

1 The influence of facultative endosymbionts on honeydew carbohydrate and
2 amino acid composition of the black bean aphid *Aphis fabae*

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20 **Abstract**

21 The facultative endosymbionts *Hamiltonella defensa* and *Regiella insecticola* are commonly found in
22 aphids. They are linked with various ecological benefits but generally occur at low prevalence, which
23 indicates a possible harboring cost. Little is known about how the presence of facultative
24 endosymbionts is reflected in honeydew composition. Honeydew is the key mediator of the aphids'
25 mutualism with tending ants. The present study examines whether endosymbionts have an influence
26 on aphid honeydew quality by comparing the amino acid and carbohydrate concentrations between
27 infected and uninfected aphids. To this end, two genetic lines of the aphid *Aphis fabae* Scopoli were
28 experimentally infected with different strains of *Hamiltonella* and *Regiella*. Infected aphids were
29 shown to have reduced concentrations of amino acids in the honeydew compared to uninfected
30 aphids. However, the presence of endosymbionts had no effect on the absolute amount of
31 carbohydrates produced. Nevertheless, interclonal variation in honeydew composition between
32 aphid genotypes was observed for both carbohydrate and amino acid production. These results imply
33 that the nutritional value of honeydew depends on aphid genotype as well as on the presence of
34 secondary bacterial endosymbionts, which suggests there is a physiological cost of harboring
35 endosymbionts and which could also impact aphid attractiveness to tending ants.

36

37 **Keywords:** Aphids, Honeydew, Endosymbiosis, Mutualism, *Aphis fabae*, *Hamiltonella*, *Regiella*

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39

40 **Introduction**

41 Insects have formed facultative or obligate relationships with multiple bacteria. This is called
42 endosymbiosis when the bacteria reside inside the host (Buchner, 1965). Endosymbiotic bacteria
43 have no free-living state and cannot propagate when taken out of their host (Hinde, 1971). In aphids,
44 several endosymbioses have been well characterized, with almost all aphids harboring at least one
45 symbiotic prokaryote, *Buchnera aphidicola* (Munson *et al.*, 1991). This symbiont plays a prominent
46 role in nutrition by aiding in synthesis of nutrients that are lacking in the diet, mainly providing
47 essential amino acids (Buchner, 1965; Douglas, 1996). Aside from the obligate mutualistic symbiosis
48 between aphids and the intracellular bacteria *Buchnera*, which is commonly referred to as the
49 primary symbiont (Buchner, 1965), aphids can also have several species of facultative secondary
50 symbionts. The relationship of these secondary symbionts with their host can be parasitic,
51 commensal or mutualistic, depending on the bacterial species and the environment (Douglas *et al.*,
52 2001; Zytynska & Weisser, 2015).

53 Beneficial ecological influences of the secondary endosymbionts *Hamiltonella defensa* and *Serratia*
54 *symbiotica* have been shown to be related to heat tolerance (Chen *et al.*, 2000; Montllor *et al.*, 2002;
55 Russell & Moran, 2006), whereas *Regiella insecticola* can increase fungal pathogen resistance
56 (Scarborough *et al.*, 2005) and change host plant suitability (Tsuchida *et al.*, 2004). Furthermore,
57 particular strains of all aforementioned endosymbionts can also increase parasitoid resistance (Oliver
58 *et al.*, 2003; Vorburger *et al.*, 2009; Vorburger *et al.*, 2010; Asplen *et al.*, 2014). Recently, a
59 combination of the aforementioned benefits was also discovered to be associated with carrying
60 strains of an endosymbiotic bacterium known as X-type, which belongs to the Enterobacteriaceae
61 (Heyworth & Ferrari, 2015). Parasitic influences are related to septicemia of tissues leading to death
62 of the host (Costechareyre *et al.*, 2012) and negative effects on growth, fecundity and longevity of
63 the host (Fukatsu *et al.*, 2001; Simon *et al.*, 2011; Vorburger & Gousskov, 2011). From the experiments
64 mentioned above, it is clear that the impact of the endosymbionts, and whether they are beneficial
65 or detrimental, is highly species and genotype dependent (Koga *et al.*, 2007; Asplen *et al.*, 2014;

66 Vorburger, 2014) and sometimes even reliant on symbiont by symbiont interaction effects (Oliver *et*
67 *al.*, 2006; Tsuchida *et al.*, 2014). Secondary symbionts can also play a role in aphid nutrition. The
68 symbiont *Serratia*, for example, has been shown to synthesize several essential amino acids, thereby
69 successfully replacing the function of *Buchnera* (Douglas & Prosser, 1992; Koga *et al.*, 2003), and it
70 has even evolved into a co-obligate symbiont in aphids of the genus *Cinara* (Lamelas *et al.*, 2011).
71 However, symbionts *Regiella* and *Hamiltonella* have lost all essential amino acid biosynthetic
72 pathways except for those relating to the production of two essential amino acids, threonine and
73 lysine (Degnan *et al.*, 2009; Degnan *et al.*, 2010).

74 Aphids are very successful and often considered as pest species because of their impact on
75 agricultural crops, causing damage by direct feeding and by transmission of plant diseases. Aphids
76 are plant-sucking insects and can become very numerous thanks to their predominantly asexual
77 mode of reproduction. The diet of the aphids consists of the phloem sap of plants, a sugar-rich but
78 nitrogen and amino acid-poor fluid. As a consequence of this diet aphids excrete honeydew, a waste
79 product that contains large amounts of both simple and complex carbohydrates and also a small
80 amount of amino acids and vitamins (Mittler, 1958). Osmoregulation is widely accepted as the main
81 reason for honeydew and complex carbohydrate production (Downing, 1978; Fisher *et al.*, 1984;
82 Wilkinson *et al.*, 1997; Douglas, 2003), but honeydew composition can be influenced by several
83 factors. Honeydew carbohydrate composition is variable between aphid species (Volkl *et al.*, 1999),
84 aphid genotypes (Vantaux *et al.*, 2011; Katayama *et al.*, 2013), aphid instars (Fischer *et al.*, 2002),
85 host plants (Volkl *et al.*, 1999; Fischer & Shingleton, 2001; Fischer *et al.*, 2005), seasons (Wool *et al.*,
86 2006) and can be influenced by ant-tending (Fischer & Shingleton, 2001; Yao & Akimoto, 2002).
87 Amino acid composition is variable between aphid species (Sandström & Moran, 2001; Woodring *et*
88 *al.*, 2004), aphid instars and the presence or absence of primary endosymbiont *Buchnera* (Sasaki *et*
89 *al.*, 1990) and can increase in response to ant-tending (Yao & Akimoto, 2002). However, there is no
90 indication of a different amino acid composition between host plants (Sandstrom & Pettersson,
91 1994) or in function of seasonal differences in phloem sap composition (Yao & Akimoto, 2002).

92 Honeydew is a key factor that drives the mutualism between ants and aphids. It is a high nutrition
93 food source for ants and in return the ants protect aphids against natural enemies (Nixon, 1951;
94 Banks, 1962; Buckley, 1987). The mutualistic interaction between ants and aphids could also exert a
95 selective pressure on the composition of honeydew, to maximize the attractiveness and nutritional
96 value for the ants (Volkl *et al.*, 1999) as the aphids benefit from the visits of ants (Hölldobler &
97 Wilson, 1990; Flatt & Weisser, 2000).

98 This study investigates the role of the facultative secondary endosymbionts *Hamiltonella* and *Regiella*
99 on the honeydew composition of *Aphis fabae* Scopoli, and thereby evaluates if harboring
100 endosymbionts could influence ant-aphid mutualisms. Presence of endosymbionts would be
101 expected to affect honeydew composition if the endosymbionts compete for (i) host niche with
102 *Buchnera*, thereby resulting in reduced amino acid production by *Buchnera* (Koga *et al.*, 2003; Sakurai
103 *et al.*, 2005) and/or (ii) essential amino acids and carbohydrates (Chandler *et al.*, 2008). Our results
104 demonstrate that presence of the secondary endosymbionts decreased total amino acid
105 concentrations in the honeydew, but that they had no effect on carbohydrate concentrations in the
106 honeydew. We suggest that the decrease in amino acid concentration could indicate a physiological
107 or ecological cost of harboring the secondary endosymbionts.

108

109 **Material and methods**

110 *Study organism*

111 The studied black bean aphid (*Aphis fabae*) is a common aphid species in the northern hemisphere. It
112 is a polyphagous species and has a wide range of secondary host plants ranging from cultivated to
113 wild plants (Stroyan, 1984). Based on those secondary host plants it is possible to distinguish four
114 different subspecies of *A. fabae*. In this study, we focused on *A. fabae fabae*, which has broad bean
115 (*Vicia faba*) as the main summer host plant. The winter host plants, mainly the European spindle tree

116 (*Euonymus europaeus*), are used in autumn for winter survival of eggs deposited after sexual
117 reproduction. In spring the eggs hatch and viviparous, parthenogenetic females start reproducing,
118 after which offspring migrates to the secondary host plants where they reproduce asexually for the
119 remainder of the spring and summer. The honeydew of *A. fabae* is highly dependent on genotype,
120 with especially the production of the trisaccharide melezitose being highly variably across aphid
121 genotypes (Vantaux *et al.*, 2011). The studied aphids belong to two clones collected in Switzerland on
122 *Chenopodium album*, hereafter referred to as clone A and B. Clone A can be categorized as a low
123 melezitose clone with melezitose levels <1%, and clone B as a high melezitose clone with melezitose
124 levels >20% in honeydew samples.

125 A PCR screening showed that both clones were clear of any of the known secondary symbionts
126 (Vorburger *et al.*, 2009). Subsequently, they were experimentally infected with one of each of two
127 strains of *Regiella* (R49 and R5.15) or one of each of four strains of *Hamiltonella* (H30, H323, H76,
128 HAF6). This generated 13 different lines, as infection of R5.15 only succeeded in one of the aphid
129 genotypes. Hereafter, the different infected lines are referred to respectively as R1, R2, H1, H2, H3
130 and H4. Infection occurred via microinjection, as described in Vorburger *et al.* (2010). The clonal lines
131 were propagated asexually on *Vicia faba* plants (seeds from Somers Seeds NV, Mechelen, Belgium)
132 under controlled laboratory conditions (LD 16:8 h photocycle, 20 ± 1 °C and 65-70% humidity).

133

134 *Collection of honeydew*

135 *Vicia faba* plants were grown from seeds in universal garden soil. Two weeks after sowing, the plants
136 were inoculated with aphids to collect honeydew as previously described in Vantaux *et al.* (2011).
137 Ten aphids (adults or fourth instar) were transferred to an apical leaf enclosed in a plastic box
138 (50x25x30 mm). After 24h the numbers of aphids that survived were counted to take into account
139 how many aphids had successfully settled on the plant and therefore had contributed to the
140 production of the honeydew. This survival measurement does not distinguish between a settlement

141 cost and survival costs related to the presence of endosymbionts. The box and leaf were rinsed with
142 400 µL of a 0.02% sodium azide solution. The obtained mixture was boiled for 5 min, dried out
143 overnight in an oven at 60 °C, re-dissolved in 200µL of 0.02% sodium azide solution and stored at -20
144 °C until further analysis. For every aphid line 5 to 7 samples were obtained following this protocol.

145

146 *Carbohydrate and amino acid analysis*

147 Before carbohydrate analysis, all samples were passed through a mixed-bed Dowex column to obtain
148 a neutral fraction (Van den Ende *et al.*, 1996). This was done by allowing 50 µl of sample solution
149 followed by 1.2 mL of distilled water to pass through the column. The samples were then centrifuged
150 for 5 min and stored at -20 °C until they could be chemically analysed. To analyse the amino acids,
151 each sample solution was diluted 5 times before the chemical analyses.

152 High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-
153 PAD, Dionex ICS 3000, Sunnyvale, California) was used to analyse the carbohydrates and amino acids
154 present in all honeydew samples (see protocol described in Vantaux *et al.* (2011) and Gijbels *et al.*
155 (2014). The molar concentrations of the different carbohydrates and amino acids in each analysed
156 sample were estimated by comparing the area under the chromatogram peaks with standards using
157 the software package Chromeleon (Dionex, Sunnyvale, California). Overall we could detect 8
158 different carbohydrates and all 20 primary amino acids in the honeydew samples. Due to column
159 interference only 17 amino acids were used in the final analyses. The missing amino acids had only
160 minor contributions to the total amino acid concentration in the honeydew samples.

161

162 *Statistical analysis*

163 To analyse the absolute concentrations of all compounds found in the honeydew we used linear
164 mixed models (Bates *et al.*, 2014) with a Gaussian distribution on log transformed data to fulfill the
165 assumptions. The full model consisted of a random intercept model with aphid genotype, symbiont
166 species and their interaction as fixed factors, the number of aphids coded as a continuous covariate
167 and symbiont strain nested within the species and replicate coded as random intercepts, to take into
168 account repeated measurement of the same aphid genotypes (Schank & Koehnle, 2009). A backward
169 stepwise model simplification based on the Akaike information criterion (AIC) was used to arrive at a
170 minimum adequate model considering the structure of the experimental setup. This resulted in
171 omitting the interaction term for all compounds. All results are displayed as least square means and
172 95% CI. Species differences were corrected for multiple comparisons with a Tukey's post hoc test. All
173 statistical analyses were performed in R 3.2.0 (R core team, 2015).

174

175 **Results**

176 *Amino acids*

177 A general pattern of reduced total amino acid concentration was observed in the lines infected with
178 secondary endosymbionts. A decrease of 51 % was detected for *Hamiltonella* (347.72 CI [280.14-
179 431.6]; P-value=0.008) and a 47 % decrease for *Regiella* (373.05 CI [261.13-532.93]; P-value=0,04)
180 compared to the uninfected lines (706.95 CI [461.73-1082.41]) (Fig. 1a). For 9 of the 17 individual
181 amino acids this trend was significant for at least one endosymbiont species (Fig. 2).

182 The major amino acid compounds (>10% of total amino acid concentration) were arginine,
183 asparagine and glycine for both aphid genotypes. Minor amino acid compounds (<1% of total amino
184 acid concentration) were glutamine, ornithine and methionine, with methionine absent from all
185 honeydew samples. For 11 of the 17 individual amino acids there were significant differences in the
186 concentration between the aphid genotypes (Fig. 3) with a general trend of lower concentrations in

187 clone B, resulting in a 36 % lower total concentration of amino acid in the honeydew compared to
188 clone A (clone A: 506.13 CI [355.91-621.97]; clone B: 324.40 CI [240.27-391.21]; P-value: 0.01) (Fig.
189 1b).

190 *Carbohydrates*

191 The two aphid clones showed significant differences in concentration of several carbohydrates. Clone
192 A showed increased fructose (P-value= 0.03), maltose (P-value= 0.005) and maltotriose (P-value=
193 0.02) production but a decreased melezitose (P-value <0.001) production compared to clone B. The
194 total concentration of carbohydrates did not show significant differences between the two clones
195 (Fig. 4). The monosaccharide fructose was the most abundant sugar followed by glucose for both
196 aphid clones and as already known the trisaccharide melezitose was only detected in clone B.
197 However, the endosymbionts showed no significant influence on the concentration of the
198 carbohydrates found in the honeydew (P-value=0.54).

199

200 **Discussion**

201 Little is known about how the presence of facultative endosymbionts *Hamiltonella* and *Regiella*
202 influences the composition of aphid honeydew. The present results clearly demonstrate that
203 secondary endosymbionts have an impact on honeydew quality, thus suggesting that there is a
204 significant cost to harbor them. Indeed, the amount of amino acids found in the honeydew of
205 infected aphids was consistently reduced for both *Hamiltonella* and *Regiella* infected aphids
206 compared to uninfected aphids, while the total amount of carbohydrates was not affected. A
207 harboring cost is also consistent with the fact that secondary endosymbionts occur at moderate or
208 low frequencies despite conferring specific benefits (Oliver *et al.*, 2006; Vorburger & Gousskov, 2011;
209 Zytynska & Weisser, 2015). Presence of endosymbionts would be expected to be costly if they
210 compete for host niche and/or limited resources with *Buchnera*, resulting in reduced *Buchnera*

211 densities and amino acid production. Both primary and secondary endosymbionts are hosted in
212 special cells and declining densities of *Buchnera* with aphid age have been observed when a
213 coinfection occurred with *Serratia*, whereas *Serratia* densities increased until a plateau was reached
214 (Koga *et al.*, 2003). Similarly, co-infection with an endosymbiont of the genus *Rickettsia*, another
215 common facultative endosymbiont in aphids, resulted in lower densities of *Buchnera* (Sakurai *et al.*,
216 2005). An additional cost can arise if secondary endosymbionts compete for essential amino acids.
217 The infection of *A. fabae* with *Hamiltonella* and *Regiella* showed a diet-dependent impact on aphid
218 fitness with a reduced performance when reared on diets with lower amino acid concentrations
219 (Chandler *et al.*, 2008). Further research, however, would be required to determine which of these
220 factors best explain the reduced amino acid secretion in the honeydew of *A. fabae*.

221 Depending on aphid species, different essential amino acids may be absent from the honeydew
222 (Woodring *et al.*, 2004). In our experiment, for example, the essential amino acid methionine was
223 absent from all honeydew samples. This is a common pattern, as methionine has also been shown to
224 be absent from the honeydew of other aphid species feeding on wheat (Sandström & Moran, 2001),
225 broad bean plants (Sasaki *et al.*, 1990) and tansy (Woodring *et al.*, 2004). The absence of essential
226 amino acids from honeydew suggest the presence of resource limitations, and resource limitation
227 combined with competition from secondary endosymbionts has earlier been shown to result in
228 significant fitness costs in *A. fabae* (Chandler *et al.* 2008).

229 Besides the intraspecific variation in melezitose production, this study reveals an aphid genotype-
230 dependant variation in amino acid concentration as well. More genotypes should be studied to know
231 if the detected decrease of amino acid concentration found in the honeydew of the low melezitose
232 genotype is linked to the lack of melezitose production or to aphid genotype-dependent population
233 sizes of the primary endosymbiont *Buchnera* (Zhang *et al.*, 2016). Alternatively, the significant
234 difference in amino acid concentration in the honeydew could indicate a difference in resilience to
235 the presence of secondary endosymbionts (Scarborough *et al.*, 2005; Russell & Moran, 2006; Łukasik
236 *et al.*, 2013) .

237 Finally, melezitose concentrations also showed aphid genotype-related variation, as had previously
238 been observed by Vantaux *et al.* (2011). The lack of melezitose production in clone A, however, was
239 completely compensated for by increased production of the other carbohydrates, as there was no
240 notable difference in total amount of carbohydrates found in the honeydew of the two aphid clones.
241 Vantaux *et al.* (2011) compared the honeydew of several low- and high-melezitose *A. fabae* clones
242 and found interclonal differences in glucose, melezitose and total carbohydrate production but no
243 general trends to explain the lack of melezitose in low-melezitose clones. In our study, clone A
244 seemed to compensate the lack of melezitose production mainly by excreting the monosaccharide
245 fructose. The presence of oligosaccharides, e.g. melezitose, in the honeydew of aphids are generally
246 seen as a mechanism to deal with the osmotic stress related to the ingestion of large amounts of
247 phloem sap (Downing, 1978; Fisher *et al.*, 1984; Wilkinson *et al.*, 1997; Douglas, 2003). However,
248 from our results, it appears that clone A must have evolved a different strategy to deal with osmotic
249 pressure without affecting honeydew production or fitness (Vantaux *et al.*, 2011; Vantaux *et al.*,
250 2015). In the future, this intraspecific variation may be exploited as a model for further studies into
251 the regulation of gut osmolarity in aphids.

252 Previous results have shown that melezitose is also an important carbohydrate in the ant-aphid
253 mutualism (Kiss, 1981; Volkl *et al.*, 1999; Woodring *et al.*, 2004; Detrain *et al.*, 2010). Higher
254 melezitose concentrations in honeydew, for example, have been shown to increase ant attendance
255 (Volkl *et al.*, 1999; Woodring *et al.*, 2004). The ant – aphid interaction is mainly based on honeydew
256 as a carbohydrate source and even though for many honeydew collecting ant species this source
257 seems to fulfill most nutritional demands (Alsina *et al.*, 1988; Fiedler *et al.*, 2007; Domisch *et al.*,
258 2009), predation of aphids is also observed (Sakata, 1994; Offenberg, 2001; Billick *et al.*, 2007).
259 Besides variability in carbohydrates, amino acid variability is proposed to play a role in ant preference
260 (Blüthgen & Fiedler, 2004). The nutritional requirements of ants, especially the protein demands, can
261 have seasonal fluctuations that go along with brood production and increased predation rate on
262 aphids (Abril *et al.*, 2007). In general, ants prefer a carbohydrate solution with amino acids over a

263 pure carbohydrate solution (Blüthgen & Fiedler, 2004) and ant-tended aphid species have higher
264 amino acid concentrations (Lanza *et al.*, 1993; Woodring *et al.*, 2004). Some ant species have also
265 been shown to prefer amino acid-rich food sources (Lanza *et al.*, 1993; Lee Cassill & Tschinkel, 1999).
266 In addition, aphids appear to be able to maintain a constant amino acid concentration across
267 different seasons (Yao & Akimoto, 2002) and when reared on different host plants (Sandstrom &
268 Pettersson, 1994), and the drepanosiphid aphid (*Tuberculatus quercicola*) produced more amino
269 acid-rich honeydew when ant tended (Yao & Akimoto, 2002). Overall, these results indicates that the
270 ant-aphid mutualism exerts positive selection for an increased nutritional value of honeydew.
271 Consequently, the presence of secondary endosymbionts and concomitant changes in the nutritional
272 value of the honeydew would also be expected to affect the ant-aphid mutualism and ant
273 attractiveness. Earlier, volatile compounds from bacterial origin have also been shown to affect the
274 ant attractiveness of aphids (Leroy *et al.*, 2011; Fischer *et al.*, 2015). Finally, the presence of
275 secondary endosymbionts could induce a higher predation rate, to compensate the reduced amino
276 acid concentration in the honeydew and satisfy the nutritional requirements of the tending ants. In
277 the future, these ideas would be interesting to test further in controlled laboratory trials.

278 Overall, our study shows that aphid honeydew composition is affected not just by aphid genotype
279 but also by the presence or absence of secondary endosymbionts. These results suggest that
280 harboring secondary endosymbionts carries significant fitness costs. Future studies could be aimed at
281 testing whether the altered nutritional value of the honeydew also impacts the ant-aphid mutualism.

282

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286

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481 **Figure Legends**

482 **Fig. 1.** Total amino acid concentration found in honeydew of *Aphis fabae* for (a) the uninfected
483 (none) and infected lines with *Regiella insecticola* (R) and *Hamiltonella defensa* (H) and (b) the two
484 aphid genotypes. Significant differences after post-hoc test (Tukey) are indicated with * for $P \leq 0.05$.

485 **Fig. 2.** Mean concentration of the minor (<1%), intermediate (<10%), and major (>10%) amino acid
486 components found in honeydew of *Aphis fabae* for the uninfected (none) and infected lines with
487 *Regiella insecticola* and *Hamiltonella defensa* (% of total amino acid concentration). Significant
488 differences after post-hoc correction (Tukey) are indicated with * for $P \leq 0.05$, ** $P \leq 0.01$ and with
489 *** for $P \leq 0.001$.

490 **Fig. 3.** Mean concentration of the minor (<1%), intermediate (<10%), and major (>10%) amino acid
491 components found in honeydew of *Aphis fabae* for the two aphid genotypes (% of total amino acid
492 concentration). Significant differences between the genotypes for individual components are
493 indicated with * for $P \leq 0.05$, ** $P \leq 0.01$ and with *** for $P \leq 0.001$.

494 **Fig. 4.** Mean concentration of the (a) minor (<5%) and (b) major (>5%) compounds and the total
495 carbohydrate concentration found in honeydew of *Aphis fabae* for the two aphid genotypes (% of
496 total carbohydrate concentration). Significant differences between the genotypes for individual
497 compounds is indicated with * for $P \leq 0.05$, ** $P \leq 0.01$ and with *** for $P \leq 0.001$.

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