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ORIGINAL ARTICLE

An aphid symbiont confers protection against a specialized RNA virus, another increases vulnerability to the same pathogen

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Abstract

Insects often harbour heritable symbionts that provide defence against specialized natural enemies, yet little is known about symbiont protection when hosts face simultaneous threats. In pea aphids (*Acyrthosiphon pisum*), the facultative endosymbiont *Hamiltonella defensa* confers protection against the parasitoid, *Aphidius ervi*, and *Regiella insecticola* protects against aphid-specific fungal pathogens, including *Pandora neoaphidis*. Here, we investigated whether these two common aphid symbionts protect against a specialized virus *A. pisum virus* (APV), and whether their antifungal and antiparasitoid services are impacted by APV infection. We found that APV imposed large fitness costs on symbiont-free aphids and these costs were elevated in aphids also housing *H. defensa*. In contrast, APV titres were significantly reduced and costs to APV infection were largely eliminated in aphids with *R. insecticola*. To our knowledge, *R. insecticola* is the first aphid symbiont shown to protect against a viral pathogen, and only the second arthropod symbiont reported to do so. In contrast, APV infection did not impact the protective services of either *R. insecticola* or *H. defensa*. To better understand APV biology, we produced five genomes and examined transmission routes. We found that moderate rates of vertical transmission, combined with horizontal transfer through food plants, were the major route of APV spread, although lateral transfer by parasitoids also occurred. Transmission was unaffected by facultative symbionts. In summary, the presence and species identity of facultative symbionts resulted in highly divergent outcomes for aphids infected with APV, while not impacting defensive services that target other enemies. These findings add to the diverse phenotypes conferred by aphid symbionts, and to the growing body of work highlighting extensive variation in symbiont-mediated interactions.

KEYWORDS
Acyrthosiphon pisum virus, defensive symbiosis, endosymbiont, *Hamiltonella defensa*, heritable symbiont, pea aphid, *Regiella insecticola*, symbiont

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1 | INTRODUCTION

Aphids (Hemiptera: Aphididae) are plant sap-feeding insects that interact with a range of microbial mutualists and pathogens. Almost all aphid species carry the obligate nutritional symbiont, *Buchnera aphidicola*, which is maternally transmitted and upgrades the nutritional profile of plant phloem (Douglas, 2009). Nine facultative, heritable symbionts have also been identified in different species that can confer conditional benefits including defence against parasitic wasps and fungal pathogens (Guo et al., 2017; Oliver et al., 2010; Oliver & Martinez, 2014; Vorburger, 2014). Aphids vector a number of plant viruses (Brault et al., 2010) and are infected by a number of other viruses including several single-stranded (ss) DNA viruses in the family Parvoviridae (Piccovirales) and positive-sense ssRNA viruses in the families Picornaviridae and Dicistroviridae (Picornavirales; Asgari & Johnson, 2010; Liu et al., 2016; Moon et al., 1998; Ryabov, 2007; Ryabov et al., 2009; Van der Wilk et al., 1997; van Munster et al., 2003). However, little is known about the interactions among the many protective symbionts in aphids and pathogenic viruses. In addition to harming aphid fitness directly, viruses may modulate symbiont-mediated protection. In turn, aphid facultative symbionts potentially confer resistance to viruses or lessen associated costs as seen for strains of *Wolbachia* in *Drosophila* that protect against RNA viruses (Hedges et al., 2008; Pietri et al., 2016).

Acyrthosiphon pisum virus (APV), is a picorna-like virus that persistently infects the pea aphid, *Acyrthosiphon pisum* (Van den Heuvel et al., 1997). The 10 kb, positive-stranded RNA genome of APV contains two open reading frames (P1, P2) encoding a protease, helicase, RNA-dependent RNA polymerase and capsid protein (Van der Wilk et al., 1997). While primarily detected in epithelial cells of the gut and salivary glands, APV has also been weakly detected in the ovaries of pea aphids (Lu et al., 2020; Van den Heuvel et al., 1997). APV is vertically transmitted at moderate rates, and can be horizontally transferred from aphids to plants (Lu et al., 2020; Van den Heuvel et al., 1997), but no studies have shown aphid acquisition of APV from plants. APV exerts variable effects on aphid growth, survival and reproduction (Lu et al., 2020; Van den Heuvel et al., 1997), but how pea aphid genotype and facultative symbionts influence APV infection and transmission has not previously been investigated.

Hamiltonella defensa (Yersiniaceae: γ-Proteobacteria) is one of the most studied facultative symbionts in pea aphids because certain strains confer high levels of resistance against parasitoid wasps like *Aphidius ervi* (Hymenoptera: Braconidae; Oliver & Higashi, 2019). Protective strains of *H. defensa* further host specific variants of a bacteriophage named APSE which as a provirus expresses virulence genes that have been implicated in disabling parasitoid development (Boyd et al., 2021; Brandt et al., 2017; Chevignon et al., 2018; Lynn-Bell et al., 2019; Oliver et al., 2009; Rouïl et al., 2020). *H. defensa* strains infected by APSE-3 confer high levels of protection (>85% of parasitized aphids survive) while strains infected by APSE-2 or APSE-8 provide moderate protection (40%–60%; Oliver & Higashi, 2019; Weldon et al., 2013). Aphid genotype also contributes to resistance to parasitoids (Martinez et al., 2018). Another facultative symbiont associated with pea aphids, *Regiella insecticola*, is closely related to *H. defensa* (Patel et al., 2019) and confers protection against specialist entomopathogenic fungi like *Pandora neoaphidis* (Entomophthorales; Parker et al., 2013; Scarborough et al., 2005). Levels of protection conferred by *R. insecticola* also vary with symbiont strain and host genotype (Parker et al., 2017).

We recently discovered APV in certain pea aphid lines maintained in our laboratory. We produced five new APV genomes and investigated virus transmission to better understand the basic biology of this pathogen. To elucidate interactions between APV and common aphid facultative symbionts, we examined a range of fitness parameters in the presence and absence of enemy challenge by levering genetically homogeneous lines that controlled aphid and *Buchnera* genotypes while manipulating facultative symbiont presence and APV infection. We report that APV adversely affected the fitness of aphids lacking facultative symbionts, effects which were worsened in aphid carrying *H. defensa*. In contrast, negative fitness impacts were ameliorated in aphids hosting *R. insecticola*. Overall, our results identify heretofore unknown interactions between facultative symbionts and a pathogenic virus that strongly influence host fitness.

2 | MATERIALS AND METHODS

2.1 | Aphid, parasitoid and fungus cultures

Acyrthosiphon pisum is cyclically parthenogenetic which enables clonal lines to be produced and maintained in the laboratory with continued exposure to a long-day-length photoperiod. Lines used in this study were established from single, parthenogenetic females and reared as earlier described (Oliver et al., 2003) on *Vicia faba* Broad Windsor seedlings in Percival biological incubators under 20±1°C on a 16h light (L):8 h dark (D) photoperiod. Offspring of clonal line were regularly screened for all known pea aphid facultative symbionts using previously published PCR-based diagnostics (Martinez et al., 2014; Russell et al., 2013). An *A. ervi* culture was established from commercially produced (Syngenta Bioline Ltd.) and field-collected wasps from Dane County (WI, USA), which are maintained on susceptible pea aphid lines lacking facultative symbionts as previously reported (Oliver et al., 2003). Adult parasitoids were kept at 20±1°C under a 16h L: 8 h D h photoperiod and provided a diet of honey and water. *P. neoaphidis* genotype ARSEF 2588 used in this study originated from the USDA-ARS Collection of Entomopathogenic Fungal Culture and was maintained on susceptible aphid lines (WI-48 and 5D-AB). Desiccated fungal cadavers were stored at 4°C with low humidity for no more than twelve weeks within an airtight container. Prior to experimental use, cadavers were rehydrated to induce sporulation (described in later section).

2.2 | APV identification and sequencing

Acyrthosiphon pisum virus was first discovered in our laboratory during an RNAseq study that compared five clonal lines that hosted

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different strains of *H. defensa*. The RNAseq data set that originally identified APV in some of our cultures was used to assemble complete genomes for five of the infected laboratory lines. In brief, this data set was generated by extracting total RNA from fourth instar aphids from five clonal lines (three biological replicates consisting of five individual aphids per line) using the mirVana miRNA Isolation Kit (Ambion, Thermo Fisher Scientific). After DNase treatment using the TURBO DNA-free Kit (Ambion, Thermo Fisher Scientific) and ethanol precipitation in the presence of glycogen, RNA templates were quality checked by the Georgia Genomics and Bioinformatics Core using an Agilent 2100 Bioanalyser (Agilent Technologies) and Fragment Analyser (Advanced Analytical). cDNA libraries were then synthesized using the Kapa Stranded RNA-seq library preparation kit (Kapa Biosystems) and 75 bp paired-end sequenced using Illumina NextSeq (150 cycles). APV genomes were assembled by pooling *A. pisum* unaligned reads with APV reads for de novo assembly with SPAdes version 3.15.3 (Prjibelski et al., 2020) using the parameter rnaviral. The first APV genome that was sequenced (NC_003780; Van der Wilk et al., 1997) was used as a reference to identify APV contigs using Minimap2 version 2.22 with parameter "x splice" (Li, 2018). The resulting APV genomes were then aligned using MAFFT version 7.450 (Kuraku et al., 2013) in Geneious Prime version 2022.0.2 (https://www.geneious.com) with default parameters and compared to other sequenced APVs including: 1 from Europe (AF024514; Van der Wilk et al., 1997), six from China (MH301282–MH301287) and four related RNA viruses; YYSMMV1 (*Sitobion miscanthi* virus 1; MK733235), two rosy apple aphid virus isolates (Riboviria; DQ286292, MW929927), and one avian-associated Riboviria (MT138201). The same viruses were also used to construct a maximum-likelihood phylogenetic tree using PhyML version 3.3.20180621 (Guindon et al., 2010) implemented in Geneious Prime (version 2022.0.2 https://www.geneious.com) with substitution model HK85 and 1000 bootstraps.

Following the discovery of APV, we established a diagnostic real-time quantitative PCR (qPCR) assay for rapid, routine screening of APV in all laboratory-held aphid lines. RNA was extracted from whole aphids using a modified protocol for virus detection that bypasses the RNA isolation step (Xu et al., 2017). Briefly, individual aphids from each line were chilled on ice for 5 min and homogenized with a sterile pipette tip in lysis buffer (10 mM Tris-Cl pH 8.2, 1 mM EDTA, 25 mM NaCl) in 0.2 ml tubes. Samples were then centrifuged at 4°C for 20 min at 2200 *g*. After centrifugation, 11 μl of supernatant was transferred to new 0.2 ml tubes which served as a template for complimentary DNA (cDNA) synthesis using SuperScript IV First-Strand Synthesis Reaction Kit (Invitrogen) following the manufacturer's protocol. We then conducted qPCR to detect APV with primers APV P1 diagF (5′ ACCATCCGAACTTGAACAGG 3′); APV P1 diagR (5′ TGAAAGAACAACGCCTGTGA 3′) designed from the AS3-AB APV genome (see above) that are diagnostic for this virus. Ten microlitre reactions were run using 5 μl of Quantabio PerfeCTa SYBR Green FastMix chemistry, 0.5 μM of each primer, 2 μl of $H_2O$and 1 μM of cDNA template on an Analytik Jena qTower[3] thermal cycler. For all PCR reactions, cDNA from APV+ aphids was used as template representing positive controls, while cDNA from APV− aphids were used as template for negative controls. We also used water as a separate "no-template" negative control. Cycling conditions were 95°C for 5 min; 40 cycles of 95°C for 10 s, 58°C for 10 s, 72°C for 10 s, and a final extension at 72°C for 2 min. The primer amplification efficiency, determined through linear regression obtained through the amplification of serially diluted cDNA extracts was determined to be 1.10 for this reaction as calculated using the formula $10^{(-1/\text{slope})}$. Reaction specificity was confirmed using a melting curve analysis at the end of each qPCR run.

For all laboratory colonies testing positive ($N = 12$) for APV using our qPCR diagnostics, we next PCR amplified and Sanger sequenced an ~890 bp region to examine sequence variation among APV isolates. We used primers that amplified a variable portion of the P1 ORF (APVp1seqF 5′ GATTGCGGTTTTCCAT TTGT 3′; APVp1seqR 5′ GGGGTTTTGCCCTATAGCAT 3′). PCRs were carried out in a 30 μl mixture using EconoTaq Plus master mix (Lucigen) that included 15 μl of Taq polymerase, 0.3 μM of forward and reverse primers, 50 ng of template DNA and nuclease-free water up to 30 μl. The PCR conditions were 95°C for 3 min (1 cycle), 95°C for 30 s, 60°C for 30 s and 72°C for 1 min (35 cycles), followed by 72°C for 7 min. Resulting products were visually examined on 1% agarose gels and then purified using the cycle pure Kit (Omega Bio-Tek) before Sanger sequencing (Eurofins).

2.3 | Experimental aphid lines

We engineered aphid lines that varied in colour, facultative symbiont status, and APV infection while controlling for aphid and *Buchnera* genotypes (Table 1). Across five aphid genotypes, each naturally susceptible to *A. ervi* and *P. neoaphidis*, we first produced eight APV− lines that were either green or pink colour morphs that either lacked facultative symbionts or hosted *R. insecticola* or *H. defensa* (Doremus et al., 2018; Parker et al., 2013). Within each aphid genotype, sublines with facultative symbionts were generated by either microinjection (*H. defensa*) or selective curing with antibiotics (*R. insecticola*; Doremus & Oliver, 2017). For example, *H. defensa* carrying phage variant APSE-3 was transferred from donor line MM12 into the symbion and APV− recipient aphid line, ND18 (named ND18.H3) and *H. defensa*/APSE-2 was transferred from the donor line 82B into the APV− and symbiont-free aphid line 5D-AB (named 5D-AB.H2). We then infected aphids from each of the above lines with APV to produce a total of 16 lines (8 noninfected lines [APV−], and eight infected lines [APV+]; Table 1). APV was first established in line ND18 (ND18-APV+) from another infected line called AS3-AB, a commonly used aphid background. After successfully establishing and maintaining APV in ND18-APV+, it was used as the APV donor to subsequently infect all other experimental lines (Table 1). APV infections were established by either: (1) collecting haemolymph from APV+ aphids lacking facultative symbionts and injecting it into aphids from each of our APV− lines, or (2) allowing aphids from our APV− lines to feed on a diet inoculated with a homogenate prepared

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TABLE 1 Experimental aphid lines established for use in this study

Aphid line	Aphid colour	Symbiont donor or cured	Facultative symbiont status	Expected resistance to wasp (W) or fungus (F)	APV infection status	APV donor
ND18	Green		None	Low W	Negative	
ND18-APV+			None		Positive	AS3-AB
ND18.H3		MM12	*H. defensa*/APSE-3	High W	Negative	
ND18.H3-APV+		MM12	*H. defensa*/APSE-3		Positive	AS3-AB
5D-AB	Pink		None	Low W	Negative	
5D-AB-APV+			None		Positive	AS3-AB
5D-AB.H2		82B	*H. defensa*/APSE-2	Moderate W	Negative	
5D-AB.H2-APV+		82B	*H. defensa*/APSE-2		Positive	AS3-AB
LSR1-AB	Pink	Cured Ri	None	Low F	Negative	
LSR1-AB-APV+		Cured Ri	None		Positive	AS3-AB
LSR1.Ri			*R. insecticola*	High F	Negative	
LSR1.Ri-APV+			*R. insecticola*		Positive	AS3-AB
WI246-8	Pink		None	Low W	Negative	
WI246-8-APV+			None		Positive	AS3-AB
WI576N-27	Green		None	Low W	Negative	
WI576N-27-APV+			None		Positive	AS3-AB

Abbreviation: APV, *Acyrthosiphon pisum* virus.

from APV+ aphids (Van den Heuvel et al., 1997). For the latter, 8–10 fourth instar APV+ aphids were homogenized in 500 μl of aphid diet (Febvay et al., 1988) in a 1.5 ml centrifuge tube. The homogenate was then spun down at 6000 *g* for 1 min to remove aphid debris followed by mixing the supernatant with ~1 ml of aphid diet. The mixture was then sandwiched between two stretched parafilm layers on a 35 × 10 mm petri dish followed by the addition of 10–15 s instar uninfected aphids that were allowed to feed for 2 days. The aphids were then transferred to a fresh *V. faba* plant to develop into adults. Each line used in experiments was then generated from a single parthenogenetic female that was maintained for a minimum of eight generations before use in any assay. The presence of APV and expected facultative symbionts for each experimental line was tested throughout the line creation process and reconfirmed just prior to performing bioassays using diagnostic PCR, as described above, on ≥ three, individual third instar aphid nymphs.

2.4 | APV transmission assays

To measure maternal transmission rates and whether *H. defensa* impacts vertical transmission, we reared APV+ adult aphids of the same genotype with (ND18.H3) or without (ND18) *H. defensa* individually in petri dishes (55 × 15 mm) containing a single *V. faba* leaf. Adult aphids were monitored for the production of offspring approximately every 30 min. Since APV can be transferred from infected aphids to plants during feeding we replaced fava leaves every 2–3 h. Nymphs were collected 1–30 min after birth were then surface sterilized in a 1% bleach solution and transferred to a new fava leaf containing Petri dish. Newborn aphids were individually reared to adulthood to prevent possible aphid to aphid horizontal transmission through the leaves. We allowed the first-generation cohort to produce offspring and develop into third-fourth instar nymphs before screening for APV infection using the diagnostics previously described. Fisher's exact test was used to compare rates of maternal APV transmission among lines. To rule out rapid horizontal transmission in our Petri dish arenas that would potentially inflate estimates of vertical transmission, we also conducted a control assay, mimicking the conditions described above by allowing single APV+ adults to feed on a single *V. faba* leaf in a Petri dish. Adults were allowed to feed continuously for 1 h before removing them and any offspring they produced. We then added 8–10 s instar APV− aphids, which were allowed to feed for 30 min on the leaves previously fed upon by APV+ before being separated and reared individually in petri dishes with a fresh *V. faba* leaf. These aphids were then allowed to develop into fourth instars and screened by PCR for the presence of APV.

Horizontal transmission of APV through plants was assessed by placing a single *V. faba* in cup cages with three APV+ aphids (donor) and three APV− aphids (recipient) which were distinguished by using 4 different donor and recipient lines lacking facultative symbionts that differed in colour (pink or green morphs). Cup cage arenas were maintained at 20°C under 16 h L: 8 h D photoperiod. Eight third or fourth instar donor and recipient aphids were then collected after 1 or 3 weeks and screened by qPCR as described above to assess APV infection status. We also conducted assays to determine if oviposition by *A. ervi* could horizontally transfer APV from infected to uninfected aphids. A female *A. ervi* was allowed to oviposit into an APV+ aphid and then immediately moved to a separate arena and allowed to oviposit into three APV− aphids in rapid succession. The three parasitized APV− aphids were identified by the order in which

oviposition occurred and then placed into separate petri dishes with a single *V. faba* leaf. We allowed parasitized APV− recipient aphids to develop into fourth instars before screening them for APV infection as above.

2.5 | Fitness measures

Aphid fecundity in different lines of APV+ and APV− aphids was estimated by allowing cohorts of five fourth instar aphids to develop into adults on a single *V. faba* (equals 1 replicate). The number of offspring produced in each cup cage was carefully removed and counted every 3 or 4 days. In total, there were nine replicates for each aphid line. Aphid mortality was also recorded and used to assess 50% survivorship. Aphid reproduction was analysed using Analyses of Variance (ANOVA) with Tukey's HSD to compare means among aphid lines. Aphid survival data was fit to a lognormal distribution to estimate 50% survival time.

2.6 | Enemy challenge assays

Cohorts of 20 aphids that were 48–72 h old (second instars) were singly parasitized by a mated *A. ervi* female and then placed onto a fresh *V. faba* plant in a cup cage (=1 replicate). A total of eight replicates were conducted for each experimental aphid line (160 parasitized aphids per line). After parasitism, cup cages were maintained at 20°C under a 16 h L:8 h D photoperiod. Ten days post-parasitism, we recorded the number of aphids that survived, mummified (a pupating wasp), or both aphid and wasp died (dual mortality; Oliver et al., 2012). Results were then analysed by logistic regression analyses. Since parasitoid fitness is often linked to host health, we measured hind tibia length to estimate the size of *A. ervi* eclosing from APV+ and APV− aphids, which served as a proxy for wasp quality (Godfray & Godfray, 1994; van Lenteren, 2003). Then, 24 h old adult *A. ervi* were frozen overnight (approximately 16 h) at −20°C, dried at 60°C for 24 h before measuring hind tibia length using an Olympus SZX16 stereomicroscope equipped with CellSens software (version 1.4.1). Pairwise comparisons (*t*-test) were performed to compare tibia measurements taken from female *A. ervi* that emerged from parasitized APV+ and APV− aphids from the following paired lines WI246-8 & WI246-8APV+, WI576N-27 & WI576N-27APV+, and ND18 & ND18APV+ (Table 1). The measurements for female wasps that emerged from the following aphid lines 5D-AB, 5D-ABAPV+, 5D-AB.H2, and 5D-AB.H2APV+ was analysed by one-way analyses of variance (ANOVA) to compare mean tibia length between APV+ and APV− aphids with or without *H. defensa*/APSE-2.

To assess whether APV infection affects fungal protection conferred by *R. insecticola*, we challenged aphids with *P. neoaphidis* as previously described (Weldon et al., 2020). Ten cohorts of ten 9-day-old (early adult) aphids (total 100 aphids) from each *R. insecticola* experimental line (Table 1) were then exposed to two sporulating aphid cadavers placed in a 35 mm diameter deep Petri dish with 1.5% agar for 90 min. Fungal plates were inverted over aphids to mimic a natural spore shower and rotated every 15 min between replicates to normalize spore exposure. Each cohort was then placed onto a fresh *V. fava* plant and kept at 20°C with 100% humidity (via an unvented cup lid) for 24 h under 16:8 L:D hour light cycle. After 24 h, the unvented lid was replaced with a vented lid. Aphids were monitored every 24 h for 10 days post-exposure for aphid survival, dual mortality (aphid and pathogen), and fungal sporulation. The results were analysed using logistic regression.

2.7 | APV and symbiont abundance

To estimate symbiont abundance and APV RNA abundance, APV+ and APV− adult aphids from experimental lines were placed in separate cup cages with a fresh *V. faba* plant and allowed to reproduce for approximately 24 h. Thereafter, all adults were removed and offspring were allowed to develop. Aphids were then sampled at 2, 4, 8 and 16 days old. We generated APV cDNA templates from 6 to 8 individual aphids at each time point (biological replicates) as described above followed by duplicate qPCR (technical replication) for each sample using the APV-specific primers and reaction conditions as described above. APV RNA abundance per sample was then estimated by plotting the data against a standard curve generated by serial dilution of a plasmid containing the APV amplicon and normalized using a single copy aphid gene (*Ef-1α*). We used previously published protocols to estimate the relative genome copy number for *H. defensa* and our two APSE variants at the same time points using primers that amplify regions of the single-copy genes *dnaK* and P2 gene, respectively (Martinez et al., 2014; Weldon et al., 2013). Relative genome copy number for *R. insecticola* was estimated using primers designed from the genome of the LSR1 *R. insecticola* strain (#NZ_ACYF00000000) to amplify a portion of the *dnaK gene* (Reg_dnaK_Q_F: 5′-TGGTGCAGCAAAAAGTG AAG-3′ and Reg_dnaK_Q_R: 5′-CACCCATGGTTTCAATACCC-3′). *Cycle conditions for the R. insecticola primers were* 95°C for 5 min; 40 cycles of 95°C for 10 s, 60°C for 10 s, 72°C for 10 s, and a final extension at 72°C for 2 min. Whole aphid DNA extracted from symbiont infected (either *R. insecticola* or *H. defensa*) and symbiont-free colonies was used as positive and negative controls, respectively, and water was used as a no-template control. The amplification efficiency, as determined above, for this reaction was 110% and the relative abundance of each symbiont was then determined by the $2^{-(\Delta CT)}$ method (Livak & Schmittgen, 2001). Reaction specificity was confirmed using a melting curve analysis. Results were log10 transformed, and the distributions of symbiont titres in each experimental line at each time point were checked for normality using the Goodness-of-fit test. Transformed titres were then compared at each time point by *t*-test. Both analyses as well as all other statistical tests performed during the study were performed using JMP Pro version 14.0 (SAS Institute Inc.).

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3 | RESULTS

3.1 | Discovery of APV in several laboratory lines of pea aphids

We discovered that APV infected some laboratory-held aphid cultures through an RNAseq data set we had earlier generated (Chevignon et. al., 2021) in which a substantial portion of total reads (15%–26%) mapped to an APV genome in three clonal lines of *A. pisum* named AS3, AS3AB and ZA17 that hosted different strains of *H. defensa* (Table S1). Less than 0.2% of reads also mapped to APV in two other lines named A2C and NY26 that hosted other strains of *H. defensa* (Table S1). After developing a PCR-based diagnostic assay, rescreening confirmed infection of the AS3, AS3AB and ZA17 lines, but did not detect APV in the A2C or NY26 lines which suggested low level infection at the time we made the RNAseq libraries had been lost. PCR screening all the other aphid cultures in the laboratory indicated that 39% (23/59) carried APV infection. Sequencing a domain within the APV P1 open reading frame suggested the APVs present in our laboratory were very similar with only a few single nucleotide polymorphisms (SNPs) identified. The RNAseq data we generated further enabled us to assemble complete genomes for the APVs in the AS3, AS3AB, ZA17, A2C and NY26 lines. Alignment to other APV genomes in public databases showed high overall similarity (Figure S1). A distance matrix computed from amino acid sequences (Figure S2) and a maximum likelihood phylogeny (Figure 1) indicated the APVs from our laboratory were nearly identical to one another (>99.6%–100%) but less similar (<92.6%) to several APV isolates from China.

3.2 | APV is both vertically and horizontally transmitted

We first assessed the efficacy of vertical transmission by determining the proportion of offspring infected females produce that are also infected. Using APV+ aphids (ND18 genotype), we observed that 30%–40% of offspring each female produced carried the virus with no significant differences detected between aphids with or without *H. defensa* (Table S2A). This finding clearly indicated that maternal transmission occurs at moderate rates, but also showed most progeny were not infected. We thus examined two mechanisms for horizontal transmission. We first tested transmission from APV+ aphids to APV− aphids feeding on the same host plant using aphid lines that differed in colour, including recipient lines with *H. defensa* or *R. insecticola*. Half or more of the APV− aphids were infected after 1 week while nearly all were infected after 3 weeks (Table S2B). The presence of *H. defensa* or *R. insecticola* did not prevent aphid acquisition of APV. The second assay tested whether *A. ervi* could horizontally transmit APV by first ovipositing into APV+ aphids without

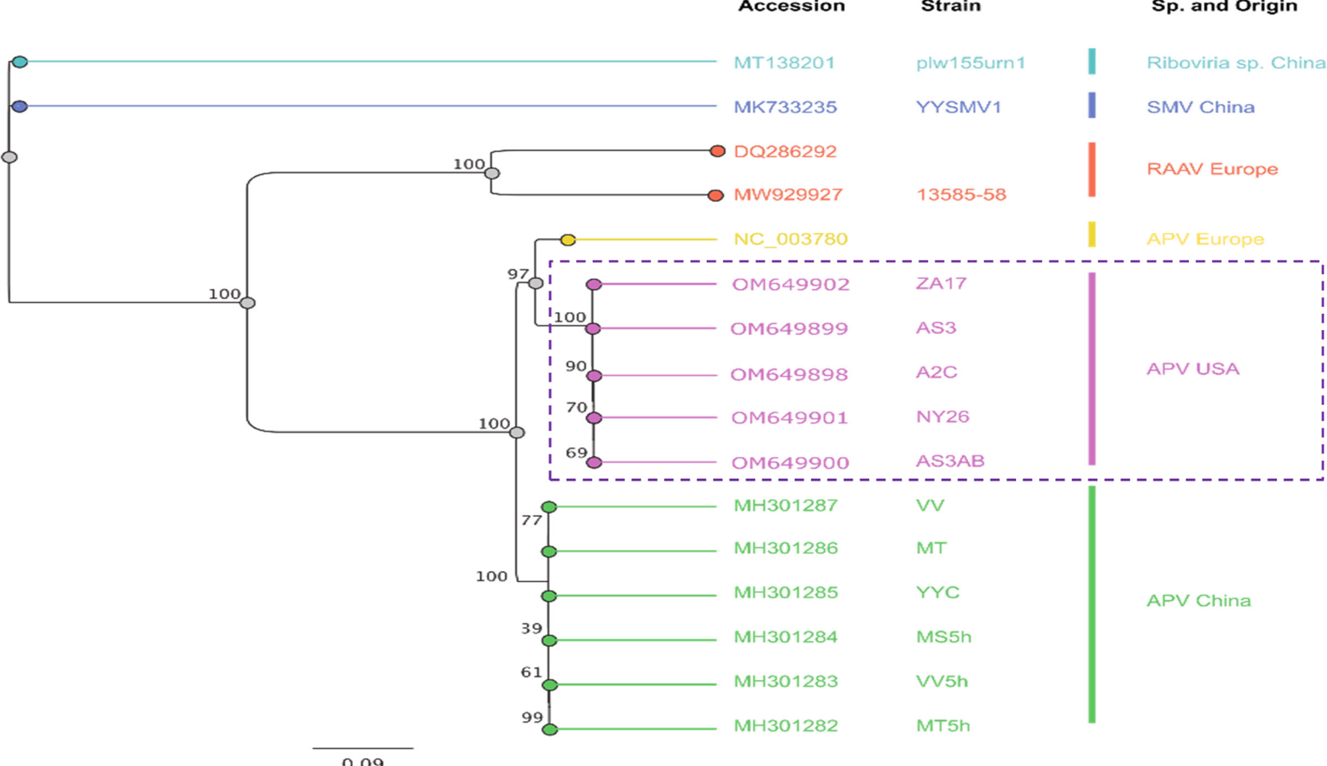

FIGURE 1 Maximum likelihood phylogeny of *Acyrthosiphon pisum virus* (APV) isolates previously reported in NCBI and those characterized in this study (outlined by the dashed box). Maximum-likelihood phylogenetic tree were constructed using PhyML version 3.3.20180621 implemented in Geneious prime (version 2022.0.2 https://www.geneious.com) with the substitution model HK85 and 1000 bootstraps (values are displayed next to nodes).

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facultative symbionts and then being allowed to oviposit into three APV− aphids with or without *H. defensa*. Only three of the 48 (6%) recipient aphids were infected: two ND18 aphids that had no facultative symbionts and one ND18.H3 aphid hosting *H. defensa*/APSE3 (Table S3). We also noted the order of attack (1–3) in recipient aphids which showed that each of the aphids that were infected by a wasp were first in the order of attack.

3.3 | *Regiella insecticola* reduces fitness costs associated with APV infection while *H. defensa* increases costs

Across all five aphid genotypes, lifetime fecundity assays showed that APV+ aphids without facultative symbionts produce fewer offspring than noninfected aphids (Table 2). APV infection also reduced aphid longevity in all genotypes except WI576N-27 (Table 2B). Since *R. insecticola* and *H. defensa* confer protection against specialized fungi and parasitoids (Parker et al., 2013; Scarborough et al., 2005), we next asked if either affected the fitness costs associated with APV infection. We found that aphids hosting *R. insecticola* (genotype LSR1) produced far more offspring and lived longer than control lines without *R. insecticola* when infected by APV (Table 2C). The cumulative fecundity of the *R. insecticola* subline with a persistent APV infection was nearly identical to that of the *R. insecticola* subline without APV. In the absence of APV, we did not observe significant reductions in fecundity or longevity in aphids harbouring *R. insecticola* compared to symbiont-free controls (Table 2C).

In contrast to *R. insecticola*, APV+ aphid that also hosted *H. defensa* exhibited significantly larger reductions in fecundity and longevity relative to isogenic aphids that hosted *H. defensa* alone or that had no facultative symbionts but were APV infected (Table 2D,E). The elevated costs observed in aphids carrying both microbes could result from additive (i.e., the cost of APV + the cost of *H. defensa*) or synergetic (i.e., super-additive) effects in which, for example, *H. defensa* presence worsens the costs of APV infection. We first quantified the average magnitude of direct effects (Higashi et al., 2020; Schmitz et al., 2000) which showed that the effect of housing both APV and *H. defensa* (E_{AB}) on aphid fecundity neared or exceeded the cost of housing the sum of each ($E_A + E_B$; Figure S3). To determine whether these effects were additive, synergistic or antagonistic, we examined effect magnitudes using a Bliss independence model ($E'_{AB} = E_A + E_B - E_A E_B$; Bliss, 1939; Coors & De Meester, 2008). We found that the 95% confidence interval values for aphids housing both microbes (E_{AB}) exceeded the Bliss expected values (E'_{AB}; red line Figure S3) for each isogenic aphid line with *H. defensa*, which is indicative of synergistic interactions. These analyses also indicated that *R. insecticola*/APV interactions were antagonistic (Figure S3), which is consistent with above analyses. Thus, *R. insecticola* reduced the fitness costs of APV infection while *H. defensa* probably increased them. In the absence of APV, both *H. defensa* lines exhibited reductions in fecundity and longevity when compared to controls without facultative symbionts (Table 2D,E).

3.4 | APV infection does not alter symbiont-mediated protection against other mortality agents

We next examined whether the protective effects of *R. insecticola* against *P. neophidis* or *H. defensa* against *A. ervi* were influenced by APV. Results strongly indicated the protective effects of *R. insecticola* against *P. neoaphidis* were not reduced by APV infection. Aphids with *R. insecticola* exhibited lower rates of fungal sporulation and higher survival when compared to isogenic symbiont-free controls (Figure 2, Table S4). In aphids with no facultative symbionts, APV reduced aphid survival and increased mortality when challenged with *Pandora* (Figure 2a,c). In contrast, APV infection did not significantly reduce aphid survival in those carrying *R. insecticola*. While APV presence resulted in fewer sporulating *R. insecticola* carrying aphids, this was explained by increases in dual mortality rather than differences in aphid survival (Figure 2a,b). Hence, the benefit of carrying *R. insecticola* is relatively larger when the aphid is challenged with *both* APV and *Pandora* because *R. insecticola* provides similar protection against *Pandora* with and without APV but eliminates fitness losses associated with APV infection.

The high-level protective effects of *H. defensa*/APSE-3 (ND18.H3) and moderate protective effects of *H. defensa*/APSE-2 (5D-AB.H2) against *A. ervi* were also not lowered by APV infection (Figure 3; Table S5). For aphid lines without facultative symbionts, aphid survival was very low, while successful wasp development (mummification) was high, but these did not generally differ between APV+ and APV− lines (Figure 3; Figure S4; Table S5). The one exception to this latter trend was the WI246-8 line, which produced fewer mummies if infected with APV (W1246-8 APV+) but this outcome was also associated with more aphids dying while also not producing a parasitoid (dual mortality) rather than an increase in aphid survival (Table S5; Figure S4E,F).

Since parasitoid fitness is known to be influenced by host quality, we also examined whether *A. ervi* developing in hosts infected by APV exhibited reduced fitness by estimating the size of emerging female wasp offspring. We found that smaller female *A. ervi* were produced from APV+ aphids in three of the four assayed aphid lines that lacked facultative symbionts (Table 3A,B). We did not measure the size of emerging wasps from aphids hosting *H. defensa*/APSE-3 because very few mummies were produced due to the high level of protection this strain confers. However, we did measure the size of female wasp offspring that developed in aphids hosting *H. defensa*/APSE-2. Interestingly, no differences were detected between the size of wasps that emerged from aphids with *H. defensa*/APSE-2 that were infected with APV versus aphids that were not (Table 3B).

3.5 | APV RNA abundance is reduced by the presence of *R. insecticola* but not *Hamiltonella defensa*

Given that fitness costs to APV infection were lower in aphids with *R. insecticola* and higher in those with *H. defensa*, we hypothesized that the former reduces APV RNA abundance while the latter does

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TABLE 2 Aphid fecundity and 50% survival in the absence of enemy challenge

Panel	Line	APV infection	Symbiont infection	18 day fecundity ± SE ($n = 45$; 9 reps)	50% adult survivorship[a] (days; $n = 45$; 9 reps)
A	WI246-8[b]	APV−	None	307.0 ± 20.4 a	15.6 a
		APV+	None	118.4 ± 11.2 b	10.5 b
B	WI576N-27[b]	APV−	None	298.6 ± 24.3 a	12.8 a
		APV+	None	174.3 ± 20.7 b	10.8 a
C	LSR1-AB[c]	APV−	None	462.1 ± 16.5 a	17.9 a
	LSR1-AB-APV+	APV+	None	308.0 ± 12.7 b	12.5 c
	LSR1.Ri	APV−	*R. insecticola*	388.4 ± 26.4 a	15.7 ab
	LSR1.Ri-APV+	APV+	*R. insecticola*	389.1 ± 21.8 a	14.9 b
D	5D-AB[c]	APV−	None	426.5 ± 14.6 a	16.8 a
	5D-AB-APV+	APV+	None	272.1 ± 11.7 c	14.2 b
	5D-AB.H2	APV−	*H. defensa*/APSE-2	366.0 ± 16.4 b	14.2 b
	5D-AB.H2-APV+	APV+	*H. defensa*/APSE-2	146.1 ± 11.3 d	9.5 c
E	ND18 UI[c]	APV−	None	435.4 ± 21.8 a	19.5 a
	ND18-APV+	APV+	None	309.6 ± 35.5 b	15.8 b
	ND18 + H3	APV−	*H. defensa*/APSE-3	250.3 ± 12.2 b	12.9 c
	ND18 + H3-APV+	APV+	*H. defensa*/APSE-3	123.9 ± 8.7 c	11.8 c

Abbreviation: APV, *Acyrthosiphon pisum virus*.

[a]Survival data was fit to lognormal distribution for estimates of 50% survival. The letter after the value indicates significant difference ($p < .05$) by Wilcoxon test.

[b]A *t*-test was used to compare fecundity for lines 246–8 and 576N-27.

[c]Fecundity between ND18 lines was compared using Tukey's post hoc test. Letter after value denotes a significant difference at $p < .05$.

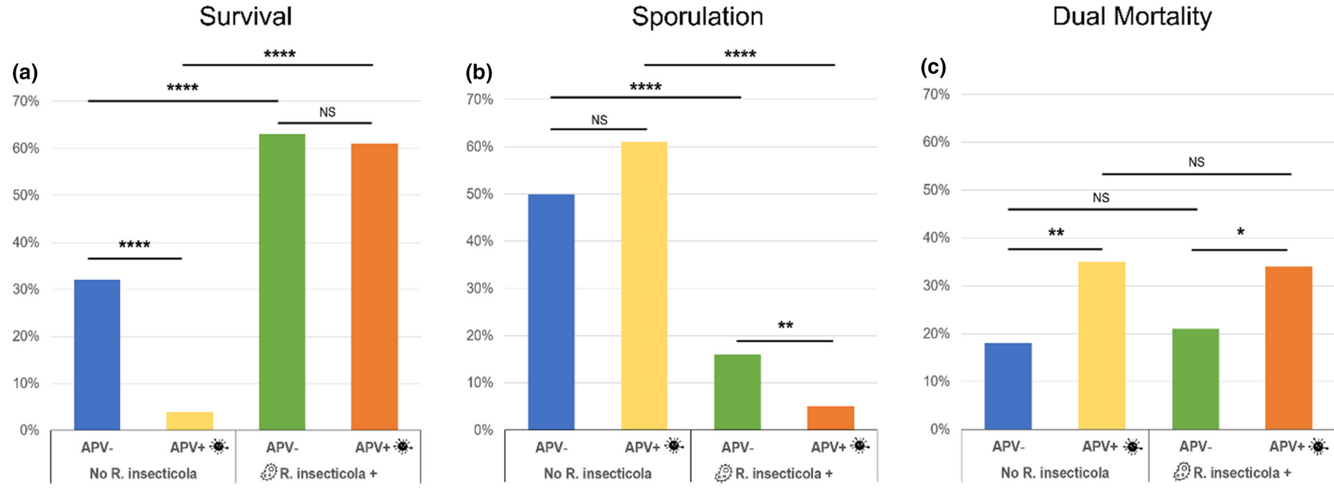

FIGURE 2 Proportion of (a) aphid survival, (b) fungal sporulation and (c) dual mortality for experimental line LSR1 following challenge by the fungal pathogen *Pandora neoaphidis*. $N = 10$ replicates of 10 individual aphids for each line. Outcomes of fungal exposure were contrasted between aphid with and without APV (virus icon) as well as aphids with and without *Regiella insecticola* (symbiont icon). Brackets above indicate contrasts between lines. Asterisk(s) above brackets indicates level of significant difference (NS, $p > .05$; *$p \le .05$; **$p \le .01$; ***$p \le .001$; ****$p \le .0001$).

not. We also investigated whether APV influenced facultative symbiont titres. Focusing first on the facultative symbionts, we compared their relative abundance in aphids that were infected with APV to aphids that were not starting from day 2, when nymphs were second instars, to day 16 when they were mature adults. While *R. insecticola* abundance progressively increased in both APV+ and APV− aphids, symbiont abundance was significantly higher in aphids without APV until day 16 when titres were similar (Figure 4a; Table S6). *H. defensa* with APSE-2 also progressively increased in abundance with aphid age, while *H. defensa* with APSE-3 exhibited little change in abundance until day 16 (Figure 4b,c). However, no differences in these trajectories were found between APV+ and APV− aphids (Tables S7,

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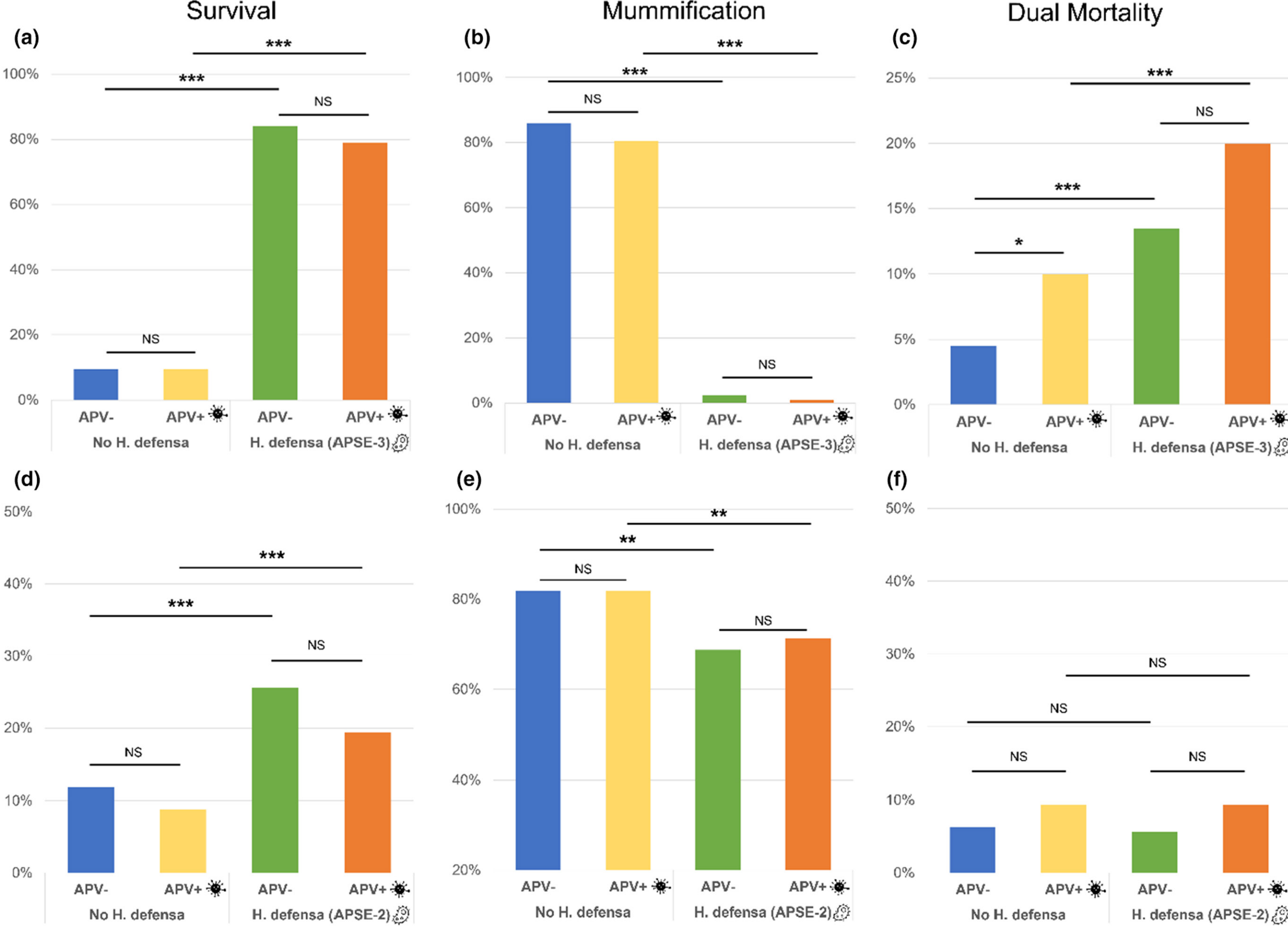

FIGURE 3 Proportion of aphid survival, mummification and dual-mortality for aphid experimental lines ND18 (a–c) and 5D-AB (d–f) following parasitism by the parasitoid wasp *Aphidius ervi*. $N = 8$ replicates of 20 individual aphids for each line. Parasitism outcomes were contrasted between aphid with and without APV as well as between aphids with and without *Hamiltonella defensa*. Brackets above indicate contrasts between sublines. Asterisk(s) above bars indicate significant differences (NS = $p > .05$; *$p \le .05$; **$p \le .01$; ***$p \le .001$; ****$p \le .0001$).

S8). We also measured relative abundances of APSE-2 and APSE3, which only modestly increased with aphid age and exhibited almost no differences between APV+ and APV− aphids (Figure 4d,e). We thus concluded that APV infection overall had modest effects on the relative abundance of *R. insecticola* and no effect on *H. defensa* abundance.

All known positive-sense RNA viruses proceed through a negative-strand replication intermediate (Modrow et al., 2013). As a result, standard reverse transcription quantitative polymerase chain reaction (RT-qPCR) protocols cannot distinguish among the multiple species of viral RNAs that are present in host samples infected by positive-sense RNA viruses like APV. However, since our interest was in assessing the relative abundance of APV in aphids that hosted *R. insecticola*, *H. defensa* or had no facultative symbionts, we used standard RT-qPCR methods to compare APV RNA abundances in our experimental aphid lines. For the LSR1 aphid genotype, APV RNA abundance progressively increased with aphid age but titres were significantly lower at days 8 and 16 aphids in aphids with *R. insecticola* (LSR1.Ri) versus isogenic controls with no facultative symbionts (LSR1-AB; Figure 5a; Table S9). For the ND18 and 5D-AB lines, APV RNA abundance increased more rapidly than in the LSR1-AB line, but did not differ from aphids that hosted *H. defensa*/APSE-3 (Table S10), *H. defensa*/APSE-2 (Table S11) or were symbiont-free (Figure 5b,c). Aphid genotype had little impact on APV infection trajectories (Figure 5).

4 | DISCUSSION

4.1 | *Regiella insecticola* reduces fitness costs associated with APV infection, while *H. defensa* likely exacerbates them

Persistent infection by APV was previously reported to reduce pea aphid fitness (Lu et al., 2020; Van den Heuvel et al., 1997). However, these studies did not control for aphid genotype or the presence of facultative symbionts, which occur in most pea aphids and are known to confer protection to specialized natural enemies (Oliver

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TABLE 3 Mean (±SE) length of right hind tibia of female adult *A. ervi* produced from (A) *Acyrthosiphon pisum virus* (APV) infected and APV free aphids lacking *H. defensa* and (B) female adult *A. ervi* produced from APV infected and APV free aphids with or without *H. defensa*/APSE-2

Line	Infection status	N[a]	Mean tibia length (μm) ± SE	*df*	*t*-Ratio	*p*-Value
(A)						
ND18	APV−/Ham−	30	911.92 ± 8.41	57.34	2.38	.021*
	APV+/Ham−	30	881.96 ± 9.31			
WI246-8	APV−/Ham−	30	878.69 ± 8.27	46.43	3.41	.001*
	APV+/Ham−	20	833.32 ± 10.44			
WI576N-27	APV−/Ham−	30	903.20 ± 8.71	42.42	0.95	.346
	APV+/Ham−	23	899.09 ± 11.97			
(B)						
5D-AB[b]	APV−/Ham−	23	865.38 ± 12.00 a	3	3.26	.025*
5D-AB-APV+	APV+/Ham−	20	816.87 ± 12.87 b			
5D-AB.H2	APV−/APSE-2	21	860.62 ± 12.56 ab			
5D-AB.H2-APV+	APV+/APSE-2	22	862.23 ± 12.27 ab			

[a]Tibia measurements were generated from a single aphid cohort (*N*) of parasitized aphids from each line.

[b]Tukey's post hoc test was used to compare wasp tibia length emerging from parasitized 5D-AB sublines. Letter after value denotes a significant difference *at* $p < .05$.

*Indicates a significant difference by one-way ANOVA.

et al., 2014; Russell et al., 2013). Here, we generalize prior findings by showing that persistent APV infection similarly reduces aphid fecundity and survival across multiple pea aphid genotypes lacking facultative symbionts (Table 2). In aphids without facultative symbionts, we also found that APV RNA abundance exhibited similar trajectories over aphid lifespan, which is consistent with the infection costs we observed (Figure 5).

When we examined our experimental lines with and without two common and closely related protective facultative symbionts *H. defensa* and *R. insecticola*, we found that the fitness of aphids with persistent APV infections varied dramatically depending on which symbiont was present. In aphids carrying *R. insecticola*, costs to persistent infection with APV were largely eliminated (Figure 2a,b, Table 2). Not only were fitness estimates similar between *R. insecticola* carrying aphids with and without APV, but aphids with both APV and *R. insecticola* produced statistically similar numbers of offspring compared to the control line (no APV or symbiont). APV abundance was also lower in aphids with *R. insecticola* versus those without this symbiont, although significantly so only in older aphids (Figure 5a). Taken together, these results indicate that *R. insecticola* provides substantial protection against infection with APV. A potential caveat of our study is that we only examined a single strain of *R. insecticola* in one aphid background. However, the aphid strain we used is by far the most common in N. American pea aphids on alfalfa (Peng, 2022).

To our knowledge, *R. insecticola* represents only the second heritable symbiont known to confer protection against viral pathogens. Some strains of the ubiquitous *Wolbachia* symbiont confer protection against specialized RNA viruses in natural hosts (Hedges et al., 2008; Pimentel et al., 2021; Teixeira et al., 2008), but antiviral effects have also been shown to occur in association with introducing certain *Wolbachia* strains into new species (Nazni et al., 2019; O'Neill et al., 2019). *Wolbachia's* pathogen blocking mechanisms remain poorly understood in both natural and novel associations, but hypotheses include immune priming, resource competition, or modification of the host cell environment (Lindsey et al., 2018; Terradas & McGraw, 2017). The same proposed mechanisms may also contribute to *R. insecticola's* beneficial effects to aphids in response to APV. However, our results also differ from *Wolbachia* in the sense that *R. insecticola* does not prevent aphids from being infected by APV nor does it prevent APV RNA abundance from increasing after infection. Instead, the presence of *R. insecticola* reduces APV RNA abundance when compared to aphids without this symbiont. However, whether this benefit is due to directly reducing APV replication, increasing aphid tolerance to APV infection or other processes is unknown. As earlier noted, prior findings also indicate *R. insecticola* improves pea aphid fitness in the presence of specialized fungal pathogens when compared to aphids with no facultative symbionts or other symbionts like *H. defensa* and *Spiroplasma* (Mathé-Hubert et al., 2019; Parker et al., 2013; Weldon et al., 2020). Thus, the mechanisms by which *R. insecticola* benefits aphids could involve processes that do not involve directly interacting with pathogens. Thus, addressing the mechanism(s) underlying how *R. insecticola* promotes aphid survival in response to at least two pathogens is an important future goal. Having a second model of antiviral symbiosis to complement studies in *Wolbachia* could also provide a broader perspective about how heritable symbionts interact with other microbes that are insect pathogens.

In contrast to *R. insecticola*, *H. defensa* did not influence APV RNA abundance (Figure 5). APV infection costs were also significantly higher in aphid lines carrying *H. defensa* relative to aphids that were infected with only *H. defensa* or that were infected with APV but had no facultative symbiont (Table 2C,D). Interaction analyses further

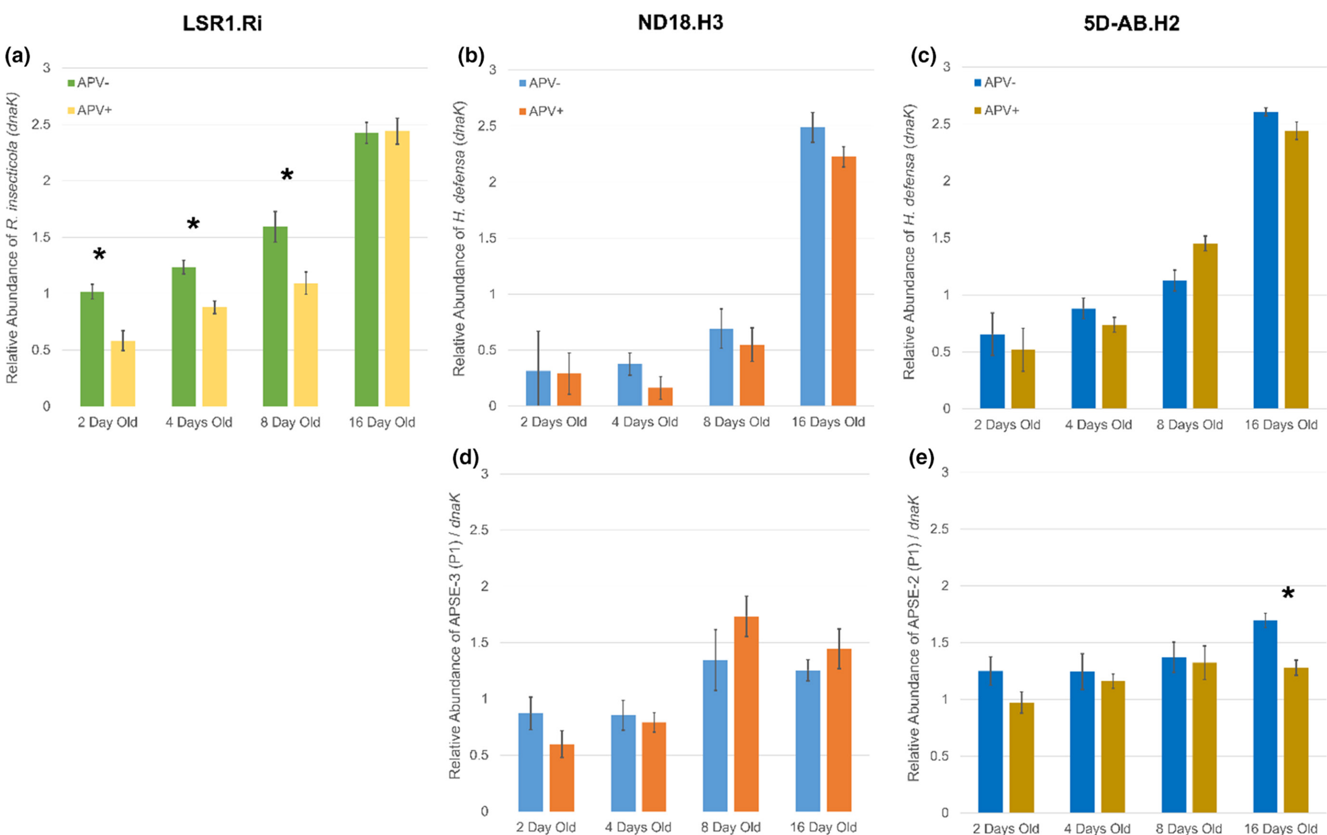

FIGURE 4 Genome copy number (±SE) of *Regiella insecticola*, *Hamiltonella defensa* and *H. defensa*-associated APSEs in three lines of developing aphids that were APV-free (APV−) or APV-infected (APV+). Abundance estimates were determined for: (a) *R. insecticola* in the aphid LSR1 line (=LSR1.Ri); (b) *H. defensa* in the aphid ND18 line (=ND18.H3); (c) *H. defensa* in the aphid 5D-AB line (=5D-AB.H2); (d) APSE3 which infects *H. defensa* in the ND18.H3 line and (e) APSE2 that infects *H. defensa* in the 5D-AB.H2 line. Bacterial symbiont abundance was estimated using the single copy gene *dnak* relative to the aphid gene *elf-α*. APSE genome copy number was estimated using the structural P2 gene relative to *H. defensa dnaK*. $N = 8$ individual aphids per time point for LSR1.Ri and 5D-AB.H2, $N = 6$ individual aphids per time point for ND18.H3. Asterisks indicate significant differences (*$p < .05$).

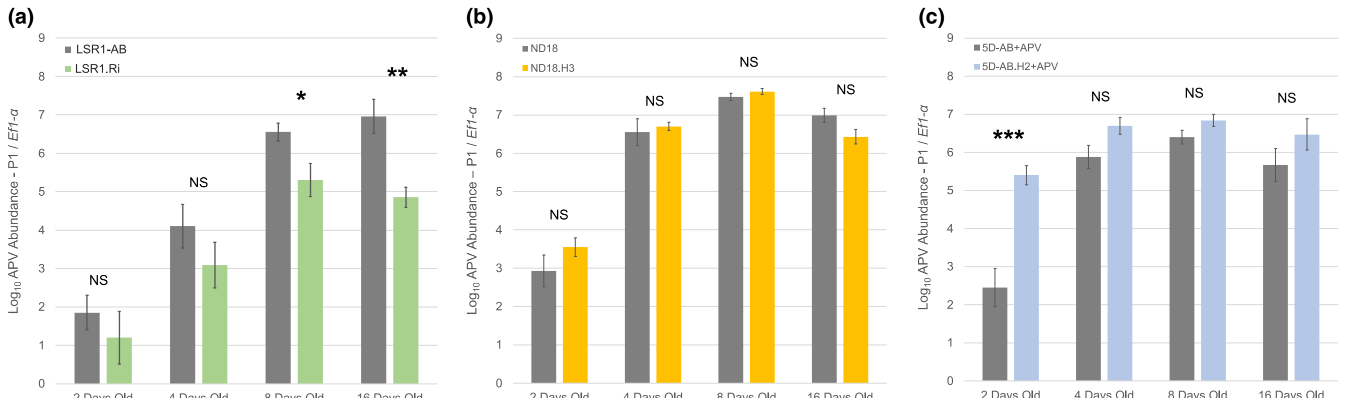

FIGURE 5 *Acyrthosiphon pisum virus* (APV) RNA abundance (± SE) in three APV-infeced aphid lines that were either symbiont free or hosted *R. insecticola* or *H. defensa*. (a) the LSR1 line with or without *R. insecticola*. (b) the ND18 line with or without *H. defensa*/APSE-3. (c) the 5D-AB lines with or without *H. defensa*/APSE-2. APV RNA abundance was determined by amplifying a region of the APV genome within the P1/P2 open reading frame and normalized to the aphid the housekeeping gene *Ef-1α*. $N = 8$ individual aphids per time point for LSR1. Ri and 5D-AB.H2. $N = 6$ individual aphids per time point for ND18.H3. Samples were performed with two technical replicates. APV RNA abundances in the presence and absence of symbionts at each time point were compared using a *t*-test. Asterisk(s) above bars indicate significant differences (NS, $p > .05$; * $p \leq .05$; **$p \leq .01$; ***$p \leq .001$).

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suggest that *H. defensa* interacts with APV in a synergistic, rather than additive fashion (Figure S3). While symbionts that protect hosts receive the most interest, those that enhance pathogen infection are nonetheless important for natural symbiont maintenance and disease dynamics (Amuzu et al., 2018; Graham et al., 2012).

4.2 | The presence of APV does not impact symbiont defensive phenotypes

Little is known about the performance of defensive symbionts when challenged with simultaneous threats (Hrček et al., 2016; Smith et al., 2021). Here, we found aphids carrying *H. defensa* were similarly susceptible to parasitism by *A. ervi* regardless of APV infection and APV had no effect on *R. insecticola* conferred protection against *P. neoaphidis* (Figure 2). While protection levels conferred by defensive symbionts are known to vary depending on abiotic factors (Doremus et al., 2018; Guay et al., 2009; Higashi et al., 2020), host genotypes (Łukasik et al., 2013; Parker et al., 2017; Vorburger & Gouskov, 2011; Weldon et al., 2020), symbiont strain (Cayetano et al., 2015; McLean et al., 2018, 2020; Oliver & Higashi, 2019), or co-occurrence with other symbionts (Weldon et al., 2020), our results indicate that APV does not alter defensive phenotypes. In contrast, APV had variable impacts on endogenous defences against these specialized enemies. Aphid lines free of facultative symbionts were equally susceptible to parasitism by *A. ervi* with and without APV (Figure S4), but those challenged with the fungus *P. neoaphidis* performed significantly worse when APV was present (Figure 2). The latter suggests that the aphid immune system may not be able to effectively respond to simultaneous pathogen challenges. This result also indicates that the antifungal benefits of carrying *R. insecticola* were greater when APV was present. Aphids carrying *R. insecticola* are likely to benefit from both enhanced antifungal benefits and tolerance to APV, spreading at the expense of symbiont-free aphids or those with *H. defensa*.

4.3 | Facultative symbionts did not influence APV transmission

We found that vertical transmission rates of APV were about 35% and not impacted by *H. defensa* (Table S2A). This rate is similar with prior reports for APV (Lu et al., 2020) and other aphid viruses (Laubscher & Von Wechmar, 1992; van Munster et al., 2003). It was also previously reported that feeding by aphids resulted in the horizontal transfer of APV to plants, with the virus persisting up to 7 days without replication in plant tissues (Lu et al., 2020). Here, we confirmed lateral transmission of APV through food plants by showing that that APV− aphids readily acquired the virus through phloem feeding on plants previously fed on by APV+ aphids (Table S2B). We further showed that APV acquisition rates were not affected by the presence of *H. defensa* or *R. insecticola*. Thus, despite reducing APV abundance and improving tolerance to infection as described above, *R. insecticola* did not impede APV acquisition. We also found that *A. ervi* can transfer APV via oviposition. Rates of wasp-mediated transfer of APV were low and only occurred when oviposition immediately followed contact with a virus-infected aphid. Parasitoid-mediated transfer of ascoviruses has been previously shown to occur in lepidopteran hosts via mechanical transfer by the ovipositor (Glynn Tillman et al., 2004; Hamm et al., 1985; Li et al., 2016). These observations, along with those showing parasitoids can move heritable endosymbionts horizontally between aphid hosts (Gehrer & Vorburger, 2012) suggests that microbial transfer via the contaminated ovipositors of wasps may be an underappreciated route of microbe exchange among multicellular eukaryotes.

Together these results show moderate vertical transmission and horizontal transfer through food plants are the major routes of APV spread. That APV can promote aphid feeding and colonization by modulating plant defences (Lu et al., 2020) suggests this virus may employ tactics that facilitate its spread as seen for plant viruses vectored by sap-feeding insects (Roossinck, 2015).

4.4 | The effects of APV may extend to higher trophic levels

Host infection with viral pathogens can impact parasitoid fitness (Dupont et al., 2020; Flick et al., 2016). We found that wasps developing from aphids that are infected with APV were smaller wasps from APV-free aphids in three of four lines lacking *H. defensa*. In the single *H. defensa* line we examined, wasps that survived symbiont defences were similar in size regardless of APV infection. This result is perplexing given that aphids with both *H. defensa* and APV exhibited the poorest fitness overall (Table 3B) combined with earlier studies finding that wasps emerging from aphids with *H. defensa* were smaller (Dion et al., 2011; Schmid et al., 2012).

5 | CONCLUSIONS

Little is known about the ecology of APV in natural aphid populations. But given the infection costs identified here and elsewhere, along with the rapid spread of this virus through multiple mechanisms, outbreaks may occur which significantly alter aphid population dynamics with effects that reverberate through the food web (Ban et al., 2008; Dupont et al., 2020; Gupta et al., 2017; Jiang et al., 2014; Laubscher & Von Wechmar, 1993). Absent other factors, APV outbreaks would probably select for aphids carrying *R. insecticola*, and against those with *H. defensa*, which would reduce the populations' potential to respond to subsequent increases in parasitism pressure, while enhancing protection against fungal pathogens. Alternatively, high parasitism rates, which select for *H. defensa* (Hrček et al., 2016; Ives et al., 2020; Oliver et al., 2008; Smith et al., 2015) potentially limit this aphid's capacity to respond to APV outbreaks. Finally, of practical concern, we note from recent experience that APV infections spread readily in pea aphids held under

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common laboratory conditions. While APV infections did not influence symbiont-mediated protective phenotypes, they did impact fitness measures in the absence of enemy challenge and hence have the potential to impact a range of laboratory-based studies. We note that the low rates of vertical transmission, combined with PCR-based screening, allow for the ready elimination of APV from valuable experimental lines without the need to discard them.

AUTHOR CONTRIBUTIONS

Clesson H.V. Higashi, Suzanne E. Allison, Germain Chevignon, and Kerry M. Oliver conceived and design experiments. Clesson H.V. Higashi, Suzanne E. Allison, Germain Chevignon, Kyungsun L. Kim, Vilas Patel, and William L. Nichols provided technical assistance with experiments. Clesson H.V. Higashi, Vilas Patel, Germain Chevignon, and Kerry M. Oliver analysed the data. Clesson H.V.Higashi, Michael R. Strand, and Kerry M. Oliver wrote the manuscript.

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CONFLICT OF INTEREST

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

Data sets on aphid parasitism, fecundity and survivorship assays have been made available in Dryad Digital Repository doi:10.5061/dryad.jdfn2z3f8. Sequences generated for a portion of the APV P1 domain that were generated from the 12 laboratory aphid lines were submitted to GenBank under the accession nos. OM649898–OM649902. Assembled APV genomes were submitted into Genbank under the accession nos. OM649898–OM649902. SRA data generated from RNAseq used to assemble the APV genomes were deposited under BioProject PRJNA803168.

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