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The strepsipteran endoparasite *Xenos vesparum* alters the immunocompetence of its host, the paper wasp *Polistes dominulus*

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ABSTRACT

It is unexplained how strepsipteran insects manipulate the physiology of their hosts in order to undergo endoparasitic development without being entrapped by the innate immune defences of the host. Here we present pioneering work that aimed to explore for the first time several components of the cellular and humoral immune response among immature stages of the paper wasp *Polistes dominulus*, in both unparasitized insects and after infection by the strepsipteran endoparasite *Xenos vesparum*. We carried out hemocyte counts, phagocytosis assays *in vitro* and antibacterial response *in vivo*. On the whole, hemocyte load does not seem to be drastically affected by parasitization: a non-significant increase in hemocyte numbers was observed in parasitized wasps as respect to control, while the two dominant hemocyte types were present with similar proportions in both groups. On the other hand, phagocytosis was significantly reduced in hemocytes from parasitized wasps while the antibacterial response seemed to be less effective in control. These somewhat unexpected results are discussed, along with the implications of a multiple approach in immune response studies.

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1. Introduction

The paper wasp *Polistes dominulus* Christ is the primary host of the strepsipteran endoparasite *Xenos vesparum* Rossi (Hughes et al., 2004a). Coexistence between the two organisms may occur from the first larval instars of the wasp, the target of infection (Hughes et al., 2003), till the emergence of the parasite, if male, or for months, if female; i.e. the sex of the parasite affects host lifespan (Beani, 2006). The *Xenos* neotenic female, living permanently in the wasp abdomen, is the vehicle of infective 1st instar larvae to the next colony cycle (Hughes et al., 2004b). Due to this prolonged association, it is appropriate to include *X. vesparum* within the specific category of "koinobiont endoparasitoids" (Brodeur and Boivin, 2004). These organisms penetrate into sessile host stages and their development is finely tuned to the development of the holometabolous host, which continues to grow and metamorphoses. Thus, the damage is reduced in comparison to other parasitoids (Pennacchio and Strand, 2006), but the host is unable to reproduce because it is rendered sterile. In our model system, the wasp is both "softly invaded" by the parasite (Manfredini et al., 2007a) and not heavily depleted during its development (Hughes and Kathirithamby, 2005).

Host–parasitoid associations have been described sometimes as a "physiological race": the outcome depends on a balance between host's potential to mount an immune response and parasitoid potential to disrupt this response (Prevost et al., 2005). Susceptible hosts provide all the requirements for successful parasitoid development, while resistant hosts are capable of avoiding or eliminating undesired intruders through both cellular and humoral factors (for example, encapsulation and melanization) as well as by means of behavioural, chemical and genetic defences (Cremer et al., 2007; Stow and Beattie, 2008). On the other hand, parasitoids are able to escape host immune responses through passive evasion and/or active suppression (Strand and Pech, 1995), i.e. they avoid triggering host defences without compromising directly the components of its immune system or by temporarily inhibiting/destroying it. Passive evasion strategies have also already been described in strepsipteran insects; for example, they develop within the host inside a "bag" derived from the host epidermis (Kathirithamby et al., 2003), and the profile of their cuticular hydrocarbons is similar to that of their hosts (Beani et al., 2005). On the other hand, parasitoids may also employ active immunosuppressive factors, primarily affecting hemocytes through inhibition of spreading (reorganization of the cytoskeleton), changes in their number, alterations of putative hematopoietic tissues and induced apoptosis: this occurs in *Drosophila melanogaster* and *Spodoptera littoralis* parasitized by *Leptopilina boulardi* and *Chelonus inanitus*, respectively (Labrosse et al., 2005; Stettler et al., 1998). A second target may be the humoral

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components of host immunity: a reduction in hemolymph viscosity or in phenoloxidase activity occurs in *Heliothis virescens* after parasitization by *Toxoneuron nigriceps* (Consoli et al., 2005). Finally, parasitoids may also cause depression of host phagocytosis of bacteria and encapsulation, as has been recently observed in the system *Cotesia plutellae*/*Plutella xylostella* (Ibrahim and Kim, 2006).

This study is focused on cellular and humoral changes in the innate immunity of *P. dominulus* immatures induced by *X. vesparum*. Similarly to hymenopteran endoparasitoids (Schmidt et al., 2001), *Xenos* must overcome host defences to achieve successful development but also to preserve the host from premature death, thus controlling its own virulence. After the invasion of *X. vesparum* (Manfredini et al., 2007b), as a first point the encapsulation process is not suppressed but delayed: hemocytes start aggregating only 48 h after infection and complete capsules are visible within the following week. Second, the capsule surrounds a pseudo-target, which is the empty exuvium of the 1st instar-infective stage, while the real target – the living parasite from 2nd larval instar till mature stages – is not trapped inside. Third, no melanization reaction is visible anywhere, either within the formed capsule or in the proximity of the wound site. Thus *X. vesparum* appears to adopt a complex strategy, involving both active suppression and passive evasion of host defence reaction, but it is not clear yet which molecular processes underlie these mechanisms. Here, by investigating both the cellular and humoral components of the immunity of wasp larval stages, we obtained the first data about hemocyte counts, *in vitro* phagocytosis and *in vivo* antibacterial response after parasitization by strepsipterans.

2. Materials and methods

2.1. Animal rearing, hemolymph collection and laboratory infections with the parasite

Hibernating clusters of *P. dominulus* were collected in San Gimignanello (Siena, Italy) at the end of the winter. They were placed in groups of three individuals inside 20 cm × 20 cm × 20 cm Plexiglas cages with sugar, water and *Sarcophaga* sp. larvae *ad libitum*, under 15L/9D and 28 ± 2 °C, to allow colony foundation (24 large nests after 4–6 weeks). No more than 2–3 larvae of the same stage were selected from the same colony, in order to reduce the risk of pseudo-replications due to relatedness. Our sources of *X. vesparum* 1st instar larvae, the infective stages of the parasite (otherwise called triungulins), were 12 overwintered wasps coming from the same hibernating clusters as above and parasitized by a single (rarely 2) *Xenos* female. These females, extruding their cephalothorax through wasp abdomen, released batches of triungulins after 4 weeks at 15L/9D and 28 °C, i.e. when wasp larvae began to develop inside nests.

Hemolymph was collected from immatures of *P. dominulus* by making a small incision in the cuticle of chilled larvae and collecting the oozing hemolymph drop with a precision microliter pipette; samples were kept in ice to prevent hemolymph coagulation and cell impairment (Manfredini et al., 2008). This procedure was followed for 3rd (“small”), 4th and 5th (“large”) instars larvae, both naïve (“control”) and parasitized by *X. vesparum*.

To obtain parasitized larvae in the lab, we performed artificial (i.e. laboratory) infections from mid-June to mid-July; thus we presumably infected early and late workers. The procedure was the same as described in a previous work (Manfredini et al., 2007a): after the temporary removal of adult wasps from the colony, the nest was placed under a stereomicroscope. Using a needle, small groups of triungulins (around five) were transferred from the abdomen of parasitized adult wasps to different larval stages, without removing them from the nest. We used a pool of triungulins from several parasitized wasps for each test, to simulate a natural infection (Beani and Massolo, 2007). After 10 min the nest was then tied in its cage, so the adult wasps would not abandon the colony. The cells containing larvae of the same stage, parasitized and not (control) were colour-marked. After hemolymph collection, we verified for the successful entry of at least one parasite, i.e. for the presence of triungulins’ exuvia and/or 2nd instar parasites.

2.2. Hemocyte counts, types and functional tests

For hemocyte counts we selected a pool of 9 nests. From each of a total of 30 insects (15 small and 15 large larvae, 16 control and 14 parasitized, 48 h post-infection) 5 μl hemolymph was sampled and diluted 4× in a mixture of Grace’s Insect Medium (Sigma–Aldrich) and Mead’s Anticoagulant Buffer (NaOH 98 mM, NaCl 145 mM, EDTA 17 mM, citric acid 41 mM; pH 4.5), added in equal proportions. Thereafter, 16 μl of the mixture were transferred in a haemocytometer (Bürker), loading 8 μl of solution per spot; after 10 min at room temperature (RT) in the dark, samples were observed under a Leica DMRB light microscope in phase contrast and for each specimen the number (THC = total hemocyte counts) and type (DHC = differential hemocyte counts) of observed hemocytes were registered. Based on previous work (Manfredini et al., 2008), we could easily distinguish between “granulocytes” (small, very refractive in phase contrast, spherical) and “plasmatocytes” (larger, less refractive and elongated). Prohemocytes, the third hemocyte type, were not considered in this count.

Additional aliquots of hemolymph were used for functional tests, after dilution in the same Grace–Mead solution as above. To evaluate mortality, samples were treated with Trypan Blue (Sigma–Aldrich) at the final concentration of 0.04 mg/ml and placed on a glass slide for 5 min in a wet chamber; for spreading activity (i.e. adhesion modality on glass), diluted hemolymph was directly settled on the glass slide for 30 min. Thereafter the slide was observed with the phase contrast microscope. In the former test (7 control and 13 parasitized larvae), we counted how many cells out of 100 randomly observed in one field were dead (intensively blue-coloured after Trypan Blue pre-treatment). Spreading activity (6 control and 13 parasitized) was evaluated by means of changes in cell conformation, from a roundish-traditional shape (no spreading performed) to an elongated-active form, typical of plasmatocytes and clearly distinguishable due to bidirectional fibroblastic extensions (Manfredini et al., 2008).

2.3. Phagocytosis assay

To carry out phagocytosis assays, we used 10 *P. dominulus* nests. Total hemolymph from pools of five immatures of 3rd, 4th and 5th instar clumped together (n = 75 at all, 40 control and 35 parasitized larvae, i.e. 8 and 7 pools, respectively) was collected 72 h post-infection and diluted in 200 μl of the Grace’s/Mead’s (1:1) mixture. We spotted 100 μl of the final solution onto Teflon®-printed microwell glass slides (VWR International); 1 μl of a solution of fluorescent beads (total 5×10^5 beads, Sigma–Aldrich) in phosphate buffered saline (PBS) 1× was added to each sample. Slides were kept for 1 h at 30 °C in wet and dark conditions, and then samples were fixed for 10 min in paraformaldehyde 4% in PBS 1× at 4 °C. Thereafter, samples were resuspended in Trypan Blue, to quench signal from beads not internalized, and analyzed by flow cytometry using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Data were acquired using CellQuest software: the output was the percentage of phagocytosis, i.e. the increase of fluorescence in the population of hemocytes due to the presence of fluorescent beads within the cytoplasm. Cell populations ranging from 2000 to 10,000 hemocytes were included in this analysis.

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Two additional pools of *P. dominulus* immatures (10 at all, 5 control and 5 parasitized) were used for observation at the fluorescence microscope: these samples underwent a prolonged fixation (20 min at RT) in the same conditions as above. As a blocking solution (to reduce background staining) we used fetal bovine serum (FBS) 1% in PBS 1× for 20 min at RT in the dark; to enhance the process slides were covered with a parafilm layer. We performed permeabilization with FBS 1% + Triton X-100 0.1% in PBS 1× for 3 h; during this step primary anti-actin antibody (1:50) (Sigma–Aldrich) was added. At this point samples were incubated with secondary antibody for 1 h; 10 min before this step was concluded, we added 1 μg/ml of the DNA-specific dye HOECHST 33258 (Sigma–Aldrich) for nuclei staining. Finally, samples were mounted on glycerol for observation under a Leica DMRB light microscope equipped with UV light source, fluorescein and UV filters.

2.4. Antibacterial response

Antibacterial response was measured by challenging *P. dominulus* larvae from 5 nests with both Gram positive and Gram negative bacteria, which are normally associated with the digestive tract and absent from the hemolymph. For this purpose, we chose two bacteria which have been commonly used for experimental challenge of insect immune systems, i.e. *Staphylococcus aureus*, strain ATCC 25923 and *Escherichia coli*, strain ATCC 23739. They were grown overnight at 37 °C in Luria-Bertani Broth (LB) and used for immune challenge at the concentration of 1.6×10^6 (*E. coli*) and 2.6×10^6 (*S. aureus*) colony forming units (CFU) μl^{-1}. Both bacterial solutions were put together and then reduced to a thick pellet by centrifugation. At this point, a thin needle was sterilized in 70% ethanol and repeatedly dipped into the bacterial pellet; this needle was gently sunk into wasp larvae, without removing them from their nest cells. Totally, 19 non-parasitized 3rd to 4th instar larvae (control) and 23 larvae 24 h after artificial infection were pricked with the needle bearing bacteria. Then, wasp nests were left in standard conditions for 24 h.

The next day, 5 μl hemolymph was collected from each larva after surface sterilization with 70% ethanol; next, aliquots were diluted in 200 μl PBS 1× and then plated on LB agar (50 μl of the original solution and the same amount of the 1000× dilution). Plates were maintained for 1 day at 30 °C, thereafter CFU were optically recorded. The morphology of the two bacterial strains after plating was different enough to easily distinguish them. The same procedure was followed for hemolymph samples collected from 6 non-parasitized non-challenged larvae ("negative controls"). As a culture medium we decided to use LB agar, which is usually indicated for *Enterobacteriacea* for two reasons: first, it is a suitable medium for the growth of both *E. coli* and *S. aureus* colonies second, in order to prevent the growth of resident constitutive bacteria, i.e. the microbial flora potentially associated to the hemolymph of the wasp.

2.5. Statistical analysis

Statistical analysis was carried on by means of SPSS statistical package for Windows. Normality was tested using the Kolmogorov–Smirnov 1-sample test (Siegel and Castellan, 1988), while homogeneity of variances was tested by Levene test of Equality of Error Variances for the 2-way ANOVA. The combined effects of parasite and larval size on the hemocyte parameters (THC, DHC, mortality and spreading) have been compared between treatment groups using a 2-way ANOVA (Sokal and Rohlf, 1995); one case has been excluded from analyses due to anomalous hemocyte count (outlier).

3. Results

3.1. Hemocyte counts, types and functional tests

Due to the high intra-group variability of both control and parasitized wasps, specimens analyzed by hemocytometer were split for their size into two subgroups, i.e. small larvae (10 control and 5 parasitized) and large larvae (6 and 9, respectively). In fact, preliminary observations in our model (pers. obs.) as well as in other insects (Silveira et al., 2003) suggested that the total hemocyte count (THC) could depend on the animal size/instar. THC (Fig. 1(A)) was not significantly different for any variables considered, i.e. size (2-way ANOVA, $F = 1.969$, df = 1; $P = 0.173$), treatment ($F = 0.882$, $P = 0.357$) and interaction ($F = 0.007$, $P = 0.934$). Concerning the functional tests (Fig. 1(B)), on average the percentage of dead cells did not significantly differ among hemocyte populations of control and parasitized wasps ($F = 1.794$, $P = 0.199$), neither for their size ($F = 3.852$, $P = 0.067$) nor interaction ($F = 1.221$, $P = 0.286$). As previously observed (Manfredini et al., 2008), the most common spreading type was the plasmatocyte, both in control and parasitized wasps (61% and 64%, respectively, out of the total number of spreading cells). Spreading activity (see Fig. 3(B), (E) and (F)) did not significantly differ for any variables (treatment $F = 0.387$, $P = 0.543$; size $F = 0.325$, $P = 0.577$; interaction $F = 0.449$, $P = 0.513$).

The relative abundance of two principal cell types (DHC) was monitored in 28 specimens at phase contrast, as described elsewhere (Manfredini et al., 2008). Granulocytes were predominant, on average, in both parasitized and control wasps (Fig. 1(C)), in line with data formerly obtained in other insect models (Ibrahim and Kim, 2006). The hemolymph composition (frequency of the two cell types) was not significantly different for all the variables here considered (treatment $F = 3.486$, $P = 0.074$; size $F = 1.814$, $P = 0.191$; interaction $F = 0.132$, $P = 0.720$).

3.2. Phagocytosis assay

FACS analysis revealed the level of phagocytosis to be significantly lower in parasitized wasps of different larval stages (Fig. 2), where it was observed on average in 9% of cell populations *vs* 14.9% of control (Student T: $t = 2.86$, df = 13, $P = 0.013$). Samples analyzed by fluorescence microscopy (Fig. 3) confirmed what had been observed using FACS. We counted the same numbers of hemocytes both in parasitized wasps ($n = 262$) and control ($n = 261$) and calculated the percentage of phagocytizing cells; the value was 9.5% for parasitized wasps and 13.8% for control. Due to similar numbers of fluorescent beads phagocytized by a single cell (1.72 in parasitized specimens and 1.86 in control, on average), the higher value recorded in control wasps is evidently the result of higher numbers of control hemocytes undertaking phagocytosis rather than the presence of larger numbers of beads within each cell.

3.3. Antibacterial response

In all six negative controls, hemolymph contained hardly any bacteria able to form colonies when plated out (on average, 0.44 CFU μl^{-1}), but viable bacteria were recovered from both control, i.e. non-parasitized wasps challenged with bacteria, and challenged parasitized wasps (Fig. 4(A)). On average, in the range of 10^1 CFU μl^{-1} were recovered from parasitized wasps, while around 10^2 CFU μl^{-1} were recovered from unparasitized controls. The difference was just significant (Student T: $t = 2.5$, df = 28, $P = 0.018$), i.e. injected bacteria were less well able to survive in parasitized wasps, or these wasps were more able to restrict bacterial growth. In general, colonies of *E. coli* were less abundant

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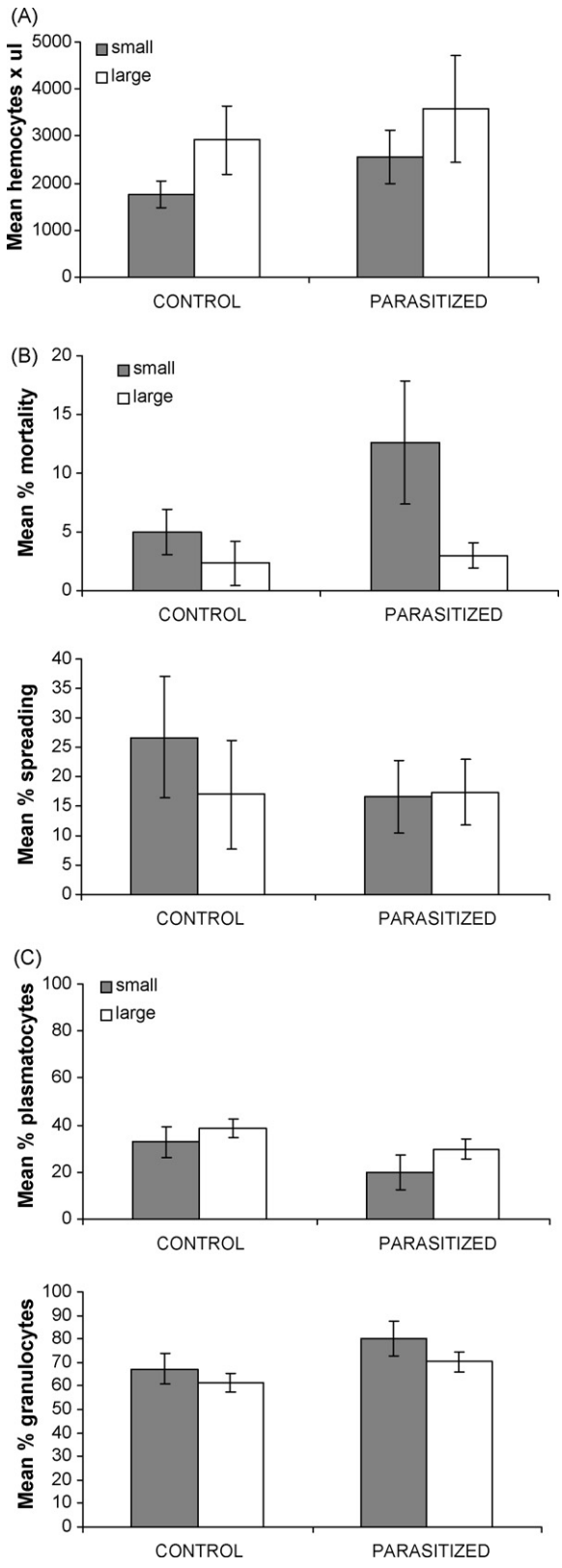

Fig. 1. Hemocyte counts, types and functional tests. (A) Total hemocyte counts split for 3rd instar larvae (small) and 4th to 5th instar larvae (large) in both control (10 small and 6 large individuals) and parasitized (5 small and 9 large) wasps. (B) Mortality % after Trypan Blue staining (top) and spreading % after 30 min adhesion on a glass slide (bottom). (C) Differential hemocyte counts: plasmatocytes (top) and granulocytes (bottom). Histograms represent means ± SE.

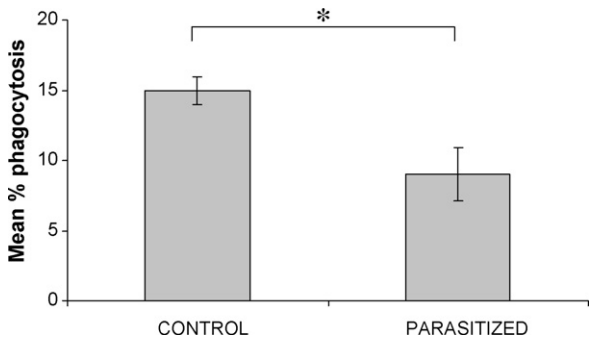

Fig. 2. *In vitro* phagocytosis of fluorescent microspheres (mean ± SE) as detected by FACScan flow cytometry for 8 and 7 pools (5 specimens each) of control (40) and parasitized (35) wasp larvae (3rd, 4th and 5th instars clumped together). (*) = $P < 0.05$.

than *S. aureus* on our agar plates (Fig. 4(B)), nevertheless there was a significant reduction in the recovery of CFU from hemolymph of 24-h-parasitized wasps for both bacterial species when parasitized insects were compared with unparasitized wasps (*E. coli* $t = 2.068$, df = 23, $P = 0.006$; *S. aureus*: $t = 2.048$, df = 28, $P = 0.022$). Because fewer colonies of *E. coli* were recovered than of *S. aureus* it appears that the Gram negative bacteria were more susceptible than the Gram positive bacteria to the immune responses of the wasps in both the parasitized and unparasitized groups. No other microbial colonies capable of growing on LB agar were noted.

4. Discussion

4.1. Hemocyte counts, types and functional tests

Cellular responses are the first line of defence in insects against macroparasites. The adaptability of hemocytes to quickly changeable conditions in the "insect environment" is realized through the alteration of some important features such as density, morphology, spreading activity and release of immune factors. Through the evaluation of these parameters, it is possible to gain some idea of the immunocompetence of the insect, i.e. the potential of the immune system to respond (Sadd and Schmid-Hempel, 2009), which is a good indicator for its fitness. The total hemocyte count (THC), in particular, is extremely suggestive as a general measure of immunocompetence: in fact, it is a reliable cue of the overall capacity of the organism to respond to an immune challenge (Schmid-Hempel, 2003). Fluctuations in THC are primarily determined by the insect developmental stage (Silveira et al., 2003; Wilson-Rich et al., 2008) and the availability of resources (Schmid-Hempel, 2005), but are also strongly influenced by parasitic infections, hemocytes being the actors of encapsulation and phagocytosis responses (Hultmark, 2003). In particular, when an insect is infected by a parasite, its final THC is due to a balance between the host reaction – an increased number of circulating hemocytes for a more effective response – and its reduction by the parasite in order to escape immunity (Prevost et al., 2005; Stettler et al., 1998).

In our host–parasite system, regardless of the larval stage, we recorded higher but non-significant THC in 48-h-parasitized wasps, in line with hemocyte increase towards hymenopteran parasitoids (Dubuffet et al., 2008; Labrosse et al., 2005). A minimal conclusion from this observation is at least that the strepsipteran parasite does not markedly suppress host hemocyte numbers. Nevertheless individual variability is high, mainly among parasitized specimens, even in laboratory conditions and within the same nest (pers. obs.): additional factors of variability are probably the genetic profile and the sex of the parasite, which could play an important role in triggering different responses in

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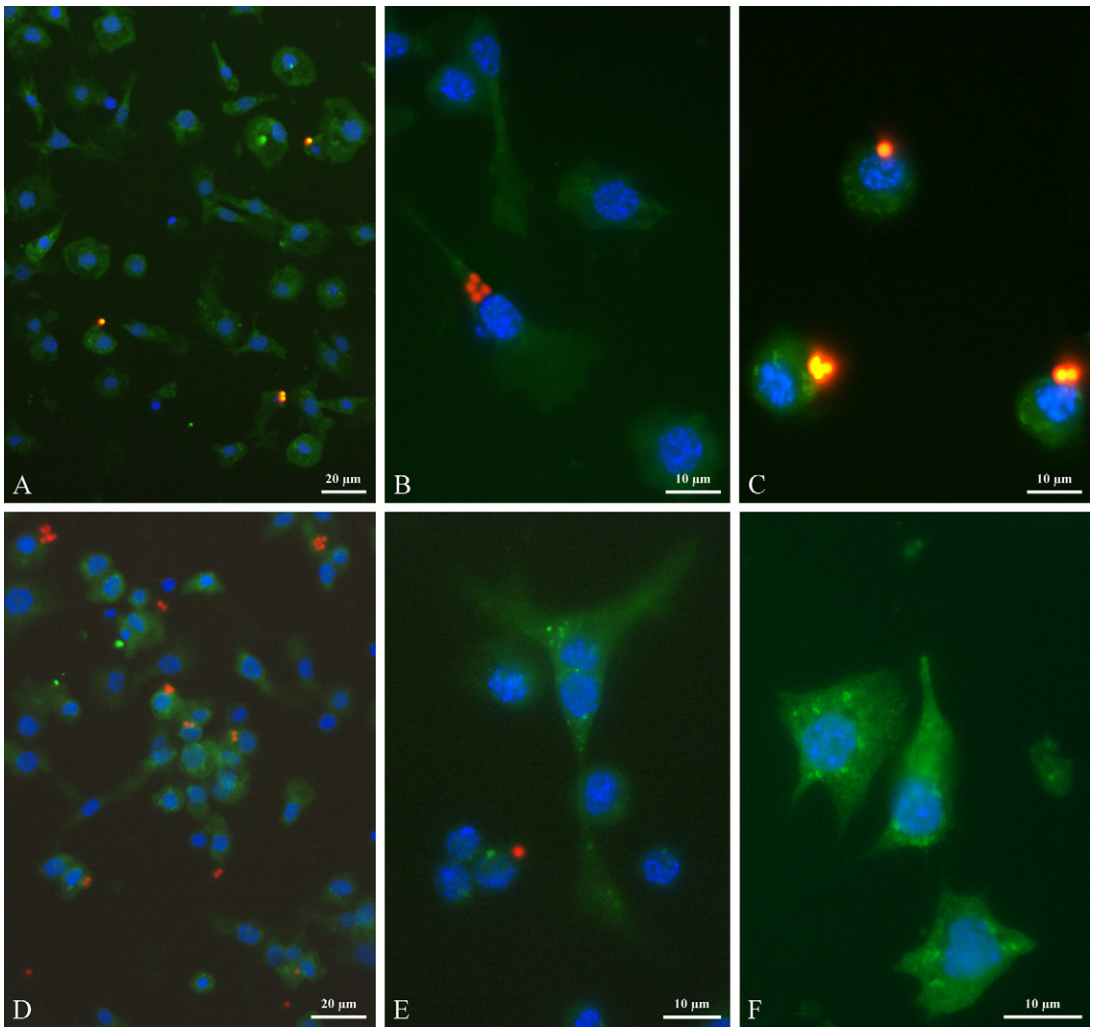

Fig. 3. Phagocytosis assay as observed by fluorescence microscopy. (A–C) Phagocytosis in hemolymph samples extracted from control *P. dominulus* larvae and (D–F) from parasitized wasps at 72 h post-infection. Higher fluorescence is noticeable in A–C than in D–F. Spreading activity of hemocytes is evident in B, E and F.

the host. Moreover, different generations of *Polistes* larvae (early and late workers) could be differently equipped in their immune responses. In the future we will investigate individual variability in relation to host castes (although *Polistes* are primitively eusocial wasps and castes are very flexible), host sex and parasite load.

A further approach in our future analysis will be to monitor the temporal dynamics of the cellular response during the initial phase of the infection process, which is "a particularly relevant but often neglected aspect" (Korner and Schmid-Hempel, 2004). We selected 48 h post-infection for THC and DHC because at this time the 2nd instar of the parasite abandons its exuvia to successfully establish inside the host (Manfredini et al., 2007a). In *Galleria mellonella* infected with *Steinerma* nematodes, hemocyte numbers decrease in the first 4 h, but then increase gradually over the next 12 h, and finally decline to values lower than control: here time spans are brief (within first 24 h) and the final decline in hemocyte numbers is associated with approaching host death (Bergin et al., 2003).

In our *Xenos–Polistes* system, the general hemocyte profile of the host is rather stable: spreading activity, mortality and relative abundances of two main morpho-types are not dramatically affected by the presence of the parasite, as well as THC, and this is in line with the requirement for *X. vesparum* to maintain the host alive and healthy (i.e. capable of fighting other possible invading pathogens), in the perspective of a long and sustainable coexistence (Hughes and Kathirithamby, 2005). This fine-tuning of host immune responses suggests that instead of decreasing the host's capacity to mount immune responses by destroying or otherwise interfering with the production of immune cells, the parasite concentrates on avoiding, whether by evasion or suppression, the responses that the hemocytes would normally mount.

4.2. Phagocytosis assay

Though parasitoids are too large to be phagocytized, phagocytosis analysis by means of synthetic microspheres is a good parameter for evaluating several mechanisms of the innate immunity (Williams et al., 2006; Wood and Jacinto, 2007). In fact, phagocytosis is an actin-dependent mechanism, requiring a proper cytoskeleton to internalize the foreign body. When this process is compromised by the action of a pathogen, further actin-dependent mechanisms are affected, in particular encapsulation and wound healing, since they rely on the ability of hemocytes to perform migration and conformational changes. Moreover, the ability to undertake phagocytosis may be considered symptomatic for the general health condition of the insect, being involved in related signalling pathways of non-cellular immune compartments, for example antimicrobial peptides production (Hoffmann, 2003; Hultmark, 2003).

After parasitization by *X. vesparum*, phagocytosis *in vitro*, relatively low in our insect model, is inhibited by about one-third,

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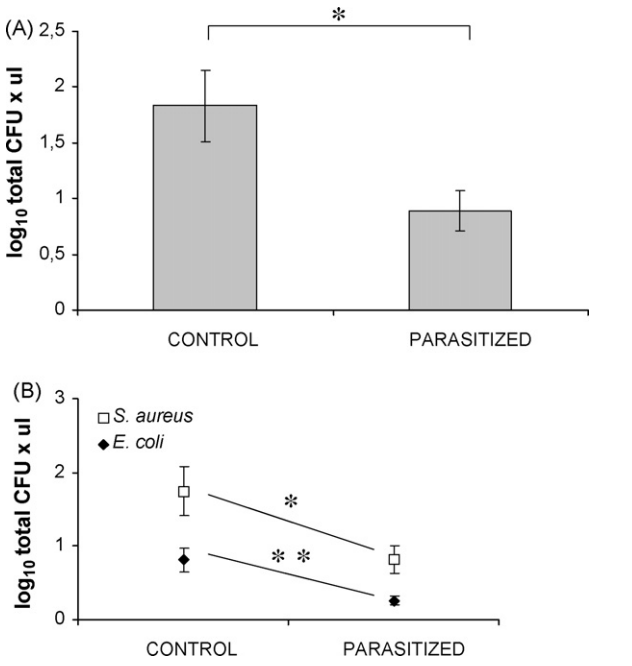

Fig. 4. Antibacterial response. (A) Total CFU ($\log_{10}$ transformed) per μl of hemolymph sampled from 19 non-parasitized larvae challenged with bacteria (control) and 23 larvae challenged with bacteria at 24 h post-infection with *Xenos* (parasitized). (B) Separate analysis for the two bacterial components: *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*). Means ± SE. (*) = $P < 0.05$; (**) = $P < 0.01$.

the number of engulfed particles being reduced from 14% in control to 9% in parasitized larvae. The inhibition does not appear as extensive as has been noted to occur in other host–parasitoid systems. For example, the parasitic wasp *Eulophus pennicornis* triggers a 87% reduction of phagocytosis in the lepidopteran host *Lacanobia oleracea* (Richards and Edwards, 2002). Again, what observed in our model is in line with the strategy of the parasite, which might be disadvantaged by drastically compromising the immunity of its host. Thus, phagocytosis is not totally suppressed, since this mechanism is important for the clearance of potentially pathogenic bacteria which could jeopardize host survival. Moreover, the similar number of fluorescent beads internalized per cell within the two treatments suggests that *X. vesparum* reduces phagocytosis by reducing the number of host hemocytes undertaking phagocytosis. How this accomplished is unclear; the data do not suggest that the parasite acts directly on any specific phagocytic cell type, as indicated by DHC and spreading activity.

There are many possible routes whereby *X. vesparum* – similarly to parasitoid insects – might interfere with *P. dominulus* phagocytosis and the literature of host–parasite systems is full of interesting examples. A peculiar family of proteins could be of great interest for our future analyses, since they are regulators of the master signalling Rho GTPases (Rho, Rac and Cdc42), highly conserved across both invertebrate and vertebrate animals, key cellular factors in cytoskeleton regulation and vesicle trafficking (Qualmann and Mellor, 2003; Takai et al., 2001). This family of proteins, belonging to the Rho-GAPs (Ras homologous GTPase Activating Protein) domain, may be exploited by different parasites to disrupt host cellular response (Labrosse et al., 2005). In this perspective, encapsulation and phagocytosis appear strictly connected. In *Drosophila* mutants defective for the protein Rac2, for example, hemocytes recognize and attach to the eggs of the parasitoid wasp *L. boulardi* but fail to spread around them and capsules fail to melanize (Williams, 2007). The similarity between what happens in *Drosophila* mutants and the peculiar immune response of *P. dominulus* towards *X. vesparum* infective larvae (delayed encapsulation and absence of melanization) is striking.

4.3. Antibacterial response

Among the immature stages of social insects, two effective barriers towards bacteria are represented by social prophylaxis, i.e. the cleaning behaviour by nursing adults and the "nest environment" itself (Cremer et al., 2007; Stow and Beattie, 2008). Our experimental challenge with bacteria not already present in the hemolymph of larval wasps has shown a significant but not striking decrease in bacterial survival in 24-h-parasitized wasps *vs* control. Unfortunately, the "pricking system" does not allow to monitor the precise number of bacterial colonies introduced in each specimen, thus we do not know the total power of *P. dominulus* immatures to respond to a bacterial challenge. The reproducibility of the procedure among our experimental groups, however, is sufficient to support a reliable comparison.

The more effective antibacterial response after bacterial challenge in parasitized *vs* unparasitized wasps is apparently inconsistent with the decreased phagocytosis capability shown by the former group. We are not currently able to account for this. It should be noted however that we observed phagocytosis dynamics *in vitro* while the antibacterial response was measured *in vivo*, where additional variables may be involved (for example, the pricking itself). Secondly, check points were fixed at different times of the infection process and immune dynamics rapidly evolve during parasitism: phagocytosis was measured at 72 h post-infection, while bacterial challenge was performed at 24 h post-infection and measured 24 h later (i.e. 48 h post-infection). Third, phagocytosis is not the sole mechanism adopted by insects to clear bacteria from the hemolymph: among other routes, the production of antimicrobial peptides (AMPs) and reactive oxygen species (ROS), both contributing to bacterial killing and cooperating with the cellular response (Elrod-Erickson et al., 2000). It is possible that in parasitized wasps these pathways are already activated by the presence of *X. vesparum* larvae, thus they are prompter in responding against the bacterial challenge. An additional possibility is that when the injected bacteria are less efficiently cleared by phagocytosis, then they may be more effective in eliciting the synthesis of AMPs.

Finally, we should not forget that it is possible that the *X. vesparum* parasites may also fight, together with their host, against inoculated microbes. We have already hypothesized (Manfredini et al., 2007a) that *X. vesparum* 1st instar larvae, similar to other insect parasitoids, release some secretions into the host to allow entrance and settlement into the hemocoel. In several cases the antibiotic power of such secretions has been demonstrated (Doury et al., 1995; Richards and Edwards, 2002). In our system, they could either kill bacteria directly or create an unsuitable environment for their growth. From this perspective, the action of *X. vesparum* parasites would be extremely intriguing: they "softly" inactivate wasp immune response either to undergo their own first developmental steps within the host, or to preserve the defence reactions of host larvae against other pathogens. Moreover, *X. vesparum* evidently at least cooperates with its host in eliminating invading bacteria, which could be doubly dangerous, being a possible source of infection for both the parasite and the host. It will be our plan in the future to identify which component(s) of the wasp immune system is (are) mainly responsible for the differential antibacterial activity (whether cellular or humoral) and to elucidate the mechanisms whereby the parasite is actually operating within the hemolymph of the host. To understand this long-term, complex parasite–host association requires assessment of more than one index of immune status, strictly connected to each other: hemocyte counts, phagocytosis and antibacterial response.

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