WILEY InterScience® DISCOVER SOMETHING GREAT

JOURNAL OF MORPHOLOGY 268:588–601 (2007)

Developmental Strategy of the Endoparasite *Xenos vesparum* (Strepsiptera, Insecta): Host Invasion and Elusion of Its Defense Reactions

Fabio Manfredini,[1] Fabiola Giusti,[1] Laura Beani,[2] and Romano Dallai[1]*

[1]*Dipartimento di Biologia Evolutiva, University of Siena, Siena 53100, Italy*
[2]*Dipartimento di Biologia Animale e Genetica "Leo Pardi", University of Florence, Florence 50125, Italy*

ABSTRACT To successfully complete its endoparasitic development, the strepsipteran *Xenos vesparum* needs to elude the defense mechanisms of its host, the wasp *Polistes dominulus*. SEM and TEM observations after artificial infections allow us to outline the steps of this intimate host–parasite association. Triungulins, the mobile 1st instar larvae of this parasite, are able to "softly" overcome structural barriers of the larval wasp (cuticle and epidermis) without any traumatic reaction at the entry site, to reach the hemocoel where they settle. The parasite molts 48 h later to a 2nd instar larva, which moves away from the 1st instar exuvium, molts twice more without ecdysis (a feature unique to Strepsiptera) and pupates, if male, or develops into a neotenic female. Host encapsulation involves the abandoned 1st larval exuvium, but not the living parasite. In contrast to the usual process of encapsulation, it occurs only 48 h after host invasion or later, and without any melanization. In further experiments, first, we verified *Xenos vesparum*'s ability to reinfect an already parasitized wasp larva. Second, 2nd instar larvae implanted in a new host did not evoke any response by hemocytes. Third, we tested the efficiency of host defense mechanisms by implanting nylon filaments in control larval wasps, excluding any effect due the dynamic behavior of a living parasite; within a few minutes, we observed the beginning of a typical melanotic encapsulation plus an initial melanization in the wound site. We conclude that the immune response of the wasp is manipulated by the parasite, which is able to delay and redirect encapsulation towards a pseudo-target, the exuvia of triungulins, and to elude hemocyte attack through an active suppression of the immune defense and/or a passive avoidance of encapsulation by peculiar surface chemical properties. J. Morphol. 268:588–601, 2007 © 2007 Wiley-Liss, Inc.

KEY WORDS: endoparasitic development; host defense elusion; encapsulation; melanization

Host–parasite relationships are important experimental models for understanding how the insect "innate" immune system (Schmidt et al., 2001) recognizes a variety of invaders (e.g., endoparasitic wasps) and how parasites—more or less closely related to the host—overcome the insect immune response. Hemocytes, the cellular component of the insect immune system (Lavine and Strand, 2002), play a major role in defense against metazoan parasites. Small pathogens are phagocytized by hemocytes, whereas parasitoids and other macroparasites are encapsulated: hemocytes recognize the foreign invader as nonself and change from freely circulating cells to adhesive cells, forming a multilayered sheath of cells. From an evolutionary perspective, relatively few studies have focused on the behavioral ecology of immature parasites: "exploitative organisms that live in continuous, intimate association with their host," forced to adapt themselves to a changing environment (Brodeur and Boivin, 2004). In this chain of events both cellular and humoral components of host immune response might be compromised, and profound changes in life-history parameters are likely to occur.

Xenos vesparum belongs to Strepsiptera (Insecta), a cosmopolitan order of obligate endoparasites of other insects. Strepsiptera have a direct life cycle; therefore, the first organism infected is the final host, which may be infected at different developmental stages by the 1st instar larva of the parasite, the so-called triungulin (the infective motile stage). The primary host of *X. vesparum* (Stylopidae) is *Polistes dominulus* (Vespidae), a primitively eusocial paper wasp. Like other members of the order (except Mengenillidae), *X. vesparum* shows a striking sexual dimorphism. Males, after the 4th larval instar, follow a complete holometabolous development: they pupate and emerge as free-living winged adults from the puparium, which extrudes through the host abdominal tergites. During their short life-span (4–6 h), they actively search for a receptive female. Neotenic larviform females are the result of an involutive process. At the end of the 4th instar, they extrude

Contract grant sponsor: PAR.

*Correspondence to: Prof. Romano Dallai, Department of Evolutionary Biology, University of Siena, Via A. Moro 2, Siena I-53100, Italy. E-mail: dallai@unisi.it

Published online 16 April 2007 in
Wiley InterScience (www.interscience.wiley.com)
DOI: 10.1002/jmor.10540

© 2007 WILEY-LISS, INC.

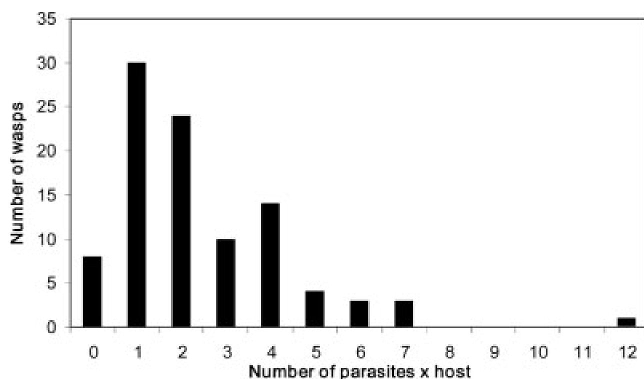

Fig. 1. Distribution of 235 living *Xenos vesparum* parasites (or exuvia in absence of living parasites) in 89 *Polistes dominulus* wasps (late larvae, pupae and adults).

with their anterior region, the cephalothorax, through the host cuticle. They are larviparous permanent endoparasites, which at maturity fill most of the wasp hemocoelic cavity, and lack distinct antennae, mouthparts, eyes, wings, legs, and external genitalia. The opening of the ventral canal in the cephalothorax allows both the extragenital insemination and the escape of the triungulins (Beani et al., 2005a).

This characteristic protrusion of the cephalotheca of the puparium or the sclerotized anterior portion of cephalothorax is called "stylopization" of the host (from the family Stylopidae). Stylopized wasps, regardless of their putative caste, neither work nor reproduce. Instead they desert the colony early on to form, first, summer extra-nidal aggregations, where parasite mating is likely to occur (Hughes et al., 2004b), then autumn-winter diapausing groups, where the fertilized *Xenos vesparum* females overwinter inside their hosts to infect new colonies the next spring (Beani, 2006). These females may be used as long-term dispensers of triungulins to infect wasp larvae in the laboratory.

We have monitored the endoparasitic development of *Xenos vesparum* inside the body of *Polistes dominulus* by means of artificial infections. First, we focus on how the triungulin manages to penetrate the wasp larva, which is protected by compact overlapping cuticle layers, and to evade the wound healing reaction at the entry site (Rowley and Ratcliffe, 1981). Second, we describe step-by-step the developmental stages of the parasite in the hemocoel, up to its 4th instar which is well synchronized with the pupal-imaginal stages of the wasp. Third, we discuss the implications of the compromised defense reaction of the host: the delayed encapsulation of a pseudo-target (the exuvia of triungulins) and the absence of melanization. Through various experimental trials (reinfections, subcuticle applications of 2nd instar larvae and nylon implants, a nonliving triungulin-sized stimulus) we have tested the immune response of *P. dominulus* larvae and the ability of *X. vesparum* to elude this response.

Recently, an unusual immune defense has been described in Strepsiptera (Kathirithamby et al., 2003): the parasite *Stichotrema dallatorreanum* manipulates the tissues of the hosts *Segestidae novaeguineae* and *S. defoliaria defoliaria* (Orthoptera, Tettigonidae) to build a "host-derived epidermal bag," a sort of "camouflage" against the host defense reaction. The immune elusion mechanisms of *Xenos vesparum* could exploit similar components of the host's defense repertoire; alternatively they may represent a different adaptive strategy which has evolved during an ancient and deep association with its host.

MATERIALS AND METHODS

Study Animals

Nests of *Polistes dominulus* Christ were collected during late spring from various locations in Tuscany (Italy). Each colony was housed in a 20 × 20 × 20 cm Plexiglas cage (with sugar, water and *Sarcophaga sp.* larvae *ad libitum*) for 1 month, to verify that nests were not yet infected by strepsipterans in the field. In all, nine colonies were artificially infected in laboratory with 1st instar larvae (triungulins) of *Xenos vesparum* Rossi. Our sources of triungulins were 13 wasps, collected from two hibernating groups (Impruneta and Volterra), parasitized by a single female of *X. vesparum.* These females, extruding their cephalothorax from wasp abdomen, released triungulins after 2–3 weeks at 12L/12D and 28°C.

Artificial Infections and Dissections

Infections (15 in all) were started at mid-June and extended until the beginning of August. After the temporary removal of adult wasps from the experimental colony, the nest was placed under a stereo microscope. Using a needle, two to four triungulins were transferred (Fig. 2A–D) from the abdomen of stylopized wasps to "late" larval stages of *Polistes dominulus* (4th, 5th and sometimes 3rd instars, Fig. 2A), without removing them from the nest and marking the cells with a distinct color. Triungulins were generally placed in the anterior portion of the wasp body, dorsally and posterior to the mandibles. After 10 min the nest was then tied in its cage, so the wasps would not abandon the colony.

The majority of stylopized adult wasps in the field present a single extruded parasite (Hughes et al., 2004a, b), although after dissections higher numbers of immature parasites were recorded (personal observations); moreover the parasite load per brood in naturally infected nests is higher and variable (Hughes et al., 2003). We tried to simulate a natural infection pattern, in which several triungulins are likely to reach one larva. For this reason, and in order to increase the probability of successful infections (at least two parasites per host), we placed from two to five triungulins in each wasp larva. For the purpose of this study, we selected hosts with a maximum of three parasites although the pattern was quite variable (Fig. 1). We omitted the cases of superparasitism (more than three) because competition for nutrients could interfere with normal parasitic development. Although every immature stage of the host was susceptible (Hughes et al., 2003), we focused on the "late" larval stages.

We dissected wasp specimens after 24 h, 30 h, 48 h, 3 days, 1 week, 2 weeks, 3 weeks, and 1 month, to monitor *Xenos vesparum* endoparasitic development, under a stereo microscope,

in 0.1 M phosphate buffer pH 7.2 containing 3% sucrose (PB). All measurements are means ± SD.

Reinfection Trials

To determine whether *Xenos vesparum* can parasitize an already infected host, about 2 days after the first infection, a reinfection of five *Polistes dominulus* larvae and one pupa was done following the same procedure described above. We chose this time span to be sure that the wasp defense reaction had already been activated by the first entry of triungulins at the moment of the second infection.

Polistes dominulus dissections were performed at the following times: one larva was dissected about 3 days after the first infection (24 h from the second infection); four larvae and one pupa were dissected 5 days after the first infection (3 days from the second infection).

Implantation of 2nd Instar Larvae

To verify whether *Xenos vesparum* 2nd instar larvae evoke an attack from *Polistes dominulus* hemocytes when implanted in naive hosts, two preliminary trials were carried out. We obtained 2nd instar *X. vesparum* larvae by dissecting specimens which had been infected 3 days previously. Using a glass capillary, 2nd instar larvae were implanted into naive specimens, previously extracted from their nest cells. We infected two 5th instar wasp larvae with one parasite, seven with two and two pupae with three. To prevent dehydration, the organisms were placed in glass pipes, which were wrapped in aluminum foil, and kept at room temperature. Dissections were done 24 h (three larvae and two pupae) and 3 days (six larvae) after implantation.

Nylon Implantation Trials

To test the normal *Polistes dominulus* defense against foreign objects, we implanted 1 × 0.25 mm nylon filaments in five "late" larvae and two pupae. The alcohol-sterilized filaments were inserted into the hemocoel of *P. dominulus* specimens previously extracted from their nest cells, and organisms were prevented from dehydration as described above. We dissected the wasps at 24 and 48 h after implantation. Presence or absence of capsules and melanization was observed by light stereo microscopy. These specimens were used as control animals for the efficiency of the normal immune system and compared with parasitized wasps.

Scanning Electron Microscopy

For SEM observations, samples were fixed in 2.5% glutaraldehyde in PB for 2 days at 4°C, postfixed in 1% osmium tetroxide in PB for 2 h at 4°C, rinsed in PB several times, and dehydrated in a graded series of ethanol to 100% ethanol. The material was processed by critical point drying in a Balzer's CPD 030. Samples were gold-evaporated in a Balzer's MED 010 sputterer and observed in a Philips XL20 scanning electron microscope operating at 10 kV.

Transmission Electron Microscopy

After dissection, the material was fixed and rinsed as described above. Ethanol dehydration was followed by a step in propylene oxide and embedding in Epon-Araldite. Ultrathin sections and serial sections, obtained with a Reichert Ultracut II E and a LKB Ultratome III, were routinely stained and observed with a TEM Philips CM 10 at 80 kV. Semithin sections were stained with 1% toluidine blue and observed with a Leica DMRB light microscope.

RESULTS

The Parasite's Perspective

Artificial infections with 1st instar larvae. In *Xenos vesparum* development, four larval instars can be distinguished, based on changes in shape, size and cuticles. Our data are in agreement with histo-morphological studies carried out in other Strepsiptera: *Elenchus tenuicornis* (Kathirithamby et al., 1984), *Stichotrema dallatorreanum* (Kathirithamby, 2000) and *Pseudoxenos iwatai* (Maeta et al., 2001). The 1st instar larva (Fig. 2D) has the general appearance of the triungulin previously described for this order (Kathirithamby, 1989), although SEM data show some species-specific features (Pohl, 1998). It is a free-living organism (length about 270 μm), with antennae, eyes and mouthparts (mandibles and labium) on the head, slender legs terminating in filiform tarsi, serrated sternites, and tarsal expansions on pro- and meso-thoracic legs, used for clinging to the host or the substrate. Their jumping ability (personal observations) is probably due to abdominal caudal appendages (setae).

The timing of emergence of 1st instar larvae is quite variable, depending on environmental conditions (principally light and temperature). Every *Xenos vesparum* female of our group was able to give rise to hundreds of triungulins between May and July (in line with 3,000–4,000 oocytes in the hemocoel). Triungulins, being extremely motile organisms, were difficult to follow after their transfer onto the larva, because they could move from one nest cell to another. At dissection, we sometimes discovered a higher number of parasites than we had placed onto the larva.

Generally, laboratory infections were successful and stylopized wasps emerged from each artificially infected nest. Figure 1 shows the trend of parasite distribution. We dissected a total of 89 infected *Polistes dominulus* specimens: 69 larvae, 9 pupae, and 12 adults. We counted 235 *Xenos vesparum* within the wasps: 181 living parasites with their 1st instar exuvia; 46 living parasites without their exuvia; 8 exuvia in absence of visible living parasites. Only rarely did we find a dead triungulin on the wasp larva cuticle or outside in its nest cell (6 cases).

Entry into the host. When the triungulins made contact with the cuticle of the wasp larva, they moved randomly for a while. Then they stopped and with their heads repeatedly hit the external coating of the wasp at an oblique angle. There was no evidence of a specific entry target: in the head, not far from the mandibles (Fig. 2A,B), but also in the middle of the body or at its posterior extremity. Usually they avoided the sclerotized

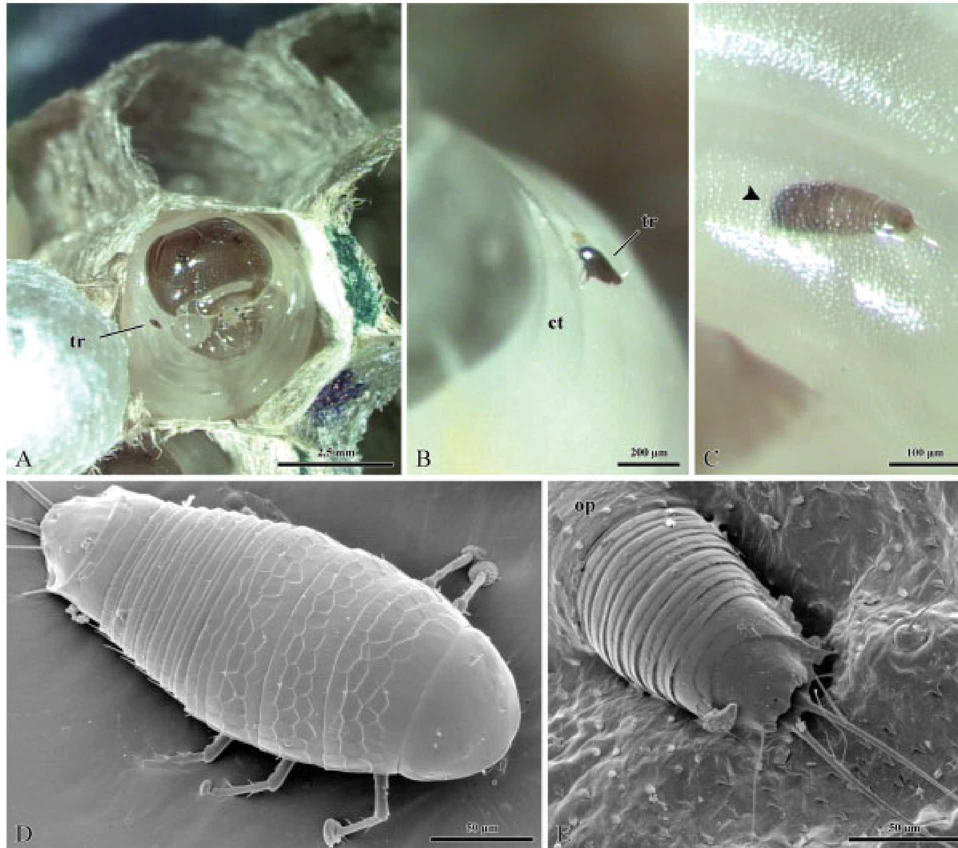

Fig. 2. **A–C**: Infection of the host. A: *Polistes dominulus* 4th instar larva in the nest, with a *Xenos vesparum* triungulin (tr) near the wasp mandibles, on the left. B: Triungulin (tr) making contact with the cuticle (ct) of the wasp larva. C: Triungulin 6 h after the beginning of the infection. Note that it has not yet fully entered: the anterior portion is already under the wasp cuticle (arrowhead) while the posterior region is still outside. **D**, **E**: The triungulin. SEM. D: Dorsal view. E: Triungulin partially penetrated into the host with its anterior region. Note the small opening (op) at the level of the wasp's cuticle.

buccal apparatus of our experimental "late" larvae; in a few cases, we observed wasp larvae chewing triungulins.

First instar larvae needed about 6–24 h to cross both exocuticle and endocuticle (Fig. 2C). This picture was taken 6 h after infection, when the triungulin has not fully entered: its anterior portion is already under the wasp cuticle, opaque and with many microtubercles; its posterior region is still outside and appears bright and smooth (Fig. 2D). Triungulins created a small opening in the cuticle (Fig. 2E), which we were unable to distinguish under microscope after a few minutes, and crossed exocuticle and then endocuticle (Fig. 3). We observed a mechanical detachment in the outer layers of the host cuticle and a not-yet-defined substance, probably secreted by the anterior region of triungulins (Fig. 4A,B). Noticeably, the entry hole in the larval coating did not melanize.

Once triungulins reached the epidermis (Fig. 4C), they began to sink into the hemocoel by passing through the epidermic layer (Fig. 5A), which completely surrounded them (Fig. 5B,C), until they were finally released in the wasp hemocoel. A transient and incomplete host epidermal infolding surrounds the parasite at this stage. We did not find any preferred region of the wasp hemocoel where triungulins settled, e.g., near the epidermis as well as deeper inside, in the fat tissues or close to nervous ganglia. Either head or dorsal/ventral body regions were susceptible.

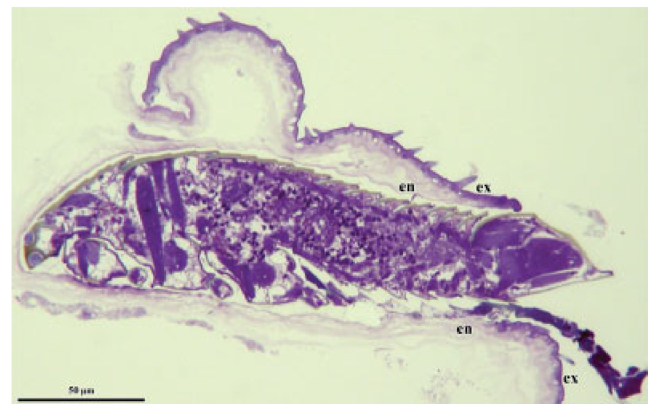

Fig. 3. Semithin sagittal section through the body of a triungulin which has not fully entered into the wasp larva. Note the mechanical detachment of both the exocuticle (ex) and the endocuticle (en).

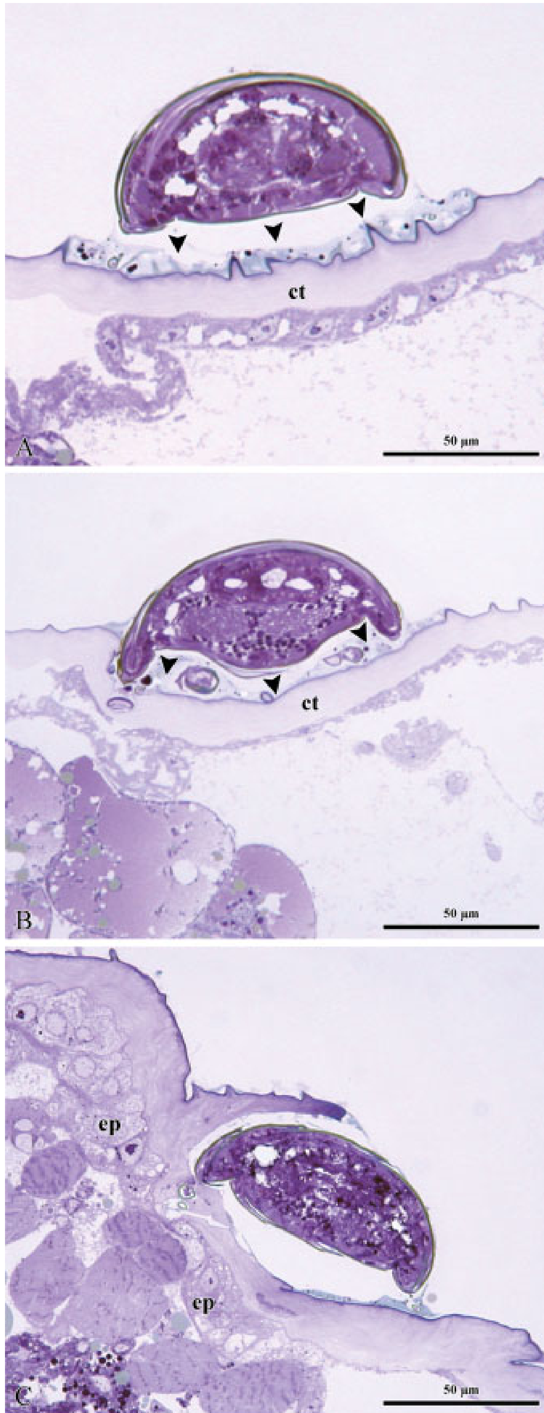

Fig. 4. Semithin serial cross sections through the anterior portion of a *Xenos vesparum* 1st instar larva infecting a new host: early steps. **A**, **B**: Triungulin probably secreting a substance (arrowhead) while making contact with the wasp cuticle (ct). **C**: Triungulin reaching the epidermis (ep) of the wasp larva.

First molt 48 h post-infection. Both 24- and 30-h postinfection dissections revealed intact triungulins inside the body of the host. Some were still moving in the hemocoel, as could be seen with the stereoscope through the thin cuticle of the wasp larva. Neither 1st instar exuvia nor 2nd instar larvae of the parasite was present: molting had not occurred yet. By 2 days after infection, young 2nd instar larvae were easily detectable

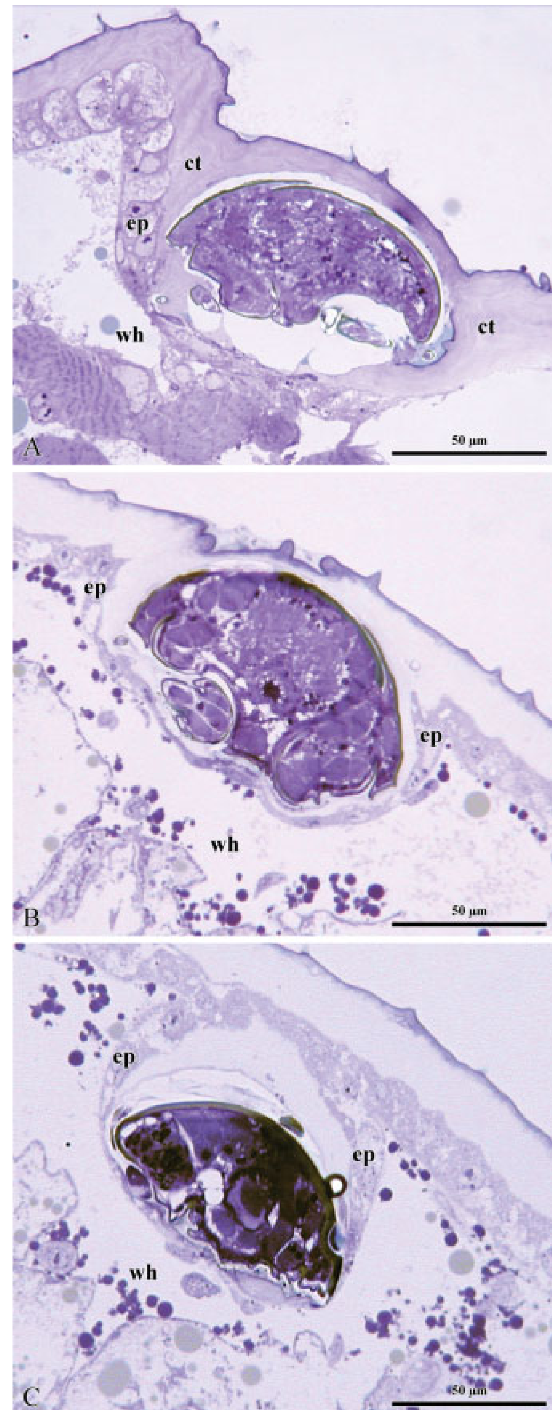

Fig. 5. Semithin serial cross sections through the anterior portion of a *Xenos vesparum* 1st instar larva infecting a new host: later events. **A**: The triungulin has crossed the cuticle (ct) and it starts breaking the epidermic layer (ep). **B**: Formation of an incomplete host epidermal infolding (ep) around the parasite while it is sinking into the wasp hemocoel (wh). **C**: Triungulin fully surrounded by the epidermal infolding.

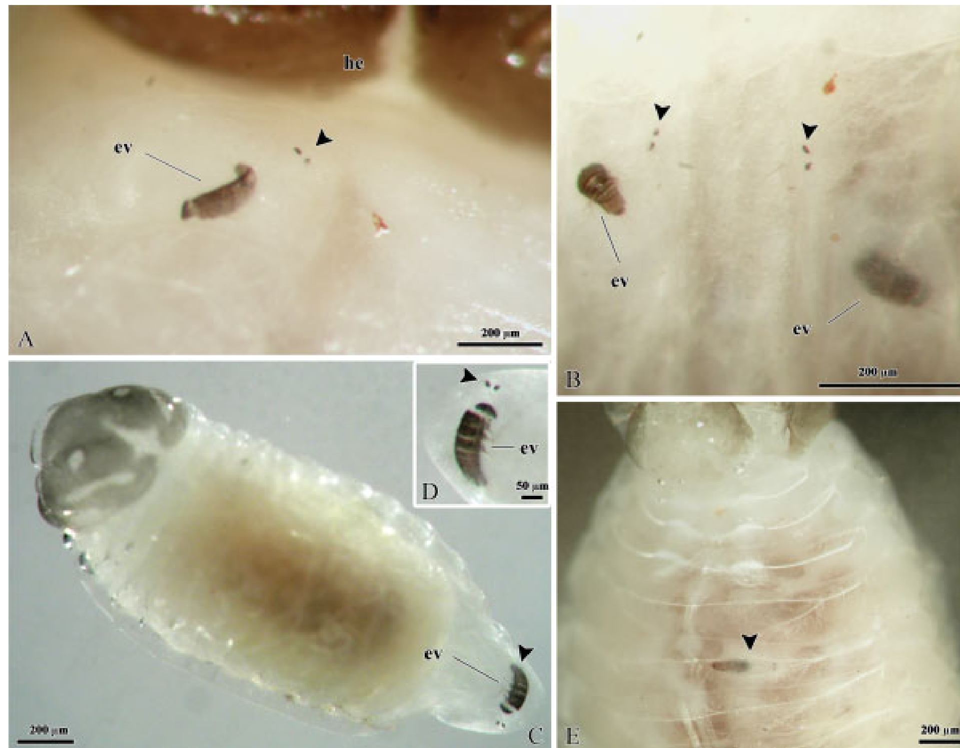

Fig. 6. First molt of *Xenos vesparum*. **A–D**: Young 2nd instar larvae (arrowheads) are visible near the exuvia (ev) of the triungulins. Note the black eyes of the molted parasites. A: 2nd instar larva and triungulin exuvium near the head (he) of the larval wasp. B: Two molted parasites settled in the fat tissues of the host. C: *Polistes dominulus* 3rd instar larva with a *X. vesparum* specimen inside its posterior region. D: Detail. **E**: Ventral view of *P. dominulus* 4th instar larva with a *X. vesparum* molting triungulin (arrowhead) under the cuticle.

near the exuvia (Fig. 6A,D). In a few cases, we were able to observe the motile larva abandoning the 1st instar exuvium through a dorsal opening, just behind the head (Figs. 6E, 7A,B).

Second instar larvae (Fig. 8A) lack body appendages (they keep only short protrusions of the three pairs of legs) and are unpigmented, except for two black spots on the dorsal side of the head (the eyes) that facilitate their identification. This developmental stage (183 ± 8 μm in length) is characterized by a soft thin cuticle (0.6 ± 0.4 μm). Many minute folds of the epicuticle (Fig. 8B) increase its surface. In a space of 7.5 μm, we have counted ~30-folds (max length: 0.75 μm) of different shape and size.

We usually found a correspondence between the number of endoparasitic larvae and the number of triungulin exuvia inside the wasp body, although sometimes there were more exuvia, probably because we were unable to localize the small living parasites when they were encompassed by the host fatty tissues. Exuvia were often free in the hemocoel at 48 h post-infection, but some were already encapsulated. Generally, we have not seen capsules around the larvae nor signs of melanization.

Third instar larva and later developmental stages. Third instar larvae appeared about 3 days after infection. At this stage the parasite appeared very similar to 2nd instar larvae (Fig. 8C), but the second molt was revealed by the presence of a double cuticle layer (Fig. 8D), easily recognizable around the posterior portion of early 3rd instar larva. The outer layer is due to a unique molting process, labeled as "apolysis without ecdysis" by Kathirirthamby et al. (1984), who first described it in *Elenchus tenuicornis* (Strepsiptera, Elenchidae).

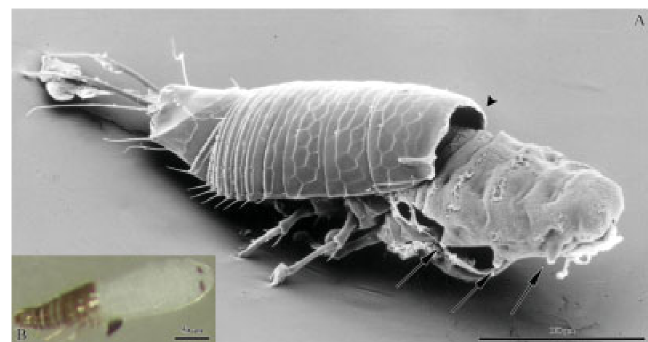

Fig. 7. A,B: Molting *Xenos vesparum* 1st instar larva. The parasite is abandoning the triungulin's exuvium through a dorsal opening (arrowhead), just behind the head. **A**: The short protrusions (arrows) of the three pairs of legs are visible in the young 2nd instar larva. SEM. **B**: Light microscope view of the same stage.

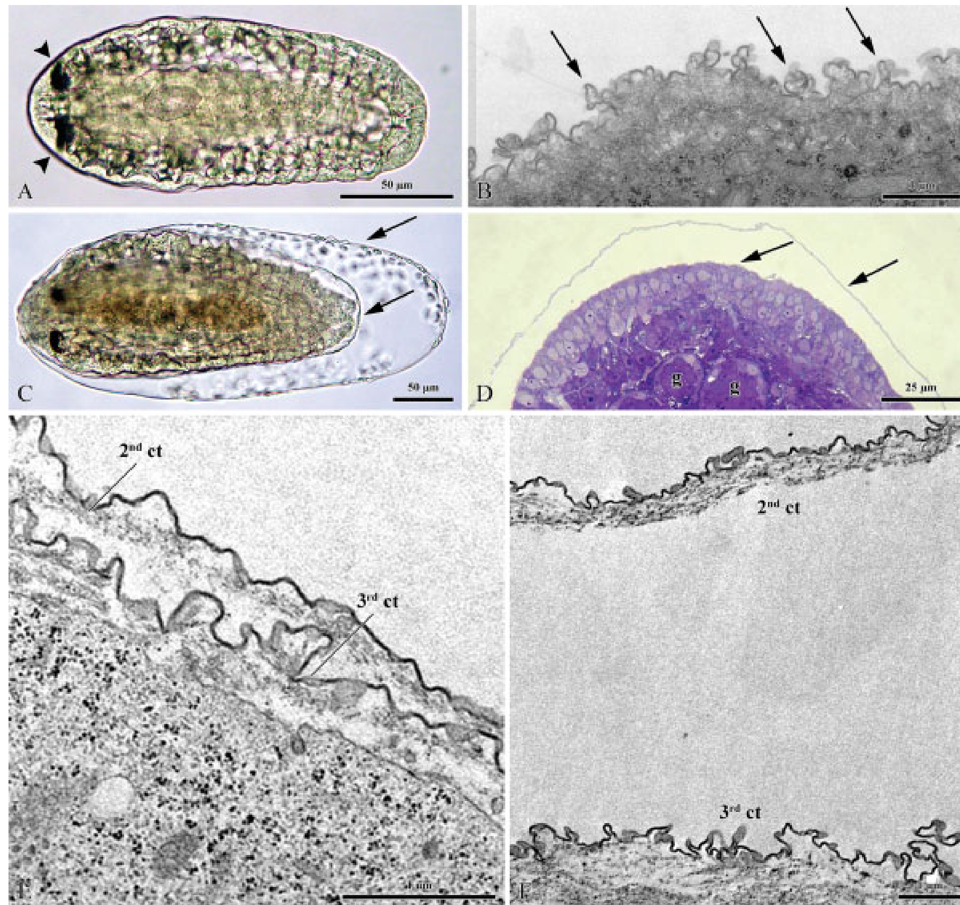

Fig. 8. Endoparasitic larval stages. **A**, **B**: 2nd instar larva. A: Whole parasite. Note the black eyes (arrowheads). B: Detail of the epicuticle with many minute folds (arrows). TEM. **C**–**F**: 3rd instar larva. C: Just molted parasite. This stage is very similar to the previous one except for the presence of a double cuticle layer (arrows). D: Semithin cross section: the double cuticle layer (arrows) and the differentiating gonads (g) are visible. E, F: Two overlapping cuticles (2nd ct and 3rd ct) soon after (E) and few days (F) from molting. TEM.

The parasite makes a new cuticle but it does not abandon the previous exuvium, which becomes the outer layer (Fig. 8E,F). As a consequence of this process, the parasite is progressively surrounded by a growing number of cuticle layers, but the one corresponding to the 2nd larval instar will always remain outside as well as in the neotenic female.

During their continued development, 3rd instar larvae were characterized by visible body growth (early 3rd instar larvae 324 ± 41 μm in length), the loss of all appendages, and the depigmentation of the two dark spots, now light red. In addition, the intestinal tract became more and more evident and gonads started to differentiate. As described earlier by Maeta et al. (2001) in *Pseudoxenos iwatai*, when transferred into buffer late 3rd instar larvae of *Xenos vesparum* settled with the ventral part of the body upward; they twisted their bodies up and downwards, until they closed like a ring. In *P. iwatai* parasite larvae migrated to the final position of future extrusion from the host eumenid wasp at just this stage.

The early 4th instar larva appeared when the wasp was in the pupal stage or in adulthood, 1–2 weeks after wasp emergence. At this stage, the two sexes were clearly recognizable. Females kept a larviform body (7.5 ± 1.5 mm in length). Male pupae, which were smaller (5.25 ± 0.75 mm), usually extruded a few days before female cephalothoraxes.

The Host Response

Encapsulation. The total number of capsules observed in wasps' hemocoel was 113, but encapsulation, which is common in parasite–insect interactions, presented some unusual features in our study.

First, capsules were formed only around empty exuvia (Fig. 9A): neither the living triungulins, nor the developing endoparasitic instars were targets of encapsulation. Second, the temporal dynamic of the process was peculiar, because encapsulation of exuvia was delayed. Wasp hemocytes began to aggregate around the triungulin exuvium

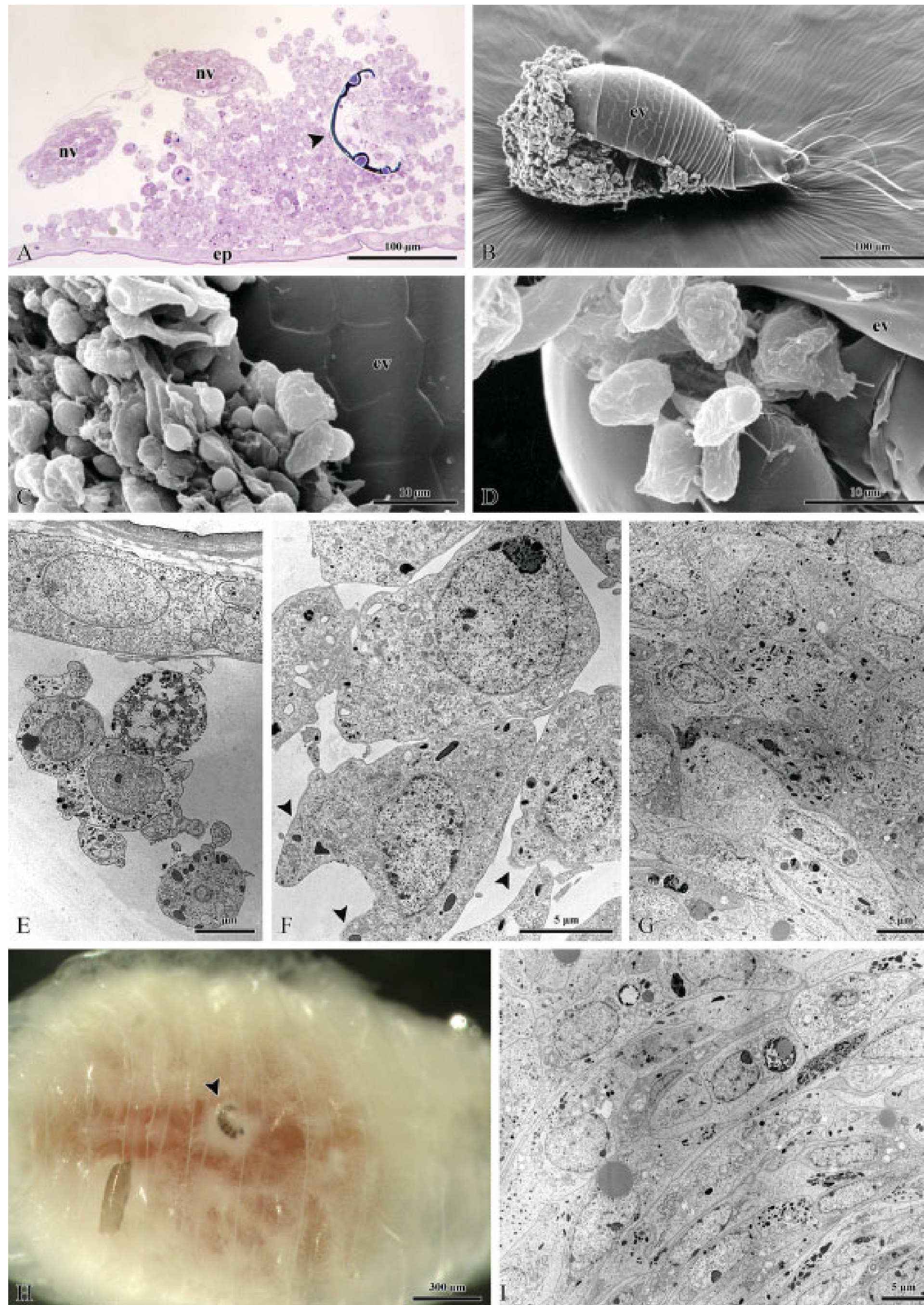

Fig. 9. The host defense reaction. **A**: Semithin section of a capsule forming around the empty exuvium (arrowhead) of a triungulin, between the epidermis (ep) and two nerves (nv) of a *Polistes dominulus* larva. **B–D**: *Polistes dominulus* hemocytes aggregating around the triungulin exuvium (ev). SEM. C, D: Detail showing the hemocytes pseudopodia contacting the exuvium. **E–G**: First steps in the formation of a capsule: round hemocytes approach the target (E), they change in shape and protrude pseudopodia (arrowheads) while aggregating around the target (F) and finally form the capsule (G). TEM. **H, I**: Mature capsules (8–10 days postinfection). H: A round milky capsule (arrowhead) visible through the cuticle of a *P. dominulus* 4th instar larva. I: Section through a mature compact capsule: note the extremely flattened hemocytes. TEM.

48 h after infection, without any specific adhesion site: they attacked the head as well as the back of the exuvia (Fig. 9B,D). At the beginning, the process involved medium- and large-sized (8–15 μm diameter) hemocytes, round to ovoid cells with a central nucleus accounting for ~40–60% of the cell volume (Fig. 9E). Capsules were not tightly packed at this stage. During the first week we observed the growing recruitment of large hemocytes (10–20 μm). These irregularly shaped cells (Fig. 9F–G)

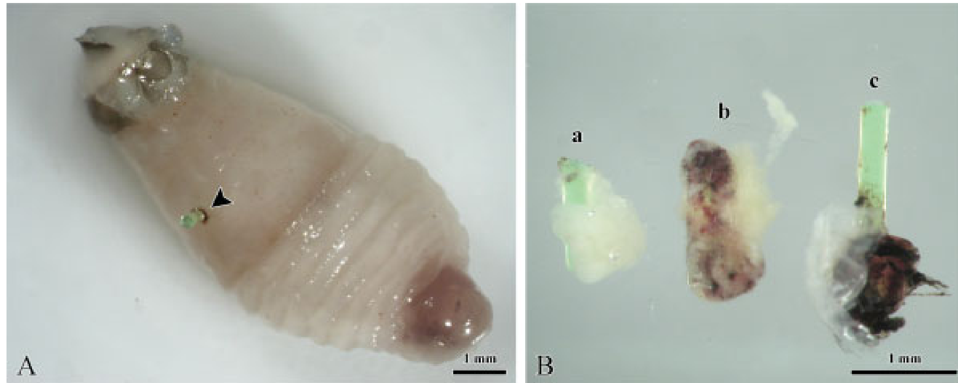

Fig. 10. Nylon implants in *Polistes dominulus* 4th instar larvae. **A**: The implant site of the nylon filament is melanized (arrowhead). **B**: Three capsules around nylon filaments, 24 h after implantation in three different specimens: capsules were not melanized (a), partially (b) or totally melanized (c).

were characterized by a few to many variable extensions (pseudopodia) and a central nucleus occupying ~40% of the cell volume.

About 8–10 days postinfection, 700-μm diameter mature capsules were visible, with a round shape and milky color (Fig. 9H). At this stage, hemocytes were flattened and capsules more compact (Fig. 9I). We found this kind of capsule deep inside the body of the wasp larva, embedded in the fatty tissue. More superficially, partial capsules and not yet encapsulated exuvia occurred. We never observed capsules in adult wasps, but sporadically free exuvia close to living parasites.

Melanization. After infection of *Polistes dominulus* larvae with *Xenos vesparum* specimens, the melanization response, a common feature of humoral insect immunity (Lavine and Strand, 2002), was absent at both the entering point and around the encapsulated exuvia (in 64 cases), while in 3 cases we recorded an apparent beginning of melanization. In 2 further cases we noticed the deposit of a melanin-like substance on the host cuticle at the entry of the triungulin, but this could be due to the contact with the forceps, as Hughes and Kathirithamby mentioned (2005) when using a similar technique.

Reinfection trials. A second infection, 55 h after the first one, confirmed the pattern described above. Dissections at either 1 or 3 days post-reinfection, revealed that triungulins were able to enter an already parasitized host, molt and give rise to the usual developmental stages. As in the first infections, capsules were found only around triungulins' exuvia and there was no evidence of melanization.

Implantation with 2nd instar larvae. Subcuticle applications of *Xenos vesparum* 2nd instar larvae (3 days) in naive hosts did not elicit any defense reactions during the first 24 h. The parasites (5 specimens) were still alive after dissection of the wasps and not encapsulated; no signs of melanization were visible. Three days after implantation, we recorded a high mortality of the *Polistes dominulus* larvae removed from their nests.

Nylon implantation trials. The filament-insertion trials gave variable host-response patterns, regardless of the wasp developmental stage. Twenty-four hours after implantation of nylon filaments in the host hemocoel, full capsules were observed in five of seven trials; in one case the encapsulation was incomplete and in another the filament did not elicit any reaction.

We also recorded melanization both at the site of implantation (Fig. 10A) and around the capsule (Fig. 10B) in five of seven trials.

Failure of parasite development. We found three exceptions to the general rules of immune response above outlined. SEM observations showed two young larvae partially encapsulated close to the triungulin exuvium (Fig. 11A,B). TEM observations revealed another case of abnormal molting, i.e., a young specimen encapsulated in the same way and partially melanized (Fig. 11C): this organism showed two additional coating layers (exuvia) overlapping the larval cuticle. Wasp hemocytes were visible close to the outer exuvium (Fig. 11C, the top of the micrograph). Noticeably, a secretion of unknown origin was found between the two outer layers. Due to its similarity to the midgut contents of the same specimen (Fig. 11D), we hypothesize it could be an outpouring of such contents.

DISCUSSION

To better understand the life-history of the system *Xenos vesparum*-*Polistes dominulus*, we have looked at it from two angles: the way in which *X. vesparum* 1st instar larvae infect a new host (the parasite's perspective) and the consequent reaction of the wasp (the host's perspective). The *P. dominulus* larva, the target of infection, is a

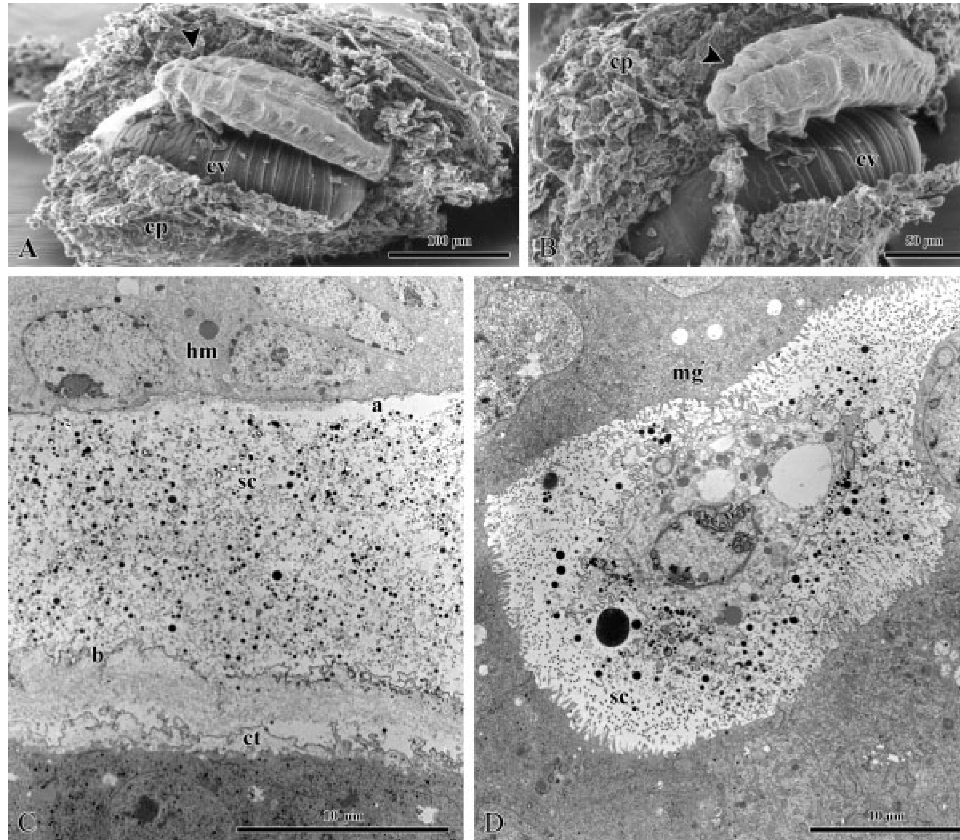

Fig. 11. Unsuccessful parasite development. **A**, **B**: Both micrographs show a young *Xenos vesparum* larva (arrowheads), probably a 2nd instar, close to the triungulin exuvium (ev) and partially encapsulated. Part of the capsule (cp) was removed. SEM. **C**: Detail of the two additional coating layers (a, b) overlapping the larval cuticle (ct) from a young *X. vesparum* specimen encapsulated and partially melanized. Wasp hemocytes (hm) are visible close to the outer exuvium, at the top of the micrograph. Note the presence of a secretion (sc) of unknown origin between the two outer layers. **D**: Cross section of the midgut (mg) of the same *X. vesparum* larva showing the midgut lumen filled with a secretion (sc) similar to that mentioned above.

changing environment and goes through great physiological and morphological reorganization (i.e., pupation). The endoparasitic development of *X. vesparum* has to be finely tuned to the development of its holometabolous host, similarly to koinobiont parasitoids (Vinson and Iwantsch, 1980). It is therefore not surprising that profound alterations occur in the endocrine equilibrium of the host environment: castration, the most relevant consequence of stylopization, is well known (Strambi and Strambi, 1973), and further studies on more subtle physical changes, i.e., epicuticular hydrocarbons and fat bodies, are in progress (Beani et al., 2005b; Beani, 2006). In our system, we suspect that the physiological damage to the host is less dramatic than in organisms affected by parasitoids (Pennacchio and Strand, 2006). *Xenos vesparum*—an obligate endoparasite, permanent if female—would gain no advantage in killing the host to satisfy its nutritional needs. On the contrary, its fitness comes from the exploitation of a living wasp, first as a substrate for its growth, then to facilitate its mating inside abnormal aggregations of stylopized wasps (Hughes et al., 2004b), and later as a vector for releasing infective 1st instar larvae. Consequently, one would expect the effects of the parasite on the defensive responses of the host to be limited.

From an evolutionary perspective, the refined and reciprocal adaptation of both *Xenos vesparum* and *Polistes dominulus* to this forced long-term coexistence (1 year, if a wasp infected by a *Xenos* female overwinters and survives to the next summer), suggests an ancient host–parasite interaction. Moreover, the large variety of organisms parasitized by Strepsiptera (Kathirithamby, 1989) highlights the plasticity of this group, which had to evolve many different modes of infection. This provides further support for the antiquity of this insect order, in agreement with recent findings of specimens in Cretaceous amber (Grimaldi et al., 2005).

The Parasite's Perspective: A "Soft" Entry Into the Host

According to the studies on the interactions between immature parasitoids and their hosts

(Brodeur and Boivin, 2004), the host-seeking stage of *Xenos vesparum*, the free-living mobile triungulin, undergoes a chain of "hierarchical steps" to achieve successful parasitism: it must locate, evaluate and penetrate the host, evade or overcome the host immune response and adapt to (or regulate) the constantly changing host environment.

Polistes dominulus larvae are typically aggregated, so in this case host selection may be a relevant step, whereas parasitoids are unlikely to have a choice of several hosts (Feener and Brown, 1997). Once a triungulin has reached a wasp nest, either on a foraging wasp (phoretic transport) or by means of a stylopized wasp which directly visits a nest (Hughes et al., 2003), a wide range of potential new hosts is available. The mechanism of host discrimination cannot be investigated by artificial infections, although a certain motility of the triungulins in the nest, even when they were put directly on a wasp larva, suggests a nonrandom choice, possibly influenced by the developmental stage, the nutritional state or even the sex of the larval wasp. We were able to successfully infect hosts of different developmental stages, naive or already parasitized, and even some wasp males, which are less parasitized than females in nature (Hughes et al., 2004a,b). The process of successful infection relies on a perfect tuning between the life cycles of host and parasite, and in the field host discrimination may be constrained by many factors: first of all, by the presence of adult wasps which actively remove triungulins moving on the larvae. In the laboratory, according to Schmid-Hempel and Ebert (2003), the "behavioral defenses," a first line of the defense cascade, are "largely eliminated."

The second step, the evasion of the host immune response, takes place as soon as the triungulin begins to penetrate the coating layers of the wasp larva, the cuticle and the epidermis. We expected that this process would evoke a reaction of melanization in the entry point, as a consequence of coagulum formation during wound-healing. In *Galleria mellonella* (Lepidoptera, Pyralidae) melanization in the wound-site was recorded 6–12 h after wounding (Rowley and Ratcliffe, 1981); it occurred after 24 h in our nylon implants. Contrary to expectations, the entry point of *Xenos vesparum* does not melanize even several days after infection; we infer that the parasite does not produce any wound.

The process of entry into the host has already been described in other Strepsiptera by Maeta et al. (2001) and Kathirithamby (1989). According to the former, the triungulins of *Pseudoxenos iwatai* (Strepsiptera, Stylopidae) "gnawed the chorion with their mandibles" while invading the eggs of a eumenid wasp. Kathirithamby instead, quoting Riek (personal communications), describes a considerable amount of liquid secreted by *Pseudoxenos* sp., and suggests an enzymatic softening of the cuticle of the host *Sceliphron laetrum* (Sphecidae, Hymenoptera). In turn, what we have observed for the *Xenos vesparum* penetration of the *Polistes dominulus* larva is a mechanical detachment of the outer layers in the host cuticle and a thin layer of a substance (its function is unknown) between the larva and the wasp's cuticle, which could be a product of the triungulin's salivary glands or the contents of its foregut.

Since the parasite does not produce a wound, there is no "injury factor" (Gupta, 1985) to trigger the coagulation and melanization of the hemolymph, and no hemocytes migrate to the entry site. To avoid the rupture of the wasp epidermal layer, which would represent the "injury factor" mentioned above, the triungulin initially penetrates the host epidermis without breaking it, inducing the formation of a transient and incomplete epidermal infolding. This process, observed in transverse serial semithin sections at the moment of entry into the host cuticle (Figs. 4A–C, 5A–C), is similar to the recently described ability of another strepsipteran to wrap itself in "a host-derived epidermal bag" (Kathirithamby et al., 2003): the epidermal cells of the hosts *Segestidea novaeguineae* and *S. defoliaria defoliaria* (Orthoptera, Tettigonidae), when detached from the endocuticle, lengthen and surround the 1st instar larva of *Stichotrema dallatorreanum* (Strepsiptera, Myrmecolacidae). This bag protects the parasite, "masqueraded as self," from the attack of the hemocytes during its entire endoparasitic development and, at the same time, allows the passage of nutrients from host hemolymph. This mechanism could explain another peculiar feature of Strepsiptera, "ecdysless molting": the parasite, while growing, cannot abandon the exuvia, because it is "closely fitted in its bag" (Kathirithamby et al., 2003).

In the case of *Xenos vesparum,* this process stops earlier and leads to an incomplete and transient "bag," or better, an epidermal infolding. This structure doesn't surround the parasite during its whole endoparasitic development, but only at its nontraumatic entry into the host, when it allows the triungulin to cross the cuticle and to reach the hemocoel without breaking the epithelial cell layer and evoking the defense reaction of the host (Schmid-Hempel, 2005). In the absence of any permanent and constraining bag wrapping the parasite, "apolysis without ecdysis" (Kathirithamby et al., 1984) has to be differently explained.

The Host's Response: Encapsulation of a Pseudo-Target, the Exuvium of the Triungulin

Hosts and parasites coexist in a "labile equilibrium" which is the result of evolutionary optimization mechanisms. This equilibrium balances itself between two extremes: ineffective parasites, unable to produce enough offspring to survive, and too-effective parasites, which reduce the survival chance of the host, thus compromising their own

existence (Götz and Boman, 1985). In this context, resistance is all-or-nothing, because if the host fails to actuate an effective immune response, it will die (Rolff and Siva-Jothy, 2003).

In the case of metazoan parasites, eggs and immature parasitoids, or xenobiotics (e.g., nylon filaments), the typical defense reaction in insects is a melanotic encapsulation which takes place in two distinct phases. First, hemocytes aggregate around the target, then lyse and release substances into the hemolymph to attract other hemocytes, until a tight capsule forms. This capsule then melanizes and kills the parasite. The process starts within a few minutes after invasion or implantation (only 1 min is required for the attachment of hemocytes to foreign objects) and within 2–24 h the typical three-layered cellular envelope has formed around the parasite (Götz and Boman, 1985). The immune response of *Polistes dominulus* after the invasion of *Xenos vesparum* differs from this general plan: first, the encapsulation is delayed (incomplete capsules were seen only 48 h after infection); second, the capsule surrounds a pseudo-target, the triungulin's exuvium, while the real target—the living parasite—is not affected. Control experiments of nylon implants (a widespread technique, due to its "spectacular" effects, Siva-Jothy et al., 2005) have shown that the immune system of *P. dominulus* immatures is able to encapsulate and melanize foreign objects after 24 h. "The use of nonliving immune stimuli allows focusing on the unfolding of the immune response as such, excluding effects due to the dynamic behavior of a real parasite"; moreover, this approach is useful to understand "the complex temporal dynamics" of host–parasite interaction, "a particularly relevant but often neglected aspect" (Korner and Schmid-Hempel, 2004).

As far as timing, the first 48 h are critical: during this time, triungulins generally undergo 1st molting without any interference. After that, encapsulation does take place but it involves a pseudo-target, hence it is ineffective; the living parasite is not recognized as nonself, therefore it is not attacked by hemocytes during its subsequent endoparasitic development. Moreover, the proteolytic cascade that leads to the production and the deposit of melanin must be locally compromised, because we didn't observe any sign of melanization either around the living parasite or within the capsule.

We hypothesize that *Xenos vesparum* may adopt a complex strategy of immunity elusion, involving both active suppression and passive avoidance of the host defense reaction. The former is critical at the beginning of infection: the parasite is able to delay and transiently inhibit encapsulation, and to locally suppress melanization. The specificity of this host–parasite interaction allows the wasp to defend itself from other injuries during its larval development and through adulthood: xenobiotics, but also bacteria, fungi, viruses and other metazoan parasites. In a similar way, the hemocytes of the host *Ephestia kühniella* (Lepidoptera, Pyralidae) may be transiently altered in proximity to the eggs of *Venturia canescens* (Hymenoptera, Ichneumonidae) but otherwise they activate the immune defense during the development of this parasitoid (Kinuthia et al., 1999; for a specific active suppression by hymenopteran parasitoids, see also Schmidt et al., 2001; Pennacchio et al., 2003; Giron and Strand, 2004).

In regard to passive avoidance, there may be surface chemical properties that allow *Xenos vesparum* to safely inhabit the *Polistes dominulus* hemocoel from 2nd instar to the extrusion of the female cephalothorax or the male puparium. Mechanisms of passive elusion of insect immunity are well-documented in several host–parasitoid systems: from the development of parasitoids in locations protected from circulating hemocytes and encapsulation (like the nerve ganglion), to "surface features that prevent the host from recognizing the parasitoid as nonself" (Schmidt et al., 2001). In our case, laboratory dissections did not reveal any fixed site where *X. vesparum* settles. Consequently, the elusion of cellular encapsulation might depend on peculiar features present on the outer surface of the parasite cuticle. Suitable examples of these mechanisms are the proteins that coat the eggs of parasitoids in the genus *Cotesia* (Hymenoptera, Braconidae) (Asgari et al., 1998) or the "fibrous layer" protecting the eggs of the parasite *Macrocentrus cingulum* (Hymenoptera, Braconidae) when deposited into the larval host *Ostrinia furnacalis* (Lepidoptera, Piralidae; Hu et al., 2003).

From this perspective, the "ecdysless molting" in Strepsiptera could be read as a "preadaptation for an endoparasitic lifestyle" (Kinuthia et al., 1999). The cuticle of the *Xenos vesparum* 2nd instar larva does not elicit the immune reaction of the host and, without ecdysis, this outer layer remains the unique host–parasite interface during the whole endoparasitic development. Moreover, the multilayered envelope of the parasite (three or more, depending on sex) makes it unlikely that immunosuppressive substances are released through this thick cuticle; it is more likely that the parasite's permanent cuticle adsorbs self-substances from the wasp hemolymph. In our experiments, specimens that have already undergone 1st molting are able to avoid the host defense reaction if implanted in a naive larval wasp, suggesting that they could have an innate camouflage mechanism, a cuticular chemical insignificance. Preliminary studies on the epicuticular hydrocarbon profiles of *X. vesparum* (Beani et al., 2005b) agree with a chemical mimicry hypothesis: the chemical profiles of cephalothorax and cephalotheca were more similar to host

hydrocarbons than the profiles of the 1st instar larvae and the adult males of *X. vesparum*, the only free living stages of this parasite.

Failure of Parasite Development

The three encapsulated *Xenos vesparum* specimens (see Results) can be seen as an imbalance of the "labile equilibrium" mentioned above: in these cases, the first molting is anomalous and the parasite's 2nd instar larva fails to move away quickly enough from the triungulin's exuvium, and is thus entrapped in the forming capsule. Dissections of naturally infected larvae of *Polistes dominulus* (Hughes et al., 2003) have shown a partial encapsulation of "molting 1st instars" in few specimens (2 of 18), and the authors were doubtful as to whether it was an example of successful defense; certainly, in the case of fully encapsulated 2nd instar larvae, the immune response of the host is effective since, within a capsule, an organism almost always dies (Schmidt et al., 2001), because it cannot take up nutrients and oxygen.

In these samples of unsuccessful parasitism, TEM observation of one specimen (probably a 2nd instar, being next to the triungulin's exuvium) revealed two additional exuvia overlapping the larval cuticle. We hypothesize that the additional cuticle layers are the result of two unexpected "ecdysless" moults, actuated by the parasite in order to strengthen the defense barrier against host hemocytes: an ultimate attempt to confront the encapsulation process. It becomes clear that too precise expectations and comparisons with other parasites are unfeasible, because "strepsipterans are the only parasitic insects (including parasitoids) to sequentially parasitize disparate stages of the same holometabolous host" (Hughes and Kathirithamby, 2005) and so large a spectrum of hosts: it follows that the strategies adopted by these organisms have to be flexible and often lie outside the schemes of the other host–parasite systems.

ACKNOWLEDGMENTS

We are grateful to Drs. David Mercati and Eugenio Paccagnini and to Prof. Pietro Lupetti for their help in the preparation of the experiments.

LITERATURE CITED

Asgari S, Theopold U, Wellby C, Schmidt O. 1998. A protein with protective properties against the cellular defense reactions in insects. Proc Natl Acad Sci USA 95:3690–3695.

Beani L. 2006. Crazy wasps: When parasites manipulate the *Polistes* phenotype. Ann Zool Fennici 43:564–574.

Beani L, Giusti F, Mercati D, Lupetti P, Paccagnini E, Turillazzi S, Dallai R. 2005a. Mating of *Xenos vesparum* (Rossi) (Insecta: Strepsiptera) revisited. J Morphol 265:291–303.

Beani L, Theodora P, Dallai R, Turillazzi S. 2005b. Epicuticular hydrocarbons of *Xenos vesparum* (Strepsiptera Stylopidae), parasite of *Polistes dominulus* (Hymenoptera Vespidae): Preliminary data. Redia 87:167–169.

Brodeur J, Boivin G. 2004. Functional ecology of immature parasitoids. Annu Rev Entomol 49:27–49.

Feener DH, Brown BV. 1997. Diptera as parasitoids. Annu Rev Entomol 42:73–97.

Giron D, Strand MR. 2004. Host resistance and the evolution of kin recognition in polyembryonic wasps. Proc Biol Sci 6: S395–S398.

Götz P, Boman HG. 1985. Insect immunity. In: Kerkut GA, Gilbert LI, editors. Comprehensive Insect Physiology, Biochemistry and Pharmacology. Oxford: Pergamon. pp 453–485.

Grimaldi D, Kathirithamby J, Schawaroch V. 2005. Strepsiptera and triungula in Cretaceous amber. Insect Syst Evol 36: 1–20.

Gupta AP. 1985. Cellular elements in the hemolymph. In: Kerkut GA, Gilbert LI, editors. Comprehensive Insect Physiology, Biochemistry and Pharmacology. Oxford: Pergamon. pp 400–451.

Hu J, Zhu XX, Fu WJ. 2003. Passive evasion of encapsulation in *Macrocentrus cingulum* Brischke (Hymenoptera: Braconidae), a polyembryonic parasitoid of *Ostrinia furnacalis* Guenée (Lepidoptera: Pyralidae). J Insect Physiol 49:367–375.

Hughes DP, Kathirithamby J. 2005. Cost of strepsipteran macroparasitism for immature wasps: Does sociality modulate virulence? Oikos 110:428–434.

Hughes DP, Beani L, Turillazzi S, Kathirithamby J. 2003. Prevalence of the parasite Strepsiptera in *Polistes* as detected by dissection of immatures. Insect Soc 50:62–69.

Hughes DP, Kathirithamby J, Beani L. 2004a. Prevalence of the parasite Strepsiptera in adult *Polistes* wasps: Field collection and literature overview. Ethol Ecol Evol 16:363–375.

Hughes DP, Kathirithamby J, Turillazzi S, Beani L. 2004b. Social wasps desert the colony and aggregate outside if parasitized: Parasite manipulation? Behav Ecol 15:1037–1043.

Kathirithamby J. 1989. Review of the order Strepsiptera. Syst Entomol 14:41–92.

Kathirithamby J. 2000. Morphology of the female Myrmecolacidae (Strepsiptera) including the apron and an associated structure. Zool J Linn Soc 128:269–287.

Kathirithamby J, Spencer Smith D, Lomas MB, Luke BM. 1984. Apolysis without ecdysis in larval development of a strepsipteran. *Elenchus tenuicornis* (Kirby). Zool J Linn Soc 82:335–343.

Kathirithamby J, Ross LD, Johnston JS. 2003. Masquerading as self? Endoparasitic Strepsiptera (Insecta) enclose themselves in host-derived epidermal bag. Proc Natl Acad Sci USA 100:7655–7659.

Kinuthia W, Li D, Schmidt O, Theopold U. 1999. Is the surface of endoparasitic wasp eggs and larvae covered by a limited coagulation reaction? J Insect Physiol 45:501–506.

Korner P, Schmid-Hempel P. 2004. In vivo dynamics of an immune response in the bumble bee *Bombus terrestris*. J Invertebr Pathol 87:59–66.

Lavine MD, Strand MR. 2002. Insect hemocytes and their role in immunity. Insect Biochem Mol Biol 32:1295–1309.

Maeta Y, Gôukon K, Kitamura K, Miyanaga R. 2001. Factors that determine the positions where *Pseudoxenos iwatai* Esaki (Strepsiptera: Stylopidae) extrudes from the host abdomen. Symposium papers on Strepsiptera presented at the XXI International Congress of Entomology, Foz do Iguaçu, August 20–26, 2001.

Pennacchio F, Strand R. 2006. Evolution of developmental strategies in parasitic Hymenoptera. Annu Rev Entomol 51:233–258.

Pennacchio F, Tranfaglia A, Malva C. 2003. Host-parasitoid antagonism in insects: New opportunities for pest control? Agro Food Industry Hi Tech 14:53–56.

Pohl H. 1998. Die Primärlarven der Fächerflügler—Evolutionäre Trends (Insecta, Strepsiptera), PhD Dissertation, I–V, Technische Universität Darmstadt, pp 1–252.
Rolff J, Siva-Jothy MT. 2003. Invertebrate ecological immunology. Science 301:472–475.
Rowley AF, Ratcliffe NA. 1981. Insects. In: Ratcliffe NA, Rowley AF, editors. Invertebrate Blood Cells, Vol 2. London: Academic Press. pp 421–488.
Schmid-Hempel P. 2005. Evolutionary ecology of insect immune defenses. Annu Rev Entomol 50:529–551.
Schmid-Hempel P, Ebert D. 2003. On the evolutionary ecology of specific immune defense. Trends Ecol Evol 18:27–32.
Schmidt O, Theopold U, Strand M. 2001. Innate immunity and its evasion and suppression by hymenopteran endoparasitoids. BioEssays 23:344–351.
Siva-Jothy M, Moret Y, Rolff J. 2005. Insect immunity: An evolutionary ecology perspective. Adv Insect Physiol 32:1–48.
Strambi C, Strambi A. 1973. Influence du développement du parasite *Xenos vesparum* Rossi (Insecta, Strepsiptère) sur le système neuroendocrinien des femelles de *Polistes* (Hyménoptère, Vespide) au début de leur vie imaginale. Arch Anat Microsc Morphol Exp 62:39–54.
Vinson SB, Iwantsch GF. 1980. Host regulation by insect parasitoids. Q Rev Biol 55:143–165.