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Host aphid immunosuppression by *Aphidius ervi* venom

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Abstract

The host immunosuppression by parasitic wasps is an important component of the host regulation strategy. The venom injected at the oviposition is one of the key-factors involved in this host alteration and, in some parasitoids, its immunosuppressive role is complemented by wasp's symbionts. Most studies in this research area are related to hosts belonging to Lepidoptera and Diptera, for which a strong immune response is observed, whereas little is known for hemimetabolous host species, characterized by apparently much weaker defense barriers. To fill this research gap, here we focus on the host-parasitoid system *Acyrtosiphon pisum* (Harris) (Hemiptera: Aphididae) – *Aphidius ervi* Haliday (Hymenoptera: Braconidae). We functionally characterized a serine protease homolog (AeSPH) protein in vivo, identified in the venom of the aphid endoparasitoid *A. ervi*, generating AeSPH-depleted female wasps by RNA interference and evaluating their capacity to successfully parasitize the host. Parasitism success rate was negatively affected by AeSPH knockdown and associated with an increased phenoloxidase (PO) cascade activation in aphids, scored by measuring PO enzymatic activity and the expression of *phenoloxidase activating factor 2*, a proPO-activating gene upregulated in response to *A. ervi* parasitism. Our results indicate that AeSPH contributes to parasitism success by inhibiting the melanization response of the host, which is therefore an important component of the defense barriers involved in the parasitoid egg suppression. The ongoing studies on other virulence factors in *A. ervi* venom will allow to further characterize the immunosuppression strategy and its possible broader role in the host regulation through its action on aphid symbiont development.

KEYWORDS

Acyrtosiphon pisum, aphid immunity, immune suppression, parasitoid venom, RNA interference, serine protease homologs, Hemiptera, Aphididae, Hymenoptera, Braconidae, hemimetabolous host species, endoparasitoid

INTRODUCTION

Parasitic Hymenoptera are the largest group of insect antagonists, showing an impressive diversity of host exploitation strategies, which parallels the diversity of colonized hosts (Godfray, 1994; Pennacchio & Strand, 2006). Starting from ancestral ectophagous wasps, endophagy evolved independently in multiple lineages, leading to increasingly complex host regulation strategies, especially in koinobionts which develop in hosts that

continue to move and feed (Pennacchio & Strand, 2006; Cuny & Poelman, 2022). The major functional constraints faced by parasitic wasps during their evolution are the need to suppress the host immune response and the need to redirect metabolism in favor of their developing progeny (Pennacchio & Strand, 2006). In ectophagous parasitoids the immune suppression, required to prevent any reaction by the host to feeding wounds interfering with food uptake and used by the wasp's larvae, and the host metabolic alterations, enhancing its nutritional

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suitability for the wasp's juveniles, are largely induced by venom and, in some cases, by host regulation factors of larval origin (Richards & Edwards, 2002; Rivers et al., 2002; Becchimanzi et al., 2017, 2020). These interactions are mostly associated with host paralysis and developmental arrest, which characterize the idiobiont lifestyle (Pennacchio & Strand, 2006). In endophagous parasitoids, these host regulation factors are complemented by additional factors produced by viral symbionts (polydnaviruses) and by cells of embryonic origin (teratocytes), which allow a finely tuned host regulation over time (Beckage & Gelman, 2004; Pennacchio & Strand, 2006; Pennacchio et al., 2014). These wasps are mostly koinobionts that do not cause host paralysis but induce a number of physiological alterations essential to enable the survival of juvenile wasps within the harsh host environment, where the immune defense systems would immediately kill them in the absence of immunosuppression (Moreau & Guillot, 2005; Pennacchio & Strand, 2006; Pennacchio & Mancini, 2012; Mrinalini & Werren, 2015).

The immunosuppressive effects are therefore, for various reasons, essential both for ectophagous and endophagous parasitoids, with the venom injected at oviposition playing a key-role in most cases. Different venom proteins often target different steps of the immune reaction, disabling both the humoral and cellular components (Asgari & Rivers, 2011; Moreau & Asgari, 2015). The study of the underlying mechanisms of host immunosuppression is of particular interest because it sheds light on the amazing molecular biodiversity of virulence factors and allows the discovery of new functional aspects of the insect immune response to be made. For example, the study of the immunosuppressive strategy of the braconid wasp *Toxoneuron nigriceps* (Viereck) facilitated the discovery of an immune gene encoding a precursor of amyloid fibrils. These are involved in the strict localization of melanization on the surface of a foreign invader, thus playing an important role in the modulation of the immune response of the noctuid moth host larvae (Falabella et al., 2012; Di Lelio et al., 2014; Caccia et al., 2016).

Most of the functional studies on parasitoid-induced host immunosuppression are focused on lepidopteran hosts, whereas very little and only fragmented information is available for other host insect groups (Asgari & Rivers, 2011; Moreau & Asgari, 2015). Here we address this research gap by focusing on the endoparasitoid *Aphidius ervi* Haliday (Hymenoptera: Braconidae) and the pea aphid host, *Acyrtosiphon pisum* (Harris) (Hemiptera: Aphididae), a model system used for studying the role of venom in host castration (Digilio et al., 1998, 2000; Falabella et al., 2007). A recent in vivo study showed that the major bioactive component of *A. ervi* venom, a γ -glutamyl transpeptidase (Ae- γ -GT), not only induces host castration but is also involved in controlling the proliferation of the aphid primary bacterial symbiont, *Buchnera aphidicola* Munson et al., stimulated by other unidentified venom components (Russo et al., 2023). Among the many other venom components whose function has not yet been investigated in vivo so far

(Colinet et al., 2014), we focused on those potentially able to interfere with host immunity, which in aphids is poorly studied and considered to be substantially weaker than in other insect groups, particularly the cellular response (Gerardo et al., 2010; Schmitz et al., 2012), starting with serine protease homologs (SPHs). Five SPHs were previously identified in a proteo-transcriptomic analysis of *A. ervi* venom, but only one (Cl9Contig1) was classified as a venom protein (Colinet et al., 2014). SPHs are endopeptidases that lack one or more residues at the conserved catalytic sites (Yu et al., 2003; Polgár, 2005) and are commonly found in insect hemolymph, where they promote melanization by interacting with proPO and proPO-activating proteinases (Yu et al., 2003; Zhang et al., 2004). Interestingly, SPHs identified in the venom of parasitic wasps were shown to act as negative modulators of the melanization response when injected in the host's haemocoel (Asgari et al., 2003), likely acting through competition with endogenous SPHs (Zhang et al., 2004; Thomas & Asgari, 2011).

Here, we explore the role of *A. ervi* venom SPH (AeSPH) in promoting parasitism success and interfering with melanization in the host *A. pisum*, using an RNAi-based approach (Siebert et al., 2015; Wang et al., 2020a; Russo et al., 2023). We generated AeSPH-depleted female wasps, by microinjecting dsRNA in pupae, and assessed parasitism success as well as PO activation in response to controlled parasitization. The results obtained shed new light on the immunosuppression strategy adopted by *A. ervi* and on the important role of melanization in aphid immunity.

MATERIALS AND METHODS

Sequence analysis and phylogeny reconstruction

Putative homologous protein sequences of AeSPH were identified by sequence similarity searches through a BlastP analysis versus the non-redundant (nr) NCBI (National Center for Biotechnology Information, Bethesda, MD, USA) database and Swiss-Prot. Additional rounds of BLASTp analyses were performed selecting '*Acyrtosiphon pisum*' in the nr NCBI collection. Most representative hits selected below the E-value threshold of 10⁻⁵ were aligned using Muscle v.3.8 (Edgar, 2004), with default settings. Protein alignment was plotted using Jalview v.2 (Waterhouse et al., 2009), and sequences were analyzed with ScanProsite (de Castro et al., 2006) and visually inspected, to identify active sites and conserved patterns.

To reconstruct phylogeny, alignments were manually trimmed to avoid comparisons of non-conserved regions present only in a subset of the taxa. Best-fit model of amino acid substitution and phylogenetic reconstruction was performed using RAXML v.8.2.12 (Stamatakis, 2006). The maximum-likelihood tree was run for 1000 bootstrap replicates and the tree figure was plotted using FigTree v.1.4.3.

Insects

Laboratory colonies of *A. pisum* and its parasitoid *A. ervi* were established from populations collected on alfalfa plants, *Medicago sativa* L. (Fabaceae), in Eboli (Salerno province), southern Italy. A single population of *A. pisum*, which reproduces parthenogenetically, was maintained on potted broad bean plants, *Vicia faba* L. (Fabaceae), at $20 \pm 1^\circ\text{C}$, $75 \pm 5\%$ r.h., and L16:D8 photoperiod. *Aphidius ervi* was reared on pea aphids, maintained on potted broad bean plants, and kept in a separate climate room at $25 \pm 1^\circ\text{C}$, $75 \pm 5\%$ r.h., and L16:D8 photoperiod. Both insect colonies were annually refreshed with field-collected material.

RNA extraction and dsRNA synthesis

Female wasps were anaesthetized on ice and dissected in sterile PBS (phosphate buffered saline: 137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.4) by grasping the ovipositor tip with fine forceps, under a stereoscope (SteREO Discovery V8, Zeiss). Total RNA was isolated from the venom glands and the rest of the body using TRIzol reagent (Thermo Fisher Scientific), according to the manufacturer's instructions. The RNA concentration and purity were measured by determining the absorbance ratio of 260/280 nm using Varioskan Flash Multimode Reader spectrophotometer (Thermo Fisher Scientific). The RNA extracted was then retrotranscribed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), following the manufacturer's protocol. The gene-specific primers used for amplification of *AeSPH* (accession number GBCU01000007.1) were designed to include the T7 polymerase promoter sequence (TAATACGACTCACTATAGGGAGAA). The T7-flanking primer sequences, shown in Table S1, were designed to amplify a 400-bp fragment. The PCR reaction mixes for in vitro transcription of double-stranded RNA (dsRNA) molecules were prepared using the Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) master mix, according to the following PCR parameters: an initial denaturation step at 98°C for 30 s, followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 20 s, and extension at 72°C for 20 s, with a final extension step at 72°C for 5 min. PCR products were purified using the PureLink PCR Purification Kit (Thermo Fisher Scientific), quantified, and used as template for subsequent dsRNA synthesis. The dsRNA targeting *AeSPH* (dsSPH) was in vitro synthesized using the MEGAscript RNAi kit (Thermo Fisher Scientific), following the manufacturer's protocol. A dsRNA targeting *GFP* (dsGFP) was similarly produced starting from the cloning vector pcDNA 3.1/CT-GFP TOPO (Thermo Fisher Scientific), as described elsewhere (Caccia et al., 2020). Synthesized dsRNA was eluted in the elution buffer provided by the manufacturer and quantified by measuring its absorbance at 260 nm with a Varioskan Flash Multimode Reader (Thermo Fisher Scientific), and purity was evaluated by assessing 260/280 nm absorbance ratio. Finally, the

dsRNA fragments produced were checked for integrity on a 1% agarose gel and stored at -20°C until further use.

dsRNA microinjection in *Aphidius ervi* females

To silence the *AeSPH* gene during the adult stage, dsRNA was microinjected into female pupae of *A. ervi*, following the methodology described by Russo et al. (2023). The injections were performed on day-1 yellow pupae, a developmental stage that precedes pigmentation of compound eyes. At this stage, the venom apparatus is not fully developed, and venom secretion has not yet started. Synchronously mummified aphids harboring parasitoid pre-pupae were monitored daily by dissecting a few individuals. Once the pupae reached the desired day-1 stage, mummies were ventrally glued to the lid of a Petri dish using water-soluble Entofix glue (Omnes Artes) and then opened gently with an entomological needle, to prepare them for injection. A volume of 40 nL of dsRNA solution ($2.2 \mu\text{g} \mu\text{L}^{-1}$) was injected between the last two abdominal segments, under a stereomicroscope (SteREO Discovery V8), using a FemtoJet 4x microinjection device (Eppendorf). Capillary needles (1.0×0.75 mm glass pipettes with a 7–9 mm taper, a 0.8 – $0.5 \mu\text{m}$ tip, and a 40–80 M Ω resistance) were pulled out on a Sutter P-97 laser micropipette puller (Sutter Instrument, Novato, CA, USA) and manually cut using microforceps. The program used for needle preparation was: Line=1, Heat=535, Pull=80, Vel=70, Del=80, Press=200. The injection conditions were set to 115 hPa injection pressure (pi), 12 hPa compensation pressure (pc), and 0.5 s injection time (t). A dsGFP (green fluorescent protein) solution was administered to an equal number of *A. ervi* control pupae. After injection, parasitoid pupae were kept in a Petri dish under the same laboratory conditions described above until adult emergence.

Parasitization protocol

Newly emerged wasps from each RNAi treatment group were individually introduced into a 35×10 mm Petri dish, fed ad libitum with a 50% honey-water solution (vol/vol) and allowed to mate with males for about 4 h. Then, third instar *A. pisum* were singly offered to experimental (dsSPH) or control (dsGFP) *A. ervi* females and allowed to receive two oviposition strokes. All aphids parasitized by the same *A. ervi* female were isolated on a *V. faba* plant, separated from the rest of the population, and maintained at $20 \pm 1^\circ\text{C}$, $75 \pm 5\%$ r.h., and L16:D8 photoperiod. Female wasps used for parasitization were immediately processed for RNA extraction and qRT-PCR analysis to assess gene silencing.

qRT-PCR analysis

Differential relative expression of *AeSPH* was assessed by SYBR Green qRT-PCR to check gene silencing efficiency. All

primers (Table S1) were designed using Primer Express, v1.0 software (Applied Biosystems). mRNA levels were measured by one-step qRT-PCR using the Power SYBR Green RNA-to-Ct 1-Step Kit (Applied Biosystems) and 1 μ L of RNA template (100 ng), according to the manufacturer's instructions. The thermal cycling conditions were set as follows: 50°C for 30 min for reverse transcription, 95°C for 10 min for initial denaturation, and 40 cycles of 95°C for 15 s and 60°C for 1 min for amplification. The analysis was performed using the Step One Plus Real-Time PCR System (Applied Biosystems). Relative gene expression data were analyzed using the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001). For validation of the $\Delta\Delta C_T$ method the difference between the C_T value of *AeSPH* and the C_T value of the *A. ervi* ribosomal protein *28S β 2* transcripts [$\Delta C_T = C_T(\text{AeSPH}) - C_T(28S\beta 2)$] was plotted versus the log of two-fold serial dilutions (200, 100, 50, 25, and 12.5 ng) of the purified RNA samples. The plot of log total RNA input versus ΔC_T displayed a slope < 0.1 , indicating that the efficiencies of the two amplicons were approximately equal.

Effect of *AeSPH* knockdown on *Aphidius ervi* parasitism success

To investigate the impact of *AeSPH* deficient venom on *A. ervi* parasitism success, we compared the rate of mummy formation for female wasps with *AeSPH* depleted venom (SPH $-$) and control females with a complete venom blend (SPH $+$). The rate of mummy formation was assessed 10 days after parasitism. Moreover, we scored the rate of adult emergence from formed mummies.

Phenoloxidase activating factor expression in aphids

To investigate the role of *AeSPH* in regulating immunity of the host we focused on *Phenoloxidase activating factor 2* (*PAF2*, XM_001950945.5), a gene that in *A. pisum* is upregulated in response to *A. ervi* parasitism (McLean & Parker, 2020) and is likely involved in proPO cascade triggering melanization (Xu et al., 2019; Ma et al., 2020a).

To determine the relative expression levels of *PAF2*, total RNA was extracted from each aphid (as described above), at 2 and 24 h post parasitization (see above), and qRT-PCR analysis was performed as described above, using *rpl7* as the endogenous control gene for normalization. The primer sequences specific for *PAF2* and *rpl7* are indicated in Table S1.

Phenoloxidase activity

Phenoloxidase (PO) activity in aphids was assessed through the catalytic conversion of L-dopa (3,4-dihydroxy-L-phenylalanine, colorless) to dopachrome (red brown), measured photometrically as described elsewhere (Laughton et al., 2011; Martin et al., 2023). Briefly,

aphids, sampled at 6 and 24 h post-parasitization, were individually placed in an Eppendorf tube and homogenized with a pestle in 150 μ L PBS solution. Samples were centrifuged at 10000 *g* for 10 min at 4°C, and protein concentration of the supernatant was quantified using Bradford's (1976) method. Samples were diluted to a concentration of 30 ng μ L $^{-1}$ of total proteins and 100 μ L of each sample was transferred to a 96-well plate (Costar 3595, Corning). In each well, 100 μ L of L-dopa (2 mmol L $^{-1}$ in PBS) was added and mixed. The plate was incubated at 37°C for 20 min and absorbance was read at 490 nm every minute for 90 min, using a Varioskan Flash Multimode Reader spectrophotometer (Thermo Fisher Scientific). Enzyme activity was measured as the initial rate of reaction (linear phase), expressed as a change in absorbance (Abs) per unit of time ($\Delta\text{Abs}_{490}/\text{min}$).

Statistical analysis

Data were analyzed using Student's *t* test and Mann-Whitney test for *AeSPH* knockdown efficacy and parasitism success rate, respectively. Two-way ANOVA followed by a post-hoc Tukey test was used to compare the means in phenoloxidase activation dataset. The assumption of normal distribution of data was tested and met by Shapiro-Wilk test. Each dataset was checked for homoscedasticity using Levene's test. All statistical analyses were performed using PRISM v.8.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

Sequence analysis and localization of *AeSPH* expression

The alignment of protein sequences showed that *AeSPH* (GenBank acc. nr. JAC59141.1) lacks the three catalytic residues His-Asp-Ser (H-D-S), which are conserved in active serine proteases (Figure 1A). The other SPHs, identified in *A. ervi* venom gland but not in the venom reservoir (Colinet et al., 2014), JAC59140.1 and JAC59135.1, lack one and two catalytic residues, respectively. Phylogenetic reconstruction revealed that *AeSPH* is more closely related to SPHs from another *Aphidius* species, *Aphidius gifuensis* Ashmead (KAF7991450.1, 33% identity, 63% coverage), and from *A. pisum* than to other SPHs identified in *A. ervi* (Figure 1B). qRT-PCR confirmed that *AeSPH* is specifically expressed in *A. ervi* venom glands (Figure 1C).

Effect of *AeSPH* knockdown on *Aphidius ervi* parasitism success

Experimental *A. ervi* females injected with dsSPH showed a significantly reduced expression of the target gene in the emerged adults, with expression being reduced by 87% (Figure 2A). The observed *AeSPH* knockdown resulted in a reduced rate of parasitism success (Figure 2B). SPH $-$ females

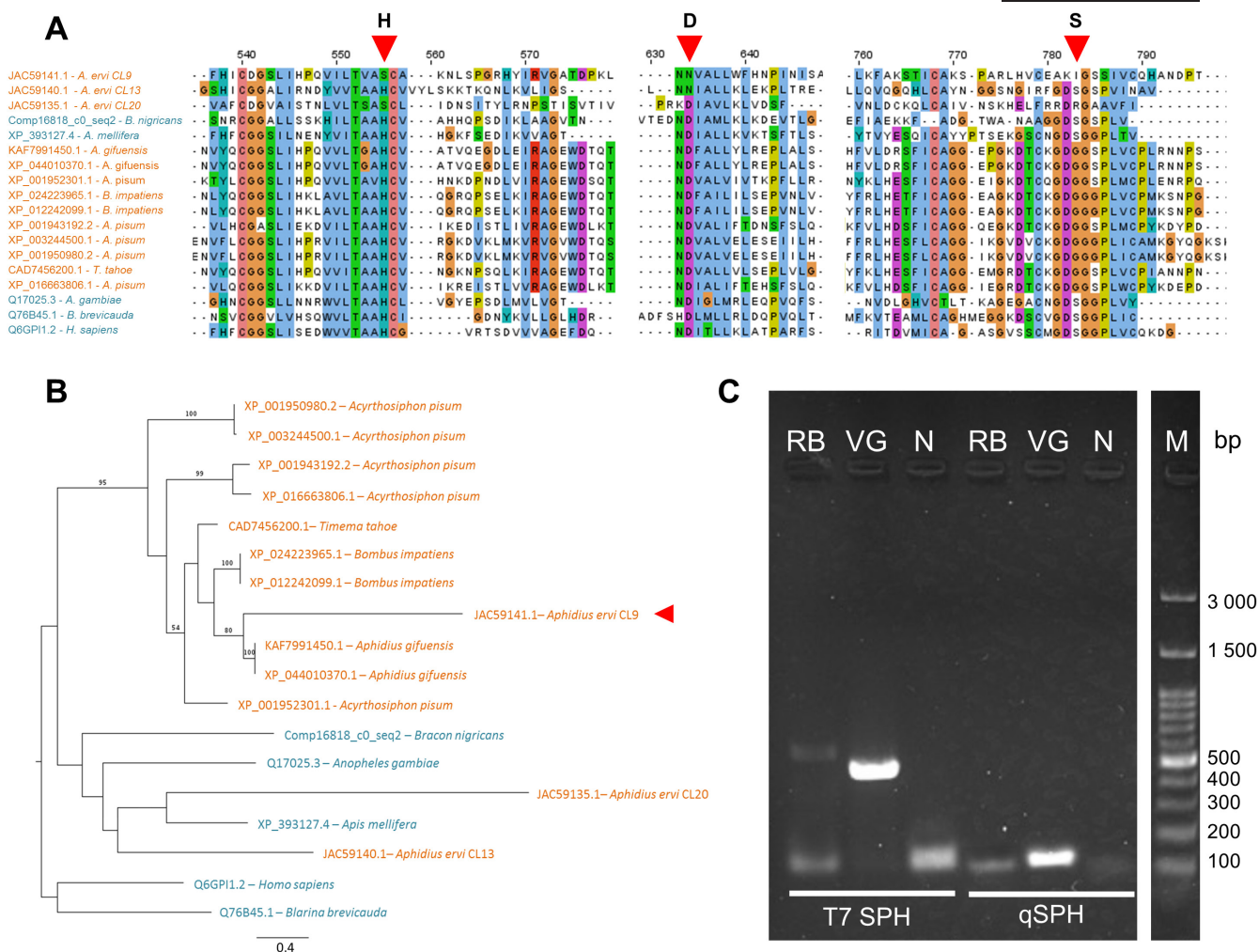


FIGURE 1 AeSPH is a putatively inactive serine protease homolog specifically expressed in *Aphidius ervi* venom glands. (A) SPHs of *A. ervi* venom were aligned with putative homologs and active serine proteases (SPs). SPH CL9 is likely to be enzymatically inactive, due to the lack of the three catalytic residues His (H), Asp (D), and Ser (S), indicated by a red arrowhead. SPHs and SPs are indicated in orange and blue, respectively. (B) Phylogenetic tree based on maximum likelihood analysis of SPHs and SPs identified by Blast searches against nr GenBank db, using AeSPH sequence as query. AeSPH, indicated by a red arrowhead, is more closely related to SPHs from *Aphidius gifuensis* and *Acyrtosiphon pisum* than to other SPHs identified in *A. ervi* proteo-transcriptomics. Bootstrap support values $\geq 50\%$ are indicated at each node. The scale bar indicates the number of amino acid substitutions per site. (C) Electrophoretic analysis of PCR products obtained using two primer pairs (T7 SPH and qSPH) targeting AeSPH on samples collected from venom glands and body carcass of *A. ervi* females. BC: body carcass; VG: venom glands; N: negative sample; M: DNA ladder and its size range (bp). The whole gel is in [Figure S2](#).

were able to sting *A. pisum* 3rd instars but their parasitism success was reduced by approximately 60% compared to SPH+ females (Figure 2B), as indicated by the significantly lower incidence of mummified hosts. The ratio of adult parasitoids emerging from the formed mummies was close to 95% for both experimental groups (Figure S1).

Effect of *AeSPH* knockdown on phenoloxidase cascade activation in the host

Phenoloxidase activation in the host is controlled by the presence of AeSPH in the venom of the parasitoid at both the transcriptional and enzymatic level. Indeed, time, treatment (non-parasitized, SPH+, and SPH-), and their interaction had an effect on *PAF2* expression (Table S2) and PO

enzyme activity (Table S3). Mean relative expression of *PAF2* was more than 2-fold higher in SPH- compared to SPH+ aphids at 24 h, whereas no significant increase in expression was observed 2 h after parasitization (Figure 3A). In parasitized aphids, *PAF2* expression significantly increased from 2 to 24 h following parasitization (Figure 3A). A similar pattern was observed in enzymatic activity of PO performed on aphids. Mean PO activity was more than 2-fold higher in SPH- compared to SPH+ aphids at 24 h after parasitization (Figure 3B).

DISCUSSION

We explored the functional role of AeSPH, a serine protease homolog present in the venom of the aphid endoparasitoid

A. ervi, by RNAi-mediated silencing of its coding gene, and by assessing the impact on parasitization success and host's melanization response. Sequence analysis indicated that AeSPH is a member of the serine protease homolog (SPH) family, which includes immune proteins similar to serine proteases, but lacking one or more residues of the catalytic triad (Yu et al., 2003; Felföldi et al., 2011). As with other SPH family members, AeSPH lacks all the three catalytic residues of serine proteases (Polgár, 2005) and, thus, is likely to be enzymatically inactive. In contrast, other SPHs, present in the venom gland transcriptome but not translated as proteins in the venom reservoir (Colinet

et al., 2014), display some of the catalytic triad residues of SPs and are likely phylogenetically distant to AeSPH. A similar situation seems to occur in *Pteromalus puparum* (L.), with the presence in its genome of 38 SPHs, of which only two are specifically expressed in the venom glands (Yang et al., 2017).

The phylogenetic analysis pointed out that AeSPH has a putative ortholog in *A. gifuensis*, although displaying low identity scores, which suggests a fast evolution of the gene. However, more genomic/transcriptomic data on different species of aphid parasitoids are needed to infer evolutionary relationships in this venom protein family. SPH family members have been identified in the venom blends of several parasitoid species, such as *Chelonus inanitus* (L.) (Vincent et al., 2010), *Cotesia rubecula* (Marshall) (Asgari et al., 2003), *Nasonia vitripennis* (Waker) (de Graaf et al., 2010), *P. puparum* (Zhu et al., 2010), and *Scleroderma guani* Xiao et Wu (Wu et al., 2020), suggesting a conserved important role in the suppression of host immunity, which seems to occur also in *A. ervi*. Indeed, AeSPH is expressed only in the venom glands, clearly indicating high tissue-specificity, a common trait shared by genes encoding the main bioactive components of venom blends (Becchimanzi et al., 2020).

To assess whether AeSPH has a role in determining parasitism success, we generated AeSPH-deficient wasps through dsRNA injection and performed controlled parasitization of *A. pisum*. Microinjection of dsRNA in pupae resulted in a strongly reduced expression in emerging adults, as observed in analogous studies (Siebert et al., 2015; Martinson et al., 2016; Russo et al., 2023), and allowed in vivo phenotypic analysis. Thus, we performed controlled parasitization studies and used the successful formation of mummies as a proxy for parasitism success, which was reduced by ca. 60% when AeSPH was knocked down. In contrast, no adverse effects of AeSPH knockdown were observed on adult emergence, suggesting that AeSPH plays a role in early suppression

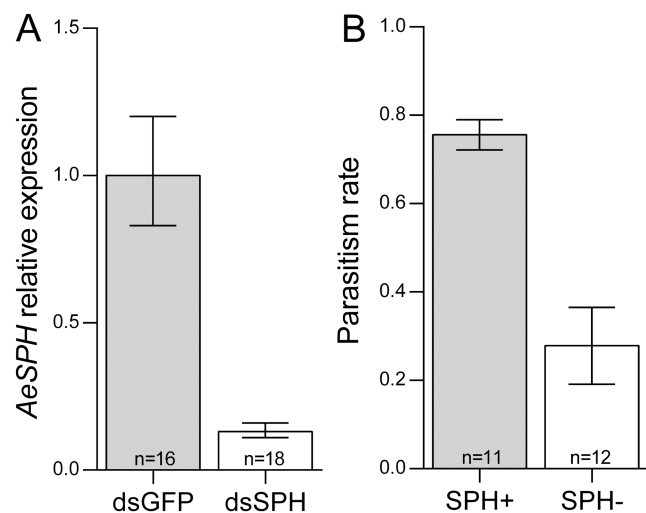


FIGURE 2 Knockdown of AeSPH in ovipositing *Aphidius ervi* wasps reduces parasitism success. (A) Mean ($\pm$ SE) relative expression of AeSPH in females used in controlled parasitization experiments. AeSPH expression was significantly reduced in females injected with dsSPH compared to dsGFP-injected controls (Student's *t* test: $t = 7.769$, d.f. = 32, $P < 0.0001$). (B) Knockdown of AeSPH (SPH-) was associated with a reduced mean ($\pm$ SE) parasitism rate (Mann Whitney test: $U = 16$, $P = 0.0011$). n: sample size.

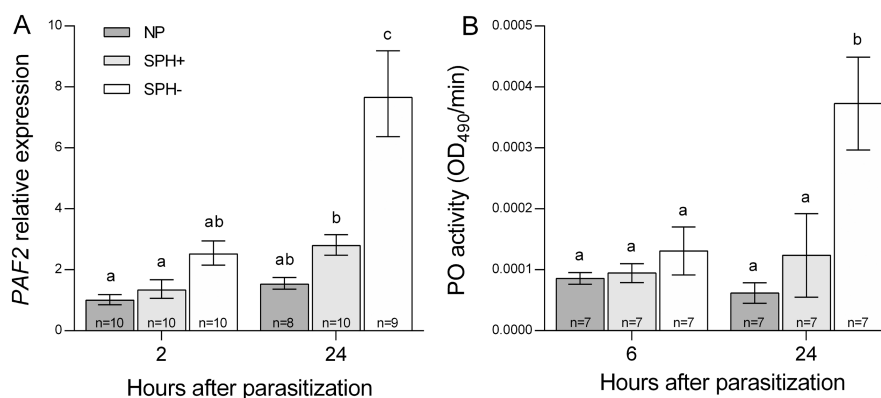


FIGURE 3 Phenoloxidase (PO) activation is enhanced in pea aphids parasitized by female *Aphidius ervi* wasps producing AeSPH-depleted venom. (A) Mean ($\pm$ SE) relative expression of *phenoloxidase activating factor 2* (PAF2) in aphids used in controlled parasitization experiments and non-parasitized controls (NP). PAF2 expression increased over time (see Table S2 for ANOVA results) and was higher in aphids parasitized by AeSPH-deficient females (SPH-) compared to aphids parasitized by females with a whole venom blend (SPH+) at 24 h from parasitization. (B) Phenoloxidase activity increased over time and was higher in SPH- compared to SPH+ aphids at 24 h hours from parasitization. n: sample size. Means within a panel capped with different letters are statistically different (Tukey's post-hoc test: $P < 0.05$).

of the immune response. This result suggests that when injected by *A. ervi* during oviposition along with other venom bioactive molecules, AeSPH plays a crucial role in protecting the wasp's egg from the immune response of the host, likely interfering with the serine protease cascade which triggers melanization.

To assess whether the impact of AeSPH on parasitism success was related to its putative involvement in the evasion of the host melanization response, as observed in other studies on the role of SPHs in parasitoid venoms (Asgari et al., 2003; Wu et al., 2020), we measured the expression of a PO activating factor (PAF2) and PO activity in aphids parasitized either by *A. ervi* females with whole venom blend or those which were AeSPH-deficient. The reduced parasitism success rate observed was associated with an increase of PO activation, both at the transcriptional and enzymatic levels, suggesting that AeSPH has a role in protecting the parasitoid egg from the melanization response by the aphid host. Indeed, in aphids parasitized by AeSPH-deficient wasps, PO enzymatic activity was more than 2-fold higher than controls at 24 h post parasitization, likely contributing to egg suppression.

In one of the first studies on venom SPHs, Asgari et al. (2003) proposed a model that describes how these proteins may interfere with the activation of the PO cascade, at the onset of the melanization response. SPHs are present in the hemolymph of several insects and function as co-factors for proPO-activating proteinases (PAPs, such as PAF2 in aphids), likely interacting with proPO to facilitate its proteolytic cleavage (Yu et al., 2003; Zhang et al., 2004). A SPH identified in the venom of *C. rubecula* (Vn50) is an agonist of the endogenous SPHs of the lepidopteran host *Pieris rapae* (L.), which prevents proPO activation (Asgari et al., 2003). Indeed, purified or recombinant Vn50 completely inhibited melanization of *P. rapae* hemolymph in a dose-dependent manner (Asgari et al., 2003). This mechanism of competition appears to be related to both sequence and structural similarities to the host's SPHs (Asgari et al., 2003; Thomas & Asgari, 2011). Such similarities between venomous and non-venomous proteins, also identified by our phylogenetic analysis of AeSPH, is not uncommon and depends on the evolutionary mechanisms of venom proteins in parasitic wasps, often based on co-option of single copy genes with physiological functions for external (venom) purposes (Martinson et al., 2017).

In addition to SPHs, the melanization response of the host is targeted in many different ways by several other venom proteins present in parasitic wasps, such as calreticulin (Siebert et al., 2015), super oxide dismutase (Colinet et al., 2011; Liu et al., 2018), and serine protease inhibitors (serpins) (Colinet et al., 2009; Yan et al., 2017; Zhou et al., 2023). The mechanisms of PO activation and blockage have been extensively characterized in Lepidoptera (Yu et al., 2003; Kanost et al., 2004; Wang et al., 2020b), whereas only limited information is available for aphids (Xu et al., 2019), which rely on both intrinsic

and symbiont-based strategies to defend themselves against parasitic wasps (Oliver et al., 2003; Luo et al., 2021). Compared to Lepidoptera and other insect orders, aphids are known to have a reduced intrinsic immunocompetence (Altincicek et al., 2011; Laughton et al., 2011) and genome analysis revealed that they lack some important immune genes (e.g., antimicrobial peptides, PGRP, IMD pathway components) (Gerardo et al., 2010; International Aphid Genomics Consortium, 2010). However, several studies demonstrate that aphids display an active melanization response against invaders (Schmitz et al., 2012; Xu et al., 2019; Ma et al., 2020b; Im et al., 2022), which varies depending on nutritional factors (Zhang et al., 2016; Martin et al., 2023) and genotypes, and is likely stimulated in response to *A. ervi* parasitization (McLean & Parker, 2020). Our experimental evidence highlights the key-importance of melanization for immune defense in aphids, and the inhibitory role played by AeSPH. This finding stimulates future studies on other venom proteins that putatively interfere with melanization, such as serpins and neprilysin-like proteins (Colinet et al., 2014).

The central importance of melanization in the aphid immune response is further corroborated by a similar virulence strategy adopted by fungal aphid pathogens. Indeed, *Metarhizium anisopliae* (Metchnikoff) Sorokin produces destruxin A, a secondary metabolite that inhibits melanization in aphids by suppressing the scavenger receptor B (a pattern recognition receptor) (Han et al., 2022).

In conclusion, the present study expands our knowledge on the role of *A. ervi* venom in host regulation, shedding new light on the host immunosuppression strategy. Previous studies on the venom from this parasitic wasp focused on its castration activity and metabolic redirection (Falabella et al., 2007; Russo et al., 2023). Further functional studies aimed at characterizing specific parasitoid venom components will facilitate a more comprehensive understanding of aphid immunity. Moreover, these investigations will elucidate the venom's role in modulating the symbiont development, which is a crucial aspect influencing host regulation by *A. ervi* (Russo et al., 2023).

AUTHOR CONTRIBUTIONS

Elia Russo: Conceptualization (equal); formal analysis (equal); investigation (equal); writing – original draft (equal); writing – review and editing (equal). **Andrea Becchimanzi:** Conceptualization (equal); formal analysis (equal); investigation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Giulia Magoga:** Data curation (equal); formal analysis (equal); writing – review and editing (equal). **Matteo Montagna:** Data curation (equal); formal analysis (equal); writing – review and editing (equal). **Ilaria Di Lelio:** Conceptualization (equal); data curation (equal); formal analysis (equal); writing – review and editing (equal). **Francesco Pennacchio:** Conceptualization (equal); funding acquisition (lead); methodology (equal);

project administration (lead); supervision (lead); writing – review and editing (equal).

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DISCLOSURE

All authors contributed critically to the drafts of this manuscript and gave final approval for publication. All authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Table S1 Primers used in the present study.

Table S2 Two-way ANOVA of ΔC_T values recorded in aphids (PAF2 relative expression, [Figure 2A](#))

Table S3 Two-way ANOVA of initial rate of reaction values recorded in aphids (PO activity, [Figure 2B](#))

Figure S1 Mean ($\pm$ SE) emergence rate of *Aphidius ervi* adults from aphid mummies (Mann Whitney test: $U=51$, $P=0.49$).

Figure S2 Whole gel belonging with [Figure 1C](#).

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