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1   **A fitness cost resulting from *Hamiltonella defensa* infection is associated with altered probing and**

2   **feeding behaviour in *Rhopalosiphum padi***

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4   **Running title**

5   Symbiont effects on aphid feeding

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19   **Summary**

20   Reduced performance of aphids infected with a common facultative endosymbiont on poor quality

21   plants may be explained by changes in aphid probing behaviour and decreased phloem sap

22   ingestion.

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28   **Abstract**

29   Aphids frequently associate with facultative endosymbiotic bacteria which influence aphid

30   physiology in myriad ways. Endosymbiont infection can increase aphid resistance against parasitoids

31   and pathogens, modulate plant responses to aphid feeding, and promote aphid virulence. These

32   endosymbiotic relationships can also decrease aphid fitness in the absence of natural enemies or

33   when feeding on less suitable plant types. Here, we use the Electrical Penetration Graph (EPG)

34   technique to monitor feeding behaviour of four genetically-similar clonal lines of a cereal-feeding

35   aphid, *Rhopalosiphum padi,* differentially infected (+/-) with the facultative protective

36   endosymbiont, *Hamiltonella defensa,* to understand how physiological processes at the aphid-plant

37   interface are affected by endosymbiont infection. Endosymbiont-infected aphids exhibited altered

38   probing and feeding patterns compared with uninfected aphids, characterised by a two-fold increase

39   in the number of plant cell punctures, a 50% reduction in the duration of each cellular puncture, and

40   a greater probability of achieving sustained ingestion of plant phloem. Feeding behaviour was

41   altered further by host plant identity: endosymbiont-infected aphids spent less time probing into

42   plant tissue, required twice as many probes into plant tissue to reach plant phloem, and showed a

43   44% reduction in phloem ingestion when feeding on the partially-resistant wild relative of barley,

44   *Hordeum spontaneum* 5, compared with a commercial barley cultivar. These observations might

45   explain reduced growth of *H. defensa*-infected aphids on the former host plant. This study is the first

46   to demonstrate a physiological mechanism at the aphid-plant interface contributing to

47   endosymbiont effects on aphid fitness on different quality plants through altered aphid feeding

48   behaviour.

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Introduction

60 Aphids are major pests of agricultural and horticultural crops with worldwide distribution (Van
61 Emden and Harrington, 2017). To feed they probe into plants using specialised mouthparts known as
62 stylets, with the aim of establishing a feeding site in the plant phloem. While probing into plant
63 tissues, aphids can transmit plant viruses (Powell, 2005), which are the main cause of economic crop
64 losses resulting from aphid infestation. Most aphid species harbour the obligate bacterial
65 endosymbiont *Buchnera aphidicola,* which supplies aphids with essential amino acids they are
66 unable to biosynthesise (Sasaki et al., 1991; Douglas and Prosser, 1992). Additional co-obligatory
67 symbiotic relationships have been described with other endosymbiont species, including *Wolbachia*
68 *sp.* in the banana aphid, *Pentalonia nigronervosa* (De Clerck et al., 2015)(although see Manzano-
69 Marín, 2019 preprint for updated analysis), and with *Serratia symbiotica* in multiple species of the
70 *Cinara* genus (Meseguer et al., 2017). In most other aphid species, however, these co-infecting
71 endosymbionts are not essential for survival. Alongside obligatory endosymbiotic relationships,
72 aphids can form facultative (non-essential) endosymbiotic relationships with a range of
73 microorganisms.

74 The diversity and frequency of infection with facultative endosymbionts can vary considerably
75 between and within aphid species (de la Peña et al., 2014; Henry et al., 2015; Zytynska and Weisser,
76 2016; Guo et al., 2019). The eleven most common facultative endosymbionts are *Hamiltonella*
77 *defensa, Regiella insecticola, S. symbiotica, Rickettsia sp., Ricketsiella sp,* the *Pea Aphid X-type*
78 *Symbiont (PAXS), Spiroplasma sp., Wolbachia sp., Arsenophonus sp., Sitobion miscanthis L-type*
79 *Symbiont (SMLS),* and *Orientia-Like Organism (OLO)* (Oliver et al., 2006; Castañeda et al., 2010;
80 Tsuchida et al., 2010; Łukasik et al., 2013a; de la Peña et al., 2014; Leybourne et al., 2018). A meta-
81 analysis of endosymbiont infection frequencies by Zytynska and Weisser (2016) assessed the
82 proportion of aphid species shown to harbour *H. defensa, R. insecticola, S. symbiotica, Rickettsia sp.,*
83 *Spiroplasma sp., PAXS, Arsenophonus sp.* and *Wolbachia sp.* infections. The number of aphid species
84 tested for each endosymbiont varied from 14 aphid species tested for PAXS infection, to 190 aphid
85 species tested for *Wolbachia* infections (Zytynska and Weisser, 2016). This analysis found that the
86 most frequently detected endosymbiont in aphids is *Serratia symbiotica* (47% of the 156 aphid
87 species tested were infected) with *Arsenophonus sp.* the least frequently detected (7% of the 131
88 aphid species tested were infected) (Zytynska and Weisser, 2016).

89 The benefits of aphid infection with nine of these endosymbionts has recently been reviewed by Guo
90 et al. (2017) and a meta-analysis of the costs and benefits of facultative endosymbiont infection has

91   recently been conducted by Zytynska et al. (2019 preprint). Beneficial traits conferred to the aphid

92   by the endosymbionts include protection against parasitism by Braconid wasps (*H. defensa* and *R.*

93   *insecticola;* Hansen et al. (2012); Leybourne et al. (2018)), protection against entomopathogenic

94   fungi (*R. insecticola, Rickettsia sp., Ricketsiella sp.* and *Spiroplasma sp.;* Łukasik et al. (2013b)), host-

95   plant adaptation (*Arsenophonus sp.;* Wagner et al. (2015)), heat tolerance (*S. symbiotica* and *H.*

96   *defensa,* alongside *B. aphidicola* mutations; Russell and Moran (2006); Dunbar et al. (2007)),

97   morphological changes in insect colour (*Ricketsiella sp.*; Tsuchida et al. (2010); Nikoh et al. (2018)),

98   and enhanced aphid virulence (mixed symbiont communities; Luna et al. (2018)). Infection with

99   endosymbionts can, however, result in negative fitness consequences for the aphid host, including

100   decreased growth (*Rickettsia sp.;* Sakurai et al., 2005), reduced fecundity (*Spiroplasma sp., H.*

101   *defensa,* and *S. symbiotica; Chen et al. (2000); Castañeda et al. (2010); Li et al. (2018); Mathé-Hubert*

102   *et al. (2019)),* shorter aphid longevity (*Spiroplasma sp.* and *S. symbiotica; Chen et al. (2000); Mathé-*

103   *Hubert et al. (2019)),* lower adult mass (*S. symbiotica*; Skaljac et al. (2018)), and increased

104   susceptibility to insecticide (*S. symbiotica*; Skaljac et al. (2018)).

105   Around one-third of 154 aphid species assessed for endosymbiont presence have been reported to

106   harbour *H. defensa* (Zytynska and Weisser, 2016). Amongst cereal-feeding aphids, the proportion of

107   the bird-cherry oat aphid, *Rhopalosiphum padi,* populations infected with the defensive

108   endosymbiont, *H. defensa,* is around 10.8% (63/585 individuals; Guo et al., (2019)). The primary trait

109   conferred to aphids infected with *H. defensa* is protection against parasitism by Braconid wasps

110   (Oliver and Higashi, 2019). This protective phenotype often depends on the association of *H. defensa*

111   with virulent strains of the bacteriophage *APSE (Acyrthosiphon pisum Secondary Endosymbiont)*

112   which secretes toxins that arrest the development of the parasitoid wasp larvae (Brandt et al., 2017).

113   A recent study has shown that *H. defensa* infection can alter interactions at the aphid-plant interface

114   by influencing aphid probing behaviour (Angelella et al., 2018). This observation suggests that

115   altered aphid probing behaviour could affect aphid fitness by altering feeding success and thus

116   potentially impacting on aphid nutrition. Indeed, *H. defensa*-infection has been shown to have

117   consequences for aphid fitness (Castañeda et al., 2010; Vorburger and Gouskov, 2011; Li et al., 2018;

118   Zytynska et al., 2019 preprint), and examining these symbiont effects in relation to aphid probing

119   behaviour could elucidate the mechanistic processes contributing towards symbiont-associated

120   fitness consequences.

121   The Electrical Penetration Graph (EPG) technique is an electrophysiological tool used to monitor the

122   probing and feeding behaviour of sap-feeding insects (Tjallingii, 1985; Tjallingii and Esch, 1993; Prado

123   and Tjallingii, 1994) and has been used successfully to monitor the feeding and probing behaviour of

124   aphids (Greenslade et al., 2016), whiteflies (Chesnais and Mauck, 2018), psyllids (Civolani et al.,

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125   2011) and planthoppers (He et al., 2011). The technique is based on an electrical circuit which is

126   made by inserting conductive copper probes into the soil around the plant and adhering conductive

127   wire onto the dorsum of the aphids (Tjallingii, 1978; Tjallingii, 1985). Both probes are connected to a

128   data logger and computational software. An electrical current is passed through the circuit, which is

129   closed when the aphid stylet comes into contact with plant tissue, and the resulting electrical

130   waveforms can be characterised to provide information on aphid stylet activities (probing and

131   feeding behaviour) (Kimmins and Tjallingii, 1985; Tjallingii and Esch, 1993; Prado and Tjallingii, 1994;

132   Tjallingii et al., 2010). Different electrical waveforms obtained from EPG recordings in aphids

133   correspond with stylet activities in specific cell layers (Sarria et al., 2009), including in the mesophyll

134   tissue (pathway, C, phase), xylem (G phase – xylem ingestion), and phloem (E phase, split into E1 –

135   salivation into the phloem, E2 – ingestion of phloem sap, and sE2 – prolonged ingestion of phloem

136   for a period of time > 10 mins). Alongside these main phases, waveforms can also relate to

137   difficulties in stylet penetration of plant tissue (F phase), salivation into the extracellular space (E1e),

138   and stylet puncture of cells within the mesophyll tissue (pd) (Sarria et al., 2009; Tjallingii et al., 2010).

139   A primary use of the EPG technique has been to identify plant tissue types involved in plant

140   resistance against sap-feeding pests (Alvarez et al., 2006; Greenslade et al., 2016). However, the EPG

141   technique can also be employed to examine insect physiological responses to a myriad of biotic and

142   abiotic factors, such as environmental stress (Ponder et al., 2000), plant disease status (Angelella et

143   al., 2018), plant association with mycorrhiza (Simon et al., 2017), and disruption of the obligate

144   aphid endosymbiont *B. aphidicola* (Machado-Assefh and Alvarez, 2018).

145   In the current study we use the EPG technique to examine aphid feeding on a susceptible modern

146   cultivar of barley, *Hordeum vulgare* cv. Concerto, and a wild relative of barley with partial-resistance

147   against aphids, *H. spontaneum* 5 (Hsp5) (Delp et al., 2009). Hsp5 is particularly unsuitable as a host

148   for *H. defensa*-infected *R. padi* (Leybourne et al., 2018). We analysed aphid feeding behaviour to test

149   two complementary hypotheses: 1) that infection with *H. defensa* can lead to altered aphid probing

150   and feeding behaviour; 2) that differential aphid probing and feeding behaviour between uninfected

151   and *H. defensa*-infected aphids is a key contributor towards the decreased fitness of *H. defensa*-

152   infected aphids feeding on partially-resistant Hsp5. In support of the first hypothesis we observed

153   that, irrespective of plant type, aphids infected with *H. defensa* exhibited differential physiological

154   behaviour relating to stylet interactions with the mesophyll tissue and the phloem sap. For example,

155   infected aphids probed intracellularly within mesophyll tissue more frequently, salivated into the

156   phloem for a shorter period of time, and were more likely to establish sustained phloem feeding.

157   Furthermore, in support of the second hypothesis, we also observed that, compared with uninfected

158   aphids, *H. defensa*-infected aphids on Hsp5 spent less time probing into plant tissue, required twice

159   as many tissue probes in order to reach the phloem sap, and showed a significant reduction in time

160   spent ingesting phloem. Our work provides novel information about the mechanisms (decreased

161   probing into plant tissue and reduced phloem access) that contribute towards the fitness

162   consequences for *H. defensa*-infected aphids feeding on a less suitable plant host.

163   **Materials and Methods**

164   ***Plant growth and aphid rearing conditions***

165   Barley seeds, *Hordeum vulgare* cv. Concerto (Linnaeus) (Concerto), and wild barley seeds, *H.*

166   *spontaneum* (Linnaeus) 5 (Hsp5) were surface sterilised by washing in 2% (v/v) hypochlorite solution

167   and rinsed in deionised water. Concerto seeds were kept in the dark at room temperature for 48 h

168   to germinate whereas Hsp5 seeds were incubated at 4°C in the dark for 14 days to synchronise

169   germination. Plants were grown to the true-leaf stage of development (1.2 on the scale described in

170   Zadoks et al. (1974)) before use in aphid fitness and EPG experiments.

171   Asexual laboratory clonal cultures of the bird cherry-oat aphid, *Rhopalosiphum padi* (Linnaeus), were

172   reared on one week old barley seedlings (cv. Optic) contained in ventilated cups and maintained at

173   18 ± 2°C and 16h:8h (day:night). *R. padi* lines were previously genotyped and characterised for the

174   presence of facultative endosymbionts (Leybourne et al., 2018). Prior to experimentation, the

175   presence of the defensive endosymbiont *Hamiltonella defensa* (Moran et al., 2005) was confirmed

176   by PCR on a ProFlex PCR system (Applied Biosystems, UK) with PCR conditions as follows: 95°C for 2

177   min followed by 35 cycles of 95°C for 30s, 55°C for 30s and 72 °C for 3 min with a final extension

178   stage of 72°C for 5 min; the final reaction solution consisted of 1X Green GoTaq® reaction buffer

179   (Promega, UK) containing 1 μmol forward primer (PABSF: 5' – AGCGCAGTTTACTGAGTTCA – 3'

180   (Darby and Douglas, 2003), 1 μmol reverse primer (16SB1 5' - TACGGYTACCTTGTTACGACTT -3'

181   (Fukatsu et al., 2000), 1.25 U GoTaq® DNA Polymerase (Promega, UK) and 1.5 mmol $MgCl_2$.

182   ***Electrical Penetration Graph (EPG) aphid feeding assessment***

183   The DC-EPG technique (Tjallingii, 1978; Tjallingii, 1988) was employed to monitor probing and

184   feeding behaviour of four *R. padi* lines representing one aphid genotype with differential infection

185   with the aphid endosymbiont, *H. defensa*: DL 16/04 (*Hd+*), DL 16/05 (*Hd+*), DL 16/06 (*Hd-*) and DL

186   16/13 (*Hd-*). Recordings were taken over a six-hour period using a Giga-4 DC-EPG device (EPG

187   Systems, The Netherlands). Aphids were adhered to aphid probes (a copper wire, 3 cm x 0.2 cm,

188   soldered to a brass pin, tip width 0.2 cm) by attaching 3 cm of gold wire (20 μm diameter; EPG

189   Systems, The Netherlands) to the aphid probe using water-based silver glue (EPG Systems, The

190   Netherlands) and adhering the free end of the wire to the aphid dorsum using the same water-based

191   adhesive. A plant probe (copper rod approximately 5 cm x 0.5 cm) was created by soldering the

192   copper rod to electrical wire extending from the plant voltage output of the Giga-4 device. The wired

193   aphid was attached to the Giga-4 device by placing the end of the brass pin into the EPG probe and

194   the copper rod was then placed into the plant soil. Recordings were taken with a 1G Ω input

195   resistance and a 50 x gain (Tjallingii, 1988), for six hours per read. The order of *R. padi* – plant

196   combinations and allocation to EPG probe was randomised, and Stylet+D software (EPG Systems,

197   The Netherlands) was used for data acquisition. Aphids were lowered onto plant leaves immediately

198   after the recording started. All EPG recordings were taken in a grounded Faraday cage. Ten

199   replicates were recorded for *H. defensa*-infected aphids on Concerto, 11 for *H. defensa*-infected

200   aphids on Hsp5, 14 for uninfected aphids on Concerto, and 14 for uninfected aphids on Hsp5

201   EPG waveforms were annotated using Stylet+A software (EPG Systems, The Netherlands) by

202   assigning waveforms to np (non-probing), C (stylet penetration/pathway), pd (potential-

203   drop/intercellular punctures), the pd sub-phases (pd-II1, pd-II2, pd-II3), E1e (extracellular saliva

204   secretion), E1 (saliva secretion into phloem), E2 (saliva secretion and passive phloem ingestion), F

205   (penetration difficulty) or G (xylem ingestion) phases (Tjallingii, 1988; Alvarez et al., 2006).

206   Annotated waveforms were converted into time-series data using the excel workbook for automatic

207   parameter calculation of EPG data (Sarria et al., 2009).

208   ***Aphid fitness experiments***

209   The aphid fitness study was split into two temporal blocks with seven fully-randomised sub-blocks

210   within each temporal block; each sub-block consisted of one replicate for each plant-aphid-

211   combination: two plant types (Hsp5, Concerto) x four aphid treatments (DL 16/04, DL 16/05, DL

212   16/06, DL 16/13) giving eight treatments with 14 replicates each. One apterous *R. padi* adult from

213   the four aphid lines described above was taken from culture, placed in a perspex clip-cage

214   (MacGillivray and Anderson, 1957), attached to the first true leaf and left to reproduce overnight.

215   After 24 h the adult was removed and the resulting progeny were retained on the plant leaf; the

216   mean mass of two nymphs was recorded at 48 h and 114 h and used to calculate the nymph mass

217   gain over this 96 h period. We have previously characterised *R. padi* fitness in relation to *H. defensa*-

218   infection (nymph mass, fecundity, survival) and detected a fitness consequence for nymph mass gain

219   in *H. defensa*-infected aphids (Leybourne et al., 2018). As such, we only measured nymph mass in

220   this current study.

221   ***Statistical analysis***

222   All statistical analyses were carried out using R Studio Desktop version 1.0.143 running R version

223   3.4.3 (R Core Team, 2017), with additional packages car v.2.1-4 (Fox and Weisberg, 2011), ggplot2

224   v.2.2.1 (Wickham, 2009), ggpubr v. 0.1.2 (Kassambara, 2017), lme4 v.1.1-13 (Bates et al., 2015),

225   lmerTest v.2.0-33 (Kuznetsova et al., 2017), lsmeans v.2.27-62 (Lenth, 2016), multcomp v.1.4-8

226   (Hothorn et al., 2008), pastecs v.1.3.21 (Grosjean and Ibanez, 2014), and vegan v.2.4-6 (Oksanen et

227   al., 2013).

228   Data were combined into two endosymbiont treatments: *H. defensa*-infected (comprising the DL

229   16/04 and DL 16/05 clonal lines) and *H. defensa*-uninfected (comprising the DL 16/06 and DL 16/13

230   clonal lines). Aphid feeding behaviour was first assessed globally by fitting a permutated multiple

231   analysis of variance to the dataset. Individual feeding parameters from the EPG experiment and

232   aphid juvenile mass gain from the aphid fitness experiment were then analysed in individual linear

233   mixed effects models. Within each model, aphid clonal line was included as a nested factor within

234   endosymbiont infection status to account for the use of multiple clonal lines of genetically similar

235   aphids with differential endosymbiont infection status, as done previously (Leybourne et al., 2018).

236   For the individual EPG parameters, EPG run (blocking factor) and the EPG probe used were included

237   as random factors (there were 3 EPG probes used over the lifetime of the experiment). For the

238   juvenile mass gain model, experimental block and temporal block were incorporated as random

239   factors. All data were modelled against host plant, aphid endosymbiont infection status, and the

240   interaction. χ^2 analysis of deviance tests were used to analyse the final models for the individual EPG

241   parameters and analysis of variance with type III Satterthwaite approximation for degrees of

242   freedom was used to analyse the final aphid fitness model. Calculation of the Least Squares Means

243   was used as a *post-hoc* test on all models with a significant interaction term. All final models were

244   checked for model suitability by observing the fitted-residual plots.

245   **Results**

246   We used the EPG technique to monitor the probing and feeding behaviour of genetically-similar

247   *Rhopalosiphum padi* clonal lines with and without *H. defensa* infection when feeding on two host

248   plants: susceptible barley (Concerto) and the partially-resistant wild relative (Hsp5). We tested two

249   complementary hypotheses: 1) that infection with *H. defensa* can lead to altered aphid probing and

250   feeding behaviour; 2) that differential aphid probing and feeding behaviour between uninfected and

251   *H. defensa*-infected aphids is a key contributor towards the decreased fitness of *H. defensa*-infected

252   aphids when feeding on partially-resistant Hsp5. We obtained 72 individual feeding parameters from

253   the EPG analysis (displayed in tables 1 and 2, and supplementary tables 1 and 2). Global analysis of

254   aphid feeding patterns indicated that aphid feeding behaviour was significantly affected by plant

255   identity ($F_{1,43}$ = 3.19; p = 0.017) and the interaction between endosymbiont presence and plant

256   identity ($F_{1,43}$ = 2.71; p = 0.037). From the 72 parameters obtained, seven parameters were affected

257   by plant identity alone (Table S1), however these parameters are not presented or discussed in

258   detail here as we recently characterised *R. padi* feeding responses to plant identity in a separate

259   study (see Leybourne et al. 2019). In support of hypothesis 1, a total of 11 parameters were

260   primarily influenced by endosymbiont infection, and these were mainly associated with stylet

261   intracellular punctures and interaction with the phloem (Table 1; Fig. 1). A further 15 parameters

262   were differentially affected by the endosymbiont infection x host plant interaction (supporting

263   hypothesis 2), and these involved stylet interactions with the plant surface, the mesophyll tissue,

264   and the phloem (Table 2; Fig 2). The remaining 39 non-significant parameters are displayed in Table

265   S2.

266   ***Cellular punctures and phloem feeding are more frequent in aphids infected with the defensive***

267   ***facultative endosymbiont Hamiltonella defensa***

268   During the six-hour EPG recording, 11 feeding parameters were affected similarly by endosymbiont

269   infection for aphids feeding on Concerto and Hsp5 (Table 1), supporting our first hypothesis that

270   infection with *H. defensa* can lead to altered aphid probing and feeding behaviour. Most of these

271   feeding parameters related to aphid stylet activities in the mesophyll tissue, specifically the

272   frequency and duration of the exploratory intracellular punctures (EPG waveform pd) performed by

273   aphids while probing into plant tissue, and stylet interaction with phloem sap. The average duration

274   of each C phase (stylet interaction with and movement through the mesophyll tissue) was around

275   25-30% shorter in *H. defensa*-infected aphids compared with uninfected aphids (Table 1; Fig. 1A).

276   The total number of intracellular punctures (pd) made by *H. defensa* infected aphids was around

277   two-fold higher than those made by uninfected aphids (Table 1; Fig 1B). Furthermore, following the

278   first stylet probe into plant tissue, the first intracellular puncture (pd) occurred more rapidly for

279   infected aphids (Table 1). Although the frequency of intracellular punctures increased in *H. defensa*

280   infected aphids (Table 1; Fig. 1B), the duration of these intracellular punctures was on average 50%

281   shorter for infected aphids compared with uninfected aphids (Table 1; Fig. 1C). The frequency of

282   these intracellular punctures was highest and their duration shortest in the first hour (Table 1).

283   Following this, the frequency of intracellular punctures in the second to sixth hours was not affected

284   by endosymbiont presence, although the duration of intracellular punctures was influenced by

285   symbiont presence in the second and sixth hours of EPG monitoring (Table 1).

286   Three aphid feeding parameters relating to stylet activity in the vascular tissue were affected by

287   endosymbiont presence (Table 1; Fig. 1D-F): aphids infected with *H. defensa* showed a 50%

288   reduction in time spent salivating into the phloem during stylet contact with the phloem (Table 1;

289   Fig. 1D), displayed a 33% increase in phloem ingestion during stylet contact with the phloem (Table

290   1; Fig. 1E) and had a higher proportion of phloem sap ingestion phases (E2 phases) containing a

291   period of sustained phloem sap ingestion (sE2 – a period of ingestion > 10 mins) (Table 1; Fig. 1F).

Interactive effects of plant partial-resistance against aphids with aphid endosymbiont infection

293   In line with previous findings (Leybourne et al., 2018), the mass gain of *R. padi* nymphs was reduced

294   when feeding on the partially-resistant wild relative of barley Hsp5 compared with aphids feeding on

295   the susceptible modern cultivar of barley Concerto (ANOVA plant species: $F_{1,93}$ =122.57; p = <0.001;

296   Fig. S1), although endosymbiont presence/absence alone did not affect aphid fitness (ANOVA

297   endosymbiont: $F_{1,93}$ = 0.42; p = 0.514). The growth of aphids infected with *H. defensa* was further

298   reduced by 22% when aphids were feeding on Hsp5 (ANOVA plant species x endosymbiont

299   interaction: $F_{1,93}$ =6.35; p = 0.013; Fig. S1). To examine whether alterations in aphid probing and

300   feeding behaviour contributed towards this fitness cost, we identified EPG parameters responding

301   differentially to endosymbiont infection on each plant type.

302   Fifteen EPG parameters were significantly affected by the endosymbiont infection x plant type

303   interaction (Table 2). Eleven of these were differentially affected by *H. defensa*-infection for aphids

304   contained on Hsp5 (Table 2). These data indicated that, in support of our second hypothesis (that

305   differential aphid probing and feeding behaviour between uninfected and *H. defensa*-infected aphids

306   is a key contributor towards the decreased fitness of *H. defensa*-infected aphids feeding on partially-

307   resistant Hsp5), altered aphid probing and feeding behaviour could contribute towards decreased

308   fitness of *H. defensa*-infected aphids on this less suitable plant (Fig. S1). When interacting with Hsp5,

309   infected aphids spent 9% less time probing into plant tissue compared with uninfected aphids (Table

310   2), resulting in an overall reduction in the total time spent probing into plant tissue (Table 2; Fig. 3A).

311   Although there was no difference in the number of non-probing phases between *H. defensa*-infected

312   and uninfected aphids when feeding on Hsp5 (Table 2), *H. defensa*-infected aphids spent longer

313   periods not probing into the plant tissue (Table 2). Furthermore, the duration of the first probe into

314   plant tissue by *H. defensa*-infected aphids feeding on Hsp5 was around six-fold shorter compared

315   with uninfected aphids (Table 2; Fig. 2B), and *H. defensa*-infected aphids required twice as many

316   probes into plant tissue before the phloem was reached compared with uninfected aphids (Table 2;

317   Fig. 2C). The total time spent ingesting phloem was reduced by 44% for *H. defensa*-infected aphids

318   feeding on Hsp5 compared with uninfected aphids (Table 2; Fig. 2D) and the longest observed period

319   of phloem ingestion was three-fold shorter for *H. defensa*-infected aphids compared with uninfected

320   aphids when feeding on Hsp5 (Table 2; Fig. 2E).

321 Infection with *H. defensa* also altered the feeding behaviour of *R. padi* when probing into the

322 susceptible barley cv. Concerto: *H. defensa*-infected aphids achieved sustained phloem sap ingestion

323 two-fold faster than uninfected aphids (Table 2; Fig. 2F), however this did not affect aphid growth

324 (Fig. S1).

325 **Discussion**

326 By analysing aphid feeding behaviour, our study provides novel mechanistic insights into the

327 consequences of *Hamiltonella defensa* infection for interactions at the aphid-plant interface and

328 shows that *H. defensa*-infection can alter aphid probing behaviour, irrespective of host plant

329 suitability, with potential consequences for insect fitness. In addition to this, our data show that

330 these interactions can be influenced by plant susceptibility to, or resistance against, aphids and we

331 provide novel evidence showing that aphid physiological processes are differentially affected by

332 endosymbiont presence and host plant suitability which, at least in part, explains a fitness cost

333 associated with *H. defensa*-infection for *R. padi* when feeding on a poor quality (partially-resistant)

334 host plant.

335 *Endosymbiont infection alters aphid exploratory probing into plant cells and promotes phloem*

336 *ingestion*

337 When probing into plant tissue, *H. defensa*-infected aphids displayed a characteristic pattern of

338 more frequent and shorter exploratory intracellular punctures (EPG waveform pd) than uninfected

339 aphids. The precise cause of this symbiont-associated effect on aphid probing is not clear, although a

340 similar pattern was recently reported in *H. defensa*-infected cowpea aphids, *Aphis craccivora*

341 (Angelella et al., 2018), and it is likely that changes in intracellular puncture frequency will affect the

342 transmission of plant viruses (Fereres and Collar, 2001; Powell, 2005). A key difference between our

343 study and the previous work of Angelella et al. (2018) was that *R. padi* infected with *H. defensa* also

344 showed differential feeding behaviour caused by altered stylet activities within the phloem. *H.*

345 *defensa*-infected aphids spent less time salivating into the phloem and showed an overall increase in

346 the percentage of phloem phases which contained phloem ingestion, including an increased

347 proportion of these containing periods of sustained phloem ingestion. Altered aphid probing and

348 feeding behaviour did not appear to affect aphid fitness directly as no overall effect of *H. defensa*

349 infection on *R padi* growth, development, fecundity, or longevity was detected (present study;

350 Leybourne et al., 2018). However, *H. defensa*-infection can affect aphid fitness in other species

351 (Zytynska et al., 2019 preprint) and differential feeding behaviour in *H. defensa*-infected aphids

352 could be associated with these altered aphid phenotypes, including an increased adult body mass

353 and enhanced offspring production in black bean aphids, *A. fabae* (Castañeda et al., 2010).

354 Endoymbiont-induced changes in feeding behaviour might be due to indirect effects of the

355 bacterium on stylet activities mediated by bacterium-derived salivary factors (Su et al., 2015; Frago

356 et al., 2017).

357 The extent of these endosymbiont-derived fitness consequences can often be dependent on aphid

358 clonal line or aphid genotype (Castañeda et al., 2010) and it is important to note that endosymbiont-

359 conferred traits vary between different aphid lines, aphid genotypes, and aphid species (Castañeda

360 et al., 2010; Vorburger and Gouskov, 2011; Leybourne et al., 2018): indeed, *H. defensa*-infection can

361 also reduce *A. fabae* reproductive rate and survivorship (Vorburger and Gouskov, 2011) and

362 decrease *S. avenae* fecundity (Li et al., 2018). Altered probing behaviour might also explain

363 differential plant responses to infestation by aphids infected with *H. defensa*, including changes in

364 the emission of Herbivore Induced Plant Volatile (HIPV) compounds (Frago et al., 2017) and reduced

365 dry matter allocation to roots (Hackett et al., 2013; Bennett et al., 2016). A focus for future research

366 should include the consequences of aphid species and genotype for *H. defensa*-associated

367 modifications to aphid probing and feeding behaviour to fully elucidate their effects on aphid pest

368 status, virus transmission, and plant responses to aphid infestation.

369 ***Endosymbiont infection reduces aphid feeding on a poor quality host plant***

370 When probing into the partially-resistant plant, Hsp5, aphids infected with *H. defensa* showed a

371 differential physiological feeding pattern compared with uninfected aphids, including a reduction in

372 the time spent probing into plant tissue, an increase in the number of plant tissue probes required to

373 reach the phloem tissue, and a decrease in total phloem ingestion. This was linked with decreased

374 fitness in *H. defensa*-infected aphids compared with uninfected aphids when feeding on Hsp5, in line

375 with our previous findings (Leybourne et al., 2018). A decrease in the duration of the first probe into

376 plant tissue, and an overall reduction in time spent probing into the plant tissue, are representative

377 of mesophyll- and epidermal-derived factors which inhibit and impede the penetration of the aphid

378 stylet through the plant tissue, as highlighted by Alvarez et al. (2006). A similar fitness cost

379 associated with *H. defensa*-infected aphids has been observed previously in *A. fabae* feeding on

380 different quality plant species *(*Chandler et al., 2008*)*, although it is not known if this was linked with

381 altered aphid probing and feeding behaviour.

382 We recently characterised the partial-resistance mechanism of Hsp5 (Leybourne et al., 2019) and

383 reported that partial-resistance involves mesophyll and phloem traits. These included an increased

384 abundance of defensive thionins and a reduction in the availability of essential amino acids as

385 mesophyll-derived and phloem-derived partial-resistance factors, respectively (Leybourne et al.,

386 2019). These factors could underlie the decreased time aphids spent probing into plant tissue and

387   the shorter duration of the initial probe into plant tissue, although the underlying processes causing

388   these differential feeding patterns are currently unclear. A key factor which likely contributes

389   towards this decrease in aphid fitness is our observation that *H. defensa*-infected aphids showed a

390   44% reduction in time spent ingesting phloem on Hsp5 compared with uninfected aphids. It is

391   probable that this substantial decrease in phloem ingestion contributes significantly to the 22%

392   reduction in nymph growth we detected. Indeed, a previous study using the peach-potato aphid,

393   *Myzus persicae,* have shown that a 58% decrease in ingestion rate can result in a 10% reduction in

394   aphid growth (Karley et al., 2002). We also detected differential feeding patterns between *H.*

395   *defensa*-infected aphids feeding on Hsp5 and Concerto: *H. defensa*-infected aphids feeding on

396   Concerto achieved sustained phloem feeding more rapidly than those feeding on Hsp5. A faster

397   initiation of sustained feeding could explain the higher mass of *H. defensa*-infected nymphs on

398   Concerto. However, it is likely that our observed reduction in nymph mass for both infected and

399   uninfected nymphs when feeding on Hsp5 compared with those feeding on Concerto is due

400   increased aphid resistance in Hsp5 (Leybourne et al., 2019). The rapid initiation of sustained feeding

401   could be associated with other aphid fitness effects which are currently uncharacterised, such as the

402   transmission or acquisition of phloem-limited viruses.

403   **Conclusion**

404   In this study, two hypotheses were tested: 1) that infection with *H. defensa* can lead to altered aphid

405   probing and feeding behaviour; 2) that differential aphid probing and feeding behaviour between

406   uninfected and *H. defensa*-infected aphids is a key contributor towards the decreased fitness of *H.*

407   *defensa*-infected aphids feeding on partially-resistant Hsp5. *R. padi* infected with the defensive

408   facultative endosymbiont, *H. defensa,* showed altered probing and feeding behaviour compared

409   with uninfected aphids, irrespective of plant type, including an increase in the number of

410   intracellular punctures and in phloem ingestion, supporting our first hypothesis. Furthermore, in

411   support of our second hypothesis, our EPG data highlight novel mechanistic processes which

412   contribute towards an observed fitness cost arising from *H. defensa*-infection in *R. padi* feeding on

413   the partially-resistant plant, Hsp5, which was associated with a reduction in the time aphids probe

414   into the plant tissue, an increase in the number of plant tissue probes required to reach the phloem,

415   and a 44% reduction in total phloem ingestion. Together, our results show that aphid facultative

416   endosymbionts can influence aphid-plant interactions in more subtle ways than previously realised

417   and indicate that plant suitability can exacerbate these effects.

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421   **Competing Interests**

422   The authors declare no competing interests.

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429   **Author Contributions**

430   AJK and DJL conceived and designed the experiments. DJL performed the experiments and analysed

431   the data. All authors contributed to data interpretation. DJL wrote the manuscript with input from

432   all authors. All authors read and approved the final manuscript.

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Figure Legends

Fig. 1: Aphid feeding parameters in the absence (-) and presence (+) of *Hamiltonella defensa*. - shows the combined data for both *H. defensa*-uninfected lines (DL 16/06; DL 16/13) and + shows the combined data for both *H. defensa*-infected lines (DL 16/04; DL 16/05). A-C: parameters associated with stylet puncturing of plant cells (intracellular punctures); D-F: parameters associated with stylet interaction with phloem sap. The black cross ("x") on each plot shows the mean value.

Fig 2: Aphid feeding parameters that were differentially affected by the absence (-) and presence (+) of *Hamiltonella defensa* infection on two plant hosts (susceptible modern barley cv. Concerto and the wild relative Hsp5). - shows the combined data for both *H. defensa*-uninfected lines (DL 16/06; DL 16/13) and + shows the combined data for both *H. defensa*-infected lines (DL 16/04; DL 16/05). Letters indicate which groups are significantly different based on pairwise comparisons using general linear hypotheses testing with single-step p-value adjustment. The black cross ("x") on each plot shows the mean value.

Tables

Table 1: Aphid feeding parameters (mean value ± standard error) that were significantly affected by the absence (-) or presence (-) of *Hamiltonella defensa* infection. Numbers in parenthesis indicate the total number of individuals which displayed each parameter, and the total number of individuals tested is indicated at the top of the column. Data marked with † display median alongside the upper and lower interquartile ranges.

Description of Response Variable Assessed (transformation used)	Absence (-) or presence (+) of Hamiltonella defensa infection		Statistical results for each Explanatory Variable (generalised least square estimation models)					
	Hd –ve (max = 28)	*Hd +ve (max = 21)*	Plant		Endosymbiont		Plant x Endosymbiont	
Mean duration of each C (mesophyll pathway) phase (sqrt)	*860.14 ± 94.77 s (28)*	620.02 s ± 70.71 s (21)	$X^2_1 = 0.25$	p = 0.611	$X^2_1 = 4.16$	p = 0.041	$X^2_1 = 0.97$	p = 0.323
Total number of pd: potential drops (intracellular punctures) (sqrt)	72.85 ± 6.83 *(28)*	163.57 ± 26.02 *(21)*	$X^2_1 = 0.52$	P = 0.470	$X^2_1 = 18.49$	P = <0.001	$X^2_1 = 3.78$	P = 0.051
Mean duration of each pd (not transformed)	4.53 s ± 0.17 s *(28)*	2.66 s ± 0.28 s *(21)*	$X^2_1 = 0.03$	P = 0.843	$X^2_1 = 40.99$	P = <0.0.01	$X^2_1 = 0.74$	P = 0.389
Time from start of aphid probe into plant tissue to first pd (not transformed)	529.41 ± 206.43 *(28)*	117.75 s ± 36.49 s *(21)*	$X^2_1 = 2.09$	P = 0.147	$X^2_1 = 2.87$	P = 0..008	$X^2_1 = 1.58$	P = 0.208
Total number of pd in first hour (sqrt)	15.35 ± 2.29 *(28)*	61.52 ± 14.58 *(21)*	$X^2_1 = 0.02$	P = 0.871	$X^2_1 = 14.67$	P = 0.001	$X^2_1 = 1.25$	P = 0.262
Mean duration of each pd in first hour (not transformed)	4.19 s ± 0.29 s *(28)*	2.39 s ± 0.32 s *(21)*	$X^2_1 = 0.01$	P = 0.977	$X^2_1 = 16.62$	P = <0.001	$X^2_1 = 0.001$	P = 0.994
Mean duration of each pd in second hour (not transformed)	*4.81 ± 0.30 s (21)*	3.14 ± 0.47 *(17)*	$X^2_1 = 0.59$	p = 0.439	$X^2_1 = 10.01$	p = 0.001	$X^2_1 = 1.62$	p = 0.202
Mean duration of each pd in sixth hour (not transformed)	4.84 ± 0.19 *(13)*	3.57 ± 0.29 *(13)*	$X^2_1 = 0.92$	p = 0.335	$X^2_1 = 5.13$	p = 0.023	$X^2_1 = 4.32$	p = 0.057
Time spent in E1 (salivation into phloem) as a percentage of the total time spent in all phloem phases (not transformed) †	16.37% LQR: 6.90% UQR: 45.61% (26)	8.93% LQR: 4.63% UQR: 22.12% (20)	$X^2_1 = 1.13$	P = 0.285	$X^2_1 = 18.20$	P = <0.001	$X^2_1 = 2.02$	P = 0.154
E2 (phloem ingestion) index (not transformed) †	29.44 % LQR: 5.25% UQR: 76.85% (24)	41.36% LQR: 8.95% UQR: 78.67% (20)	$X^2_1 = 0.01$	P = 0.987	$X^2_1 = 6.72$	P = 0.009	$X^2_1 = 0.94$	P = 0.329
% of E2 phases which contained a period of sustained (>10min) phloem ingestion (not transformed) †	45.00% LQR: 27.08% UQR: 100.00% (20)	100.00% LQR: 50.00% UQR: 100.00% (17)	$X^2_1 = 0.37$	P = 0.541	$X^2_1 = 3.21$	P = 0.047	$X^2_1 = 0.90$	P = 0.341

Table 2: Aphid feeding parameters (mean value ± standard error) that were differentially affected by plant type and *Hamiltonella defensa* infection. Letters indicate which groups are significantly different based on pairwise comparisons using differences in the least square means analysis. Numbers in parenthesis indicate the total number of individuals which displayed each parameter, and the total number of individuals tested is indicated at the top of the column. Data marked with † display median alongside the upper and lower inter quartile ranges.

Description of Response Variable Assessed (transformation used)	*H. defensa* infection – plant type combination (n)				Statistical results for each Explanatory Variable					
	Hd –ve Concerto (max = 14)	*Hd +ve Concerto (max = 10)*	*Hd –ve Hsp5 (max = 14)*	*Hd +ve Hsp5 (max = 11)*	Plant		Endosymbiont		Plant x Endosymbiont	
% of time spent probing into plant tissue † (not transformed)	91.66% ab LQR: 91.66 UQR: 93.82 (14)	93.68% a LQR: 91.68 UQR: 95.45 (10)	97.89% a LQR: 93.32 UQR: 99.53 (14)	89.15% b LQR: 75.04 UQR: 98.78 (11)	$X^2_1 = 0.09$	P = 0.752	$X^2_1 = 0.947$	P = 0.330	$X^2_1 = 0.548$	P = 0.019
Total time spent probing plant tissue (not transformed)	19048.02 s ± 474.76 s a (14)	19792.08 s ± 248.27 s a (10)	19647.97 s ± 444.58 s a (14)	16376.19 s ± 1664.46 s b (11)	$X^2_1 = 2.08$	P = 0.148	$X^2_1 = 1.31$	P = 0.251	$X^2_1 = 6.84$	P = 0.008
Number of C (mesophyll pathway) phases (sqrt)	12.85 ± 1.96 ab (14)	10.50 ± 1.51 ab (10)	8.50 ± 1.26 a (14)	17.18 ± 4.53 b (11)	$X^2_1 = 0.03$	p = 0.846	$X^2_1 = 1.56$	p = 0.210	$X^2_1 = 4.40$	p = 0.035
Number of brief probes (< 3 mins) into plant tissue (sqrt)	1.35 ± 0.30 a (11)	0.50 ± 0.26 b (3)	0.78 ± 0.23 ab (7)	1.36 ± 0.38 ab (7)	$X^2_1 = 0.02$	p = 0.878	$X^2_1 = 0.09$	p = 0.751	$X^2_1 = 5.03$	p = 0.024
Number of probes into plant tissue (not transformed)	6.57 ± 1.06 a (14)	4.30 ± 0.74 ab (10)	3.99 ± 0.73 b (14)	6.36 ± 1.02 ab (11)	$X^2_1 = 0.88$	P = 0.347	$X^2_1 = 0.04$	P = 0.827	$X^2_1 = 6.04$	P = 0.013
Total duration of the first probe into plant tissue (not transformed)	4004.58 s ± 1585.71 s ab (14)	6790.5 s ± 2413.59 s a (10)	7499.64 s ± 2138.37 s a (14)	1307.63 s ± 399.07 s b (11)	$X^2_1 = 0.03$	P = 0.845	$X^2_1 = 0.97$	P = 0.322	$X^2_1 = 5.93$	P = 0.014
Number of probes into plant tissue in the second hour (log)	2.00 ± 3.77 b (14)	1.30 ± 0.21 ab (10)	1.21 ± 0.15 a (14)	2.09 ± 0.43 b (11)	$X^2_1 = 0.19$	p = 0.657	$X^2_1 = 0.05$	p = 0.816	$X^2_1 = 5.70$	p = 0.016
Total time spent not probing plant tissue (sqrt)	2222.85 s ± 455.44 s ab (14)	1348.83 s ± 210.27 s ab (10)	1190.47 s ± 453.83 s a (14)	3174.05 s ± 1051.14 s b (11)	$X^2_1 = 0.07$	P = 0.780	$X^2_1 = 0.498$	P = 0.479	$X^2_1 = 3.89$	P = 0.048
Total number of non-probing phases (not transformed)	5.57 ± 1.06 a (14)	3.30 ± 0.74 ab (10)	2.92 ± 0.73 b (14)	5.36 ± 1.02 ab (11)	$X^2_1 = 0.88$	P = 0.347	$X^2_1 = 0.04$	P = 0.827	$X^2_1 = 6.04$	P = 0.013
Number of probes into plant tissue until first E (phloem) phase (phloem contact) (log)	2.50 ± 0.34 a (14)	1.80 ± 0.29 a (10)	2.57 ± 0.71 a (14)	5.00 ± 1.10 b (11)	$X^2_1 = 4.87$	P = 0.027	$X^2_1 = 0.92$	P = 0.336	$X^2_1 = 7.56$	P = 0.005
Time from first probe into plant tissue until first sE2 (sustained phloem feeding phase) phase (sqrt)	12284.97 s ± 2033.97 s a (11)	5243.38 s ± 1634.65 s b (10)	127729.06 s ± 1975.25 s a (9)	15901.41 s ± 1786.35 s a (7)	$X^2_1 = 7.77$	P = 0.005	$X^2_1 = 0.92$	P = 0.337	$X^2_1 = 9.11$	P = 0.002
Total time spent in E2 phase (not transformed)	5879.69 s ± 1660.46 s a (14)	8042.94 s ± 1749.09 s a (10)	7369.52 s ± 1561.35 s a (12)	4934.72 s ± 1281.47 s b (10)	$X^2_1 = 0.05$	P = 0.820	$X^2_1 = 0.13$	P = 0.715	$X^2_1 = 4.27$	P = 0.038
Longest period of E2 (not transformed)	4800 ± 1562.49 s ab (14)	6256.68 ± 1530.16 s ab (10)	6237.50 ± 1504.46 s a (12)	2941.27 ± 944.83 s b (10)	$X^2_1 = 0.08$	P = 0.771	$X^2_1 = 0.50$	P = 0.478	$X^2_1 = 3.93$	P = 0.047
Total number of pd in the third hour (sqrt)	15.71 ± 4.16 a (11)	10.40 3.00 a (7)	16.35 ± 3.57 a (12)	54.27 ± 18.85 b (10)	$X^2_1 = 7.30$	P = 0.006	$X^2_1 = 2.63$	P = 0.104	$X^2_1 = 7.82$	P = 0.005
Total number of pd in the fourth hour (sqrt)	14.85 ± 4.70 ab (8)	5.10 ± 2.75 a (3)	5.50 ± 2.06 a (9)	35.09 ± 14.30 b (8)	$X^2_1 = 0.90$	P = 0.341	$X^2_1 = 0.60$	P = 0.437	$X^2_1 = 6.88$	P = 0.006