Journal of Insect Science, 2026, **26**(5), ieag102
DOI: https://doi.org/10.1093/jisesa/ieag102
Research

Endosymbiont-mediated resistance: effects of *Hamiltonella defensa* (Enterobacterales: Enterobacteriaceae) on parasitism of *Aphidius colemani* and *Praon volucre* (Hymenoptera: Braconidae) in *Aphis craccivora* (Hemiptera: Aphididae)

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Subject Editor: Rachid Hanna

The present study investigated the influence of the *Hamiltonella defensa*—APSE association, a functional defensive unit in aphids, on the performance of 2 aphidiine parasitoids, *Aphidius colemani* Viereck, 1912 and *Praon volucre* (Haliday, 1833), attacking the cowpea aphid, *Aphis craccivora*, on alfalfa. To assess the effects of *H. defensa*, naturally infected cowpea aphids with this bacterium (Hd+) were treated with a combination of antibiotics to eliminate the symbiont, generating uninfected cowpea aphids (Hd−). The results showed that *A. colemani* had similar parasitism success in Hd+ and Hd− cowpea aphids, suggesting that *H. defensa* had a limited effect on the overall parasitism success of this species. However, *H. defensa* significantly prolonged the time to mummification of *A. colemani* developing in Hd+ aphids, while post-mummification emergence and the mummification-to-emergence interval were not significantly affected. In *P. volucre*, *H. defensa* had a strong negative effect on parasitoid performance. Hd+ aphids showed significantly lower parasitism success and post-mummification emergence, together with significantly longer time to mummification and mummification-to-emergence interval. Neither female sex ratio nor body size showed a treatment effect that met the Bonferroni-corrected significance threshold in either parasitoid species. These findings show that the *H. defensa*—APSE association can influence several key components of parasitoid performance and highlight the potential importance of defensive symbionts in shaping aphid–parasitoid interactions.

Keywords: aphids, biological control, Enterobacteriaceae, endosymbiotic bacteria, parasitoid wasps

Introduction

The cowpea aphid, *Aphis craccivora* Koch (Hemiptera: Sternorrhyncha), is a major pest of numerous legume crops and has a worldwide distribution, causing substantial damage in alfalfa (*Medicago sativa* L., Fabales: Fabaceae) production systems (Brady et al. 2014). In Iran, *A. craccivora* is considered probably native and occurs on a wide range of host plants (Rakhshani et al. 2005, Whittaker 2015, Momeni Shahraki et al. 2019). This aphid damages crops through direct feeding and the transmission of plant viruses, resulting in stunted growth, reduced yields, and significant economic losses (Franz et al. 1998, Descamps et al. 2015). Natural enemies, especially aphidiine parasitoid wasps, can help suppress *A. craccivora* and are therefore important for biological control of this pest (Henter and Via 1995, Desneux et al. 2006, Hance et al. 2017). However, the outcome of this interaction is not determined by the aphid and parasitoid alone. Bacterial endosymbionts carried by the aphid can also affect aphid–parasitoid interactions and may influence parasitoid performance (McLean et al. 2020, Heidari Latibari et al. 2022). The cowpea aphid harbors a diverse assemblage of bacterial endosymbionts, several of which can shape host ecology and interactions with parasitoids

Received: 1 April 2026. **Revised:** 21 August 2026. **Accepted:** 21 August 2026

(Tsuchida et al. 2006, Bayendi Loudit et al. 2018). The obligate symbiont, *Buchnera aphidicola* (Enterobacterales: Erwiniaceae), is essential for cowpea aphid survival, as it synthesizes essential amino acids that are deficient in the phloem-based diet of its host. Conversely, facultative (secondary) symbionts can offer a variety of ecological benefits under stressful environmental conditions, including increased heat tolerance and protection against natural enemies (McLean et al. 2016, Heidari Latibari et al. 2025). These facultative symbionts can substantially influence the ecological performance and adaptive potential of cowpea aphids across diverse environments, thereby affecting their population dynamics and interactions within agricultural systems (Oliver et al. 2010, Zytynska and Weisser 2016).

The study of endosymbiotic relationships in insects has profound implications for evolutionary biology, ecology, and pest management. Facultative bacterial symbionts, such as *Hamiltonella defensa*, exemplify how microbial associates can mediate host-parasitoid interactions. This symbiont is well known for protecting aphid hosts against parasitoid attack by reducing parasitism success and impairing parasitoid development (Oliver et al. 2005, Oliver and Higashi 2019). Residing primarily in the extracellular space of the hemocoel, *H. defensa* has attracted considerable attention due to its pivotal role in the defense of agriculturally important aphid species (Oliver and Higashi 2019, Boyd et al. 2021).

The presence of *H. defensa* within aphids decreases their susceptibility to certain parasitoid species, thereby reshaping the population dynamics of both aphid pests and their natural enemies (Oliver et al. 2010, Vorburger et al. 2010). Crucially, *H. defensa* is typically infected by a tailed, double-stranded (ds) DNA bacteriophage known as APSE (*Acyrthosiphon pisum* secondary endosymbiont). APSE phages carry diverse toxin cassettes and are strongly associated with the protective phenotype observed in aphids (Boyd et al. 2021). Such symbiotic protection has the potential to destabilize pest management systems, underscoring the importance of understanding these symbiotic relationships for the development of sustainable agricultural practices (Vorburger 2018, Postic et al. 2020). The impact of *H. defensa*, a primary defensive symbiont in aphids (Vorburger 2022), on biological control agents raises important questions about the efficacy of parasitoid wasps and highlights the potential role of endosymbionts in mediating pest resistance to biocontrol strategies.

Previous studies have shown that *H. defensa*-mediated protection is highly specific and depends on the aphid host, the symbiont/APSE strain, and the parasitoid species involved. In *A. craccivora*, *H. defensa* has been reported to provide strong protection against some aphidiine parasitoids, including *Binodoxys* species, but not against *A. colemani* or *Lysiphlebus orientalis* (Asplen et al. 2014, Dykstra et al. 2014). Thus, the *A. craccivora–A. colemani* association provides an important comparison for assessing whether this parasitoid can overcome *H. defensa*-associated defenses. In contrast, the effect of *H. defensa* on *P. volucre* has not previously been tested in *A. craccivora*. In *Acyrthosiphon pisum*, McLean et al. (2020) found no evidence that the tested *H. defensa* strains protected against *P. volucre*, suggesting that protection against this parasitoid may depend strongly on the aphid host background and/or the particular *H. defensa*—APSE strain involved.

The current study aims to investigate the effect of the *H. defensa*—APSE association in the cowpea aphid on the performance of 2 aphidiine endoparasitoids, *Aphidius colemani* Viereck (tribe Aphidiini) and *Praon volucre* (Haliday) (tribe Praini). The objective of this research is to gain insights into the defensive role of *H. defensa*—APSE and improve understanding of how defensive symbionts shape interactions between crop pests and their natural enemies, with possible implications for parasitoid-based biological control.

Materials and Methods

Sampling Sites

Sampling was conducted during the typical alfalfa growth period in the northwestern region of Iran, from mid-March to mid-October in 2021 and 2022. The cowpea aphids and their endoparasitoids were collected from 3 alfalfa farms in Guilan Province, Iran. The locations in question were as follows: (a) Rudsar County (37°07′48.0″N, 50°16′48.0″E); (b) Astara County (38°24′57.8″N, 48°51′30.2″E); and (c) Rezvanshahr County (37°32′37.1″N, 49°08′35.5″E).

Species Identification

The alfalfa plant and the cowpea aphid species were identified in accordance with the taxonomic keys established by Lesins and Lesins (1979) and Blackman and Eastop (2017), respectively. Parasitoid wasp specimens were prepared and labeled following the protocols described by Ghafouri Moghaddam et al. (2017, 2018). Identification of the emerged parasitoids was confirmed using the taxonomic keys of Starý (1979) and Rakhshani et al. (2019).

Aphid Rearing

A total of 9 cowpea aphid colonies (designated I-IX) were established from specimens collected randomly from alfalfa leaves. Each colony was initiated by placing a single young apterous female (foundress) onto a potted alfalfa plant and rearing it under controlled growth-chamber conditions at 21 ± 1 °C, 60 ± 5% relative humidity (RH), and a 16 L:8D photoperiod. To prevent overcrowding and the induction of winged morphs (alates), apterous parthenogenetic females were transferred to fresh alfalfa plants as needed. All cowpea aphid colonies were maintained under identical environmental conditions from their establishment until the start of the experiments. Experimental alfalfa plants were housed in cylindrical mesh cages (35 × 20 cm; height × diameter) to prevent cowpea aphid escape.

PCR Screening for *H. defensa* and Its Associated APSE Bacteriophage (Hd+)

A total of 9 parthenogenetic cowpea aphid females were individually reared on alfalfa leaves to establish 9 isofemale lines. DNA was extracted from pooled cowpea aphids consisting of 10 individuals per line (4 first-instar, 3 second-instar, and 3 third-instar nymphs) using the RNA/DNA Geneaid Blood and Tissue Kit (Geneaid Biotech Ltd., New Taipei City, Taiwan) following the manufacturer's protocol. To characterize the symbiont composition of each line, a targeted diagnostic screening was conducted for the facultative bacterial symbionts most commonly reported in *A. craccivora* and known to influence aphid–parasitoid interactions. All lines were screened by polymerase chain reaction (PCR) for *Arsenophonus* sp., *Serratia symbiotica*, *Regiella insecticola*, and *H. defensa* and its associated bacteriophage APSE

using specific primer sets (Supplementary Table S1) (Douglas et al. 2006, Degnan and Moran 2008, Jousselin et al. 2013). The screening confirmed the *H. defensa* and APSE infection status of the experimental cowpea aphid lines while excluding major facultative symbiont infections that could potentially confound the interpretation of the parasitoid bioassay results. PCR assays targeting facultative symbionts were performed in a total reaction volume of 14 µL containing 1 µL of DNA template, 7.52 µL of nuclease-free water, 2.8 µL of 5× reaction buffer stock (final concentration 1×), dNTPs (10 mM each), primers (10 µM each), and DNA polymerase (1.25 U/µL). Amplifications were performed with an initial denaturation at 93 °C for 3 min, followed by 30 cycles of denaturation (93 °C, 30 s), annealing (54 °C, 30 s), extension (72 °C, 1 min), and a final extension at 72 °C for 5 min. APSE detection was carried out in 10 µL reactions containing 0.70 µL of DNA template, 5.42 µL of nuclease-free water, 1 µL of 10× buffer (final concentration 1×), 1 µL of dNTPs, 0.20 µL of $MgCl_2$, 0.80 µL of each primer, and 0.08 µL of DNA polymerase. Amplifications were carried out under the following thermal conditions: an initial denaturation at 94 °C for 2 min, followed by 10 cycles of denaturation (95 °C, 30 s), annealing at 56 °C (decreasing by 1 °C per cycle) for 50 s, and extension (72 °C, 75 s) subsequently, 24 cycles of denaturation (95 °C, 30 s), annealing (46 °C, 50 s), and extension (72 °C, 75 s), followed by a final extension at 72 °C for 6 min (Hopper et al. 2018). PCR products were visualized by electrophoresis on 1% agarose gels and subsequently purified using the Geneaid PCR purification kit (Geneaid Biotech Ltd., New Taipei City, Taiwan), according to the manufacturer's protocol.

Hamiltonella defensa-Removed Infection (Hd–)

To generate *H. defensa*- free (Hd–) cowpea aphid lines, second-instar nymphs from each of the 9 cowpea aphid clones (I–IX) were exposed to an antibiotic treatment. A mix of ampicillin (100 mg mL^{-1}), cefotaxime (50 mg mL^{-1}), and gentamicin (50 mg mL^{-1}) (Research Products International, IL, USA) was used to target *H. defensa*, a Gram-negative γ-proteobacterial facultative symbiont (McLean et al. 2011). Excised alfalfa stalks bearing 3 leaves were placed individually in 1.5 ml Eppendorf tubes containing the antibiotic solution. The space between the stalks and the tube opening was sealed with Parafilm to minimize evaporation of the antibiotic solution and reduce contamination. Treated leaves were maintained under controlled environmental conditions (21 ± 1 °C, 60 ± 5% RH). Five second-instar aphid nymphs were transferred to each treated leaf and allowed to feed for 3 to 4 days. Following the initial treatment, diagnostic PCR screening indicated that *H. defensa* had not been completely eliminated from all cowpea aphid lines (Supplementary Fig. S1). Therefore, a second antibiotic treatment was administered 2 days later following the same protocol, allowing sufficient time for recovery between treatments. After treatment, cowpea aphids were transferred to untreated alfalfa leaves and maintained under standard rearing conditions. To minimize potential physiological effects of antibiotic exposure, experimental colonies were propagated for 6 generations before use in bioassays. The infection status of each line was subsequently verified by diagnostic PCR. Cowpea aphid lines showing no detectable *H. defensa* amplification (absence of band in the gel; Supplementary Fig. S1) were designated as cured (Hd–) and used in the subsequent experiment.

Parasitoid Rearing

The most abundant endoparasitoid wasp species recovered from the sampling sites were *Aphidius colemani* Viereck and *Praon volucre* (Haliday), both belonging to the subfamily Aphidiinae (Braconidae). Specimens were obtained from naturally parasitized cowpea aphids collected in alfalfa fields. Parasitized aphids, identified by the presence of mummies, were transported to the laboratory and maintained in a controlled-environment chamber at 21 ± 1 °C, 60 ± 5% RH, and 16L:8D. Each field-collected mummy was isolated individually in a glass dish containing an alfalfa leaf placed on moist filter paper until parasitoid emergence. Emerged adults were supplied with honey and water, and males and females of each species were allowed to mate. Subsequently, individual mated females were transferred separately to alfalfa plants infested with newly emerged *H. defensa*-free (Hd–) cowpea aphids. The absence of *H. defensa* was confirmed by diagnostic PCR using a pooled sample of 10 aphids representing different nymphal stages. For each parasitoid species, one laboratory isofemale line was established. Each isofemale line was founded by a single mated female and maintained separately for at least 6 generations, with mating restricted to individuals originating from the same line. This approach preserved traceable maternal origin, minimized uncontrolled mixing of field-derived parasitoid backgrounds, and standardized parasitoid populations prior to bioassays. Maintaining the isofemale lines under laboratory conditions across multiple generations also reduced field-derived environmental effects and allowed acclimation to standardized rearing conditions, as commonly recommended in parasitoid performance studies (Vorburger and Rouchet 2016, Wei et al. 2022). Adults from these laboratory-established isofemale lines were subsequently used in all bioassays.

Parasitism Assay

Following molecular confirmation of *H. defensa*—APSE infection in all 9 cowpea aphid lines and successful elimination of the symbiont from the antibiotic-treated lines, cowpea aphid lines were pooled according to infection status to establish 2 experimental stocks: infected (Hd+) and cured (Hd–). Pooling was performed to minimize potential line-specific effects and provide a broader genetic background for evaluating parasitoid responses. The pooled Hd+ and Hd– experimental stocks were maintained separately for at least 6 generations before the bioassays. Before the bioassays, the infection status of both pooled stocks was re-verified by diagnostic PCR, confirming the presence of *H. defensa* in the Hd+ stock and its absence from the Hd– stock. Aphids were selected from different areas of the corresponding rearing stock and from different positions on the alfalfa stems. Neighboring aphids from the same local feeding cluster were avoided whenever possible to reduce local sampling bias caused by clonal aggregation. The same selection procedure was applied to both Hd+ and Hd– stocks. For each replicate, 80 second- and third-instar cowpea aphid nymphs were established on a 10-cm alfalfa stem and maintained in a mesh-covered plastic container (20 cm × 15 cm × 5 cm). To evaluate the effect of *H. defensa* on parasitism performance, a single male-female pair of either *A. colemani* or *P. volucre* was introduced into each container and allowed to forage and oviposit

for 24 h, after which the adults were removed. Each container represented an independent experimental unit and contained only one aphid treatment, either Hd+ or Hd–. Parasitoid exposures for all 320 replicate containers were initiated on the same assay day, with 80 Hd+ and 80 Hd– replicates for each parasitoid species. Newly selected parasitoid pairs were randomly assigned to Hd+ or Hd– treatment containers, and no parasitoid pair was reused. Following parasitoid exposure, cowpea aphid individuals were maintained under controlled environmental conditions (21 ± 1 °C, 60 ± 5% RH) using fresh alfalfa leaf disks and monitored until mummy formation. All emerging parasitoids were examined to confirm that no other primary parasitoid or hyperparasitoid species were present in the experimental colonies.

Parameters Measured

To assess parasitoid performance, the number of mummified aphids was recorded for each replicate container. Parasitism success was defined as the number of mummified aphids relative to the 80 aphids initially exposed to parasitoids. All mummies from the Hd+ and Hd– treatments were collected and maintained under the previously described laboratory conditions until adult parasitoid emergence (Mackauer and Chow 2016). Upon emergence, the total number of adult parasitoids was recorded, and individuals were sexed as female or male. Post-mummification emergence was defined as the number of emerged adults relative to the number of mummies. Adult emergence per aphid exposed (emerged adults/80 exposed aphids) was retained only as a descriptive composite endpoint because it combines successful mummification and subsequent adult emergence. Aphid survival was operationally defined as the number of non-mummified aphids (80 – mummies). Because no separate third-fate category was recorded, survival was the exact complement of mummification and was therefore treated descriptively only. Time to mummification was recorded as the number of days between parasitoid exposure and mummy formation. Time to adult emergence was recorded as the number of days between parasitoid exposure and adult emergence and was used together with time to mummification to calculate the post-mummification developmental interval. The mummification-to-emergence interval was calculated for each replicate container as time to adult emergence minus time to mummification. Female sex ratio was calculated as the number of emerged females divided by the total number of emerged adults in each replicate container. For body-size measurements, one female and one male were selected from each replicate container. Thus, for each parasitoid species, each of the 80 Hd+ and 80 Hd– replicate containers contributed one female and one male, yielding 160 females (80 Hd+ and 80 Hd–) and 160 males (80 Hd+ and 80 Hd–), for a total of 320 measured adults per parasitoid species. No replicate container contributed more than one individual of a given sex to the corresponding sex-specific body-size analysis. Body-size measurements were therefore conditional on successful adult emergence. Measurements were obtained using a Leica M205 C microscope (Wetzlar, Germany) equipped with the Leica Application Suite (LAS X) software. Body length was measured from the anterior margin of the head, excluding the antennae, to the tip of the abdomen (eg Ghafouri Moghaddam et al. 2025). Identical experimental procedures were applied to both parasitoid species throughout the assay. Developmental-time and body-size responses were conditional on parasitoids reaching the relevant developmental stage. However, all 80 Hd+ and all 80 Hd– replicate containers for each parasitoid species produced at least one mummy and at least one emerged adult. All containers also produced at least one female and one male. Consequently, all 80 containers per treatment contributed to the corresponding container-level developmental-time and sex-specific body-size analyses.

Statistical Analysis

Statistical analyses were conducted separately for *A. colemani* and *P. volucre*, with the replicate container treated as the experimental unit. Hd+ and Hd– replicate containers were independent rather than paired. Two biologically distinct binomial parasitoid-performance outcomes were retained for confirmatory inference: parasitism success (mummified aphids/80 exposed aphids) and post-mummification emergence (emerged adults/mummified aphids). Female sex ratio (females/all emerged adults) was analyzed. These responses were analyzed using beta-binomial regression with a logit link fitted using the glmmTMB package in R, thereby allowing extra-binomial variation among replicate containers. Treatment effects were evaluated using likelihood-ratio tests (LRTs) comparing models with and without infection status, with the test statistic reported as LR χ^2 with 1 degree of freedom. Developmental-time and body-size responses were analyzed at the replicate-container level. The mummification-to-emergence interval was calculated for each container as time to adult emergence minus time to mummification. Body size was analyzed separately by sex; because only one adult of a given sex was selected from each container, each sex-specific analysis contained one observation per experimental unit. All 80 Hd+ and 80 Hd– replicate containers contributed to each developmental-time and sex-specific body-size analysis. Treatment effects on continuous responses were tested using linear models. Model assumptions were assessed from residual diagnostics. The confirmatory family comprised 14 planned tests: for each parasitoid species, parasitism success, post-mummification emergence, time to mummification, mummification-to-emergence interval, female sex ratio, female body length, and male body length. The Bonferroni-corrected significance threshold was therefore $\alpha = 0.05/14 = 0.0036$. All statistical analyses were conducted in R v.4.4.1 (R Core Team 2024). Beta-binomial models were fitted using the glmmTMB package (Brooks et al. 2017), and continuous responses were analyzed using linear models implemented in the base R stats package. Estimated marginal means and treatment contrasts were obtained using emmeans (Lenth 2024) for model summarization and effect estimation. Model diagnostics were assessed using DHARMa for the beta-binomial models and performance for additional model-assumption checks (Lüdecke et al. 2021, Hartig 2024). Data handling and figure preparation were performed using tidyverse and ggplot2 (Wickham 2016, Wickham et al. 2019).

Results

Prevalence of the Facultative Bacterial Symbiont

Diagnostic PCR screening confirmed the presence of *H. defensa* and its associated bacteriophage APSE in all 9 cowpea aphid lines examined. In contrast, *Arsenophonus* sp., *Serratia symbiotica*, and *Regiella insecticola* were absent from all cowpea aphid lines.

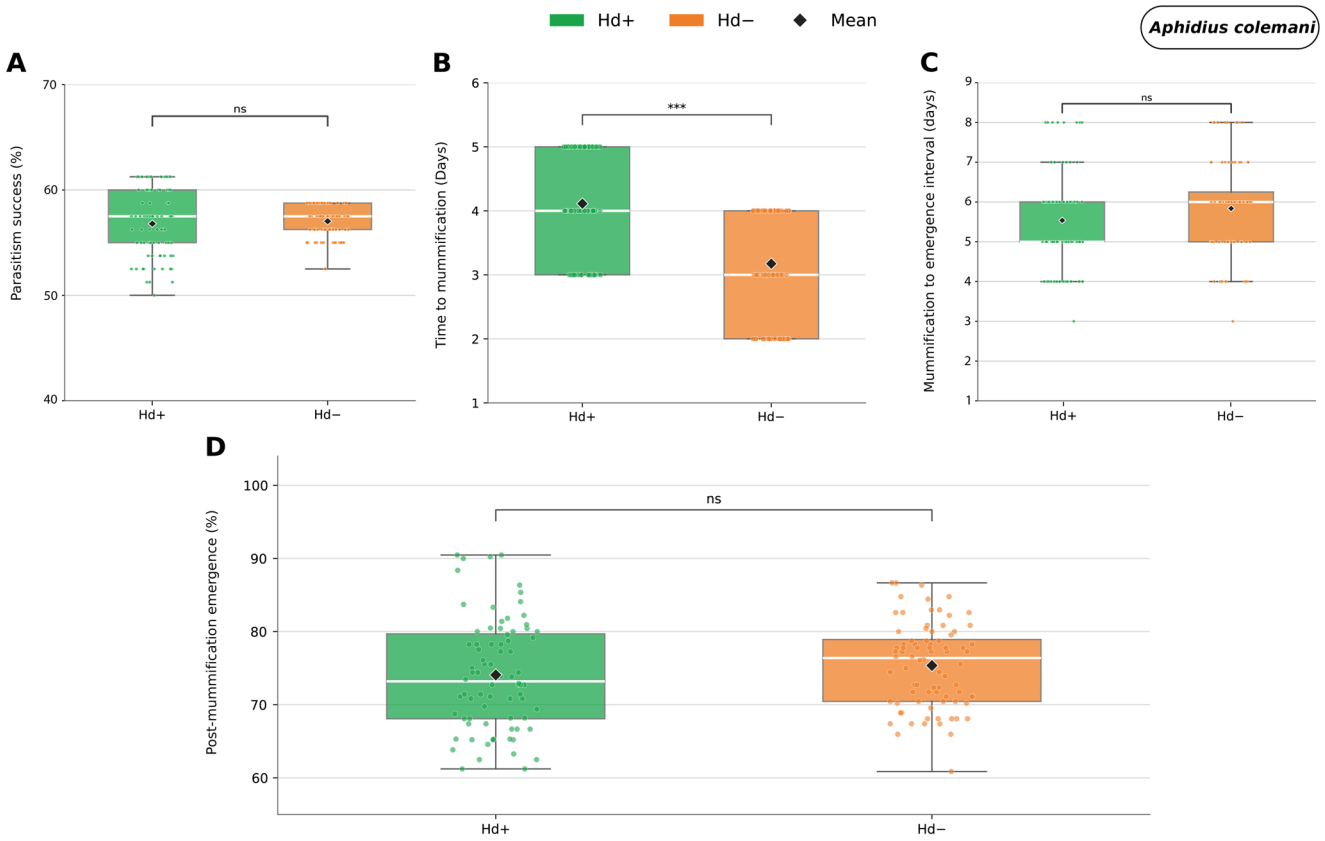

Fig. 1. Effects of *Hamiltonella defensa* infection status on the performance of *Aphidius colemani* developing in *Aphis craccivora*. A) Parasitism success (mummified aphids/80 exposed aphids), B) time to mummification (days), C) mummification-to-emergence interval (days), D) post-mummification emergence (emerged adults/mummies). Hd+ = aphids naturally infected with *H. defensa*; Hd− = *H. defensa*-free aphids. *** = statistically significant differences between treatments ($P < 0.0036$), NS = a non-statistically significant ($P \geq 0.0036$).

Effect of *Hamiltonella defensa*—APSE on *Aphidius colemani* Performance

The *H. defensa*—APSE association did not significantly affect parasitism success in *A. colemani*. Parasitism success was 56.80% in Hd+ containers and 57.03% in Hd− containers (LR $\chi^2 = 0.07$, df = 1, $P = 0.788$; Fig. 1A; Table 1). Time to mummification was significantly longer in Hd+ than Hd− aphids (4.11 ± 0.80 vs 3.18 ± 0.82 days; $t = -7.32$, df = 158, $P < 0.001$; Fig. 1B; Table 2). In contrast, the mummification-to-emergence interval did not differ significantly between treatments (5.54 ± 1.20 vs 5.84 ± 1.18 days; $t = 1.59$, df = 158, $P = 0.114$; Fig. 1C; Table 2). Post-mummification emergence was also similar between treatments (73.84% in Hd+ vs 75.34% in Hd− containers; LR $\chi^2 = 1.99$, df = 1, $P = 0.158$; Fig. 1D; Table 1). The number of emerged adults and adult emergence per initially exposed aphid are shown in Supplementary Figs. S2A and S3A, respectively; adult emergence per initially exposed aphid was 41.94% in Hd+ and 42.97% in Hd− containers. Aphid survival was 43.20% in Hd+ and 42.97% in Hd− containers (Supplementary Fig. S4A). Thus, the detectable developmental response in *A. colemani* was associated with time to mummification rather than with the subsequent post-mummification interval.

Effect of *Hamiltonella defensa*—APSE on *Praon volucre* Performance

The *H. defensa*—APSE association strongly affected parasitism by *P. volucre*. Parasitism success was substantially lower in Hd+ than Hd− aphids (27.97% vs 76.72%; LR $\chi^2 = 607.10$, df = 1, $P < 0.001$; Fig. 2A; Table 1). Time to mummification was significantly longer in Hd+ than Hd− aphids (5.11 ± 0.76 vs 2.86 ± 0.74 days; $t = -18.91$, df = 158, $P < 0.001$; Fig. 2B; Table 2), and the mummification-to-emergence interval was also significantly longer (6.93 ± 1.11 vs 4.18 ± 1.12 days; $t = -15.57$, df = 158, $P < 0.001$; Fig. 2C; Table 2). Post-mummification emergence was significantly lower in Hd+ than Hd− containers (59.78% vs 90.22%; LR $\chi^2 = 406.62$, df = 1, $P < 0.001$; Fig. 2D; Table 1). The number of emerged adults and adult emergence per initially exposed aphid are shown in Supplementary Figs. S2B and S3B, respectively; adult emergence per initially exposed aphid was 16.72% in Hd+ and 69.22% in Hd− containers. Aphid survival was 72.03% in Hd+ and 23.28% in Hd− containers (Supplementary Fig. S4B).

Sex Ratio

Female sex ratio did not differ significantly between Hd+ and Hd− treatments in either parasitoid species. In *A. colemani*, females accounted for 58.16% of emerged adults in Hd+ containers and 57.20% in Hd− containers (LR $\chi^2 = 0.51$, df = 1, $P = 0.474$; Fig. 3A; Table 1). In *P. volucre*, the corresponding proportions were 65.98% and 66.25% (LR $\chi^2 = 0.03$, df = 1, $P = 0.866$; Fig. 3B; Table 1).

Body Size

Adult body size did not meet the Bonferroni-corrected significance threshold in either parasitoid species. In *A. colemani*,

Table 1. Effect of the facultative symbiont *Hamiltonella defensa* on cowpea aphids (*Aphis craccivora*) feeding on alfalfa and parasitized by *Aphidius colemani* Viereck and *Praon volucre* (Haliday) (Braconidae: Aphidiinae). Proportional responses were analyzed using beta-binomial regression

Parasitoid species	Response variable	Model	Effect estimate (OR, Hd–/Hd+)	df	LR χ^2	*P*	Bonferroni $\alpha = 0.0036$
Aphidius colemani	Parasitism success	Beta-binomial regression	OR = 1.01	1	0.07	0.788	ns
	Post-mummification emergence	Beta-binomial regression	OR = 1.08	1	1.99	0.158	ns
	Female sex ratio	Beta-binomial regression	OR = 0.96	1	0.51	0.474	ns
Praon volucre	Parasitism success	Beta-binomial regression	OR = 8.49	1	607.10	<0.001	Significant
	Post-mummification emergence	Beta-binomial regression	OR = 6.21	1	406.62	<0.001	Significant
	Female sex ratio	Beta-binomial regression	OR = 1.01	1	0.03	0.866	ns

Hd+ = cowpea aphids naturally infected with *H. defensa*; Hd– = *H. defensa*-free; OR = odds ratio (Hd–/Hd+); LR χ^2 = likelihood-ratio chi-square; df = degrees of freedom; $N = 80$ independent replicate containers per treatment. "Significant" indicates $P < 0.0036$; "ns" indicates not significant.

Table 2. Effect of the facultative symbiont *Hamiltonella defensa* in cowpea aphids (*Aphis craccivora*) feeding on alfalfa and parasitized by Aphidius *colemani* Viereck and *Praon volucre* (Haliday) (Braconidae: Aphidiinae). Developmental time and body size responses were analyzed using linear models (LM)

Parasitoid species	Response variable	Model	Mean difference Δ (95% CI)	df	*P*	*T*	Bonferroni $\alpha = 0.0036$
Aphidius colemani	Time to mummification (days)	LM	Δ = –0.94 (–1.19 to –0.68)	158	<0.001	–7.32	Significant
	Mummification-to-emergence interval (days)	LM	Δ = 0.30 (–0.07 to 0.67)	158	0.114	1.59	ns
	Female body size (mm)	LM	Δ = 0.00 (–0.06 to 0.06)	158	0.938	0.08	ns
	Male body size (mm)	LM	Δ = –0.01 (–0.06 to 0.03)	158	0.572	–0.57	ns
Praon volucre	Time to mummification (days)	LM	Δ = –2.25 (–2.49 to –2.01)	158	<0.001	–18.91	Significant
	Mummification-to-emergence interval (Days)	LM	Δ = –2.75 (–3.10 to –2.40)	158	<0.001	–15.57	Significant
	Female body size (mm)	LM	Δ = –0.04 (–0.07 to –0.01)	158	0.020	–2.35	ns
	Male body size (mm)	LM	Δ = –0.02 (–0.07 to 0.02)	158	0.268	–1.11	ns

Hd+ = cowpea aphids naturally infected with *H. defensa*; Hd– = *H. defensa*-free; LM = linear model; Δ = mean difference (Hd– – Hd+); df = degrees of freedom. $N = 80$ independent replicate containers per treatment. "Significant" indicates $P < 0.0036$; "ns" indicates not significant.

female body length ($t = 0.08$, df = 158, $P = 0.938$; Fig. 4A; Table 2) and male body length ($t = -0.57$, df = 158, $P = 0.572$; Fig. 4B; Table 2) showed no supported treatment effect. In *P. volucre*, females emerging from Hd+ aphids were numerically larger than those emerging from Hd– aphids ($t = -2.35$, df = 158, $P = 0.020$; Fig. 4C; Table 2), but this contrast did not meet the Bonferroni-corrected significance threshold of $\alpha = 0.0036$. Male body length also did not meet the corrected threshold ($t = -1.11$, df = 158, $P = 0.268$; Fig. 4D; Table 2).

Discussion

The study of the endosymbiont *H. defensa* in aphids reveals a complex interaction between defensive symbionts, aphid hosts, and parasitoid wasps. This intricate relationship holds substantial ramifications for the efficacy of biological control strategies (Vorburger 2018, Wu et al. 2024). It is established that *H. defensa* confers protection to aphids from parasitoid attacks by producing toxins that disrupt the development of parasitoid larvae (Oliver et al. 2005). However, these protective phenotypes are limited to *H. defensa* strains that harbor specific APSE bacteriophages, which are essential for conferring resistance to parasitoid attack (Degnan and Moran 2008, Oliver et al. 2009, Weldon et al. 2013, Lynn-Bell 2021). Nevertheless, this protective effect is highly specific, depending on both the strain of *H. defensa* and the identity of the aphid and parasitoid species involved (Dykstra et al. 2014, Oliver and Higashi 2019). The present study provides further evidence of this specificity. *H. defensa* reduced the parasitism success of *P. volucre* in cowpea aphids, whereas no comparable effect was detected against *A. colemani*. This contrast was also reflected in cowpea aphid survival following parasitoid exposure. Cowpea aphid survival was similar in Hd+ and Hd– containers following exposure to *A. colemani*, consistent with the similar parasitism success observed between treatments. Similar patterns have been reported in other systems, where certain parasitoid species are able to overcome or tolerate symbiont-mediated defenses (Dykstra et al. 2014, Oliver and Higashi 2019).

In *P. volucre*, aphid survival was higher in Hd+ than Hd– containers, consistent with the lower parasitism success observed

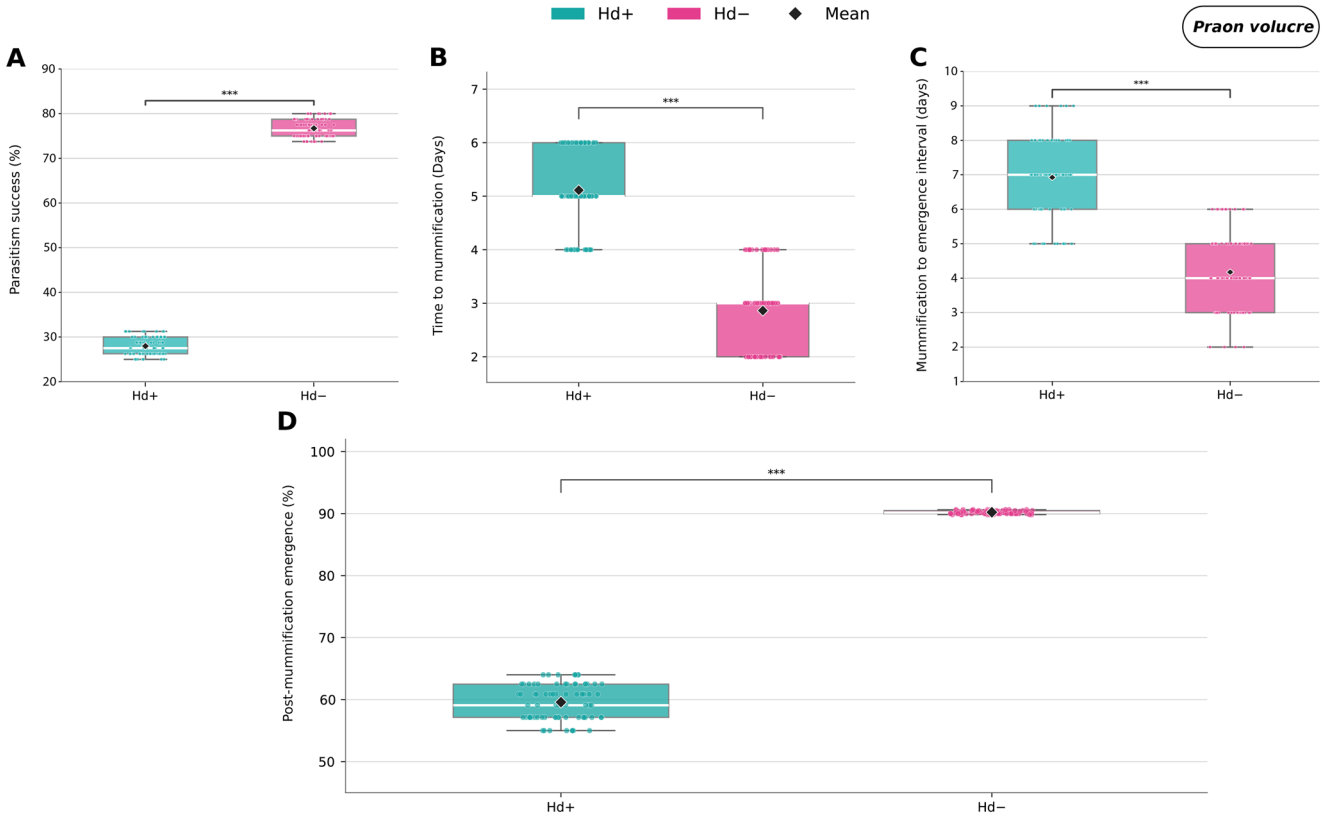

Fig. 2. Effects of *Hamiltonella defensa* infection status on the performance of *Praon volucre* developing in *Aphis craccivora*. A) Parasitism success (mummified aphids/80 exposed aphids), B) time to mummification (days), C) mummification-to-emergence interval (days), D) post-mummification emergence (emerged adults/mummies). Hd+ = aphids naturally infected with *H. defensa*; Hd− = *H. defensa*-free aphids. *** = statistically significant differences between treatments ($P < 0.0036$), NS = a non-statistically significant ($P \geq 0.0036$).

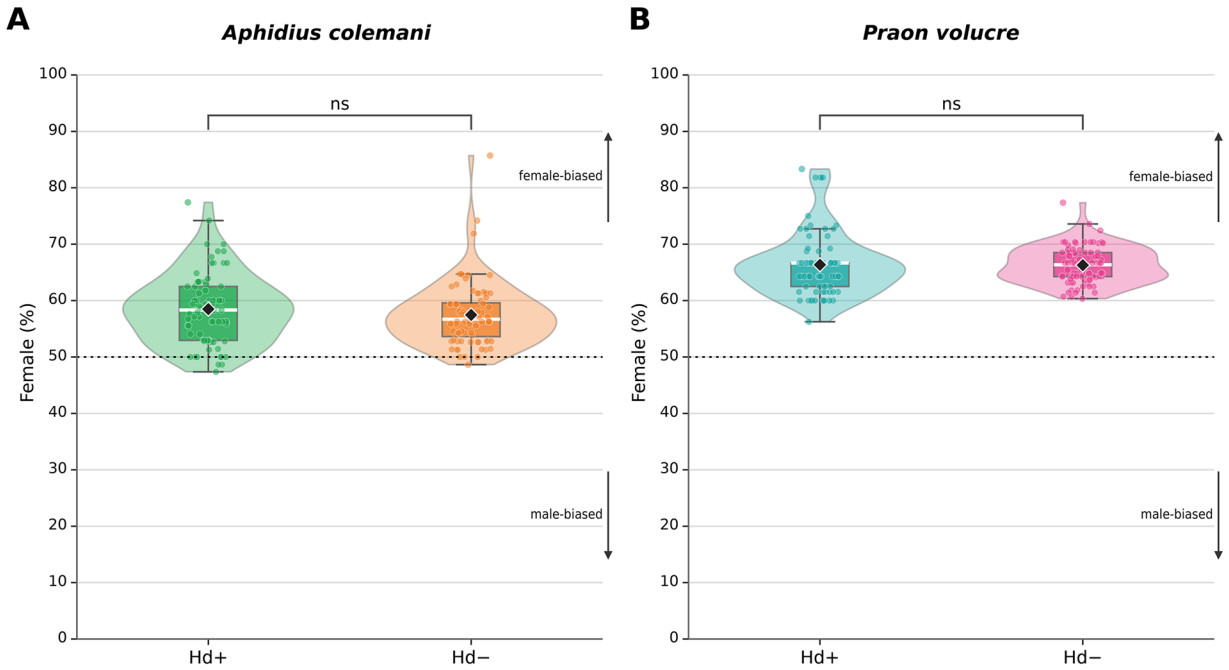

Fig. 3. Sex ratio of Aphidiinae parasitoid wasps emerged from cowpea aphids (female), *Aphis craccivora* Koch (Hemiptera: Aphididae) under 2 treatment conditions: Hd+ (naturally infected with *H. defensa*) and Hd− (*H. defensa*-free). A) *Aphidius colemani* Viereck, B) *Praon volucre* (Haliday). The dotted 50% line denotes a balanced (1:1) sex ratio; values above and below indicate female- and male-biased sex ratios, respectively. ns = non-statistically significant differences between treatments ($P \geq 0.0036$).

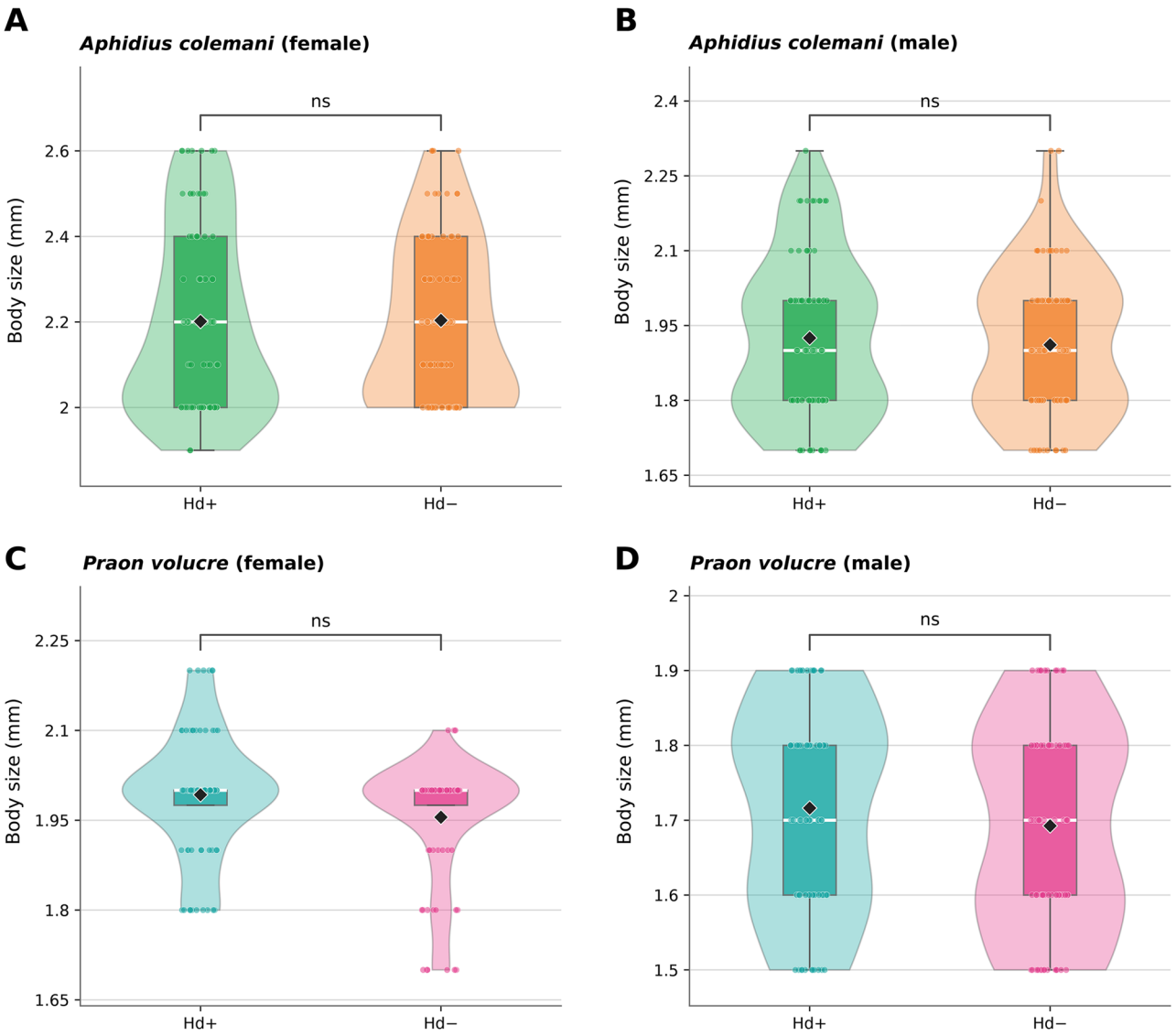

Fig. 4. Body size (mm) of adult female and male Aphidiinae parasitoids emerged from cowpea aphids, *Aphis craccivora* Koch (Hemiptera: Aphididae) under 2 treatment conditions: Hd+ (naturally infected with *H. defensa*) and Hd− (*H. defensa*-free). A-B) *Aphidius colemani* Viereck. A) female. B) Male. C-D) *Praon volucre* (Haliday). C) Female. D) Male. ns = non-statistically significant differences between treatments ($P \geq 0.0036$).

in Hd+ aphids, in line with the lower mummification and adult emergence recorded for this parasitoid. The presence of *H. defensa* was associated with a prolonged parasitoid development, including a longer period between mummification and adult emergence of *P. volucre*. These developmental delays were accompanied by a marked reduction in parasitoid success. Adult emergence of *P. volucre* was more than 4-fold lower from Hd+ than from Hd− cowpea aphids. APSE-associated toxins have been shown in other systems to interfere with parasitoid development (Oliver et al. 2009, Weldon et al. 2013). However, because APSE was not manipulated separately, the results are considered at the level of the *H. defensa*—APSE association, rather than as evidence for an independent APSE effect.

Body size is often linked to parasitoid performance and fitness, including host-searching ability and reproductive potential (Godfray 1994, Segoli and Rosenheim 2015). We therefore expected parasitoids emerging successfully from *H. defensa*-protected aphids to differ in body size from those emerging from Hd− aphids. However, no body-size contrast met the Bonferroni-corrected significance threshold. Female *P. volucre* emerging from Hd+ aphids were slightly larger on average, but this difference did not meet the corrected threshold. Overall, these results provide no supported evidence that *H. defensa* affects adult parasitoid body size.

Our results for *A. colemani* are consistent with previous studies showing little or no protection by *H. defensa* against this parasitoid (Asplen et al. 2014). In contrast, we found a strong reduction in the success of *P. volucre* in Hd+ *A. craccivora*. To our knowledge, this is the first experimental evidence of *H. defensa*-associated protection against *P. volucre* in this aphid species. Notably, McLean et al. (2020) found no evidence of protection against *P. volucre* in *Acyrthosiphon pisum* across 5 genetically diverse *H. defensa* strains. This contrast may reflect differences among *H. defensa*—APSE associations, aphid host backgrounds, or specific host × symbiont interactions that modify protection against the same parasitoid. Because the *H. defensa* strain and APSE variant in our study were not characterized, these alternatives cannot be

distinguished. Recent evidence further supports the importance of symbiont genotype and APSE identity in determining protection against *Praon* species. In pea aphids (*Acyrthosiphon pisum*), Patel et al. (2026) showed that a C11 strain of *H. defensa* carrying APSE11 conferred protection against *Praon pequodorum*, whereas a closely related C9 strain protected against *Aphidius ervi* but not against *P. pequodorum*. Furthermore, the loss of APSE11 eliminated protection against *P. pequodorum*, providing strong evidence that APSE-associated factors can determine resistance against at least some *Praon* species. The *H. defensa*—APSE association in our *A. craccivora* population substantially reduced the success of *P. volucre*. However, this effect should not be generalized to the *P. volucre–A. craccivora* system as a whole, because the *H. defensa* strain, APSE variant, and broader genetic diversity of the parasitoid lines were not characterized. Given the strong strain- and line-specificity of symbiont-mediated protection, broader sampling will be needed to assess the generality of this effect. Because the 9 aphid lines were pooled into Hd+ and Hd− stocks, treatment effects should be interpreted as responses of the pooled stocks rather than as line-specific effects. Future studies maintaining the lines separately and including line identity in the analysis would help assess the consistency of these effects across aphid genetic backgrounds. Although propagation for 6 generations after antibiotic treatment likely reduced potential carry-over effects, residual physiological effects cannot be completely excluded. Nevertheless, diagnostic PCR before the bioassays verified the expected *H. defensa* status in both stocks, confirming that treatment identities remained stable after 6 generations.

Susceptibility of *P. volucre* to symbiont-mediated defense is also evident from other aphid–symbiont systems. McLean et al. (2020) found that most *Spiroplasma* strains examined in pea aphids conferred strong protection against *P. volucre*, substantially reducing parasitoid success. This result is particularly relevant because it shows that susceptibility of *P. volucre* is not limited to a single defensive symbiont or aphid system. Rather, multiple facultative symbionts can impair the performance of this parasitoid, although the magnitude and mechanism of protection may differ among symbiont strains and host backgrounds.

The absence of an effect of *H. defensa* on the sex ratio of *P. volucre* indicates that symbiont-mediated protection was not sex-specific. Although *H. defensa* reduced parasitoid emergence and increased aphid survival, the proportion of male and female offspring remained unchanged. Thus, the protective effect appeared to affect both sexes similarly.

In contrast, *H. defensa* did not provide detectable protection against *A. colemani* parasitism, as evidenced by the absence of significant effects on parasitoid emergence, sex ratio, and adult body size. However, parasitoids developing in Hd+ cowpea aphids required more time to reach mummification than those developing in antibiotic-treated cowpea aphids, indicating that *H. defensa* and its associated APSE bacteriophage impose developmental costs on *A. colemani* despite having little effect on overall parasitoid success. Prolonged development may reduce parasitoid fitness by increasing generation time and delaying reproduction, although the demographic consequences of this delay were not evaluated in the present study. The response patterns observed for the 2 parasitoid species are consistent with the context-dependent nature of symbiont-mediated defense. Whereas *P. volucre* experienced substantial reductions in emergence and increased host survival, *A. colemani* appeared largely capable of completing development successfully at the cost of a modest developmental delay. One possible explanation is that *A. colemani* possesses physiological or evolutionary adaptations that reduce its susceptibility to *H. defensa*-associated defenses. Similar patterns of tolerance have been reported in parasitoid lineages exposed to defensive symbionts over evolutionary time (Dion et al. 2011). However, because the mechanisms underlying this apparent tolerance were not investigated, this hypothesis remains speculative. Together, these findings suggest that symbiont-mediated defense can affect different parasitoid species through distinct fitness components, ranging from severe reductions in survival to more subtle developmental costs.

The effects of *H. defensa* extended beyond reducing parasitoid success and included changes in developmental performance. Both *A. colemani* and *P. volucre* required more time to form mummies when developing in cowpea aphids naturally infected with *H. defensa*, indicating that the symbiont can impose sublethal fitness costs even when parasitoid development is ultimately completed. Such delays may have ecological consequences because longer developmental times increase generation length and can reduce the rate of parasitoid population growth. These developmental costs were particularly evident in *P. volucre*, for which the mean interval between mummification and adult emergence was prolonged by 2.75 days (65.90%) in Hd+ aphids. This prolonged developmental period, combined with the marked reduction in parasitoid emergence, suggests that *H. defensa* strongly interferes with the development of this species. In contrast, although *A. colemani* also required more time to form mummies, its emergence success, sex ratio, and adult body size were unaffected. These results suggest that *A. colemani* can largely complete development in *H. defensa*-infected aphids despite experiencing a developmental delay. Thus, the 2 species showed different response patterns: *A. colemani* was mainly affected in time to mummification, whereas *P. volucre* showed strong effects on parasitism success and post-mummification emergence. The ability of *A. colemani* to complete development in Hd+ aphids could enhance its effectiveness as a biological control agent in aphid populations where defensive symbionts are common, although this hypothesis should be evaluated under field conditions.

The specificity of *H. defensa*-mediated protection highlights the complex coevolutionary dynamics that can arise among aphids, defensive symbionts, and parasitoids (Degnan and Moran 2008). Several studies have shown that some parasitoid species are able to overcome or tolerate *H. defensa*-mediated defenses, often following prolonged evolutionary association with symbiont-protected hosts. For example, *Aphelinus certus* (Chalcidoidea) exhibits reduced susceptibility to *H. defensa* compared with other *Aphelinus* species (Hopper et al. 2018). Experimental evolution studies have shown that an *Aphidius ervi* population exposed to *H. defensa*-protected *A. pisum* evolved increased virulence, and eventually achieved parasitism rates comparable to those observed in unprotected hosts, but at the cost of reduced adult body size (Dion et al. 2011). This example demonstrates how defensive symbionts can act as important selective agents, driving the evolution of parasitoid resistance or tolerance over time. The reduced effectiveness of some introduced parasitoid species in combating *H. defensa*-protected aphids has likewise been attributed to a lack

of prior evolutionary association with symbiont-bearing hosts (Wu et al. 2022). In the present study, both aphids and parasitoids were field collected from natural populations, yet the 2 parasitoid species showed different response patterns to *H. defensa* and its associated APSE bacteriophage. Whereas *A. colemani* retained its parasitism ability, *P. volucre* experienced substantial reductions in emergence and prolonged development. These different outcomes may reflect differences in their susceptibility to symbiont-mediated defenses as a consequence of their ecological and evolutionary histories. However, because the present study did not directly examine local adaptation or evolutionary responses, alternative explanations, including species-specific physiological differences in susceptibility to *H. defensa*-associated defenses, cannot be excluded.

Adding further complexity to these interactions, the prevalence of *H. defensa* varies considerably among aphid populations and is influenced by a range of ecological and evolutionary pressures. By collecting aphids and their parasitoids from geographically proximate populations within the same climatic region, the present study reduced the likelihood that observed differences were driven primarily by environmental or geographical variation. Consequently, the contrasting response patterns observed for *A. colemani* and *P. volucre* may reflect biological differences in their interactions with *H. defensa* and its associated APSE bacteriophage. The ecological consequences of these interactions are further shaped by the costs and benefits associated with symbiont infection. Although *H. defensa* can provide substantial protection against susceptible parasitoids, symbiont carriage may also impose fitness costs on aphids, including reduced reproductive performance and imperfect vertical transmission to offspring in the absence of parasitoid pressure (Gimmi and Vorburger 2021). As a result, the prevalence of *H. defensa* in natural populations is likely shaped by the balance between the benefits and costs of symbiont infection, together with other ecological factors. Although parasitoid pressure may contribute to these dynamics, field evidence suggests that its effect can be limited. Smith et al. (2021) found no significant relationship between parasitoid abundance and *H. defensa* prevalence in natural populations, while temperature was the strongest predictor of symbiont prevalence.

The ecological consequences of *H. defensa* extend beyond the pairwise aphid–parasitoid interactions and may influence higher-order trophic dynamics. By reducing the success of susceptible parasitoid species, symbiont infection has the potential to weaken natural biological control and thereby alter aphid population regulation under conditions where defensive symbionts are prevalent. However, the magnitude of these effects is expected to depend strongly on the composition of the parasitoid community, particularly on the presence of species capable of developing successfully in symbiont-harboring hosts. From an applied perspective, these findings suggest that the effectiveness of biological control programs may vary depending on the compatibility between parasitoid species and symbiont-infected aphid populations (Brandt et al. 2017, Oliver and Higashi 2019). However, such implications remain contingent on field-level validation of parasitoid performance and aphid population dynamics. Therefore, rather than prioritizing specific species a priori, a more robust approach would involve selecting parasitoids based on demonstrated performance against *H. defensa*-infected hosts. *Aphidius colemani* and *P. volucre* belong to different aphidiine tribes and differ in host-range breadth (Sanchis et al. 2000, Rakhshani et al. 2019; for details of the *COI*-Based molecular identification of Aphidiinae and host records, see Supplementary Section S1 and Supplementary Fig. S5), but whether these ecological differences influence responses to *H. defensa* remains unclear.

In summary, *H. defensa* can influence the effectiveness of biological control by reducing the susceptibility of aphids to certain parasitoid species. However, its effects are parasitoid-specific rather than universal, highlighting the importance of considering defensive symbionts when evaluating parasitoid performance and predicting biological control outcomes (Oliver and Higashi 2019, Gimmi et al. 2023).

Conclusion

This study provides new insights into the interactions among *A. craccivora*, the *H. defensa*—APSE association, and 2 aphidiine parasitoids. In *A. colemani*, the *H. defensa*—APSE association mainly affected developmental timing. In *P. volucre*, it was associated with substantially reduced parasitism success and post-mummification emergence, together with prolonged development. These findings have important implications for biological control, as parasitoid effectiveness may be influenced by the occurrence of defensive symbionts within aphid populations. A better understanding of the ecological and evolutionary factors underlying variation in susceptibility to symbiont-mediated defenses will improve predictions of biological control outcomes and support the development of more effective and sustainable pest management strategies.

Acknowledgements

The authors wish to express their profound gratitude to the following individuals for their invaluable contributions in the form of advice and insights: Kerry Oliver (University of Georgia, USA), Jan Hrček, Eva Nováková and Anna Mácová (Biology Centre CAS, Czech Republic), Keith Hopper (United States Department of Agriculture, USA), Ailsa McLean (University of Oxford, UK), Christoph Vorburger (ETH Zürich, Switzerland), Julia Ferrari (University of York, UK), Olivier Duron (FNCS, France), and Ehsan Rakhshani (University of Zabol, Iran). We sincerely thank the Subject Editor, Rachid Hanna, and the 3 anonymous reviewers for their constructive and valuable comments, which have greatly improved the manuscript.

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[equal]), Chawatat Thanoosing (Formal analysis [equal], Funding acquisition [equal], Project administration [equal], Resources [equal], Validation [equal], Writing—review & editing [equal]), Xue-xin Chen (Methodology [equal], Resources [equal], Writing—review & editing [equal]), Buntika Areekul Butcher (Methodology [equal], Resources [equal], Writing—review & editing [equal]), and Natapot Warrit (Formal analysis [equal], Funding acquisition [equal], Project administration [equal], Resources [equal], Supervision [equal], Validation [equal], Writing—review & editing [equal])

Supplementary Material

Supplementary material is available at *Journal of Insect Science* online.

Funding

M.H.L. was financed by the Second Century Fund (C2F), Chulalongkorn University, Thailand. C.T. is financed by the New Faculty Funding, Ratchadapisek Somphot Fund, Chulalongkorn University (DNS_67_082_2300_012). T.H. received funding from SPW-Feder-Interreg for the TransPest project (grant number 0100226).

Conflicts of Interest

The authors declare no conflict of interest.

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https://doi.org/10.1093/jisesa/ieag102
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