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

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

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16 sterility, symbiont, real-time PCR, transgenerational effect, temperature, Hemiptera,
17 Aphididae

18

19

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 *Acyrtosiphon 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.
6 First-instar aphids received a single heat shock (35 °C for 6 h), repetitive heat shocks (repeat
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 (EF1 α) genes,
10 respectively. Heat shock negatively affected aphid body length and *Buchnera* and EF1 α gene
11 densities. Heat-shocked aphids contained lower densities of *Buchnera* and EF1 α genes than
12 control aphids when body length was kept constant. When *Buchnera* and EF1 α 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 EF1 α 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 α 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
22 supplies essential amino acids, vitamins, and an essential protein, this could reduce aphid
23 development and reproduction and possibly leads to extinction of local populations.

24

25

1 **Introduction**

2 Climate change under global warming will reportedly increase temperature variability,
3 leading to a substantial increase in daily maximum temperature, with extreme heat events
4 (Easterling et al., 2000; IPCC, 2013; Ma et al., 2015). Global warming is expected to alter the
5 distribution, phenology, and life-history traits of insects in the coming decades (Hoffmann et
6 al., 2013; Kingsolver et al., 2013; Auad et al., 2015; Peng et al., 2019). Thus, increase in the
7 frequency, intensity, and duration of high temperatures has become an area of focus for
8 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).

23 When pea aphids, *Acyrtosiphon pisum* (Harris) (Hemiptera: Aphididae), have been
24 exposed to 37 °C for 3 h as young nymphs, they were not able to synthesize symbionin,
25 resulting in severely retarded growth and sterility due to damaged endosymbionts (Ohtaka &
26 Ishikawa, 1991). Chen et al. (2009) quantified *Buchnera* density in four nymphal and three
27 adult stages of *Aphis craccivora* Koch that were reared at each of six temperatures ranging
28 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.

23 The present study first aimed to examine the impacts of heat shock on the pea aphid and
24 its obligate endosymbiont *Buchnera* to evaluate the quantitative relationships among aphid
25 nuclear gene density, *Buchnera* gene density, and aphid body size. In addition, to evaluate the
26 relationship between the frequency of heat shock and aphid growth, two types of heat shock
27 were studied. The single heat shock treatment (at 35 °C for 6 h) was considered because of the
28 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 (EF1 α) 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**

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

21

22 **Experiment 1: heat shock to first instar**

23 Twenty apterous pea aphid females were randomly selected from the stock culture and their
24 reproduction was checked daily. Five first instars of similar size were selected and transferred
25 onto a new broad bean seedling within 6 h after birth. Seedlings with five nymphs were
26 randomly allocated to one of three treatments: the single heat shock (SH, n = 6), repetitive
27 heat shock (RH, n = 12), or control treatment (CT, n = 8). In the SH treatment, first instars
28 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**

4 Prior to extracting DNA, the images of the preserved aphids were captured using a Dino-lite
5 digital microscope (10-55× magnification; AnMo Electronics, New Taipei City, Taiwan).

6 Based on the images, we measured body length (distance from the vertex to the tip of the
7 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 (EF1α) 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.

22 A portion of the *Buchnera* 16S-rRNA gene was amplified by the primer-probe method,
23 using a primer set of AP-Buch-16S-0806F (5'-GGA-TGA-ACC-CAG-ACG-AGA-TTA-G-3')
24 and AP-Buch-16S-0806R (5'-GTT-CCA-GTG-TGG-CTG-GTT-AT-3') with a probe of AP-
25 Buch-16S-0806Pr (5'-CCA-AGG-CAA-CGA-TCT-CTA-GCT-GGT-3'). The probe was
26 labeled with FAM on the 5' and TAM on the 3'. The 10 µl of reaction mixture included 5 µl
27 of Kapa Probe Fast qPCR Master Mix, 0.2 µl of Kapa Rox Low (Nippon Genetics), 0.2 µl of
28 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

1 extracted DNA. PCR conditions were as follows: 95 °C for 3 min followed by 35 cycles at
2 95 °C for 3 s, annealing at 50 °C for 20 s, and 60 °C for 20 s. For the 16S-rRNA gene, the
3 manufacture-made oligonucleotide standard did not work well. Instead, a PCR product of the
4 gene was selected arbitrarily, quantified with the Qubit dsDNA HS Assay Kit (Thermo Fisher,
5 Yokohama, Japan) and the plate reader function of the qPCR system, and used for making a
6 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 EF1 α gene. The 10 μ l of reaction mixture contained 5 μ l 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 μ l 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 EF1 α 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, ln-transformed gene densities were averaged over the technical
26 replicates, and the average (the logarithm of the geometric mean) was used as the value of
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 EF1 α 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 EF1 α 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,
12 USA). The Tukey-Kramer method was performed using the package ‘emmeans’ in R v. 3.4.2
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).

20 The estimation of the variance components indicated that the within-aphid component
21 for *Buchnera* densities and EF1 α densities, i.e., variance due to experimental errors,
22 accounted only for 0.13 and 0.15 %, respectively, of the total variance in experiment 1, and
23 0.39 and 1.39 %, respectively, in experiment 2, suggesting that experimental errors were very
24 low. Thus, in the later analyses, ln-transformed gene densities were averaged over the
25 technical replicates.

26 Comparisons of body length showed that fourth instar aphids subjected to the RH
27 treatment were significantly smaller than those subjected to the CT treatment (Figure 1A).
28 EF1 α 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 EF1 α and *Buchnera* densities. Throughout
6 experiment 1, body length (BL), ln(*Buchnera* gene titer), and ln(EF1 α titer) were positively
7 correlated [BL vs. ln(*Buchnera*): $r = 0.72$; BL vs. ln(EF1 α): $r = 0.66$; ln(*Buchnera*) vs.
8 ln(EF1 α): $r = 0.79$, all $P < 0.001$; $n = 58$).

9 In experiment 2, in the offspring generation of the aphids subjected to heat shock,
10 significant differences were detected in all three measured traits. The offspring of the RH-
11 exposed aphids were significantly smaller than the offspring of the CT-exposed aphids
12 (Figure 1D). The offspring of the aphids exposed to the RH treatment contained lower
13 densities of both EF1 α (Figure 1E) and *Buchnera* (Figure 1F) than the aphids exposed to the
14 CT and SH treatments. Compared to the aphids subjected to heat shock treatments, their
15 offspring generation had reduced EF1 α 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 α 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 α 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
26 the elevation did vary among the treatments (Table 1). When evaluated at equal body length,
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 *Buchnera* densities on EF1 α 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 α densities were compared
11 among the offspring from the aphids exposed to the three treatments, the slopes did not differ
12 ($F_{2,46} = 0.84$, $P = 0.44$) and neither did the elevations ($F_{2,46} = 0.03$, $P = 0.97$) (Figure 2B).

13 When *Buchnera* and EF1 α gene densities were represented on a log-log scale, the
14 regression slope of *Buchnera* on EF1 α 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 EF1 α 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 EF1 α 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
26 the RH treatment, $r = -0.03$, $P = 0.89$; $n = 19$) (Figure 3A).

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 α (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 EF1 α density, ANCOVA indicated that aphids exposed to a
9 single heat shock contained lower EF1 α 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 α 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).

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

1 EF1 α density, and body length in the offspring. This is probably because nymphs contain
2 developing embryos in their abdomen, so that heat shock to nymphs simultaneously has
3 adverse effects on their embryos. Temperature conditions used in the present study would
4 readily occur; e.g., in Sapporo, the daily maximum temperature ranged from 32-34 °C for five
5 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 α densities than their heat-shocked mothers. Similarly, the offspring of the
9 control aphids had greatly reduced EF1 α 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 EF1 α 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 EF1 α 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 EF1 α 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 α
21 density in *A. pisum* is liable to change, reflecting food conditions and population density
22 during aphid development.

23 When *Buchnera* gene and EF1 α densities are represented on a log-log scale, the
24 regression slope corresponds to the allometry coefficient. In the present study, the allometry
25 coefficients of the control aphids of the current generation and their offspring were less than
26 unity, showing that *Buchnera* gene density had negative allometry to EF1 α density. As an
27 index of the relative amount of *Buchnera* cells to the host cells, several authors have used a
28 ratio index, i.e., the number of *Buchnera* gene copies divided by the number of aphid gene

1 (EF1 α) 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).

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

1 bacteriocytes) (Whitehead & Douglas, 1993; Koga et al., 2012). A study attempting to count
2 aphid symbionts using flow cytometry indicated that although the number of *Buchnera*
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 α
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 EF1 α 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 EF1 α density).

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

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

1 2013). However, repetitive heat shocks would rapidly reduce the number of *Buchnera* cells,
2 and thereby could negatively affect the development of the next generation as well as the
3 current generation. We conclude that frequent occurrences of high temperatures accompanied
4 by global warming have a great impact on aphid development and reproduction and could
5 possibly lead to population extinction at a local scale.

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1

2

3 **Figure captions**

4 **Figure 1** Mean (\pm SE) (A, D) body length (mm), (B, E) ln[elongation factor 1-alpha (EF1 α)
5 gene density], and (C, F) ln(*Buchnera aphidicola* gene density) in fourth instars of the (A-C)
6 current generation (n = 19-20) and (D-F) offspring generation (n = 14-22) of *Acyrtosiphon*
7 *pisum* exposed to a single heat shock (SH), repeated heat shocks (RH), or control treatment
8 (CT). Different letters near the means within a panel indicate significant differences between
9 treatments (Tukey-Kramer method: P<0.05).

10

11 **Figure 2** Relationship between ln(*Buchnera aphidicola* gene density) and ln[elongation factor
12 1-alpha (EF1 α) gene density] in fourth instars of the (A) current generation (n = 19-20) and
13 (B) the offspring generation (n = 14-22) of *Acyrtosiphon pisum* exposed to a single heat
14 shock (SH), repeated heat shocks (RH), or control treatment (CT). Based on ANCOVA, the
15 common slope is displayed for the three treatments. SH and RH treatments rendered some
16 aphids sterile; gene densities of sterile adults are superimposed, but not included in analyses
17 (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 *Acyrtosiphon 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 (EF1 α) and
 2 *Buchnera aphidicola* gene densities on fourth-instar pea aphid body length

Independent variable	Dependent variable						
	d.f.	EF1 α			<i>Buchnera</i>		
		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

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).

Current generation

Offspring generation





