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ORIGINAL ARTICLE

Influence of *Hamiltonella defensa* infection and predator type on anti-predator behaviours in pea (*Acyrthosiphon pisum*) and potato aphids (*Macrosiphum euphorbiae*)

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Abstract

Facultative endosymbionts can induce benefits and costs to their aphid hosts. In the pea aphid, *Acyrthosiphon pisum* (Harris) (Hemiptera: Aphididae), infection with the γ-proteobacterium *Hamiltonella defensa* Moran et al. can confer resistance against parasitoids, but may also reduce the frequency of aggressive and escape behaviours exhibited in response to predators. In potato aphids, *Macrosiphum euphorbiae* (Thomas) (Hemiptera: Aphididae), *H. defensa* does not appear to influence susceptibility to parasitism, but its impact on anti-predator behaviours remains unexplored. Here we investigated defensive behaviours in two pea aphid lines (differing in *H. defensa*-infection status) and four potato aphid lines (that additionally differed in genotype-associated parasitism susceptibility) when faced with foraging ladybirds – *Adalia bipunctata* (L.) (Coleoptera: Coccinellidae) – and lacewings – *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae). In response to ladybirds, symbiont-infected pea aphids exhibited proportionately fewer evasive defences (dropping and walking away) than non-infected (cured) pea aphids, but more frequent aggressive kicking. Ladybirds provoked more evasive, aggressive, and total defensive behaviours than lacewings. For potato aphids, symbiont status, predator type, and aphid genotype (i.e., assumed parasitism susceptibility) all influenced behavioural repertoires. Overall, infected lines showed greater differentiation in behaviours in response to the two predators than the uninfected lines. The presence of the symbiont *H. defensa* may be a key determinant of aphid anti-predator behaviours, but the fitness consequences of this are unresolved. In our study, neither symbiont infection status nor aphid genotype affected the number of aphids consumed by predators.

KEYWORDS
anti-predator behaviours, Aphididae, Chrysopidae, Coccinellidae, Coleoptera, facultative endosymbionts, *Hamiltonella defensa*, Hemiptera, Neuroptera, parasitism resistance, predators, symbionts

INTRODUCTION

Aphids (Hemiptera: Aphididae) are important crop pests (van Emden & Harrington, 2007; Dedryver et al., 2010). Long-time considered as helpless, thin-skinned, sedentary prey exhibiting no self-defence against predation (Imms, 1947), aphids are now appreciated as having a wide range of defensive behaviours (Dixon, 1958). These include: remaining motionless to avoid detection (Dixon, 1958, 1985; Brodsky & Barlow, 1986), kicking (Dixon, 1958; Hartbauer, 2010; Dion

et al., 2011; Polin et al., 2014), backing-up and walking away (Dixon, 1958; Clegg & Barlow, 1982; Brodsky & Barlow, 1986), and dropping from plants (Dixon, 1958; Roitberg & Myers, 1978; Clegg & Barlow, 1982; Agabiti et al., 2016; Harrison & Preisser, 2016). Dropping from plants is thought to be a widespread, common, and effective response of aphids to perceived threats (Dixon, 1985; Montgomery & Nault, 1977; Gross, 1993; Humphreys & Ruxton, 2019). However, dropping can itself be costly, via ground-foraging predators, high ground temperatures, or subsequent failure to locate a new, suitable host plant (Roitberg & Myers, 1979; Roitberg et al., 1979; Losey & Denno, 1998b; Gish & Inbar, 2006). The use of dropping relative to other defences, therefore, is expected to be context-dependent.

Many studies have explored external factors that can influence the occurrence of aphid dropping, including variables relating to the predatory threat – such as size, speed, and foraging tactic (e.g., lunging ambushers or active cruisers) (Brown, 1974; Brodsky & Barlow, 1986; Losey & Denno, 1998a; Day et al., 2006; Hoki et al., 2014). Less attention has been paid to characteristics of the prey in this context, but one relatively new field involves consideration of how facultative endosymbionts might influence anti-predator behaviours (Dion et al., 2011; Polin et al., 2014). Endosymbionts are microbes that form associations with insects and are located intracellularly in the host (Clark et al., 2010). Most aphids possess the obligate bacterial endosymbiont *Buchnera aphidicola* Munson et al., which synthesizes essential amino acids for its host (Douglas, 1998; Gündüz & Douglas, 2009), but aphids can also harbour additional facultative bacterial endosymbionts, whose effects are diverse and variable (Guo et al., 2017; Vorburger, 2018). Some effects are clearly beneficial to the aphid host (such as increased stress tolerance or protection against natural enemies), but some are detrimental (such as reduced fecundity and lifespan), and the same symbionts can have multiple effects [see Guo et al. (2017), Vorburger (2018), and references therein].

One symbiont that appears to affect both aphid resistance to parasitism and behavioural responses to predators is the facultative endosymbiont *Hamiltonella defensa* Moran et al. *Hamiltonella defensa* is a γ-proteobacterium that provides resistance against *Aphidius ervi* Haliday parasitoid wasps in pea aphids, *Acyrthosiphon pisum* (Harris) (Hemiptera: Aphididae) (Oliver et al., 2003, 2006; Guay et al. 2009; Donald et al. 2016), bird cherry-oat aphids [*Rhopalosiphum padi* (L.)] (Leybourne et al., 2018), cowpea aphids (*Aphis craccivora* C.L. Koch) (Asplen et al., 2014), and black bean aphids (*Aphis fabae* Scopoli) (Schmid et al., 2012) – though not against all species of parasitoid wasps (Cayetano & Vorburger, 2015).

Hamiltonella defensa is also the symbiont most heavily implicated in influencing aphid behaviour (Vorburger, 2018). Pea aphids infected with *H. defensa* exhibit a reduced frequency of defensive behaviours in the presence of a parasitoid (Dion et al., 2011). This could make infected aphids more vulnerable to other natural enemies if the change is not specific to parasite-based cues (Sochard et al., 2020). Exploration of the interaction of symbionts and cues from predators will be the focus of our study. Polin et al. (2014) demonstrated that pea aphids hosting *H. defensa* exhibited aggressive and evasive behaviour less frequently in the presence of ladybird predators and suffered higher predation. However, the consequences of *H. defensa* infection on the anti-predator behavioural responses of pea aphids feeding on live plants – which might represent a more naturalistic context in terms of, for example, physical structure, gravitational forces, and volatiles than the excised leaves used by Polin et al. (2014) – and pea aphids encountering predators with different foraging characteristics have not yet been tested, but will be the focus of our study.

It is also unknown whether the observed impact of *H. defensa* infection on the behavioural responses of aphids is due to the presence of the symbiont itself or its associated parasitism resistance. One means by which this could be explored is through the testing of aphid species that vary in their genotypic susceptibility to parasitism and/or for which *H. defensa* does not provide protection from parasitism. Clarke et al. (2017) suggested that there was little evidence that *H. defensa* provided strong protection from parasitism in the potato aphid, *Macrosiphum euphorbiae* (Thomas) (Hemiptera: Aphididae). Lines in which *H. defensa* occurred also did not appear to experience the fitness costs sometimes associated with *H. defensa* infection (Oliver et al., 2006; Simon et al., 2011; Vorburger & Gouskov, 2011) or parasitism resistance; in fact, they showed faster development, higher survival, and greater fecundity (Clarke et al., 2017). Whether *H. defensa* generally does not improve parasitism resistance in potato aphids, or whether this only applies to the particular strains typically found infecting potato aphids or the parasitoid species tested (*A. ervi*), has not been investigated. Further, the relative uses of various behavioural responses by potato aphids of parasitism-resistant or -susceptible genotypes with different *H. defensa* infection statuses during encounters with natural enemies have not been explored.

In this study we address three questions. (1) Can the effects of *H. defensa* infection on anti-predator defence be observed in pea aphids faced with predators on live plants? (2) Does symbiont infection differentially affect pea aphid behaviour against two predator types? And (3) is any predator-induced effect due to symbiont presence per se or parasitism resistance generally? This study exposed aphids to lacewing larvae – *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) – and ladybird adults – *Adalia bipunctata* (L.) (Coleoptera: Coccinellidae) – on live plants. Whereas ladybird adults forage vigorously on plants, with their relatively large body sizes and energetic searching behaviour, the larvae of lacewings and other predatory insects are much slower foragers (Brodsky & Barlow, 1986; Losey & Denno, 1998a); these predators were therefore expected to represent various predator 'types'.

To address research questions 1 and 2, two pea aphid lines were studied, both of the same genotype but one

naturally infected with *H. defensa* and the other cured of *H. defensa*. To address research question 3, four potato aphid lines were studied, differing in both *H. defensa* infection status and in genotype (parasitism-resistant or -susceptible, as suggested by previous laboratory tests). *Hamiltonella defensa* is not thought to confer strong protection against parasitism in potato aphids (Clarke et al., 2017) so, for example, if the responses of potato aphids of both genotypes (with their respective assumed parasitism resistance and susceptibility) were affected by this symbiont in a similar way to pea aphids this might indicate that the behavioural consequence is due to the symbiont's presence itself rather than general resistance to parasitism.

MATERIALS AND METHODS

Plants were grown from seed (faba bean; *Vicia faba* L. cv. The Sutton) or tubers (potato; *Solanum tuberosum* L. cv. Désirée) in compost (sand-perlite-peat mix containing N:P:K 17:10:15; William Sinclair Horticulture, Lincoln, UK) in a glasshouse with supplementary light, at L16(20 °C):D8(15 °C) photo-thermoperiod. Immature bean plants 2–4 weeks from planting and immature potato plants 2–3 weeks from planting were used in experiments.

Aphid lines were reared on excised leaf material in containers comprising one Perspex cup (5 × 15 cm) inside another; plant material was inserted through a ca. 5-mm-diameter circular hole in the base of the inner cup into ca. 1 cm deep water in the base of the outermost cup, and the cup surface was sealed with a mesh-ventilated lid. Containers were maintained at 18 ± 2 °C and L16:D8 photoperiod. Pea aphids (*A. pisum*) were cultured on leaves from 2–3-week-old faba bean plants; potato aphids (*M. euphorbiae*) on leaves from 3-week-old potato plants. Age-synchronized cohorts of third-fourth instars were produced daily for experiments; these were 6–7 days old for *A. pisum* and 8–9 days old for *M. euphorbiae*.

Ladybird adults (*A. bipunctata*) and lacewing larvae (*C. carnea*) were supplied by Dragonfli (Halstead, UK) and Ladybird Plantcare (Brighton, UK). Predators were kept in ventilated Perspex cages (35 × 45 × 90 cm) housed within a MicroClima growth chamber (Snijders Scientific, Tilburg, The Netherlands) maintained at L16(15 °C):D8(10 °C) photothermoperiod and 60–70% r.h. Dry paper towels provided non-slippery substrate and shelter, and predators were supplied daily with 2–3-week-old faba bean plants infested with an excess of pea aphids of a clonal line shown to be free of facultative bacterial endosymbionts (including *H. defensa*) and of a different genotype to experimental aphid lines. Ladybirds were additionally supplied with dilute sugar water-soaked cotton wool. Two days prior to assays, predators were transferred (along with pea aphid-infested leaf material) to ventilated containers and placed in the glasshouse where assays took place; predators were then isolated into paper-towel lined ventilated cups and starved for 24–32 h.

Pea aphid lines were supplied by Dr. Julia Ferrari (University of York, York, UK). They belonged to the same genotype, with one line possessing *H. defensa* (GT218-*H. defensa*) and one line having been artificially cured of *H. defensa* (GT218-cured; see Heyworth et al., 2020) (Table S1). Confirmatory genotyping and facultative endosymbiont detection were conducted following standard procedures (see Supporting Information, Tables S2, S3, S6, S8, S9).

Potato aphids were obtained from cultures at the James Hutton Institute (Dundee, UK). Two of the potato aphid lines had previously been found to be parasitism resistant (termed 'genotype 1'); of these, line RB15/11 was naturally infected with *H. defensa* when obtained from the wild whereas MW16/67 was not. The other two potato aphid lines belonged to a parasitism-susceptible genotype (genotype 2); of these, line AK14/01 was naturally infected with *H. defensa* when obtained from the wild whereas MW16/48 was not (Table S1). After the experiment was completed, routine checks of the stock lines cast some uncertainty on the proportion of aphids infected with *H. defensa* for the RB15/11 line (see Supporting Information, Table S4, S5, S7, S10, S11 for details of genotyping and symbiont screening checks conducted). We have analysed our data on the assumption that *H. defensa* infection rates were complete or high for the individuals used in our experiment (see Discussion).

Assays were conducted in a glasshouse with L16(20 °C):D8(14 °C) photo-thermoperiod. For each assay, a plant pot (15 cm diameter) with two plant seedlings was encased by a mesh net supported by a metal frame and infested with 16 wingless immature aphids, placed evenly across the two seedlings. Seedlings were trimmed pre-infestation to prevent contact with the mesh. Aphid-infested plants were left undisturbed overnight. Ten minutes before each assay, the pot was placed into a plastic tray (60 × 39 × 6 cm) and the frame and mesh net were removed. An Everio HDD GZ-MG330AE hard disk camcorder (JVC, Yokohama, Japan) fixed to a 49 × 135 cm TR-654 tripod (Ex-Pro, Reading, UK) was positioned to give a planar view of the pot.

Throughout trials, the experimenter wore a surgical mask to prevent plants or insects from being disturbed by air movement. All insects were transferred using a fine artist's paintbrush. Each assay started at either 09:00, 11:30, 14:00, or 16:30 hours. When filming began, the number of aphids remaining on plants was recorded using a telescopic inspection mirror (129472; Silverline, Yeovil, UK) to determine whether any aphids had escaped overnight and/or fallen onto the soil. A predator (ladybird adult or lacewing larva) was then added to the base of a randomly selected seedling.

For each assay, the predator was observed for 30 min, with predator movements and aphid anti-predator behaviours on each plant section (considered as stem, petiole, petiolule, upper leaf, or lower leaf) being recorded by dictating to the camera. Aphid behaviours were classified as: drop, walk, kick, shift, and no response. 'Drop' was where an aphid dropped from its current plant section,

usually to the ground. 'Walk' included behaviours ranging from backing-up a pace or two to running quickly away. 'Kick' described the quick motion/flicking of one or two legs in the direction of the predator. 'Shift' was defined as when aphids moved their body or antennae in response to the stimulus but did not take a step anywhere. 'No' was where the aphid continued to feed despite the predator having made contact.

If aphids departed from the pot by walking during the 30-min observation period, they were not replaced. If an aphid dropped off the side of the pot, it was replaced on the substrate at the edge of the pot. If a predator climbed the rim of the pot, it was moved to the base of the seedling it had visited least recently (or not at all). If the predator did not encounter an aphid for 5 min, it was also moved to the base of the least recently explored (or unexplored) seedling. If a predator successfully captured an aphid, it was given 10 min to consume its prey and continue moving before it was replaced entirely with a predator of the same species. If a predator was motionless for two uninterrupted minutes or (ladybird-specific) flew away, it was also replaced with a fresh predator of the same species. After any occasion where a predator needed to be replaced, the next predator was introduced to the least recently explored (or unexplored) seedling. The timing of the observation period was paused while predators were being replaced, and restarted when the new predator was on a seedling. Each predator was used only once. The observation period for each assay continued until 30 min had passed.

Pea aphid trials took place in May 2019. Each of the two lines were tested across eight trials (n = 8), and the predator type used in each trial was randomized (n = 2–6 for each line–predator combination). Potato aphid trials took place in July 2019. Each of the four aphid lines was tested across eight assays (n = 8), with predator type assigned equally but in a random order (n = 4 for each line–predator combination).

Evasive and aggressive defences made up the majority of recorded responses. Thus, the focal dependent variables (per trial) analysed for this study were: total number of defensive responses to predators, total number of evasive responses (dropping or backing-up/walking away), total number of aggressive responses (kicking), and proportion of the overall behavioural responses represented by evasive defences (see Supporting Information for details and results of other metrics). Generalized linear mixed models (GLMMs) were fitted using the 'lme4' package (Bates et al., 2015) in R v.3.6.3 (R Core Team, 2020), assuming either a Poisson (for count data) or binomial (for proportions) error, with log- or logit-link functions, respectively. For the pea aphid trials, the effect of aphid symbiont status (presence or absence of *H. defensa*) and predator type (lacewing larva or ladybird adult) on each dependent variable was tested, with time of day and date considered as potential random factors. For the potato aphid trials, aphid genotype (parasitism-resistant or -susceptible) was added to the model. Details of how the best-fitting models were selected are given in the Supporting Information.

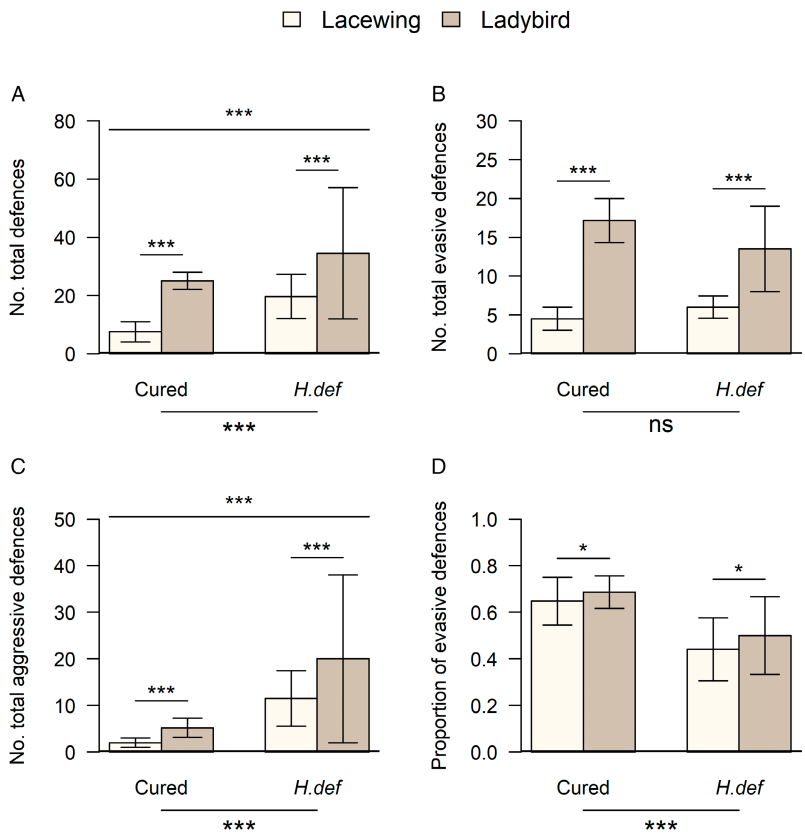

FIGURE 1 Mean (± SE) effect of *Hamiltonella defensa* infection status [cured or infected (*H.def*)] and predator type (lacewing larva or ladybird adult) on the behavioural responses of pea aphids (line GT218): (A) number of total counts of defences, (B) total counts of evasive defences, (C) total counts of aggressive defences, and (D) proportion of evasive defences relative to the total defences recorded. The horizontal lines at the top of panels A and C show the significance of the interaction between symbiont status and stimulus. Lines above paired bars in all panels show the significance of stimulus, and lines below panels show the significance of symbiont status (GLMM: ***P<0.001, *0.01<P<0.05; ns, P>0.05; n = 2–6 for each line–predator combination).

Final models for the results reported below were checked using the 'DHARMa' package function 'testResiduals' (Hartig, 2020) and found to have no issues with the distribution of residuals, outliers, or dispersion of data.

RESULTS

Can the effects of *Hamiltonella defensa* infection on anti-predator defence be observed in pea aphids faced with predators on live plants?

The *H. defensa*-infected pea aphid line exhibited a higher number of total defences to predators on faba bean plants than the uninfected (cured) line (Figure 1A, Table 1). Considering evasive responses alone (dropping and walking away/backing-up), symbiont status did not influence these responses [likelihood ratio test (LRT): $\chi^2 = 0.028$, d.f. = 1, P = 0.87; Figure 1B, Table 1), but the uninfected (cured) line utilized proportionately more evasive defences ($\chi^2 = 10.85$, d.f. = 1, P<0.001; Figure 1D, Table 1). The *H. defensa*-infected line showed higher numbers of aggressive defences (kicking) (Figure 1C, Table 1).

Does symbiont infection differentially affect pea aphid behaviour against two predator types?

Ladybirds provoked more total defensive responses than lacewings (Figure 1A, Table 1). Ladybirds also provoked more evasive responses (LRT: $\chi^2 = 23.159$, d.f. = 1, P<0.001; Figure 1B, Table 1) and aggressive responses (Figure 1C, Table 1), as well as proportionately more evasive responses ($\chi^2 = 4.138$, d.f. = 1, P = 0.042; Figure 1D, Table 1).

An interaction between symbiont status and predator type was a significant predictor of both the total counts of all responses (LRT: $\chi^2 = 30.469$, d.f. = 1, P<0.001; Figure 1A, Table 2) and the total counts of aggressive responses ($\chi^2 = 35.38$, d.f. = 1, P<0.001; Figure 1C, Table 2).

Only one pea aphid was captured by a predator. The aphid was from the uninfected (cured) line, and was predated by a ladybird after an attempted drop escape, which landed it only millimetres away onto a petiole directly below.

Are the observed effects in pea aphid due to symbiont presence per se or parasitism resistance generally?

Similar experiments as with pea aphid were performed with potato aphid, in which, reportedly, *H. defensa* does not provide strong protection from parasitism. Infection with *H. defensa*, potato aphid genotype (LRT: $\chi^2 = 53.683$, d.f. = 1, P<0.001), predator type (Table 3), and the interaction

TABLE 1 Summary table outputs from the best fit pea aphid GLMMs for the individual fixed effect terms included in all models: symbiont status (presence or absence of *Hamiltonella defensa*) and stimulus (lacewing larva or ladybird adult). Also shown for each dependent variable is the random effect(s) included in the best fit model and the estimate value produced for the intercept

Dependent variable (aphid behavioural response)	Random effects		Intercept	Symbiont status				Stimulus			
	Time	Date		Estimate	SE	z	P	Estimate	SE	z	P
Total defensive	Yes	Yes	1.480	1.949	0.372	5.238	<0.001	2.041	0.361	5.659	<0.001
Total evasive	Yes	Yes	1.745	−0.035	0.206	−0.168	0.87	1.052	0.225	4.671	<0.001
Total aggressive	Yes	Yes	−0.949	4.287	0.767	5.592	<0.001	3.036	0.736	4.122	<0.001
Proportion evasive	No	Yes	0.225	−1.049	0.310	−3.390	<0.001	0.651	0.322	2.022	0.043

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TABLE 2 Summary table outputs from the best fit pea aphid GLMMs for the interaction term included in some models: the interaction between symbiont status (presence or absence of *Hamiltonella defensa*) and stimulus (lacewing larva or ladybird adult)

Dependent variable (aphid behavioural response)	Symbiont status*stimulus			
	Estimate	SE	z	P
Total defensive	−3.378	0.668	−5.060	<0.001
Total evasive	n/a	n/a	n/a	n/a
Total aggressive	−6.664	1.352	−4.928	<0.001
Proportion evasive	n/a	n/a	n/a	n/a

n/a = interaction term not included in the best fit GLMM.

between symbiont status and predator type ($\chi^2 = 78.368$, d.f. = 1, P<0.001; Table 4) were all significant predictors of the total counts of all responses (Figure 2A). Across both parasitism-resistant (genotype 1) and parasitism-susceptible (genotype 2) genotypes, the lines infected with *H. defensa* (RB15/11 and AK14/01) showed greater differentiation than the uninfected lines (MW16/67 and MW16/48) in the number of responses they exhibited towards the two predator types, with ladybirds eliciting more responses than lacewings. Aphids from parasitism-resistant genotypes exhibited higher total numbers of responses than their symbiont status-equivalent susceptible lines when faced with the same predator type (Figure 2A).

These trends also held when the total counts of only evasive defences (dropping or backing-up/walking away) were considered (Figure 2B). Again, the significant predictor variables consisted of symbiont status, potato aphid genotype (LRT: $\chi^2 = 30.645$, d.f. = 1, P<0.001), predator type (Table 3), and the interaction between symbiont status and predator type ($\chi^2 = 17.587$, d.f. = 1, P<0.001; Table 4). The lines not infected with *H. defensa* showed significantly higher mean counts of evasive defences in response to ladybirds than lacewings (Figure 2B). However, looking at the proportion of the different lines' behavioural responses that constituted evasive defences (Figure 2D), symbiont status did not have an effect (LRT: $\chi^2 = 1.133$, d.f. = 1, P = 0.29). Predator type and aphid genotype (Table 3) were both significant predictors of the proportion of evasive defences utilized, though, as was the interaction between stimulus and genotype (LRT: $\chi^2 = 4.522$, d.f. = 1, P = 0.033; Table 4). The parasitism-susceptible genotype lines exhibited a slightly greater differentiation in the proportion of evasive responses used against the two predator types, but across all lines, the predator type was clearly the most important predictor; all lines used proportionately more evasive defences in response to ladybirds compared to lacewings (Figure 2D).

Considering the total counts of aggressive defences (kicking), infection with *H. defensa*, aphid genotype (LRT: $\chi^2 = 22.326$, d.f. = 1, P<0.001), predator type (Table 3), and the interaction between symbiont status and predator type ($\chi^2 = 78$, d.f. = 1, P<0.001; Table 4) were all significant predictors (Figure 2C). Both parasitism-resistant genotype lines appeared to differentiate the number of aggressive responses dependent on predator type, but with the line negative for *H. defensa* (MW16/67) exhibiting more kicks towards lacewings than ladybirds and the line harbouring *H. defensa* (RB15/11) exhibiting more kicks towards ladybirds than lacewings. The *H. defensa*-positive parasitism-susceptible genotype line (AK14/01) also appeared to differentiate the number of aggressive defences used against the two predator types, kicking more against ladybirds than lacewings, but the *H. defensa*-negative parasitism-susceptible genotype line (MW16/48) did not differ greatly in its mean number of kicks towards the two stimuli, although ladybirds provoked more kicks than lacewings (Figure 2C).

Seven potato aphids with the parasitism-resistant genotype were captured and consumed by predators, three of which did not harbour *H. defensa* (line MW16/67) and four of which did (line RB15/11). Additionally, 10 potato aphids belonging to the parasitism-susceptible genotype were captured by predators, five of which were infected with *H. defensa* (line AK14/01) and five of which were not (line MW16/48). Aphids from each line were captured by both predator types, and there was at least one example from each line where an aphid was captured ahead of showing any response to the predator.

DISCUSSION

Pea aphids infected with *H. defensa* exhibited proportionately fewer evasive behaviours (dropping and walking away) than cured aphids. This supports the finding of Polin et al. (2014) that pea aphids – for whom *H. defensa* infection can provide parasitism resistance (Oliver et al., 2003, 2006; Guay et al., 2009; Donald et al., 2016) – exhibit fewer evasive behaviours to ladybird predators. However, infected pea aphids also exhibited greater frequencies of all recorded defences and, specifically, aggressive kicking defence in response to ladybirds than cured pea aphids. This contrasts with previous findings of *H. defensa*-infected aphids exhibiting relatively fewer aggressive defences towards natural enemies (Dion et al., 2011; Polin et al., 2014). Whereas *H. defensa*, or the parasitism-resistance associated with it in pea aphids (Oliver et al., 2003, 2006; Guay et al., 2009; Donald et al., 2016), may reduce aphids' propensity to utilise particular behaviours, perhaps it does not cause an overall reduction in defence. Pea aphids harbouring *H. defensa* here

TABLE 3 Summary table outputs from the best fit potato aphid GLMM for the individual fixed effect terms included in all models: symbiont status (presence or absence of *Hamiltonella defensa*), genotype (parasitism resistant or susceptible), and stimulus (lacewing larva or ladybird adult). Also shown for each dependent variable is the random effect(s) included in the best fit model and the estimate value produced for the intercept

Dependent variable (aphid behavioural response)	Random effects		Intercept	Symbiont status				Genotype				Stimulus			
	Time	Date		Estimate	SE	z	P	Estimate	SE	z	P	Estimate	SE	z	P
Total defensive	Yes	Yes	2.023	1.200	0.190	6.314	<0.001	−0.686	0.094	−7.262	<0.001	1.899	0.204	9.293	<0.001
Total evasive	Yes	Yes	1.758	0.549	0.239	2.293	0.022	−0.652	0.120	−5.436	<0.001	1.443	0.245	5.891	<0.001
Total aggressive	Yes	Yes	−0.182	2.503	0.401	6.235	<0.001	−0.989	0.218	−4.531	<0.001	3.088	0.452	6.826	<0.001
Proportion evasive	Yes	No	−0.151	−0.202	0.190	−1.065	0.29	0.666	0.332	2.005	0.045	0.913	0.272	3.358	<0.001

showed a tendency to utilize fewer energetically costly defences relative to cured pea aphids, but still exhibited many defensive responses, including a greater tendency towards aggressive kicking. This finding could result from the more naturalistic set-up on live plants compared to the excised leaves of previous work (Dion et al., 2011; Polin et al., 2014), as well as the consideration of 'shifting' behaviour beyond evasive and aggressive activity alone.

Predators forage in characteristic ways, which presumably provide different cues to their prey and can consequently provoke different behavioural responses. In our study, ladybird adults provoked more evasive, aggressive, and total counts of behaviours than lacewing larvae. Ladybirds were observed to be much faster-moving and appeared to actively forage more than lacewing larvae. For pea aphids, then, perhaps utilizing a greater frequency of defensive behaviours (including dropping as the most energetically costly option) is necessary in order to avoid predation by more active predators.

Strains of *H. defensa* can vary in the strength of protection conferred to pea aphids against parasitoids (Chevignon et al., 2018). We did not test for parasitism resistance in the *H. defensa*-infected lines that we used. If resistance to parasitoids and behaviour are linked, reduced protection against parasitism could mean that the frequency of defensive behaviours exhibited by infected hosts (towards parasitoids, predators, or both) should, in turn, be expected to be less reduced. Protection from parasitism depends on the strain of *H. defensa* and the symbiotic consortium of the host (Leclair et al., 2016), as well as the attacking parasitoid species and pea aphid biotype (McLean & Godfray, 2015). Even in pea aphids it is possible to find strains that provide little or no protection from parasitism to their aphid hosts (McLean & Godfray, 2015; Leclair et al., 2016). Łukasik et al. (2013) found that infection with *H. defensa* did not reduce the susceptibility of the grain aphid *Sitobion avenae* (Fabricius) to two species of parasitoids, although parasitoid females did appear to preferentially oviposit into uninfected hosts. More recently, and most relevant to this current study, Sochard et al. (2020) assessed the effects of various strains of *H. defensa* on both parasitism resistance and defensive behaviours against parasitoids in pea aphids. Strains of *H. defensa* provided various levels of protection against parasitism and varied in their effects on aphid behaviours. Whereas some strains reduced all considered behavioural defences, consistent with earlier studies (Dion et al., 2011; Polin et al., 2014), other strains reduced aggressiveness only if they protected their hosts completely against parasitoids. Reduced frequencies of aphid defensive behaviours were not related to the level of resistance conferred by the secondary symbionts (Sochard et al., 2020). Sochard et al. (2020) suggested that, given that symbiont effects on defensive behaviours and resistance to parasitism were not linked, the reduction of behaviours in infected aphids could simply be a by-product of the infection. Further exploration of this would be valuable.

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TABLE 4 Summary table outputs from the best fit potato aphid GLMM for the interaction terms included in some models: the interaction between symbiont status (presence or absence of *Hamiltonella defensa*) and stimulus (lacewing larva or ladybird adult), and the interaction between genotype (parasitism resistant or susceptible) and stimulus

Dependent variable (aphid behavioural response)	Symbiont status*stimulus				Genotype*stimulus			
	Estimate	SE	z	P	Estimate	SE	z	P
Total defensive	−2.413	0.268	−9.004	<0.001	n/a	n/a	n/a	n/a
Total evasive	−1.384	0.342	−4.048	<0.001	n/a	n/a	n/a	n/a
Total aggressive	−4.776	0.609	−7.839	<0.001	n/a	n/a	n/a	n/a
Proportion evasive	n/a	n/a	n/a	n/a	−0.844	0.398	−2.119	0.034

n/a = interaction term not included in the best fit GLMM.

In light of the work by Sochard et al. (2020), it is possible that our results regarding infected pea aphids' use of various behaviours and the relative susceptibility to predation differ from earlier literature (Dion et al., 2011; Polin et al., 2014) because of some variation in the pea aphid biotypes tested or strains of *H. defensa* present. Important to reiterate is the fact that we did not assess the level (or specificity) of parasitism-resistance that the strain of *H. defensa* provided our infected pea aphids with, nor whether this is related to the pea aphid biotype, meaning that we could not confidently suggest that the *H. defensa* here would definitely act as a 'protective symbiont' against parasitoids. If the symbiont strain in our study offered little or no protection from parasitism, then infected aphids would not be expected to exhibit reduced defences unless such a reduction was merely a by-product of infection. Whether the symbiont strain offered some protection against parasitism or not, infected pea aphids in this study showed a reduced use of evasive responses, but this did not appear to increase susceptibility to predation – in contrast to previous work (Polin et al., 2014). This is unlikely to be because the mechanism of protection against parasitism is the same as the mechanism of protection against predation (if *H. defensa* did indeed act as a 'protective symbiont' against parasitoids). It might, instead, be that the strain of *H. defensa* in our infected pea aphid line reduced dropping behaviour but not aggressiveness, enabling aphids to still defend themselves sufficiently. However, our study had a relatively small sample size and a short timescale, so it is possible that bigger-scale or longer-term effects of *H. defensa* on aphids' abilities to evade predation were not detected. Sochard et al. (2020) argue that conferring some protection against parasitism should not be enough for a symbiont to be labelled 'protective', rather it should "[limit] the development of the enemy without reducing its host's fitness drastically."

The full effects of symbionts on host fitness and behaviours during encounters with all their common natural enemies must be considered before its overall benefits and costs to its host can be determined. *Hamiltonella defensa* infection also has detrimental effects on host fitness in pea aphids by reducing host survival and reproductive success (Oliver et al., 2006; Simon et al., 2011; Vorburger & Gouskov, 2011).

One component that also could be valuable in understanding the role of *H. defensa* infection in its hosts is identifying whether any effects it appears to have on behaviour in other aphid species also are due to its presence alone or more generally due to the parasitism resistance it confers. To contribute to this, our study tested potato aphids, a species for which there has been little evidence for *H. defensa* providing strong protection from parasitism; instead, parasitism resistance is associated with aphid genotype (Clarke et al., 2017). However, following subsequent checks on the potato aphid lines, we have reason to think that an unknown fraction of the RB15/11 aphids used in this experiment may not have been infected with *H. defensa*, and so the following results regarding infection status are conservative, as we implicitly assumed that all individuals of this line used in our experiment were infected.

Hamiltonella defensa infection status, predator type, and aphid genotype all appeared to influence potato aphid defences, with a possible interaction between infection status and predator type having a significant influence on some behavioural components of potato aphids' anti-predator repertoire. Symbiont-infected potato aphids showed greater differentiation in the frequency of behaviours (particularly evasive behaviours). As *H. defensa* does not provide strong protection to potato aphids from parasitism (Clarke et al., 2017), these findings indicate that the presence of the symbiont itself, rather than any influence it may have on parasitism resistance, may be a key determinant of defensive behaviours in aphids. This supports the suggestion of Sochard et al. (2020) that changes in aphid behaviour as a result of infection by *H. defensa* are by-products of the infection, and that they are not linked with the parasitism resistance sometimes associated with the symbiont in pea aphids. However, we cannot be confident that all of the RB15/11-line potato aphids used in this experiment were infected with *H. defensa*, and parasitism resistance was not tested for any of the lines, so these findings require strengthening with further testing. It is also worth noting that predator type had the most significant effect when the proportion rather than the frequency of evasive behaviours was considered; as with the pea aphids, ladybird adults elicited a higher proportion of dropping and walking away. This again supports the idea

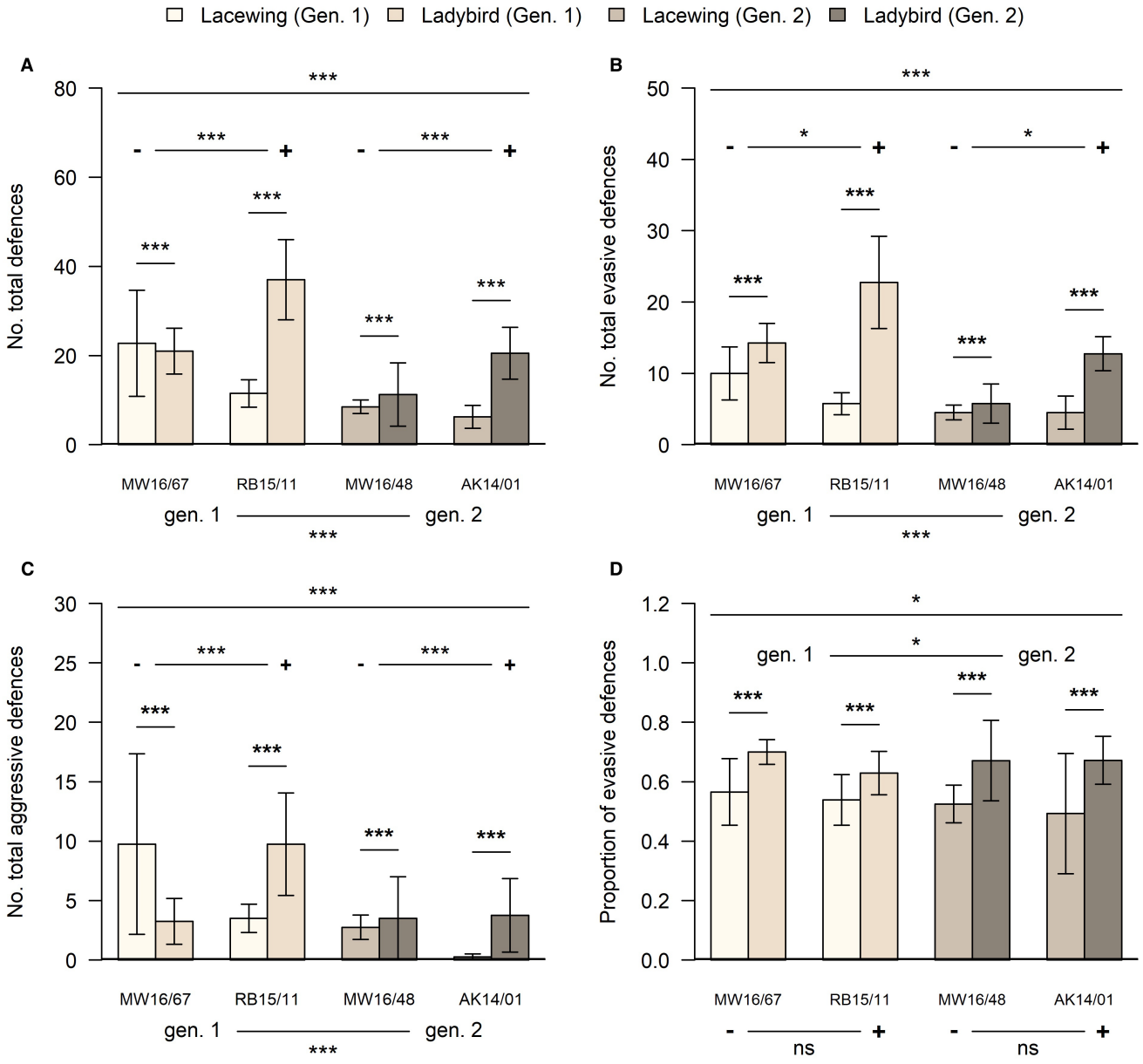

FIGURE 2 Mean (± SE) effect of *Hamiltonella defensa* infection status (–, uninfected; +, infected), genotype (gen. 1, parasitism resistant; gen. 2, parasitism susceptible), and predator type (lacewing larva or ladybird adult) on the behavioural responses of potato aphids (lines MW16/67, RB15/11, MW16/48, and AK14/01). (A) Number of total counts of defences, (B) total counts of evasive defences, (C) total counts of aggressive defences, and (D) proportion of evasive defences relative to the total defences recorded. The horizontal lines above paired bars in all panels show the significance of stimulus. Horizontal lines with - and + symbols show the significance of symbiont status, and horizontal lines with ends labelled 'gen. 1' and 'gen. 2' show the significance of aphid genotype. In panels A-C, the horizontal lines at the top represent the significance of the interaction between symbiont status and stimulus; in panel D, this line indicates an interaction between genotype and stimulus (GLMM: ***P<0.001, *0.01<P<0.05; ns, P>0.05; n = 4 for each line–predator combination).

that predator types have characteristic foraging styles, which are responded to differently by aphids (Brodsky & Barlow, 1986; Losey & Denno, 1998a).

Given the significant economic impact aphids can have on agricultural production (Dedryver et al., 2010), increasing understanding of the factors that affect their behaviours and subsequent fitness is important as a guide for effective management strategies. In particular, defensive symbioses are of interest where they may compromise the effectiveness of biological control with parasitoids (Oliver et al., 2005; Vorburger, 2018), and here understanding how such symbioses affect interactions with other aphid natural enemies (i.e., predators) could be invaluable when devising effective biocontrol strategies. By exploring the influence of *H. defensa* infection on the anti-predator defensive behaviours of two major pest species of aphid, this study aimed to contribute to current understanding with regards to the association of pea aphids with *H. defensa* and to offer novel findings about the behaviours seen in potato aphid lines with and

without the symbiont. Both aphid species tested here differentiated their defensive behaviours depending on the type of predator they encountered. The frequency and types of responses a predator elicits can have an indirect influence on a prey individual's survival, leading to knock-on consequences for pest populations; this is particularly true of costly defences such as dropping. Facing aphids with predators enables observation of aphid anti-predator behaviours that can be considered important non-consumptive effects of natural enemies (Nelson & Rosenheim, 2006). Studies that adopt such methodologies will continue to be of great use in developing biological pest control and informing selection of the most effective natural enemies.

AUTHOR CONTRIBUTIONS

Rosalind Kay Humphreys: Conceptualization (equal); data curation (lead); formal analysis (lead); investigation (lead); methodology (lead); project administration (lead); visualization (lead); writing – original draft (lead); writing – review and editing (equal). Graeme Douglas Ruxton: Conceptualization (equal); methodology (supporting); supervision (lead); writing – review and editing (supporting). Ali Karley: Conceptualization (equal); methodology (supporting); supervision (supporting); writing – review and editing (equal).

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