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A fitness cost resulting from *Hamiltonella defensa* infection is associated with altered probing and feeding behaviour in *Rhopalosiphum padi*

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Keywords

Electrical penetration graph (EPG), facultative endosymbiont, insect physiology, symbiosis

Summary

Reduced performance of aphids infected with a common facultative endosymbiont on poor quality plants may be explained by changes in aphid probing behaviour and decreased phloem sap ingestion.

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Abstract

Many herbivorous arthropods, including aphids, frequently associate with facultative endosymbiotic bacteria, which influence arthropod physiology and fitness. In aphids, endosymbionts can increase resistance against natural enemies, enhance aphid virulence, and alter aphid fitness. Here, we use the Electrical Penetration Graph technique to uncover physiological processes at the insect-plant interface affected by endosymbiont infection. We monitored the feeding and probing behaviour of four independent clonal lines of the cereal-feeding aphid *Rhopalosiphum padi* derived from the same multilocus genotype containing differential infection (+/-) with a common facultative endosymbiont, *Hamiltonella defensa*. Aphid feeding was examined on a partially-resistant wild relative of barley known to impair aphid fitness and a susceptible commercial barley cultivar. Compared with uninfected aphids, endosymbiont-infected aphids on both plant species exhibited a two-fold increase in the number of plant cell punctures, a 50% reduction in the duration of each cellular puncture, and a two-fold higher probability of achieving sustained phloem ingestion. Feeding behaviour was also altered by host plant identity: endosymbiont-infected aphids spent less time probing plant tissue, required twice as many probes to reach the phloem, and showed a 44% reduction in phloem ingestion when feeding on the wild barley relative compared with the susceptible commercial cultivar. Reduced feeding success could explain the 22% reduction in growth of *H. defensa*-infected aphids measured on the wild barley relative. This study provides the first demonstration of mechanisms at the aphid-plant interface contributing to physiological effects of endosymbiont infection on aphid fitness, through altered feeding processes on different quality host plants.

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Introduction

Infection with facultative (non-essential) endosymbiotic bacteria is widespread within herbivorous arthropod populations, including aphids (Luna et al., 2018), whiteflies (Ghosh et al., 2018), psyllids (Hansen et al., 2012), mites (Zhu et al., 2018), and weevils (Morera-Margarit et al., 2019). Many of these herbivorous arthropods are important agricultural and horticultural pests, and, therefore, understanding the effects of symbiont infection on arthropod fitness, arthropod pest status, and the interactions between multiple trophic levels is a dynamic area of research (Oliver et al., 2014; McLean et al., 2016; Zytynska and Meyer, 2019). A wealth of studies have focussed on aphids which, due to their abundance and near-worldwide distribution (Van Emden and Harrington, 2017), are a particularly relevant group for elucidating the role of facultative endosymbionts in herbivorous pests. While many studies have described the effects on aphid fitness of several endosymbiotic bacteria, (Oliver et al., 2003; Moran et al., 2005; Russell and Moran, 2006; Degnan and Moran, 2008; Vorburger and Gouskov, 2011; Wulff and White, 2015; Mathé-Hubert et al., 2019), few have attempted to elucidate the underpinning physiological mechanisms. Recent observations of endosymbiont-associated changes in aphid probing (Angelella et al., 2018), plant resource allocation (Bennett et al., 2016), plant phytohoromone accumulation (Li et al., 2019), and plant volatile emission (Frago et al., 2017) have hinted that endosymbionts could alter processes at the aphid-plant interface with similar observations reported for other herbivorous arthropods, including whiteflies (Su et al., 2015). More detailed comparative studies are needed, however, to confirm the mechanism(s) through which facultative endosymbionts manipulate physiological interactions between herbivorous pests and their host plants.

To feed, aphids probe plants using specialised piercing and sucking mouthparts known as stylets, with the aim of establishing a feeding site in the plant phloem. While probing into plant tissues, aphids can transmit plant viruses (Powell, 2005; Moreno et al., 2012). Infection with aphid-transmitted viruses is a significant cause of economic crop loss, often resulting in yield losses in excess of 80% (Perry et al., 2000; Murray and Brennan, 2010). Most aphid species harbour the obligate bacterial endosymbiont *Buchnera aphidicola,* which supplies aphids with essential amino acids they are unable to biosynthesise (Sasaki et al., 1991; Douglas and Prosser, 1992; Shigenobu et al., 2000; Hansen and Moran, 2011). Additional co-obligatory symbiotic relationships have been described with other endosymbiont species, including *Wolbachia sp.* in the banana aphid, *Pentalonia nigronervosa* (De Clerck et al. 2015; Manzano-Marin, 2019 preprint) and with *Serratia symbiotica* in multiple species of the *Cinara* genus (Meseguer et al., 2017). In most other aphid species, however, these co-infecting endosymbionts are not essential for survival. Alongside obligatory endosymbiotic

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relationships, aphids can form facultative endosymbiotic relationships with a range of microorganisms.

The diversity and frequency of infection with facultative endosymbionts can vary considerably between and within aphid species (de la Peña et al., 2014; Henry et al., 2015; Zytynska and Weisser, 2016; Guo et al., 2019). The eleven most common facultative endosymbionts are *Hamiltonella defensa, Regiella insecticola, S. symbiotica, Rickettsia sp., Ricketsiella sp,* the *Pea Aphid X-type Symbiont (PAXS;* occasionally classified as *Candidatus* Fukatsuia symbiotica)*, Spiroplasma sp., Wolbachia sp., Arsenophonus sp., Sitobion miscanthis L-type Symbiont (SMLS),* and *Orientia-Like Organism (OLO)* (Oliver et al., 2006; Castañeda et al., 2010; Tsuchida et al., 2010; Łukasik et al., 2013a; de la Peña et al., 2014; Leybourne et al., 2020). Several of these facultative endosymbionts, including *H. defensa* and *Rickettsia sp.,* can infect other important agricultural pests, including whiteflies (Brumin et al., 2011; Su et al., 2015; Zytynska et al., 2019 preprint). An analysis of the endosymbiont infection frequencies of aphids by Zytynska and Weisser (2016) assessed the proportion of aphid species shown to harbour *H. defensa, R. insecticola, S. symbiotica, Rickettsia sp., Spiroplasma sp., PAXS, Arsenophonus sp.* and *Wolbachia sp.* infections, and showed that the frequency of endosymbiont infection can vary widely, with *Serratia symbiotica* as the most frequently detected endosymbiont in aphids (47% of the 156 aphid species tested were infected) and *Arsenophonus sp.* as the least frequently detected (7% of the 131 aphid species tested were infected) (Zytynska and Weisser, 2016).

The benefits of aphid infection with nine of these endosymbionts has recently been reviewed by Guo et al., 2017 and a meta-analysis of the costs and benefits of facultative endosymbiont infection has recently been conducted by Zytynska et al., 2019 preprint. Beneficial traits conferred to the aphid by the endosymbionts include protection against parasitism by Braconidae wasps (*H. defensa* and *R. insecticola;* Hansen et al., 2012; Leybourne et al., 2020), protection against entomopathogenic fungi (*R. insecticola, Rickettsia sp., Ricketsiella sp.* and *Spiroplasma sp.;* Łukasik et al., 2103b), host-plant adaptation (*Arsenophonus sp.;* Wagner et al., 2015), heat tolerance (*S. symbiotica* and *H. defensa,* alongside *B. aphidicola* mutations; Russell and Moran, 2006; Dunbar et al., 2007), morphological changes in insect colour (*Ricketsiella sp.*; Tsuchida et al., 2010; Nikoh et al., 2018), and enhanced aphid virulence (mixed symbiont communities; Luna et al., 2018). Infection with endosymbionts can, however, result in negative fitness consequences for the aphid host, including decreased growth (*Rickettsia sp.;* Sakurai et al., 2005), reduced fecundity (*Spiroplasma sp., H. defensa,* and *S. symbiotica;* Chen et al., 2000*;* Castañeda et al., 2010; Li et al., 2018; Mathé-Hubert et al., 2019*),* shorter aphid longevity (*Spiroplasma sp.* and *S. symbiotica;* Chen et al., 2000; Mathé-Hubert et al., 2019*),* lower adult mass (*S. symbiotica*; Skaljac et al., 2018), and

increased susceptibility to insecticides (*S. symbiotica*; Skaljac et al., 2018). Endosymbiont effects can depend on host plant factors, including the nutritional quality of the host plant (Chandler et al., 2008), host plant resistance against aphids (Leybourne et al., 2020), and other plant traits that underpin aphid biotype specialisation on particular host plant species (Wagner et al., 2015; Sochard et al., 2019). These observations highlight the importance of investigating how endosymbiont-infection and host plant suitability for aphids interact to influence processes at the aphid-plant interface.

Around one-third of 154 aphid species assessed for endosymbiont presence have been reported to harbour *H. defensa* (Zytynska and Weisser, 2016). Amongst cereal-feeding aphids, the proportion of bird-cherry oat aphid, *Rhopalosiphum padi,* populations infected with the defensive endosymbiont, *H. defensa,* is around 10.8% (63/585 individuals; Guo et al., 2019). The primary trait conferred to aphids infected with *H. defensa* is protection against parasitism by Braconidae wasps (Oliver and Higashi, 2019). Additionally, *H. defensa*-infection can have a more direct effect on aphid biology: A recent study has shown that *H. defensa* infection can alter the interactions which occur between aphids and their host plants by influencing aphid probing behaviour (Angelella et al., 2018). This observation suggests that altered aphid probing behaviour could affect aphid fitness by altering feeding success. Indeed, *H. defensa*-infection has been shown to have consequences for aphid fitness (Castañeda et al., 2010; Vorburger and Gouskov, 2011; Li et al., 2018; Zytynska et al., 2019 preprint). Examining these symbiont effects in relation to aphid probing behaviour could elucidate the mechanistic processes which contribute towards symbiont-associated fitness consequences, including those which are only observed when host plant nutritional quality decreases (e.g. Chandler et al. (2008) and Leybourne et al. (2020)).

The Electrical Penetration Graph (EPG) technique is an electrophysiological tool used to monitor the probing and feeding behaviour of sap-feeding insects (Tjallingii, 1985; Tjallingii and Esch, 1993; Prado and Tjallingii, 1994) and has been used successfully to monitor the feeding and probing behaviour of aphids (Greenslade et al., 2016), whiteflies (Chesnais and Mauck, 2018), psyllids (Civolani et al., 2011) and planthoppers (He et al., 2011). The technique is based on an electrical circuit which is made by inserting conductive copper probes into the soil around the plant and adhering conductive wire onto the dorsum of the aphids (Tjallingii, 1978; Tjallingii, 1985). Both probes are connected to a data logger and computational software. An electrical current is passed through the circuit, which is closed when the aphid stylet comes into contact with plant tissue, and the resulting electrical waveforms can be characterised to provide information on aphid stylet activities (probing and feeding behaviour) (Kimmins and Tjallingii, 1985; Tjallingii and Esch, 1993; Prado and Tjallingii, 1994; Tjallingii et al., 2010). Different electrical waveforms obtained from EPG recordings correspond with stylet

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activities within different plant tissues (Sarria et al., 2009), including the mesophyll and vascular tissue (these are detailed in materials & methods below). A primary use of the EPG technique has been to identify plant tissue types involved in plant resistance against sap-feeding pests (Alvarez et al., 2006; Greenslade et al., 2016; Leybourne et al., 2019). However, the EPG technique can also be employed to examine insect physiological responses to a myriad of biotic and abiotic factors, such as environmental stress (Ponder et al., 2000), plant disease status (Angelella et al., 2018), plant association with mycorrhiza (Simon et al., 2017), and disruption of the obligate aphid endosymbiont *B. aphidicola* (Machado-Assefh and Alvarez, 2018).

In the current study, we use the EPG technique to examine aphid feeding on two host plant species of contrasting quality for aphids: a susceptible modern cultivar of barley, *Hordeum vulgare* cv. Concerto, and a wild relative of barley with partial-resistance against aphids, *H. spontaneum* 5 (Hsp5) (Delp et al., 2009). The mechanism of partial-resistance against aphids in Hsp5 has been characterised previously, with resistance factors located in the mesophyll (elevated defence gene and phytohormone-regulated gene expression) and phloem (decreased phloem sap nutritional quality) (Leybourne et al., 2019). Hsp5 is particularly unsuitable as a host for *H. defensa*-infected *R. padi* causing impaired aphid growth (Leybourne et al., 2020), although the mechanism of decreased fitness of *H. defensa*-infected aphids on the partially-resistant plant host has not been investigated empirically. Comparative studies of endosymbiont effects on aphid physiology depend on access to aphid lines with a common genetic background that are differentially infected with the target symbiont, achieved either by curing or transfecting aphids (Wagner et al., 2015; Mathé-Hubert et al., 2019) or, as in this study, making use of aphid genotypes that vary naturally in their infection status (Sochard et al., 2019; Leybourne et al., 2020). Here, we analysed aphid feeding behaviour to test two complementary hypotheses: 1) that infection with *H. defensa* can lead to altered aphid probing and feeding; 2) that differential aphid probing and feeding between uninfected and *H. defensa*-infected aphids is a key contributor towards the decreased fitness of *H. defensa*-infected aphids on partially-resistant Hsp5. We discuss how the findings enhance our understanding of the mechanisms underpinning plant-insect herbivore interactions and the influence of insect endosymbionts on the outcome of these interactions.

Materials and Methods

Plant growth and aphid rearing conditions

Barley seeds, *Hordeum vulgare* cv. Concerto (Linnaeus) (Concerto), and wild barley seeds, *H. spontaneum* (Linnaeus) 5 (Hsp5) were surface sterilised by washing in 2% (v/v) hypochlorite solution and rinsed in deionised water. Concerto seeds were kept in the dark at

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room temperature for 48 h to germinate whereas Hsp5 seeds were incubated at 4°C in the dark for 14 days to synchronise germination. Plants were grown to the true-leaf stage of development (1.2 on the scale described in Zadoks et al. (1974); *c.* seven-to-ten days-old) before use in aphid fitness and EPG experiments.

Asexual laboratory clonal cultures of the bird cherry-oat aphid, *Rhopalosiphum padi* (Linnaeus)*,* were reared on one week old barley seedlings (cv. Optic) contained in ventilated cups and maintained at 18 ± 2°C and 16h:8h (day:night). Aphid lines were reared on a different barley cultivar to prevent prior habituation to the experimental treatments. *R. padi* lines were previously genotyped and characterised for the presence of facultative endosymbionts (Leybourne et al., 2020). Aphid lines were represented by one aphid genotype (E) with differential infection with the aphid endosymbiont, *Hamiltonella defensa* (Moran et al., 2005): DL 16/04 (*Hd*+), DL 16/05 (*Hd*+), DL 16/06 (*Hd*-) and DL 16/13 (*Hd*-). Prior to experimentation, DNA was extracted from aphid cultures using the Qiagen Plant DNA Extracttion Kit (Qiagen, UK) and the presence of *H. defensa* was confirmed by PCR on a ProFlex PCR system (Applied Biosystems, UK) with PCR conditions as follows: 95°C for 2 min followed by 35 cycles of 95°C for 30s, 55°C for 30s and 72 °C for 3 min with a final extension stage of 72°C for 5 min; the final reaction solution consisted of 1X Green GoTaq® reaction buffer (Promega, UK) containing 1 µmol forward primer (PABSF: 5' – AGCGCAGTTTACTGAGTTCA – 3' (Darby and Douglas, 2003), 1 µmol reverse primer (16SB1 5' - TACGGYTACCTTGTTACGACTT -3' (Fukatsu et al., 2000), 1.25 U GoTaq® DNA Polymerase (Promega, UK) and 1.5 mmol $MgCl_2$.

Electrical Penetration Graph (EPG) aphid feeding assessment

The DC-EPG technique (Tjallingii, 1978; Tjallingii, 1988) was employed to monitor probing and feeding behaviour of the four *R. padi* lines described above. Recordings were taken over a six-hour period using a Giga-4 DC-EPG device (EPG Systems, The Netherlands). Aphids were adhered to aphid probes (a copper wire, 3 cm x 0.2 cm, soldered to a brass pin, tip width 0.2 cm) by attaching 3 cm of gold wire (20 µm diameter; EPG Systems, The Netherlands) to the aphid probe using water-based silver glue (EPG Systems, The Netherlands) and adhering the free end of the wire to the aphid dorsum using the same water-based adhesive. A plant probe (copper rod approximately 5 cm x 0.5 cm) was created by soldering the copper rod to electrical wire extending from the plant voltage output of the Giga-4 device. The wired aphid was attached to the Giga-4 device by placing the end of the brass pin into the EPG probe and the copper rod was then placed into the plant soil. Recordings were taken with a 1G Ω input resistance and a 50 x gain (Tjallingii, 1988), for six hours per read. The order of *R. padi* – plant combinations and allocation to EPG probe was randomised, and Stylet+D software (EPG Systems, The Netherlands) was used for data acquisition. Aphids were lowered onto plant

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leaves immediately after the recording started. All EPG recordings were taken in a grounded Faraday cage. Sample size was determined by previous experience (Leybourne et al., 2019): ten replicates for *H. defensa*-infected aphids on Concerto, 11 for *H. defensa*-infected aphids on Hsp5, 14 for uninfected aphids on Concerto, and 14 for uninfected aphids on Hsp5. For each EPG run, age-synchronised cohorts of young adult apterae were produced (between seven and ten days old, with each run of three EPG replicates containing aphids of the same age). Additionally, fresh plant material was used for each EPG recording (i.e. each recording consisted of a unique biological replicate consisting of one unique aphid and one unique plant).

EPG waveforms were annotated using Stylet+A software (EPG Systems, The Netherlands) by assigning waveforms to np (non-probing), C (stylet penetration/pathway; epidermal and mesophyll tissue), pd (potential-drop/intercellular punctures; mesophyll tissue), the pd sub-phases (pd-II1, pd-II2, pd-II3), E1e (extracellular saliva secretion; mesophyll tissue), E1 (saliva secretion into phloem; vascular tissue), E2 (saliva secretion and passive phloem ingestion; vascular tissue), F (penetration difficulty; mesophyll tissue) or G (xylem ingestion; vascular tissue) phases (Tjallingii, 1988; Alvarez et al., 2006). Using the excel workbook for automatic parameter calculation of EPG data (Sarria et al., 2009), annotated waveforms were converted into time-series data containing information about the duration of each waveform during the EPG run.

Aphid fitness experiments

The aphid fitness study was split into two temporal blocks with seven fully-randomised sub-blocks within each temporal block; each sub-block consisted of one replicate for each plant-aphid- combination: two plant types (Hsp5, Concerto) x four aphid treatments (DL 16/04, DL 16/05, DL 16/06, DL 16/13) giving eight treatments with 14 replicates each. One apterous *R. padi* adult from the four aphid lines described above was taken from culture, placed in a perspex clip-cage (MacGillivray and Anderson, 1957), attached to the first true leaf and left to reproduce overnight. After 24 h, the adult was removed and the resulting progeny were retained on the plant leaf; the mean mass of two nymphs was recorded at 48 h (second instar) and 114 h (fourth instar) and used to calculate the nymph mass gain over this 96 h period. We have previously characterised *R. padi* fitness in relation to *H. defensa*-infection (nymph mass, fecundity, survival) and detected a fitness consequence for nymph mass gain in *H. defensa*-infected aphids (Leybourne et al., 2020). As a result, we measured the most relevant fitness parameter (nymph mass gain) in this study to allow us to estimate the effect of endosymbiont infection and host plant quality on aphid fitness. As with the EPG experiment, each replicate used in the performance experiment consisted of a unique plant replicate which had not been previously exposed to aphid infestation.

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Statistical analysis

All statistical analyses were carried out using R Studio Desktop version 1.0.143 running R version 3.4.3 (R Core Team, 2017), with additional packages car v.2.1-4 (Fox and Weisberg, 2011), ggplot2 v.2.2.1 (Wickham, 2009), ggpubr v. 0.1.2 (Kassambara, 2017), lme4 v.1.1-13 (Bates et al., 2015), lmerTest v.2.0-33 (Kuznetsova et al., 2017), lsmeans v.2.27-62 (Lenth, 2016), multcomp v.1.4-8 (Hothorn et al., 2008), pastecs v.1.3.21 (Grosjean and Ibanez, 2014), and vegan v.2.4-6 (Oksanen et al., 2013).

Data were combined into two endosymbiont treatments: *H. defensa*-infected (comprising the DL 16/04 and DL 16/05 clonal lines) and *H. defensa*-uninfected (comprising the DL 16/06 and DL 16/13 clonal lines). Aphid feeding behaviour was first assessed globally by fitting a permutational multiple analysis of variance to the dataset. Individual feeding parameters from the EPG experiment and aphid juvenile mass gain from the aphid fitness experiment were then analysed in individual linear mixed effects models. Within each model, aphid clonal line was included as a nested factor within endosymbiont infection status to account for the use of four independently initiated clonal lines of the same multilocus genotype which contain differential endosymbiont infection status, as done previously (Leybourne et al., 2020). For the individual EPG parameters, EPG run (blocking factor) and the EPG probe used were included as random factors (there were 3 EPG probes used over the lifetime of the experiment). For the juvenile mass gain model, experimental block and temporal block were incorporated as random factors. All data were modelled against host plant, aphid endosymbiont infection status, and the interaction. χ^2 analysis of deviance tests were used to analyse the final models for the individual EPG parameters and analysis of variance with type III Satterthwaite approximation for degrees of freedom was used to analyse the final aphid fitness model. Calculation of the Least Squares Means was used as a *post-hoc* test on all models with a significant interaction term. All final models were checked for model suitability by observing the fitted-residual plots.

Results

We obtained 72 individual feeding parameters from the EPG analysis (displayed in Tables 1 and 2, and Tables S1 and S2). Global analysis of aphid feeding patterns indicated that aphid feeding behaviour was significantly affected by plant identity ($F_{1,43} = 3.19$; $p = 0.017$) and the interaction between endosymbiont presence and plant identity ($F_{1,43} = 2.71$; $p = 0.037$). From the 72 parameters obtained, seven parameters were affected by plant identity alone (Table S1), however these parameters are not presented or discussed in detail here as we recently characterised *R. padi* feeding responses to plant identity in a separate study (Leybourne et al. 2019). In support of hypothesis 1, a total of 11 parameters were primarily influenced by

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endosymbiont infection, and these were mainly associated with stylet intracellular punctures and interaction with the phloem (Table 1; Fig. 1). A further 15 parameters were differentially affected by the endosymbiont infection × host plant interaction (supporting hypothesis 2), and these involved stylet interactions with the plant surface, the mesophyll tissue, and the phloem (Table 2; Fig 2). The remaining 39 non-significant parameters are displayed in Table S2.

More frequent cellular punctures and phloem feeding in* Hamiltonella defensa*-infected aphids

During the six-hour EPG recording, 11 feeding parameters were affected similarly by endosymbiont infection for aphids feeding on Concerto and Hsp5 (Table 1), supporting our first hypothesis that infection with *H. defensa* can lead to altered aphid probing and feeding behaviour. Most of these feeding parameters related to aphid stylet activities in the mesophyll tissue, specifically the frequency and duration of the exploratory intracellular punctures (EPG waveform pd) performed by aphids while probing into plant tissue, and stylet interaction with phloem sap. The average duration of each C phase (stylet interaction with and movement through the mesophyll tissue) was around 25-30% shorter in *H. defensa*-infected aphids compared with uninfected aphids (Table 1; Fig. 1A). The total number of intracellular punctures (pd) made by *H. defensa* infected aphids was around two-fold higher than those made by uninfected aphids (Table 1; Fig 1B). Furthermore, following the first stylet probe into plant tissue, the first intracellular puncture (pd) occurred more rapidly for infected aphids (Table 1). Although the frequency of intracellular punctures increased in *H. defensa* infected aphids (Table 1; Fig. 1B), the duration of these intracellular punctures was on average 50% shorter for infected aphids compared with uninfected aphids (Table 1; Fig. 1C). The frequency of these intracellular punctures was highest and their duration shortest in the first hour (Table 1). Following this, the frequency of intracellular punctures in the second to sixth hours was not affected by endosymbiont presence, although the duration of intracellular punctures was influenced by symbiont presence in the second and sixth hours of EPG monitoring (Table 1).

Three aphid feeding parameters related to stylet activity in the vascular tissue were affected by endosymbiont presence (Table 1; Fig. 1D-F): aphids infected with *H. defensa* showed a 50% reduction in time spent salivating into the phloem during stylet contact with the phloem (Table 1; Fig. 1D), displayed a 33% increase in phloem ingestion during stylet contact with the phloem (Table 1; Fig. 1E) and had a higher proportion of phloem sap ingestion phases (E2 phases) containing a period of sustained phloem sap ingestion (sE2 – a period of ingestion > 10 mins) (Table 1; Fig. 1F).

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Differential endosymbiont effects on aphid feeding on a partially-resistant plant

In line with previous findings (Leybourne et al., 2020), the mass gain of *R. padi* nymphs was reduced when feeding on the partially-resistant wild relative of barley Hsp5 compared with aphids feeding on the susceptible modern cultivar of barley Concerto (ANOVA plant species: $F_{1,93}$ =122.57; p = <0.001; Fig. S1), although endosymbiont presence/absence alone did not affect aphid fitness (ANOVA endosymbiont: $F_{1,93}$ = 0.42; p = 0.514). The growth of aphids infected with *H. defensa* was further reduced by 22% when aphids were feeding on Hsp5 (ANOVA plant species × endosymbiont interaction: $F_{1,93}$ =6.35; p = 0.013; Fig. S1). To examine whether alterations in aphid probing and feeding behaviour contributed towards this fitness cost, we identified EPG parameters responding differentially to endosymbiont infection on each plant type.

Fifteen EPG parameters were significantly affected by the endosymbiont infection x plant type interaction (Table 2). Eleven of these were differentially affected by *H. defensa*-infection for aphids contained on Hsp5 (Table 2). These data indicated that, in support of our second hypothesis, altered aphid probing and feeding behaviour could contribute towards decreased fitness of *H. defensa*-infected aphids on this less nutritious host plant (Fig. S1). When interacting with Hsp5, infected aphids spent 9% less time probing into plant tissue compared with uninfected aphids (Table 2), resulting in an overall reduction in the total time spent probing into plant tissue (Table 2; Fig. 2A). Although there was no difference in the number of non-probing phases between *H. defensa*-infected and uninfected aphids when feeding on Hsp5 (Table 2), *H. defensa*-infected aphids spent longer periods not probing into the plant tissue (Table 2). Furthermore, the duration of the first probe into plant tissue by *H. defensa*-infected aphids feeding on Hsp5 was around six-fold shorter compared with uninfected aphids (Table 2; Fig. 2B), and *H. defensa*-infected aphids required twice as many probes into plant tissue before the phloem was reached compared with uninfected aphids (Table 2; Fig. 2C). The total time spent ingesting phloem was reduced by 44% for *H. defensa*-infected aphids feeding on Hsp5 compared with uninfected aphids (Table 2; Fig. 2D) and the longest observed period of phloem ingestion was three-fold shorter for *H. defensa*-infected aphids compared with uninfected aphids when feeding on Hsp5 (Table 2; Fig. 2E).

Infection with *H. defensa* also altered the feeding behaviour of *R. padi* when probing into the susceptible barley cv. Concerto: *H. defensa*-infected aphids achieved sustained phloem sap ingestion two-fold faster than uninfected aphids (Table 2; Fig. 2F), however this did not affect aphid growth (Fig. S1).

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Discussion

By analysing aphid feeding behaviour, our study provides novel mechanistic insights into the consequences of *Hamiltonella defensa* infection for interactions at the aphid-plant interface and shows that *H. defensa* infection can alter aphid probing behaviour irrespective of host plant suitability. In addition to this, our data show that these interactions can be influenced by plant susceptibility to, or resistance against, aphids and we provide novel evidence showing that aphid physiological processes are differentially affected by endosymbiont presence and host plant suitability which, at least in part, explains a fitness cost associated with *H. defensa*-infection for *R. padi* when feeding on a poor quality (partially-resistant) host plant. More broadly, our findings provide new information about the mechanisms by which facultative endosymbionts can influence insect physiology by altering feeding processes at the herbivore-plant interface and highlight further avenues of research which could be explored in other insect-symbiont systems.

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

When probing into plant tissue, *H. defensa*-infected aphids displayed a characteristic pattern of more frequent and shorter exploratory intracellular punctures (EPG waveform pd) than uninfected aphids. The precise cause of this symbiont-associated effect on aphid probing is not clear, although a similar pattern was recently reported in *H. defensa*-infected cowpea aphids, *Aphis craccivora* (Angelella et al., 2018). Intracellular punctures have been associated with the transmission of non-persistent and semi-persistent plant viruses (Fereres and Collar, 2001; Powell, 2005; Moreno et al., 2012) and it is likely that changes in intracellular puncture frequency will affect the transmission of these virus types. Although *R. padi* is not a significant vector of semi-persistent or non-persistent plant viruses, it would be possible to test this hypothesis if increased frequencies of intracellular punctures were detected in other *H. defensa*-infected aphid species or other sap-feeding insect pests. For example, the cabbage aphid, *Brevicoryne brassicae*, has been reported to form facultative endosymbiont associations with *H. defensa* (Desneux et al., 2017) and vectors several semi-persistent and non-persistent plant viruses (Moreno et al., 2012), including cauliflower mosaic virus and turnip mosaic virus. Furthermore, infection with *H. defensa* has been reported in the whitefly species *Bemisia tabaci,* (Su et al., 2015) which is a vector of multiple devastating plant viruses, including the Begomoviruses (Czosnek et al., 2017). Examining whether *H. defensa*-infection alters probing behaviour in these organisms could highlight opportunities for altering the efficiency of virus vectoring by economically important insect pests.

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A key difference between our study and the previous work of Angelella et al. (2018) was that *R. padi* infected with *H. defensa* (this study) also showed differential feeding behaviour caused by altered stylet activities within the phloem. *H. defensa*-infected aphids spent less time salivating into the phloem and showed an overall increase in the percentage of phloem phases which contained phloem ingestion, including an increased proportion of phases containing periods of sustained phloem ingestion. This could potentially influence *R. padi* transmission or acquisition of phloem-limited plant viruses, such as barley yellow dwarf virus (BYDV), which are acquired during phloem ingestion and transmitted during salivation (Ng and Perry, 2004). Altered aphid probing and feeding behaviour did not appear to affect aphid fitness directly as no overall effect of *H. defensa* infection on *R padi* growth, development, fecundity, or longevity was detected (present study; Leybourne et al., 2020). However, *H. defensa*-infection can affect aphid fitness in other species (Zytynska et al., 2019 preprint) and differential feeding behaviour in *H. defensa*-infected aphids could be associated with these altered aphid phenotypes, including increased adult body mass and enhanced offspring production in black bean aphids, *A. fabae* (Castañeda et al., 2010). Endosymbiont-induced changes in feeding behaviour might be due to indirect effects of the bacterium on stylet activities mediated by bacterium-derived salivary factors (Su et al., 2015; Frago et al., 2017).

The extent of these endosymbiont-derived fitness consequences can often be dependent on aphid clonal line or aphid genotype (Castañeda et al., 2010) and it is important to note that endosymbiont-conferred traits vary between different aphid lines, aphid genotypes, and aphid species (Castañeda et al., 2010; Vorburger and Gouskov, 2011; Leybourne et al., 2020): indeed, *H. defensa*-infection can also reduce *A. fabae* reproductive rate and survivorship (Vorburger and Gouskov, 2011) and decrease *S. avenae* fecundity (Li et al., 2018). Altered probing behaviour might also explain differential plant responses to infestation by aphids infected with *H. defensa*, including changes in the emission of Herbivore Induced Plant Volatile (HIPV) compounds (Frago et al., 2017), reduced dry matter allocation to roots (Hackett et al., 2013; Bennett et al., 2016), and reduced accumulation of salicylic and jasmonic acids (Li et al., 2019). A focus for future research should include the consequences of aphid species and genotype for *H. defensa*-associated modifications to aphid probing and feeding behaviour to fully elucidate their effects on aphid pest status, virus transmission, and plant responses to aphid infestation.

Endosymbiont infection reduces aphid feeding on a poor quality host plant

When probing into the partially-resistant plant, Hsp5, aphids infected with *H. defensa* showed a differential physiological feeding pattern compared with uninfected aphids, including a reduction in the time spent probing into plant tissue, an increase in the number of plant tissue

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probes required to reach the phloem tissue, and a decrease in total phloem ingestion. This was linked with decreased fitness in *H. defensa*-infected aphids compared with uninfected aphids when feeding on Hsp5, in line with our previous findings (Leybourne et al., 2020). A decrease in the duration of the first probe into plant tissue, and an overall reduction in time spent probing into the plant tissue, are representative of mesophyll- and epidermal-derived factors which inhibit and impede the penetration of the aphid stylet through the plant tissue, as highlighted by Alvarez et al. (2006). Similar fitness costs associated with *H. defensa*-infected aphids have been observed previously in *A. craccivora* (Wagner et al., 2015) and in *A. fabae* when feeding on different quality plant species (Chandler et al., 2008), although, in these cases, it is not known if this was linked with altered aphid probing and feeding behaviour.

We recently characterised the partial-resistance mechanism of Hsp5 (Leybourne et al., 2019) and reported that partial-resistance involves mesophyll and phloem traits. These included an increased abundance of defensive thionins and a reduction in the availability of essential amino acids as mesophyll-derived and phloem-derived partial-resistance factors, respectively (Leybourne et al., 2019). These factors could underlie the decreased time aphids spent probing into plant tissue and the shorter duration of the initial probe into plant tissue, although the underlying processes causing these differential feeding patterns are currently unclear. A key factor which likely contributes towards this decrease in aphid fitness is our observation that *H. defensa*-infected aphids showed a 44% reduction in time spent ingesting phloem on Hsp5 compared with uninfected aphids. It is probable that this substantial decrease in phloem ingestion contributed significantly to the 22% reduction in nymph growth we detected. Indeed, a previous study using the peach-potato aphid, *Myzus persicae,* showed that a 58% decrease in ingestion rate can result in a 10% reduction in aphid growth (Karley et al., 2002). We also detected differential feeding patterns between *H. defensa*-infected aphids feeding on Hsp5 and Concerto: *H. defensa*-infected aphids feeding on Concerto achieved sustained phloem feeding more rapidly than those feeding on Hsp5. A faster initiation of sustained feeding could explain the higher mass of *H. defensa*-infected nymphs on Concerto. However, it is likely that the observed reduction in nymph mass for both infected and uninfected nymphs when feeding on Hsp5 compared with those feeding on Concerto is due to increased aphid resistance in Hsp5 (Leybourne et al., 2019). The rapid initiation of sustained feeding could be associated with other aphid fitness effects which are currently uncharacterised, such as the transmission or acquisition of phloem-limited viruses.

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Conclusion

In this study, two hypotheses were tested: 1) that infection with *H. defensa* can lead to altered aphid probing and feeding behaviour; 2) that differential aphid probing and feeding behaviour between uninfected and *H. defensa*-infected aphids is a key contributor towards the decreased fitness of *H. defensa*-infected aphids feeding on partially-resistant Hsp5. *R. padi* infected with the defensive facultative endosymbiont, *H. defensa,* showed altered probing and feeding behaviour compared with uninfected aphids, irrespective of plant type, including an increase in the number of intracellular punctures and in sustained phloem ingestion, supporting our first hypothesis. Furthermore, in support of our second hypothesis, we present novel EPG data providing the first demonstration of mechanisms at the aphid-plant interface which contribute towards a fitness cost arising from *H. defensa*-infection in *R. padi* feeding on the partially-resistant plant, Hsp5. The physiological effects of endosymbiont infection were associated with a reduction in the time aphids probe into the plant tissue, an increase in the number of plant tissue probes required to reach the phloem, and a 44% reduction in total phloem ingestion. Together, our results show that aphid facultative endosymbionts can influence aphid-plant interactions in more subtle ways than previously realised and indicate that plant quality can exacerbate these effects. One pertinent question arising from our study, which is broadly applicable to all piercing and sucking insects, is whether the feeding processes associated with facultative endosymbiont infection affect insect propensity to move through the plant canopy, which has potential consequences for the level of feeding damage and virus transmission inflicted by economically-important crop pests. Answering this question will be key to forecasting the dynamics of insect pests and insect-vectored viruses in the field. More broadly, our findings highlight avenues of research for other herbivorous insects to understand the processes by which facultative endosymbionts affect insect success and pest status.

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Competing Interests

The authors declare no competing interests.

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Author Contributions

AJK and DJL conceived and designed the experiments. DJL performed the experiments and analysed the data. All authors contributed to data interpretation. DJL wrote the manuscript with input from all authors. All authors read and approved the final manuscript.

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Figures

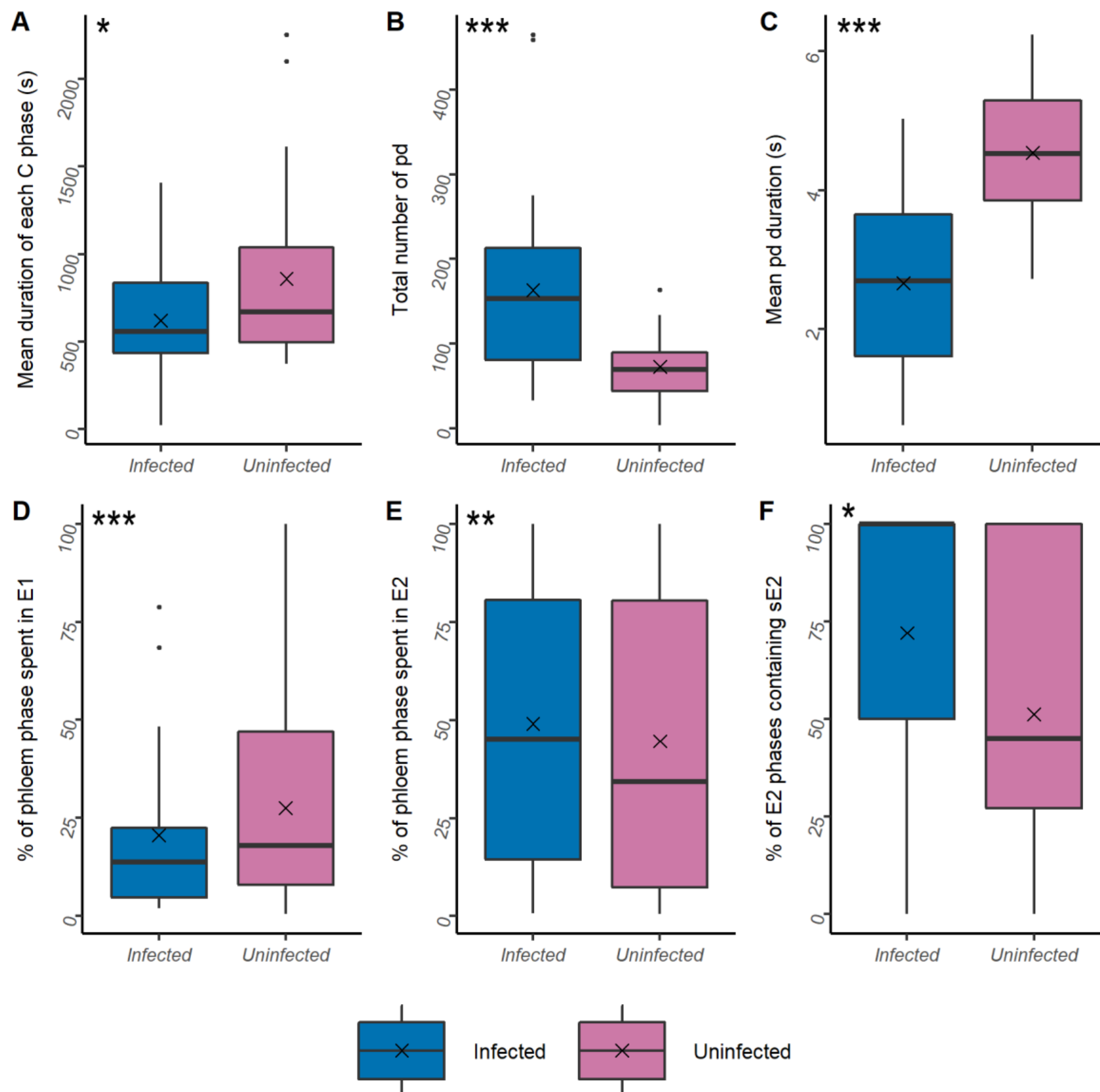

Fig. 1: Aphid feeding parameters that were differentially affected by the presence (infected) and absence (uninfected) of *Hamiltonella defensa*. Box plot characteristics: median = thick line, interquartile range (IQR) = the box, 1.5 IQR = the whiskers, outliers = points, mean = the black cross ("×"). Infected: combined data for both *H. defensa*-infected lines (DL 16/04; DL 16/05). Uninfected: combined data for both *H. defensa*-uninfected lines (DL 16/06; DL 16/13). A-C: parameters associated with stylet puncturing of plant cells (intracellular punctures); D-F: parameters associated with stylet interaction with phloem sap. Feeding and probing parameter abbreviations: C (stylet penetration/pathway phase), pd (potential-drop/intercellular punctures), E1 (saliva secretion into phloem), E2 (saliva secretion and passive phloem ingestion), sE2 (sustained phloem ingestion). Level of statistical significance: * <0.05, ** <0.01, *** <0.001. The number of replicates for each experimental group (number of aphids from which each parameter was observed at least once during the six hour recording) are as follows: for panels A-C n=21 for *H. defensa*-infected aphids and n=28 for uninfected aphids. Panel D: n = 20 and 26 for *H. defensa*-infected and uninfected aphids, respectively. For panel

E: n = 20 (*H. defensa*-infected) and 24 (*H. defensa*-uninfected), and for panel F: n = 17 (*H. defensa*-infected) and 20 (*H. defensa*-uninfected). Each replicate is taken from a unique aphid and represents a single biological replicate.

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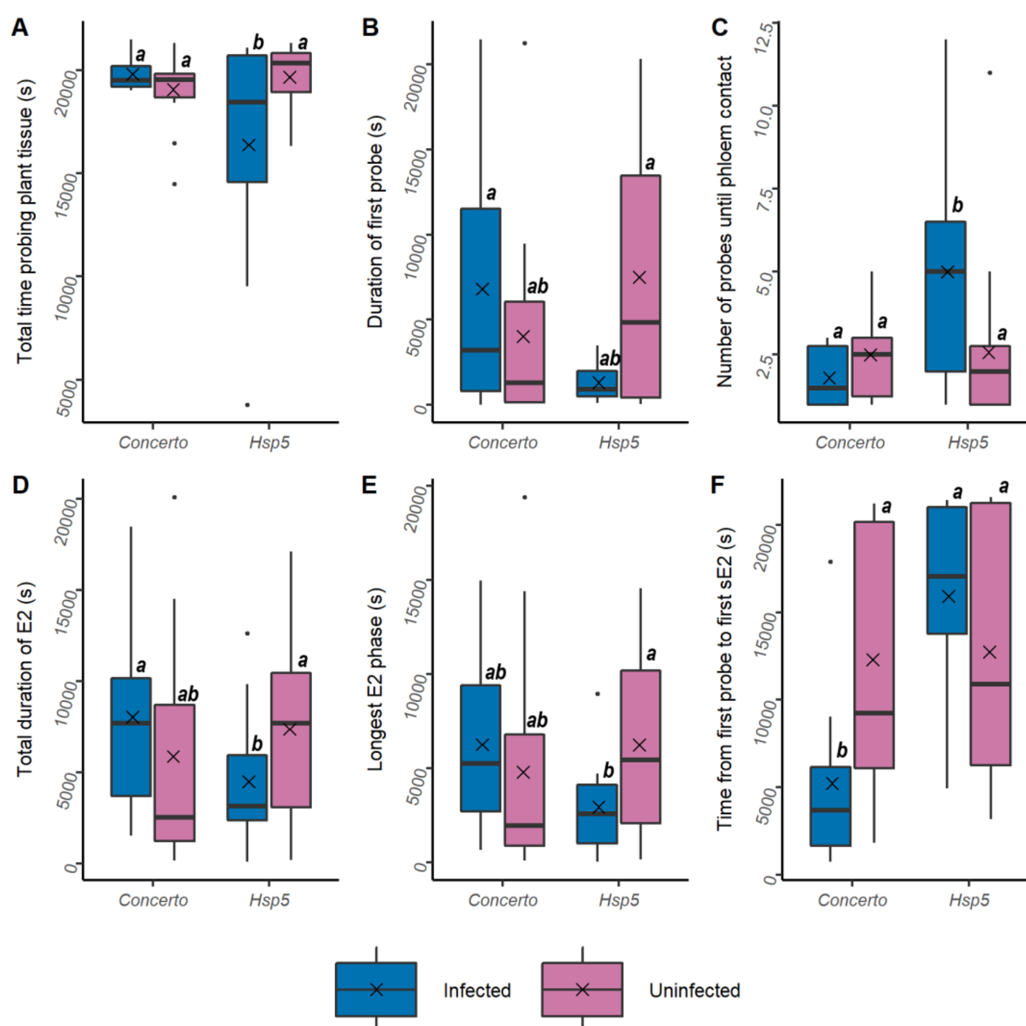

Fig 2: Aphid feeding parameters that were differentially affected by the presence (infected) and absence (uninfected) of *Hamiltonella defensa* infection on two plant hosts (susceptible modern barley cv. Concerto and the wild relative Hsp5. Box plot characteristics: median = thick line, interquartile range (IQR) = the box, 1.5 IQR = the whiskers, outliers = points, mean = the black cross ("×"). Infected: combined data for both *H. defensa*-infected lines (DL 16/04; DL 16/05). Uninfected: combined data for both *H. defensa*-uninfected lines (DL 16/06; DL 16/13). Letters indicate which groups are significantly different based on pairwise comparisons using general linear hypotheses testing with single-step p-value adjustment. Feeding and probing parameter abbreviations: E2 (saliva secretion and passive phloem ingestion), sE2 (sustained phloem ingestion). The number of replicates for each experimental group (number of aphids from which each parameter was observed at least once during the six hour recording) are as follows: for panels A-C: n = 14 (*H. defensa*-infected on Concerto), 14 (*H. defensa*-uninfected on Concerto), 11 (*H. defensa*-infected on Hsp5), and 10 (*H. defensaa*-uninfected on Hsp5). For panels D-E: n = 14 (*H. defensa*-infected on Concerto), 12 (*H. defensa*-uninfected on Concerto), 10 (*H. defensa*-infected on Hsp5), and 10 (*H. defensaa*-uninfected on Hsp5). For panel F: n = 11 (*H. defensa*-infected on Concerto), 9 (*H. defensa*-uninfected on Concerto), 7 (*H. defensa*-infected on Hsp5), and 10 (*H. defensaa*-uninfected on Hsp5). Each replicate is taken from a unique aphid and represents a single biological replicate.

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Tables

Table 1: Aphid feeding and probing parameters (mean value ± standard error) that were significantly affected by the absence (*Hd*-ve) or presence (*Hd*+ve) 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.

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 × 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 = <0.001 ***	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

Data marked with † display median alongside the upper and lower inter quartile ranges; level of statistical significance: * <0.05, ** <0.01, *** <0.001. Feeding and probing parameter abbreviations: C (stylet penetration/pathway phase), pd (potential-drop/intercellular punctures), E1 (saliva secretion into phloem), E2 (saliva secretion and passive phloem ingestion), sE2 (sustained phloem ingestion).

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Table 2: Aphid feeding and probing 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.

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 × 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 *

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Time from first probe into plant tissue until first sE2 (sustained phloem feeding phase) phase (sqrt)	12284.97 s ± 2033.97 [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 [ab] *(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 *

Data marked with † display median alongside the upper and lower inter quartile ranges**.** level of statistical significance: * <0.05, ** <0.01, *** <0.001. Feeding and probing parameter abbreviations: C (stylet penetration/pathway phase), pd (potential-drop/intercellular punctures), E (an observed phloem phase), E1 (saliva secretion into phloem), E2 (saliva secretion and passive phloem ingestion), sE2 (sustained phloem ingestion).

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Table S1: Aphid feeding and probing parameters (mean value ± standard error) that were significantly affected by the different plant types (Hsp5 and Concerto). Numbers in parenthesis indicate the total number of individuals which displayed each parameter, the total number of individuals tested is indicated at the top of the column.

Description of Response Variable Assessed (transformation used)	Host plant: Concerto *(max = 24)*	Host plant: Hsp5 *(max = 25)*	Statistical results for each Explanatory Variable: Plant		Endosymbiont		Plant × Endosymbiont	
Minimum recorded time to E1 (salivation into phloem) from first probe (sqrt)	1286.76 s ± 287.82 s *(24)*	3546.65 s ± 572.45 s *(23)*	X^2_1 = 16.69	P = <0.001 ***	X^2_1 = 0.09	P = 0.757	X^2_1 = 0.14	P = 0.699
Mean time to first E1 phase from first probe into plant tissue (sqrt)	3986.44 ± 527.44 s *(24)*	8992.00 ± 1257.19 s *(23)*	X^2_1 = 13.59	P = <0.001 ***	X^2_1 = 0.01	P = 0.945	X^2_1 = 2.22	P = 0.135
Number of E1 phases (sqrt)	4.79 ± 0.60 *(24)*	3.28 ± 0.56 *(23)*	X^2_1 =4.28	P = 0.030 *	X^2_1 = 0.01	P = 0.946	X^2_1 = 0.22	P = 0.635
Mean time from first probe into plant tissue until first E2 phase (sqrt)	5490.25 ± 917.45 s *(24)*	10773.24 ± 1286.41 s *(22)*	X^2_1 = 12.70	P = <0.001 ***	X^2_1 = 0.33	P = 0.565	X^2_1 = 2.41	P = 0.119
Number of E2 phases (sqrt)	2.33 ± 0.24 *(24)*	1.52 ± 0.23 *(22)*	X^2_1 = 6.76	P = 0.009 *	X^2_1 = 0.01	P = 0.975	X^2_1 = 0.01	P = 0.930
Total number of E phases (including E1, E2, and sE2) (sqrt)	4.75 ± 0.61 *(24)*	3.20 ± 0.54 *(23)*	X^2_1 = 4.38	P = 0.036 *	X^2_1 = 0.01	P = 0.993	X^2_1 = 0.18	P = 0.666
Number of sE2 phases (sqrt)	1.20 ± 0.14 *(21)*	0.80 ± 0.15 *(16)*	X^2_1 = 4.13	P = 0.042 *	X^2_1 = 2.25	P = 0.133	X^2_1 = 2.80	P = 0.094

Level of statistical significance: * <0.05, ** <0.01, *** <0.001

Table S2: Aphid feeding and probing parameters which were not significantly affected by any treatment factors.

Description of Response Variable Assessed (transformation used)	Statistical results for each Explanatory Variable					
	Plant		Endosymbiont		Plant x Endosymbiont	
Mean duration of each non-probing period (sqrt)	$X^2_1 = 0.11$	P = 0.732	$X^2_1 = 1.19$	P = 0.274	$X^2_1 = 1.19$	P = 0.273
Duration of the second probe into plant tissue (log)	$X^2_1 = 0.01$	P = 0.999	$X^2_1 = 0.01$	P = 0.935	$X^2_1 = 0.555$	P = 0.456
Number of stylet probes into plant tissue in the first hour (not transformed)	$X^2_1 = 0.09$	p = 0.758	$X^2_1 = 0.63$	p = 0.424	$X^2_1 = 2.32$	p = 0.127
Number of stylet probes into plant tissue in the third hour (not transformed)	$X^2_1 = 0.50$	p = 0.477	$X^2_1 = 0.01$	p = 0.993	$X^2_1 = 2.81$	p = 0.093
Number of stylet probes into plant tissue in the fourth hour (not transformed)	$X^2_1 = 2.49$	p = 0.114	$X^2_1 = 1.05$	p = 0.304	$X^2_1 = 0.01$	p = 0.971
Number of stylet probes into plant tissue in the fifth hour (not transformed)	$X^2_1 = 2.25$	p = 0.132	$X^2_1 = 0.82$	p = 0.365	$X^2_1 = 0.01$	p = 0.898
Number of stylet probes into plant tissue in the sixth hour (not transformed)	$X^2_1 = 0.15$	p = 0.694	$X^2_1 = 1.77$	p = 0.182	$X^2_1 = 0.146$	p = 0.226
Number of brief probes into plant tissue (< 3 mins) following first phloem contact (not transformed).	$X^2_1 = 0.80$	p = 0.368	$X^2_1 = 0.52$	p = 0.466	$X^2_1 = 2.09$	p = 0.147
Total duration of all C (mesophyll pathway) phases (not transformed)	$X^2_1 = 0.49$	P = 0.482	$X^2_1 = 0.77$	P = 0.379	$X^2_1 = 1.20$	P = 0.277
Mean number of pd: potential drop (intracellular punctures) per probe into plant tissue (log)	$X^2_1 = 2.53$	P = 0.111	$X^2_1 = 2.89$	P = 0.088	$X^2_1 = 1.08$	P = 0.298
Number of pd in second hour (sqrt)	$X^2_1 = 0.18$	P = 0.665	$X^2_1 = 0.50$	P = 0.479	$X^2_1 = 2.47$	P = 0.115
Number of pd in fifth hour (sqrt)	$X^2_1 = 3.02$	P = 0.082	$X^2_1 = 1.08$	P = 0.296	$X^2_1 = 0.16$	P = 0.685
Number of pd in sixth hour (sqrt)	$X^2_1 = 0.16$	p = 0.684	$X^2_1 = 2.35$	p = 0.125	$X^2_1 = 1.25$	p = 0.261
Total duration of all pd's (sqrt)	$X^2_1 = 0.11$	p = 0.739	$X^2_1 = 0.21$	p = 0.646	$X^2_1 = 1.95$	p = 0.162
Mean duration of each pd in third hour (not transformed)	$X^2_1 = 1.29$	P = 0.255	$X^2_1 = 1.50$	P = 0.219	$X^2_1 = 0.64$	P = 0.420
Mean duration of each pd in fourth hour (not transformed)	$X^2_1 = 0.03$	P = 0.860	$X^2_1 = 0.04$	P = 0.832	$X^2_1 = 1.44$	P = 0.228
Mean duration of each pd in fifth hour (not transformed)	$X^2_1 = 0.34$	p = 0.554	$X^2_1 = 2.97$	p = 0.084	$X^2_1 = 0.01$	p = 0.999
Total duration of pd sub-phase 1 (not transformed)	$X^2_1 = 3.25$	P = 0.071	$X^2_1 = 0.50$	P = 0.477	$X^2_1 = 0.55$	P = 0.457
Total duration of pd sub-phase 2 (sqrt)	$X^2_1 = 2.65$	P = 0.103	$X^2_1 = 0.131$	P = 0.717	$X^2_1 = 0.143$	P = 0.705
Total duration of pd sub-phase 3 (sqrt)	$X^2_1 = 2.11$	P = 0.145	$X^2_1 = 0.90$	P = 0.342	$X^2_1 = 0.98$	P = 0.321
Number of xylem ingestion phases (not transformed)	$X^2_1 = 0.30$	p = 0.577	$X^2_1 = 0.01$	p = 0.982	$X^2_1 = 3.02$	p = 0.081
Number of (phloem) E phases which only contain E1	$X^2_1 = 1.55$	P = 0.212	$X^2_1 = 0.01$	P = 0.899	$X^2_1 = 0.95$	P = 0.328
Total duration of all E phases which only contain E1 (salivation into phloem)	$X^2_1 = 0.15$	P = 0.697	$X^2_1 = 0.17$	P = 0.679	$X^2_1 = 1.47$	P = 0.224
Combined duration of E1 (sqrt)	$X^2_1 = 0.03$	p = 0.852	$X^2_1 = 0.25$	p = 0.611	$X^2_1 = 0.21$	p = 0.639
Mean duration of each E1 phase (log)	$X^2_1 = 0.78$	p = 0.375	$X^2_1 = 0.01$	p = 0.899	$X^2_1 = 0.01$	p = 0.981
Mean duration of E1 phase when followed by E2 (phloem ingestion) phase (log)	$X^2_1 = 0.39$	p = 0.530	$X^2_1 = 0.01$	p = 0.946	$X^2_1 = 0.05$	p = 0.806
Mean duration of E1 phase when followed by sE2 (sustained phloem ingestion) phase (log)	$X^2_1 = 0.02$	p = 0.867	$X^2_1 = 0.04$	p = 0.823	$X^2_1 = 0.33$	p = 0.561
Total duration of all E1 phases before first E2 (log)	$X^2_1 = 0.59$	p = 0.442	$X^2_1 = 0.137$	p = 0.710	$X^2_1 = 1.56$	p = 0.221
Total duration of all E1 phases before first sE2 (sqrt)	$X^2_1 = 0.76$	p = 0.383	$X^2_1 = 0.115$	p = 0.734	$X^2_1 = 0.01$	p = 0.992
Duration of first E phase (log)	$X^2_1 = 0.01$	p = 0.899	$X^2_1 = 0.34$	p = 0.554	$X^2_1 = 3.19$	p = 0.073
Duration of first E2 phase (log)	$X^2_1 = 0.12$	p = 0.722	$X^2_1 = 0.01$	p = 0.889	$X^2_1 = 1.09$	p = 0.295
Mean duration of each E2 phase (sqrt)	$X^2_1 = 0.01$	P = 0.979	$X^2_1 = 0.01$	P = 0.984	$X^2_1 = 1.80$	P = 0.178
Percentage of probe spent in C phase (not transformed)	$X^2_1 = 0.15$	p = 0.689	$X^2_1 = 0.38$	p = 0.537	$X^2_1 = 3.15$	p = 0.075
Percentage of probe spent in E1 phase (sqrt)	$X^2_1 = 0.19$	p = 0.661	$X^2_1 = 0.27$	p = 0.597	$X^2_1 = 0.98$	p = 0.320

Percentage of probe spent in E2 phase	$X^2_1 = 0.90$	P = 0.340	$X^2_1 = 0.44$	P = 0.503	$X^2_1 = 1.22$	P = 0.268
Percentage of probe spent in xylem phase	$X^2_1 = 1.37$	P = 0.241	$X^2_1 = 0.41$	P = 0.517	$X^2_1 = 0.78$	P = 0.376
Total duration of all E phases (sqrt)	$X^2_1 = 1.15$	P = 0.283	$X^2_1 = 0.06$	P = 0.792	$X^2_1 = 1.84$	P = 0.174
Total time spent ingesting xylem (sqrt)	$X^2_1 = 1.17$	P = 0.278	$X^2_1 = 1.31$	P = 0.252	$X^2_1 = 1.14$	P = 0.283
Mean duration of each xylem ingestion phase (sqrt)	$X^2_1 = 1.24$	P = 0.265	$X^2_1 = 0.20$	P = 0.652	$X^2_1 = 0.341$	P = 0.559

Feeding and probing parameter abbreviations: np (non-probing), C (stylet penetration/pathway phase), pd (potential-drop/intercellular punctures), the pd sub-phases (pd-II1, pd-II2, pd-II3), E1e (extracellular saliva secretion), E1 (saliva secretion into phloem) E2 (saliva secretion and passive phloem ingestion), F (penetration difficulty) or G (xylem ingestion).

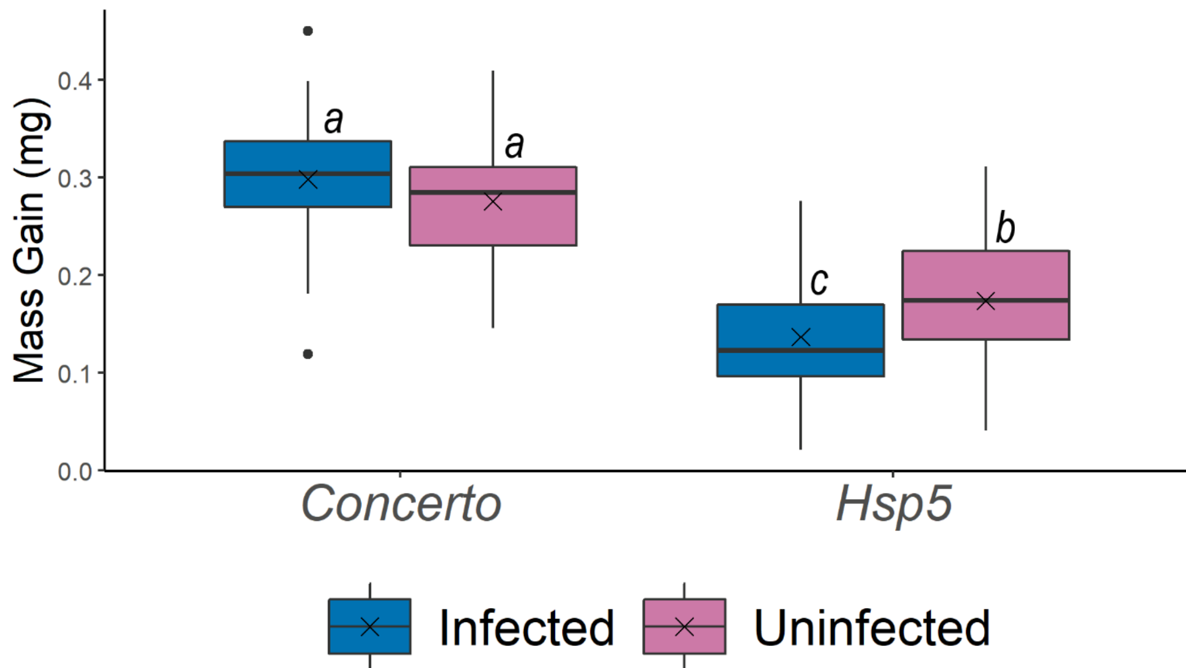

Fig. S1: Juvenile *R. padi* mass gain over a 96 h period in the presence (infected) and absence (uninfected) of *Hamiltonella defensa* infection on Concerto and Hsp5. Box plots indicate the median as a thick line, the interquartile range (IQR) as a box, 1.5 IQR as whiskers and the outliers as points outside the whisker range. Infected reports the combined data for both *H. defensa*-infected lines (DL 16/04; DL 16/05) and Uninfected reports the combined data for both *H. defensa*-uninfected lines (DL 16/06; DL 16/13). Letters indicate which groups are similar to each other using Least Squares Means *post-hoc* analysis. The black cross ("×") on each plot shows the mean value. The number of replicates for each experimental group was 14 and comprised two experimental blocks, each containing seven replicates. Each replicate was taken from a unique aphid and represents a single biological replicate. See methods for details of the statistical analysis.

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