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

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9 **Keywords:** *Acyrtosiphon pisum*; *Buchnera aphidicola*; facultative symbiont; heat stress; insect
10 symbionts; quantitative PCR; symbiosis

11 Abstract

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

30 1 Introduction

31 It is well established that infection with bacterial symbionts can affect an insect host's biology.
32 Reproductive fitness, insect behavior, immune pathway function, and responses to natural enemies
33 may all be influenced by the presence of endosymbionts (Dion et al., 2011; Gerardo and Parker,
34 2014; Vorburger, 2014; Martinez et al., 2015). By improving the ecological fitness of a host through
35 raising its immunity to natural enemies, or by enhancing its tolerance to environmental stress, a

36 vertically transmitted symbiont increases its own fitness (Oliver et al., 2005; Brownlie and Johnson,
37 2009).

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

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

57 Instead of indirectly affecting the host's stress or immune responses, symbionts may themselves
58 produce and release heat shock proteins or metabolites that directly protect the host or other microbes
59 that the host depends on (i.e. obligate symbionts) (Burke et al., 2010a). Obligate symbionts are often
60 a thermal "weak link" and more susceptible to temperature extremes than their hosts (Corbin et al.,
61 2017; Shan et al., 2017; Zhang et al., 2019). Shielding an obligate symbiont from thermal damage
62 would therefore benefit both the host and all of its symbionts. For example, in pea aphids that
63 experience heat shock, the density of the obligate nutritional symbiont *Buchnera aphidicola* is
64 usually reduced, but is maintained at near normal levels in aphids that carry the facultative symbiont
65 *Candidatus Serratia insecticola* (hereafter *Serratia*) (Burke et al., 2010). It is also plausible that
66 facultative symbionts might be directly protecting the host by replacing an obligate symbiont that is
67 no longer able to perform its function. When the obligate symbiont *Buchnera* is removed using
68 antibiotics at benign temperatures, *Serratia* in pea aphids moved into the bacteriocytes vacated by
69 *Buchnera* and subsequently allow the stressed aphid to survive and reproduce (Koga et al., 2003,
70 2007).

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

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

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

95 2 Materials and Methods

96 2.1 Aphids and symbionts

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

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

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

126 from infected donor aphids (Table S1). Hemolymph was extracted from donor aphids under a
127 microscope using glass needles and re-injected into the appropriate aphid line and surviving aphids
128 raised to adulthood. Glass needles were pulled from Kwik-Fil™ borosilicate glass capillaries
129 (1B100-4, World Precision Instruments, 1 mm diameter) using a P-97 Flaming/Brown micropipette
130 Puller (Sutter Instrument Co.). The offspring of the surviving aphids were tested for the successful
131 establishment of the new infection when they were adults, and these aphid lines were retested
132 regularly to ensure the maintenance of the new aphid-symbiont combinations. All injected lines had
133 been maintained in the laboratory for at least a year before being used for experimental assays.

134 2.2 Heat shock protocol

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

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

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

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

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

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

177 **2.3 qPCR protocol**

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

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

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

209 **2.4 Statistical analysis**

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

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

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

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

235 For all general linear models, post-hoc tests were only performed when the factor or interaction was
236 significant in the main analysis. This was conducted using the R package “phia” (Helios and Fox,
237 2015), with Holm’s correction for multiple comparisons.

238 **3 Results**

239 **3.1 Effects of facultative symbionts on fecundity after heat shock**

240 We exposed aphids to a short spike of high temperature and measured facultative and obligate
241 symbiont densities and aphid fitness after one and eleven days. As expected, heat shock decreased
242 the number of offspring produced in an aphid’s lifetime in all three sets of lines (*Regiella* $F_{1,19} =$
243 103.23 , $P < 0.001$; *Fukatsuia*: $W = 109$, $P = 0.03$, *Hamiltonella*: $W = 136.5$, $P = 0.001$; Figure 1).
244 However, the extent of this decrease was modified by the presence of *Regiella* and *Fukatsuia*
245 (*Regiella*, symbiont \times heat treatment: $F_{1,19} = 5.78$, $P = 0.03$; *Fukatsuia*: heat treatment in uninfected
246 lines $W = 36$, $P = 0.004$ and in infected lines $W = 13$, $P = 0.48$). *Fukatsuia* provided the greatest
247 protection from heat as there was no difference in the fecundity of the infected lines in the control
248 and heat shock treatment, whereas there was a greater reduction in fecundity in the uninfected aphids
249 than in the infected aphids for the *Regiella* lines. In contrast, there was a similar decrease in
250 fecundity for both uninfected and infected *Hamiltonella* lines following heat shock (uninfected: $W =$
251 36 , $P = 0.004$; infected: $W = 34.5$, $P = 0.008$; Figure 1). At benign temperatures, two of the
252 symbionts also affected lifetime fecundity: the presence of *Hamiltonella* increased lifetime fecundity
253 ($W = 4$, $P = 0.03$), whereas *Fukatsuia* decreased it ($W = 34$, $P = 0.013$), and there was no difference
254 for *Regiella* (Figure 1).

255

256 3.2 Facultative symbiont densities after heat shock

257 We measured the densities of the three facultative symbionts at two time points after exposure to
 258 heat, 24-26 hours and 11 days post-heat shock (Figure 2). Compared to non-heat shocked controls
 259 the densities of two of the symbionts, *Fukatsuia* and *Hamiltonella*, were lower on the day after heat
 260 shock (*Fukatsuia*: $F_{1,8} = 65.05$, $P < 0.001$, *Hamiltonella*: $F_{1,8} = 6.64$, $P = 0.03$), whereas densities of
 261 *Regiella* are unaffected ($W = 28$, $p=0.13$; but note that this is significant in a less conservative
 262 parametric test). By the second time point, taken when the aphids were young adults, there was no
 263 difference between population densities in heat stressed or control aphids for any of the three
 264 facultative symbionts (*Fukatsuia*: $F_{1,8} = 0.35$, $P = 0.57$, *Hamiltonella*: $F_{1,7} = 0.95$, $P = 0.36$, *Regiella*:
 265 $F_{1,6} = 0.01$, $P = 0.91$; Figure 2), suggesting that heat did not have long-term effects on facultative
 266 symbiont populations.

267 3.3 Obligate symbiont densities under heat shock

268 Compared to the control treatment densities of *Buchnera* were decreased on the day after heat shock
 269 in each of the three pairs of lines, regardless of facultative symbiont infection (*Regiella* lines: $F_{1,19} =$
 270 80.36 , $P < 0.001$, *Fukatsuia* lines: $F_{1,18} = 70.71$, $P < 0.001$, *Hamiltonella* lines: $F_{1,18} = 56.07$, $P <$
 271 0.001 ; Figure 3). Regardless of treatment, *Buchnera* densities were higher in the lines harbouring
 272 *Fukatsuia* ($F_{1,18} = 33.86$, $P < 0.001$) or *Hamiltonella* ($F_{1,18} = 24.52$, $P < 0.001$) compared to
 273 uninfected lines, an effect that was not seen in the *Regiella* lines ($F_{1,19} = 0.15$, $P = 0.71$). For the
 274 *Fukatsuia* and *Hamiltonella* lines there was also a significant interaction between symbiont presence
 275 and heat treatment (*Fukatsuia*: $F_{1,18} = 9.57$, $P = 0.006$; *Hamiltonella*: $F_{1,18} = 4.80$, $P = 0.04$): in both
 276 cases, *Buchnera* densities in the control treatment were higher in lines with facultative symbionts
 277 compared to uninfected aphids but there was no difference between these lines after heat shock. The
 278 interaction was not significant for the *Regiella* lines ($F_{1,19} = 2.58$, $P = 0.13$) where the extent of the
 279 loss of *Buchnera* did not differ between infected and uninfected lines.

280 At the later time point, when aphids were young adults, heat shock again reduced *Buchnera* densities
 281 on average (*Regiella* lines: $W = 105$, $P = 0.004$; *Fukatsuia* lines: $F_{1,19} = 6.85$, $P = 0.02$; *Hamiltonella*
 282 lines: $F_{1,17} = 24.84$, $P < 0.001$). *Fukatsuia* presence on average also significantly increased the
 283 density of *Buchnera* regardless of treatment which was due to high *Buchnera* densities in the heat
 284 shocked aphids ($F_{1,19} = 7.89$, $P = 0.01$); a difference that was not found for *Regiella* ($W = 46$, $P =$
 285 0.37) or *Hamiltonella* presence ($F_{1,17} = 0.17$, $P = 0.69$). Importantly, there was a significant
 286 interaction between symbiont infection and temperature in the *Fukatsuia* lines ($F_{1,19} = 7.55$, $P = 0.01$)
 287 and an equivalent effect in the *Regiella* lines (heat treatment in uninfected lines: $W = 36$, $P = 0.004$
 288 and in infected lines: $W = 17$, $P = 0.42$) that was not seen for the *Hamiltonella* lines ($F_{1,17} = 1.95$, $P =$
 289 0.18): *Buchnera* densities after heat shock were significantly reduced in uninfected aphids but not
 290 when *Fukatsuia* or *Regiella* were present.

291 4 Discussion

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

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

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

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

319 The patterns of obligate and facultative symbiont densities observed here suggest that a different
320 mechanism underlies the heat protection provided by *Regiella* and *Fukatsuia* compared to that
321 provided by *Serratia* (Montllor et al., 2002; Burke et al., 2010a). After heat shock, *Serratia* in pea
322 aphids lyse and this coincides with metabolomic changes (Burke et al., 2010a). At the same time
323 *Buchnera* densities are maintained at similar levels to those at benign temperatures (Burke et al.,
324 2010a). In our experiments, in aphids with *Regiella* or *Fukatsuia*, *Buchnera* densities initially
325 decrease. This demonstrates that the protection provided by the facultative symbionts is not instant
326 and suggests that the protection is probably not due to a constitutively activated aphid stress
327 response, but it is still possible that the facultative symbionts prime a stress response by the aphid
328 that helps recovery later on.

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

341 Some strains of *Buchnera* are more resistant to heat than others (Dunbar et al., 2007; Moran and Yun,
342 2015) and it is possible that the two aphid genotypes here carry different *Buchnera* genotypes.
343 Aphids in laboratory populations often carry *Buchnera* strains with the *ibpA*¹² mutant allele that are
344 more sensitive to heat but have higher fitness (Burke et al., 2010b). We did not sequence *ibpA* in the

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

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

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

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

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

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

405 **5 Conflict of Interest**

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

408 **6 Author Contributions**

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

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419

420 **9 References**

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629 **10 Data Availability Statement**

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

632 **11 Figure Legends**

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

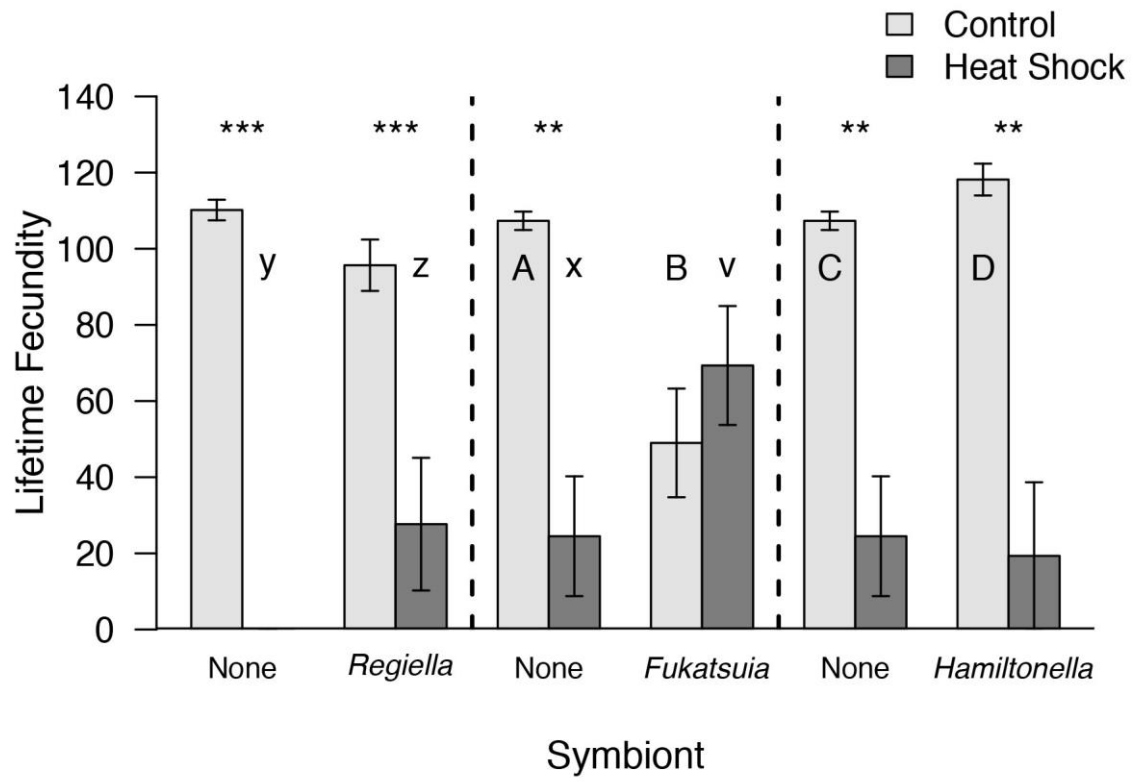
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644 **Figure 2. Densities of facultative symbionts in pea aphids after heat shock or in the control**
645 **treatment (A) 24-26 hours after the onset of the heat shock and (B) eleven days after heat**
646 **shock.** Densities are shown as the copy number of the *gyrB* gene of the facultative symbiont relative
647 to copy number of the aphid gene *EFL-α*. Means and standard errors are shown. Asterisks denote
648 differences between heat treatments for aphids carrying a given symbiont (* $P < 0.05$, *** $P <$
649 0.001).

650

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

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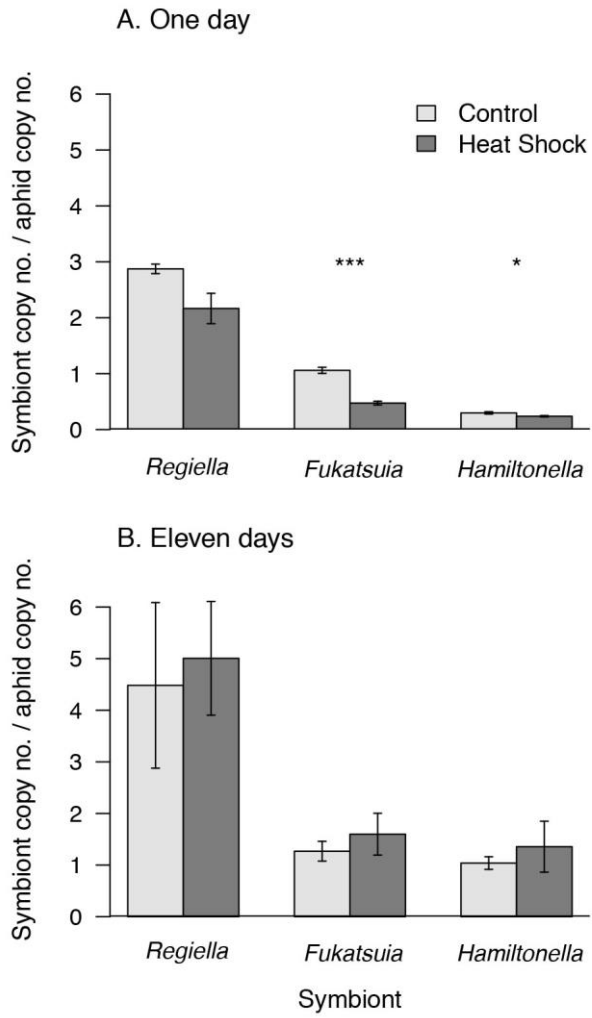


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666 Figure 1

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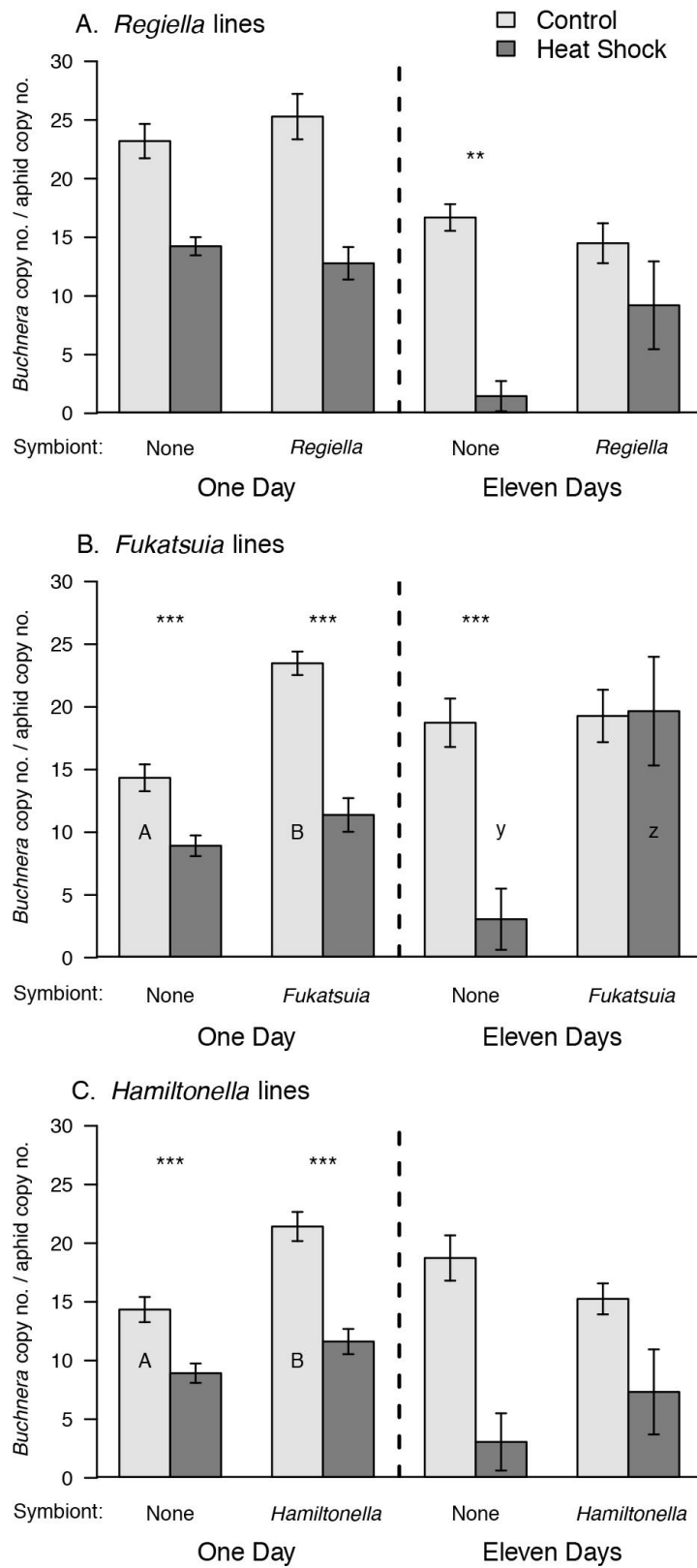


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670 Figure 2

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673 Figure 3