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1 **Physiological costs of facultative endosymbionts in aphids assessed** 2 **from energy metabolism**

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9

10 **Abstract**

- 11 1. Many insect species harbor heritable bacterial endosymbionts. Some facultative
12 endosymbionts provide benefits to their hosts under certain environmental conditions.
- 13 2. Facultative endosymbionts are expected to impose additional energetic expenditures to
14 their host, reducing host fitness. While there is accumulating evidence in plant sucking
15 insects that facultative endosymbionts reduce the fitness of their host under permissive
16 conditions, no direct energy costs associated with facultative endosymbionts have been
17 identified.
- 18 3. Using the standard metabolic rate (SMR) as a measure of the energy cost of self-
19 maintenance, we investigated whether two common facultative endosymbionts
20 *Hamiltonella defensa* or *Regiella insecticola* increase the maintenance cost of the pea
21 aphid *Acyrtosiphon pisum* and which could translate into host fitness reduction
22 ('compensation hypothesis'). In addition, we tested if there was a link between SMR
23 and the aphid fitness and whether it depended on endosymbiont density and aphid
24 energetic reserves. Finally, we measured SMR at different temperatures to assess the
25 impact of suboptimal thermal conditions on physiological cost of endosymbionts.
- 26 4. In the presence of facultative endosymbionts, aphids expressed generally a lower fitness
27 and a higher SMR compared to uninfected ones, in accordance with the 'compensation
28 hypothesis'. However, the SMR difference between infected and uninfected aphids
29 tended to decrease with increasing temperature. Complex host genotype-by- symbiont
30 genotype- by- temperature interactions on SMR were also revealed.
- 31 5. Energetic budget of adult aphids appeared weakly influenced by the aphid genotype and
32 endosymbiont species, suggesting that facultative endosymbionts primarily impact the
33 consumption of energy resources rather than their acquisition. Density of facultative

34 endosymbionts varied largely among aphid lines but was not associated with the fitness
35 nor metabolic rate of aphids.

36 6. This work provides a better understanding of the maintenance of facultative associations
37 and raises new questions about the effect of facultative endosymbionts on the energy
38 metabolism of their host.

39 **Keywords**

40 Symbiosis ; standard metabolic rate ; aphid ; temperature ; *Hamiltonella* ; *Regiella*

41 **1 Introduction**

42 The evolution and ecology of insects are influenced by their associations with heritable
43 bacterial endosymbionts (Feldhaar, 2011; McFall-Ngai et al., 2013). Herbivorous insects in
44 particular are renowned for hosting complex microbial communities that enlarge their
45 ecological niche and influence their interactions with environmental factors (Frago et al., 2012).
46 Over time, some associations evolved co-dependency between the endosymbionts and their
47 insect hosts, resulting in obligate mutualistic relationships. For instance, aphids typically harbor
48 the strict intracellular bacteria *Buchnera aphidicola* that provides nutrients lacking in the plant
49 phloem sap (Douglas, 1998). Other associations are facultative, however, such that the
50 symbiont is not essential for host viability but can confer fitness benefits under certain
51 environmental conditions (Bénard et al., 2020; Feldhaar, 2011; Oliver et al., 2010). For
52 instance, different endosymbionts are involved in the cuticle formation of their host in beetles,
53 thereby ameliorating fitness under dry environments (Anbutsu et al., 2017; Engl et al., 2018;
54 Vigneron et al., 2014). In sap-feeding insects, some facultative symbionts have been described
55 to provide host plant adaptation (Tsuchida et al., 2004; Wagner et al., 2015), heat-tolerance
56 (Brumin et al., 2011; Burke et al., 2010) and protection from natural enemies (Hendry et al.,
57 2014; Oliver et al., 2003).

58 Compared to obligate associations, facultative ones are generally more dynamic across
59 spatial and temporal scales (Carpenter et al., 2021; Leclair et al., 2021; Smith et al., 2021;
60 Zytynska & Weisser, 2016). For instance, a survey on pea aphids has shown that the frequency
61 of the facultative endosymbiont *Hamiltonella defensa* increased from 5% to 80% in field
62 populations within only 6 weeks, corresponding to few generations, and declined rapidly just
63 after (Smith et al., 2021). Balancing selection has been proposed to explain these fluctuations
64 in prevalence, such that the ~~maintenance~~ frequency of facultative endosymbionts in host
65 populations is expected to be mainly governed by the net fitness benefit provided to the host,

66 *i.e.* the balance between the benefits and the costs of hosting facultative symbionts according
67 to environmental conditions (Kwiatkowski & Vorburger, 2012; Oliver et al., 2014). Hence,
68 when selective pressures that favor the associations with facultative symbionts are weak the
69 costs of carrying facultative symbionts can exceed the benefits, thereby reducing the
70 competitive ability of infected insects compared to uninfected ones, and reciprocally when
71 selective pressures are strong (Dykstra et al., 2014; Oliver et al., 2008). Thus, the frequency
72 variations of some endosymbiont have been recently linked to temporal fluctuations of
73 environmental stressors, like climate and natural enemies (Carpenter et al., 2021; Leclair et al.,
74 2021; Smith et al., 2021). Other factors such as stochastic processes are predicted to play a role
75 (Mathé-Hubert et al., 2019) but strong parallelism of frequency fluctuations observed across
76 various localities in field studies tends to minimize their importance (Carpenter et al., 2021).
77 During the last two decades, evidences of fitness costs associated with facultative
78 endosymbionts have been accumulating, especially in plant sucking insects (for a meta-analysis
79 Zytynska et al., 2021). For instance, the endosymbiont *H. defensa*, that provides protection
80 against parasitoid wasps, shortens the longevity of the black bean aphid, *Aphis fabae*, resulting
81 in a lower lifetime reproduction (Vorburger & Gouskov, 2011). Fitness costs and benefits of
82 carrying facultative endosymbionts also present complex variations resulting from host by
83 symbiont genetic interactions (Cayetano et al., 2015; Russell & Moran, 2006; Vorburger &
84 Gouskov, 2011) as well as from environmental factors (Liu et al., 2019; Zytynska et al., 2021).

85 Such fitness costs are expected to result from additional expenditures due to hosting
86 endosymbionts that relate to at least two non-mutually exclusive processes. First,
87 endosymbionts consume nutrients from the surrounding host cell and hence represent a
88 nutritional sink, except when they recycle host by-products (Ankrah et al., 2018). Based on
89 genomic predictions, facultative endosymbionts appear be generally auxotrophic for several
90 essential resources, in particular amino acids, and rely on their insect host to sustain their
91 metabolism (Degnan et al., 2009; Renoz et al., 2021). Negative correlations between host
92 fitness traits and facultative endosymbiont densities observed in aphids and *Drosophila* tend to
93 support the hypothesis of energetic demand imposed by the symbionts that further depress their
94 host fitness (Cayetano et al., 2015; Leclair et al., 2017; Parker, 2021; Weldon et al., 2013).
95 Second, in facultative associations, hosts present generally heightened immune related-
96 functions to control bacterial density (Laughton et al., 2014). These immune responses come
97 probably at the cost of an increased energetic demand (Ardia et al., 2012; Blow et al., 2020;

98 Dolezal et al., 2019). However, these observations are only indirect, therefore demonstrations
99 of physiological costs associated with facultative endosymbionts are lacking.

100 The standard metabolic rate (SMR) is a widely measured physiological trait that has
101 been linked to important traits, such as reproductive output, longevity and immunity (Brown et
102 al., 2004; Koch et al., 2021). It can be interpreted as an integrative measure of the energy cost
103 of self-maintenance in a given environment (Burton et al., 2011). Under the ‘compensation
104 hypothesis’ (or ‘allocation hypothesis’), a higher SMR suggests a fitness disadvantage as a
105 result of higher maintenance costs, and thereby a lower allocation of energy to growth and/or
106 reproduction (Nilsson, 2002). Hence, if facultative endosymbionts increase the maintenance
107 costs of their host, it would result in a higher SMR compared to uninfected host. By contrast, a
108 higher metabolic rate can contribute to increased energy intake (e.g. higher feeding rate) for
109 reproduction (so-called ‘increased intake hypothesis’) (Careau & Garland, 2012). The increase
110 of the metabolic rate observed in female mosquitoes *Aedes aegypti* carrying a life-shortening
111 strain of *Wolbachia* (wMelPop) tends to support the ‘compensation hypothesis’ (Evans et al.,
112 2009), although the fitness consequence was not directly evaluated. To our knowledge this
113 study is the only one investigating the impact of facultative endosymbionts on host metabolic
114 rate. Measurements of metabolic rates need to be associated with assessments of fitness cost or
115 benefit to determine which mechanistic hypothesis prevails (compensation or increased
116 uptake). Such approach has never been applied to facultative associations, thereby limiting our
117 ability to understand the nature and extent of the physiological costs associated with facultative
118 endosymbionts.

119 We applied this approach to investigate whether facultative endosymbionts entail a
120 fitness cost in the pea aphid *Acyrtosiphon pisum* and whether the SMR of aphids relates to this
121 fitness cost. Aphids are a relevant model to explore this question as they are well known to host
122 diverse species of facultative endosymbionts, associated with ecological benefits but also
123 fitness costs (Guo et al., 2017; Oliver et al., 2010; Zytynska et al., 2021). We measured
124 metabolic rate and fitness traits in several associations involving two common bacterial
125 endosymbionts, *H. defensa* and *Regiella insecticola*. We compared different pea aphid
126 genotypes infected either by *H. defensa* or *R. insecticola* to their uninfected counterparts
127 (ending with 9 different combinations) to test whether endosymbiont effect varies according to
128 bacterial species and host genotype. This experimental design evaluates whether the
129 compensation versus increased intake hypotheses prevail by investigating the link between
130 SMR and fitness of aphids. In addition, we measured the endosymbiont density and energetic

131 reserves of adult aphids to test whether they are related to aphid SMR and fitness. Under the
 132 increased intake hypothesis, we expected that energetic reserves of adult aphids should be
 133 positively correlated to SMR, whereas aphid SMR should not be influenced by energetic
 134 reserves under the compensation hypothesis. Finally, ectotherm metabolic rate increases
 135 exponentially with temperature (Angilletta, 2009) and therefore the impact of facultative
 136 endosymbionts may be seen at suboptimal temperatures and not under optimal thermal regimes
 137 for the host. We explored this thermal dependency by measuring the SMR of the 9 aphid-
 138 symbionts combinations under different temperature regimes spanning optimal and suboptimal
 139 conditions. The obligate endosymbiont, *B. aphidicola*, is particularly vulnerable to high
 140 temperature and restricts thermal tolerance of their aphid host (Dunbar et al., 2007; Zhang et
 141 al., 2019). Therefore, we predicted that warm suboptimal temperatures would exacerbate any
 142 potential physiological cost induced by the endosymbionts as they may approach the thermal
 143 stress zone (i.e., higher SMR).

144 2 Materials and Methods

145 2.1 Experimental lines

146 Four pea aphid (*Acyrtosiphon pisum*) genotypes collected in France, United Kingdom
 147 and Japan were used in this study (Table 1). Lines infected and uninfected by facultative
 148 endosymbionts were compared to assess the effects of endosymbionts on aphid traits.

149 **Table 1.** Characteristics of the pea aphid lines used in the experiments

Genotype	Origin	Collection	Natural host plant	Line	Secondary endosymbiont	Aphid genotype-endosymbiont combination
YR2	United Kingdom	2002	<i>Trifolium sp.</i>	YR2-acg	None	Artificial
				YR2+Hd ^{L100}	<i>H. defensa</i>	Artificial
				YR2+Reg ^{YR2}	<i>R. insecticola</i>	Naturally-occurring
L9MS-07	France	2011	<i>Medicago sativa</i>	L9MS-07acg	None	Artificial
				L9MS-07acg+Hd ^{L9MS-07}	<i>H. defensa</i>	Naturally-occurring
LL01	France	1987	<i>Medicago sativa</i>	LL01	None	Naturally-occurring
				LL01+Reg ^{A-AA-01}	<i>R. insecticola</i>	Artificial
TUt	Japan	2001	<i>Trifolium sp.</i>	TUt-amp	None	Artificial
				TUt-amp+Reg ^{Tut}	<i>R. insecticola</i>	Naturally-occurring

150

151 These different aphid genotypes are all distinct on the basis of 7 polymorphic microsatellite loci
 152 (Peccoud et al., 2008) and were checked before starting the experiments. Nine lines carrying

153 either *H. defensa* or *R. insecticola* were established by antibiotic treatments followed by
154 microinjection of bacteria-containing hemolymph using the procedure described in Sochard et
155 al. (2020) (Table 1). It is worth noting that due to experimental variation in the success rates of
156 symbiont introduction and maintenance, we could not have all the aphid lineages allowing a
157 full-factorial design. Further, different strains of *H. defensa* and *R. insecticola* have been
158 injected in the different aphid genotypes, precluding the investigation of the endosymbiont
159 strain effect. Before and after experiments, the symbiotic status of aphids of each line was
160 verified by diagnostic PCR using specific primers (Table S1). Prior to experiments, the aphid
161 lines have been maintained parthenogenetically for at least 80 generations at 18°C on broad
162 bean, *Vicia faba* L. (Fabaceae), under a L16:D8 photoperiod. Plants were grown individually
163 in plastic pots filled with commercial seed raising mixture and placed in a greenhouse under
164 long days. To control the age of the aphids in all the experiments, adult aphids were placed on
165 young healthy plants to reproduce for 24 hours and were then removed to let only newborns of
166 the same age (\pm 12 hours). Cages made from clear plastic cylinders and fine mesh were placed
167 on plants to prevent aphid escape.

168 2.2 Fitness costs associated with facultative endosymbionts

169 The lifetime reproduction and adult longevity of aphids were measured for all nine lines.
170 For this, unwinged adult aphids were kept individually on the abaxial part of broad bean leaves
171 using clip-cages (diameter: 2.5cm) and maintained at 18°C in a climatic chamber (PHCBI
172 model MIR-254-PE) with a photoperiod of L16:D8. The introduction of aphids into the clip-
173 cages was done before their first reproduction. Survival and fecundity were observed every 2-
174 3 days and the offspring were counted and removed from the clip-cages after each observation.
175 When the adult aphids died between two observations, the median date was taken as the date
176 of death. The maximum observation time was 18 days. The data from individuals that survived
177 >18 days were considered censored in the statistical analyses (see below). Between three and
178 five clip-cages were placed on the same plant, and aphids on the same plant were from the same
179 line to avoid putative plant-mediated horizontal transfer of endosymbionts (Li et al., 2018; Pons
180 et al., 2019). Location of the plants in the climatic chamber was rotated after each observation.
181 At least 11 individuals were observed for each line.

182

183 2.3 Standard metabolic rate of aphids

184 Standard metabolic rate (SMR) of aphids was determined by measuring their oxygen
185 consumption through time in a closed respiratory system. We used two 24-well multi-dishes

186 (80µl volume, Loligo Systems, Denmark) integrated to a fluorescence-based multichannel
187 respirometer (SDR SensorDish Reader, PreSens, Germany). This system offers a non-invasive
188 and non-destructive method to assess real time oxygen consumption of small organisms by
189 measuring the amount of oxygen in percent saturation in wells over time. Measurements were
190 carried out on unwinged adult aphids (10-14 days-old) because it is expected to be the stage at
191 which endosymbiont densities reach a peak (Laughton et al., 2014; Simonet et al., 2016) and
192 so putatively the most relevant stage to measure symbiont-associated costs. Aphids of the
193 different lines were randomly distributed between and within each plate and were transferred
194 individually into wells sealed with adhesive PCR film (Thermo Scientific, USA). The
195 respirometer was used following the manufacturer's recommendations. Measurements were
196 carried out at three different temperatures (18°, 22° and 26°C) by placing microplates in a
197 climatic chamber (PHCBI, model MIR-254-PE) and the temperature of the microplates has
198 been verified with a thermocouple data logger (Hobo UX120, Onset). This range of temperature
199 is representative of the conditions encountered by *A. pisum* during the growing season under
200 temperate climates (Carpenter et al., 2021) and is expected to encompass optimal (18°-22°C)
201 and suboptimal conditions (26°C) (Lamb, 1992). Oxygen concentration inside wells was
202 measured every 30 seconds during 60 minutes. During this period, aphids were not handled and
203 kept in darkness. This observation period is short enough to limit the effects of aphid
204 dehydration and avoid deleterious stress for the aphids (Kouame & Mackauer, 1992; Nelson,
205 2007, personal observation). SMR was calculated as the slope of the oxygen depletion over
206 time (MO_2 in $\text{pmol}\cdot\text{min}^{-1}$) after truncation of both extremities to keep the most linear portion.
207 For each series of measurements, four wells of each microplate were left empty and used as
208 negative controls. At least 30 individuals were analyzed for each lineage and each temperature
209 – different individuals were used for each temperature exposure. As the metabolic rate is related
210 to body mass (Gillooly et al., 2001), fresh mass of aphids was measured at the end of each trial
211 on an electronic scale (Sartorius balance, ± 0.001 mg). Aphids were then kept individually at -
212 20 ° C for subsequent molecular and physiological analyses (see below).

213

214 2.4 Energetic budget quantification

215 The whole body energy content was calculated for each individual aphid in the 9 lines
216 exposed to 18°C. The amounts of proteins, free sugars, lipids, and glycogen were quantified
217 individually using an adjusted version of the protocol of Foray et al. (2012). Briefly, aphids
218 were first solubilized in a phosphate-lysis buffer and total proteins were then assayed using

219 Bradford's reagent (B6916, Sigma-Aldrich) with BSA as a standard. In a second step, a
220 chloroform–methanol mixture was added to the aqueous phase and carbohydrates (free sugars
221 and glycogen) were quantified using Anthrone reagent (319899, Sigma-Aldrich) and D-glucose
222 was used as standard, whereas total lipid quantification was performed using Vanillin (V1104,
223 Sigma -Aldrich), with triolein as a standard. Optical density measurements were performed
224 using a spectrophotometer (NanoQuant, TECAN). The total energy budget of aphids was then
225 computed using the following equivalence: 16 Joules per mg carbohydrates or proteins and
226 37.65 Joules per mg lipids (Giron & Casas, 2003; Jervis et al., 2008). At least 18 aphids were
227 analyzed per aphid line.

228

229 2.5 Quantification of facultative endosymbiont densities

230 To evaluate the possible effect of endosymbiont density on fitness-related traits and
231 SMR of aphids, we estimated *H. defensa* and *R. insecticola* densities relative to aphid gene
232 copies using real-time quantitative PCR. DNA was extracted individually from five adult aphids
233 (12-13 days-old) of each line using the NucleoSpin DNA Insect kit (Macherey-Nagel). Primers
234 specifically amplifying the *gyrB* gene from *H. defensa* and the *tufB* gene from *R. insecticola*
235 were used (Table S1). The relative abundance of *H. defensa* or *R. insecticola* was determined
236 after normalization to aphid single copy *CtpL* gene using specific primers (Table S1). The qPCR
237 reaction mixture included 6.5 μ l qPCR MasterMix for SYBR green (Eurogentec), 1.25 μ l of
238 each primer (300nM), 0.25 μ l ddH₂O and 5 μ l of template DNA. The qPCRs were run in
239 triplicate (technical replicates) on a QuantStudio 6 Flex Real-Time PCR System (Life
240 Technologies). Copy number of each gene was estimated based on standard curves prepared
241 with 10-fold dilutions of genomic DNA ranging from 10^2 to 10^8 copies/5 μ l.

242

243 2.6 Statistical analyses

244 In all analyses, the two main explanatory variables tested were the genotype (four
245 modalities) and the symbiotic status (three modalities corresponding to ‘without facultative
246 endosymbiont’, ‘infected by *H. defensa*’ and ‘infected by *R. insecticola*’). The temperature was
247 also introduced as an explanatory variable to analyze the SMR. In preliminary analyses, SMR
248 did not vary according to the plate ($p=0.88$) and the between plate effect was not included in
249 subsequent analyses. Linear models were used to analyze SMR and energetic reserves. The
250 number of offspring was analyzed by means of a generalized linear mixed model with a Poisson
251 error distribution (log link function) and with the plant identity as random effect. To compare

252 the adult longevity of aphids, we used a parametric survival analysis assuming a Weibull
 253 distribution and allowing censored data (*i.e.* individuals still alive after 18 days) to be
 254 considered. The variation of symbiont density among infected lines was analyzed by means of
 255 a Kruskal-Wallis rank sum test on averaged values of technical triplicates. Spearman’s rank
 256 correlation tests were also used to analyze the correlation between various traits. All statistical
 257 analyzes and graphs were conducted using R version 4.0.3 (R Development Core Team, 2007)
 258 with the ‘lme4’, ‘survival’ and ‘ggplot2’ packages.

259 3 Results

260 3.1 Influence of facultative symbionts on aphid fitness

261 Both lifetime reproduction and adult survival of aphids were affected by the genotype
 262 and the symbiotic status of aphids (Fig. 1). Genotype and symbiotic status had an interactive
 263 effect on the lifetime reproduction of aphids ($Z = -4.71$, $p < 0.001$, Fig. 1a). Facultative
 264 endosymbionts reduced the number of offspring produced by aphids, except in the LL01
 265 genotype ($Z = -0.476$, $p = 0.634$). On average, the lifetime reproduction of aphids infected by *H.*
 266 *defensa* and *R. insecticola* was 59% and 39% lower than their uninfected counterparts,
 267 respectively. The survival analyses revealed that the presence of facultative endosymbionts
 268 shortens the adult longevity of aphids for the YR2 and TUt genotypes (YR2: $|z| = 3.44$, $p < 0.001$;
 269 TUt: $|z| = 2.08$, $p < 0.037$, Fig. 1b) but not for the LL01 and L9MS07 genotypes (LL01: $|z| = 0.59$,
 270 $p = 0.55$; L9MS07: $|z| = 1.51$, $p = 0.13$, Fig. 1b).

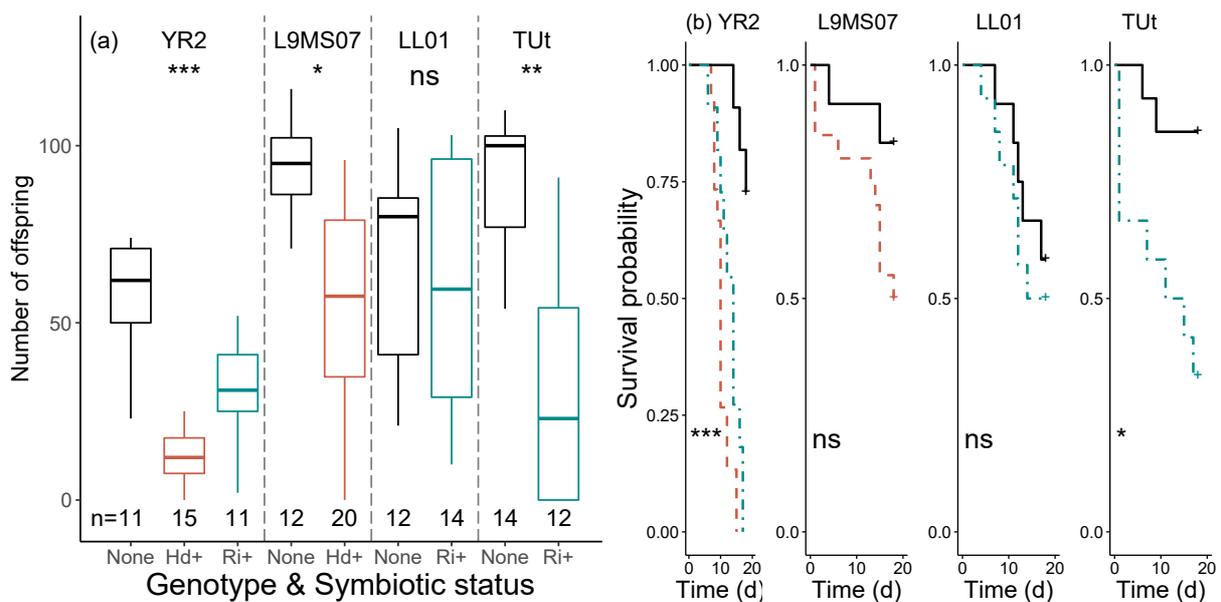


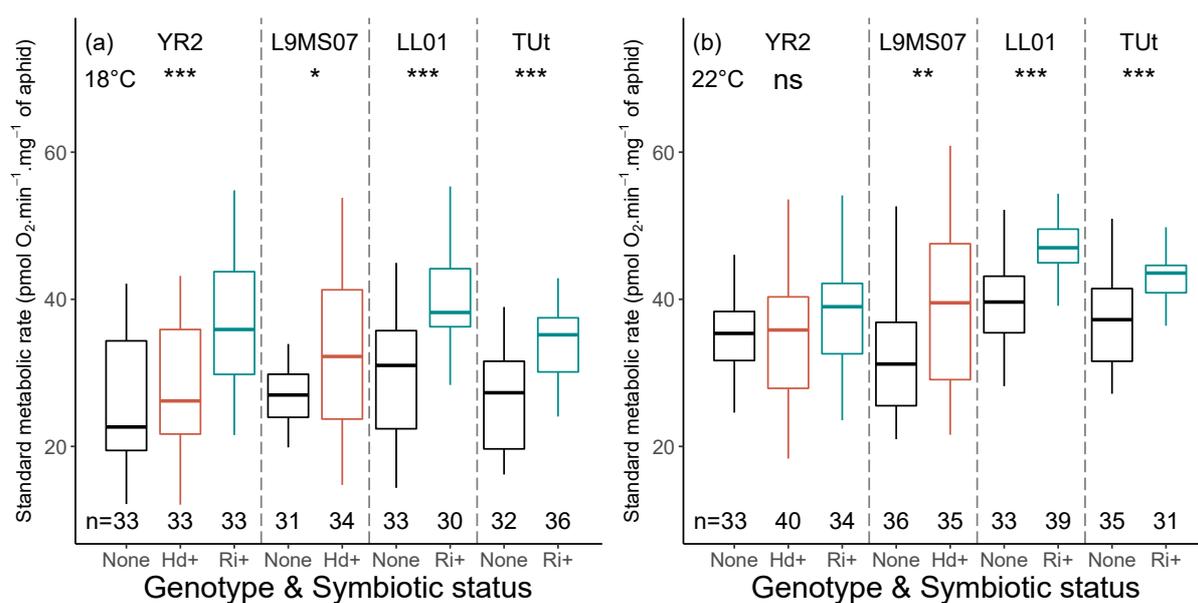
Fig. 1. Lifetime reproduction (a) and adult survival (b) of aphids without facultative endosymbiont (“None”, black and solid line), infected by *H. defensa* (“Hd+”, red and dashed line) and infected by *R. insecticola* (“Ri+”, blue

and dash-dotted line). The lower and upper edges of the boxplots represent 25% and 75% quantiles; the intermediate lines are the median; and the whiskers indicate the smallest and largest non-outlier observations. In the survival curves, crosses represent censored data. Sample size is denoted by “*n*”. Asterisks and “ns” indicate significant (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$) and non-significant ($p > 0.05$) differences according to aphid’s symbiotic status.

271

272 3.2 Aphid standard metabolic rate

273 The effect of facultative endosymbionts on the standard metabolic rate (SMR) of aphids
 274 varied according to the aphid genotype and temperature as highlighted by the significant
 275 interactive effect between the genotype, temperature and symbiotic status on SMR ($F_{3,932} =$
 276 3.98, $p = 0.008$, Fig. 2). In general, facultative endosymbionts significantly increased aphid
 277 SMR, except in the YR2 genotype at 22°C ($F_{2,104} = 2.03$, $p = 0.14$, Fig. 2a) and LL01 and TUt
 278 genotypes at 26°C (LL01: $t = -0.67$, $p = 0.49$; TUt: $t = -0.82$, $p = 0.42$, Fig. 2c). Conversely, in the
 279 L9MS07 genotype at 26°C, aphids hosting *H. defensa* had a lower SMR than uninfected ones
 280 ($t = -6.62$, $p < 0.001$, Fig. 2c). Overall, *R. insecticola* seemed to have a larger effect on aphid
 281 SMR than *H. defensa*, as the infection by *R. insecticola* modified the rate of oxygen
 282 consumption on average by +17.5 % whereas the mean variation of SMR induced by *H. defensa*
 283 was +11.3%. As expected, overall, the SMR increased with increasing temperature ($F_{1,932} =$
 284 651.1, $p < 0.001$; mean \pm se: 18°C: 31.02 ± 0.53 , 22°C: 38.42 ± 0.47 , 26°C: 45.62 ± 0.38).
 285 Moreover, the effect of endosymbionts on aphid SMR was larger at lower temperature ($F_{2,932} =$
 286 24.98, $p < 0.001$, Fig. 2), with the variation of SMR induced by endosymbionts ranging from
 287 10.2% to 39.8% at 18°C and from 0.6% to 19.2% at 26°C.



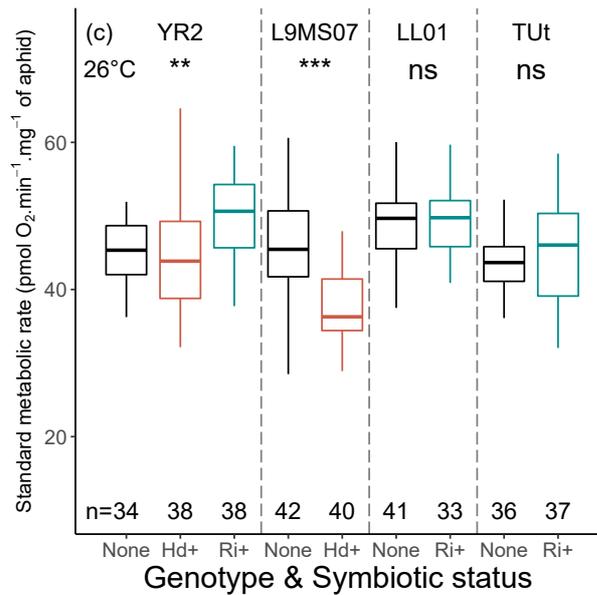


Fig. 2. Standard metabolic rate (SMR) of aphids without facultative endosymbiont (“None”, black), infected by *H. defensa* (“Hd+”, red) and infected by *R. insecticola* (“Ri+”, blue) at 18°C (a), 22°C (b) and 26°C (c). The lower and upper edges of the boxplots represent 25% and 75% quantiles; the intermediate lines are the median; and the whiskers indicate the smallest and largest non-outlier observations. Sample size is denoted by “n”. Asterisks and “ns” indicate significant (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$) and non-significant ($p > 0.05$) differences according to aphid’s symbiotic status.

288

289 The increase in metabolic rate was not associated with a reduction of the lifetime
 290 reproduction and adult lifespan of aphids at 18°C (lifetime reproduction: $r = -0.43$, $p = 0.25$;
 291 lifespan: $r = -0.63$, $p = 0.076$) (Fig. 3). However, the power of this statistical test is low given
 292 that it is based on only 9 values.

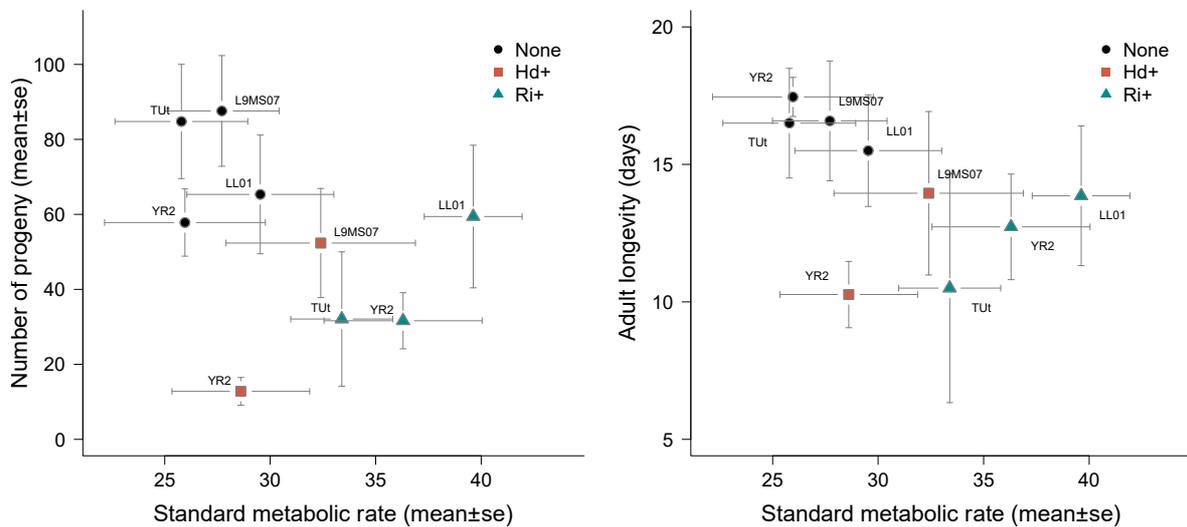


Fig. 3. Relationship between the standard metabolic rate and the lifetime reproduction (a) and the adult lifespan (b) of aphids measured at 18°C.

293

294 3.3 Aphid energetic budget

295 The energetic budget of aphids depended on the interaction between their genotype and
296 their symbiotic status ($F_{3, 158} = 7.62, p < 0.001$). This effect appeared principally associated with
297 the larger energy content in the LL01 genotype carrying *R. insecticola* compared to their
298 uninfected counterpart ($t = -4.14, p < 0.001$, Fig. 4). The energetic budget did not vary
299 significantly according to the symbiotic status in the other genotypes (YR2: $F_{2, 51} = 1.18, p =$
300 0.32 ; L9MS07: $t = -1.83, p = 0.07$; Tut: $t = -0.22, p = 0.83$). In contrast, when analyzed separately,
301 protein, lipid, free sugar and glycogen contents revealed complex variations according to the
302 genotype and symbiotic status of aphids (Table S2, Fig S1). In general, the energetic budget
303 was positively correlated to the SMR ($r = 0.42-0.75, p < 0.05$), except in L9MS-07acg ($r = 0.13,$
304 $p = 0.51$).

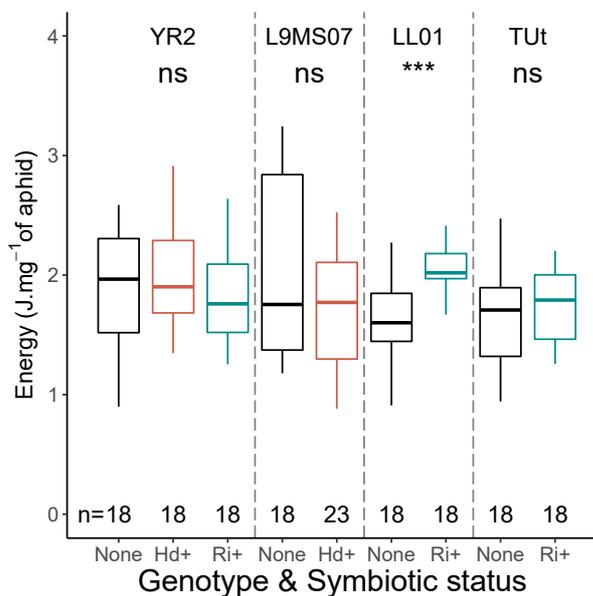


Fig. 4. Whole-body energy budget of adult aphids without facultative endosymbiont (“None”, black), infected by *H. defensa* (“Hd+”, red) and infected by *R. insecticola* (“Ri+”, blue). The lower and upper edges of the boxplots represent 25% and 75% quantiles; the intermediate lines are the median; and the whiskers indicate the smallest and largest non-outlier observations. Sample size is denoted by “n”. Asterisks and “ns” indicate significant (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$) and non-significant ($p > 0.05$) differences according to aphid’s symbiotic status.

305

306 3.4 Endosymbiont densities

307 The symbiont titers varied significantly among aphid lines (Kruskal-Wallis χ^2 test: $Z =$
308 $20.95, p < 0.001$). Particularly, the average density of *H. defensa* and *R. insecticola* were 38 and
309 8 times higher in YR2 genotype than in other genotypes, respectively (Fig. 5). The difference
310 of mean lifetime reproduction between infected aphids compared to their uninfected
311 counterparts was not correlated to the mean symbiont density ($r = -0.3, p = 0.68$). In addition,
312 the difference of mean SMR between infected aphids and their uninfected counterparts was not
313 correlated to the symbiont density whatever the temperature at which SMR was measured
314 ($18^\circ\text{C}: r = -0.4, p = 0.52$; $22^\circ\text{C}: r = -0.9, p = 0.08$; $26^\circ\text{C}: r = -0.1, p = 0.95$).

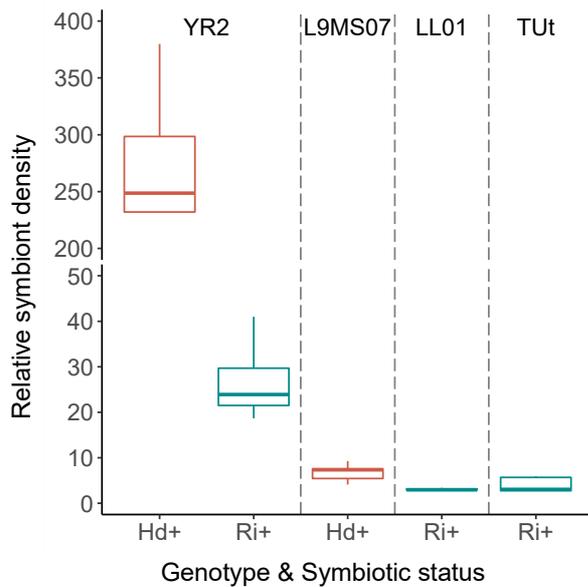


Fig. 5. Relative densities of *H. defensa* (“Hd+”, red) and *R. insecticola* (“Ri+”, blue), *i.e.* the ratio between symbiont gene copy numbers and host gene copy numbers, in adult aphids according to aphid genotype. The lower and upper edges of the boxplots represent 25% and 75% quantiles; the intermediate lines are the median; and the whiskers indicate the smallest and largest non-outlier observations.

315

316 4 Discussion

317 The physiological cost of hosting endosymbionts has rarely been determined
 318 concomitantly with fitness cost. Measurements of metabolic rates need to be associated with
 319 assessments of fitness cost or benefit to determine whether an increase in metabolic rate
 320 translates into a decrease of the host fitness or is balanced by an increased feeding uptake. We
 321 showed a clear cost of facultative symbionts on their hosts’ fitness in many instances,
 322 confirming earlier studies (Oliver et al., 2014; Zytynska et al., 2021). This fitness cost is linked
 323 to some extent to the negative impact of endosymbionts on the SMR of aphids under favorable
 324 thermal conditions. Therefore, under optimal temperature, the facultative endosymbionts cause
 325 an increase of the host metabolic expenses, and the insect host is not able to fully compensate
 326 this energy loss by increasing nutrient uptake for instance. In addition, we confirmed that aphid
 327 phenotype displays complex variations depending on bacterial species, host genotype and
 328 environmental conditions (Oliver et al., 2006; Russell & Moran, 2006). This complexity was
 329 particularly obvious at elevated temperature because in one association the SMR was reduced
 330 in presence of endosymbionts, suggesting that a physiological benefit of hosting microbes may
 331 happen under challenging thermal conditions.

332 4.1 Energetic cost of hosting facultative endosymbionts

333 Our study highlighted that aphids harboring *H. defensa* and *R. insecticola* generally
 334 experienced a decrease in adult lifespan and lifetime reproduction. This is in line with a recent
 335 meta-analysis concluding that, under benign conditions, the hosting of these two facultative

336 endosymbionts generally resulted in fitness costs in *A. pisum* (Zytynska et al., 2021). Naturally
337 occurring associations with facultative endosymbionts are expected to be less costly than
338 experimental infection, due to purifying selection against novel and more costly endosymbionts
339 (Oliver et al., 2006; Zytynska et al., 2021). Our results were not in accordance with this trend
340 as the LL01 aphid line did not suffer any fitness reduction when infected by *R. insecticola*
341 whereas it is the unique line in our study that is naturally deprived of any facultative
342 endosymbionts. Conversely, the fitness of YR2 aphids is strongly affected by the infection with
343 *R. insecticola* whereas this association (YR2+Reg^{YR2}) was established from field collection.
344 This result showed that new symbiotic associations are not systematically counter-selected and
345 can have a high probability of increasing in frequency within natural host populations. It could
346 be interesting to investigate whether the absence of infection cost depends on host or symbiont
347 genetic factors. Despite the establishment and maintenance of the lines used in this study for
348 several years in the laboratory, they are representative of the preferential associations between
349 the clover biotype of *A. pisum* and the endosymbiont *R. insecticola*, as well as the association
350 between the alfalfa biotype and *H. defensa* commonly found in natural populations of *A. pisum*
351 in various countries (Ferrari et al., 2011; Russell et al., 2013; Simon et al., 2003) and over years
352 (Leclair et al., 2021; Smith et al., 2015). However, it has been recently highlighted that
353 symbiont-associated fitness costs can fluctuate through long-time period in lab culture
354 putatively due to variations in symbiont densities (Kaeck et al., 2022), suggesting that our
355 results may under- or over-estimate symbiont-associated fitness costs of current natural
356 populations depending on changes in symbiont population sizes since collection.

357 Standard metabolic rate is classically interpreted as the energy cost of an organism to
358 live in a given environment (Burton et al., 2011). Thus, in many species including aphids, a
359 variation in SMR is interpreted as a variation in the energy costs associated with self-
360 maintenance (Castañeda et al., 2009). In the presence of facultative endosymbionts, aphids
361 generally expressed a higher SMR than uninfected ones under optimal thermal conditions (18-
362 22°C). This pattern is consistent with the hypothesis that infected aphids invest more energy to
363 self-maintenance, due to additional expenditures to hosting facultative endosymbionts. Such a
364 cost could arise from the metabolites and structures provided by the hosts to their
365 endosymbiotic partners (Degnan et al., 2009). Prior to our study, only the work of Evans et al.,
366 (2009) on *A. aegypti* infected with *Wolbachia* revealed a facultative endosymbiont-induced
367 increase in metabolic rate. Similar effects likely arise in *Drosophila* as well, since different
368 strains of *Wolbachia* either increase or decrease the flies' locomotor activity (Hague et al.,

369 2021), which necessarily implies variations in their metabolic rate. There are few studies
370 suggesting that *Wolbachia* could interact with its host's aerobic metabolism, either by
371 supplementing or hijacking it (Darby et al., 2012; Uribe-Alvarez et al., 2019). Such sub-cellular
372 effects of facultative endosymbionts need to be investigated in other symbiotic associations,
373 such as *H. defensa* and *R. insecticola* with aphids.

374 The comparison of several aphid lines revealed complex variations of the SMR
375 depending on the aphid and bacterial genotypes. Such phenotypic variations have been widely
376 reported for different traits and are more likely the result of complex host genotype-by-
377 symbiont genotype interactions (Parker et al., 2017; Vorburger & Gouskov, 2011). In various
378 insect-symbiont associations, fitness costs induced by endosymbionts have been positively
379 correlated with the bacterial density (Cayetano et al., 2015; Nichols et al., 2021). In our study,
380 nor fitness costs nor SMR were positively correlated with endosymbiont density. This
381 discrepancy might be due to insufficient statistical power in our study (N=5) or suggest
382 additional factors driving endosymbiont costs. It has been recently demonstrated that
383 endosymbiont density in *A. pisum* was controlled by the host immune system and that this was
384 influenced by host genetic factors, such that some aphid genotypes experienced high-density
385 symbiont titer due to reduced immune gene expression, whereas other genotypes had a strong
386 immune response, maintaining symbionts at a low density (Nichols et al., 2021). Future studies
387 should integrate the immune response of aphids to better understand the variations in bacterial
388 density and its consequence for hosts. Indeed, we can hypothesize contrasted strategies among
389 aphid lines used in our study, with some aphid genotypes regulating strongly endosymbiont
390 density at the expense of a strong immune response, whereas others let the endosymbionts reach
391 a high density at the cost of a significant uptake of resources by the bacteria. In both cases, we
392 expect that infection by endosymbionts results in an increase in metabolic rate and a decrease
393 of the host fitness.

394 4.2 Link between SMR and fitness

395 The “compensation hypothesis” proposes that a higher SMR corresponds to a higher
396 allocation of energy to self-maintenance, and thereby a lower amount of energy remaining to
397 fuel growth and reproduction (Nilsson, 2002). This hypothesis was supported by the differences
398 observed between infected and uninfected aphids as aphids carrying facultative endosymbionts
399 had a higher SMR in general and presented lower fitness-related traits than their uninfected
400 counterparts (but see below for exception). However, the expected negative correlation between
401 aphid fitness and SMR was not clear when comparing the different genotype-symbiont

402 associations (Fig. 3), probably due to the large variability among infected lines relative to the
403 number of associations (N=9). This discrepancy feeds the debate around the link between SMR
404 and fitness in ectotherms (Burton et al., 2011; Careau & Garland, 2012) and could be explained
405 by alternative hypotheses. The "increased intake" hypothesis proposed that organisms with a
406 higher SMR would have a better (or unchanged) fitness thanks to a higher energy intake (Hayes
407 et al., 1992). LL01 aphids carrying *R. insecticola* presented a higher SMR without fitness
408 reduction compared to their uninfected counterpart, suggesting that "increased intake"
409 hypothesis holds for this line. The fact that this line is also the only one with a higher energetic
410 budget than its uninfected counterpart tends to support this hypothesis too. Further studies on
411 feeding behavior (e.g. EPG monitoring) and aphid intake (e.g. honeydew production assay)
412 would confirm whether aphids of this line increase their feeding intake (quantitatively and
413 qualitatively by consuming either xylem or phloem). Conversely, fitness of YR2 aphids
414 infected by *H. defensa* was strongly reduced without variation in their SMR compared to
415 uninfected aphids. This suggests pathogenic effects of this symbiotic strain putatively
416 associated with its over-replication, leading to local cellular and tissue dysfunctions and
417 damages, like *Wolbachia* wMelPop in *D. melanogaster* (Min & Benzer, 1997). We cannot
418 exclude however that the density of the obligate endosymbiont, *B. aphidicola*, varies among
419 aphid lines, with possible effect on aphid fitness and on the functions endorsed by the facultative
420 endosymbionts. Indeed, the facultative endosymbiont *Serraria symbiotica* can have a
421 nutritional role when *B. aphidicola* does not fully fulfill this function (Koga et al., 2003;
422 Lamelas et al., 2011). It is also important to note that our study is based on only one time point
423 measurement of SMR and an integrated measure of metabolic rate over lifetime could improve
424 our understanding of the relationship between SMR and fitness, by taking into account potential
425 age-related variations of the metabolic rate (Terblanche et al., 2005).

426 4.3 Impact of thermal conditions on facultative symbiosis

427 The effect of endosymbionts on aphid SMR varied according to the temperature. The
428 difference in SMR between infected and uninfected aphids tended to decrease with increasing
429 temperature. In one genotype (L9MS07), we even observed a lower SMR in infected aphids
430 than uninfected ones at 26°C, to the point that their SMR became slightly below the SMR of
431 infected aphids at 22°C. We can exclude the hypothesis that the variations in metabolic rate
432 according to the temperature are due to variations in the density of primary and secondary
433 endosymbionts because the aphids experienced these different temperatures only during the
434 SMR assay (i.e. one hour). This result suggests that the presence of endosymbionts can alter

435 the temperature-dependency of the metabolic rate, a hypothesis that requires to consider a wider
436 temperature range. Reduced sensitivity of the metabolic rate to temperature (Q_{10}) and metabolic
437 depression could be adaptive responses in the face of challenging elevated temperatures to
438 minimize the energetic costs associated with thermal stress (González-Tokman et al., 2020;
439 Storey & Storey, 2004). They could explain, at least partially, the beneficial role of *H. defensa*
440 under heat stress (Heyworth et al., 2020) and its higher prevalence after hot periods within field
441 populations (Carpenter et al., 2021; Smith et al., 2021). However, in our study, we focused only
442 on the constitutive costs of facultative endosymbionts for their host, but the fitness benefits of
443 facultative endosymbionts can also vary according to the temperature (Cayetano & Vorburger,
444 2013). Thus, the co-variation of benefits and costs of endosymbionts appears to be a central
445 question for understanding the stability and evolution of these symbiotic associations under
446 changing thermal environments. Such investigations are particularly important to predict the
447 impact of climate change on symbiotic association (Iltis et al., 2021; Renoz et al., 2019).
448 Interestingly, this concept of co-variation of benefits and energetic costs certainly also applies
449 to other types of associations. Some plant viruses provide benefits to their aphid vectors in
450 terms of host plant suitability (Bosque-Pérez & Eigenbrode, 2011) and tolerance to high
451 temperatures (Porrás et al., 2020). Viral infections have been associated with metabolic
452 modifications in *D. melanogaster* (Arnold et al., 2013) but their consequences in aphids remain
453 unknown. Thus, this framework raises new questions about the effect of facultative
454 endosymbionts or plant viruses on the energy metabolism of their host and ultimately on their
455 response to environmental challenges.

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766 **Supporting information**

767 Table S1 List of primers used in the study

768 Table S2 Statistical analyses of energetic components

769 Figure S1 Protein, lipid, free sugar and glycogen contents