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Variations on a protective theme: *Hamiltonella defensa* infections in aphids variably impact parasitoid success

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Protective mutualisms are common in nature and include insect infections with cryptic symbionts that defend against pathogens and parasites. An archetypal defensive symbiont, *Hamiltonella defensa* protects aphids against parasitoids by disabling wasp development. Successful defense requires *H. defensa* infection with bacteriophages (APSEs), which play other key roles in mutualism maintenance. Genomes of *H. defensa* strains are highly similar in gene inventories, varying primarily in mobile element content. Protective phenotypes are highly variable across aphid models depending on *H. defensa*/APSE, aphid and wasp genotypes. Infection frequencies of *H. defensa* are highly dynamic in field populations, influenced by a variety of selective and non-selective factors confounding biological control implications. Overall, *H. defensa* infections likely represent a global aphid protection network with effects radiating outward from focal interactions.

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Cryptic protective mutualisms in insects

Protection mutualisms, in which one partner receives shelter or nutrients in exchange for providing protection against additional species are widespread in nature [1]. Although many of the best known protective mutualisms involve macroscopic players (e.g. ant-tending of aphids), cryptic defensive symbioses involving inconspicuous microbes certainly represent the bulk of examples. Over the preceding two decades, the importance of the resident microbiota in shaping the ecology and evolution of animals, including insects, has become widely recognized [2]. Symbionts are often sources of evolutionary novelty that play critical roles in resource acquisition and defense, but they also can be costly to hosts and exploited by cheaters [3,4].

Microbe-mediated defense of insects spans diverse interaction types varying in degree of specificity, specialization, localization, transmission and state or age of host-restriction. Examples include environmentally acquired microbes that establish in the gut and provide protection against ingested pathogens [5], ectosymbionts that protect broods or food supply via antibiotic production [6,7] and host-restricted, maternally-transmitted endosymbionts associated with diverse defensive phenotypes. The latter include *Wolbachia* infections in natural and transinfected hosts that protect against a range of microbial pathogens and *Spiroplasma*-mediated protection against entomopathogenic nematodes and parasitoids in *Drosophila* [8].

Diverse defensive symbioses in aphids

Aphids are phloem-feeding herbivores and all individuals are infected with an obligate nutritional symbiont, usually *Buchnera aphidicola*, to supplement their nitrogen-limited diet [9]. In addition, at least nine heritable facultative symbiont (HFS) species regularly occur in aphids [10–12]. While some HFS have roles in expanding dietary breadth [13], affecting reproduction during aphids' sexual phase [14], or transitioning to obligate roles to make up for decaying *Buchnera* function [15•], most characterized phenotypes involve protection against biotic threats and abiotic stresses [16].

Aphids are great models for the study of defensive symbiosis. Clonal reproduction combined with the ability to manipulate HFS infections allows isolation of symbiont effects during enemy challenge [10]. The pea aphid, *Acyrthosiphon pisum*, is the most intensively studied aphid model using these experimental approaches, with all seven naturally-occurring HFS species reported to impact aphid defense.

Here we focus on the bacterial symbiont *Hamiltonella defensa*, for which there is clear experimental evidence for symbiont-based protection against specific parasitoids across multiple aphid species (Table 1). While not all *H. defensa* strains have conferred protection against the specific parasitoid species they were tested against [e.g. 17], known target specificity (see below) indicates that additional studies are needed to determine whether these are truly non-protective or instead target different species [18]. Another common aphid HFS, *Serratia symbiotica* was

Table 1

Five aphid systems where *H. defensa* confers variable protection (P; NP, no protection) against braconid (dark gray box) or aphelinid (light gray) parasitoids [70–74]

Aphids	Parasitoids	APSE type	P or NP	Refs
Acyrthosiphon pisum	*Aphidius ervi*	APSE1-like	P	70
			P	48
		APSE 2	P	
		APSE 3	P	
	Praon pequodorum	APSE 8	NP	54
		APSE 3	NP	
		APSE 8		
	Aphelinus abdominalis	Unknown	P	70
	Aphelinus atriplicis	APSE 3	NP	71
Aphis craccivora	*Binodoxys communis*	APSE 4	P	72
	Binodoxys koreanus	APSE 4	P	
	Lysiphlebus orientalis	APSE 4	NP	
	Aphidius colemani	APSE 4	NP	
	Lysiphlebus testaceipes	Unknown	NP	73
	Aphelinus atriplicis	APSE 4	NP	71
	Aphelinus glycinis	APSE 4	NP	
Aphis fabae	*Lysiphlebus fabarum*	APSE 3	P	50
		APSE 6	P	51
	Aphidius colemani	Unknown	NP	
	Binodoxys angelicae	Unknown	NP	
	Aphelinus chaonia	Unknown	NP	50
Rhopalosiphum padi	*Aphidius colemani*	Unknown	P	74
Sitobion avenae	*Aphidius ervi*	Unknown	NP	17
	Ephedrus plagiator	Unknown	NP	

reported to reduce rates of successful parasitism in pea aphids, both as a single infection and co-infection infection alongside *H. defensa* [19,20]. However, no direct benefits to infections were found as surviving aphids were largely castrated, indicating that protection against parasitoids is likely not the basis of *S. symbiotica* persistence in pea aphid populations. Also, *Regiella insecticola* from *Myzus persicae* when transferred to *Aphis fabae* provided protection against parasitoids [21], but strains tested from pea aphids did not [19,22] and it remains unclear whether anti-parasitoid defense is a common role for this HFS.

A brief history of *Hamiltonella defensa*

Hamiltonella defensa was first reported from pea aphids in 2001 as 'PABS' (pea aphid *Bemisia*-like symbiont) and as 'T-type' from multiple aphid species [23,24]. In a common pea aphid genetic background this symbiont was shown to provide protection against the dominant parasitoid of pea aphids in North America, the braconid wasp *Aphidius ervi* [19] and was named *H. defensa* in 2005 after evolutionary biologist W.D. Hamilton in conjunction with these recently discovered defensive properties [25]. A member of the Yersiniaceae (Order Enterobacteriales: Class Gammaproteobacteria), *H. defensa* is most closely related to two other aphid HFS, *R. insecticola* and *Fukatsuia symbiotica* (formerly X-type/PAXS) (Figure 1a) [3,26]. In the pea aphid, *H. defensa* primarily persist extracellularly in the hemocoel, although some cells occur in the cytoplasm of bacteriocytes (aphid cells housing *Buchnera*) and surrounding sheath cells [25,27••]. Although screening effort varies among species, 34% (53/154) of examined species are reported to carry *H. defensa* [11]. Intraspecific infection frequencies are typically intermediate, but infection appears fixed in some aphids, including *Uroleucon ambrosiae*, possibly indicating transition to co-obligate status as seen for other HFS in *Cinara* (Lachninae) aphids [10,15•,24]. Outside of aphids, *H. defensa* occurs in the *Bemisia tabaci* species complex where it is restricted to bacteriocytes and likely has non-defensive roles [28–30] and in psyllids and mealybugs where roles are unknown [24,31].

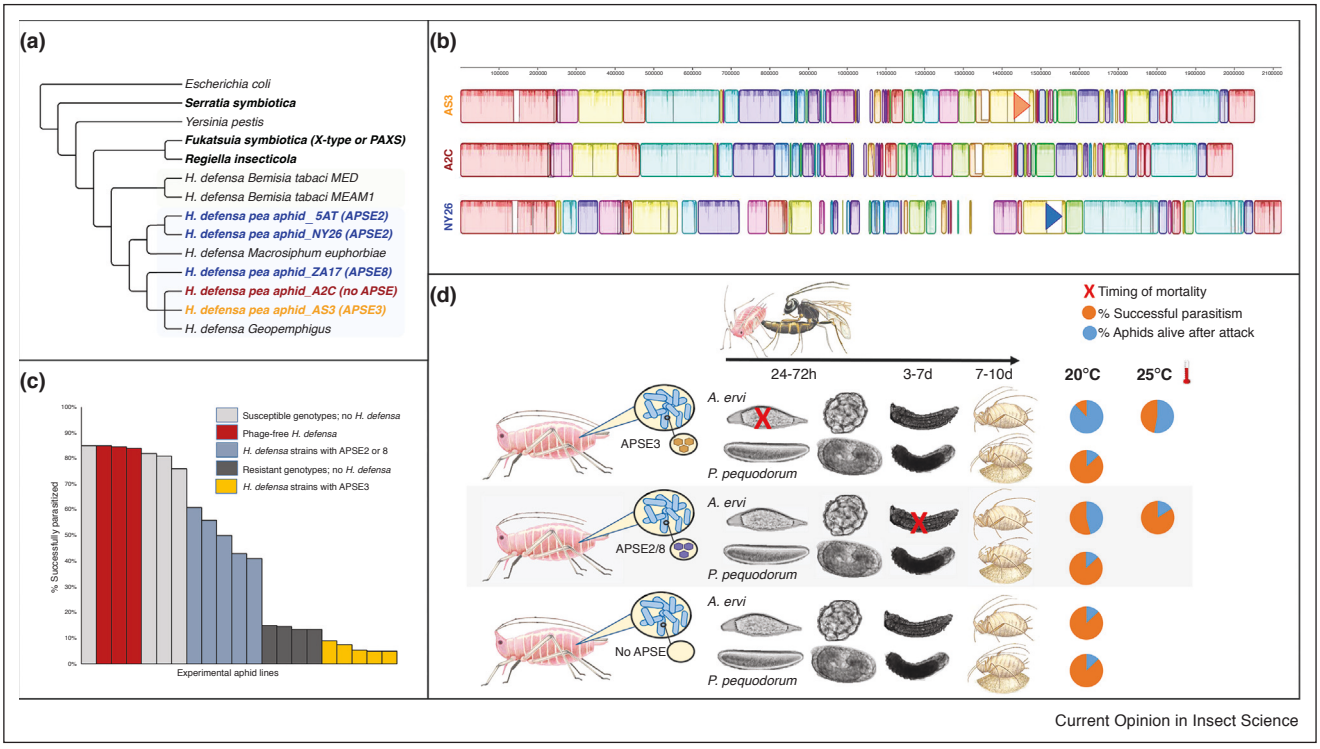
Rates of maternal transmission of *H. defensa* are very high under lab conditions, but are lower under field conditions and vary with aphid genotype and co-infection states with other HFS [32]. Though maternal transmission is the primary route to new hosts, phylogenetic studies show that *H. defensa* occasionally moves horizontally within and among species potentially resulting in the instant acquisition of novel defensive capabilities (Figure 1a) [10,33,34•,35]. The contaminated ovipositors of parasitoids and aphid food plants are likely routes of lateral transfer [36,37] but the contribution of this phenomenon to *H. defensa* prevalence and distributions in field populations remains to be determined.

Genomics and mechanism of *H. defensa*-based protection

Preliminary genome sequencing identified numerous pathogenicity factors on the *H. defensa* chromosome as agents of interest in disabling wasp development [38]. Most intriguing were intact podovirus-like bacteriophages called APSEs (for *A. pisum* secondary endosymbiont) encoding putative eukaryotic toxins. While APSEs were later experimentally shown to be required for protection [39], the specific factors that harm wasps have not been identified. In addition to bringing protective traits that enhance bacterial and aphid fitness, APSEs influence the within-aphid abundance of *H. defensa* [40] and can move protective traits horizontally between *H. defensa* strains [27••]. While APSEs are vertically transmitted at high rates, phage loss can lead the rapid breakdown of the defensive symbiosis due to loss of protection in conjunction with large fitness costs associated with increased *H. defensa* titers [40].

Recent advances in the ability to culture *H. defensa* and parasitoid tissues *in vitro* confirms that *H. defensa* requires APSE to harm wasps, and that this combination can do so in the absence of aphid factors [27••]. The ability to culture key players in this defensive mutualism opens exciting avenues to directly study function, including via

Figure 1



(a) Phylogenetic relationships of *H. defensa* from aphids and whiteflies relative to other aphid HFS (bolded) and related bacteria. Colored *H. defensa* strains indicate APSE infection type in pea aphid-associated strains (blue: APSE2, orange: APSE3, red: No APSE). **(b)** Depiction of Mauve-generated [75] locally collinear blocks for three *H. defensa* strains: AS3 and A2C are closely related strains with shared synteny but the former encodes highly-protective APSE3 (▶); The genome of NY26 is rearranged relative to A2C and AS3 and encodes moderately-protective APSE2 (▶). **(c)** Variable protection against *Aphidius ervi* by *H. defensa* depending on APSE presence/type (red, blue, yellow) or aphid genotypes without *H. defensa* infection (light or dark gray). **(d)** The timing of mortality to developing *A. ervi* varies depending on *H. defensa* strain; the same strains have no effect on the development of *Praon pequodorum*. Moderate increase in temperature (20 vs 25 °C) compromise *H. defensa*-based protection. Insect illustrations by Rebecca K Neher.

the manipulation of *H. defensa*, and through the ability to produce RNA and DNA template with minimal eukaryotic contamination. Such culture-assisted approaches were recently used to produce high-quality genomes from four pea aphid-acquired strains of *H. defensa* varying in protective phenotype to complement an existing genome [34•,41]. Together these show that *H. defensa* is an aerobic heterotroph with strains showing large overlap in the inventory of genes involved in metabolism, housekeeping functions and nutrient acquisition. Strains differ largely in the content of mobile genetic elements (MGEs) and resulting genomic rearrangements (Figure 1b) [34•]. These genomic studies are consistent with key roles for APSEs in defense. For example, two closely related strains, one highly protective and the other non-protective, were nearly identical in gent content, including most MGEs, except for the presence of APSE3 in the former. Across strains, *H. defensa* cannot make many essential nutrients and the presence of transporters for missing pathways suggests this symbiont is an obligate parasite of their insect hosts + obligate symbionts, and a conditional mutualist when parasitoids are present. Variable costs to infection with *H. defensa* have been identified in multiple aphid species which may reflect constituent costs associated with resource parasitism, but also pathogenicity when invading new tissues, or induced costs following mobilization of wasp-killing factors that also harm aphids [42–44]. Two additional *H. defensa* genomes from *B. tabaci* indicate that whitefly-infecting strains are slightly reduced (1.74 vs 2.27 Mb) relative to those occurring in aphids and lack some pathogenicity factors, including inactivation of APSEs, consistent with non-defensive roles [34•,45,46].

Highly variable and specialized *H. defensa*-mediated defense against parasitoids

One of the most striking features of *H. defensa*-mediated defense is the tremendous variation in protective phenotype observed depending on aphid genotype, *H. defensa*/APSE strain, and wasp genotype. For instance, distinct *H. defensa* strains confer widely varying levels of protection (0–100% relative to uninfected controls) against particular natural enemies (Figure 1c,d). Variation largely correlates with APSE type [47,48], but since *H. defensa* strain and

APSE variant are typically co-inherited [34•,49] additional studies are required to partition relative contributions. In the *Aphis fabae* (black bean aphid) interaction, which also features a parthenogenetic parasitoid *Lysiphlebus fabarum*, the specificity of the interaction is determined by wasp genotype and *H. defensa* strain rather than aphid genotype; a phenomenon potentially mediated by APSEs [50,51]. Additional variation occurs as some aphid genotypes lacking *H. defensa* are also resistant to parasitoids (Figure 1c) [52,53] and aphids may have both endogenous and symbiont-based resistance [43].

Across multiple aphid species, particular *H. defensa* strains protect only against specific parasitoid species (Table 1). In pea aphids, for instance, several *H. defensa*/APSE strains tested showed significant protection against *A. ervi*, but no protection again the related *Praon pequodorum* (both aphidiine braconids) (Figure 1d) [54]. Such specificity can mediate competition between rival parasitoids affecting parasitoid community composition [55•,56,57•]. For example, the introduction of *A. ervi* to control pea aphids in North America resulted in the competitive displacement of numerous native and introduced wasps, except for *P. pequodorum*. Population cage studies showed that *A. ervi* displaced *P. pequodorum* in aphid populations without *H. defensa*, but in cages containing 20% (or more) *H. defensa*-infected aphids, *P. pequodorum* not only persisted, but typically displaced *A. ervi* [58].

Are *H. defensa* effective defenders in the field? Implications for biological control

The rapid spread of symbiont-mediated resistance in response to wasp-induced mortality, as demonstrated in simplified lab-based population cages [37], ostensibly poses a serious challenge to biological control. However, field surveys show that while *H. defensa* infections fluctuate rapidly, and often dramatically, over spatial and temporal scales, they do not reliably track parasitoid-induced mortality [37,59]. Instead, a range of selective and non-selective factors likely serve to attenuate defensive symbiont services under 'real-world' conditions or otherwise serve to create mismatches between parasitoid-driven mortality and *H. defensa* infection frequencies. As noted above, protection levels and enemy specificity vary among strains, such that mortality caused by the wasp *P. pequodorum*, for example, would not favor the spread of *H. defensa* in pea aphid populations. Further, the protective benefits of any given *H. defensa* strain depend on environmental conditions as several strains of *H. defensa* failed to protect pea aphids at only moderately warmer temperatures while endogenous defenses remained robust [60]. Thus, defensive symbionts may be less effective depending on wasp species/genotype or in warmer locations or times of year inhibiting symbiont spread. Costs of infection can also vary independent of benefits, such as when *H. defensa* infections occur in resistant host genotypes resulting in redundant services but increased costs [43,61].

Similarly, *H. defensa* infection may modify wasp behaviors in ways that weaken links between parasitism pressure and infection frequency. For example, *A. ervi* were shown to selectively superparasitize *H. defensa*-infected aphids, allowing them to partially overcome symbiont defenses [62]. And in an amazing example of a very extended phenotype, a recent study [63••] found that plants fed on by *H. defensa*-infected pea aphids emitted fewer induced volatiles and attracted fewer parasitoids compared with plants fed on by aphids without *H. defensa*.

Of course, parasitoids are not the only natural enemies exerting selection pressure on aphids. Although, *A. pisum* and *A. fabae* with *H. defensa* are protected against parasitoids in the field, the abundance of infected aphids did not necessarily increase relative to uninfected ones because of mortality from other agents such as fungal pathogens [56,64]. Interestingly, field-collected pea aphids harboring at least one HFS, typically have two or more HFS, suggesting there may be selection for 'generalist' aphids that are resistant to multiple enemies routinely encountered [32]. Consistent with this hypothesis, co-infection studies show that having multiple HFS can maintain or even improve protective phenotypes, although costs can also be enhanced [20,65]. Community context will also matter as *H. defensa* services may be less important for ant-attended aphids [66,67] or impacted by the infection status of other aphid species [57•,68].

Finally, the importance of non-selective factors, including migration, vertical and horizontal transmission rates, on *H. defensa* infection frequencies are largely unknown under field conditions, although co-infection context can impact transmission rates [32] and APSEs infecting the most protective *H. defensa* strains are lost more readily than those in moderately protective strains [40].

Together, diverse and specialized symbiont defenses and wasp counter defenses, combined with mitigating environmental factors, unpredictable costs and non-selective factors limit its efficacy and invasion potential, which in turn, may support the retention of attenuated biological control services. These same factors also set the stage for coevolutionary interactions that may contribute to the substantial diversity observed in aphid, wasp and symbiont genotypes [69•].

Conclusions

More than 1700 aphid species (5000 × 34%) are estimated to be infected with *H. defensa*. Assuming protective roles against parasitoids for most, this would represent an extensive, global defensive mutualism network that affects herbivore–parasitoid interactions across an even more diverse suite of host plants. Although many features

of protective symbiosis will be shared among aphid–parasitoid systems, given the extensive phenotypic diversity and complexity of interactions observed in the few systems explored to date, we can expect additional surprising variation. We also expect that phenotypes other than anti-parasitoid defense will be discovered for *H. defensa*, and it will be interesting to uncover the distribution of this symbiont inside and outside of hemipteran insects as 16s rRNA amplicon studies accumulate. Studies investigating *H. defensa*-based protection under field conditions, and how infection effects radiate through food webs are underway and producing intriguing results. These coupled with modeling will lead to a better understanding of *H. defensa* dynamics in natural populations that may inform control strategies. In turn, lab-based studies leveraging the advent of culturing protocols are likely to advance our understanding of the mechanisms of *H. defensa* protection and may lead to manipulations that enhance control services.

Conflict of interest statement

Nothing declared.

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- •• of outstanding interest

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