

Cascading effects of herbivore protective symbionts on hyperparasitoids

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Abstract. 1. Microbial symbionts can play an important role in defending their insect hosts against natural enemies. However, researchers have little idea how the presence of such protective symbionts impacts food web interactions and species diversity.

2. This study investigated the effects of a protective symbiont (*Hamiltonella defensa*) in pea aphids (*Acyrtosiphon pisum*) on hyperparasitoids, which are a trophic level above the natural enemy target of the symbiont (primary parasitoids).

3. Pea aphids, with and without their natural infections of *H. defensa*, were exposed first to a primary parasitoid against which the symbiont provides partial protection (either *Aphidius ervi* or *Aphelinus abdominalis*), and second to a hyperparasitoid known to attack the primary parasitoid species.

4. It was found that hyperparasitoid hatch rate was substantially affected by the presence of the symbiont. This effect appears to be entirely due to the removal of potential hosts by the action of the symbiont: there was no additional benefit or cost experienced by the hyperparasitoids in response to symbiont presence. The results were similar across the two different aphid–parasitoid–hyperparasitoid interactions we studied.

5. It is concluded that protective symbionts can have an important cascading effect on multiple trophic levels by altering the success of natural enemies, but that there is no evidence for more complex interactions. These findings demonstrate that the potential influence of protective symbionts on the wider community should be considered in future food web studies.

Key words. Aphid, hyperparasitoid, parasitoid, symbiont, symbiosis, trophic cascade.

Introduction

Symbiotic associations with bacteria or fungi can allow animals to exploit otherwise inaccessible niches (Douglas, 2011; McFall-Ngai *et al.*, 2013). Microbial symbiosis is particularly important in insects where it can allow the exploitation of otherwise nutrient-inadequate diets (Douglas, 2006, 2009) or lead to improved tolerance of heat shock and other abiotic challenges (Montllor *et al.*, 2002). Different bacterial symbionts have also been found to reduce insect mortality from fungal pathogens (Kaltenpoth *et al.*, 2005; Scarborough *et al.*, 2005; Łukasik *et al.*, 2013), parasitic nematodes (Jaenike *et al.*, 2010), parasitoids

(Oliver *et al.*, 2005; Xie *et al.*, 2010) and predators (Kellner, 1999; Lopanik *et al.*, 2004). Acquisition of defensive capabilities through infection with a novel symbiont – perhaps from a ‘horizontal gene pool’ of potential symbionts with different defensive capabilities (Jaenike, 2012; Henry *et al.*, 2013) – is likely to have consequences for the wider food web within which the insect is embedded through the direct and indirect effects of reducing the incidences of specific natural enemies. Protective symbionts could potentially alter community diversity and food web structure, but we currently have little idea of the magnitude or importance of these potential effects (McLean *et al.*, 2016).

A food web cascade occurs when a perturbation at one trophic level leads to further changes at multiple trophic levels either above or below the one initially affected. Food web cascades have been reported in a variety of contexts and at different spatial

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scales (Pace *et al.*, 1999). In communities comprising plants, insect herbivores and insect predators, both bottom-up cascades (e.g. Härrä *et al.*, 2008; Hartley *et al.*, 2015) and top-down cascades (e.g. Dyer & Letourneau, 1999; Schmidt *et al.*, 2003) have been observed. Perturbations in food webs can also indirectly affect species in the same food web. A good example of this is provided by experiments in mesocosms perturbing a stable community of multiple aphid species feeding on a single host plant species but each with a specialist parasitoid (Sanders *et al.*, 2013, 2015). Following removal of one parasitoid species, its prey species was able to increase in numbers and outcompete the other aphid species present, leading to secondary extinctions among the parasitoids.

Aphids provide an excellent experimental model to explore how microbial symbiosis affects food web ecology (Rothacher *et al.*, 2016) because their protective symbionts (Oliver *et al.*, 2014) and their natural enemy complex (van Veen *et al.*, 2006) have been well characterised. Aphids are attacked by a broad suite of natural enemies, including fungal pathogens and hymenopteran parasitoids. Quantitative food webs describing these interactions have been constructed (e.g. Müller *et al.*, 1999; van Veen *et al.*, 2008; Lohaus *et al.*, 2013) and studied using manipulation experiments in the field (e.g. Morris *et al.*, 2001; Bukovinszky *et al.*, 2008; Harmon *et al.*, 2009). In terms of the symbionts, almost all aphids host the obligate nutritional symbiont *Buchnera aphidicola*, while a variety of facultative symbionts are present in different species. Five facultative symbiont species have been shown to provide protection against aphid-specific fungal pathogens (Scarborough *et al.*, 2005; Lukasik *et al.*, 2013; Parker *et al.*, 2013; Heyworth & Ferrari, 2015), while four increase resistance to hymenopteran parasitoids (Oliver *et al.*, 2003; Vorburger *et al.*, 2010; Heyworth & Ferrari, 2015). Of the species providing protection against parasitoids, *Hamiltonella defensa* is the best studied (Oliver *et al.*, 2010, 2014). It has been shown that the resistance requires the presence of a bacteriophage (Oliver *et al.*, 2009) (Weldon *et al.*, 2013) that carries genes for putative eukaryote toxins which are presumed to act against the developing parasitoid (Degnan & Moran, 2008a, 2008b). *Hamiltonella defensa* strains vary considerably in the protection they provide (Moran *et al.*, 2005), and there is evidence of both parasitoid-species and parasitoid-genotype specificity (Rouchet & Vorburger, 2012; Asplen *et al.*, 2014; McLean & Godfray, 2015). A recent population cage experiment using a model aphid–parasitoid food web demonstrated that the presence or absence of *H. defensa* in one of the aphid species can affect community stability and dynamics (Rothacher *et al.*, 2016; Sanders *et al.*, 2016).

Aphid parasitoids are themselves hosts for a considerable number of secondary parasitoids (hyperparasitoids). These can be broadly divided into ‘true hyperparasitoids’ (koinobionts that develop on the primary parasitoid while it is inside the living aphid) and ‘mummy parasitoids’ (idiobionts that attack their host after the death of the aphid host, while the primary parasitoid is pupating) (Sullivan & Völkl, 1999). Hyperparasitoids form an important component of aphid food webs, often occurring at high prevalences (Müller *et al.*, 1999; Morris *et al.*, 2001). Given the negative effects that aphid protective symbionts can exert on primary parasitoids (Schmid *et al.*, 2012), the

presence of symbionts may lead to a trophic cascade affecting secondary parasitoids. Bottom-up cascades have been demonstrated in another insect herbivore system where secondary parasitoid abundance was influenced by changes in food plant quality three trophic levels below (Harvey *et al.*, 2003). However, we are unaware of any studies that have investigated whether and how protective symbionts affect multiple trophic levels above that of their host species.

Here we examine the potential effects of aphid protective symbionts on secondary parasitoids in two food chains. We identified three ways in which protective symbionts could impact on hyperparasitoids (Fig. 1). First, the presence of symbionts could reduce the number of potential primary parasitoids, leading to a decrease in the absolute numbers of hyperparasitoids per aphid present (Fig. 1a,b). Second, the interaction between hyperparasitoid and primary parasitoid inside the aphid could be affected. This may favour the hyperparasitoid if the presence of the protective symbiont weakens the primary parasitoid and makes it less able to defend itself from attack (Fig. 1c). Alternatively, the defensive symbiont could have a direct or indirect negative effect on the hyperparasitoid (Fig. 1d).

We tested these hypotheses using two pea aphid (*Acyrthosiphon pisum*) clones, each of which hosted a specific strain of *H. defensa* that provided protection against a different species of primary parasitoid. For each clone we generated uninfected aphid lines using antibiotics. Aphids from lines with and without symbionts were exposed to a primary parasitoid female, and then either allowed to develop without further intervention or exposed to a hyperparasitoid female of an appropriate species 3 days later. We recorded aphid survival, parasitism rates and the hatch rates for the primary and secondary parasitoids. This allowed us to investigate whether protective symbionts: (i) alter hyperparasitoid hatch rates indirectly; (ii) modify hyperparasitoid interactions with their primary parasitoid host; and (iii) have direct negative effects on the hyperparasitoid.

Materials and methods

Experimental organisms

We used two different clonal lines of pea aphids, both founded from single individuals collected from the field in the south of England, U.K. Clone 132 was collected from *Lotus pedunculatus* in 2003 while clone 302 was collected from *Medicago sativa* in 2012. Both aphids were found to carry the symbiont *H. defensa* using diagnostic polymerase chain reaction (Henry *et al.*, 2013). Clone 302 also carried the symbiont known as ‘X-type’, and no other known secondary symbionts were present (Henry *et al.*, 2013). Lines without *H. defensa* (designated C132 and C302) were established by oral administration of antibiotics which do not affect the primary symbiont *Buchnera* (cefotaxime, gentamicin and ampicillin; Douglas *et al.*, 2006), following the protocols laid out in McLean *et al.* (2011) and McLean and Godfray (2015). The ‘cured’ lines were established more than 10 generations before the experiments were carried out. In the case of clone 302, the X-type infection remained in the cured lines, and thus experimental comparisons are made between

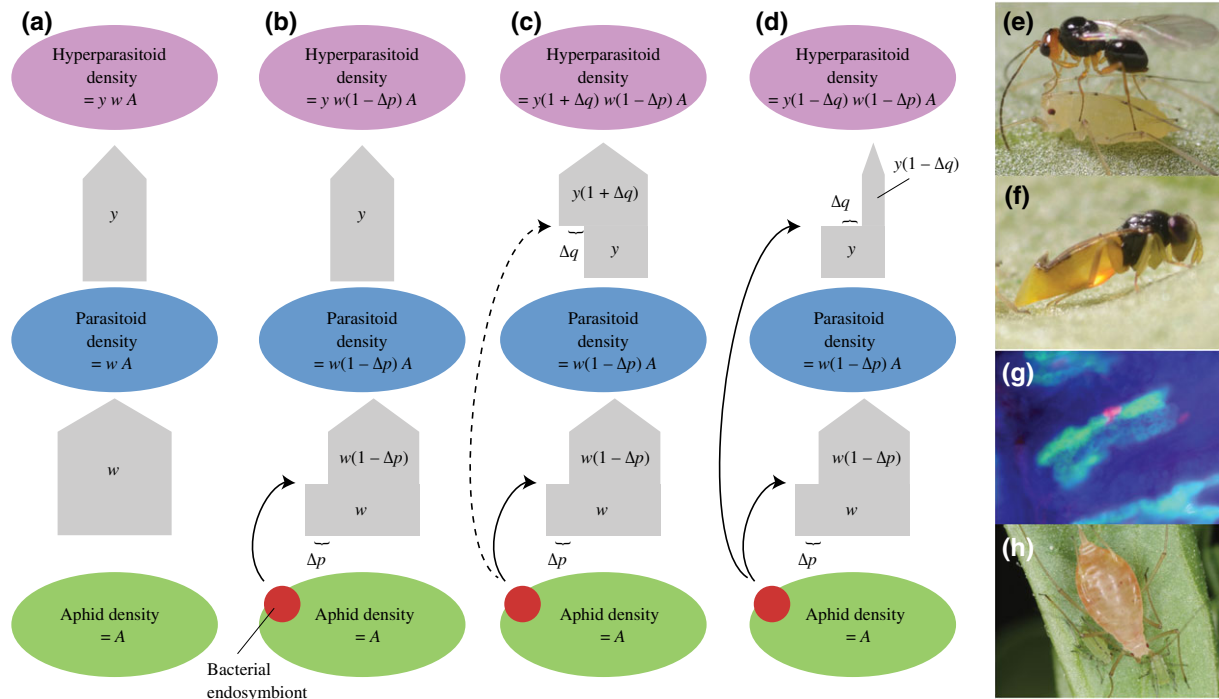


Fig. 1. Potential effects of protective symbionts on hyperparasitoids. (a) In the absence of symbionts an aphid is attacked by primary parasitoids at rate w , and the primary parasitoid is attacked by hyperparasitoids at rate y . (b) A protective symbiont reduces the success rate of primary parasitoids by a proportion p (as a result there are fewer primary parasitoids available for hyperparasitoids). (c) A protective symbiont decreases primary parasitoid fitness and thus reduces its ability to resist hyperparasitoids, increasing the rate of success for the hyperparasitoid by a proportion q . (d) Protective symbionts have an additional negative effect on hyperparasitoid survival, decreasing the rate of success by a proportion q . This effect could be direct (e.g. via toxins produced) or indirect (e.g. because size of surviving primary parasitoids is insufficient to support hyperparasitoid development). The players in the food web studied here were: (e) hyperparasitoids in the genus *Alloxysta* (in this case, *Alloxysta victrix*); (f) primary parasitoids of aphids (in this case, *Aphelinus abdominalis*); (g) the protective bacterial symbiont *Hamiltonella defensa* shown in a developing aphid embryo: bacteriocytes containing *Buchnera* are coloured in green, *H. defensa* are coloured pink; and (h) aphids *Acyrthosiphon pisum*. Photographs: Jan Hrček (e, f); Melanie Smee (g); Kevin Foster (h). [Colour figure can be viewed at wileyonlinelibrary.com].

individuals with and without *H. defensa* in the presence of the X-type. We knew from previous work that the *H. defensa* strain carried by clone 132 confers near-perfect resistance to *Aphelinus abdominalis* Dalman (Hymenoptera, Aphelinidae) and partial protection against *Aphidius ervi* Haliday (Hymenoptera, Braconidae), while that in clone 302 provides a high level of resistance to *A. ervi* and partial protection against *A. abdominalis* (McLean & Godfray, 2015). Aphids were routinely maintained at 14 °C and LD 16:8 h in 9 cm Petri dishes containing a single leaf of *Vicia faba* with the petiole inserted in 2% agar gel, and were transferred to a fresh dish once a week. All experiments were carried out at 20 °C and aphids were moved to this temperature one generation beforehand.

The primary parasitoids *A. abdominalis* and *A. ervi* were maintained as inbred stocks in the laboratory at 20 °C and LD 16:8 h (for over 5 years in the case of *A. ervi* and for over a year for *A. abdominalis*). The wasps were reared exclusively on a susceptible pea aphid stock clone different from those used in our experiments and which does not possess any described facultative endosymbionts. Two solitary hyperparasitoid species [*Alloxysta victrix* Westwood (Hymenoptera, Figitidae) and a second *Alloxysta* species (hereafter '*Alloxysta* sp. 2') identified

to be near *Alloxysta brevis* Thomson (Ferrer-Suay *et al.*, 2013)] (Hymenoptera, Figitidae) were collected from pea aphids in the field in southern England during summer 2013, and established as breeding populations in the laboratory on the primary parasitoids *A. ervi* (*A. victrix*) and *A. abdominalis* (*Alloxysta* sp. 2). These cultures were established six or more generations before the parasitoids were used in the experiments.

Before use in experiments, individual wasps from both species of primary parasitoids were exposed to susceptible stock aphids to allow oviposition experience. The hyperparasitoid species were both given experience of pre-parasitised aphids containing their preferred primary host. All wasps were provided with a 1:6 honey:water solution while searching for hosts. Primary parasitoids used in the experiments were virgins (thus ensuring the primary hosts were all male and so as uniform as possible between treatments); secondary parasitoid females were provided with males and were assumed to be mated.

Effects of *Hamiltonella defensa* on *Alloxysta victrix*

Parasitised aphids from the two lines of clone 132 that carried (132) or did not carry (C132) the symbiont were obtained by

exposing 14 third-instar aphids, placed on a *V. faba* leaf in a 9 cm Petri dish, to one female *A. ervi* wasp for 2 h. The parasitised aphids were then transferred to fresh *V. faba* leaves in new dishes. Aphids were distributed amongst the new dishes such that no individual primary parasitoid treatment contributed more than two aphids per dish, to give a total of eight to 10 aphids per dish. Six dishes from each aphid line were set aside as controls to assess levels of primary parasitism (these contained eight to 10 aphids). Seventy-two hours after primary parasitism, female *A. victrix* hyperparasitoids were placed in each of the remaining 14 dishes (each containing exactly 10 aphids) and allowed to oviposit for 4 h. After exposure, aphids were maintained in groups of five or fewer on *V. faba* leaves that were refreshed every 3 days (when any dead aphids were recorded) until 11 days after primary parasitism. At this point, the number of parasitoid mummies, live aphids and dead aphids were counted, and the mummies transferred to 'size 0' gelatine capsules until adult wasps emerged (around 20 days after secondary parasitism).

Effects of *Hamiltonella defensa* on *Alloxysta* sp. 2

The procedures were the same as those described in the last section except as follows. To produce primary-parasitised aphids, 18 third-instar aphids of clone 302 (with or without symbiont) were placed on a *V. faba* leaf in a 9 cm Petri dish and allowed 2–4 h to settle and begin feeding. One female *A. abdominalis* was then introduced for 24 h. *Aphelinus abdominalis* uses some hosts as food ('host-feeding') and these aphids always die shortly afterwards (Bai & Mackauer, 1990); host-fed aphids can be identified (such aphids either have visible injury with haemolymph escaping, or are shrivelled due to the loss of haemolymph) and were discarded before exposure to hyperparasitoids. Aphids were redistributed as described for *A. victrix*, with every dish containing 10 aphids. Seventy-two hours after *A. abdominalis* exposure, female *Alloxysta* sp. 2 were introduced to the hyperparasitism treatment dishes to oviposit for 24 h (controls without hyperparasitoid exposure were also maintained). Longer exposure times were used for these wasps because pilot studies had shown they required more time to achieve similar levels of parasitism. This experiment was carried out over three temporal blocks.

Statistical analysis

For each aphid exposed to hyperparasitoids, there are five potential outcomes: the aphid could: (i) survive; (ii) hatch into a primary parasitoid; (iii) hatch into a hyperparasitoid or die of other, unknown causes; (iv) before mummification; or (v) after mummification. We looked at the effects of symbionts on the proportion of aphids experiencing these different outcomes, comparing aphids that were exposed to hyperparasitoids with controls where hyperparasitoids were not present. We asked: (i) what proportion of all exposed aphids survived (a measure of aphid fitness); (ii) what proportion of all exposed aphids produced hatched primary parasitoids (a measure of primary parasitoid fitness); (iii) what proportion of all pupating parasitoids hatched (a measure of fitness costs of symbionts to

later parasitoid development; Schmid *et al.*, 2012); and (iv) what proportion of all hatching wasps were hyperparasitoids (a measure of hyperparasitoid fitness). All data were analysed using generalised linear modelling implemented in R v 3.0.2 (R Development Core Team, 2013), using a quasi-binomial error distribution to account for overdispersion in the data, with temporal block entered into the model (where relevant) before the explanatory factors (symbiont and hyperparasitoid presence/absence) and their interaction term. The full model was fitted in all cases. *Post hoc* testing using Tukey's honest significant differences (HSD) was performed with the 'multcomp' R package (Hothorn *et al.*, 2008).

Results

Effects of *Hamiltonella defensa* on *Alloxysta victrix*

The number of aphids surviving to the end of the experiment was low (~20%) in all treatments. Overall aphid survival was not improved by the presence of the symbiont ($F_{1,24} = 0.660$, $P = 0.425$; Fig. 2a), or affected by hyperparasitoid attack ($F_{1,23} = 0.028$, $P = 0.869$, Fig. 2a). However, the proportion of aphids from which primary parasitoids emerged was significantly lower in the presence of the symbiont ($F_{1,24} = 13.702$, $P = 0.001$), and when attacked by hyperparasitoids ($F_{1,23} = 10.666$, $P = 0.004$; Fig. 2b). The interaction between symbionts and hyperparasitoids is not significant ($F_{1,22} = 1.855$, $P = 0.187$; Fig. 2b), but a *post hoc* comparison of means (using Tukey's HSD adjustments) shows that symbiont-free aphids not exposed to hyperparasitoids produce significantly more primary parasitoids than any of the other treatment combinations (Fig. 2b, Table S1).

Developing in the presence of the symbiont increased the overall combined mortality rate of wasps during the mummy stage (i.e. the proportion mummies which failed to hatch into either primary parasitoid or hyperparasitoid; $F_{1,24} = 19.634$, $P < 0.001$; Fig. 2c), although mummies in the hyperparasitoid treatment were slightly more likely to hatch (producing either a primary parasitoid or hyperparasitoid wasp) ($F_{1,23} = 7.381$, $P = 0.012$). The proportion of hatching wasps that were hyperparasitoids was unaffected by the presence of the symbiont ($F_{1,21} = 0.043$, $P = 0.839$; Fig. 2d). A full breakdown of the values for different parasitism outcomes and results of the statistical analysis is given in Tables S2 and S3.

Effects of *Hamiltonella defensa* on *Alloxysta* sp. 2

The presence of *H. defensa* significantly improved aphid survival in the presence or absence of the hyperparasitoids ($F_{1,51} = 35.167$, $P < 0.001$). There was no main effect of hyperparasitoid attack on aphid survival ($F_{1,50} = 1.509$, $P = 0.225$) and no significant interaction between the symbiont status and hyperparasitoid treatment (interaction term: $F_{1,49} = 1.956$, $P = 0.168$) (Fig. 3a). Primary parasitoid success was reduced by both symbiont presence ($F_{1,51} = 9.856$, $P = 0.003$) and hyperparasitoids ($F_{1,50} = 11.196$, $P = 0.002$) (Fig. 3b); again, the symbiont-free aphids without hyperparasitoids produced

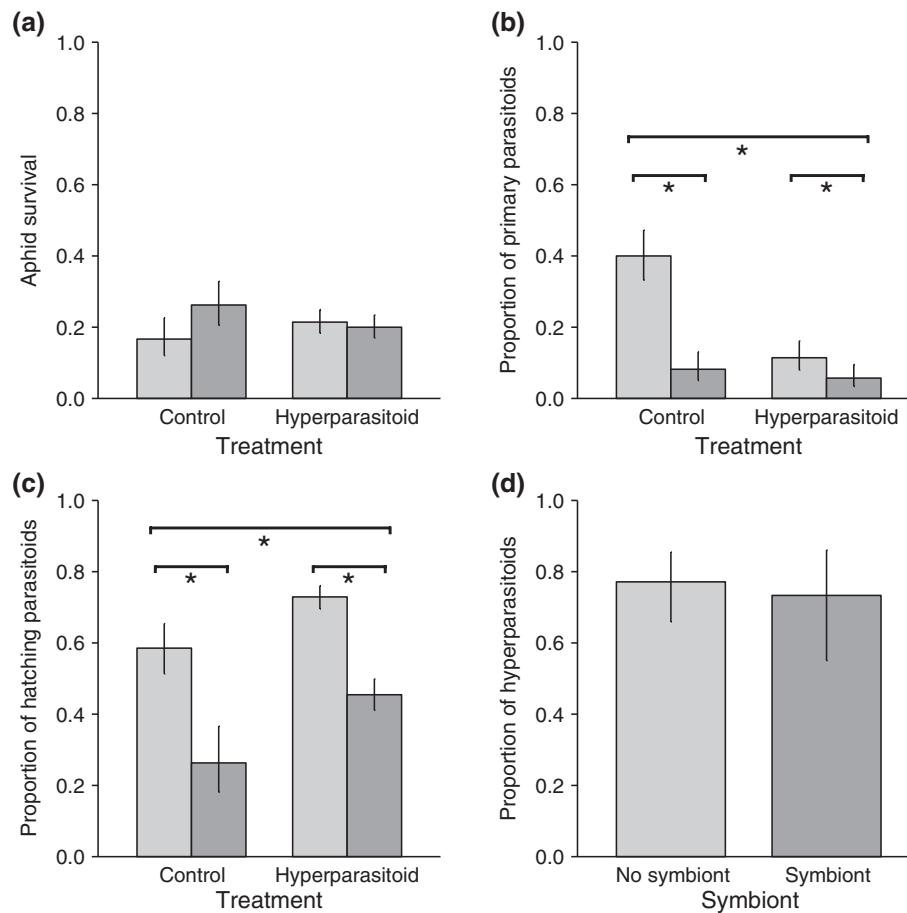


Fig. 2. Effects of *Hamiltonella defensa* on *Aphidius ervi* in the presence and absence of the hyperparasitoid *Alloxysta victrix*. (a) Proportion aphids surviving; (b) proportion of potential aphid hosts producing primary parasitoids; (c) proportion of mummies hatching; (d) proportion of hatching wasps that were hyperparasitoids. Dark bars represent aphids with *H. defensa*; light bars represent aphids lacking *H. defensa*. Error bars denote standard error of the mean and asterisks indicate significant differences between values, where relevant (other differences not significant).

more primary parasitoids than any of the other treatments (comparisons using Tukey's HSD adjustment), driving this effect (Table S4).

We found no effect of the presence of the symbiont ($F_{1,51} = 0.583$, $P = 0.449$) or the hyperparasitoid ($F_{1,50} = 1.022$, $P = 0.317$) on the probability that a wasp of either species emerged from a mummy, indicating that for these parasitoids the effects of the symbiont on parasitoid survival occurred pre- rather than post-pupation (Fig. 3c). The proportion of hyperparasitoids amongst wasps emerging from mummies was not influenced by the presence of the symbiont ($F_{1,23} = 0.070$, $P = 0.794$, Fig. 3d). A full breakdown of the values for different parasitism outcomes and results of statistical analysis is given in Tables S5 and S6.

Discussion

We investigated whether the protective effects of a defensive facultative symbiont in pea aphids can impact the hyperparasitoids two trophic levels above them, using two species of primary parasitoids and two species of hyperparasitoids. We found that

both the protective symbiont and hyperparasitoids reduce the numbers of hatching primary parasitoids. However, symbionts did not affect the outcome of the interaction between primary parasitoid and hyperparasitoid: the proportions of secondary wasps hatching were identical from aphids with and without symbionts (Figs 2d and 3d). We therefore conclude that the presence of symbionts affects hyperparasitoids by reducing the number of potential primary parasitoid hosts available (Fig. 1b), but not by affecting the hyperparasitoid directly or by altering the interaction between the hyperparasitoid and its host (Fig. 1c,d).

There are some notable differences between the two food chains we investigated, relating to the timing of aphid mortality and the influence of symbionts. For the *A. abdominalis*–*A. brevis*-complex food chain, we found that aphid survival was significantly improved by the presence of *H. defensa* (Fig. 3). Once a primary parasitoid had successfully pupated, there was no difference in mummy-stage mortality between treatments with and without symbionts, or with and without hyperparasitoids. By contrast, we did not find the symbiont to provide an overall survival benefit to the aphid for the *A. ervi*–*A. victrix* food chain. This was mostly due to considerable aphid mortality

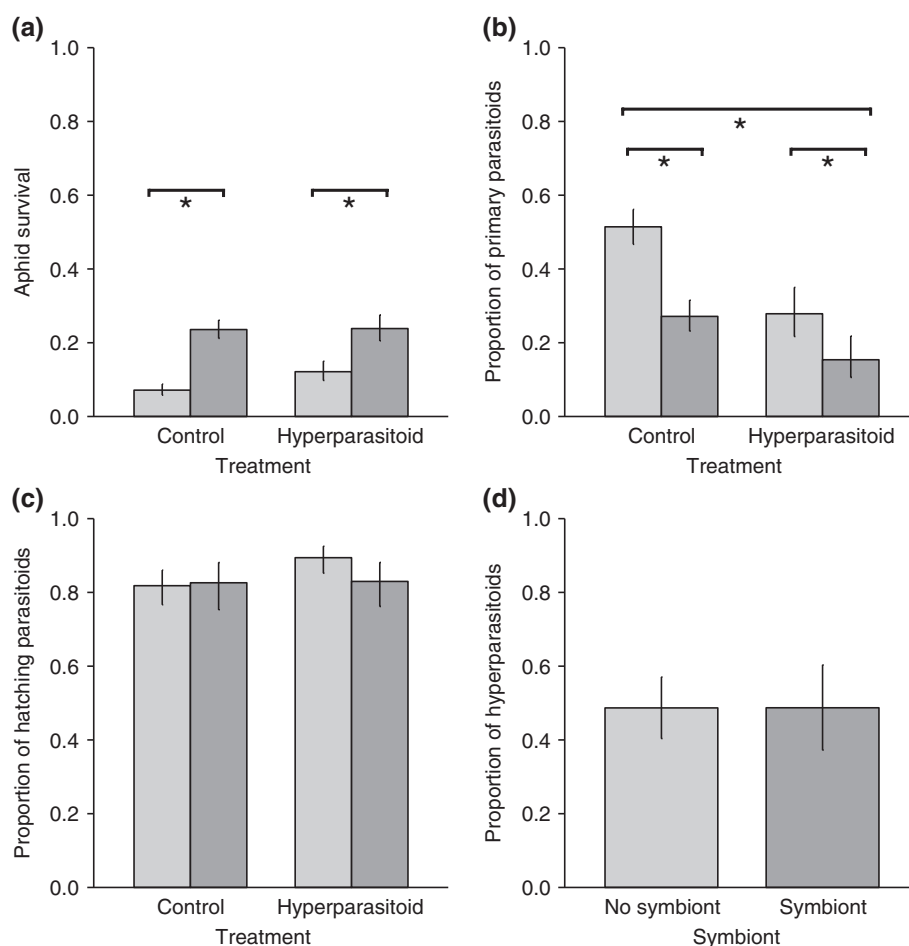


Fig. 3. Effects of *Hamiltonella defensa* on *Aphis abdominalis* in the presence and absence of the hyperparasitoid *Alloxysta* sp. 2 (*brevis-complex*). (a) Proportion of aphids surviving; (b) proportion of potential aphid hosts producing primary parasitoids; (c) proportion of mummies hatching; (d) proportion of hatching wasps that were hyperparasitoids. Dark bars represent aphids with *H. defensa*; light bars represent aphids lacking *H. defensa*. Error bars denote standard error of the mean and asterisks indicate significant differences between values, where relevant (other differences not significant).

before parasitoid pupation and we suggest that in some cases the partially protective symbiont may not have been capable of preventing fatal damage to the aphid host. However, the presence of symbionts led to a considerable cost to both parasitoid and hyperparasitoid during the mummy phase: for aphids exposed to hyperparasitoids, hatch rates of mummies were approximately 45% from the aphids with symbionts, and 73% in the symbiont-free aphids. These figures were significantly higher than hatch rates for the primary parasitoids alone (26% and 59%, respectively). This suggests that *A. ervi* were able to complete development on *A. ervi* individuals which would not have been viable. We note that aphids in all treatments for this food chain hosted the symbiont X-type, which has recently been shown to affect *A. ervi* development negatively (Heyworth & Ferrari, 2015), and that this might also impact aphid survival in our experiments.

Our study used single strains of symbionts and aphid genotypes in each food chain, and our parasitoid populations were inbred to limit genetic diversity. We therefore cannot be certain our results will generalise to all

symbiont–aphid–parasitoid–hyperparasitoid interactions. For example, considerable variation between strains of the same ‘species’ of aphid endosymbiont has been observed (Oliver *et al.*, 2005; McLean & Godfray, 2015) and different genotypes of aphids show different levels of innate resistance to parasitoids (Martinez *et al.*, 2014; McLean & Godfray, 2015). In addition, inbreeding in parasitoids can significantly influence their biology, including host selection behaviour (Sepúlveda *et al.*, 2016) and it is possible that outbred individuals may respond differently to endosymbiont defence or to hyperparasitoid attack. However, field experiments suggest that aphids are protected by symbionts against natural parasitoid populations in a similar manner to that observed in the laboratory (Hrček *et al.*, 2016; Rothacher *et al.*, 2016), and it is likely that the results reported in this study will also pertain in natural conditions.

We have shown that protective symbionts, by reducing the numbers of specific hosts available for parasitism, can cause a cascading reduction in hyperparasitoid emergence rates. This effect is, in turn, likely to have wider effects for the aphid–parasitoid food web. No laboratory or field cage

experiments previously undertaken in the aphid symbiont system have yet included hyperparasitoids, although they can be an extremely important source of parasitoid mortality in the field (Müller *et al.*, 1999). A previous field experiment has shown no differences between aphids with and without symbionts in their rates of secondary parasitism (Hrček *et al.*, 2016; Rothacher *et al.*, 2016), although hyperparasitoid sample sizes were small. The fact that the relative benefits of a symbiont can vary depending on the wider community present shows that we need to consider the food web context when seeking to explain the patterns of symbiont distribution we observe in nature (Ferrari *et al.*, 2012; Russell *et al.*, 2013).

We predict that protective symbionts could actually provide an advantage to the primary parasitoids attacking other aphid species in the same local area, by reducing hyperparasitoid numbers (and we predict that 'mummy parasitoids' are likely to be impacted in a similar way to the hyperparasitoids, exacerbating this effect). In addition, if a protective symbiont causes a local reduction in an otherwise abundant primary parasitoid, it may allow increased food web diversity by providing opportunities for other primary parasitoids. High numbers of specialist hyperparasitoids have been shown to suppress dominant primary parasitoids in the field (Nofemela, 2013), allowing other primary parasitoid species to flourish. Likewise, different primary parasitoids have different levels of vulnerability to protection provided by different strains of *H. defensa* (Asplen *et al.*, 2014; Cayetano & Vorburger, 2015; McLean & Godfray, 2015), and this asymmetry means that some primary parasitoids actually gain a relative advantage in the presence of the symbiont, as it releases them from competition (Rothacher *et al.*, 2016). Hyperparasitoids may, in some instances, impact on symbionts by reducing the survival advantage that protective symbionts supply and so weakening the selection pressure for symbiont maintenance. For both food chains we studied, reduction in primary parasitoids when hyperparasitoids and protective symbionts were both present was less than the sum of the costs of these factors alone (Figs 2b and 3b). Finally, in addition to their direct effects on aphid and parasitoid survival, symbionts may have indirect effects on food webs, e.g. via manipulating aphid behavioural traits (Dion *et al.*, 2011) in a manner that affects hyperparasitoid foraging success.

Our results demonstrate that to understand effects of symbionts in nature, we need to have a full picture of their influence on food web interactions at higher trophic levels. Protective symbionts are known to be widespread in nature (Brownlie & Johnson, 2009) and are therefore likely to be hidden but important players in many insect food webs (Ferrari & Vavre, 2011; McLean *et al.*, 2016). We believe that there is now a need to assess whether community composition and diversity, and food web characteristics such as connectivity, are affected by herbivore symbionts in the field.

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Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/een.12424

Table S1. Rates of primary parasitoid *Aphidius ervi* hatching per aphid: comparisons for aphids with and without symbionts, with and without hyperparasitoids. Comparisons made using Tukey's honest significant differences.

Table S2. Sum of outcomes across replicates for aphids with and without symbionts under control (*Aphidius ervi* primary parasitoids only) and hyperparasitism (*Alloxysta victrix*) treatments.

Table S3. Results of the analysis of deviance for the food chain *Acyrtosiphon pisum*–*Aphidius ervi*–*Alloxysta victrix*. Significant effects are highlighted in bold.

Table S4. Rates of primary parasitoid *Aphelinus abdominalis* hatching per aphid: comparisons for aphids with and without symbionts, with and without hyperparasitoids. Comparisons made using Tukey's honest significant differences.

Table S5. Sum of outcomes across blocks and replicates for aphids with and without symbionts under control (*Aphelinus abdominalis* primary parasitoids only) and hyperparasitism (*Alloxysta brevis*-complex) treatments.

Table S6. Results of the analysis of deviance for the food chain *Acyrtosiphon pisum*–*Aphelinus abdominalis*–*Alloxysta* sp. 2 (*brevis* complex). Significant effects are highlighted in bold.

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