

Climate change under global warming will reportedly increase temperature variability, leading to a substantial increase in daily maximum temperature, with extreme heat events (Easterling et al., 2000; IPCC, 2013; Ma et al., 2015). Global warming is expected to alter the distribution, phenology, and life-history traits of insects in the coming decades (Hoffmann et al., 2013; Kingsolver et al., 2013; Auad et al., 2015; Peng et al., 2019). Thus, increase in the frequency, intensity, and duration of high temperatures has become an area of focus for ecological research (Hansen et al., 2006; Bauerfeind & Fischer, 2014).

The detrimental effects of high temperatures on the survival, development, and reproduction of aphid species have been well documented (Lamb et al., 1987; Wang et al., 1997; Chen et al., 2000; Shingleton et al., 2003; Ma et al., 2004; Kuo et al., 2006; Russell & Moran, 2006; Lu & Kuo, 2008; Moran & Yun, 2015). Heat stress has harmful effects on aphids, primarily because of the heat sensitivity of the aphid obligate endosymbiont *Buchnera aphidicola* Munson et al., whose density in the host aphid decreases under high-temperature conditions (Montllor et al., 2002; Chen et al., 2009; Burke et al., 2010; Lu et al., 2014). *Buchnera* supplies essential amino acids, vitamins, and a specific protein [symbionin, a member of the GroEL (Hsp60) family] for the embryonic and nymphal development of the host aphid (Douglas, 1998; Wilkinson & Ishikawa, 2000; Montllor et al., 2002; Bermingham et al., 2009; Vinuelas et al., 2011; Rabatel et al., 2013); thus, experimental elimination of symbiotic bacteria from host aphids has resulted in serious negative impacts on aphid growth, reproduction, and life span (Sasaki et al., 1991; Douglas, 1992, 1996; Douglas & Prosser, 1992; Koga et al., 2003).

When pea aphids, *Acyrthosiphon pisum* (Harris) (Hemiptera: Aphididae), have been exposed to 37 °C for 3 h as young nymphs, they were not able to synthesize symbionin, resulting in severely retarded growth and sterility due to damaged endosymbionts (Ohtaka & Ishikawa, 1991). Chen et al. (2009) quantified *Buchnera* density in four nymphal and three adult stages of *Aphis craccivora* Koch that were reared at each of six temperatures ranging from 10 to 35 °C. The density of the *Buchnera* gene relative to the density of the host aphid

gene decreased with increasing temperatures, and the net reproductive rate was greatly reduced in aphids reared at 30 or 35 °C. The effect of ambient temperatures on the relative density of *Buchnera* gene to aphid gene in *A. pisum* was investigated by Lu et al. (2014), who found that relative *Buchnera* gene density decreased with increasing temperatures and later aphid developmental stages. In an attempt to replace the native *Buchnera* symbionts in a recipient *A. pisum* with exogenous *Buchnera* from a donor *A. pisum*, Moran & Yun (2015) exposed recipient aphids to 35 °C for 4 h to reduce the density of the native *Buchnera* and then injected a genetically distinct *Buchnera*. Interactions between *Buchnera* and the facultative symbiont *Serratia symbiotica* Moran et al. within the host aphid have also been reported; e.g., although heat stress decreased *Buchnera* densities, if the host aphid was simultaneously infested with *S. symbiotica*, *Buchnera* densities remained high under heat stress (Burke et al., 2010).

Climatic factors influence insect body size, resulting in changes in reproduction and survival through body size-mediated effects (Kingsolver & Huey, 2008). High ambient temperatures generally reduce body size in aphids (Dixon, 1985; Blackman & Spence, 1994; Humphreys & Douglas, 1997), and this phenotypic plasticity is adaptive in some species (Partridge et al., 1994; David et al., 1997; Fischer et al., 2003). Therefore, previous studies have suggested that high temperatures negatively affect the cell proliferation of both aphids and their symbiont *Buchnera* (Ohtaka & Ishikawa, 1991; Chen et al., 2009; Burke et al., 2010; Lu et al., 2014). However, few studies have addressed how the aphid-*Buchnera* relationship can be altered by a sudden increase in ambient temperature and to what extent the offspring recover from the impacts of heat shock imposed on their mother.

The present study first aimed to examine the impacts of heat shock on the pea aphid and its obligate endosymbiont *Buchnera* to evaluate the quantitative relationships among aphid nuclear gene density, *Buchnera* gene density, and aphid body size. In addition, to evaluate the relationship between the frequency of heat shock and aphid growth, two types of heat shock were studied. The single heat shock treatment (at 35 °C for 6 h) was considered because of the maximum temperature in the locality the aphid clone was collected, whereas the repetitive

heat shock treatment (repeat of the single heat shock for 3 days) was considered to simulate actual temperatures in mid-summer in the locality. In our study, aphid nuclear gene density and *Buchnera* gene density were estimated by the number of copies of the single-copy nuclear gene elongation factor 1-alpha (EF1α) and the *Buchnera* 16S-rRNA gene, respectively. The second objective was to evaluate the impacts of heat shock on the next generation, to gain an understanding of the recovery process. Thus, this paper examined (1) the relationship of aphid body length with *Buchnera* and aphid gene density after single and repetitive heat shocks, (2) the effects of single and repetitive heat shocks on the relationship between aphid and *Buchnera* gene density, and (3) the extent to which offspring could recover from heat shock imposed on the maternal generation. The latter addresses whether aphid plasticity is transgenerational and adaptive under global warming scenarios.

Materials and methods

Insects and plants

An *A. pisum* clone (Sap08Ms2) was collected in 2008 from alfalfa (*Medicago sativa* L.) on the riverbed of the Toyohira river, Sapporo, Japan (43°00'36.4"N, 141°20'55.7"E). A clonal lineage, founded from a single female, has been maintained as a stock culture using broad bean (*Vicia faba* L.) seedlings as the host plant, at 20 °C and L16:D8 photoperiod. The stock culture has been maintained by transferring five newborn nymphs onto a new seedling, each seedling caged in a cylindrical container (3 cm diameter, 10 cm high).

Experiment 1: heat shock to first instar

Twenty apterous pea aphid females were randomly selected from the stock culture and their reproduction was checked daily. Five first instars of similar size were selected and transferred onto a new broad bean seedling within 6 h after birth. Seedlings with five nymphs were randomly allocated to one of three treatments: the single heat shock (SH, n = 6), repetitive heat shock (RH, n = 12), or control treatment (CT, n = 8). In the SH treatment, first instars were transferred from 20 to 35 °C for 6 h and thereafter returned to 20 °C. In the RH

treatment, first instars were transferred to 35 °C for 6 h per day, which was repeated for three consecutive days, with intervals at 20 °C. After the heat shock treatments, surviving first instars were individually transferred onto a new seedling and were maintained at 20 °C. In the CT treatment, first instars were continuously maintained at 20 °C. Replicates from the three treatments were prepared on the same day, and they were subjected to the heat shock treatments simultaneously. We used fourth instar nymphs for molecular analyses. Our observations showed that heat shock delayed larval development. Thus, to obtain fourth instar individuals, 7-day-old aphids in the CT (n = 19), 8-day-old aphids in the SH (n = 20), and 10-day-old aphids in the RH (n = 19) treatment were collected, preserved in 80% ethanol, and used for body size measurements and molecular analyses (current generation) as described below. Aphid mortality and survival to adulthood were also recorded.

Experiment 2: effects of heat shock on the next generation

In a separate experiment, after the heat shock treatments performed as described above (SH, RH, and CT), first-instar pea aphids that survived were individually transferred onto a new broad bean seedling and maintained at 20 °C. The matured aphids were allowed to reproduce for 7 days, during which early-born offspring reached the fourth instar. The offspring of the heat shock-treated aphids developed normally at 20 °C. One fourth instar was randomly selected from each of the colonies. The CT treatment resulted in a colony of 44.1 ± 3.86 (mean ± SE) nymphs, but the colony size was smaller as a result of the two heat shock treatments (for the offspring of the SH treatment, 21.8 ± 3.30; for the offspring of the RH, 5.30 ± 4.05). Thus, aphids subjected to the control treatment were grown in more crowded conditions than aphids subjected to the heat shock treatments. A total of 22 aphids in the CT, 16 in the SH, and 14 in the RH treatment were used for body size measurements and molecular analyses (offspring generation) as described below.

Some of the heat-shocked aphids did not produce nymphs for three or more days after the last molt and thus were considered sterile. To evaluate the effect of *Buchnera* and nuclear gene density on aphid sterility, 18 sterile adults (four in the RH and 14 in the SH treatment)

were randomly selected and used for molecular analyses.

Body length

Prior to extracting DNA, the images of the preserved aphids were captured using a Dino-lite digital microscope (10-55× magnification; AnMo Electronics, New Taipei City, Taiwan). Based on the images, we measured body length (distance from the vertex to the tip of the cauda) using Image J 2.0.0-rc-69/1.52p (Abramoff et al., 2004).

DNA extraction and qPCR

For 128 aphids collected, total DNA was extracted and purified using Cica Geneus total DNA preparation kits for tissue (Cica-reagent; Kanto Chemical, Tokyo, Japan) according to the manufacturer's instructions and eluted with 100 µl distilled water. The eluted DNA solution was preserved at –22 °C until analysis. We estimated *Buchnera* gene density by measuring the number of amplicons of *Buchnera* 16S-rRNA gene using a TaqMan probe (Nippon Genetics, Tokyo, Japan). Aphid gene density was estimated by measuring the number of amplicons of the single-copy nuclear gene elongation factor 1-alpha (EF1α) using SYBR Green (Nippon Genetics). For both genes, 10-fold serial dilutions were used as standards. Three technical replicates were prepared for each aphid sample in a qPCR (Mx3005P; Agilent Technologies, Tokyo, Japan) assay according to the methods of Lu et al. (2014) and Sugimoto et al. (2015), and variation among technical replicates was compared with variation among aphids.

A portion of the *Buchnera* 16S-rRNA gene was amplified by the primer-probe method, using a primer set of AP-Buch-16S-0806F (5′-GGA-TGA-ACC-CAG-ACG-AGA-TTA-G-3′) and AP-Buch-16S-0806R (5′-GTT-CCA-GTG-TGG-CTG-GTT-AT-3′) with a probe of AP-Buch-16S-0806Pr (5′-CCA-AGG-CAA-CGA-TCT-CTA-GCT-GGT-3′). The probe was labeled with FAM on the 5' and TAM on the 3'. The 10 µl of reaction mixture included 5 µl of Kapa Probe Fast qPCR Master Mix, 0.2 µl of Kapa Rox Low (Nippon Genetics), 0.2 µl of each primer (0.2 pmol μl^{-1}), 0.2 µl of probe (0.2 pmol μl^{-1}), 3.2 µl of ddH_2O, and 1 µl of

extracted DNA. PCR conditions were as follows: 95 °C for 3 min followed by 35 cycles at 95 °C for 3 s, annealing at 50 °C for 20 s, and 60 °C for 20 s. For the 16S-rRNA gene, the manufacture-made oligonucleotide standard did not work well. Instead, a PCR product of the gene was selected arbitrarily, quantified with the Qubit dsDNA HS Assay Kit (Thermo Fisher, Yokohama, Japan) and the plate reader function of the qPCR system, and used for making a standard curve.

A primer set of APEF1-alpha 107F (5′-CTG-ATT-GTG-CCG-TGC-TTA-TTG-3′) and APEF1-alpha 246R (5′-TAT-GGT-GGT-TCA-GTA-GAG-TCC-3′) (Dunbar et al., 2007) was used to amplify the EF1α gene. The 10 μl of reaction mixture contained 5 μl of Kapa SYBR Fast qPCR Mix (Nippon Genetics), 0.09 μl of each primer (0.09 pmol μl^{-1}), 3.82 μl ddH_2O, and 1 μl DNA. PCR conditions were as follows: 95 °C for 3 min followed by 35 cycles at 95 °C for 3 s, annealing at 55 °C for 20 s, and 72 °C for 11 s. Dissociation curves were generated at the end of each qPCR run to determine whether a single peak was detected in each sample. To make a standard curve for the EF1α gene, the oligonucleotide was synthesized using gBlocks Gene Fragments (IDT, Tokyo, Japan).

Statistical analysis

Buchnera and EF1α densities, consisting of count data, were ln-transformed before statistical analysis. For experiments 1 and 2, we first evaluated the extent of experimental errors among the three technical replicates per aphid by calculating variance components in all 58 and 52 aphids, respectively (Sokal & Rohlf, 1995). Differences among treatments were tested using ANOVA, in which individual aphids were treated as a random effect. The percentages of the variance components among aphids and within aphids (among technical replicates) were evaluated with the restricted maximum likelihood estimation. When experimental errors were at a negligible level, ln-transformed gene densities were averaged over the technical replicates, and the average (the logarithm of the geometric mean) was used as the value of each aphid.

We tested differences in aphid body length among treatments using ANOVA, followed

by post hoc Tukey-Kramer method to separate the means ($\alpha = 0.05$). The relationships between aphid body length, *Buchnera* gene densities, and EF1α gene densities were analyzed with ANCOVA ($\alpha = 0.05$) (Raubenheimer & Simpson, 1992; Sokal & Rohlf, 1995). The three variables were ln-transformed before analysis. We first examined whether regression lines of EF1α densities on body length could be considered as significantly different among treatments. Differences in the slopes among the treatments were tested, and if there were none, then the differences in elevation among the treatments were tested. The latter were represented by least square means (LSmeans) based on the ANCOVA model from which the interaction term was removed. ANCOVA was also applied to the relationships between aphid body length and *Buchnera* gene densities, or between *Buchnera* gene densities and EF1α densities. Most statistical analyses were performed using JMP v.13 (SAS Institute, Cary, NC, USA). The Tukey-Kramer method was performed using the package 'emmeans' in R v. 3.4.2 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Effects of heat shock on the current and offspring generation

Exposure of first-instar pea aphids to heat shock resulted in high mortality in experiment 1. In the SH and RH treatments, 66.6% (n = 30) and 50.0% (n = 60) of the aphids survived to adulthood, respectively, whereas in the CT treatment, this was 97.4% (n = 39).

The estimation of the variance components indicated that the within-aphid component for *Buchnera* densities and EF1α densities, i.e., variance due to experimental errors, accounted only for 0.13 and 0.15 %, respectively, of the total variance in experiment 1, and 0.39 and 1.39 %, respectively, in experiment 2, suggesting that experimental errors were very low. Thus, in the later analyses, ln-transformed gene densities were averaged over the technical replicates.

Comparisons of body length showed that fourth instar aphids subjected to the RH treatment were significantly smaller than those subjected to the CT treatment (Figure 1A). EF1α densities were highest in the aphids exposed to the CT treatment (set at 100%),

followed by those exposed to the SH treatment (20.2% based on the real copy number), and then those exposed to the RH treatment (0.85%) (Figure 1B). The same tendency was found in *Buchnera* densities, which were highest in the CT-exposed aphids (set at 100%), followed by the SH-exposed aphids (0.64%), and lowest in the RH-exposed aphids (0.10%) (Figure 1C). Thus, heat shock drastically reduced both EF1α and *Buchnera* densities. Throughout experiment 1, body length (BL), ln(*Buchnera* gene titer), and ln(EF1α titer) were positively correlated [BL vs. ln(*Buchnera*): $r = 0.72$; BL vs. ln(EF1α): $r = 0.66$; ln(*Buchnera*) vs. ln(EF1α): $r = 0.79$, all $P<0.001$; $n = 58$).

In experiment 2, in the offspring generation of the aphids subjected to heat shock, significant differences were detected in all three measured traits. The offspring of the RH-exposed aphids were significantly smaller than the offspring of the CT-exposed aphids (Figure 1D). The offspring of the aphids exposed to the RH treatment contained lower densities of both EF1α (Figure 1E) and *Buchnera* (Figure 1F) than the aphids exposed to the CT and SH treatments. Compared to the aphids subjected to heat shock treatments, their offspring generation had reduced EF1α and *Buchnera* densities.

Effects of heat shock on the relationships between *Buchnera* and pea aphid cells

When the regression lines of EF1α densities on body length were compared among the three treatments in the current generation, ANCOVA indicated no significant interactions between treatments (T) and body length (BL), suggesting that the slopes of the regression lines were not significantly different among the treatments (Table 1). However, the elevations of the regression lines differed significantly (Table 1). When evaluated at equal body length, the CT-exposed aphids contained the highest EF1α densities, followed by the SH-exposed and then the RH-exposed aphids (LSmeans, Table 1). Similarly, ANCOVA showed that in the regression of *Buchnera* densities on body length, the slopes did not differ significantly, but the elevation did vary among the treatments (Table 1). When evaluated at equal body length, the CT-exposed aphids contained higher *Buchnera* densities than the SH-exposed and the RH-exposed aphids (LSmeans, Table 1).

Regarding the regression of *Buchnera* densities on EF1α densities, the slopes did not differ ($F_{2,52}$ = 0.27, P = 0.77), but the elevations did differ among the treatments ($F_{2,52}$ = 6.65, P = 0.003) (Figure 2A). When evaluated at equal EF1α densities, the CT-exposed aphids contained higher *Buchnera* densities than the RH-exposed and the SH-exposed aphids (Tukey-Kramer method: P<0.05). Data of sterile adults obtained in experiment 2 were superimposed on the data of fourth instars obtained in experiment 1. These sterile aphids harbored lower *Buchnera* gene densities than 42 484 copies. The *Buchnera* densities of some of the RH-exposed aphids were very low and comparable to those of the sterile adults (Figure 2A).

When the regression lines of *Buchnera* densities on EF1α densities were compared among the offspring from the aphids exposed to the three treatments, the slopes did not differ ($F_{2,46}$ = 0.84, P = 0.44) and neither did the elevations ($F_{2,46}$ = 0.03, P = 0.97) (Figure 2B).

When *Buchnera* and EF1α gene densities were represented on a log-log scale, the regression slope of *Buchnera* on EF1α densities was 0.60 in control aphids of the current generation (Figure 2A) and 0.44 in the offspring of the control aphids (Figure 2B), which suggested a negative allometry of *Buchnera* gene densities with EF1α densities. In the offspring generation (Figure 3B), the ratios of *Buchnera* to EF1α densities decreased with increasing EF1α densities (r = –0.86, P<0.001; n = 52), suggesting that aphids with lower nuclear gene density contain a relatively larger number of *Buchnera* cells. In contrast, the majority of heat-shocked aphids of the current generation had negative values for the ln-transformed ratios of *Buchnera* to EF1α densities (Figure 3A), indicating that *Buchnera* gene density was lower than aphid nuclear gene density. For the control aphids, the ratios of *Buchnera* densities to EF1α densities were also negatively correlated with EF1α densities (r = –0.47, P = 0.04; n = 19), but there was no significant relationship between them for the aphids subjected to the SH and RH treatments (for the SH treatment, r = –0.19, P = 0.42; n = 20; for the RH treatment, r = –0.03, P = 0.89; n = 19) (Figure 3A).

Discussion

The present study attempted to evaluate the impact of heat shock, which can frequently occur in temperate regions, during the first instar on the current and subsequent generation of aphids. Our results indicated, as in previous studies (Chen et al., 2009; Burke et al., 2010; Lu et al., 2014), that heat shock had negative effects on aphid body length, *Buchnera* (symbiont) gene density, and EF1α (aphid) gene density. Repetitive heat shocks reduced the three traits more intensely than a single heat shock. However, the two heat shock treatments affected the three traits differently. Although there were positive correlations between aphid body length, *Buchnera* gene density, and EF1α density, ANCOVA indicated that aphids exposed to a single heat shock contained lower EF1α densities than control aphids when compared among individuals with equal body length, and densities were even lower when aphids were exposed to repetitive heat shocks. A similar decrease in *Buchnera* gene densities was visible in heat-shocked aphids compared to control aphids when comparing aphids with equal EF1α density. Therefore, the present study confirmed that *Buchnera* was more sensitive than aphid cells to high temperatures, which severely hinder *Buchnera* cell proliferation (Montllor et al., 2002; Chen et al., 2009; Burke et al., 2010; Lu et al., 2014; Corbin et al., 2017).

Sterile adults occurred even though nymphs were only exposed to a single heat shock (at 35 °C for 6 h), a phenomenon also reported by Ohtaka & Ishikawa (1991) and Lu et al. (2014). Aphids subjected to heat shock likely develop successfully into adults, but harbor reduced amounts of *Buchnera*, which may lead to sterility. Some fourth instars subjected to the repetitive heat shock treatment exhibited low *Buchnera* gene densities comparable to sterile adults (Figure 2A); these nymphs are thus likely to become sterile when they enter adulthood. Thus, temperature conditions in mid-summer Japan would possibly lead to reduced aphid reproduction through decreased density of *Buchnera*. Also, in the tree-dwelling aphid *Tuberculatus macrotuberculatus* Essig & Kuwana, *Buchnera* gene density as well as aphid reproductive performance and host leaf suitability are reported to decline in mid-summer compared to June (Yao, 2019).

Transgenerational effects of heat shock were detected in the offspring of heat-shocked aphids. Repetitive heat shocks resulted in a marked decrease in the *Buchnera* gene density,

EF1α density, and body length in the offspring. This is probably because nymphs contain developing embryos in their abdomen, so that heat shock to nymphs simultaneously has adverse effects on their embryos. Temperature conditions used in the present study would readily occur; e.g., in Sapporo, the daily maximum temperature ranged from 32-34 °C for five consecutive days in 2019.

In the case of a single heat shock, the offspring seemed to partly recover, as they contained higher *Buchnera* gene densities than their mothers. However, the offspring showed much lower EF1α densities than their heat-shocked mothers. Similarly, the offspring of the control aphids had greatly reduced EF1α densities compared with their control mothers. Rather than transgenerational effects, this may be due to differences in rearing conditions between experiment 1 and 2. The current generation aphids were reared on broad bean seedlings in groups of five, whereas the offspring generation aphids were reared in a crowded colony produced during 7 days by a single adult. These crowded conditions likely affected *Buchnera* and EF1α gene densities negatively. In contrast, the offspring of repetitively heat-shocked aphids developed in a colony of on average 5.3 individuals because of deteriorated fecundity in their mothers. This environment, nevertheless, did not contribute to increasing *Buchnera* or EF1α gene densities, and thus the low densities of these genes could be attributed to transgenerational effects of heat shock. Wilkinson et al. (2007) reported that EF1α density in *A. pisum* varied greatly compared to aphid fresh weight, which they ascribed to differences in the rate of ovariole development among nymphs. The present study confirmed that EF1α density in *A. pisum* is liable to change, reflecting food conditions and population density during aphid development.

When *Buchnera* gene and EF1α densities are represented on a log-log scale, the regression slope corresponds to the allometry coefficient. In the present study, the allometry coefficients of the control aphids of the current generation and their offspring were less than unity, showing that *Buchnera* gene density had negative allometry to EF1α density. As an index of the relative amount of *Buchnera* cells to the host cells, several authors have used a ratio index, i.e., the number of *Buchnera* gene copies divided by the number of aphid gene

(EF1α) copies (hereafter: relative *Buchnera* density) or its logarithm (Chen et al., 2009; Burke et al., 2010; Vogel & Moran, 2011; Lu et al., 2014; Zhang et al., 2016; Xu et al., 2020). The present study indicated that the relative *Buchnera* density was negatively correlated to EF1α density. Therefore, contrary to expectations, the offspring of the repetitively heat-shocked and the control aphids exhibited high and low relative *Buchnera* density, respectively. A similar result was obtained by Lu et al. (2014), who showed that the logarithm of relative *Buchnera* density in *A. pisum* decreased as the developmental stage advanced or as the rearing temperature increased (10-35 °C), suggesting that relative *Buchnera* density decreases with increasing aphid biomass if the aphids are reared at a constant temperature. In addition, Humphreys & Douglas (1997) indicated that rearing *A. pisum* at higher temperatures (15-25 °C) resulted in greater decrease in fresh weight and protein content per adult. However, they pointed out that the number of *Buchnera* cells per amount of aphid fresh weight or per protein content increased with decreasing body mass, suggesting that *Buchnera* density per amount of aphid biomass is negatively correlated to aphid biomass. Whitehead & Douglas (1993) reported that during the aphid developmental process, *Buchnera* cells proliferated more slowly than the host aphid cells, and resultantly that relative *Buchnera* density decreased with the growth of host aphids. These results suggest that care is needed in interpreting the relationship between relative *Buchnera* density and other aphid traits. Several authors have pointed out that analyzing ratio data can lead to a variety of problems, potentially leading to incorrect conclusions (Raubenheimer & Simpson, 1992; Beaupre & Dunham, 1995; Liermann et al., 2004). If a ratio is used as an index of relative *Buchnera* density, negative correlation could arise between relative *Buchnera* density and aphid reproductive performance (e.g., Chong & Moran, 2016). This is because there is a negative correlation between relative *Buchnera* density and aphid biomass, which is in turn positively correlated with aphid reproductive performance (Nevo & Coll, 2001; Lamb et al., 2009).

The reason why the growth rate of *Buchnera* cells is lower than that of aphid cells during aphid development remains to be clarified. In an aphid, *Buchnera* cells reside both in embryonic bacteriocytes and in bacteriocytes of the maternal compartment (maternal

bacteriocytes) (Whitehead & Douglas, 1993; Koga et al., 2012). A study attempting to count aphid symbionts using flow cytometry indicated that although the number of *Buchnera* increased rapidly at the final stage of nymphal development, almost all *Buchnera* cells were stored in maternal bacteriocytes, with ca. 5 and 12% of *Buchnera* cells contained in embryonic bacteriocytes in fourth instar nymphs and teneral adults, respectively (Simonet et al., 2016). Although the total number of *Buchnera* cells in an aphid nymph increases with nymphal development, it could be postulated that the cell proliferation rate of the embryos is much higher than that of *Buchnera*, resulting in negative allometry of *Buchnera* density with aphid nuclear gene density. Possibly, because of the different proliferation rates, the relative *Buchnera* density could be lowered when the host aphid has more developing embryos.

In future studies, the quantitative relationship between *Buchnera* gene density and EF1α density needs to be explored from a viewpoint of allometry using aphids of different clones (Raubenheimer & Simpson, 1992). The crucial point may be whether these clones have different allometry between the two gene densities, and whether this is related to variations in reproductive performance (Vogel & Moran, 2011; Chong & Moran, 2016). Provided that the relationship between *Buchnera* gene density and EF1α density is described by a single allometry curve, an aphid with relatively high *Buchnera* density could be identified as a large residual value from the allometry curve rather than a high value in the relative *Buchnera* density (*Buchnera* density per EF1α density).

Sharing of the common allometry of *Buchnera* gene density among the offspring of the CT, SH, and RH-exposed aphids suggests that the number of *Buchnera* cells was rigidly regulated and adjusted to a certain amount by the host aphids. Thus, the common allometry in the offspring generation can be considered to reflect an adaptive phenotypic plasticity in aphids, because host aphids have the ability to adjust the *Buchnera* cell number even though the developmental condition is altered by different densities and transgenerational effects.

If heat shock does not destroy the majority of the *Buchnera* cells in aphids, the offspring generation could overcome the impact of heat shock through the supply of essential nutrients from *Buchnera* (Bermingham et al., 2009; Vinuelas et al., 2011; Rabatel et al.,

2013). However, repetitive heat shocks would rapidly reduce the number of *Buchnera* cells, and thereby could negatively affect the development of the next generation as well as the current generation. We conclude that frequent occurrences of high temperatures accompanied by global warming have a great impact on aphid development and reproduction and could possibly lead to population extinction at a local scale.

Acknowledgements

We thank Dr. Hitoshi Araki for providing experimental facilities and Dr. Takuya Kubo for helping us with statistic methods. This research was supported by Grants-in-Aid for Scientific Research to IY (24570016 and 15K07210) and to SA (19K06848) from the Japan Society for the Promotion of Science.

References

Abramoff MD, Magalhaes PJ & Ram SJ (2004) Image processing with ImageJ. Biophotonics International 11: 36–42.

Auad AM, Silva SEB, Santos JC & Vieira TM (2015) Impact of fluctuating and constant temperatures on key life history parameters of *Sipha flava* (Hemiptera: Aphididae). Florida Entomologist 98: 424–429.

Bauerfeind SS & Fischer K (2014) Simulating climate change: temperature extremes but not means diminish performance in a widespread butterfly. Population Ecology 56: 239–250.

Beaupre SJ & Dunham AE (1995) A comparison of ratio-based and covariance analyses of a nutritional data set. Functional Ecology 9: 876–880.

Bermingham J, Rebatel A, Calevro F, Vinuelas J, Febvay G et al. (2009) Impact of developmental age on the transcriptome of the symbiotic bacterium *Buchnera aphidicola* in the pea aphid (*Acyrthosiphon pisum*). Applied and Environmental Microbiology 75: 7294–7297.

Blackman RL & Spence JM (1994) The effects of temperature on aphid morphology, using a

multivariate approach. European Journal of Entomology 91: 7–22.

Burke G, Fiehn O & Moran N (2010) Effects of facultative symbionts and heat stress on the metabolome of pea aphids. International Society for Microbial Ecology 4: 242–252.

Chen CY, Lai CY & Kuo MH (2009) Temperature effects on the growth of *Buchnera* endosymbiont in *Aphis craccivora* (Hemiptera: Aphididae). Symbiosis 49: 53–59.

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*. Entomologia Experimentalis et Applicata 95: 315–323.

Chong RA & Moran NA (2016) Intraspecific genetic variation in host affects regulation of obligate heritable symbionts. Proceedings of the National Academy of Sciences of the USA 113: 13114–13119.

Corbin C, Heyworth ER, Ferrari J & Hurst GDD (2017) Heritable symbionts in a world of varying temperature. Heredity 118: 10–20.

David JR, Gibert P, Gravot E, Petavy G, Morin JP et al. (1997) Phenotypic plasticity and developmental temperature in *Drosophila*: analysis and significance of reaction norms of morphometrical traits. Journal of Thermal Biology 22: 441–451.

Dixon AFG (1985) Structure of aphid populations. Annual Review of Entomology 30: 155–174.

Douglas AE (1992) Requirement of pea aphids (*Acyrthosiphon pisum*) for their symbiotic bacteria. Entomologia Experimentalis et Applicata 65: 195–198.

Douglas AE (1996) Reproductive failure and the free amino acid pools in pea aphids (*Acyrthosiphon pisum*) lacking symbiotic bacteria. Journal of Insect Physiology 42: 247–255.

Douglas AE (1998) Nutritional interactions in insect-microbial symbiosis: aphids and their symbiotic bacteria *Buchnera*. Annual Review of Entomology 43: 17–37.

Douglas AE & Posser WA (1992) Synthesis of the essential amino acid tryptophan in the pea aphid (*Acyrthosiphon pisum*) symbiosis. Journal of Insect Physiology 38: 565–568.

Dunbar HE, Wilson ACC, Ferguson NR & Moran NA (2007) Aphid thermal tolerance is

governed by a point mutation in bacterial symbionts. PLoS Biology 5: e96.
Easterling DR, Meehl GA, Parmesan C, Changnon SA, Karl TR & Mearns LO (2000) Climate extremes: observations, modeling, and impacts. Science 289: 2068–2074.
Fischer K, Brakefield PM & Zwaan BJ (2003) Plasticity in butterfly egg size: why larger offspring at lower temperatures? Ecology 84: 3138–3147.
Hansen J, Sato M, Ruedy R, Lo K, Lea DW & Medina-Elizade M (2006) Global temperature change. Proceedings of the National Academy of Sciences of the USA 103: 14288–14293.
Hoffmann AA, Chown SL & Clusella-Trullas S (2013) Upper thermal limits in terrestrial ectotherms: how constrained are they? Functional Ecology 27: 934–949.
Humphreys NJ & Douglas AE (1997) Partitioning of symbiotic bacteria between generations of an insect: a quantitative study of a *Buchnera* sp. in the pea aphid (*Acyrthosiphon pisum*) reared at different temperatures. Applied and Environmental Microbiology 63: 3294–3296.
IPCC (2013) Climate Change 2013: The Physical Science Basis. Working Group I Contribution to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change (ed. by TF Stocker, D Qin, G-K Plattner, MMB Tignor, SK Allen et al.). Cambridge University Press, New York, NY, USA.
Kingsolver JG, Diamond SE & Buckley LB (2013) Heat stress and the fitness consequences of climate change for terrestrial ectotherms. Functional Ecology 27: 1415–1423.
Kingsolver JG & Huey RB (2008) Size, temperature, and fitness: three rules. Evolutionary Ecology Research 10: 251–268.
Koga R, Meng XY, Tsuchida T & Fukatsu T (2012) Cellular mechanisms for selective vertical transmission of an obligate insect symbiont at the bacteriocyte-embryo interface. Proceedings of the National Academy of Sciences of the USA 109: 1230–1237.
Koga R, Tsuchida T & Fukatsu T (2003) Changing partners in an obligate symbiosis: a facultative endosymbiont can compensate for loss of the essential endosymbiont

Buchnera in an aphid. Proceedings of the Royal Society of London B 270: 2543–2550.

Kuo MH, Chiu MC & Perng JJ (2006) Temperature effects on life history traits of the corn leaf aphid, *Rhopalosiphum maidis* (Homoptera: Aphididae) on corn in Taiwan. Applied Entomology and Zoology 41: 171–177.

Lamb RJ, Mackay PA & Gerber GH (1987) Are development and growth of pea aphids, *Acyrthosiphon pisum*, in North America adapted to local temperatures? Oecologia 72: 170–177.

Lamb RJ, Mackay PA & Migui SM (2009) Measuring the performance of aphids: fecundity versus biomass. Canadian Entomologist 141: 401–405.

Liermann M, Steel A, Rosing M & Guttorp P (2004) Random denominators and the analysis of ratio data. Environmental and Ecological Statistics 11: 55–71.

Lu WN, Chiu MC & Kuo MH (2014) Host life stage- and temperature-dependent density of the symbiont *Buchnera aphidicola* in a subtropical pea aphid (*Acyrthosiphon pisum)* population. Journal of Asia-Pacific Entomology 17: 537–541.

Lu WN & Kuo MH (2008) Life table and heat tolerance of *Acyrthosiphon pisum* (Hemiptera: Aphididae) in subtropical Taiwan. Entomological Science 11: 273–279.

Ma CS, Hau B & Poehling HM (2004) Effects of pattern and timing of high temperature exposure on reproduction of the rose grain aphids, *Metopolophium dirhodum*. Entomologia Experimentalis et Applicata 110: 65–71.

Ma G, Hoffmann AA & Ma CS (2015) Daily temperature extremes play an important role in predicting thermal effects. Journal of Experimental Biology 218: 2289–2296.

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

Moran NA & Yun Y (2015) Experimental replacement of an obligate insect symbiont. Proceedings of the National Academy of Sciences of the USA 112: 2093–2096.

Nevo E & Coll M (2001) Effects of nitrogen fertilization on *Aphis gossypii* (Homoptera: Aphididae): variation in size, color, and reproduction. Journal of Economic Entomology 94: 27–32.

Ohtaka C & Ishikawa H (1991) Effects of heat treatment on the symbiotic system of an aphid mycetocyte. Symbiosis 11: 19–30.

Partridge L, Barrie B, Fowler K & French V (1994) Evolution and development of body size and cell size in *Drosophila melanogaster* in response to temperature. Evolution 48: 1269–1276.

Peng X, Zhao Q, Guo X, Su S, Liu L et al. (2019) Effects of variable maternal temperature on offspring development and reproduction of *Rhopalosiphum padi*, a serious global pest of wheat. Ecological Entomology 45: 269–277.

Rabatel A, Febvay G, Gaget K, Duport G, Baa-Puyoulet P et al. (2013) Tyrosine pathway regulation is host-mediated in the pea aphid symbiosis during late embryonic and early larval development. BMC Genomics 14: 235.

Raubenheimer D & Simpson SL (1992) Analysis of covariance: an alternative to nutritional indices. Entomologia Experimentalis et Applicata 62: 221–231.

Russell JA & Moran NA (2006) Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. Proceedings of the Royal Society of London B 273: 603–610.

Sasaki T, Hayashi H & Ishikawa H (1991) Growth and reproduction of symbiotic and aposymbiotic pea aphids, *Acyrthosiphon pisum* maintained on artificial diets. Journal of Insect Physiology 37: 749–756.

Shingleton AW, Sisk GC & Stern DL (2003) Diapause in the pea aphid (*Acyrthosiphon pisum*) is a slowing but not a cessation of development. BMC Developmental Biology 3: 7.

Simonet P, Duport G, Gaget K, Gayet MW, Colella S et al. (2016) Direct flow cytometry measurements reveal a fine-tuning of symbiotic cell dynamics according to the host developmental needs in aphid symbiosis. Scientific Reports 6: 19967.

Sokal RR & Rohlf FJ (1995) Biometry – The Principles and Practice of Statistics in Biological Research, 3rd edn. Freeman and Company, New York, NY, USA.

Sugimoto TN, Kayukawa T, Matsuo T, Tsuchida T & Ishikawa Y (2015) A short, high

temperature treatment of host larvae to analyze *Wolbachia*-host interactions in the moth *Ostrinia scapulalis*. Journal of Insect Physiology 81: 48–51.

Vinuelas J, Febvay G, Duport G, Colella S, Fayard JM et al. (2011) Multimodal dynamic response of the *Buchnera aphidicola* pLeu plasmid to variations in leucine demand of its host, the pea aphid *Acyrthosiphon pisum.* Molecular Microbiology 81: 1271–1285.

Vogel KJ & Moran NA (2011) Effects of host genotype on symbiont titer in the aphid-*Buchnera* symbiosis. Insects 2: 423–434.

Wang K, Tsai JH & Harrison NA (1997) Influence of temperature on development, survivorship, and reproduction of buckthorn aphid (Homoptera: Aphididae). Annals of the Entomological Society of America 90: 62–68.

Whitehead LF & Douglas AE (1993) Populations of symbiotic bacteria in the parthenogenetic pea aphid (*Acyrthosiphon pisum*) symbiosis. Proceedings of the Royal Society of London B 254: 29–32.

Wilkinson TL & Ishikawa H (2000) Injection of essential amino acids substitutes for bacterial supply in aposymbiotic pea aphids (*Acyrthosiphon pisum*). Entomologia Experimentalis et Applicata 94: 85–91.

Wilkinson TL, Koga R & Fukatsu T (2007) Role of host nutrition in symbiont regulation: impact of dietary nitrogen on proliferation of obligate and facultative bacterial endosymbionts of the pea aphid *Acyrthosiphon pisum.* Applied and Environmental Microbiology 73: 1362–1366.

Xu TT, Jiang LY, Chen J & Qiao GX (2020) Host plants influence the symbiont diversity of Eriosomatinae (Hemiptera: Aphididae). Insects 11: 217.

Yao I (2019) Seasonal changes in the density of the symbionts *Buchnera* and *Wolbachia* of the aphid *Tuberculatus macrotuberculatus* on *Quercus dentata*. Entomologia Experimentalis et Applicata 167: 261–268.

Zhang Y-C, Cao W-J, Zhong L-R, Godfray HCJ & Liu X-D (2016) Host plant determines the population size of an obligate symbiont (*Buchnera aphidicola*) in aphids. Applied and Environmental Microbiology 82: 2336–2346.

Figure captions

Figure 1 Mean (± SE) (A, D) body length (mm), (B, E) ln[elongation factor 1-alpha (EF1α) gene density], and (C, F) ln(*Buchnera aphidicola* gene density) in fourth instars of the (A-C) current generation (n = 19-20) and (D-F) offspring generation (n = 14-22) of *Acyrthosiphon pisum* exposed to a single heat shock (SH), repeated heat shocks (RH), or control treatment (CT). Different letters near the means within a panel indicate significant differences between treatments (Tukey-Kramer method: $P<0.05$).

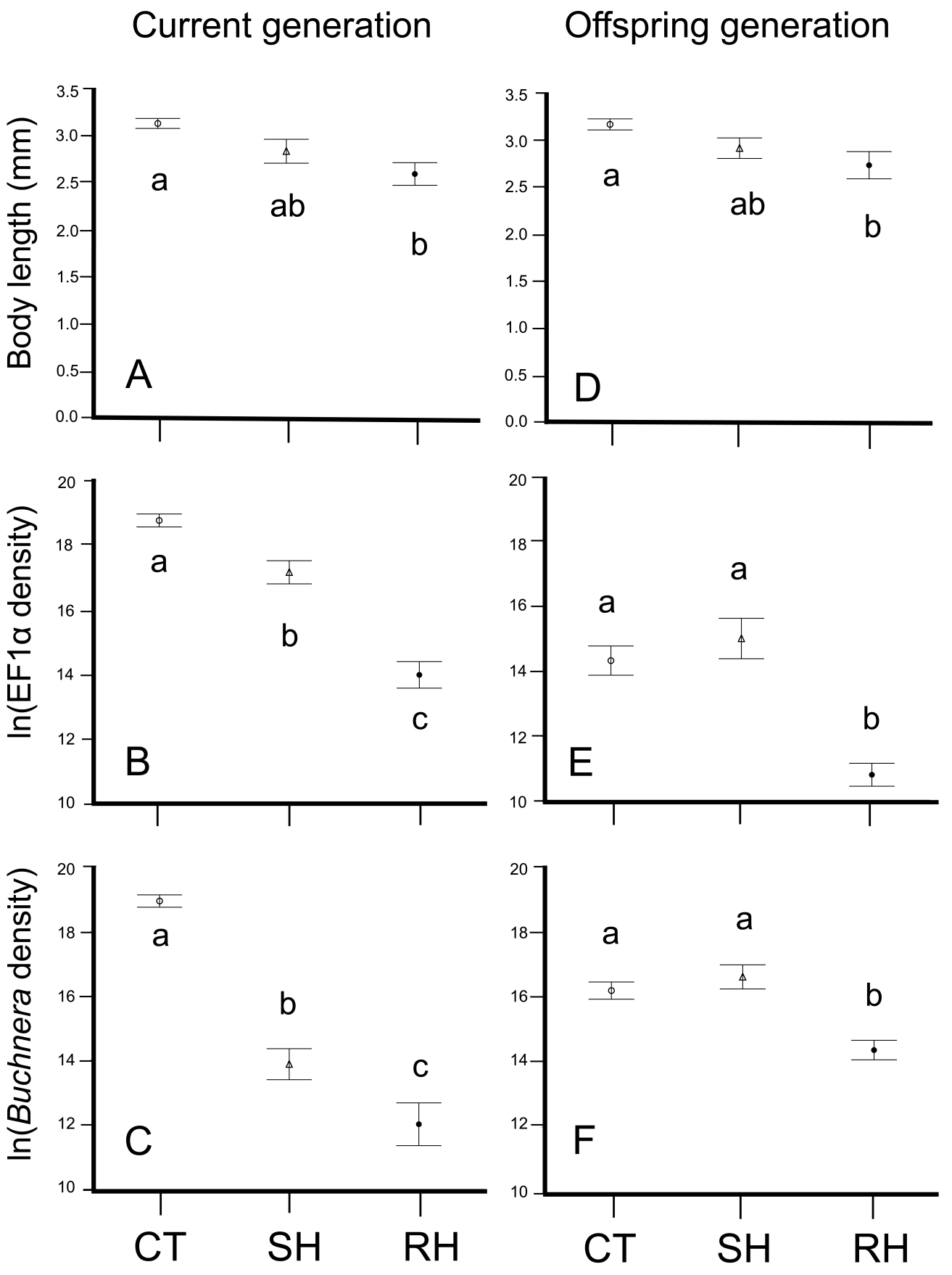
Figure 2 Relationship between ln(*Buchnera aphidicola* gene density) and ln[elongation factor 1-alpha (EF1α) gene density] in fourth instars of the (A) current generation (n = 19-20) and (B) the offspring generation (n = 14-22) of *Acyrthosiphon pisum* exposed to a single heat shock (SH), repeated heat shocks (RH), or control treatment (CT). Based on ANCOVA, the common slope is displayed for the three treatments. SH and RH treatments rendered some aphids sterile; gene densities of sterile adults are superimposed, but not included in analyses (n = 4-14).

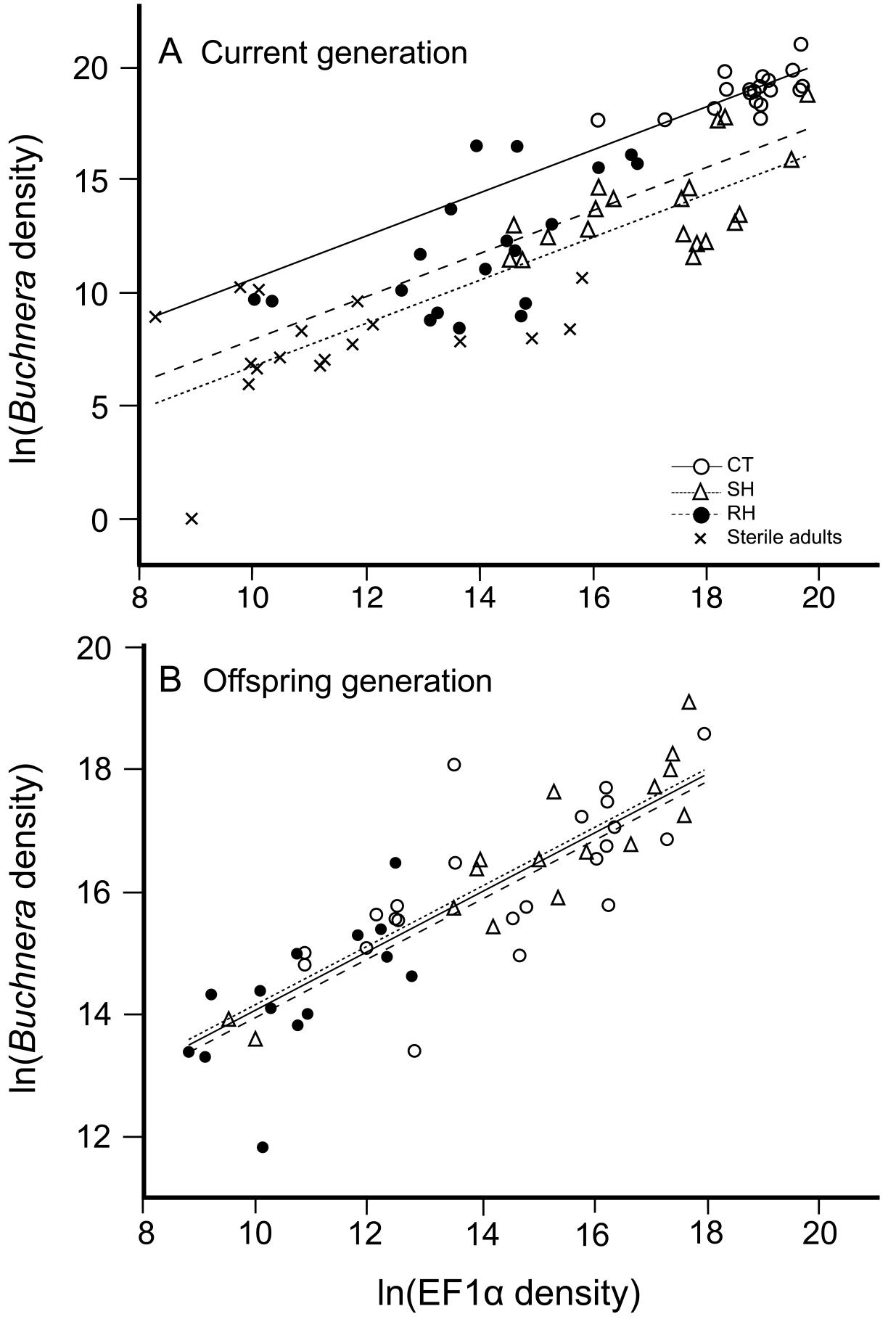
Figure 3 Relationship between ln[*Buchnera aphidicola* gene density/elongation factor 1-alpha (EF1α) gene density] and ln(EF1α density) in fourth instars of the (A) current generation (n = 19-20) and (B) offspring generation (n = 14-22) of *Acyrthosiphon pisum* exposed to a single heat shock (SH), repeated heat shocks (RH), or control treatment (CT).

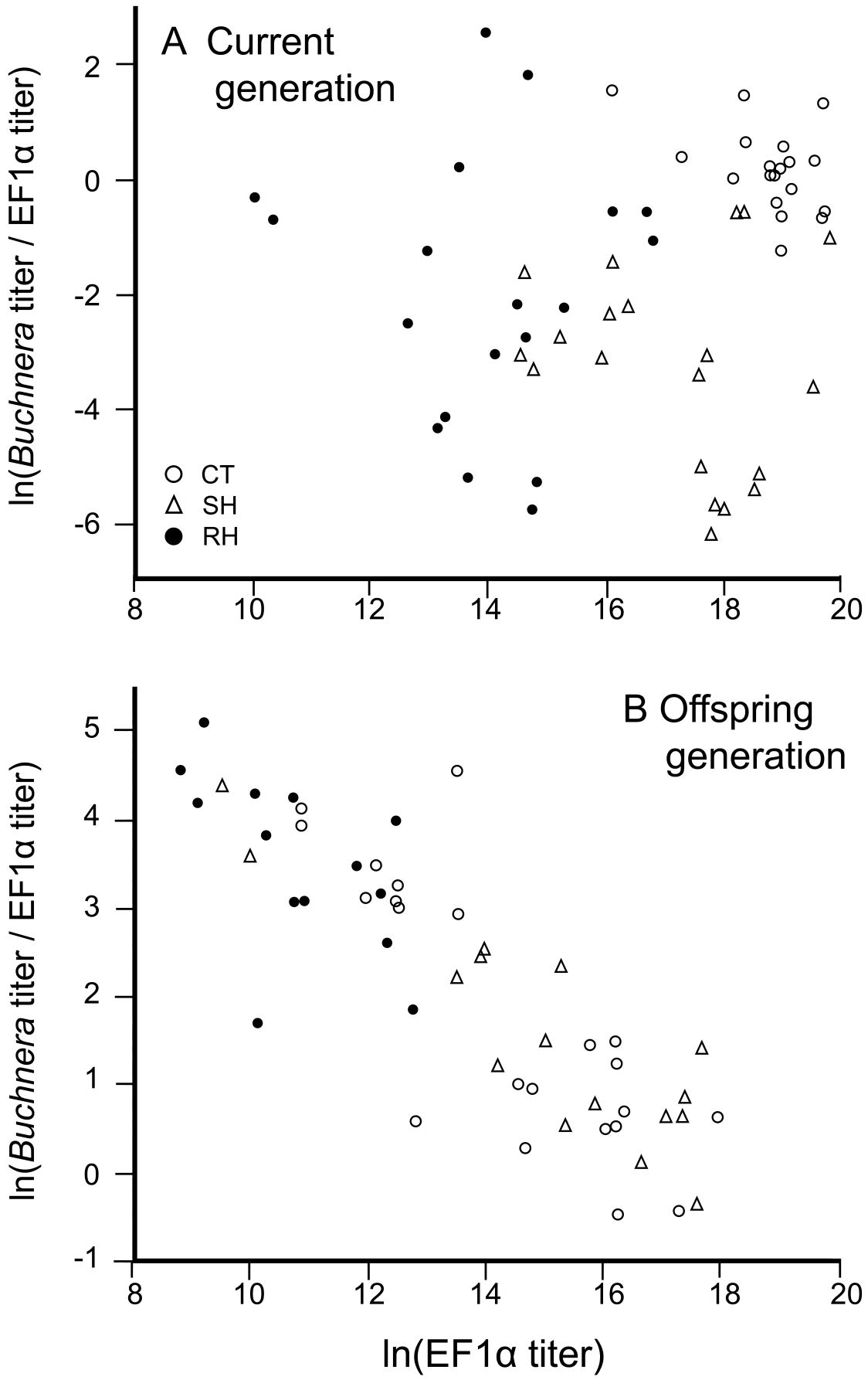
Table 1 Results of ANCOVA for the regressions of elongation factor 1-alpha (EF1α) and *Buchnera aphidicola* gene densities on fourth-instar pea aphid body length

	Dependent variable						
		EF1α			*Buchnera*		
Independent variable	d.f.	F	P	LSmeans[1]	F	P	LSmeans[1]
Treatment (T)	2,52	34.12	<0.001		29.62	<0.001	
Body length (BL)	1,52	7.72	0.008		16.41	<0.001	
T*BL	2,52	1.01	0.37		1.10	0.34	
Control treatment (CT)				18.2a			18.0a
Single heat shock (SH)				17.2b			14.0b
Repetitive heat shocks (RH)				14.5c			12.9b

[1]Least square means for EF1α and *Buchnera* densities were calculated at the grand mean of body length. Means within a column followed by different letters are significantly different between treatments (Tukey-Kramer method: $P<0.05$).


Current generation
Offspring generation
Body length (mm)
ln(EF1α density)
ln(Buchnera density)
A
B
C
D
E
F
a
ab
b
c
CT
SH
RH


A Current generation
B Offspring generation
ln(Buchnera density)
ln(EF1α density)
CT
SH
RH
Sterile adults
0
5
10
15
20
8
10
12
14
16
18
20


A Current generation
B Offspring generation
ln(Buchnera titer / EF1α titer)
ln(EF1α titer)
CT
SH
RH
2
0
-2
-4
-6
5
4
3
2
1
0
-1
8
10
12
14
16
18
20