Journal of Insect Physiology 59 (2013) 500–508

Contents lists available at SciVerse ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Hyposoter didymator uses a combination of passive and active strategies to escape from the *Spodoptera frugiperda* cellular immune response

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ARTICLE INFO

Article history:
Received 16 November 2012
Received in revised form 19 February 2013
Accepted 20 February 2013
Available online 1 March 2013

Keywords:
Hyposoter didymator
Ichneumonid
Polydnavirus
Immune evasion
Encapsulation
Spodoptera frugiperda

ABSTRACT

An endoparasitic life style is widespread among Hymenoptera, and various different strategies allowing parasitoids to escape from the host encapsulation response have been reported. Species carrying polydnaviruses (PDVs), such as the ichneumonid *Hyposoter didymator*, generally rely on the viral symbionts to evade host immune responses. In this work, we show that *H. didymator* eggs can evade encapsulation by the host in the absence of calyx fluid (containing the viral particles), whereas protection of the larvae requires the presence of calyx fluid. This evasion by the eggs depends on proteins associated with the exochorion. This type of local passive strategy has been described for a few species carrying PDVs. Immune evasion by braconid eggs appears to be related to PDVs or proteins synthesized in the oviducts being associated with the egg. We report that in *H. didymator*, by contrast, proteins already present in the ovarian follicles are responsible for the eggs avoiding encapsulation. Mass spectrometry analysis of the egg surface proteins revealed the presence of host immune-related proteins, including one with similarities with apolipophorin-III, and also the presence of three viral proteins encoded by IVSPERs (Ichnovirus Structural Protein Encoding Regions).

1. Introduction

Endoparasitoid species develop directly inside the insect host hemocele and must therefore deal with the host immune response to allow survival and development. In insects, the primary immune response directed against multicellular invaders is encapsulation, the formation of a multilayered sheath of cells around the intruder. It is often associated with the production of melanin, due to the activation of the phenoloxidase (PO) cascade, and of toxic compounds including reactive oxygen and nitrogen species (Falabella et al., 2012; Marmaras and Lampropoulou, 2009; Strand, 2012). The steps leading to the formation of the capsule in insects have not been described in detail. They are initiated by the recognition of the invader, which may involve specific humoral pattern recognition receptors (for instance immunolectins) or hemocyte membrane receptors such as integrins (Strand, 2008, 2012). This recognition then induces activation of the hemocytes and their adhesion to the foreign intruder. In *Pseudoplusia includens*, recognition leads to the activation and the attachment of granulocytes, a specific type of hemocyte, which enhance the recruitment of a second cell type, plasmatocytes (Pech and Strand, 1996; Strand, 2012). A group of cytokines called ENF peptides mediating the encapsulation process have been identified in lepidopteran insects. Examples of these ENF peptides are the plasmatocyte spreading peptide (PSP) that induces the adhesion and spreading of plasmatocytes on foreign surfaces and first described in *Pseudoplusia includens* (Strand, 2008), and the hemocyte chemotactic peptide (HCP) that stimulates aggregation and enhances clotting in the moth *Pseudaletia separata* (Nakatogawa et al., 2009; Strand, 2012).

To thwart the host encapsulation response, endoparasitoids have developed diverse strategies, both "passive" and "active". The "passive" strategies refer to the abilities of the parasitoid surface to avoid recognition by the host immune system. However, there may be active interactions between the parasitoid surface and effectors of the host immune system such that the term "passive" is not entirely appropriate; instead, the term "local active regulation" has been proposed (Moreau, 2003). A variety of examples of species relying on this type of strategy have been described. In general, properties of the parasitoid surface, particularly of the egg chorion, contribute to parasitoid evasion. For instance, in the braconid *Toxoneuron nigriceps*, the

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http://dx.doi.org/10.1016/j.jinsphys.2013.02.010

eggs are enveloped by a fibrous material of unknown origin that prevents encapsulation (Huw Davies and Vinson, 1986). In the ichneumonid *Campoletis sonorensis*, the eggs escape from recognition by *Heliothis virescens* immune system due to unknown lipid and protein components of the exochorion (Norton and Vinson, 1977). Some endoparasitoid species produce proteins sharing similarities with host immune-related proteins (molecular mimicry) such that the host immune system does not recognize them are foreign. For instance, *Macrocentrus cingulum* eggs and larvae are covered by a lectin-binding protein (HpLBP), produced in the wasp ovaries, that may play a role in their non-recognition by host hemocytes (Hu et al., 2008). Another example is the ichneumonid *Venturia canescens*: the egg surface presents a hemomucin-like protein and virus-like particles (VLPs) containing a P42 protein. Both proteins appear to share similarities with protein components of the immune system of the host *Ephestia kuhniella* (Kinuthia et al., 1999; Schmidt et al., 1990). Similarly, the eggs and also the PDV particles surface of the braconids *Cotesia kariyai* and *Cotesia rubecula* are covered by immunoevasive proteins (IEPs), IEP-1 and Crp32, respectively for the two wasp species (Hayakawa and Yazak, 1997; Tanaka et al., 2002). The protein, IEP-1, associated with *C. kariyai* eggs presents two EGF-like (epidermal growth factor) motifs and Crp32 that covers *C. rubecula* eggs contains a hyaluronan-binding motif. Although the function of these IEPs is not known, they may be involved in host molecular mimicry. Indeed, a protein sharing antigenic similarities with IEP-1 was identified in the hemolymph of the permissive *Pseudaletia separata* host but not that of non-permissive (able to encapsule the *C. kariyai* eggs) *Pseudaletia separata*. IEPs are produced by oviduct cells, and in the case of IEP-1, they are different from those producing the PDV (Tanaka et al., 2002). It was therefore suggested that the IEPs are secreted into the lumen and then attached to the eggs and PDV particle surface, thereby conferring immune protection (Asgari et al., 1998; Tanaka et al., 2002).

In parallel to the "passive" strategies, endoparasitoids display "active" strategies. These are mechanisms that alter the host immune physiology. Host regulation generally results from maternal factors produced in the venom or calyx glands and injected into the host during oviposition. Immune host regulation by endoparasitoids commonly reduces the capacity of the hemocytes to form the capsule or inhibits PO activation. For example, proteins such as serine protease inhibitors and superoxide dismutase produced in the venom gland of *Leptopilina boulardi* have been reported to inhibit the host PO activation cascade (Colinet et al., 2009, 2011; Zhang et al., 2006). The ability of hemocytes to encapsulate may be reduced by venom proteins produced by various parasitoid species. For instance, calreticulin in *C. rubecula* venom prevents hemocyte attachment to a foreign surface (Zhang et al., 2006), and their spreading behavior is disrupted by venom proteins including RhoGAP and Vrp3 produced by *Leptopilina boulardi* and *Pimpla hypochondriaca*, respectively (Dani and Richards, 2009; Labrosse et al., 2005). Finally, *Asobara japonica* venom induces the destruction of the hematopoietic organs leading to insufficient numbers of hemocytes to ensure encapsulation (Mabiala-Moundoungou et al., 2010). In braconid species, PDVs have been reported to be major factors for early parasitoid immune protection, that act, for example, by inducing hemocyte apoptosis or altering their spreading capacity (Labrosse et al., 2005; Lavine and Beckage, 1996; Strand, 2012). In contrast, in ichneumonid species, PDVs were only shown to disturb the host hemocytes spreading behavior or reduce the total hemocyte count (Provost et al., 2011; Strand, 2012).

In the present work, we investigated the strategies employed by the ichneumonid wasp *Hyposoter didymator* to escape the *Spodoptera frugiperda* immune response. This endoparasitoid belongs to the *Campopleginae* sub-family and carries the PDV *Hyposoter didymator* Ichnovirus (HdIV). As for many species carrying PDVs, the HdIV particles produced in the calyx gland and injected into the host upon oviposition are the primary factor involved in the regulation of the host immune physiology. Following HdIV injection, hemocytes are rapidly infected and their adhesion capacity is impaired (Barat-Houari et al., 2006). Nevertheless, one recent study indicates that *H. didymator* calyx fluid injections result in only a partial inhibition of the encapsulation response to beads, whether or not venom is co-injected (Dorémus et al., 2013). This suggests that *H. didymator* employs strategies in addition to calyx fluid injection to ensure survival in the host caterpillar. We injected parasitoid eggs and show that they are not encapsulated, whether they originate from the follicles, the oviducts or parasitized caterpillars. Thus, in the early stages following ovipositon, *H. didymator* eggs are protected by surface-associated proteins. Proteomic analysis of the egg surface reveals the presence of immune-related and viral proteins associated with the exochorion that may be involved in this protection. At later stages of the parasitic process, calyx fluid synergizes with this local regulation to suppress fully the risk of the parasitoid larva being encapsulated. We thus demonstrate that *H. didymator* escapes from the host cellular immune response through a combination of "passive" and "active" strategies. The "passive" strategy involves the exochorion properties and the "active" strategies probably involve the associated HdIV.

2. Materials and methods

2.1. Insect rearing and parasitism

S. frugiperda was obtained from a laboratory strain and maintained at 24 ± 2 °C, 65% relative humidity, with a 16/8 light/dark cycle on a semi-synthetic diet. The *H. didymator* strain was maintained on *S. frugiperda* larvae in the same abiotic conditions, using 2nd instar larvae for parasitism.

For experiments requiring 100% parasitism, *S. frugiperda* larvae (2nd or 3rd instar) were individually introduced into a glass vial containing ten 2-day-old *H. didymator* female wasps. The host larvae were removed immediately after being stung and maintained in an incubator as described above.

2.2. Isolation of H. didymator eggs

To obtain eggs in which embryogenesis has been activated (developing eggs), *S. frugiperda* larvae (2nd or 3rd instar) were parasitized two or three times and rapidly dissected in phosphate-buffered saline (PBS) to recover the *H. didymator* eggs (2–3 eggs per larva). The eggs were pooled in a Petri dish containing 20 ml of PBS and washed twice with 20 ml of PBS. The eggs were then stored in PBS at room temperature for less than 2 h before being injected into *S. frugiperda* larvae. To obtain eggs from the oviducts and from the ovarian follicles (non-developing eggs), reproductive tracts were dissected in PBS from ice-anesthetized *H. didymator* females and ovarioles were separated from calyces in Petri dishes. Mature eggs were extracted from the oviducts and washed three times with 20 ml of PBS. Eggs were also collected from the ovarian follicles present at the extreme base of the ovarioles. Follicle cells were removed by adding 0.5% NP40. The eggs were then washed three times in 20 ml of PBS. The absence of contamination by HdIV particles was verified by injection of two washed eggs into 3rd instar *S. frugiperda* larvae; 24 h later, a sample of 10 *S. frugiperda* larvae was withdrawn and tested for HdIV gene expression by RT-PCR (using primers specific for the M24 and P30 genes, both abundantly transcribed) to ensure absence of HdIV infection (data not shown).

2.3. Egg treatments

For egg surface treatments, eggs from the oviduct were incubated for 1 h at room temperature in 1 ml of one of the various test solutions: detergents (0.5%, 1% and 5% NP40, 0.5% Tween-20 or 5 M urea) 38% and 50% ethanol, and 0.2 M NaOH. Eggs were also subjected to digestion with N-glycosidase-F (5×10^{-4} and 5×10^{-3}U/μl; Boehringer Mannheim) and trypsin (0.2 μg/μl; TrypsinGold, Promega). After treatment, the solution was discarded and the eggs were rinsed three times with 20 ml of PBS.

2.4. Microscopy

For ultrastructure observations, eggs recovered from the follicles or the oviducts, and chemically or enzymatically treated or not treated, were fixed with 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer, pH = 7.4, overnight at +4 °C, then post-fixed with 2% (v/v) osmium tetroxide in the same buffer for 1 h at room temperature. The samples were dehydrated through an ethanol series and embedded in Epon. Ultrathin sections were cut, contrasted with uranyl acetate and lead citrate, and examined under an electron microscope (Zeiss EM 10 CR) at 80 KV.

2.5. Preparation of H. didymator calyx fluid extract

Calyx fluid (containing the PDV particles) was extracted from *H. didymator* females anesthetized on ice and dissected in PBS under a light microscope. The whole ovaries were pooled in 250 μl PCR tubes. The volume was adjusted with PBS to the desired concentration in wasp equivalents (w.e., for example: venom apparatus from 10 wasps in 25 μl of PBS for injection of 100 nl corresponds to 0.04 w.e./larva). The dose injected was chosen according to an egg load of 50 eggs at female emergence; each egg was considered to have been injected with 1/50 w.e. of maternal fluid (0.02 w.e.), and we choose to inject twice this quantity, 0.04 w.e.. Preparations were homogenized by several passages through a 20 μl micropipette cone. Tubes were centrifuged for 5 min at 5000 rpm to eliminate tissue. Supernatants containing the calyx extracts were stored on ice and injected into S. *frugiperda* larvae within 6 h.

2.6. Injections into S. frugiperda larvae

A Nanoject II™ Auto-Nanoliter Injector (Drummond) was used to give injections to *S. frugiperda* larvae anesthetized with CO_2. In all experiments, 100 nl of PBS (control), or 0.04 w.e. of calyx fluid in 100 nl was injected into 4th instar *S. frugiperda* larvae, less than 1 day old. Similarly, for all experiments, 4th instar *S. frugiperda* larvae were each injected with two eggs and/or three or four Sephadex G-75 beads (40–120 μm diameter). Previously parasitized larvae (control) were each injected with one egg during the 6 h following parasitism. The *S. frugiperda* larvae were then kept individually in 24-wells plates at 25 ± 2 °C and fed with a semi-synthetic diet.

2.7. Determinations of percentage of encapsulation of eggs, larvae and beads

S. frugiperda larvae were dissected in PBS to recover the eggs, larvae or beads at various times after injection of the eggs or beads. The percentage of encapsulation was calculated based on the ratio of the number of encapsulated foreign bodies to the number of total foreign bodies. Recovered beads were photographed under a phase contrast microscope. For each encapsulated bead, the sizes of the bead (B) and the hemocyte layer (H) were measured using Image J software. The bead covering factor (CovF) was calculated as: CovF = H/B.

2.8. Statistics

For all encapsulation percentage data (developing eggs, eggs from oviducts or from follicles, treated eggs and parasitoid larvae), logistic regression with a generalized linear model specially developed for modeling binomial data using the logistic link function was used to compare average values (McCullagh and Nelder, 1989). For developing egg and larva encapsulation percentages, the effects of PBS, calyx fluid and parasitism were tested. The difference in encapsulation percentage between eggs from oviducts and eggs from follicles was tested. All egg conditions effects on encapsulation percentage were tested. In all cases, post-hoc comparisons between conditions were done using a Tukey test. SAS software (SAS Institute Inc., 1999) was used for all computations.

2.9. Egg surface proteomics

To identify egg surface components, approximately 100 eggs recovered from oviducts were treated with trypsin (0.2 μg/μl in 200 μl of PBS for 1 h). The supernatant was collected and analyzed using a nanoESI LTQ_XL Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled to an Ultimate 3000 HPLC (Dionex, Amsterdam, Netherlands). Samples were desalted and pre-concentrated on-line on a Pepmap® precolumn (0.3 × 10 mm). A gradient of 0–40% B in 60 min and 80% B for 15 min (A = 0.1% formic acid, 2% acetonitrile in water; B = 0.1% formic acid in acetonitrile) at 300 nl/min was used to elute peptides from the capillary (0.075 × 150 mm) reverse-phase column (Pepmap®, Dionex). Nano-ESI was performed with a spray voltage of 2.4 kV, a heated capillary temperature of 200 °C, and a tube lens voltage of 140 V. A cycle of one full-scan mass spectrum (400–1600 m/z) at a resolution of 30,000, followed by five data-dependent MS/MS spectra was repeated continuously throughout the nanoLC separation. All MS/MS spectra were recorded using normalized collision energy (35%, activation Q 0.25 and activation time 30 ms), and an isolation window of 3 m/z. Data were acquired using Xcalibur software (v 2.0.7, Thermo Fisher Scientific, Waltham, MA). Proteins were identified by searching against the entries in an EST *H. didymator* ovary-specific database (Volkoff et al., 2010) with the Mascot v2.2 algorithm (Matrix Science Inc., Boston, USA). Data was submitted by ProteomeDiscoverer v1.1 (Thermo Fisher Scientific, Waltham, MA). Peptides with scores greater than the identity score ($p < 0.05$) were considered to be significant. All spectra were manually validated for proteins identified with less than three different peptides. All sequences identified by this procedure were compared to a non-redundant protein database (NCBI, release March 1, 2011) with a cut-off for the E-value >E−05 by using the Blastx algorithm.

3. Results and discussion

3.1. H. didymator eggs – but not larvae – are able to escape from encapsulation in S. frugiperda in the absence of maternal factors

Two developing *H. didymator* eggs were injected with PBS into each *S. frugiperda* 4th instar larva. Only 18% of the eggs recovered 12–40 h p.i. were encapsulated (Fig. 1A, PBS). Thus, most *H. didymator* eggs evaded the host cellular immune response in the absence of maternal factors, and in particular calyx fluid. We tested whether this ability was related to a protective characteristic of the *H. didymator* eggs themselves or to an inability of some hosts to mount a cellular response against an invader. Thus, non-developing eggs (collected from oviducts) were injected with Sephadex beads into *S. frugiperda* larvae, and the percentages of encapsula-

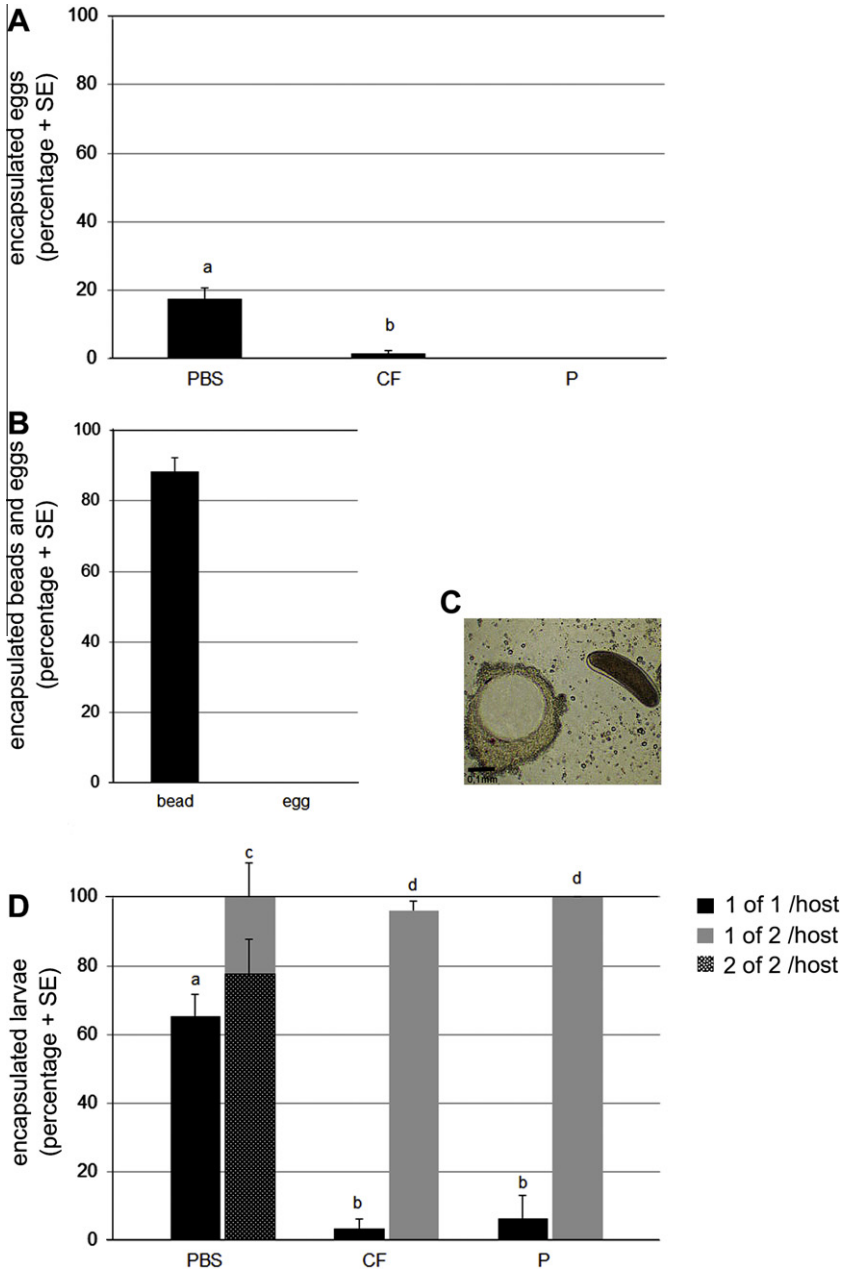

Fig. 1. Encapsulation of *H. didymator* eggs and larvae. (A) Percentage of encapsulation of parasitoid eggs injected into *S. frugiperda* 4th instar larvae with phosphate-buffered saline (PBS; $n = 165$ eggs recovered), 0.04 wasp-equivalents of calyx fluid (CF; $n = 193$ eggs recovered) or in previously parasitized larva (P; $n = 101$ eggs recovered). Observations were made from 12 to 40 h post-injection (p.i.). (B) Percentage of encapsulation 24 h p.i. of cleaned eggs collected from oviducts that were co-injected into *S. frugiperda* larvae with Sephadex beads (on $n = 69$ beads and $n = 58$ eggs recovered). (C) Light microscopy showing an encapsulated bead (left) and a non-encapsulated egg (right) recovered from the same *S. frugiperda* larva. (D) Percentage of encapsulated larvae found in *S. frugiperda* caterpillars 72–112 h p.i.. As two eggs were injected per larva, left columns correspond to caterpillars in which a single parasitoid was found ($n = 58$, 53 and 15 larvae recovered in PBS, CF and P, respectively), and right columns to caterpillars in which two parasitoids were recovered ($n = 18$, 54 and 16 larvae recovered in PBS, CF and P, respectively). Different lowercase letters specify that results are significantly different ($p < 0.05$).

tion were measured 24 h p.i. (Fig. 1B and C). The eggs recovered were not encapsulated whereas about 90% of the beads were encapsulated and covered by a thick hemocyte layer (covering factor of 0.93). This confirms that the *S. frugiperda* caterpillars can react to an invader and indicates that the parasitoid eggs are protected from the cellular immune response of these hosts. The difference between the encapsulation rates of developing eggs (18%) and non-developing eggs (0%) could be explained by a progressive loss of protection due to increase in size during embryo development for the first egg type. To test whether this protection was specific to *H. didymator* eggs, *Drosophila* eggs were also injected into *S. frugiperda* larvae, and were all encapsulated 24 h p.i. (data not shown). Therefore, the surface of *H. didymator* eggs may prevent recognition or hemocyte capsule formation.

Developing eggs that escape from encapsulation hatch on average 48 h after infection. At 48 h p.i., we found only a single larva in half of the *S. frugiperda* larva hosts, probably because in many cases one of the eggs had been lost during the injection process. In these cases, 72–112 h after injection of eggs with PBS, 65% of the larvae were encapsulated (Fig. 1D, PBS). Where two larvae were recovered per injected caterpillar, both larvae were encapsulated in 78% of cases and one of the two larvae was encapsulated in the other 22% (Fig. 1D, PBS). Thus, in absence of maternal factors, including calyx fluid, the larvae are mostly encapsulated.

3.2. The presence of calyx fluid increases the proportion of eggs that escape from the S. frugiperda immune response

To test for any complementary effect of maternal factors on the percentages of encapsulation, two developing eggs were injected with 0.04 w.e. of calyx fluid into *S. frugiperda* 4th instar larvae. As a control, one egg was injected into parasitized *S. frugiperda* larva (thus already containing one egg). Encapsulation of recovered eggs and larvae was scored 12–40 h p.i. and 72–112 h p.i., respectively. For eggs injected with calyx fluid, only 2% of the eggs recovered were encapsulated, consistent with the results for eggs injected into parasitized caterpillars (0%) (Fig. 1A, CF and P). Therefore, the presence of calyx fluid, although not required, increases the protection of the developing egg against the encapsulation response. In *S. frugiperda* hosts in which a single larva was found, the larvae were rarely encapsulated 72–112 h p.i., following injection with calyx fluid or into a parasitized caterpillar (4% and 7%, respectively; Fig. 1D, CF and P). In the caterpillars from which two larvae were recovered, encapsulation of both larvae was never observed (whereas this was found for the majority of the cases with PBS only), but one of the two was encapsulated in 98% and 100% of the caterpillars injected with calyx fluid or parasitized, respectively (Fig. 1D, CF and P). Thus, in all conditions tested (PBS, CF or the parasitized condition), if there were two parasitoid larvae in the same host, one was always encapsulated. Fights between solitary parasitoid larvae inside the same host have been reported (Quicke, 1997; Tillman and Powell, 1992), so it is possible that the larvae we found to be encapsulated had been killed by the other. These findings suggest that *H. didymator* larvae are more susceptible than the eggs to encapsulation in *S. frugiperda*, and that the presence of calyx fluid is necessary for parasitoid larva survival.

3.3. The egg acquires evasion factors in the ovarian follicles

We investigated whether the ability of *H. didymator* eggs to escape from encapsulation in *S. frugiperda* was related to their association with virus particles. *H. didymator* non-developing eggs were isolated from follicles and from oviducts, and were observed by TEM. *H. didymator* eggs present a thick endochorion, formed by a periodic multiple layer orientated perpendicularly to the egg surface, and an exochorion with numerous short projections 0.5 μm long (Fig. 2A). Note the folds of the endochorion (EnF) on the young eggs that permit extension of the growing parasitoid embryo (Fig. 2A). When observed within the oviduct lumen, where the HdIV particles are stored, a layer of flocculent material separates the exochorion from the viral particles (Fig. 2B). Therefore, no PDV particles were found associated with *H. didymator* eggs. These observations are congruent with what has been described in the *Campopleginae* wasp *C. sonorensis* (Norton and Vinson, 1977). Conversely, in two other *Campopleginae* wasps, *Tranosema rostrale* and *Venturia canescens*, projections of the exochorion are longer (respectively, 5 and 3 μm on average) and are coated with PDVs or VLPs when the eggs pass through the calyx (Cusson et al., 1998; Rotheram, 1973). It has been suggested that this coating in involved in the inhibition of capsule formation by these two species.

To confirm that immune evasion by *H. didymator* eggs is not related to their association with ovarian proteins during passage through the oviduct, eggs collected from ovarian follicles were injected into *S. frugiperda* caterpillars. These immature eggs were not significantly more encapsulated than eggs collected from the oviducts (Fig. 2C). Therefore, the eggs do not need to pass through the calyx to acquire their protective characteristics. TEM observa-

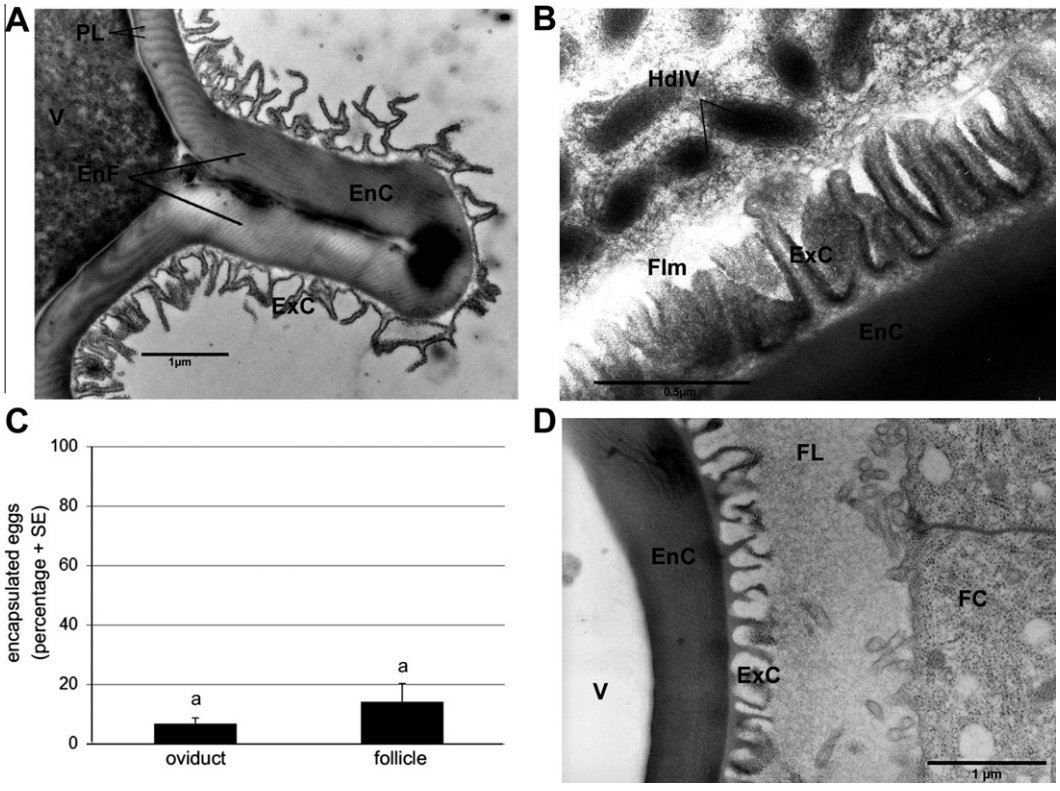

Fig. 2. *H. didymator* egg surface properties. (A) TEM view of *H. didymator* egg collected from oviducts. (B) TEM view of *H. didymator* egg within the oviduct showing the flocculent material separating the chorion from the virus particles. (C) Percentage of encapsulation of cleaned eggs collected from oviducts or from follicles 24 h after injection into *S. frugiperda* larvae. (D) TEM view of a *H. didymator* egg in ovarian follicles. E: egg; EnC: endochorion, ExC: exochorion; EnF: endochorion fold; FC: follicular cell; FL: follicular lumen; Flm: flocculent material; HdIV: virus particles; PL: periodic multiple layer; V: vitellus.

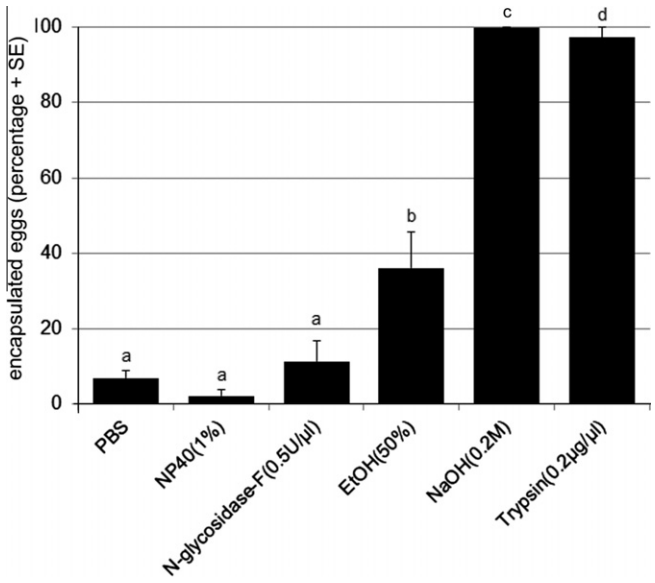

Fig. 3. Effect of chemical and enzymatic treatments on egg encapsulation. Eggs were pre-treated with various agents and injected into *S. frugiperda* 4th instar larvae, and the percentage of eggs encapsulated was scored 24 h p.i. (n = 174, 88, 35, 25, 23 and 37 eggs recovered in PBS, NP40, N-glycosidase, EtOH, NaOH and Trypsin treatments, respectively). The PBS sample corresponds to the data presented in Fig. 2C. Different lowercase letters specify that results are significantly different ($p < 0.05$).

tion of eggs within follicles reveals that the exochorion structure is similar to that of eggs in oviducts (Fig. 2D). To conclude, the factors protecting against host encapsulation seem to be produced during egg maturation in the follicles, and are most probably associated with the egg surface, and presumably exochorion.

3.4. Alteration of the egg surface restores encapsulation by S. frugiperda hemocytes

We tested whether protection of *H. didymator* eggs against the host immune response was due to surface characteristics, and tried to determine the nature of the factors involved. Eggs recovered from the oviducts were subjected to various chemical and enzymatic treatments and then injected into *S. frugiperda* larvae. Treatments with the detergents NP40 (tested at concentrations of 1% and 5%) and 0.5% Tween-20 or with 5 M urea had no effects on the encapsulation percentage 24 h p.i. (Fig. 3; data shown only for 1% NP40); similarly, glycosidase treatment did not affect the percentage of encapsulation that remained low (Fig. 3). Incubation in 50% ethanol, 2 M NaOH and 0.2 µg/µl trypsin each had a significant effect, and almost 100% of the eggs treated with NaOH or trypsin were encapsulated 24 h p.i. (Fig. 3). Eggs treated with NaOH and trypsin were observed by TEM. There were no observable ultrastructural modifications of the egg surface (data not shown), indicating that these treatments did not affect the chorion. The protection of the eggs against encapsulation thus appears to be related to surface properties or molecules. NaOH and ethanol destabilize proteins, and trypsin is a specific protein-cleaving enzyme (Gülich et al., 2000; Nick Pace et al., 2004). Therefore, these experiments suggest that proteins are responsible for egg protection, consistent with previous results for *C. sonorensis* where protease treatments of cleaned eggs results in higher levels of encapsulation (Norton and Vinson, 1977). These two closely related Campopleginae species thus appear to employ similar "passive" egg-protection strategies.

3.5. Proteins potentially involved in protection of H. didymator eggs against the S. frugiperda immune response

To identify proteins on the egg surface that might be involved in egg protection, eggs were collected from *H. didymator* oviducts and treated with 0.2 µg/µl trypsin for 1 h. The supernatant containing the tryptic peptides was directly analyzed by nano-LC–MS/MS. The peptide fragments detected were matched against a *H. didymator* ovary-specific EST library: 34 ESTs were identified (Table 1) including four sequences of particular interest.

One of the four genes is predicted to code for an apolipophorin-III (66% amino acid identity with the *Apis mellifera* apolipophorin-III-like protein). The insect apolipophorin-III is an exchangeable apolipoprotein, that facilitates lipid transport from the fat body to the flight muscle (Bolanos-Garcia and Miguel, 2003); it has also been reported to mediate the immune response in lepidopteran insects by interacting with pathogen elicitors (Gupta et al., 2010). As suggested for the hemomucin-like protein in *V. canescens* and the lectin-binding protein in *M. cingulum*, the presence of a protein at the egg surface similar to a protein in the host hemolymph may be related to the non-recognition of *H. didymator* as foreign.

Proteomic analysis also reveals the presence of peptides corresponding to the IVSPERs (Ichnovirus Structural Protein Encoding Regions) genes U23, IVSP4-1 and IVSP4-2. IVSPERs are regions in the *H. didymator* genome specialized in the synthesis of proteins associated with HdIV particles (Volkoff et al., 2010). Thus, although *H. didymator* eggs appear not to be associated with PDV particles, IVSPER proteins may be involved in the protection of the eggs against the host immune response. However, we cannot completely exclude the possibility that the three IVSPER proteins detected result from HdIV contamination, although no particle was found associated with the eggs (see above) and controls indicated no expression of viral genes in egg-injected caterpillars (data not shown). Also, egg IEPs that bind to eggs and PDV seem to be produced by oviduct cells in braconids (Tanaka et al., 2002), and this is unlike the model we describe here, in which the ability of the eggs to escape from the host immune system is acquired early, in the follicles.

Further investigations will be needed to confirm that these proteins are localized in the exochorion and that they are indeed involved in the escape of *H. didymator* eggs from encapsulation. Nonetheless, this work is the first description of the possible contribution of viral-like and host-like proteins, associated with the surface of the egg, to "local" protection of the eggs against the host cellular immune response for an endoparasitoid carrying PDV.

4. Conclusion

This study reveals that *H. didymator* uses both "passive" and "active" strategies to escape from the *S. frugiperda* cellular immune response. During the early phase of the parasitism, intrinsic properties of the exochorion ensure the absence of encapsulation, possibly by preventing recognition of the parasitoid egg as foreign. Although not essential, PDV infection – and potentially other ovarian factors – increases the protection by modifying the number and probably the adhesion properties of the hemocytes (Provost et al., 2011). Further work is needed to elucidate more fully the mechanisms by which the egg escapes from encapsulation, and in particular to identify the molecules involved and how they act on the immune cascade. Physiological studies on the natural hosts of *H. didymator*, such as *Helicoverpa armigera*, are necessary to verify that these findings are not specific to this particular host. Cleaned *C. sonorensis* eggs (another Ichneumonid carrying PDV) display similar properties of immune evasion in *H. virescens* hemolymph 24 h p.i. The experiments with *H. didymator* were continued only

Table 1
Sequences identified by comparing the peptides released by trypsin treatment of *H. didymator* eggs to the *H. didymator* ovarian EST database. Are indicated: name of the sequence (Seq_ID); its corresponding GenBank accession number (GenBank Acc #) and dbEST identity (dbEST_Id); total peptides (# PSMs) and number of different peptides (# Peptides) that matched with the sequence; annotations are as predicted using the Blast2GO tool (Conesa et al., 2005); description of the hit that gave the best BlastX match against the NCBI nr database, and E-Value.

Seq_ID	GenBank Acc #	dbEST_Id	# PSMs	# Peptides	Annotation (Blast2GO)	Hit description of better BlastX match against NCBI nr database	E-Value
RR0AAB1YB01CM1	JZ125050	77719579	9	6	Gene regulation	gi|380014528|ref|XP_003691281.1|PREDICTED: nuclease-sensitive element-binding protein 1-like [Apis florea]	1.8E−03
RR0AAD1YK16CM1	JZ125053	77719582	6	5	Hemocyte immune related	gi|380017736|ref|XP_003692803.1|PREDICTED: 14-3-3 protein zeta-like isoform 1 [Apis florea]	2.9E−149
RR0AAC6YM04CM1	JZ125058	77719587	5	3	Gene transcription	gi|307196337|gb|EFN77947.1|Elongation factor 1-alpha [Harpegnathos saltator]	6.2E−128
RR0AAB1YH03CM1	JZ125060	77719589	4	3	Development	gi|110750766|ref|XP_001120194.1|PREDICTED: protein lethal(2)essential for life-like [Apis mellifera]	4.0E−74
RR0AAD1YA19CM1	JZ125061	77719590	3	3	IVSPER, IVSP4-1	gi|297384849|gb|ADI40475.1|unknown [*Hyposoter didymator*]	1.9E−149
RR0AAD6YH18CM1	JZ125057	77719586	3	2	IVSPER, U23	gi|297384863|gb|ADI40488.1|unknown [*Hyposoter didymator*]	4.5E−159
RR0AAC2YJ08CM1	JZ125064	77719593	3	2	IVSPER, IVSP4-2	gi|297384859|gb|ADI40484.1|unknown [*Hyposoter didymator*]	4.6E−179
RR0AAB1YB02CM1	JZ125067	77719596	3	2	Gene transcription	gi|383849981|ref|XP_003700610.1|PREDICTED: uncharacterized protein LOC100878621 [Megachile rotundata]	5.6E−89
RR0AAD1YB14CM1	JZ125069	77719598	2	2	Ran GTPase binding	gi|383847263|ref|XP_003699274.1|PREDICTED: GTP-binding nuclear protein Ran-like [Megachile rotundata]	1.3E−97
RR0AAC8YC23CM1	JZ125070	77719599	2	2	Oxidoreductase activity	gi|328777124|ref|XP_393445.3|PREDICTED: peroxiredoxin 1 isoform 1 [Apis mellifera]	1.1E−34
RR0AAA1YG09CM1	JZ125071	77719600	2	2	Chaperone	gi|345485940|ref|XP_001604538.2|PREDICTED: protein lethal(2)essential for life-like [Nasonia vitripennis]	3.5E−48
RR0AAC2YL24CM1	JZ125072	77719601	3	2	Hydrolase activity	gi|383849928|ref|XP_003700585.1|PREDICTED: esterase E4-like [Megachile rotundata]	1.5E−30
RR0AAD5YK17CM1	JZ125075	77719604	2	2	Gene transcription	gi|65736550|dbj|BAD98517.1|hypotheical protein [Nasutitermes takasagoensis]	2.1E−26
RR0AAD4YK06CM1	JZ125076	77719605	2	2	Lipid transport	gi|166795901|ref|NP_001107670.1|apolipophorin-III-like protein precursor [Apis mellifera]	1.2E−36
RR0AAA1YL10CM1	JZ125077	77719606	2	2	Lyase activity	gi|283046759|ref|NP_001164327.1|fructose-bisphosphate aldolase isoform B [Nasonia vitripennis]	1.5E−159
RR0AAD2YG04CM1	JZ125078	77719607	7	2	Chaperone	gi|23820961|gb|AAN39696.1|heat shock protein [Choristoneura parallela]	1.2E−55
RR0AAD6YF13CM1	JZ125079	77719608	2	2	RNA binding	gi|383859712|ref|XP_003705336.1|PREDICTED: fragile X mental retardation syndrome-related protein 1-like [Megachile rotundata]	4.5E−28
RR0AAB1YA15CM1	JZ125080	77719609	2	2	Hypothetical protein	gi|332018854|gb|EGI59410.1|hypothetical protein G5I_12447 [Acromyrmex echinatior]	2.4E−10
Ribosomal							
RR0AAC2YG02CM1	JZ125048	77719577	7	6	Ribosomal	gi|307209216|gb|EFN86323.1|40S ribosomal protein S16 [Harpegnathos saltator]	6.3E−95
RR0AAD5YH24CM1	JZ125049	77719578	7	6	Ribosomal	gi|307177476|gb|EFN66603.1|40S ribosomal protein S8 [Camponotus floridanus]	4.7E−109
RR0AAC2YD14CM1	JZ125051	77719580	6	5	Ribosomal	gi|307207541|gb|EFN85228.1|60S ribosomal protein L28 [Harpegnathos saltator]	3.5E−72
RR0AAD8YF15CM1	JZ125052	77719581	5	5	Ribosomal	gi|285027695|gb|ADC34221.1|60S ribosomal protein L7 [Solenopsis invicta]	1.0E−147
RR0AAC3YP04CM1	JZ125055	77719584	5	4	Ribosomal	gi|383855254|ref|XP_003703131.1|PREDICTED: 60S ribosomal protein L9-like [Megachile rotundata]	1.2E−127
RR0AAC8YI24CM1	JZ125056	77719585	5	4	Ribosomal	gi|383859003|ref|XP_003704988.1|PREDICTED: 40S ribosomal protein S3a-like [Megachile rotundata]	5.6E−36
RR0AAD4YE02CM1	JZ125054	77719583	4	4	Ribosomal	gi|340725537|ref|XP_003401125.1|PREDICTED: 60S ribosomal protein L4-like [Bombus terrestris]	3.8E−116
RR0AAD3YI02CM1	JZ125062	77719591	5	3	Ribosomal	gi|340725678|ref|XP_003401193.1|PREDICTED: 60S ribosomal protein L26-like [Bombus terrestris]	6.3E−40
RR0AAD1YJ16CM1	JZ125059	77719588	4	3	Ribosomal	gi|322800518|gb|EFZ21522.1|hypothetical protein SINV_16043 [Solenopsis invicta]	6.2E−130
RR0AAC1YE03CM1	JZ125065	77719594	3	2	Ribosomal	gi|328793058|ref|XP_624620.2|PREDICTED: 60S ribosomal protein L5 [Apis mellifera]	3.3E−136
RR0AAC1YG02CM1	JZ125073	77719602	3	2	Ribosomal	gi|307194306|gb|EFN76672.1|60S ribosomal protein L22 [Harpegnathos saltator]	2.7E−61
RR0AAC8YF12CM1	JZ125066	77719595	2	2	Ribosomal	gi|156542046|ref|XP_001601390.1|PREDICTED: 40S ribosomal protein S18-like [Nasonia vitripennis]	7.7E−75
RR0AAC1YK04CM1	JZ125068	77719597	2	2	Ribosomal	gi|383851993|ref|XP_003701515.1|PREDICTED: 40S ribosomal protein S3-like [Megachile rotundata]	3.0E−55
RR0AAC6YK10CM1	JZ125074	77719603	2	2	Ribosomal	gi|229577209|ref|NP_001153326.1|ribosomal protein L7A [Nasonia vitripennis]	7.1E−144
RR0AAC2YF23CM1	JZ125063	77719592	2	2	Ribosomal	gi|322790861|gb|EFZ15546.1|hypothetical protein SINV_12532 [Solenopsis invicta]	2.5E−88

until 40 h p.i., whereas the *C. sonorensis* eggs were followed for longer: 96 h p.i., 80% of eggs were encapsulated suggesting that they lost their ability to escape encapsulation over time (Norton and Vinson, 1977). This raises questions about the mechanisms by which local protection is lost during the parasitoid egg development, and also demonstrates the necessity for calyx fluid (PDV) in later stages for successful parasitism in these models.

Proteomic analysis of the *H. didymator* egg surface revealed several proteins that may be incorporated into or associated with the exochorion during its synthesis in the ovarian follicles and before

passage through the calyx. The expression in follicle cells of the four genes encoding the candidate proteins and translocation of the proteins to the exochorion should be determined to confirm their involvement in the immune protection.

Calyx fluid appears to be necessary to avoid larva encapsulation during the later stages of parasitism. In the absence of calyx fluid, larvae are nearly always encapsulated. It would be informative to study complementarity between the calyx fluid and the characteristics of the parasitoid larvae. One puzzling issue is why, when two larvae hatch in the same host, dead larvae are encapsulated whereas surviving larvae are not, even in presence of calyx fluid. Possibly, live *H. didymator* larvae produce surface molecules that prevent hemocyte attachment, as suggested for the *Cotesia congregata* larvae that are not encapsulated after their transplantation into non-parasitized *Manduca sexta* hosts (Lavine and Beckage, 1996). Another possibility is that the mobility of the parasitoid larva is sufficient to prevent PDV-infected hemocytes to adhere. Without the addition of calyx fluid, a large proportion of *H. didymator* larvae are encapsulated, indicating that host hemocytes are able to encapsulate foreign objects including those that are mobile. Calyx fluid alters hemocyte behavior, but does not prevent them encapsulating immobile objects such as beads (Dorémus et al., 2013), although the mobile parasitoid larvae avoid this fate. Indeed, it may mostly be physical factors (notably movements) that allow *H. didymator* larvae to escape encapsulation.

We report that the *Campopleginae* wasp *H. didymator* uses complementary strategies to ensure successful parasitism. This multiple immune evasion strategy may be advantageous because it requires calyx fluid to have only relative minor effects on the host immune system. This minimizes alterations to the host, which is the food source, such that it can continue to fight against other pathogens and parasites that would otherwise be deleterious for the parasitoid (Lavine and Beckage, 1996).

Acknowledgements

We thank M. Jambart, A. Ausset, C. Gibard and G. Clabots for providing insects and/or reagents for this study. The use of English was revised by Alex Edelman & Associates. This work received support from the Génoscope (“Polydnavirus” project) and from the French National Research Agency (Paratoxose project, #ANR-09-BLAN-0243-01).

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