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Author(s)	PARVEN, NOUSHEEN
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Effects of high temperature stress on aphid growth and reproduction: responses of clones with diverse genetic backgrounds and aphid symbionts to heat stress

（高温ストレスがアブラムシの成長と繁殖に与える影響：多様な遺伝的背景を持つクローンおよびアブラムシ共生生物の熱ストレスに対する反応）

According to global warming scenarios, it is estimated that the frequency and intensity of high temperature events during summer will increase in the future. In this study, I firstly investigated the effects of high temperature stress on the growth and reproduction of aphids by selecting clones with various genetic backgrounds. Aphids are major pests causing damages to crops and trees, and thus it is important to evaluate the effects of high temperature stress from the viewpoint of pest management. In addition, aphids harbor obligate intracellular endosymbiont *Buchnera*, and symbiosis with *Buchnera* has a critical effect on aphid survival and reproduction. As a second objective, I evaluated the copy numbers of genes specific to *Buchnera* and host aphids after heat shock to analyze how high temperature stress affects the growth of both *Buchnera* and the host aphids.

In Chapter 1, I investigated the relationships among aphid clones, high temperature treatments, and temperature acclimations. Two clones of *Acyrthosiphon pisum*, collected in Sapporo and Tokyo, and three clones of *Megoura crassicauda,* collected in Sapporo, Tokyo and Nagasaki, were used for experiments. Newly hatched nymphs were treated with a single heat shock (35°C for 6 h), repetitive heat shock (35°C for 6 h for 3 consecutive days), or a control (constant 20°C). Before the heat shock treatment, the clones were reared for four or more generations at constant 20°C, constant 22°C, or increasing temperature from 22°C to 24°C in order to investigate the possibility of acquiring high temperature tolerance (acclimation effects).

I tested whether heat shock reduces the fitness traits and induces different responses between the two species and clones, and whether acclimation to higher rearing temperature mitigates the impact of heat shock. Information about the survival and fecundity was obtained from control and all heat-shock aphids and were used separately for statistical analysis. Differences in the survival after heat shock between species were tested using a generalized linear mixed model (GLMM) that treated clonal differences as a random effect, assuming a binomial error structure. After the analysis, I tested the effects of the interaction between clones and heat treatments for each species using GLMM, in which clones were treated as a random effect. The effects of rearing in a higher temperature prior to heat shock, that is acclimation effects on survival were tested

for each species using a two-factorial design (rearing at 20℃ or 22℃, and single or repetitive heat shock) with clones treated as a random effect. Finally, for each clone, differences in the survival among the five treatments were tested using Tukey-Kramer method. The results were shown in a figure with different letters indicating significant difference at P= 0.05 among treatments.

The Sapporo clone was more susceptible to the high temperature treatment than the Tokyo clone in *A. pisum*, and both survival rate and the number of offspring were significantly reduced in the repetitive heat shock treatment. *M. crassicauda* was more tolerant to heat shock treatments than *A. pisum*. Especially in the Tokyo and Nagasaki clones, no significant decrease in survival was observed. The latitudes of the collection sites were related to the thermotolerant ability of *A. pisum* and *M. crassicauda* clones. Analysis of the relationship between the pretreatments and the heat shock treatments showed that rearing at 22°C for four generations mitigated the effects of heat shock only in the Sapporo clone of *A. pisum*. This result suggests that the acclimation effect may be an adaptation in aphids inhabiting low temperature regions. However, when the temperature was increased from 22°C to 24°C for six generations to promote acclimatization, there was a significant decrease in the number of offspring compared to the control in *A. pisum*. In addition, no recovery in fecundity was observed in *M. crassicauda*. These results indicate that the aphids of both species are unlikely to acquire high temperature tolerance through acclimatization, and that the population will be more damaged by continued higher temperatures in summer.

In Chapter 2, I investigated the relationship between inbreeding/outbreeding and heat shock treatments. In aphids, the possibility of intra-clonal mating is high, and this may result in the production of clones of the selfing origin. Two clones were selected as parents, and the sexual generation was induced under a low temperature and short-day conditions to obtain eggs. New clones were established from nymphs hatching from the overwintered eggs, and two sets of the parental clone–selfed progeny clone were prepared.

To prepare selfed clones from the parental clone, an apterous adults (G0 generation) from a parental clone were transferred from a long day condition (16L:8D photoperiod) at 20℃ to a short-day condition (8L:16D photoperiod) at 15°C and allowed to reproduce. First instar nymphs produced in this condition grow to apterous adults (G1 generation), which produce male and oviparous females in addition to a small number of viviparous females. G1 females produce sexual female first and then males after a short suspension of reproduction. The produced winged males and oviparae female of the same clone were allowed for sibling mating according to the methods of Dedryver et al. (2013). Fertilized eggs produced from clonal mating for 10 days at 15°C and then transferred to 3℃ for overwintering for 3 months (Akimoto, 2006; Huang & Caillaud, 2012). The laboratory-generated selfed lines were Cracca E selfed and C07C selfed (CRA-SF and C07C-SF), which were maintained at a similar condition as their respective parent clones

(CRAE and C07C) at 20°C and, in a 16L:8D photoperiod for parthenogenetic reproduction. I have maintained the parental clones and selfed offspring clones for at least four generations at a low density by transferred five nymphs on newly seedlings of broad bean to avoid overcrowding effects before conducting the heat-stress experiment.

These clonal pairs were subjected to one of the single heat shock treatment, the repeated high temperature treatment, and the control treatment (constant 20°C). The results showed that there was no significant difference in the survival rate and the number of offspring between the parent and the selfed progeny at 20°C. However, the survival rate and the number of offspring of the selfed progeny were significantly lower than those of the parent clones under the repeated heat shock treatment. These results demonstrate for the first time in aphids that the progeny of the selfing origin are likely to be driven to extinction due to pronounced inbreeding depression under high temperature stress, even if their reproductive performance is comparable to that of the parent clone under benign conditions.

In Chapter 3, the effects of heat shock on the cell proliferation of the host aphid and the symbiont *Buchnera* were investigated. When the aphids reached the fourth instar stage after heat shock, the gene copy numbers of the host aphid and *Buchnera* were investigated. Quantitative PCR was used to count the copy number (density) of the aphid nuclear gene (elongation factor 1-alpha) and the copy number (density) of the *Buchnera* 16S-rRNA gene in the current and next generation after the heat shock treatment.

Twenty apterous pea aphid females were randomly selected from the stock culture and their reproduction was checked daily. Five first instar nymphs of similar size were selected and transferred onto a new seedling within 6 h after birth. Seedlings with five nymphs were randomly allocated to one of three treatments: the single heat shock treatment (SH, n = 6), the repetitive heat shock treatment (RH, n = 12), or the control treatment (CT, n = 8). In the SH, first instars were transferred from 20°C 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 the 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, first instars were continuously maintained at 20°C. Because mortality is high as a result of RH treatments, the initial sample size was higher. Replicates from the three treatments were prepared on the same day, and they were subjected to the heat treatments simultaneously. I used fourth instar nymphs for molecular analyses. Our observation showed that heat shock delayed larval development. Thus, to obtain fourth instars, 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 analysis (current generation) as describe below. Aphid mortality and survival to adulthood were also recorded.

In a separate experiment, after the heat shock treatments performed as described above (SH, RH, and CT) first-in-star 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 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 were used for body size measurement and molecular analyses (offspring generation) as describe below. Some of the heat-shocked aphids in the SH and the RH 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) were randomly selected and used for molecular analyses.

For 128 aphids collected, total DNA was extracted and purified using Cica Geneus total DNA preparation kits for tissue (Cica-reagent, Kanto chemical, 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. I 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 8Nippon Genetics). For both genes, 10-fold serial dilutions were used as standards. Three replicates were prepared for each aphid sample in qPCR (Mx3005P; Agilent Technologies, Tokyo, Japan) assay according to the methods of Lu et al. (2014) and Sugimoto et al. (2015), and variation among replicates was compared with variation among aphids.

A portion of the *Buchnera* 16S-rRNA gene was amplified by the primer-probe method; 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′) was used for the quantification of the *Buchnera* 16S-rRNA gene. The probe was labeled with FAM on the 5′ and TAM on the 3′. The 10 µL of reaction mixture for the 16S-rRNA gene included 5 µL of Kapa Probe Fast qPCR Master Mix, 0.2 µL of Kapa Rox Low (Nippon Genetics, Tokyo, Japan), 0.2 µL of each primer (0.2 pmol/µL), 0.2 µL of probe (0.2 pmol/µL), 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 Mx3005P qPCR system, and used for making a standard curve.

A primer set of APEF1-alpha 107 F (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 for the qPCR of EF1α gene. The 10 μL of reaction mixture for EF1α gene contained 5 μL of Kapa SYBR Fast qPCR Mix (Nippon Genetics, Tokyo, Japan), 0.09 μL of each primer (0.09 pmol/μL), 3.82 μL ddH2O, 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 20s, 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 by using gBlocks Gene Fragments (IDT, Tokyo, Japan).

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 the 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 the 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, heat shock affected the three traits differently. Although there were positive correlations between the aphid body length, *Buchnera* gene density, and EF1α density, ANCOVA indicated that aphids exposed to a single heat-shocked 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 to high temperature, 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 probably leads to sterility. Some fourth instars subjected to the repetitive heat shock treatment exhibited low *Buchnera* gene densities comparable to sterile adults; these nymphs are thus likely to become sterile when they enter adulthood. Thus, temperature conditions in mid-summer Japan would possibly lead to reduced reproduction through decreased density of *Buchnera*. Also, in the tree-dwelling aphid, *Tuberculatus macrotuberculatus*, *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 marked a 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; for example, in Sapporo, the daily maximum temperature ranged from 32°C to 34°C continued 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 in their mothers. However, the offspring showed much lower EF1α densities than did their heat-shocked mothers. Similarly, the offspring of the control aphids had greatly reduced EF1α densities compared with their mothers (control aphids). 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 a broad bean seedling in a group of five aphids, whereas the offspring fourth instars were reared in a crowded colony of (on average 44 and 22 nymphs for control and single heat-shocked treatment, respectively), produced during 7 days by a single adult. These crowded conditions likely affected *Buchnera* and EF1α 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* gene or EF1α 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 also 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 (from 10°C to

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 (from 15°C to 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 aphid fresh weight or per protein content increased with decreasing body mass, suggesting that *Buchnera* density per 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 decreases 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 wide 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 negative correlation between relative *Buchnera* density and aphid biomass, which is in turn positively correlated with 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 the 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 a nymphal aphid 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 different clones have different allometry between the two gene densities, and whether different allometry is related to difference 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 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 ability to adjust the *Buchnera* cell number even though the developmental condition is altered by different densities and transgenerational effects.

If heat shock to aphids 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.

In summary, this paper provides a multifaceted analysis of the effects of high temperature stress on aphids and provides important insights into the increase or decrease in local aphid populations under a global warming scenario.