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Original article

Parasitic infection protects wasp larvae against a bacterial challenge

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Abstract

Host antibacterial defense after Strepsiptera parasitization is a complex and rather unexplored topic. The way how these parasites interact with bacteria invading into the host insect during an infection is completely unknown. In the present study we demonstrate that larvae of the paper wasp *Polistes dominulus* are more efficient at eliminating bacteria when they are parasitized by the strepsipteran insect *Xenos vesparum*. We looked at the expression levels of the antimicrobial peptide defensin and we screened for the activity of other hemolymph components by using a zone of inhibition assay. Transcription of *defensin* is triggered by parasitization, but also by mechanical injury (aseptic injection). Inhibitory activity *in vitro* against the Gram positive bacterium *Staphylococcus aureus* is not influenced by the presence of the parasite in the wasp or by a previous immune challenge, suggesting a constitutive power of killing this bacterium by wasp hemolymph. Our results suggest either direct involvement of the parasite or that defensin and further immune components not investigated in this paper, for example other antimicrobial peptides, could play a role in fighting off bacterial infections in *Polistes*.
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Keywords: Host-parasite-bacteria interactions; Innate immunity; Wasps; Defensin; Inhibition zone assay

1. Introduction

An emerging field in immunology is the interaction between immunity and other important physiological functions, such as feeding behavior, ageing, energy use, circadian rhythm and reproduction: all these aspects can noticeably impact the outcome of an immune response [1]. In insects like bees, ants, wasps and termites, "social immunity" is an additional variable [2]. Colonies of social insects have been described as "factory fortresses" by Wilson [3], since multiple barriers are interposed between the colony and the external "abrasive environment" [4], a continuous source of predators, parasites and pathogens. Both individual immunity and cooperative hygienic behavior contribute to maintain the nest safe and stable [5]. Colony parasites need to progressively overwhelm multiple defenses, including colony-level defenses, if they want to parasitize a member of the colony inside the nest [6]. Endoparasites must accomplish an additional step to perform successful infection, which consists in overcoming host individual immunity, since they have to settle within the hemocoel, another small homeostatic fortress [7].

An insect society with numerous prey items in a single sheltered environment may be attractive for many parasites and/or pathogens at the same moment. Given the comfortable environmental temperature, the crowded living conditions and the high genetic relatedness of colony members, insect societies are highly susceptible to microbial infections [8] especially for young individuals of the colony, which have soft cuticles and limited movements. Data from the literature claim that immatures from social insects rely more than adults on molecular defence mechanisms against parasites and pathogens, since they are practically devoid of behavioural responses, being confined within a comb cell with limited possibility of movement and necessarily defended by the other colony members [9].

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The scenario depicted so far corresponds exactly to what we expect to find in annual colonies of the primitively eusocial wasp *Polistes dominulus* (Hymenoptera: Vespidae) infected by the strepsipteran endoparasite *Xenos vesparum* (Strepsiptera: Stylopidae). Infective stages of the parasite (1st instars, also called triungulins) manage to reach *Polistes* nests carried by foraging wasps (for infection modalities different from phoresy see Refs. [10,11]). Due to their non-selective host-seeking behaviour (unpublished), they quickly penetrate into immatures – thus escaping possible aggressions by adult nestmates – and reach the hemocoel, where they perform all the steps of their endoparasitic development, rendering the host unable to mount an effective immune response [12]. The cost of parasitism for wasp larvae has been shown to be irrelevant in terms of mortality and mass loss [13]. This is a good example of virulence trade-off: the host is valuable to the parasite if it lives a long life [14]. As a testament to this, coexistence between *X. vesparum* and *P. dominulus* is quite long (around one year for female parasites) compared to the limited life span of a worker wasps (usually a few weeks, although they may reproduce and/or overwinter, due to their caste flexibility [15]). *Xenos* achieves this goal by castrating the host and manipulating its behaviour in such a manner that parasitized wasps are no longer social and active on the nest but turn into simple vectors that facilitate the development, the reproduction and the spread of the parasite [16].

In a system where host and parasite are so deeply connected, it is evident that pathogens which are potentially dangerous for the host represent a secondary risk for the parasites that would clearly get important benefits by killing them straight or pre-activating the immune defense of the host. In the present study, we tested the antibacterial response of *P. dominulus* larvae against pathogenic bacteria injected in their hemocoel before and after parasitization by *X. vesparum.* From previous analyses we knew that wasp immatures are equipped with a functional immune system provided with both cellular and humoral responses [12,17,18]. We performed different measures of immunocompetence (see Ref. [19]): bacterial clearance, expression levels of the antimicrobial peptide defensin and the inhibition zone assay. This is the first time that an antimicrobial peptide – i.e. defensin – is sequenced and characterized in *P. dominulus*, a "model organism" for studies on social insects [20].

2. Material and methods

2.1. Study animals

Insect colonies for laboratory experiments were obtained from hibernating clusters of *P. dominulus* (Christ) females (i.e. future queens), which were collected at the end of the winter in three different sites in Tuscany (Italy): Impruneta (Florence), Renaccio and San Gimignanello (Siena). Specimens were split in pools of three females inside 20 × 20 × 20 cm Plexiglas cages under standard conditions (15L/9D and 28 ± 2 °C, with paper, sugar, water and *Sarcophaga* sp. larvae ad libitum) to allow polygynous colony foundation (30 large nests after 4–6 weeks).

To obtain parasitized larvae in the lab, we performed artificial (i.e. laboratory) infections. Our sources of *X. vesparum* (Rossi) triungulins were 15 wasps coming from the same hibernating clusters and parasitized by a single (rarely two) *Xenos* female. For each trial, we matched nesting and parasitized wasps coming from the same hibernation site. Parasite females, extruding their cephalothorax (top portion of the body) through a wasp's abdomen, started to release batches of triungulins after four weeks at 15L/9D and 28 °C, i.e. when wasp larvae began to develop inside nests. The procedure was the same as described in a previous work [12]: briefly, we used a thin needle to transfer *Xenos* triungulins from mothers' cephalothorax to wasp larvae at their 3rd–4th developmental stage. To simulate a natural infection we used a pool of triungulins (around 5) for each wasp larva and then we painted the respective nest cell in order to be able to find parasitized larvae at the moment of dissection. The measures of immunocompetence were performed by collecting hemolymph at 4 days post-infection. During post hoc dissections, we sought for *X. vesparum* exuvia and/or 3rd instars, all clues for the parasite's successful development. Infections were carried out from mid-June to mid-July, thus we presumably infected larvae of early and late workers, i.e. the main target for triungulins in the field [16]. For each experiment we clumped together pools of 3rd–4th instars larvae – parasitized and unparasitized – from multiple nests (15 total, randomly chosen among 30 laboratory colonies), in order to avoid any pseudo-replication due to the colony of origin. The mixed pools of our relatively limited sample prevented from doing any nested analysis, nevertheless we controlled for random effects of colony (see Section 2.7).

2.2. Injection of bacteria and bacterial clearance

As immune elicitors we used Gram positive and Gram negative bacteria, i.e. *Staphylococcus aureus* and *Escherichia coli* (strain ATCC 23739 and ATCC 25923, respectively) that are common model pathogens for insects normally absent from the wasp's hemolymph [18]: therefore, this was likely to be a novel immune challenge for *P. dominulus* larvae. Bacterial cultures were grown overnight at 37 °C in Luria-Bertani Broth (LB) to an optical density of $OD_{600} = 2$. After centrifugation, bacteria were washed twice, resuspended in phosphate-buffered saline (PBS) and diluted to the desired concentration with PBS. Then the two solutions were mixed together for inoculation in wasp larvae, in order to test the effect of both Gram positive and Gram negative bacteria in our relatively limited set of samplings. Preliminary trials were conducted to determine bacterial dosage. A higher mortality rate was registered at injections of more than 10^5 cells in both parasitized and unparasitized larvae, therefore a dose of 10^5 bacteria was chosen as our experimental challenge; a similar amount has been used previously with honey bees [21]. Under these optimized conditions, the survival rate of wasp larvae at 24 h post-injection was about 70% for both groups.

During three independent trials, *Polistes* larvae were divided into three groups and treated without being removed

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from their nest: one group was kept as a non-injected control (C), the second group was injected with PBS (PBS-i) and the third group was injected with bacteria (Bac). This procedure was followed for each of two animal pools: unparasitized wasps ($C = 3$; PBS-i = 12; Bac = 17, from eight colonies) and parasitized wasps at 3 days after infection with *X. vesparum* ($C = 7$; PBS-i = 12; Bac = 22, from seven colonies). *Polistes* larvae were challenged with 1 μl of the bacterial mixture (total = 10^5 cells) or 1 μl PBS using a microsyringe (Hamilton Microliter, series 700, ⌀ 33 gauge); before injection, the needle of the syringe was washed with 75% ethanol and then in sterile distilled water. Nests were left in standard condition for 24 h to allow recovering of larvae. The next day, 10 μl hemolymph was collected from each larva, diluted in 90 μl PBS 1× and then serially diluted as described elsewhere [18]. We plated 20 μl of the original solution and 20 μl of the 1000× dilutions on LB agar and we evaluated bacterial clearance by optically recording the total colony forming units (CFU) for both *S. aureus* and *E. coli*.

2.3. DNA and RNA isolation and cDNA synthesis

Total DNA was extracted from a *P. dominulus* naïve 4th instar larva using the Wizard SV Genomic DNA purification system (Promega Corporation, Madison, WI, USA). For RNA extraction, sample larvae were frozen in liquid nitrogen and subsequently homogenized using a Polytron homogenizer (Kinematica AG). From each sample total RNA was extracted following a standard "TRI REAGENT™" (SIGMA-Aldrich, St. Louis, MO, USA) procedure. The quantity of the extracted RNA was assessed with a Nanodrop ND-1000 UV–vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and absorbance ratio at 260/280 nm and 260/230 nm was used to assess purity of the RNA samples. For each sample, 1 μg of total RNA was used to make Oligo(dT) cDNA by means of "RevertAid H Minus First Strand cDNA Synthesis Kit" (Fermentas AB, Vilnius, LT) according to the manufacturer's specifications.

2.4. Sequencing of defensin

Full-length *defensin* (*def*) cDNA of *P. dominulus* was obtained by RT-PCR and rapid amplification of cDNA ends (RACE) method. A multiple alignment of Hymenoptera *def* sequences (Genbank source) was performed using CLUSTAL X [22] to identify highly conserved nucleotide sequence portions used to design a pair of degenerate primers (Hym_deg_FW vs Hym_deg_RV) that allowed to amplify and sequence the first portion (158 bp long) of *P. dominulus def*. Gene-specific primers (Def_RACE_5′ and Def_RACE_3′) and nested primers (Def_NEST_5′ and Def_NEST_3′) were designed from previously determined DNA sequence to get the full-length *def* cDNA by means of 5′- and 3′-RACE analyses performed using the SMART RACE cDNA amplification Kit (Clontech, Mountain View, CA, USA) and following manufacturer's instructions. For all primer sequences see Table 1. Both fragments obtained (5′- and 3′-regions) were cloned in

Table 1
Primer sequences.

Primer name	Sequence 5′→3′
Hym_deg_FW	GAACGTGCCGAYAGACAWAGAAGA
Hym_deg_RV	TTCTCGCARYGACCTCCAGCTTT
Def_RACE_5′	TTTCTCGCAATGACCTCCAGCTT
Def_RACE_3′	AACGTGCCGATAGACAAAGAAGA
Def_NEST_5′	GTCTTCTTCTTTGTCTATCGGCACGT
Def_NEST_3′	AAGCTGGAGGTCATTGCGAGAAA
Def_FW	CGTCATAGTTGCGGTCAATATGGC
Def_RV	CGCAAATACCACTGCTGCAATATCC
Def_Gen_FW	GACTTCGATAATTTTATCTAATAAC
Def_Gen_RV	GTGATACATTAAATTATCAAAATGTTG
Act_FW[a]	AGCAGGAGATGGCCACC
Act_RV[a]	TCCACATCTGCTGGAAGG

[a] Sequence obtained from Ref. [23].

the pGEM-T Easy vector (Promega Corporation, Madison, WI, USA) and three clones for each fragment were randomly chosen and sequenced in both strands, using M13 forward and reverse primers. All sequences obtained were corrected manually and assembled using Sequencer 4.2.2 (Gene Codes, Ann Arbor, MI, USA) in a complete *def* transcript, which shows a 3X coverage, at least, for each nucleotide position. Protein analysis was performed using Prosite database [24] to eventually identify conserved domains and motifs. By SignalP 3.0 software [25] the sequence was screened for signal peptide presence. A search for similarities within known genes was performed using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A pair of species-specific primers was designed on the untranslated regions, which were identified at the 5′ and 3′ (5′-UTR and 3′-UTR) of the transcript obtained to get intron sequences using DNA as a template in a PCR. The sequence of the isolated gene was submitted to GenBank with accession number GU327374.

2.5. PCR analysis of defensin

For both unparasitized and parasitized wasps (see Section 2.2) we screened the following samples to establish *def* transcription level by means of PCR: 3 C, 3 PBS-i and 9 Bac specimens (total 15 specimens for each group). The relative transcription of cytoplasmic *actin* (*act*) gene was also investigated to normalized *def* expression data (internal control). One microliter of cDNA was used as a template in a 25 μl PCR reaction combining two different primer pairs: Def_FW vs Def_RV and Act_FW vs Act_RV (Table 1). After an initial denaturation at 95 °C for 5 min, the PCR conditions were: 95 °C 60 s, 54 °C 60 s, 72 °C 45 s, for 32 cycles. PCR mixture was: 1 μM of each primers, 1 mM of each dNTPs, 1X PCR reaction buffer (Promega Corporation, Madison, WI, USA), 2.5 mM Mg^{2+} and 1U of recombinant Taq DNA polymerase (Promega Corporation, Madison, WI, USA). This allowed to amplify a 365 bp portion of the *def* gene and a 399 bp portion of the *act* gene. PCR products were run in a 1.5% agarose gel and for each sample we performed 3 experimental replicas. Densitometric quantification of unsaturated images was performed using ImageQuant 5.2 software (Molecular Dynamics).

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The optical density of each band was established based on an area (with the same size for all samples) including the band; pixel density for each area was quantified and adjusted by means of a background correction.

2.6. Inhibition zone assay

As a third measure of immunocompetence we performed an inhibition zone assay. This is a test to explore the ability of antibacterial substances present in the hemolymph to inhibit bacterial growth *in vitro*: the result is an approximately circular, clear zone around the hemolymph (where bacteria were unable to grow) with a diameter proportional to the strength of the inhibition. The procedure was the same described in Ref. [26], with a few minor modifications and as follows. Antibacterial test plates were prepared separately for *S. aureus* and *E. coli*. Live bacteria from an overnight culture were added to sterile LB containing 1% agar and maintained at 45 °C: the final concentration was 10^6 cells/ml for each bacterial strain. One percent Mead's Anticoagulant Buffer (NaOH 98 mM, NaCl 145 mM, EDTA 17 mM, citric acid 41 mM; pH 4.5) was added as melanization inhibitor [27]. The inoculated agar broths were poured onto 9 cm Petri dishes. Wells (⌀2 mm) were made by puncturing the agar with a glass capillary. Hemolymph samples (2 μl) were pipetted directly into wells and plates were then incubated for 24 h at 37 °C. We evaluated the antibacterial activity as the mean of the min and max diameters (mm) of inhibition zone per each specimen [26]. Three replicates per specimen for both bacterial strains were conducted. Antibiotics (10,000 U penicillin and 10 mg Streptomycin/ml, 2 μl per well) were added to each plate as a control sample and had an inhibition zone of approximately 36 mm diameter.

We conducted a preliminary test of the antibacterial activity of hemolymph samples from the previous year collected from 17 unparasitized *Polistes* larvae and 26 larvae parasitized by *X. vesparum*, which were diluted in a 4X mixture of Grace's Insect Medium (Sigma–Aldrich) and Mead's Anticoagulant Buffer (1:1) and immediately stored at −80 °C. Thereafter, we measured the antibacterial activity of hemolymph samples from 16 controls (C: unparasitized = 4; parasitized = 12) and 26 wasp larvae challenged with bacteria (Bac: unparasitized = 8; parasitized = 18), from the same sample groups as above (see Section 2.2). In this case, we used wasp larvae stored at −80 °C, which were placed into microcentrifuge tubes and spinned at 500*g* for 5 min at 4 °C, as described in Ref. [28].

2.7. Statistical data analysis

Descriptive statistics were computed for the central tendency and variability of each dependent variable. Barplots were used to visualize differences between parasitized and unparasitized larvae and among treatments. Datasets of bacterial clearance, expression of defensin and inhibition zone assay (further trials) had a hierarchical structure with specimens nested within colony. In order to test the effect of treatments and/or parasitization we used mixed effects models [29] to account for random effects of colony and specimen. Normality of residuals was evaluated using the Shapiro–Wilk test. When the assumption of homogeneity was not met, the final equation was weighted by a power variance function to correct for heteroskedasticity.

For the quantitative analysis of defensin expression, a measure of the repeatability of the same measurement taken three times was computed using an intraclass correlation coefficient. All analyses were performed with R version 2.9.2 [30]. Mixed effects models were conducted using the lme function in library nlme.

3. Results

3.1. Bacterial clearance

Non-injected larvae did not harbour any bacterial strain capable of growing on LB agar, as detected in three unparasitized wasps and in seven wasps parasitized by *X. vesparum* (data not shown): this is consistent with previous data [18]. Analogously, LB agar plates from PBS-i larvae did not show any evidence of bacterial presence in both pools of animals (12 specimens each, data not shown), thus we may assume that injection itself was performed in sterile conditions and no bacteria were accidentally introduced during the experimental procedure.

The pattern was different in Bac: two bacterial morphotypes were clearly distinguishable on LB agar plate 24 h after the inoculation of 10^5 bacterial cells. In 10 μl hemolymph per larva, on average 1.03×10^4 total CFU were recorded in unparasitized wasps and 1.67×10^2 in wasps parasitized by *X. vesparum* (Fig. 1A): the difference between the two pools was highly significant ($F_{(1,24)} = 13.66$, $P < 0.001$). The result is in line with previous observations on the increased bacterial clearance in 24-h-parasitized wasps [18]. Focusing on unparasitized specimens, animals were able to eliminate around 90,000 CFU within 24 h, which corresponded to a reduction of 98% of injected bacteria. This finding is consistent with data reported for mosquitoes and fruit flies [31,32].

When we performed separate statistical analyses for the two bacterial strains, we noticed that the pattern was slightly different (Fig. 1B). *E. coli* was significantly reduced in parasitized larvae ($F_{(1,24)} = 9.23$, $P < 0.01$; total CFU = 1.88×10^3 and 0.34×10^2, unparasitized and parasitized wasps, respectively) while *S. aureus* did not change significantly ($F_{(1,24)} = 1.07$, $P = 0.31$; total CFU = 1.12×10^3 and 1.05×10^2, unparasitized and parasitized wasps, respectively).

3.2. Wasp defensin

The complete cDNA sequence of *P. dominulus def* (Fig. 2) is 482 bp long and shows a poly$(A)_{26}$ sequence at the 3′-end. A predicted coding region of 104 amino acids, as well as 5′ (60 bp) and 3′ (110 bp) untranslated portions are present in this sequence. Comparison with genomic sequence reveals the presence of three exons of 73 bp (E1), 202 bp (E2) and 37 bp (E3), and two introns of 135 bp (among E1 and E2) and 141 bp

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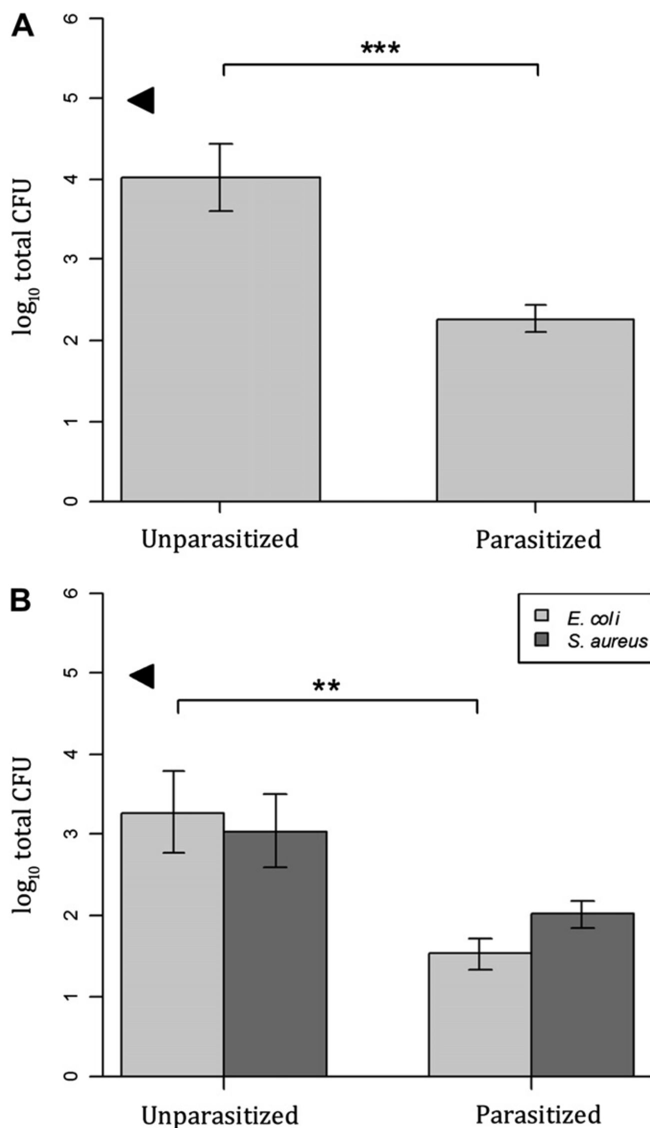

Fig. 1. Bacterial clearance. (A) Total CFU ($\log_{10}$ transformed) in 10 μl of hemolymph sampled from 17 unparasitized wasp larvae challenged with bacteria and 22 wasps challenged with bacteria at 3 days post-infection with *Xenos*. (B) Separate analysis for the two bacterial components (*E. coli* and *S. aureus*). Means ± SE. $^{**}P < 0.01$; $^{***}P < 0.001$. Arrows denote the injection dose (10^5 bacterial cells). No CFU grew in control wasps (non-injected individuals) or in wasps injected with PBS.

(among E2 and E3). Prosite database search allowed the identification of several hypothetical functional sites through the deduced amino acid sequence: two cAMP- and cGMP-dependent kinase phosphorylation sites (51-RRVT-54 and 91-RKDS-94), four N-myristoylation sites (62-GMIGSS-67, 65-GSSACA-70, 80-GGYCSS-85 and 86-GICVCR-91), one protein kinase C phosphorylation site (94-SFK-96) and one casein kinase II phosphorylation site (94-SFKD-97). Through exon 2, six cysteines were identified as involved in intra-chain disulfide bonds (positions: 55, 69, 73, 83, 88 and 90). A most likely cleavage site between positions 20 and 21 of the deduced protein (NMA-AP) was identified by SignalP 3.0 software revealing the presence of a signal peptide. Also a mature peptide cleavage site between position 52 and 53 (RR-VT) and a C-terminal amidation site between position 101 and 104 (KRF-G) were recognized, comparing the sequence with *Apis mellifera* defensin 1 protein [33]. A polyadenilation signal sequence AATAAA was identified at the C-terminal of the sequence.

BLAST analysis of the complete deduced amino acid sequence showed an amino acid identity with *A. mellifera* def 1 isoform (GenBank accession number: NP_001011616) of 58%. There is a gap of seven amino acids between *Polistes* and *Apis* sequences located at the position 38−44 in the wasp protein (FREDMVE), this portion seems to be absent only in Apoidea sequences recorded in GenBank. Regarding non-Apoidea sequences the best match (58% identity) was obtained with *Nasonia vitripennis* (Hymenoptera, Chalcidoidea) def (GenBank accession number: ACX54960).

The analysis of *P. dominulus* defensin sequence revealed several features that are typical of this gene. First, defensin shows three exons as the *A. mellifera* defensin1 isoform [33], moreover these are similar in position and length. Second, the presence of a signal peptide on the N-terminal of the deduced defensin protein indicates that this protein is secreted in the hemolymph. Third, the existence of a mature peptide cleavage site and a C-terminal amidation site suggests that the native protein is composed by 51 amino acids, in line with data from the literature showing defensin ranges in length from 32 to 51 amino acids [34]. Fourth, the six cysteines involved in hypothetical disulfide bonds are a distinctive character of invertebrate defensins, which are a family of cysteines-rich antimicrobial peptides that show six conserved cysteines, all involved in intra-chain disulfide bonds.

3.3. Expression of defensin

Defensin was constitutively expressed in C unparasitized wasp larvae (Fig. 3, left side), which showed a significant increase in transcriptional levels after the injection of PBS ($F_{(2,5)} = 51.12$, $P < 0.001$, ICC = 0.55). The relative expression of the gene remained at similar levels at 24 h following a bacterial challenge. On the other hand, the presence of *Xenos* (Fig. 3, right side) increased the expression of *def* in C parasitized individuals ($F_{(1,2)} = 17.39$, $P = 0.05$, ICC = 0.77), thus neither PBS injection nor a bacterial inoculum were able to trigger a significant boost in transcript levels, ($F_{(2,4)} = 0.27$, $P = 0.77$, ICC = 0.83).

3.4. Inhibition zone assay

No inhibitory activity was recorded against the Gram negative test bacterium *E. coli* in *Polistes* hemolymph (data not shown), whereas evident antimicrobial activity occurred against the Gram positive bacterium *S. aureus*. The inhibitory activity was strong in preliminary analyses with frozen hemolymph from untreated animals (mean diameter of the inhibition zones = 17.65 mm). No significant difference (Fig. 4A) was detectable between unparasitized and parasitized wasps ($F_{(1,41)} = 0.85$, $P = 0.36$). Further trials with hemolymph collected from frozen larvae (Fig. 4B) revealed

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  1 GACATTTGACTTCGATAATTTTATCTAATAACATTACGAATCTTTCGTAAATTATTTAAAATGACAAGACTC
  1                                                             M  T  R  L
 73 TGTATTATTCTTTCTATTTTATTCGTCATAGTTGCGGTCAATATGGCTGCTCCTGTAGAAGGTAAATTATTT
  5 C  I  I  L  S  I  L  F  V  I  V  A  V  N  M  A ↑A  P  V  E  D
145 ATTATGTGATAATAATTTTAAGAAATATATTATAAAATAGAACTTATATAGTTATTTATTACTTCAATAAAT
217 GTAATCATAAAAAATATCTGAACGAATAAATAAATAATAATGAATATTTTCAGATGACAGATTAGAAAATTTA
 26                                                      D  R  L  E  N  L
289 ATGGAACTCGATGATGAATTCAGAGAGGACATGGTCGAGGAACGAGCTGATAGACAACGTCGAGTGACCTGT
 32 M  E  L  D  D  E  F  R  E  D  M  V  E  E  R  A  D  R  Q  R  R ↑V  T  C
361 GACCTTTTATCCTTTGGTGGGATGATTGGTTCATCGGCTTGTGCGGCTAATTGTCTTTCCATGGGAAAAGCT
 56 D  L  L  S  F  G  G  M  I  G  S  S  A  C  A  A  N  C  L  S  M  G  K  A
433 GGAGGATATTGCAGCAGTGGTATTTGCGTGTGTCGCAAGTAAGCCTCGTTTTCTTTTATTTTTCTTAATTAA
 80 G  G  Y  C  S  S  G  I  C  V  C  R  K
505 TCATTATAAACTTATTATTGTGTTCTTCTCTAAAAAAAAAAAAAAAGAAAAGAAAAAAAGATCTATTAATCT
577 CTTGTCACTAATTTCCTTCCTTTTTTTGCTTTCAGAGATTCATTCAAAGATTTGTGGAATAAACGATTTGGT
 93                                    D  S  F  K  D  L  W  N  K  R  F ↑G
649 TGAAATCAACAAATATTTCTATACCGTCTCCATTCAACATTTTGATAATTTAATGTATCACTTTTTATTGTA
    *
721 AAAATACAAATAATAAAAAAATATATGTATCTTTATAC
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Fig. 2. Nucleotide sequence and deduced amino acid sequence of *P. dominulus* defensin. Open reading frames are in bold. Amino acid deduced sequences of each exon are in grey boxes. Vertical arrows mark a cleavage site for signal peptide processing, a mature peptide cleavage site and a C-terminal amidation site. In square are marked cytosines involved in disulfide bonds. Splicing signals and the polyadenylation signal sequences are underlined.

a smaller diameter of the inhibition zones (2.69 mm on average), whereas the inhibitory activity was not affected by *X. vesparum*, nor challenge with bacteria or combination of both ($F_{(1,21)} = 0.52$, $P = 0.47$).

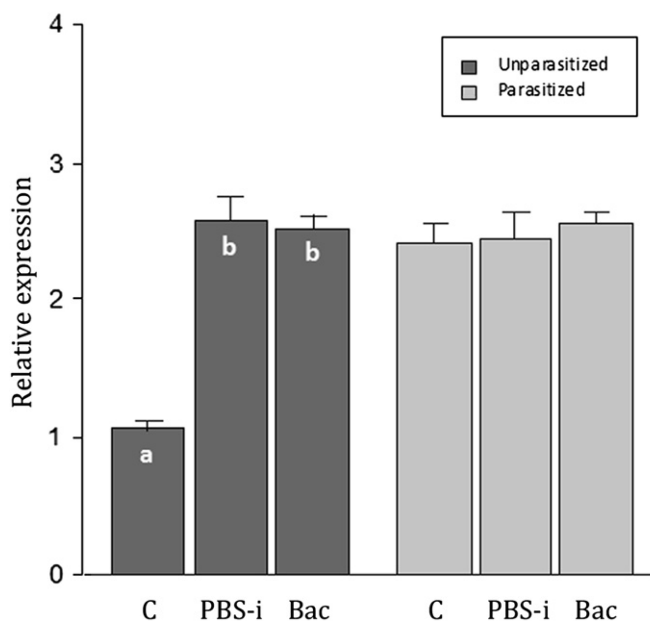

Fig. 3. Transcriptional levels for the antimicrobial peptide *defensin*. Relative expression of *defensin* in 15 unparasitized and 15 parasitized wasp larvae parted into 3 groups: non-injected controls (C, $n = 3$), injected with phosphate buffer saline (PBS-i, $n = 3$), injected with bacteria (Bac, $n = 9$). All of the values shown are mean + SE; the bars with different letters are significantly different (ANOVA, $P < 0.001$).

4. Discussion

Recent studies on host-parasite interactions have suggested that parasites do not always irreversibly damage their host [35], thus the scenario may be different compared to insects affected by hymenopteran parasitoids (for a review of the subject, see Ref. [36]). In this perspective, parasites are organisms that redirect the physiological pathways of the host to gain the energy necessary for their development and dispersal. In particular, parasitic castrators are able to exploit resources that were allocated for reproduction of the host [14]. These two aspects apply to the *X. vesparum-P. dominulus* system: *Xenos* is a permanent parasitic castrator and, as a consequence of that, parasitized hosts represent a complex “extended phenotype” (sensu Dawkins) and become members of the parasite populations.

In this playground, it may be easier to interpret our main result: *P. dominulus* larvae parasitized by *X. vesparum* are not compromised in their antibacterial response. On the contrary, they are better equipped than unparasitized specimens to face a huge bacterial challenge, as reported in previous studies [18]. The lower number of bacterial colonies in parasitized wasps (Fig. 1) may have two possible justifications: a more effective antibacterial response due to the infected wasp’s innate immune system, or a direct involvement of the parasite in killing bacteria. In this study we focus on the first possibility in order to point out which pathway/s is/are specifically targeted by the parasite. However, it is not always easy to distinguish between pathways, which may be mechanistically linked via a sophisticated network of signaling cascades (namely Toll, Imd, Jak/Stat and JNK pathways) which regulate the effectors of innate immunity [37,38].

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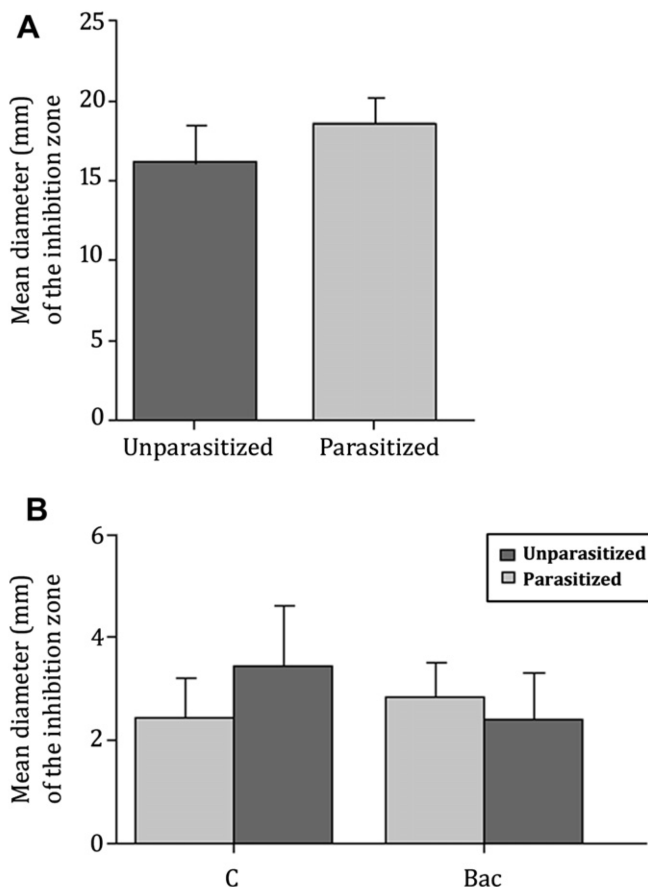

Fig. 4. Inhibition zone assay. (A) Preliminary analysis of the antibacterial activity in the hemolymph of 17 unparasitized wasp larvae and 26 individuals parasitized by *Xenos*. (B) Comparison of the antibacterial activity in the hemolymph obtained from 26 wasp larvae challenged with bacteria (Bac: unparasitized = 8; parasitized = 18) and 16 untreated controls (C: unparasitized = 4; parasitized = 12). The antibacterial activity is expressed as the zone of inhibition (mm diameter) around a drop of hemolymph on a bacterial test plate inoculated with *S. aureus*. Data are shown as means ± SE. No significant differences were found.

As a critical component of *Polistes* humoral immunity, we analyzed the *def* transcriptional rate, in combination with a bacterial challenge and infection by *X. vesparum*. A similar approach is frequently used to investigate immune mechanisms in insects which are important disease vectors [39]. Based on our results, defensin seems to play a marginal role in clearing bacteria. First, the injection itself triggers a significant increase in *def* expression (Fig. 3), as demonstrated by higher levels of transcripts during aseptic injury in unparasitized wasps (with no further increase after injection of bacteria): this is in line with dynamics reported in *Apis* [5,6,40] and *Bombus* [41]. Second, visible levels of *def* expression in unparasitized non-injected individuals suggest that this gene is constitutively expressed in wasp larvae, similarly to genes for several antimicrobial peptides in other organisms [4]. Concerning a possible effect of *Xenos* on *def* transcription levels, parasitized controls show a higher transcription rate than unparasitized ones, but the expression levels of the gene, 24 h after bacterial challenge, are not different in the two animal pools.

The most obvious explanation (that the wasp increases the production of defensin to fight *Xenos* parasites) does not appear fully appropriate for this case, since antimicrobial peptides are normally released to selectively kill unicellular invaders, such as bacteria and fungi [34] or in the case of protozoan infections [42]. Nevertheless, whether a macroparasite can induce the production of antimicrobial peptides remains an open question. For example parasitoid insects during their development seem to maintain the host free of opportunistic infections [43] while antimicrobial peptides in the hemolymph of a mollusk affected by *Schistosoma* contribute to produce lesions to the sporocysts of the parasite [44]. An alternative explanation is that the expression of *def* is just a side effect of a broad immune response activated by the parasite, although in our experience the defense reaction of the wasp is apparently silent during the first three days of parasitization. Within this time span, in fact, hemocyte numbers slightly increase [18], phagocytosis in vivo is not significantly affected by the parasite (unpublished), the encapsulation process is just starting and the melanization response is absent [12]. On the other hand, the simple entry of a foreign body into the larval host may be an elicitor of *def* expression, a sort of priming that produces an immune memory and enables parasitized wasps to respond more promptly to subsequent bacterial challenges: a similar mechanism has been tested in other insects for several stimuli [45,46]. Though in the past we described this process as “soft entry” and a “non-traumatic event” [12], nevertheless *X. vesparum* must interrupt the epidermal layer to reach the hemocoel. The expression of antimicrobial peptides is persistent for several days at the level of many epithelia [37,47].

Through the inhibition zone assay we tested the *in vitro* activity of *Polistes* hemolymph. One unexpected result is the absence of antibacterial activity towards the Gram negative bacterium *E. coli* but not against the Gram positive *S. aureus*, unlike what observed in bacterial clearance assay. This discrepancy could be due to specific antimicrobial responses. In the yellow fever mosquito *Aedes aegypti*, the clearance of *E. coli* relies principally on phagocytosis (a typical cellular mechanism), while *S. aureus* on both cellular and humoral processes such as melanization and lysis [48]. Since hemolymph aliquots used in our assay were previously frozen, we probably inhibited hemocyte activity, thus we lost the cellular component of the process. Also the smaller inhibition zone in *S. aureus* trials with frozen larvae may be linked to a different modality of hemolymph storage (see Methods and Fig. 4). On the whole, these results suggest a constitutive power of killing bacteria which is not influenced by septic injury or by the presence of *Xenos* parasites.

In conclusion, parasitization by *X. vesparum* evokes a better response in wasp larvae against a subsequent microbial challenge. This effect could be partially due to a higher level of defensin, here sequenced for the first time in a paper wasp. In this study we focused on this antimicrobial peptide and performed functional analysis following the concomitant attack of a macroparasite and bacteria. So far only two antimicrobial peptides, Dominulin A and B, have been described

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on the cuticle and in the venom of *P. dominulus* and represent a social protection against infection for the nest [49]. Further antimicrobial peptides are likely to be present in wasp hemolymph. Since each peptide may have individual patterns of expression [50], it would be incorrect to extend what we found for *def* to other genes. Moreover, the speed of the response, rather than the total amount of transcripts, could be the core of a successful defense strategy. Finally, transcript abundance does not always reflect actual protein levels [50] which are the real weapon against bacterial cells. Quantifying protein levels in the hemolymph would be the best way to link the activity of *def* gene with the bacterial clearance: but this is a different approach, which is beyond the scope of the current study. Further investigations on the expression patterns of other antimicrobial peptides as well as additional immune measures (for example the phenoloxidase system) will help elucidating whether bacteria are better eliminated in parasitized wasps due to a more effective response of their immune system or to a direct antibacterial activity of *Xenos* parasites.

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