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Aphid facultative symbionts aid recovery of their obligate symbiont and their host after heat stress

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Abstract

Environmental conditions affect insect fitness, with many species constrained by specific temperature ranges. Aphids are limited to temperate climates and it is hypothesized that this is partly due to their heat-susceptible obligate nutritional symbiont *Buchnera*. Aphids often carry additional facultative symbionts which can increase the host's fitness after heat stress. Here we used the pea aphid (*Acyrthosiphon pisum*) and three of its facultative endosymbionts (*Candidatus* Regiella insecticola, *Candidatus* Fukatsuia symbiotica (X-type; PAXS), and *Candidatus* Hamiltonella defensa) to investigate how these species respond to heat stress and whether their presence affects the fitness of the host or the obligate symbiont. We exposed aphid lines to a single high temperature event and measured lifetime fecundity and population densities of both obligate and facultative symbionts. Heat shock reduced aphid fecundity, but for aphids infected with two of the facultative symbionts (*Regiella* or *Fukatsuia*), this reduction was less than in uninfected aphids. The population density of *Buchnera* was also reduced after heat shock, and only recovered in aphids infected with *Regiella* or *Fukatsuia* but not in uninfected aphids or those with *Hamiltonella*. Although heat shock initially reduced the densities of two of the facultative symbionts (*Hamiltonella* and *Fukatsuia*), all facultative symbiont densities recovered by adulthood. Two of the facultative symbionts tested therefore aided the recovery of the obligate symbiont and the host, and we discuss possible underlying mechanisms. Our work highlights the beneficial effects of protective symbionts on obligate symbiont recovery after heat stress and how facultative symbionts may affect the wider ecological community.

1 Introduction

It is well established that infection with bacterial symbionts can affect an insect host's biology. Reproductive fitness, insect behavior, immune pathway function, and responses to natural enemies may all be influenced by the presence of endosymbionts (Dion et al., 2011; Gerardo and Parker, 2014; Vorburger, 2014; Martinez et al., 2015). By improving the ecological fitness of a host through raising its immunity to natural enemies, or by enhancing its tolerance to environmental stress, a

vertically transmitted symbiont increases its own fitness (Oliver et al., 2005; Brownlie and Johnson, 2009).

Rising global temperatures are already affecting insect populations; there is evidence of range shifts (Parmesan and Yohe, 2003), changes in phenology (Walther et al., 2002) and interactions with predators and parasitoids (Harrington et al., 1999; Schmitz and Barton, 2014). For several insect groups infection with various bacterial symbionts has been shown to enhance resistance to temperature stress (Corbin et al., 2017). These effects may be direct symbiont-mediated host protection (Montllor et al., 2002; Neelakanta et al., 2010; Brumin et al., 2011) or indirect effects of temperature on the symbiont itself (Chen et al., 2009; Bordenstein and Bordenstein, 2011).

There are a number of hypotheses for the mechanisms underlying indirect symbiont-mediated protection from heat. Infection with symbionts has been shown to increase the expression of immune system genes (Laughton et al., 2013), and it is hypothesized that this immune response controls the bacteria, restricting growth or location and protecting the host from microbial over-proliferation (Kwong et al., 2017; Maire et al., 2018). It may also bestow temperature tolerance as a by-product. For example, infection of *Rickettsia* in whiteflies leads to the upregulation of stress-response genes in the host and thus increases survival of the insect under heat shock (Brumin et al., 2011); similarly, insects infected with bacterial symbionts often produce more immune cells than those that are uninfected (Schmitz et al., 2012; Weiss et al., 2012; Laughton et al., 2013; Kim et al., 2015). There are close links between insect responses to heat and to infection - many heat shock proteins are chaperones that aid protein production and refolding post-stress, and they may also enhance immune responses (Young et al., 1993).

Instead of indirectly affecting the host's stress or immune responses, symbionts may themselves produce and release heat shock proteins or metabolites that directly protect the host or other microbes that the host depends on (i.e. obligate symbionts) (Burke et al., 2010a). Obligate symbionts are often a thermal "weak link" and more susceptible to temperature extremes than their hosts (Corbin et al., 2017; Shan et al., 2017; Zhang et al., 2019). Shielding an obligate symbiont from thermal damage would therefore benefit both the host and all of its symbionts. For example, in pea aphids that experience heat shock, the density of the obligate nutritional symbiont *Buchnera aphidicola* is usually reduced, but is maintained at near normal levels in aphids that carry the facultative symbiont *Candidatus* Serratia insecticola (hereafter *Serratia*) (Burke et al., 2010). It is also plausible that facultative symbionts might be directly protecting the host by replacing an obligate symbiont that is no longer able to perform its function. When the obligate symbiont *Buchnera* is removed using antibiotics at benign temperatures, *Serratia* in pea aphids moved into the bacteriocytes vacated by *Buchnera* and subsequently allow the stressed aphid to survive and reproduce (Koga et al., 2003, 2007).

Pea aphids, *Acyrthosiphon pisum*, are a model system for understanding how facultative symbionts protect their hosts from thermal stress. They and their obligate symbiont are typically intolerant to heat in laboratory populations (Dixon et al., 1987; Dunbar et al., 2007), but three of their eight potential facultative symbionts (*Serratia*, *Candidatus* Fukatsuia symbiotica and *Candidatus* Hamiltonella defensa; hereafter *Fukatsuia* and *Hamiltonella*, respectively) are known to improve survival or reproduction after heat shock (Montllor et al., 2002; Koga et al., 2003; Russell and Moran, 2006; Heyworth and Ferrari, 2015). The obligate symbiont *Buchnera* synthesises essential amino acids for the host, which are required for aphids to thrive on their imbalanced diet of plant phloem sap (Douglas, 1998). *Buchnera* has a highly reduced genome (Moran, 1996; Gómez-Valero et al., 2007) and some genotypes are susceptible to high temperatures; under heat stress, just five protective heat shock proteins are deployed (Wilcox et al., 2003) compared to over 75 in its free

living relative *Escherichia coli* (Carruthers and Minion, 2009) and during severe heat shock *Buchnera* can be killed.

While the costs and benefits of infection are being explored in a broad spectrum of insect species, relatively little is known about how different facultative symbionts confer increased heat tolerance to their hosts, and how these mechanisms vary depending on symbiont species. Understanding how insects can and will respond to increases in temperature is vital to accurately model current and future populations. We investigate whether three common facultative symbionts of the pea aphid (*Candidatus* Regiella insecticola (hereafter *Regiella*), *Fukatsuia* and *Hamiltonella*) protect the host and how they respond to heat stress themselves. Importantly, we test whether the protection from the effects of heat co-occur with the protection of the obligate symbiont *Buchnera*. We test whether the facultative symbionts directly protect *Buchnera*, allow it to recover after heat stress or protect the host by replacing *Buchnera* and whether this mode of protection is similar for all tested facultative symbionts.

2 Materials and Methods

2.1 Aphids and symbionts

Rapid, asexual reproduction results in clonal lines of aphids that can be kept indefinitely under long-day conditions. This allows the manipulation of facultative symbiont presence through antibiotic curing or artificial infections while maintaining an essentially identical aphid genotype. Two pea aphid genotypes were used for this study, both collected from the UK (Table S1). Genotype 218 was collected naturally infected with *Fukatsuia* and *Hamiltonella,* and was cured more than a year before use. This was achieved by feeding young aphids with broad bean leaves suspended in a tube of antibiotic solution (0.5% Gentomicin, 1% Ampicillin, 0.5% Cefotaxime in distilled water) over four days (McLean et al., 2011). Genotype 200 was collected naturally uninfected, harbouring no known facultative symbionts. All aphid lines were screened for *Hamiltonella*, *Regiella*, *Serratia symbiotica*, *Fukatsuia*, *Spiroplasma* sp., *Rickettsia* sp. and *Rickettsiella viridis* following protocols in Tsuchida et al. (2010) and Ferrari et al. (2012) to ensure that they had the appropriate symbiont infection and were not infected with any other known facultative symbionts. The symbiont infections were regularly checked to detect possible contamination. The symbiont-specific PCR primers can be found in Table S2. The PCR mix comprised 6.25 µl BioMix (Bioline), 0.1 µl (20 µM) of forward and 0.1 µl (20 µM) reverse primer, 5.55 µl distilled water and 1.0 µl sample DNA. The PCR reaction was performed at 94°C for 2 minutes, followed by 35 cycles of: 94°C for 30 seconds, 55°C for 30 seconds and 72°C for one minute. It concluded with 6 minutes at 72°C and then cooled the sample to 4°C indefinitely. PCR products were run on a 1% agarose gel and the presence of a band confirmed the presence of the symbiont.

Five aphid lines were used in the experiment, two uninfected with facultative symbionts (200 and 218) and the remainder infected singly with one of three facultative symbionts, *Regiella*, *Fukatsuia* and *Hamiltonella* (Table S1). *Regiella* was injected into aphid genotype 200, while the other two symbionts were injected singly into genotype 218. These five aphid lines comprise three pairwise comparisons between uninfected and infected aphids, with the uninfected line of 218 used in two comparisons. This design aimed to compare host fitness and symbiont densities within each pair across the same aphid genetic background and thus we conducted no analyses across multiple pairs. These specific isolates of symbiont were chosen because preliminary results indicated that they were likely to provide heat shock protection.

To produce these infections of *Hamiltonella*, *Regiella* and *Fukatsuia* we used hemolymph injections

from infected donor aphids (Table S1). Hemolymph was extracted from donor aphids under a microscope using glass needles and re-injected into the appropriate aphid line and surviving aphids raised to adulthood. Glass needles were pulled from Kwik-Fil™ borosilicate glass capillaries (1B100-4, World Precision Instruments, 1 mm diameter) using a P-97 Flaming/Brown micropipette Puller (Sutter Instrument Co.). The offspring of the surviving aphids were tested for the successful establishment of the new infection when they were adults, and these aphid lines were retested regularly to ensure the maintenance of the new aphid-symbiont combinations. All injected lines had been maintained in the laboratory for at least a year before being used for experimental assays.

2.2 Heat shock protocol

This assay was designed to understand symbiont dynamics after aphids have been exposed to heat shock. Aphids were exposed to either a single peak of high temperatures or were maintained at a steady control temperature. The 'heat shock' temperature chosen was 38.5°C, which was based on a series of pilot studies (data not shown). Our aim was to find a temperature that had a strong negative effect on fitness, but at which approximately half the individuals in an aphid population still survived. The aim was to explore the phenotypes of the symbionts and not to model natural situations directly. The temperature experienced by aphids near the ground is often considerably higher than meteorological records and depends, for example, on aspect and slope of the site, but it is likely that aphids are exposed to similarly high temperatures in Northern England on hot summer days (Bennie et al., 2008; Suggitt et al., 2011).

To produce age-controlled populations of each of the five lines, groups of young adults were placed into petri dishes (9 cm diameter) that contained a single broad bean (*Vicia faba* var. Sutton Dwarf) leaf, placed in 2% agar. *V. faba* is a host plant that almost all pea aphids perform well on (Ferrari et al., 2008). The adults were left to reproduce for 24 hours at 20°C, and the offspring were subsequently put onto two-week old *V. faba* plants in groups of 50 and enclosed in a vented, transparent cage. On the following day (aphid age 24-48 h) the populations were moved into cabinets where they were either exposed to heat stress or 20°C as a control. Temperature cabinets were rigorously checked before and during the experiments to ensure even distribution of heat and the same relative humidity (50%) in both cabinets. Plants containing aphids were also placed in a randomized block pattern within the cabinet to remove any potential effects of uneven heat distribution.

While the control treatment was left at 20°C, the temperature in the heat treatment was increased from 20°C to 38.5°C steadily over the course of 2 hours, held at 38.5°C for four hours, and then decreased back to 20°C over a further two hours. Surviving aphids from both treatments were moved onto fresh two-week-old plants on the day after heat shock to mitigate any temperature effects on the plant itself. These plants were moved into a different controlled-temperature room (20°C), where aphids from both heat treatments were kept together until being collected for analysis.

Aphids were removed to measure symbiont density at two time points. The first was 24-26 hours after the start of the peak heat shock period (when the aphids were three days old), and the second eleven days post heat shock (when the aphids were 14 days old, approximately six days after an aphid would usually begin reproducing). These two time points were chosen to investigate symbiont densities immediately after stress, and to test if recovery by the onset of reproduction was possible. *Buchnera* densities are known to decrease as aphids age (Simonet et al., 2016) and so this second time point was chosen to be the potential highest density of *Buchnera* during an aphid development.

The aphids were flash frozen using dry ice and kept at -80°C until DNA extraction. In addition, one

surviving apterous individual from each group was placed on a petri dish with a *V. faba* leaf (as above) to measure the number of offspring produced. These dishes were refreshed every 3-4 days to ensure healthy *V. faba* leaves. Offspring counts continued until all aphids had died, measuring total lifetime fecundity. There were 5-6 replicates for fecundity counts and symbiont density for each of the five aphid lines in each treatment (i.e. 10-12 replicates in total for each line), these were performed in two temporal blocks with approximately half the replicates of each treatment in each block.

2.3 qPCR protocol

DNA was extracted from the aphids after samples were defrosted at room temperature. Aphids were homogenised in a 200 µl 5% Chelex solution made in distilled water. 10 µl of proteinase K (10 mg/ml) was added per sample, and samples were incubated for 6 hours at 56°C to facilitate digestion. They were then 'boiled' at 100°C for ten minutes before being centrifuged at 13,000 rpm for 3 minutes and the supernatant containing the DNA pipetted into a clean 1.5 ml Eppendorf tube which was stored at -20°C until use. Five aphids per replicate were pooled to generate a sample for the first time point (24-26 hours after heat shock) and one aphid for the second time point (eleven days later).

Samples were run in duplicate using SYBR® Green reagent on a StepOnePlus™ Real Time PCR machine (Applied Biosystems). Each reaction consisted of 10 µl FAST SYBR 2x mastermix (Applied Biosystems), 1 µl forward primer (7 µM), 1µl reverse primer (7 µM), 6 µl nuclease-free water and 2 µl DNA sample. qPCR primers for *Regiella, Fukatsuia*, *Hamiltonella* and the aphid housekeeping gene elongation factor-1 alpha (EF-1α) (Table S2) were tested to ensure high efficiency and similarity between primer sets. Melt curves were performed on each plate to ensure the primers were specific to each target and only bound once. Cycling conditions were 95°C for 20 seconds, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. The melt curve involved a further 95°C for 15 seconds, 60°C for one minute and then a gradual increase to 95°C over 15 minutes. Each 96-well qPCR plate was analysed using StepOne Software v2.2.2 (Applied Biosystems) and Cycle threshold (Ct) values were obtained by comparing each primer sample to a single standard curve of known concentration and using identical threshold and baseline levels for each primer target across plates. Standard curves were created by amplifying positive control samples using PCR, calculating DNA concentrations using a High Sensitivity DNA Assay on a 2100 Bioanalyzer system (Agilent), and then serially diluting the sample 1:10 with distilled water to create a 5-sample curve comprising known concentrations decreasing from 10 pmol/ml. Samples with Ct values over 30 were classed as negative, confirmed by our negative controls. This corresponds to a copy number of less than 52 per 2 µl sample for all primers, and is below the threshold of detection. Where the difference in Ct values between technical replicates was greater than 1.5, the sample was either rerun or not used in the analysis.

The standard curves were used to calculate the DNA concentration of each sample, and this was converted into copy numbers per 2 µl of DNA extract. To control for aphid size and extraction quality, copy numbers for each sample were presented relative to those of a housekeeping aphid gene as a control, giving a ratio of symbiont copy numbers to aphid copy numbers.

2.4 Statistical analysis

Data were analysed using the R software v. 3.4.1 (R Core Team, 2018). Since our core question was to test whether the three symbiont species can provide heat shock protection, but not to compare the extent of this protection, the data were analysed separately for each symbiont species. Thus, in each analysis the infected line was compared with the same uninfected aphid genotype, within and across

heat treatments. The data for the uninfected line 218 was therefore used twice, paired with line 218 infected with *Hamiltonella* or *Fukatsuia*. Similarly, we analysed the two time points separately because symbiont densities change during aphid development, which would complicate the interpretation of the analysis.

Lifetime fecundity of the set of *Regiella* lines was analysed using a general linear model assuming a normal error distribution. The number of offspring was the response variable, and temporal block, facultative symbiont presence, heat treatment and the interaction between symbiont presence and heat treatment were the explanatory variables. The sets of *Fukatsuia* and *Hamiltonella* lines were analysed with a non-parametric Kruskal-Wallis test, because the model assumptions of parametric models were not met. This was followed by Wilcoxon tests to identify differences between specific treatments.

The densities of the symbionts were also analysed with a general linear model assuming a normal error distribution. This was split into six analyses, separate for the lines relating to each symbiont species at each time point, to simplify the interpretation. For *Buchnera* densities, the explanatory variables were again temporal block, facultative symbiont presence and heat treatment as well as the interaction between the latter factors. For the densities of the facultative symbionts, only block and heat treatment were explanatory variables. In most cases model assumptions were met without transforming the data, only the *Buchnera* densities at the first time point in the *Regiella* lines were log-transformed. For *Regiella* densities at the first time point and *Buchnera* densities in the set of *Regiella* lines at the second time point, Kruskal Wallis and Wilcoxon tests were used as described for the fecundity data.

For all general linear models, post-hoc tests were only performed when the factor or interaction was significant in the main analysis. This was conducted using the R package "phia" (Helios and Fox, 2015), with Holm's correction for multiple comparisons.

3 Results

3.1 Effects of facultative symbionts on fecundity after heat shock

We exposed aphids to a short spike of high temperature and measured facultative and obligate symbiont densities and aphid fitness after one and eleven days. As expected, heat shock decreased the number of offspring produced in an aphid's lifetime in all three sets of lines (*Regiella* $F_{1,19}$ = 103.23, $P < 0.001$; *Fukatsuia*: $W = 109$, $P = 0.03$, *Hamiltonella*: $W = 136.5$, $P = 0.001$; Figure 1). However, the extent of this decrease was modified by the presence of *Regiella* and *Fukatsuia* (*Regiella,* symbiont × heat treatment: $F_{1,19} = 5.78$, $P = 0.03$; *Fukatsuia*: heat treatment in uninfected lines $W = 36$, $P = 0.004$ and in infected lines $W = 13$, $P = 0.48$). *Fukatsuia* provided the greatest protection from heat as there was no difference in the fecundity of the infected lines in the control and heat shock treatment, whereas there was a greater reduction in fecundity in the uninfected aphids than in the infected aphids for the *Regiella* lines. In contrast, there was a similar decrease in fecundity for both uninfected and infected *Hamiltonella* lines following heat shock (uninfected: $W = 36$, $P = 0.004$; infected: $W = 34.5$, $P = 0.008$; Figure 1). At benign temperatures, two of the symbionts also affected lifetime fecundity: the presence of *Hamiltonella* increased lifetime fecundity ($W = 4$, $P = 0.03$), whereas *Fukatsuia* decreased it ($W = 34$, $P = 0.013$), and there was no difference for *Regiella* (Figure 1).

3.2 Facultative symbiont densities after heat shock

We measured the densities of the three facultative symbionts at two time points after exposure to heat, 24-26 hours and 11 days post-heat shock (Figure 2). Compared to non-heat shocked controls the densities of two of the symbionts, *Fukatsuia* and *Hamiltonella*, were lower on the day after heat shock (*Fukatsuia*: $F_{1,8} = 65.05$, $P < 0.001$, *Hamiltonella*: $F_{1,8} = 6.64$, $P = 0.03$), whereas densities of *Regiella* are unaffected (W = 28, p=0.13; but note that this is significant in a less conservative parametric test). By the second time point, taken when the aphids were young adults, there was no difference between population densities in heat stressed or control aphids for any of the three facultative symbionts (*Fukatsuia*: $F_{1,8} = 0.35$, $P = 0.57$, *Hamiltonella*: $F_{1,7} = 0.95$, $P = 0.36$, *Regiella*: $F_{1,6} = 0.01$, $P = 0.91$; Figure 2), suggesting that heat did not have long-term effects on facultative symbiont populations.

3.3 Obligate symbiont densities under heat shock

Compared to the control treatment densities of *Buchnera* were decreased on the day after heat shock in each of the three pairs of lines, regardless of facultative symbiont infection (*Regiella* lines: $F_{1,19} = 80.36$, $P < 0.001$, *Fukatsuia* lines: $F_{1,18} = 70.71$, $P < 0.001$, *Hamiltonella* lines: $F_{1,18} = 56.07$, $P < 0.001$; Figure 3). Regardless of treatment, *Buchnera* densities were higher in the lines harbouring *Fukatsuia* ($F_{1,18} = 33.86$, $P < 0.001$) or *Hamiltonella* ($F_{1,18} = 24.52$, $P < 0.001$) compared to uninfected lines, an effect that was not seen in the *Regiella* lines ($F_{1,19} = 0.15$, $P = 0.71$). For the *Fukatsuia* and *Hamiltonella* lines there was also a significant interaction between symbiont presence and heat treatment (*Fukatsuia*: $F_{1,18} = 9.57$, $P = 0.006$; *Hamiltonella*: $F_{1,18} = 4.80$, $P = 0.04$): in both cases, *Buchnera* densities in the control treatment were higher in lines with facultative symbionts compared to uninfected aphids but there was no difference between these lines after heat shock. The interaction was not significant for the *Regiella* lines ($F_{1,19} = 2.58$, $P = 0.13$) where the extent of the loss of *Buchnera* did not differ between infected and uninfected lines.

At the later time point, when aphids were young adults, heat shock again reduced *Buchnera* densities on average (*Regiella* lines: $W = 105$, $P = 0.004$; *Fukatsuia* lines: $F_{1,19} = 6.85$, $P = 0.02$; *Hamiltonella* lines: $F_{1,17} = 24.84$, $P < 0.001$). *Fukatsuia* presence on average also significantly increased the density of *Buchnera* regardless of treatment which was due to high *Buchnera* densities in the heat shocked aphids ($F_{1,19} = 7.89$, $P = 0.01$); a difference that was not found for *Regiella* ($W = 46$, $P = 0.37$) or *Hamiltonella* presence ($F_{1,17} = 0.17$, $P = 0.69$). Importantly, there was a significant interaction between symbiont infection and temperature in the *Fukatsuia* lines ($F_{1,19} = 7.55$, $P = 0.01$) and an equivalent effect in the *Regiella* lines (heat treatment in uninfected lines: $W = 36$, $P = 0.004$ and in infected lines: $W = 17$, $P = 0.42$) that was not seen for the *Hamiltonella* lines ($F_{1,17} = 1.95$, $P = 0.18$): *Buchnera* densities after heat shock were significantly reduced in uninfected aphids but not when *Fukatsuia* or *Regiella* were present.

4 Discussion

Our results show that different aphid symbionts can protect the aphid from heat and help the obligate symbiont to recover after heat shock. Infection with *Regiella* and *Fukatsuia* was closely linked to *Buchnera* recovery after heat shock and led to increased production of offspring compared to uninfected controls whereas there was no such protection in aphids infected with *Hamiltonella*. This pattern differs from other studies (Russell and Moran, 2006; Doremus and Oliver, 2017), which found that *Hamiltonella* but not *Regiella* or *Fukatsuia* provided heat protection. As different lines of insects and symbionts were used in these studies, it is likely that these protective effects are dependent on symbiont, host genotype or their interaction and are thus not a universal feature of

symbiont infection. The prevalence of heat protection may be overestimated here since we chose genotypes based on preliminary results.

Buchnera densities are closely linked to aphid fitness. Disrupting the obligate symbiosis by removing *Buchnera* leads to large reductions in offspring production and often host death (Koga et al., 2003; Akman Gündüz and Douglas, 2009) Overly high densities of *Buchnera* can also lead to a reduction in fitness (Chong and Moran, 2016), meaning that the relationship between density of the symbiont and number of offspring produced is not directly proportional. However, removal of *Buchnera*, via antibiotics or heat, as also shown in our results, generally leads to aphid sterility in the absence of facultative symbionts (Dunbar et al., 2007; Koga et al., 2007).

A key question that we addressed was whether the facultative symbionts protect *Buchnera* from the effects of heat, and if so whether *Buchnera* is directly protected or its recovery facilitated. The densities of both *Buchnera* and the facultative symbionts were reduced 24 hours after heat shock. In aphids carrying *Fukatsuia* or *Regiella*, these densities returned to levels that were similar to those in non-heat shocked controls, thus demonstrating a clear role of the facultative symbionts in the recovery of *Buchnera*. The most parsimonious interpretation of the observed pattern is that the facultative symbionts do not provide immediate protection, although it is possible that the decline in *Buchnera* DNA density occurs due to different processes in aphids with and without facultative symbionts. It is conceivable that *Buchnera* is only truly killed by heat inside the latter and growth is merely arrested in the former and thus some immediate protection occurs.

The patterns of obligate and facultative symbiont densities observed here suggest that a different mechanism underlies the heat protection provided by *Regiella* and *Fukatsuia* compared to that provided by *Serratia* (Montllor et al., 2002; Burke et al., 2010a). After heat shock, *Serratia* in pea aphids lyse and this coincides with metabolomic changes (Burke et al., 2010a). At the same time *Buchnera* densities are maintained at similar levels to those at benign temperatures (Burke et al., 2010a). In our experiments, in aphids with *Regiella* or *Fukatsuia*, *Buchnera* densities initially decrease. This demonstrates that the protection provided by the facultative symbionts is not instant and suggests that the protection is probably not due to a constitutively activated aphid stress response, but it is still possible that the facultative symbionts prime a stress response by the aphid that helps recovery later on.

The provision of heat-protective compounds, through either lysis or release from a live cell, is also consistent with our observations. Burke et al. (2010a) explored which metabolites are affected by heat treatment when *Serratia* lyse. They found three metabolic changes linked to the presence of the protective *Serratia* after heat stress, one of which is a decrease in concentration of the antioxidant indole-3-lactate, as well as two other unidentified metabolites (Burke et al., 2010a). The initial decline of *Fukatsuia* in our study suggest that lysis is likely in this symbiont (and possibly in *Regiella* where the decline is significant using less conservative statistics). However, the protective compounds released during this process act appear to act later than in *Serratia* (Burke et al., 2010a) as *Buchnera* also declines initially. In some cases, facultative symbionts can replace the function of an obligate symbiont (Koga et al., 2003, 2007) and this could be a way of protecting the host from the effects of heat. In our system, both *Buchnera* and the facultative symbionts recover demonstrating that this functional replacement is not a likely mechanism here.

Some strains of *Buchnera* are more resistant to heat than others (Dunbar et al., 2007; Moran and Yun, 2015) and it is possible that the two aphid genotypes here carry different *Buchnera* genotypes. Aphids in laboratory populations often carry *Buchnera* strains with the $ibpA^{12}$ mutant allele that are more sensitive to heat but have higher fitness (Burke et al., 2010b). We did not sequence *ibpA* in the

genotypes used here since we were interested in the effects of facultative symbionts and the *Buchnera* strain is the same within all our comparisons. It is worth noting that the *Fukatsuia* and *Hamiltonella* lines both had the same aphid and *Buchnera* genotype but only *Fukatsuia* protected, suggesting that the *Buchnera* strain does not bias our conclusions. However, in natural populations the absence of facultative symbiont infections is correlated with a higher incidence of this mutation (Burke et al., 2010b). Aphids thus have two mechanisms which protect *Buchnera* from heat: the absence of the heat-sensitive *ibpA*^{12} mutant allele and the presence of protective facultative symbionts. Facultative symbionts appear to confer low fitness in the presence of *ibpA*^{12} and are thus likely selected against in aphids with this mutation (Burke et al., 2010b). It thus suggests that in natural populations facultative symbionts may only be able to rescue the aphid *Buchnera* from heat in aphids that carry the heat-tolerant *ibpA*^{13} allele and this may explain the scarcity of *ibpA*^{12} in natural populations.

As well as the protective effect of *Fukatsuia* and *Regiella*, we observed interesting changes in symbiont densities at benign temperatures. *Fukatsuia* decreased fecundity, as shown previously (Heyworth and Ferrari, 2015; Doremus and Oliver, 2017); the densities of *Buchnera* confirm that this is not due to suppression of the obligate symbiont (Koga et al., 2003), which has been observed for a costly infection by *Rickettsia* in pea aphids (Sakurai et al., 2005). Surprisingly, infection with *Fukatsuia* or *Hamiltonella* leads to an increase of *Buchnera* population levels in younger aphids. This may benefit both the aphid and *Buchnera* when facultative symbionts are present, because additional nutrients may be required. It is possible that either the aphid upregulates *Buchnera* densities or that this strain of *Buchnera* responds to the presence of facultative symbionts by increasing its growth rate. In either case, the density of *Buchnera* cells in infected aphids is comparable to uninfected aphids once the aphids are adult.

The ability of facultative symbionts to protect obligate nutritional symbionts from heat stress has implications for the frequencies and spread of both the microbes and the insects themselves. Many phytophagous insects rely on obligate symbionts to provide essential nutrition, but these are often vulnerable to ecologically stressful situations (Bennett and Moran, 2015; Kikuchi et al., 2016) due to severe genome reduction during coevolution with their hosts (McCutcheon and Moran, 2011). This genome reduction probably led to the heat sensitivity that some facultative symbionts ameliorate, an example of a symbiosis rescuing another symbiosis. It seems improbable that this rescue resulted from close coevolution due to the relatively transient nature of facultative symbiosis infections (Smith et al., 2015).

As we and others (Montllor et al., 2002; Burke et al., 2010a) have shown, carrying certain isolates of facultative symbionts can protect obligate symbionts from a single, short exposure to heat, but it remains to be investigated whether this protection is also effective under long-term or regular exposure to extreme temperatures. The temperature-dependent fitness effects are likely to alter the frequencies of facultative symbionts in natural populations, but will do so in concert with other abiotic and biotic factors, including the frequencies of heat-tolerant *Buchnera* strains. The symbionts' ability to affect interactions between host and natural enemies is also well documented (Hrček et al., 2016). These interactions can be affected by a change in temperature, through an effect of temperature on the natural enemy itself (Roux et al., 2010; Nguyen et al., 2013) or on the interaction between host, symbiont and natural enemy (Guay et al., 2009; Jeffs and Lewis, 2013; Heyworth and Ferrari, 2016). In addition, both vertical and horizontal transmission frequencies of symbionts can be affected by temperature (Anbutsu et al., 2008; Osaka et al., 2008; Liu et al., 2019). A combination of temperature-dependent fitness effects and transmission dynamics is therefore a likely reason for the lack of a clear correlation between symbiont mediated benefits seen in

laboratory experiments and symbiont frequencies observed in the field (Oliver et al., 2014), and probably contributes to the geographic variation in the composition of facultative symbiont communities (Montllor et al., 2002; Tsuchida et al., 2002; Sepúlveda et al., 2017). There are, however, examples where the patterns based on laboratory experiments are observed: the frequencies of the aphid heat-protective symbiont *Serratia* are high in host populations in the warmer climes of Southern USA (Chen and Purcell, 1997; Montllor et al., 2002) and more generally in arid compared to temperate regions (Henry et al., 2013). Similarly, land temperature correlates with symbiont prevalence in midges (Morag et al., 2012).

Facultative symbionts alter insect fitness under stressful conditions and can affect not just the host, but also species that the host interacts with directly and indirectly (McLean and Godfray, 2016; Doremus et al., 2018). In extreme cases, hosting a defensive symbiont can lead to cascading extinctions and the collapse of entire communities (Sanders et al., 2016). Our work highlights how the host and its symbiont community is affected by temperature and that this temperature-dependency might result in changes of community interactions under climate change.

5 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6 Author Contributions

ERH, MRS and JF conceived the ideas and designed methodology, collected the data and wrote the manuscript and gave final approval for publication. ERH and JF analyzed the data.

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10 Data Availability Statement

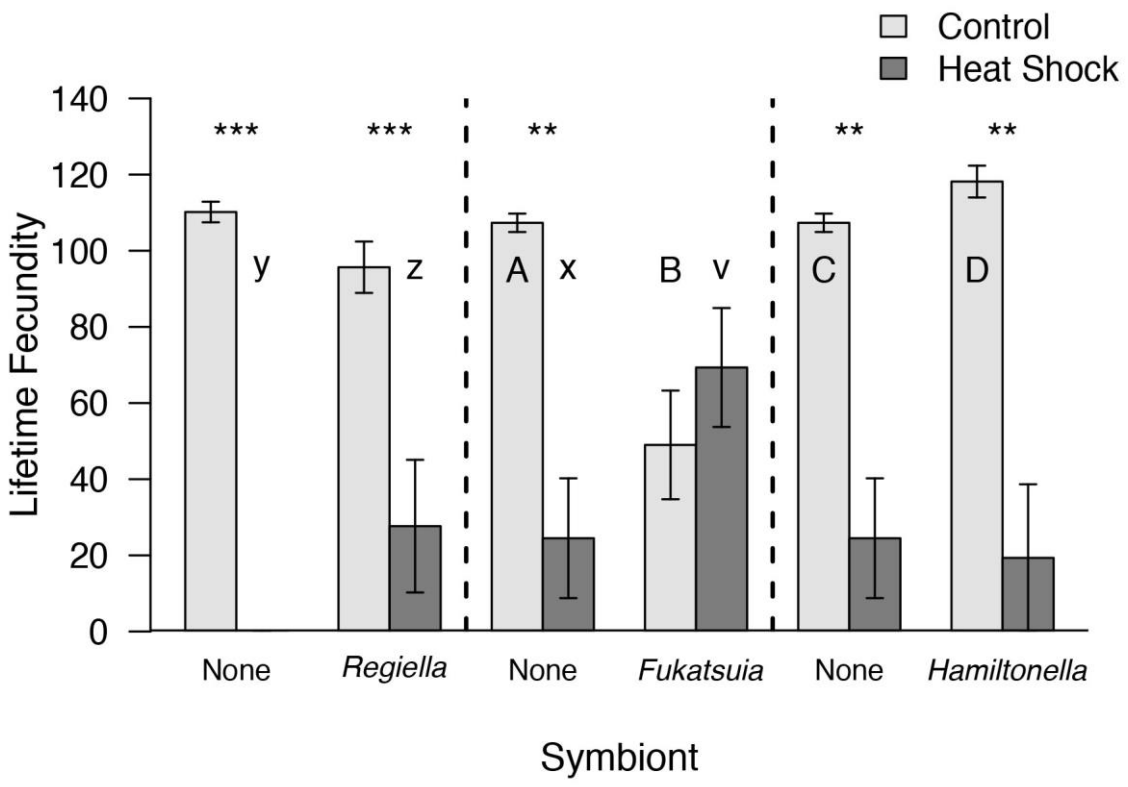
All datasets generated and analyzed for this study are included in the manuscript and the supplementary files.

11 Figure Legends

Figure 1. The effect of heat shock and facultative symbiont presence on the number of offspring produced by pea aphids. Aphids from genotype 200 that were uninfected or carrying *Regiella* are compared in the first panel. Aphids from genotype 218 that were uninfected or carrying *Fukatsuia* or *Hamiltonella* are compared separately in the second and third panels, respectively. In all comparisons, there was a significant effect of heat shock compared to controls, but no overall effect of symbiont presence. Means and standard errors are shown. Within each panel separately, upper case letters denote significant differences between aphids with different symbionts within the control treatment and lower case letters denote significant differences in the heat shock treatment The asterisks show significant differences between heat treatments for aphids of the same symbiont status (** $P < 0.01$ *** $P < 0.001$).

Figure 2. Densities of facultative symbionts in pea aphids after heat shock or in the control treatment (A) 24-26 hours after the onset of the heat shock and (B) eleven days after heat shock. Densities are shown as the copy number of the *gyrB* gene of the facultative symbiont relative to copy number of the aphid gene *EF1-α*. Means and standard errors are shown. Asterisks denote differences between heat treatments for aphids carrying a given symbiont (* $P < 0.05$, *** $P < 0.001$).

Figure 3. The effect of heat shock and facultative symbiont presence on densities of the primary symbiont *Buchnera* in pea aphids. In each panel *Buchnera* densities are shown one day or eleven days after heat shock. Uninfected aphids are compared to aphids carrying (A) *Regiella*, (B) *Fukatsuia* and (C) *Hamiltonella*. The uninfected replicates are the same for the *Fukatsuia* and *Hamiltonella* lines. Means and standard errors are shown. In all cases, there was a significant overall difference between the heat shock and control treatments. There was also a significant overall effect of symbiont presence for the Fukatsuia lines at both time points, and *Hamiltonella* lines one day after heat shock. These main effects are not illustrated on the figure. Posthoc results from significant interactions are denoted by upper case letters for significant differences between aphids with different symbionts within the control treatment, and lower-case letters denote significant differences in the heat shock treatment. The asterisks show significant posthoc differences between heat treatments for aphids of the same symbiont status (** $P < 0.01$, *** $P < 0.001$).



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666 Figure 1

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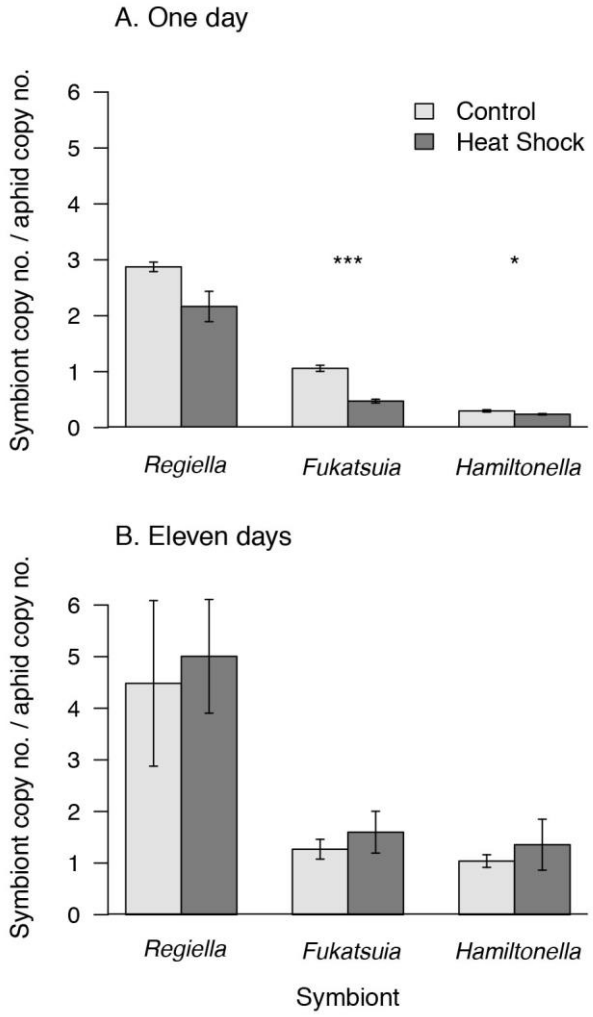

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670 Figure 2

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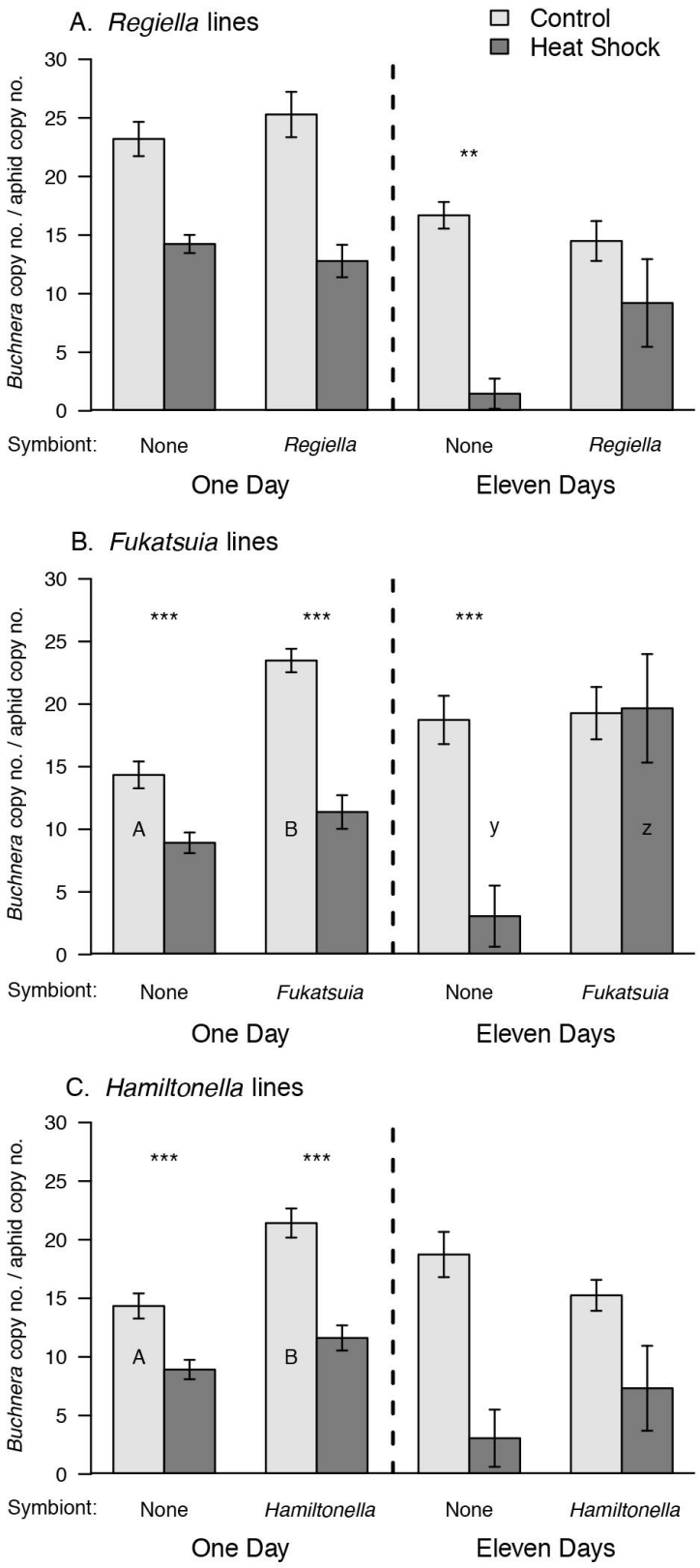

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673 Figure 3