Journal of Invertebrate Pathology 130 (2015) 88–96

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

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

Differential immunosuppression by *Campoletis chlorideae* eggs and ichnovirus in larvae of *Helicoverpa armigera* and *Spodoptera exigua*

Li-Bin Han [1], Li-Hong Yin [1], Ling-Qiao Huang, Chen-Zhu Wang *

State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, 1 Beichenxilu, Chaoyang District, Beijing 100101, PR China

ARTICLE INFO

Article history:
Received 8 March 2015
Revised 10 July 2015
Accepted 11 July 2015
Available online 13 July 2015

Keywords:
Campoletis clorideae
Polydnavirus
Egg
Immune response

ABSTRACT

The ichneumonid wasp, *Campoletis chlorideae* Uchida, successfully develops in the cotton bollworm *Helicoverpa armigera* (Hübner), but rarely survives in the beet armyworm *Spodoptera exigua* (Hübner) due to the encapsulation by host immunity. In this study, we investigated the role of *C. chlorideae* ichnovirus (CcIV) and eggs in the evasion of the host immune system. Washed eggs of different types, immature, mature, newly laid, or pretreated with protease K, were injected alone or with the calyx fluid containing CcIV into the larvae of *H. armigera* and *S. exigua*. In *H. armigera*, when injected with washed eggs alone, only 9.5% of the mature eggs were encapsulated at 24 h post-injection. This is much lower than that of the immature eggs (100%), mature eggs pretreated with protease K (100%) and newly laid eggs (54.4%). No encapsulation was observed when the washed eggs were co-injected with calyx fluid at 24 h post-injection. Conversely, the eggs in all treatments were encapsulated in *S. exigua*. Electron microscopic observations of parasitoid eggs showed structural differences between the surfaces of the mature and other kinds of eggs. The injected CcIV decreased the numbers of host hemocytes and suppressed the spreading ability of plasmatocytes and granulocytes in *H. armigera*, but had little effect on the hemocytes from *S. exigua*. In conclusion, the *C. chlorideae* egg provides a passive protection against encapsulation by itself, and CcIV supplies an active protection through disrupting host immune responses. These coordinated protections are host-specific, implying their role in host range determination.

1. Introduction

The generalist endoparasitoid wasps lay their eggs into the body cavity of a range of host species, but the level of success for parasitism may vary greatly (Godfray, 1994). Once a host is selected by a female parasitoid, the ability for a new generation to develop depends on the suitability of the selected host for the parasitoid's development. Although a number of factors determine the suitability of a host, the host defense is undoubtedly one of the most important for the endoparasitoids. In non-permissive hosts, the parasitoids are usually eliminated by encapsulation, a process in which one or more classes of hemocytes form multilayered cells around an invading organism (Carton et al., 2008; Marmaras and Lampropoulou, 2009; Strand, 2008). Conversely, in permissive hosts, the parasitoids have evolved passive and active strategies for circumventing encapsulation response. Some parasitoid species passively avoid elimination by developing in host tissues that protect the parasitoid from encapsulation, or by possessing surface features that fail to elicit an immune response. Others actively suppress host immune system by injecting some immune-suppressive factors such as polydnavirus (PDV) and venom during oviposition (Strand and Pech, 1995; Vinson, 1990).

Passive mechanisms include epitopes shared by the host, fibrous layer coated in the egg surface, and virus-like particles present on the egg surface (Kinuthia et al., 1999; Feddersen et al., 1986; Davies and Vinson, 1986). Eggs from braconid wasp *Cardiochiles nigriceps* Viereck were covered with a 0.5–1.0 m thick fibrous layer on their outer surface. The fibrous layer, together with other immune-suppressive factors, provides effective protection to eggs from being recognized by host haemocytes (Davies and Vinson, 1986). In the eggs of ichneumonid wasps *Venturia canescens* (Gravenhorst), virus-like particles (VLPs), an element associated with the egg chorion, are involved in the protection of eggs against the host defense (Feddersen et al., 1986; Rotheram, 1967; Salt, 1973). Likewise, in *Cotesia rubecula* (Marshall), a 32-kDa protein produced in the calyx cells of wasp ovaries has

* Corresponding author.
E-mail address: czwang@ioz.ac.cn (C.-Z. Wang).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.jip.2015.07.005
0022-2011/

been found to attach to the surface of the egg and prevent cellular encapsulation reactions (Asgari et al., 1998).

PDV are symbiotic proviruses in some Ichneumonid and Braconid wasps. They replicate in the calyx region of the female parasitoid's ovary and are stored with proteins as calyx fluid in the lumen of the lateral and common oviducts (Krell and Stoltz, 1980; Norton et al., 1975). During parasitization, the calyx fluid with eggs and the venom from venom glands are injected into the hemocoel of the host. For some braconidae, PDV and venom must work together to regulate host physiology (Kitano, 1982; Tanaka, 1987; Tanaka and Vinson, 1991; Yu et al., 2007). For Ichneumonidae, however, both the calyx fluid and PDV have been recognized as the main inhibitory factor, and injection of them alone has an inhibitory effect similar to that of parasitism (Edson et al., 1981; Luckhart and Webb, 1996; Webb and Luckhart, 1994; Dorémus et al., 2013).

Host cellular immune responses to parasitoid eggs appear to be one important factor determining the level of success of parasitism and restricting host range. In the susceptible *Drosophila* strain of the parasitoid wasp *Leptopilina boulardi*, the wasp develops normally, whereas in the resistant strain the parasitoid egg is recognized and encapsulated (Carton and Nappi, 1991; Vass et al., 1993; Russo et al., 2001). The same situation has been also investigated in the *Campoletis sonorensis–Spodoptera frugiperda* system (Prévost et al., 1990). Recently, correlations between parasitism success and host total and differential hemocytes counts have also been reported (Gerritsma et al., 2013; Poyet et al., 2013). *Campoletis chlorideae* Uchida (Hymenoptera: Ichneumonidae) is an important early larval endoparasitoid for many noctuid species (Lepidoptera: Noctuidae) (Wang, 2001; Liu et al., 2004; Yan and Wang, 2006; Zhang et al., 2010). In a previous study, we reported that *Helicoverpa armigera* is a permissive host for *C. chlorideae*, while *Spodoptera exigua* is considered as a non-permissive host because of a much lower parasitism rate. After parasitization, host cellular immune response to the parasitoid was significantly inhibited in *H. armigera* but did not in *S. exigua*, indicating a marked difference of the parasitoid's ability to suppress host immunity in the two species (Han et al., 2013). In this study, we further investigated the effect of CcIV and eggs on cellular immune response in *H. armigera* and *S. exigua*.

2. Materials and methods

2.1. Insects

Experiments were performed with the parasitoid wasp *C. chlorideae* and two noctuid species, *H. armigera* and *S. exigua*. Rearing conditions have been described before (Han et al., 2013). Fourth-instar *H. armigera* and *S. exigua* were reared in glass tubes with artificial diet and used in all experiments. *C. chlorideae* were maintained with *H. armigera* and fed with 30% honey solution.

2.2. CcIV preparation

The calyx fluid containing CcIV was prepared from female *C. chlorideae* as described before (Yin et al., 2003). Briefly, calyces from 20 female wasps were dissected from reproductive tracts, punctured with forceps and the contents were allowed to diffuse into Ringer's solution. After centrifugation at 10,000 rpm for 5 min, the supernatant containing calyx fluid was collected, and adjusted to the final concentration of 0.5 wasp equivalents per μl.

In the field, *C. chlorideae* parasitizes 2nd and 3rd instar *H. armigera*. However, the size of these instar larvae is too small to perform the injection experiment conveniently. In this study, we used the 4th instar larvae. It has been reported that a female of the parasitoid *Microplitis demolitor* and *C. sonorensis* injected approximately 0.02–0.05 wasp equivalents of PDV into a host at each oviposition (Strand et al., 1992; Theilmann and Summers, 1986). We found that injection of 0.02 *C. chlorideae* wasp equivalents also inhibited the development of 2nd and 3rd instar *H. armigera* larvae (unpublished data). Considering that the body weight of the fourth instar larva we used in the injection experiment is about 20 times than that of the second instar in which the parasitoid usually parasitizes, the quantity of the calyx fluid was adjusted to 0.5 wasp equivalents per injection.

2.3. Effect of C. chlorideae eggs and CcIV on encapsulation

Eggs were dissected either from the reproductive system of *C. chlorideae* or from the parasitized *H. armigera*. Immature and mature eggs were from ovarioles and from the calyx region respectively. The newly laid eggs of *C. chlorideae* were collected from the freshly parasitized larvae of *H. armigera*. After washing with Ringer's solution for three times, eggs were injected alone or with calyx fluid into the larvae of *H. armigera* or *S. exigua* with glass micropipettes in the following conditions: (1) mature eggs alone; (2) mature eggs pretreated with protease K; (3) mature eggs together with calyx fluid; (4) immature eggs alone; (5) newly laid eggs alone; (6) newly laid eggs together with calyx fluid; (7) Sephadex G-25 beads lone; (8) Sephadex G-25 beads together with calyx fluid. The injected larvae were dissected 24 h later, 10–30 eggs were recovered from each treatment and encapsulation was recorded as previously described (Han et al., 2013). For protease K treatment, mature eggs were treated with 1 mg/ml protease K (Sigma) at 25 °C for 2 h, then washed with Ringer's solution and injected into host larvae.

2.4. Scanning electron microscopy (SEM) for C. chlorideae eggs

Mature and immature eggs were isolated either from different locations of mated female wasps' reproductive tracts, or from parasitized hosts. Eggs were fixed in 2% paraformaldelyde–3% glutaraldehyde in 0.1 M PBS (pH 7.4) at 4 °C for 1 day. After dehydration through an ascending series of ethanol solutions ending to 100% ethanol, eggs were rinsed in isoamyl acetate for 30 min, critical point dried, and coated with gold using an ion sputter device (E-102) before being examined in a HITACHI S-2360N scanning electron microscope.

2.5. Transmission electron microscopy (TEM) for C. chlorideae eggs

Ovaries were dissected from the abdomens of adult female *C. chlorideae*. TEM was performed using a modified method described by Norton and Davies (Norton and Vinson, 1977; Davies and Vinson, 1986). Samples were divided into six groups: lateral oviducts, mature eggs from the calyx region, immature eggs from ovarioles, mature eggs pretreated with 1 mg/ml protease K (Sigma) at 25 °C for 2 h, eggs from host 6 h and 24 h post-parasitization in *H. armigera*. Samples were placed in 2% paraformaldelyde–5% glutaraldehyde, 0.1 M PBS, pH 7.4. After fixation for 4 h, the oviducts or eggs were rinsed several times in fresh PBS buffer and post-fixed in 2% osmium tetroxide. After serial ethanol dehydration, oviducts and eggs were embedded in Epon 812. Thin sections were doubly stained with uranyl acetate and lead citrate, and examined with a TEM H-600 electron microscope.

2.6. Effect of CcIV on host hemocyte numbers and behavior

Larvae of *H. armigera* and *S. exigua* were injected with the calyx fluid from *C. chlorideae*. After 24 h, total and differential hemocytes were counted as previously described (Han et al., 2013). Briefly,

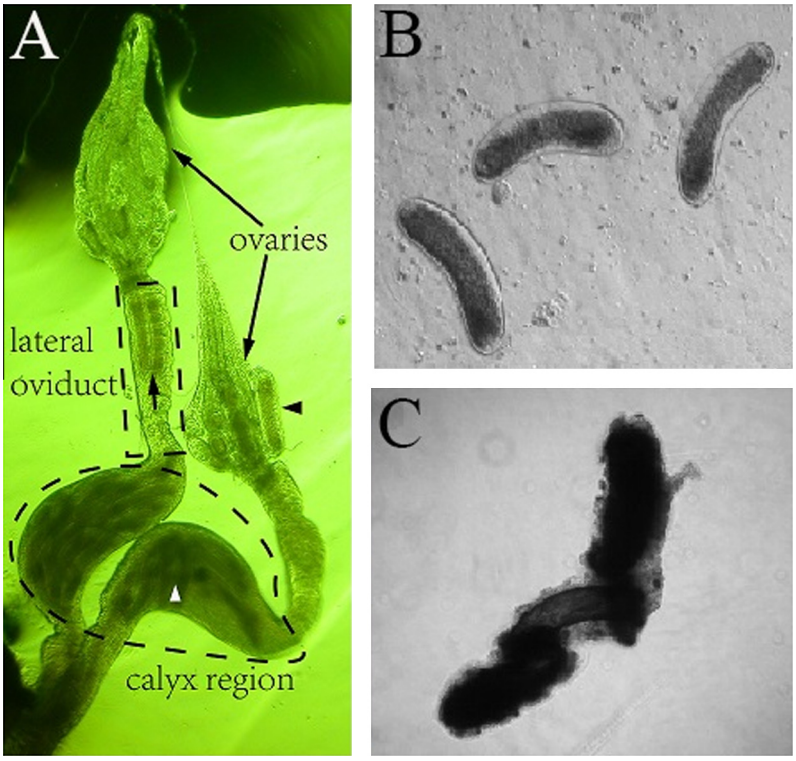

Fig. 1. (A) The reproductive system of *C. chlorideae* showing location of eggs for injection experiment and ultrastructural studies. The black triangle indicates the immature eggs in the ovaries. The black arrow indicates the mature egg in the lateral oviduct. The white triangle indicates the mature egg in the calyx region in which the eggs are immersed in a fluid containing PDV and ovarian proteins. (B) Unencapsulated egg in *H. armigera*. (C) Encapsulated egg in *S. exigua*.

Table 1
Encapsulation of *C. chlorideae* eggs and Sephadex G25 beads in *H. armigera* and *S. exigua* 24 h post-injection.

Species	Materials injected	No. egg/beads injected	No. egg/beads encapsulated	% of encapsulation
H. armigera	ME	21	2	9.5
	ME^{PK}	21	21	100
	ME + CF	25	0	0
	IE	20	20	100
	NLE	22	12	54.4
	NLE + CF	23	0	0
	Beads	30	30	100
	Beads + CF	24	3	12.5
S. exigua	ME	20	20	100
	ME^{PK}	–	–	–
	ME + CF	22	22	100
	IE	–	–	–
	NLE	22	22	100
	NLE + CF	10	10	100
	Beads	26	26	100
	Beads + CF	19	19	100

ME: mature egg; ME^{PK}: mature eggs pretreated with protease K; CF: calyx fluid; IE: immature egg; NLE: new laid egg.

1 μl samples of hemolymph from single larvae were diluted 100 times with Grace's insect medium in a 96 well cell culture plate. For total hemocyte counts, the diluted hemolymph was dripped on a Neubauer hemocytometer and the numbers of hemocytes per mL were counted. For differential hemocytes counts, the same samples were incubated at 25 °C for 1 h in a Falcon tissue culture plate and 150 to 200 hemocytes were classified under a Zeiss inverted phase-contrast microscope as described by Strand and Noda (1991). The relative numbers of plasmatocytes, granulocytes, prohemocytes and oenocytoids were recorded. The data were converted to cells/mL using the appropriate total hemocyte counts. Normal plasmatocytes and granulocytes were considered spread if they attached to the culture plate, and the spreading percentages were also recorded. Ten larvae were examined in each experiment.

2.7. Effect of CcIV and parasitism on host hemocyte cytoskeleton

To investigate the effects of CcIV or parasitism on the cytoskeleton of *H. armigera* hemocytes, we labeled hemocytes with F-actin-specific phalloidin-FITC (Sigma) from either saline/CcIV-injected or unparasitized/parasitized larvae. Fourth-instar *H. armigera* larvae were collected at 24 h post-treatment, sterilized in 75% ethanol, and bled from the proleg. The hemolymph was transferred to a glass fluoro-slide with 15 mm

etched ring. Cells were cultured in 50 μL of complete Grace's medium (containing 2 mM GSH and 5% FBS) in a humidified chamber. After 2 h incubation, hemocytes were gently washed three times with sterile PBS (pH 7.4), fixed in 3.7% formaldehyde for 10 min, washed again and extracted with acetone at −20 °C for 4 min. After air drying, the hemocytes were incubated with 20 μL of phalloidin-FITC for 40 min followed by propidium iodide (25 μg/mL) nuclear staining for 1 min. Here propidium iodide was used to stain the nuclei to detect whether the hemocytes undergo apoptosis after parasitization. Hemocytes were then examined using a Leica TCS-4D confocal laser scanning microscope (Leica, Heidelberg, Germany).

2.8. Statistical analyses

All statistical analyses were performed using the SPSS software (SPSS.18). Data were analyzed by Student *t*-test. *P*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Encapsulation of injected eggs or Sephadex G25 beads

The reproductive system of *C. chlorideae* is shown in Fig. 1A. We have found that mature eggs of *C. chlorideae* were always encapsulated in *S. exigua* (Fig. 1C), but rarely in *H. armigera* (Fig. 1B). In *H. armigera*, when mature eggs were injected alone, only 9.5% of them were encapsulated, which was much less than that of the immature eggs (100%), mature eggs pretreated with protease K (100%) and newly laid eggs (54.4%); when eggs were co-injected with calyx fluid, no encapsulation was found. However, in *S. exigua*, the eggs in all treatments were encapsulated (Table 1).

3.2. Ultrastructure of C. chlorideae eggs

Under a scanning electron microscope (SEM), we found that the surface of immature eggs from ovarioles, mature eggs from calyx region and developing eggs from host were all covered with a network of fibrous projections (Fig. 2). All of the eggs share the same characteristic of being sticky and easy to adhere to glass and dissection tools. Observations in transmission electron microscopy (TEM) indicate that the chorionic membrane of eggs from ovarioles can be divided into three regions (Fig. 3A). The morphology of innermost layer appeared to be unclear. The middle region consisted of many regular slender filaments that were arranged perpendicular to the egg surface. The outmost layer was composed of long microvilli-like projections, which extended perpendicular from the egg surface.

The surface of eggs from the calyx region was similar to those described previously in *C. sonorensis* (Cameron) (Norton and Vinson, 1977), except for the fact that the fibrous layer on the surface of the chorion was not found. The chorion of mature eggs could also be divided into three parts. The endochorion contained thin periodic tubular structures that appear to be surrounded by double membranes. The middle part consisted of a number of columns supporting a row of longitudinal chambers. Above the middle part was a number of membranous microvilli-like projections perpendicular to the egg surface (Fig. 3B). Close to fully developed eggs in the lateral oviduct lumen, we observed numerous electron-dense particles. It has been reported that these particles are polydnaviruses. These viruses were free in the oviduct lumen and did not adhere to the surface of the eggs (Fig. 3F).

The eggs dissected from host 6 h after oviposition underwent a slight morphological change. The projections decreased in length and in quantity, and the average distance between projections

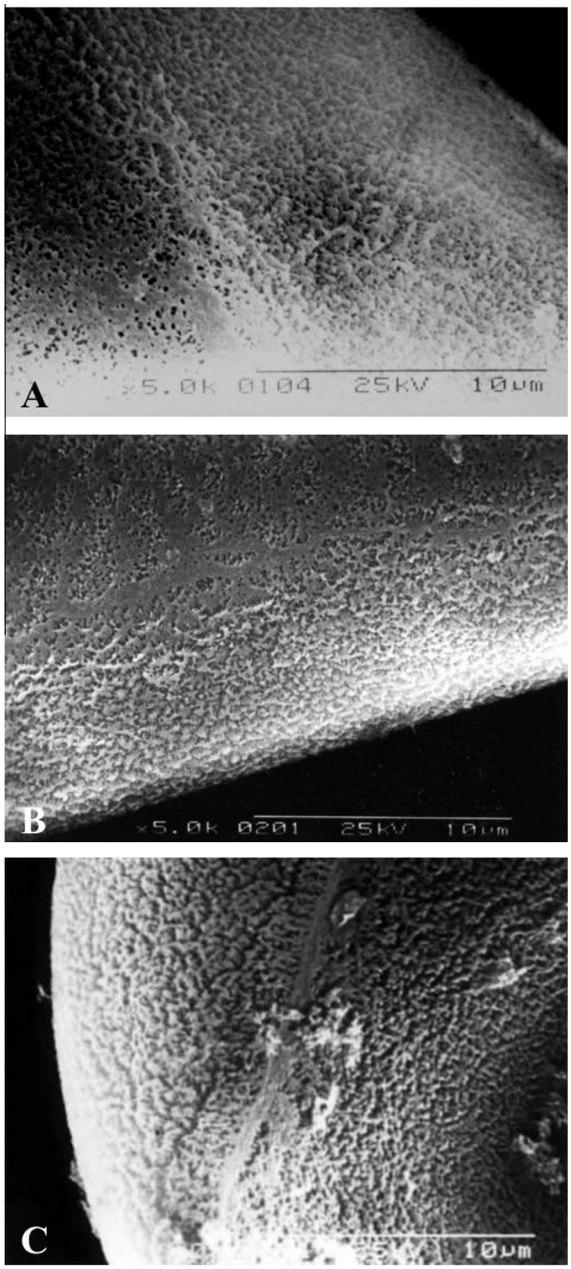

Fig. 2. Scanning electron microscopy of *C. chlorideae* egg surface. (A) Immature egg obtained from ovaroile. (B) Mature egg obtained from calyx region. (C) Newly laid egg obtained from host body cavity. The surfaces of eggs were covered with a network which was formed by fibrous projections.

increased slightly (Fig. 3C). On the surface of the 1 day-old eggs, the projections became obscure (Fig. 3D). Pretreatment with protease K also changed the morphology of eggs. The middle region was deformed and the innermost periodic layer became loose (Fig. 3E).

3.3. Effect of calyx fluid on host hemocyte numbers and behavior

In larvae of *H. armigera*, five types of hemocytes were classified under the phase-contrast microscope: prohemocytes, granulocytes, plasmatocytes, spherulocytes and oenocytoids (Lavine and Strand, 2002). Among them, the prohemocytes, spherulocytes and oenocytoids were not adhesive (Fig. 4A–C). Their morphology had no significant changes after parasitization or injection of CcIV. The plasmatocytes and granulocytes in normal larvae could fully

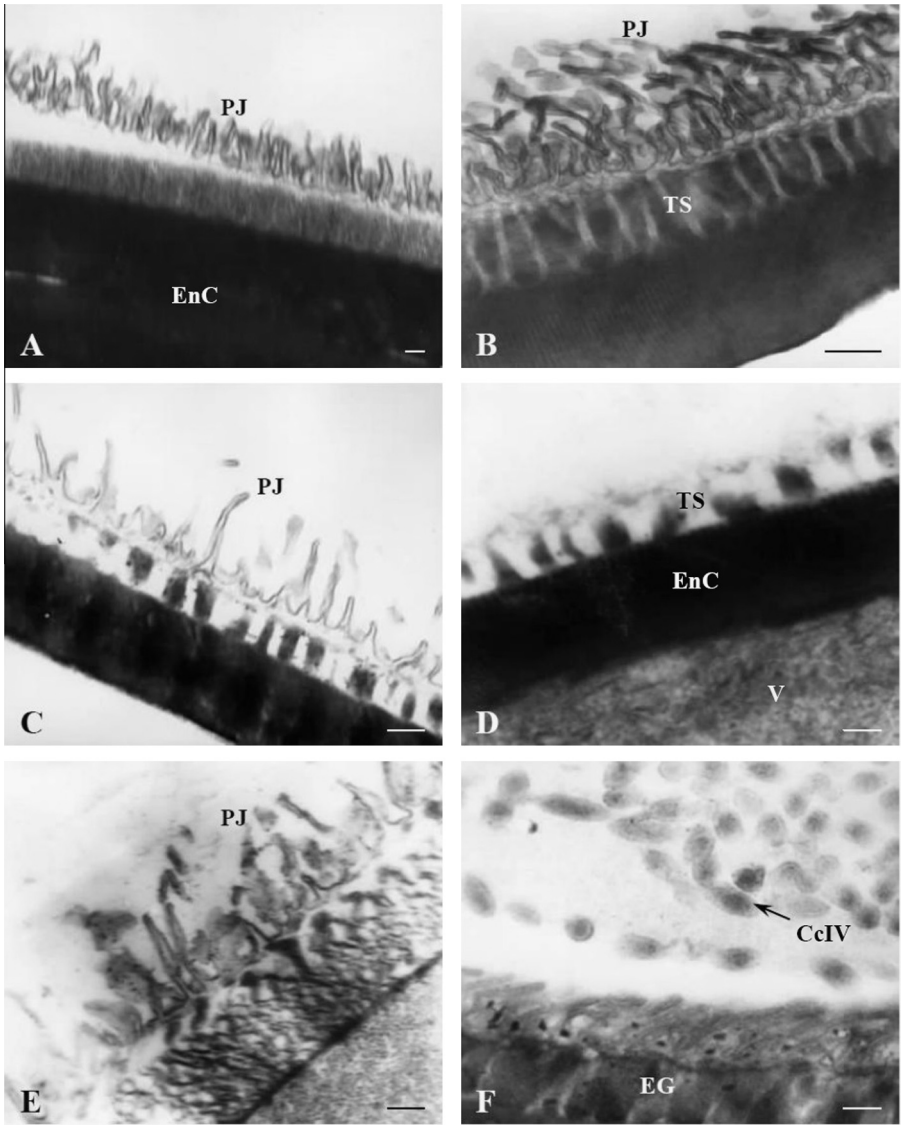

Fig. 3. Transmission electron microscopy of *C. chlorideae* eggs. (A) Immature egg obtained from ovariole. The outmost layer is composed of a number of membranous microvilli-like projections, the middle region consists of many regular filaments while the morphology of innermost layer looks unclear. (B) Mature egg obtained from the calyx region. The endochorion contains thin periodic tubular structures showing a periodicity, the middle part consists of a number of columns supporting a row of longitudinal chambers, the outmost layer is composed of a number of microvilli-like projections. (C) Egg laid for 6 h. The projections became short and their numbers also decrease. (D) Egg laid for 1 day. The projections are obscure and barely visible. (E) Mature egg treated with PK. The middle region is deformed, but the outmost layer does not show obvious changes. (F) Mature egg (EG) in calyx region and PDV in calyx fluid, this latter indicated by the arrow. PJ: projection; EnC: endochorion; TS: tubular structures; V: vitellus; CcIV: *Campoletis chlorideae* ichnovirus; EG: egg. Scale bar = 100 nm.

spread (Fig. 4C and D). After injection with CcIV, however, the filopodial elongation of plasmatocytes and granulocytes were both inhibited (Fig. 5). The injection of *C. chlorideae* calyx fluid caused about 59% of total hemocytes, 61% of plasmatocytes, and 68% of granulocytes reduction in *H. armigera*, but not in *S. exigua* (Table 2). The spreading of both plasmatocytes and granulocytes was strongly suppressed in *H. armigera*, while in *S. exigua* only the spreading of plasmatocytes was inhibited in some degree (Fig. 6).

3.4. Effect of injected calyx fluid and parasitization on host hemocyte cytoskeleton

When *H. armigera* larvae were injected with Ringer's solution, their plasmatocytes and granulocytes could normally spread and the actin cytoskeleton was clear with radial bundles or stellate actin fibers. Some punctuate "foci" of actin are also present in unpolarized plasmatocytes. Granulocytes present ruffled lamellipodia and a peripheral network. In the larvae parasitized and injected with CcIV, their spreading ability was decreased and the F-actin clumps were distributed abnormally (Fig. 7). Propidium iodide staining showed no differences of hemocytes nuclei in normal and parasitized larva, suggesting that apoptosis was not induced after parasitization.

4. Discussion

As a generalist endoparasitoid, *C. chlorideae* develops in a variety of Noctuid caterpillars. However, the level of its successful parasitism in hosts is different. Besides ecological and physiological factors, the host immune response to eggs of *C. chlorideae* is definitely an important factor limiting parasitoid development (Han et al., 2013). The direct relationship between immunoevasion and parasitoid survival prompted us to consider the importance

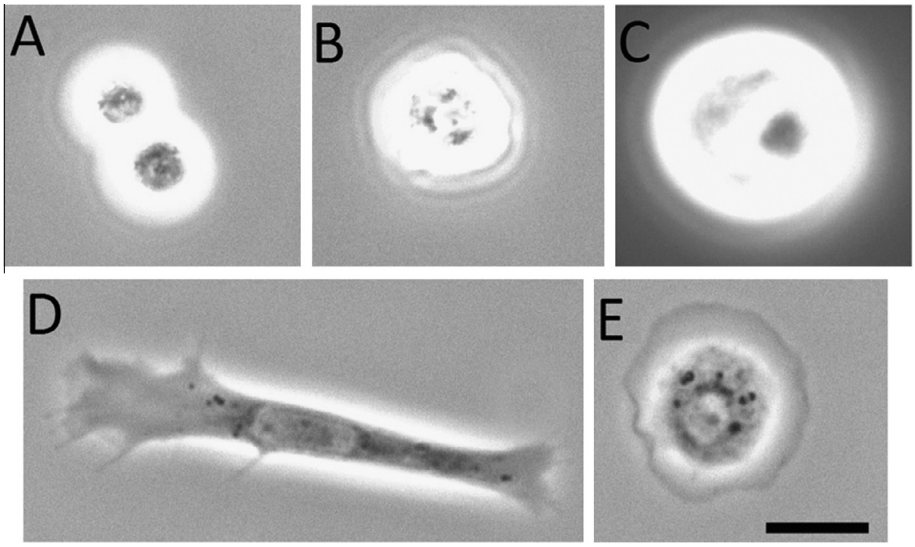

Fig. 4. Morphology of the five types of hemocytes found in the Lepidopteran *H. armigera*. (A) prohemocytes, (B) spherulocyte, (C) oenocytoid, (D) fully spreading plasmatocyte, and (E) fully spreading granulocyte. Scale bar = 10 μm.

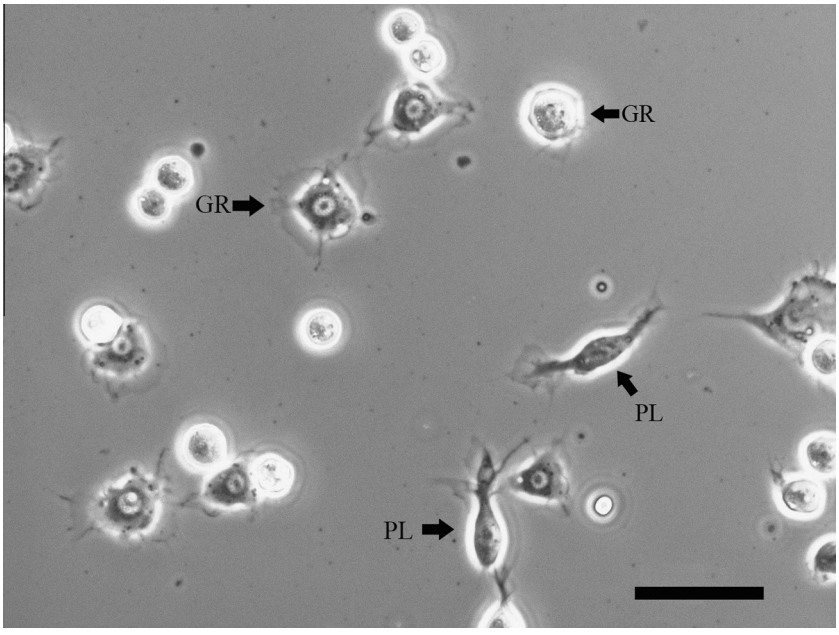

Fig. 5. Morphology of hemocytes from *H. armigera* larva injected with CcIV. The spreading ability of granulocytes (GR) and plasmatocytes (PL) was suppressed.

Table 2
Effect of *C. chlorideae* ichnovirus on the total and differential hemocyte numbers ($\times 10^4$/ml mean ± SD, $n = 10$) in the hemolymph of *H. armigera* and *S. exigua* at 24 h post-injection.

Species	Treatments	Total hemocyte counts	Differential hemocyte count			
			Plasmatocytes	Granulocytes	Prohemocytes	Oenocytoids
H. armigera	Ringer	3117 ± 579.6	630.9 ± 77.3	1375 ± 166.5	41.7 ± 16.6	163.5 ± 30.6
	CcIV	1275 ± 361.5*	250.2 ± 26.3*	450.7 ± 40.1*	16.5 ± 6.1	69 ± 12.9
S. exigua	Ringer	239 ± 29.8	47.4 ± 8.6	101.1 ± 15.8	2.3 ± 0.4	13.6 ± 2.5
	CcIV	216.5 ± 29.4	42.9 ± 6.7	89.8 ± 12.5	2.9 ± 0.6	14.1 ± 3.0

* denotes the statistical significance ($p < 0.05$) when compared between parasitized and control sample at indicated time points.

of wasp derived factors in successful parasitism. In this study, we provide evidence for both a passive evasion strategy (from eggs) and an active suppressive strategy (from polydnavirus), which protects the eggs from encapsulation.

Eggs of *C. chlorideae* different in maturity underwent different fates when they were washed and injected into host larvae. Immature eggs from *C. chlorideae* ovarioles were all encapsulated, but the mature eggs from lateral oviducts were barely

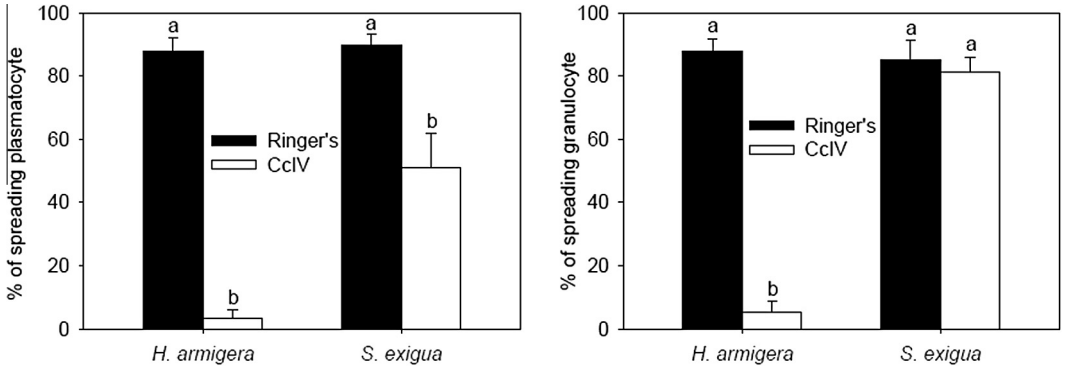

Fig. 6. Percentage of plasmatocytes and granulocytes spreading *in vitro* after injection of *C. chlorideae* calyx fluid or Ringer's solution. 0.5 wasp equivalents of calyx fluid was injected into each larva. Ten larvae were bled in each treatment for each species and cells were allowed to spread 2 h before examination. 100–200 plasmatocytes or granulocytes per larva were identified. Data are presented as means ± SD.

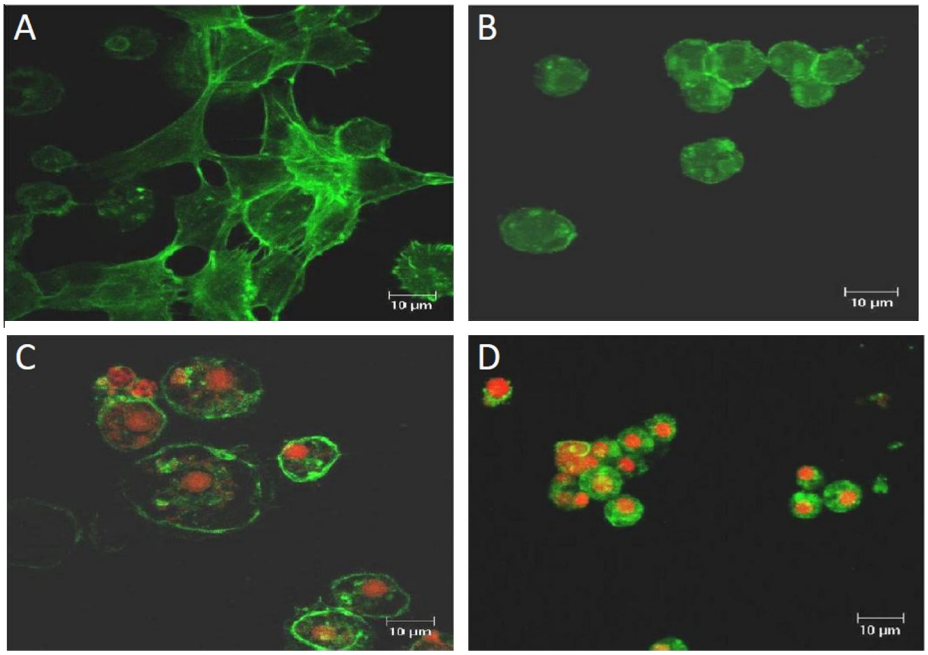

Fig. 7. Actin staining of parasitized and CcIV-injected hemocytes in *H. armigera*. (A) Unparasitized hemocytes. (B) 24 h post injection, the hemocytes are difficult to classify. F-actin clumps are present and distributed abnormally. (C) Actin and nuclear staining from unparasitized hemocyte. (D) Actin and nuclear staining from 24 post-parasitized hemocytes.

encapsulated at 24 h post injection. Instead, in the closely related species *C. sonorensis*, most washed mature eggs were encapsulated after being injected into hosts (Webb and Luckhart, 1996). We further investigated the ultrastructure of immature, mature, and laid eggs from *C. chlorideae* by SEM and TEM, and could not find obvious differences between them except for the length and quantity of membranous microvilli-like projections. On the surface of *C. chlorideae* eggs we did not find the fibrous layer that was described on the eggs of *C. nigriceps*, *C. sonorensis* and *Apanteles glomeratus* (Linnaeus) (Davies and Vinson, 1986; Kitano, 1984; Norton and Vinson, 1977). It has been reported that the fibrous layer plays a role in passive protection of mature eggs. The eggs from *C. nigriceps* ovariole could be encapsulated by *Heliothis virescens* haemocytes *in vitro* because the fibrous layer was covered by a sheath of follicle cells; when it was exposed, the fibrous layer could provide temporary protection for the eggs obtained from lateral oviducts until *C. nigriceps* bracovirus established a persistent infection in the host (Davies and Vinson, 1986).

Some substances secreted by the parasitoid or its eggs may also play a protective role. In *C. rubecula*, a 32-kDa protein (Crp 32) produced in the wasp calyx cells can attach to the surface of the eggs and prevent cellular encapsulation reaction by a local inactivation of the host defense system (Asgari et al., 1998). In *A. glomeratus*, a substance is likely secreted into the hemocoel of the host by the parasitoid eggs, which suppresses defense reactions of the host (Kitano and Nakatsuji, 1978). Kinuthia et al. (1999) found that a hemolymph component constitutes a protective mucinous layer on the surface of the parasitoid egg. Recently, it was reported that the eggs of the ichneumon *Hyposoter didymator* could evade encapsulation by the host *S. frugiperda* in the absence of calyx fluid, and mass spectrometry analysis of the egg surface protein revealed the presence of immune-related proteins (Dorémus et al., 2013). In our study, the mature eggs pre-treated with protease K were encapsulated after 24 h when they were injected into *H. armigera*, implying that the egg surface perhaps also possesses a certain protective substance.

The passive protection of eggs does not always work well. In the parasitoid *Cotesia kariyai* Watanabe, the egg surface is covered by an immunoevasive protein (IEP-1), which presents two EGF-like motifs and is thought to be involved in host molecular mimicry (Hayakawa and Yazak, 1997). A protein sharing antigenic similarities with IEP-1 was identified in the hemolymph of the permissive strain but not in that of the nonpermissive (able to encapsulate the *C. kariyai* eggs) strain of *Mythimna separata* (Walker). In our case, only 9.5% of the mature eggs from *C. chlorideae* were encapsulated in the permissive host, but all were encapsulated in the non-permissive host *S. exigua*, suggesting that the passive protection of *C. chlorideae* eggs is host specific.

Although the eggs of *C. chlorideae* have the ability to protect themselves from host encapsulation, this is not enough for the wasp to totally overcome host immune responses during development. CcIV, the unique virus from *C. chlorideae*, also provides a complemental protection by expressing specific viral genes in infected host hemocytes. In the presence of *C. sonorensis* ichnovirus (CsIV), host hemocytes are unable to encapsulate *C. sonorensis* eggs as well as other foreign targets (Edson et al., 1981; Davies et al., 1987). We found the encapsulation response was also significantly inhibited after injection of CcIV in the permissive host of *C. chlorideae*, *H. armigera*. PDV can induce apoptosis of host hemocytes and decrease the hemocytes number (Strand et al., 1995). Infection of ichnoviruses from several species of campoplegine ichneumonids results in altering circulating hemocyte numbers and reducing the ability of granulocytes and plasmatocytes to spread on foreign surfaces in the hosts (Davies et al., 1987; Guzo and Stoltz, 1987; Doucet and Cusson, 1996). Our results provide further evidence that injection of CcIV significantly decreases the numbers of host granulocytes and plasmatocytes, while suppressing their spreading ability in the permissive host *H. armigera*.

Host suitability of the parasitoid wasp is closely associated with its ability to suppress host encapsulation response (Han et al., 2013). The capsule formation in the noctuid species is a highly organized process. For example, in *Pseudoplusia includens*, granulocytes are the first cells to bind to the target, and thereafter plasmatocytes attach (Pech and Strand, 1996, 2000). Therefore, granulocytes and plasmatocytes with sufficient spreading ability are crucial in capsule formation. Prévost et al. found that the effect of parasitism by *C. sonorensis* on inhibiting the spreading ability of plasmatocytes was more significant in the susceptible larvae of *S. frugiperda* than in the resistant larvae (Prévost et al., 1990). It has been proved that the expression level of CsIV genes is higher in the permissive host than in the semi-permissive host (Cui et al., 2000). In this study, CcIV injection produces great changes in the permissive host *H. armigera*, the total hemocytes number significantly decreased and the spreading ability of granulocytes was remarkably inhibited; however, no marked effect was observed in the non-permissive host *S. exigua*. Though the inhibition to plasmatocyte spreading was also detected in *S. exigua*, the extent was much less than that in *H. armigera*. This implies that PDV infection may also occur in the non-permissive hosts, but at a lower level. Taking into account the literature data and our results, we suggest that CcIV suppress host encapsulation through altering host hemocyte number and spreading behavior, and its effects are also host specific.

Although the *C. chlorideae* ichnovirus and eggs have a role in host immunoevasion, more factors are needed for the parasitoid to ensure successful parasitism. The developmental stage of *C. chlorideae* inside the host is about 7 days. Yin et al. (2003) detected CcIV transcripts at 1–4 day post-parasitization, suggesting the partial immunosuppressive effects of infection by CcIV in *H. armigera*. We hypothesize that the wasp egg itself is responsible for its development at very early stage of parasitization, and then the CcIV plays a major role by active suppression of host immune responses. However, injection of the wasp egg and CcIV is just a mimic of the wasp parasitization. More elements from wasp reproductive tracts such as venom and ovarian proteins may also be involved in such a parasitic process. Further work will be required to define the sophisticated mechanisms of these factors in protecting wasp development.

Acknowledgments

We thank our colleague Q.-L. Qin for supplying *Spodoptera exigua* larvae, Paolo Pelosi, University of Pisa, Italy for a critical reading of the manuscript. This work was supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (grant number XDB11010300), National Basic Research Program of China (grant number 2013CB127600), the Key Technologies R&D Program of China (grant number 2012BAD19B05), and the State Key Laboratory of Integrated Management of Pest Insects and Rodents (Grant No. ChineseIPM1306).

References

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.

Carton, Y., Nappi, A., 1991. The Drosophila immune reaction and the parasitoid capacity to evade it: genetic and coevolutionary aspects. Acta Oecol. 12, 89–104.

Carton, Y., Poirie, M., Nappi, A.J., 2008. Insect immune resistance to parasitoids. Insect Sci. 15, 67–87.

Cui, L., Soldevila, A.I., Webb, B.A., 2000. Relationships between polydnavirus gene expression and host range of the parasitoid wasp *Campoletis sonorensis*. J. Insect Physiol. 46, 1397–1407.

Davies, D.H., Vinson, S.B., 1986. Passive evasion by eggs of braconid parasitoid *Cardiochiles nigriceps* of encapsulation in vitro by hemocytes of host *Heliothis virescens*. Possible role for fibrous layer in immunity. J. Insect Physiol. 32, 1003–1010.

Davies, D.H., Strand, M.R., Vinson, S.B., 1987. Changes in differential hemocyte count and in vitro behavior of plasmatocytes from host *Heliothis virescens* caused by *Campoletis sonorensis* polydnavirus. J. Insect Physiol. 33, 143–153.

Dorémus, T., Jouan, V., Urbach, S., Cousserans, F., Wincker, P., Ravallec, M., Wajnberg, E., Volkoff, A.-N., 2013a. *Hyposoter didymator* uses a combination of passive and active strategies to escape from the *Spodoptera frugiperda* cellular immune response. J. Insect Physiol. 59, 500–508.

Dorémus, T., Urbach, S., Jouan, V., Cousserans, F., Ravallec, M., Demettre, E., Wajnberg, E., Poulain, J., Azéma-Dossat, C., Darboux, I., Escoubas, J.-M., Colinet, D., Gatti, J.-L., Poirié, M., Volkoff, A.-N., 2013b. Venom gland extract is not required for successful parasitism in the polydnavirus-associated endoparasitoid *Hyposoter didymator* (Hym. Ichneumonidae) despite the presence of numerous novel and conserved venom proteins. Insect Biochem. Mol. Biol. 43, 292–307.

Doucet, D., Cusson, M., 1996. Role of calyx fluid in alterations of immunity in *Choristoneura fumiferana* larvae parasitized by *Tranosema rostrale*. Comp. Biochem. Physiol. A – Mol. Integr. Physiol. 114, 311–317.

Edson, K., Vinson, S., Stoltz, D., Summers, M., 1981. Virus in a parasitoid wasp: suppression of the cellular immune response in the parasitoid's host. Science 211, 582.

Feddersen, I., Sander, K., Schmidt, O., 1986. Virus-like particles with host protein-like antigenic determinants protect an insect parasitoid from encapsulation. Experientia 42, 1278–1281.

Gerritsma, S., de Haan, A., van de Zande, L., Wertheim, B., 2013. Natural variation in differentiated hemocytes is related to parasitoid resistance in *Drosophila melanogaster*. J. Insect Physiol. 59, 148–158.

Godfray, H.C.J., 1994. Parasitoids: Behavioral and Evolutionary Ecology. Princeton University Press, Princeton, USA.

Guzo, D., Stoltz, D.B., 1987. Observations on cellular immunity and parasitism in the tussock moth. J. Insect Physiol. 33, 19–31.

Han, L.-B., Huang, L.-Q., Wang, C.-Z., 2013. Host preference and suitability in the endoparasitoid *Campoletis chlorideae* is associated with its ability to suppress host immune responses. Ecol. Entomol. 38, 173–182.

Hayakawa, Y., Yazak, K., 1997. Envelope protein of parasitic wasp symbiont virus, polydnavirus, protects the wasp eggs form cellular immune reactions by the host insect. Eur. J. Biochem. 246, 820–826.

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.

Kitano, H., 1982. Effect of the venom of the gregarious parasitoid *Apanteles glomeratus* on its hemocytic encapsulation by the host, *Pieris*. J. Invertebr. Pathol. 40, 61–67.

Kitano, H. 1984. Role of *Apanteles glomeratus* venom during parasitism. In: XVII International Congress of Entomology, Hamburg. Abstract S4.9, 19.

Kitano, H., Nakatsuji, N., 1978. Resistance of *Apanteles* eggs to the haemocytic encapsulation by their habitual host, *Pieris*. J. Insect Physiol. 24, 261–271.
Krell, P., Stoltz, D., 1980. Virus-like particles in the ovary of an ichneumonid wasp: purification and preliminary characterization. Virology 101, 408–418.
Lavine, M.D., Strand, M.R., 2002. Insect hemocytes and their role in immunity. Insect Biochem. Mol. Biol. 32, 1295–1309.
Liu, W.-X., Wan, F.-H., Yuan, S.-T., 2004. Mass-rearing and bionomics of *Campoletis chlorideae*. Chinese J. Biol. Control 20, 17–20.
Luckhart, S., Webb, B., 1996. Interaction of a wasp ovarian protein and polydnavirus in host immune suppression. Dev. Comp. Immunol. 20, 1–21.
Marmaras, V.J., Lampropoulou, M., 2009. Regulators and signalling in insect haemocyte immunity. Cell. Signal. 21, 186–195.
Norton, W.N., Vinson, S.B., 1977. Encapsulation of a parasitoid egg within its habitual host: an ultrastructural investigation. J. Invertebr. Pathol. 30, 55–67.
Norton, W., Vinson, S., Stoltz, D., 1975. Nuclear secretory particles associated with the calyx cells of the ichneumonid parasitoid *Campoletis sonorensis* (Cameron). Cell Tissue Res. 162, 195–208.
Pech, L.L., Strand, M.R., 1996. Granular cells are required for encapsulation of foreign targets by insect haemocytes. J. Cell Sci. 109, 2053–2060.
Pech, L.L., Strand, M.R., 2000. Plasmatocytes from the moth *Pseudoplusia includens* induce apoptosis of granular cells. J. Insect Physiol. 46, 1565–1573.
Poyet, M., Havard, S., Prevost, G., Chabrerie, O., Doury, G., Gibert, P., Eslin, P., 2013. Resistance of *Drosophila suzukii* to the larval parasitoids *Leptopilina heterotoma* and *Asobara japonica* is related to haemocyte load. Physiol. Entomol. 38, 45–53.
Prévost, G., Davies, D.H., Vinson, S.B., 1990. Evasion of encapsulation by parasitoid correlated with the extent of host hemocyte pathology. Entomol. Exp. Appl. 55, 1–10.
Rotheram, S., 1967. Immune surface of eggs of a parasitic insect. Nature 214, 700.
Russo, J., Brehélin, M., Carton, Y., 2001. Haemocyte changes in resistant and susceptible strains of *D. melanogaster* caused by virulent and avirulent strains of the parasitic wasp *Leptopilina boulardi*. J. Insect Physiol. 47, 167–172.
Salt, G., 1973. Experimental studies in insect parasitism. XVI. The mechanism of the resistance of *Nemeritis* to defense reactions. Proc. R. Soc. B – Biol. Sci. 183, 337–350.
Strand, M.R., 2008. The insect cellular immune response. Insect Sci. 15, 1–14.
Strand, M.R., Mckenzie, D.I., Grassl, V., Dover, B.A., Aiken, J.M., 1992. Persistence and expression of *Microplitis demolitor* polydnavirus in *Pseudoplusia includens*. J. Gen. Virol. 73, 1627–1635.
Strand, M.R., Noda, T., 1991. Alterations in the hemocytes of *Pseudoplusia includens* after parasitism by *Microplitis demolitor*. J. Insect Physiol. 37, 839–850.
Strand, M.R., Pech, L.L., 1995. Immunological basis for compatibility in parasitoid-host relationships. Annu. Rev. Entomol. 40, 31–56.
Tanaka, T., 1987. Calyx and venom fluids of *Apanteles kariyai* (Hymenoptera: Braconidae) as factors that prolong larval period of the host, *Pseudaletia separata* (Lepidoptera: Noctuidae). Ann. Entomol. Soc. Am. 80, 530–533.
Tanaka, T., Vinson, S.B., 1991. Interaction of venoms with the calyx fluids of three parasitoids, *Cardiochiles nigriceps*, *Microplitis croceipes* (Hymenoptera: Braconidae), and *Campoletis sonorensis* (Hymenoptera: Ichneumonidae) in effecting a delay in the pupation of *Heliothis virescens* (Lepidoptera: Noctuidae). Ann. Entomol. Soc. Am. 84, 87–92.
Theilmann, D.A., Summers, M.D., 1986. Molecular analysis of *Campoletis sonorensis* virus DNA in the lepidopteran host *Heliothis virescens*. J. Gen. Virol. 67, 1961–1969.
Vass, E., Nappi, A.J., Carton, Y., 1993. Comparative study of immune competence and host susceptibility in *Drosophila melanogaster* parasitized by *Leptopilina boulardi* and *Asobara tabida*. J. Parasitol., 106–112
Vinson, S.B., 1990. How parasitoids deal with the immune system of their host: an overview. Arch. Insect Biochem. Physiol. 13, 3–27.
Wang, C.-Z., 2001. Effects of host size on oviposition and development of the endoparasitoid, *Campoletis chlorideae* Uchida. Chinese J. Biol. Control 17, 107–111.
Webb, B., Luckhart, S., 1994. Evidence for an early immunosuppressive role for related *Campoletis sonorensis* venom and ovarian proteins in *Heliothis virescens*. Arch. Insect Biochem. Physiol. 26, 147–163.
Webb, B.A., Luckhart, S., 1996. Factors mediating short- and long-term immune suppression in a parasitoid insect. J. Insect Physiol. 42, 33–40.
Yan, Z.-G., Wang, C.-Z., 2006. Similar attractiveness of maize volatiles induced by *Helicoverpa armigera* and *Pseudaletia separata* to the generalist parasitoid *Campoletis chlorideae*. Entomol. Exp. Appl. 118, 87–96.
Yin, L.-H., Zhang, C., Qin, J.-D., Wang, C.-Z., 2003. Polydnavirus of *Campoletis chlorideae*: Characterization and temporal effect on host *Helicoverpa armigera* cellular immune response. Arch. Insect Biochem. 52, 104–113.
Yu, R., Chen, Y.-F., Chen, X., Huang, F., Lou, Y., Liu, S., 2007. Effects of venom/calyx fluid from the endoparasitic wasp *Cotesia plutellae* on the hemocytes of its host *Plutella xylostella* in vitro. J. Insect Physiol. 53, 22–29.
Zhang, J.-H., Gu, L.-Q., Wang, C.-Z., 2010. Superparasitism behavior and host discrimination of *Campoletis chlorideae* (Ichneumonidae: Hymenoptera) toward *Mythimna separata* (Noctuidae: Lepidoptera). Environ. Entomol. 39, 1249–1254.