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Instructions for use

1	Heat shock alters pea aphid-Buchnera interactions: negative
2	allometry of gene densities
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1 Abstract

2 In the face of global climate change, the understanding of how aphid-symbiont relationships 3 are affected by heat shock is critical. We evaluated the effects of heat shock on the pea aphid, 4 Acyrthosiphon pisum Harris (Hemiptera: Aphididae), and its obligate endosymbiont Buchnera 5 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 6 7 of the single heat shock for 3 days), or a control treatment (constant 20 °C). We evaluated the 8 impacts on aphid body length and Buchnera and aphid gene densities, estimated from the 9 number of copies of bacterial 16S-rRNA and nuclear elongation factor 1-alpha (EF1a) genes, 10 respectively. Heat shock negatively affected aphid body length and *Buchnera* and EF1a gene 11 densities. Heat-shocked aphids contained lower densities of Buchnera and EF1a genes than 12 control aphids when body length was kept constant. When Buchnera and EF1a gene densities 13 were represented on a log-log scale, *Buchnera* densities increased with EF1 α densities in all 14 treatments, but Buchnera densities showed negative allometry with EF1a densities. Compared 15 to control aphids, heat-shocked aphids contained lower *Buchnera* densities relative to EF1 α 16 densities. Some heat-shocked aphids became sterile if their Buchnera gene density was lower 17 than a threshold (ca. 42 000 copies). The offspring of aphids subjected to a single heat shock 18 recovered the number of Buchnera, but the offspring of aphids subjected to repetitive heat 19 shocks exhibited markedly lower *Buchnera* and $EF1\alpha$ densities. Thus, heat shock negatively 20 affects both aphid and Buchnera cell proliferation, in the heat shock-treated generation as well 21 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 22 23 development and reproduction and possibly leads to extinction of local populations.

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1 Introduction

2 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).

9 The detrimental effects of high temperatures on the survival, development, and 10 reproduction of aphid species have been well documented (Lamb et al., 1987; Wang et al., 11 1997; Chen et al., 2000; Shingleton et al., 2003; Ma et al., 2004; Kuo et al., 2006; Russell & 12 Moran, 2006; Lu & Kuo, 2008; Moran & Yun, 2015). Heat stress has harmful effects on 13 aphids, primarily because of the heat sensitivity of the aphid obligate endosymbiont Buchnera 14 aphidicola Munson et al., whose density in the host aphid decreases under high-temperature 15 conditions (Montllor et al., 2002; Chen et al., 2009; Burke et al., 2010; Lu et al., 2014). 16 Buchnera supplies essential amino acids, vitamins, and a specific protein [symbionin, a 17 member of the GroEL (Hsp60) family] for the embryonic and nymphal development of the 18 host aphid (Douglas, 1998; Wilkinson & Ishikawa, 2000; Montllor et al., 2002; Bermingham 19 et al., 2009; Vinuelas et al., 2011; Rabatel et al., 2013); thus, experimental elimination of 20 symbiotic bacteria from host aphids has resulted in serious negative impacts on aphid growth, 21 reproduction, and life span (Sasaki et al., 1991; Douglas, 1992, 1996; Douglas & Prosser, 22 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

1 gene decreased with increasing temperatures, and the net reproductive rate was greatly 2 reduced in aphids reared at 30 or 35 °C. The effect of ambient temperatures on the relative 3 density of Buchnera gene to aphid gene in A. pisum was investigated by Lu et al. (2014), who 4 found that relative Buchnera gene density decreased with increasing temperatures and later 5 aphid developmental stages. In an attempt to replace the native Buchnera symbionts in a 6 recipient A. pisum with exogenous Buchnera from a donor A. pisum, Moran & Yun (2015) 7 exposed recipient aphids to 35 °C for 4 h to reduce the density of the native Buchnera and 8 then injected a genetically distinct Buchnera. Interactions between Buchnera and the 9 facultative symbiont Serratia symbiotica Moran et al. within the host aphid have also been 10 reported; e.g., although heat stress decreased *Buchnera* densities, if the host aphid was 11 simultaneously infested with S. symbiotica, Buchnera densities remained high under heat 12 stress (Burke et al., 2010).

13 Climatic factors influence insect body size, resulting in changes in reproduction and 14 survival through body size-mediated effects (Kingsolver & Huey, 2008). High ambient 15 temperatures generally reduce body size in aphids (Dixon, 1985; Blackman & Spence, 1994; 16 Humphreys & Douglas, 1997), and this phenotypic plasticity is adaptive in some species 17 (Partridge et al., 1994; David et al., 1997; Fischer et al., 2003). Therefore, previous studies 18 have suggested that high temperatures negatively affect the cell proliferation of both aphids 19 and their symbiont Buchnera (Ohtaka & Ishikawa, 1991; Chen et al., 2009; Burke et al., 2010; 20 Lu et al., 2014). However, few studies have addressed how the aphid-Buchnera relationship 21 can be altered by a sudden increase in ambient temperature and to what extent the offspring 22 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

1 heat shock treatment (repeat of the single heat shock for 3 days) was considered to simulate 2 actual temperatures in mid-summer in the locality. In our study, aphid nuclear gene density 3 and Buchnera gene density were estimated by the number of copies of the single-copy nuclear 4 gene elongation factor 1-alpha (EF1a) and the Buchnera 16S-rRNA gene, respectively. The 5 second objective was to evaluate the impacts of heat shock on the next generation, to gain an 6 understanding of the recovery process. Thus, this paper examined (1) the relationship of aphid 7 body length with *Buchnera* and aphid gene density after single and repetitive heat shocks, (2) 8 the effects of single and repetitive heat shocks on the relationship between aphid and 9 Buchnera gene density, and (3) the extent to which offspring could recover from heat shock 10 imposed on the maternal generation. The latter addresses whether aphid plasticity is 11 transgenerational and adaptive under global warming scenarios.

12

13 Materials and methods

14 **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).

21

22 **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

1 treatment, first instars were transferred to 35 °C for 6 h per day, which was repeated for three 2 consecutive days, with intervals at 20 °C. After the heat shock treatments, surviving first 3 instars were individually transferred onto a new seedling and were maintained at 20 °C. In the 4 CT treatment, first instars were continuously maintained at 20 °C. Replicates from the three 5 treatments were prepared on the same day, and they were subjected to the heat shock 6 treatments simultaneously. We used fourth instar nymphs for molecular analyses. Our 7 observations showed that heat shock delayed larval development. Thus, to obtain fourth instar 8 individuals, 7-day-old aphids in the CT (n = 19), 8-day-old aphids in the SH (n = 20), and 10-9 day-old aphids in the RH (n = 19) treatment were collected, preserved in 80% ethanol, and 10 used for body size measurements and molecular analyses (current generation) as described 11 below. Aphid mortality and survival to adulthood were also recorded.

12

13 Experiment 2: effects of heat shock on the next generation

14 In a separate experiment, after the heat shock treatments performed as described above (SH, 15 RH, and CT), first-instar pea aphids that survived were individually transferred onto a new 16 broad bean seedling and maintained at 20 °C. The matured aphids were allowed to reproduce 17 for 7 days, during which early-born offspring reached the fourth instar. The offspring of the 18 heat shock-treated aphids developed normally at 20 °C. One fourth instar was randomly 19 selected from each of the colonies. The CT treatment resulted in a colony of 44.1 ± 3.86 20 (mean \pm SE) nymphs, but the colony size was smaller as a result of the two heat shock 21 treatments (for the offspring of the SH treatment, 21.8 ± 3.30 ; for the offspring of the RH, 22 5.30 ± 4.05). Thus, aphids subjected to the control treatment were grown in more crowded 23 conditions than aphids subjected to the heat shock treatments. A total of 22 aphids in the CT, 24 16 in the SH, and 14 in the RH treatment were used for body size measurements and 25 molecular analyses (offspring generation) as described below.

26 Some of the heat-shocked aphids did not produce nymphs for three or more days after 27 the last molt and thus were considered sterile. To evaluate the effect of *Buchnera* and nuclear 28 gene density on aphid sterility, 18 sterile adults (four in the RH and 14 in the SH treatment)

- 1 were randomly selected and used for molecular analyses.
- 2

3 **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).

8

9 **DNA extraction and qPCR**

10 For 128 aphids collected, total DNA was extracted and purified using Cica Geneus total DNA 11 preparation kits for tissue (Cica-reagent; Kanto Chemical, Tokyo, Japan) according to the 12 manufacturer's instructions and eluted with 100 µl distilled water. The eluted DNA solution 13 was preserved at -22 °C until analysis. We estimated Buchnera gene density by measuring 14 the number of amplicons of Buchnera 16S-rRNA gene using a TaqMan probe (Nippon 15 Genetics, Tokyo, Japan). Aphid gene density was estimated by measuring the number of 16 amplicons of the single-copy nuclear gene elongation factor 1-alpha (EF1a) using SYBR 17 Green (Nippon Genetics). For both genes, 10-fold serial dilutions were used as standards. 18 Three technical replicates were prepared for each aphid sample in a qPCR (Mx3005P; Agilent 19 Technologies, Tokyo, Japan) assay according to the methods of Lu et al. (2014) and Sugimoto 20 et al. (2015), and variation among technical replicates was compared with variation among 21 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 ddH₂O, 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.

7 A primer set of APEF1-alpha 107F (5'-CTG-ATT-GTG-CCG-TGC-TTA-TTG-3') and 8 APEF1-alpha 246R (5'-TAT-GGT-GGT-TCA-GTA-GAG-TCC-3') (Dunbar et al., 2007) was 9 used to amplify the EF1a gene. The 10 µl of reaction mixture contained 5 µl of Kapa SYBR 10 Fast qPCR Mix (Nippon Genetics), 0.09 μ l of each primer (0.09 pmol μ l⁻¹), 3.82 μ l ddH₂O, 11 and 1 µl DNA. PCR conditions were as follows: 95 °C for 3 min followed by 35 cycles at 12 95 °C for 3 s, annealing at 55 °C for 20 s, and 72 °C for 11 s. Dissociation curves were 13 generated at the end of each qPCR run to determine whether a single peak was detected in 14 each sample. To make a standard curve for the EF1 α gene, the oligonucleotide was 15 synthesized using gBlocks Gene Fragments (IDT, Tokyo, Japan).

16

17 Statistical analysis

18 Buchnera and EF1α densities, consisting of count data, were ln-transformed before statistical 19 analysis. For experiments 1 and 2, we first evaluated the extent of experimental errors among 20 the three technical replicates per aphid by calculating variance components in all 58 and 52 21 aphids, respectively (Sokal & Rohlf, 1995). Differences among treatments were tested using 22 ANOVA, in which individual aphids were treated as a random effect. The percentages of the 23 variance components among aphids and within aphids (among technical replicates) were 24 evaluated with the restricted maximum likelihood estimation. When experimental errors were 25 at a negligible level, In-transformed gene densities were averaged over the technical replicates, and the average (the logarithm of the geometric mean) was used as the value of 26 27 each aphid.

28

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

1 by post hoc Tukey-Kramer method to separate the means ($\alpha = 0.05$). The relationships 2 between aphid body length, Buchnera gene densities, and EF1a gene densities were analyzed 3 with ANCOVA ($\alpha = 0.05$) (Raubenheimer & Simpson, 1992; Sokal & Rohlf, 1995). The three 4 variables were ln-transformed before analysis. We first examined whether regression lines of 5 EF1α densities on body length could be considered as significantly different among 6 treatments. Differences in the slopes among the treatments were tested, and if there were 7 none, then the differences in elevation among the treatments were tested. The latter were 8 represented by least square means (LSmeans) based on the ANCOVA model from which the 9 interaction term was removed. ANCOVA was also applied to the relationships between aphid 10 body length and *Buchnera* gene densities, or between *Buchnera* gene densities and EF1 α 11 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 12 13 (R Foundation for Statistical Computing, Vienna, Austria).

14

15 **Results**

16 Effects of heat shock on the current and offspring generation

17 Exposure of first-instar pea aphids to heat shock resulted in high mortality in experiment 1. In

18 the SH and RH treatments, 66.6% (n = 30) and 50.0% (n = 60) of the aphids survived to

19 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 EF1 α 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%),

1 followed by those exposed to the SH treatment (20.2% based on the real copy number), and 2 then those exposed to the RH treatment (0.85%) (Figure 1B). The same tendency was found 3 in *Buchnera* densities, which were highest in the CT-exposed aphids (set at 100%), followed 4 by the SH-exposed aphids (0.64%), and lowest in the RH-exposed aphids (0.10%) (Figure 5 1C). Thus, heat shock drastically reduced both EF1a and Buchnera densities. Throughout 6 experiment 1, body length (BL), $\ln(Buchnera \text{ gene titer})$, and $\ln(\text{EF1}\alpha \text{ titer})$ were positively 7 correlated [BL vs. $\ln(Buchnera)$: r = 0.72; BL vs. $\ln(EF1\alpha)$: r = 0.66; $\ln(Buchnera)$ vs. 8 $\ln(EF1\alpha)$: 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 RHexposed 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.

16

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

18 When the regression lines of $EF1\alpha$ densities on body length were compared among the three 19 treatments in the current generation, ANCOVA indicated no significant interactions between 20 treatments (T) and body length (BL), suggesting that the slopes of the regression lines were 21 not significantly different among the treatments (Table 1). However, the elevations of the 22 regression lines differed significantly (Table 1). When evaluated at equal body length, the CT-23 exposed aphids contained the highest $EF1\alpha$ densities, followed by the SH-exposed and then 24 the RH-exposed aphids (LSmeans, Table 1). Similarly, ANCOVA showed that in the 25 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, 26 27 the CT-exposed aphids contained higher Buchnera densities than the SH-exposed and the RH-28 exposed aphids (LSmeans, Table 1).

1	Regarding the regression of <i>Buchnera</i> densities on $EF1\alpha$ densities, the slopes did not
2	differ (F _{2,52} = 0.27, P = 0.77), but the elevations did differ among the treatments (F _{2,52} = 6.65,
3	$P = 0.003$) (Figure 2A). When evaluated at equal EF1 α densities, the CT-exposed aphids
4	contained higher Buchnera densities than the RH-exposed and the SH-exposed aphids
5	(Tukey-Kramer method: P<0.05). Data of sterile adults obtained in experiment 2 were
6	superimposed on the data of fourth instars obtained in experiment 1. These sterile aphids
7	harbored lower Buchnera gene densities than 42 484 copies. The Buchnera densities of some
8	of the RH-exposed aphids were very low and comparable to those of the sterile adults (Figure
9	2A).

10 When the regression lines of *Buchnera* densities on $EF1\alpha$ densities were compared 11 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). 12 13 When *Buchnera* and EF1 α gene densities were represented on a log-log scale, the 14 regression slope of Buchnera on EF1a densities was 0.60 in control aphids of the current 15 generation (Figure 2A) and 0.44 in the offspring of the control aphids (Figure 2B), which 16 suggested a negative allometry of *Buchnera* gene densities with EF1 α densities. In the 17 offspring generation (Figure 3B), the ratios of Buchnera to EF1a densities decreased with 18 increasing EF1 α densities (r = -0.86, P<0.001; n = 52), suggesting that aphids with lower 19 nuclear gene density contain a relatively larger number of *Buchnera* cells. In contrast, the 20 majority of heat-shocked aphids of the current generation had negative values for the ln-21 transformed ratios of Buchnera to EF1a densities (Figure 3A), indicating that Buchnera gene 22 density was lower than aphid nuclear gene density. For the control aphids, the ratios of 23 Buchnera densities to EF1 α densities were also negatively correlated with EF1 α densities (r = 24 -0.47, P = 0.04; n = 19), but there was no significant relationship between them for the aphids 25 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). 26 27

28 Discussion

1 The present study attempted to evaluate the impact of heat shock, which can frequently occur 2 in temperate regions, during the first instar on the current and subsequent generation of 3 aphids. Our results indicated, as in previous studies (Chen et al., 2009; Burke et al., 2010; Lu 4 et al., 2014), that heat shock had negative effects on aphid body length, *Buchnera* (symbiont) 5 gene density, and $EF1\alpha$ (aphid) gene density. Repetitive heat shocks reduced the three traits 6 more intensely than a single heat shock. However, the two heat shock treatments affected the 7 three traits differently. Although there were positive correlations between aphid body length, 8 Buchnera gene density, and EF1a density, ANCOVA indicated that aphids exposed to a 9 single heat shock contained lower $EF1\alpha$ densities than control aphids when compared among 10 individuals with equal body length, and densities were even lower when aphids were exposed 11 to repetitive heat shocks. A similar decrease in Buchnera gene densities was visible in heat-12 shocked aphids compared to control aphids when comparing aphids with equal $EF1\alpha$ density. 13 Therefore, the present study confirmed that *Buchnera* was more sensitive than aphid cells to 14 high temperatures, which severely hinder Buchnera cell proliferation (Montllor et al., 2002; 15 Chen et al., 2009; Burke et al., 2010; Lu et al., 2014; Corbin et al., 2017). 16 Sterile adults occurred even though nymphs were only exposed to a single heat shock

17 (at 35 °C for 6 h), a phenomenon also reported by Ohtaka & Ishikawa (1991) and Lu et al. 18 (2014). Aphids subjected to heat shock likely develop successfully into adults, but harbor 19 reduced amounts of *Buchnera*, which may lead to sterility. Some fourth instars subjected to 20 the repetitive heat shock treatment exhibited low Buchnera gene densities comparable to 21 sterile adults (Figure 2A); these nymphs are thus likely to become sterile when they enter 22 adulthood. Thus, temperature conditions in mid-summer Japan would possibly lead to reduced 23 aphid reproduction through decreased density of Buchnera. Also, in the tree-dwelling aphid 24 Tuberculatus macrotuberculatus Essig & Kuwana, Buchnera gene density as well as aphid 25 reproductive performance and host leaf suitability are reported to decline in mid-summer 26 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.

6 In the case of a single heat shock, the offspring seemed to partly recover, as they 7 contained higher Buchnera gene densities than their mothers. However, the offspring showed 8 much lower $EF1\alpha$ densities than their heat-shocked mothers. Similarly, the offspring of the 9 control aphids had greatly reduced EF1a densities compared with their control mothers. 10 Rather than transgenerational effects, this may be due to differences in rearing conditions 11 between experiment 1 and 2. The current generation aphids were reared on broad bean 12 seedlings in groups of five, whereas the offspring generation aphids were reared in a crowded 13 colony produced during 7 days by a single adult. These crowded conditions likely affected 14 Buchnera and EF1a gene densities negatively. In contrast, the offspring of repetitively heat-15 shocked aphids developed in a colony of on average 5.3 individuals because of deteriorated 16 fecundity in their mothers. This environment, nevertheless, did not contribute to increasing 17 Buchnera or EF1a gene densities, and thus the low densities of these genes could be attributed 18 to transgenerational effects of heat shock. Wilkinson et al. (2007) reported that EF1a density 19 in A. pisum varied greatly compared to aphid fresh weight, which they ascribed to differences 20 in the rate of ovariole development among nymphs. The present study confirmed that $EF1\alpha$ 21 density in A. pisum is liable to change, reflecting food conditions and population density 22 during aphid development.

When *Buchnera* gene and EF1 α 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 EF1 α 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

1 (EF1a) copies (hereafter: relative Buchnera density) or its logarithm (Chen et al., 2009; Burke 2 et al., 2010; Vogel & Moran, 2011; Lu et al., 2014; Zhang et al., 2016; Xu et al., 2020). The 3 present study indicated that the relative *Buchnera* density was negatively correlated to EF1 α 4 density. Therefore, contrary to expectations, the offspring of the repetitively heat-shocked and 5 the control aphids exhibited high and low relative Buchnera density, respectively. A similar 6 result was obtained by Lu et al. (2014), who showed that the logarithm of relative Buchnera 7 density in A. *pisum* decreased as the developmental stage advanced or as the rearing 8 temperature increased (10-35 °C), suggesting that relative Buchnera density decreases with 9 increasing aphid biomass if the aphids are reared at a constant temperature. In addition, 10 Humphreys & Douglas (1997) indicated that rearing A. pisum at higher temperatures (15-11 25 °C) resulted in greater decrease in fresh weight and protein content per adult. However, 12 they pointed out that the number of *Buchnera* cells per amount of aphid fresh weight or per 13 protein content increased with decreasing body mass, suggesting that *Buchnera* density per 14 amount of aphid biomass is negatively correlated to aphid biomass. Whitehead & Douglas 15 (1993) reported that during the aphid developmental process, Buchnera cells proliferated 16 more slowly than the host aphid cells, and resultantly that relative Buchnera density decreased 17 with the growth of host aphids. These results suggest that care is needed in interpreting the 18 relationship between relative Buchnera density and other aphid traits. Several authors have 19 pointed out that analyzing ratio data can lead to a variety of problems, potentially leading to 20 incorrect conclusions (Raubenheimer & Simpson, 1992; Beaupre & Dunham, 1995; Liermann 21 et al., 2004). If a ratio is used as an index of relative Buchnera density, negative correlation 22 could arise between relative Buchnera density and aphid reproductive performance (e.g., 23 Chong & Moran, 2016). This is because there is a negative correlation between relative 24 Buchnera density and aphid biomass, which is in turn positively correlated with aphid 25 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

1 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 2 3 increased rapidly at the final stage of nymphal development, almost all Buchnera cells were 4 stored in maternal bacteriocytes, with ca. 5 and 12% of Buchnera cells contained in 5 embryonic bacteriocytes in fourth instar nymphs and teneral adults, respectively (Simonet et 6 al., 2016). Although the total number of *Buchnera* cells in an aphid nymph increases with 7 nymphal development, it could be postulated that the cell proliferation rate of the embryos is 8 much higher than that of Buchnera, resulting in negative allometry of Buchnera density with 9 aphid nuclear gene density. Possibly, because of the different proliferation rates, the relative 10 *Buchnera* density could be lowered when the host aphid has more developing embryos.

11 In future studies, the quantitative relationship between *Buchnera* gene density and $EF1\alpha$ 12 density needs to be explored from a viewpoint of allometry using aphids of different clones 13 (Raubenheimer & Simpson, 1992). The crucial point may be whether these clones have 14 different allometry between the two gene densities, and whether this is related to variations in 15 reproductive performance (Vogel & Moran, 2011; Chong & Moran, 2016). Provided that the 16 relationship between *Buchnera* gene density and EF1a density is described by a single 17 allometry curve, an aphid with relatively high Buchnera density could be identified as a large 18 residual value from the allometry curve rather than a high value in the relative Buchnera 19 density (Buchnera density per EF1a 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.

6

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- 1
- 2

3 **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).

10

Figure 2 Relationship between $\ln(Buchnera aphidicola$ gene density) and $\ln[elongation factor 1-alpha (EF1\alpha) gene density] in fourth instars of the (A) current generation (n = 19-20) and (B) the offspring generation (n = 14-22) of$ *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).

18

19 Figure 3 Relationship between ln[*Buchnera aphidicola* gene density/elongation factor 1-

20 alpha (EF1 α) gene density] and ln(EF1 α density) in fourth instars of the (A) current

21 generation (n = 19-20) and (B) offspring generation (n = 14-22) of Acyrthosiphon pisum

22 exposed to a single heat shock (SH), repeated heat shocks (RH), or control treatment (CT).

1 **Table 1** Results of ANCOVA for the regressions of elongation factor 1-alpha (EF1α) and

	Depend	Dependent variable					
		EF1α		Buchnera			
Independent variable	d.f.	F	Р	LSmeans ¹	F	Р	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				18.2a			18.0a
Single heat shock				17.2b			14.0b
Repetitive heat shocks (RH)				14.5c			12.9b

2 Buchnera aphidicola gene densities on fourth-instar pea aphid body length

3 ¹Least square means for EF1 α and *Buchnera* densities were calculated at the grand mean of

4 body length. Means within a column followed by different letters are significantly different

5 between treatments (Tukey-Kramer method: P<0.05).





