JOURNAL OF APPLIED ENTOMOLOGY

J. Appl. Entomol.

Cytotoxic effects of parasitism and application of venom from the endoparasitoid *Pimpla turionellae* on hemocytes of the host *Galleria mellonella*

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Key words
apoptosis, *Galleria mellonella,* hemocyte viability, *Pimpla turionellae*

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Received: December 29, 2009; accepted: March 9, 2010.

doi: 10.1111/j.1439-0418.2010.01528.x

Abstract

In parasitoid species devoid of polydnaviruses and virus-like particles, venom appears to play a major role in suppression of host immunity. Venom from the pupal endoparasitoid *Pimpla turionellae* L. (Hymenoptera: Ichneumonidae) has previously been shown to contain a mixture of biologically active components, which display potent paralytic, cytotoxic, and cytolytic effects toward lepidopteran and dipteran hosts. The current study was undertaken to investigate if parasitism and/or envenomation by *P. turionellae* affects the frequency of apoptotic and necrotic hemocytes, hemocyte viability and mitotic indices in *Galleria mellonella* L. (Lepidoptera: Pyralidae) pupae and larvae. Our study indicates that parasitism and experimental envenomation of *G. mellonella* by *P. turionellae* resulted in markedly different effects on the ratio of apoptotic hemocytes circulating in hemolymph depending on the host developmental stages. The ratio of early and late apoptotic hemocytes increased in *G. mellonella* pupae and larvae upon parasitization and at high doses of venom when compared to untreated, null and Phosphate Buffered Saline (PBS) injected controls. In contrast, an increase in necrotic hemocytes was only observed in parasitized pupae at 24 h and no difference was observed in larvae. The lowest hemocyte viability values were observed with pupae as 69.87%, 69.80%, and 72.47% at 4, 8, and 24 h post-parasitism. The ratio of mitotic hemocytes also decreased in pupae and larvae upon parasitization and at high doses of venom. Staining of hemocytes with annexin V-FITC revealed green fluorescent 'halos' along the plasma membranes of venom treated cells within 15 min following exposure to venom. By 1 h post-venom – treatment, the majority of hemocytes displayed binding of this probe, indicative of early stage apoptosis. These same hemocytes also displayed a loss of plasma membrane integrity at the same time points as evidenced by accumulation of propidium iodide in nuclei.

Introduction

Parasitoids have evolved a variety of strategies as active and/or passive mechanisms in avoiding host immune responses (Schmidt et al. 2001). Passive protection can be gained by the parasitoid developing in locations that protect the parasitoid from encapsulation, or by using a type of surface display equivalent to molecular mimicry in which the wasp's eggs or larvae are not recognized as non-self

(Asgari et al. 1998; Schmidt et al. 2001; Rivers et al. 2007). However, active mechanisms involve suppression of host immunity through the activity of maternally derived factors that are injected by adult females at the time of oviposition (Rivers et al. 2007). Either alone or in combination with other maternal factors, parasitoid venoms are known to have distinct functions, including inhibition or reduction of the hemocyte responses (Schmidt et al. 2001; Beckage and Gelman 2004). In most cases, venom enhances the effects of PDV's (polydnaviruses) or calyx fluid rather than serving as separate immunological suppressants (Davies et al. 1987; Tanaka 1987; Gupta and Ferkovich 1998; Beckage and Gelman 2004). In parasitoid species devoid of PDV's or other symbiotic viruses such as *Pimpla hypochondriaca* Retzius (Hymenoptera: Ichneumonidae) (Parkinson and Weaver 1999; Richards and Parkinson 2000), *Pteromalus puparum* L. (Hymenoptera: Pteromalidae) (Cai et al. 2004; Wu et al. 2008), and *Nasonia vitripennis* Walker (Hymenoptera: Pteromalidae) (Rivers et al. 2002), venoms would alone perturb host immune defenses.

The most common feature shared in the action of wasp secretions and viral products is the induction of cell death in selected tissues of the insect host (Rivers et al. 2007). Apoptosis and/or oncosis appear to be necessary means to manipulate the host to ensure successful development of parasitoid larvae (Nakamatsu and Tanaka 2003; Zhang et al. 2005; Asgari 2006). There are some examples of PDV and/or venom mediated apoptosis in host hemocytes (Strand and Pech 1995; Teramoto and Tanaka 2004; Luo and Pang 2006; Richards and Dani 2007). The PDV's of the *Microplitis demolitor* Wilkinson (Hymenoptera: Braconidae) reduced the number of hemocytes in *Pseudoplusia includens* Walker (Lepidoptera: Noctuidae) by inducing the granular cells to undergo apoptosis (Strand and Pech 1995). Suzuki and Tanaka (2006) reported that parasitism of *Meteoris pulchricornis* Wesmael (Hymenoptera: Ichneumonidae) disrupts the host encapsulation response by VLPs (virus like particles)-induced hemocyte apoptosis. Furthermore, in the host *Pseudaletia separata* Walker (Lepidoptera: Noctuidae) parasitized by *Cotesia kariyai* Watanabe (Hymenoptera: Braconidae), PDV induced apoptosis in the circulating hemocytes (Teramoto and Tanaka 2004). Dubuffet et al. (2008) investigated the variations in immunosuppressive effects of two lines of parasitoid wasp *Leptopilina boulardi* Barbotin et al. (Hymenoptera: Figitidae) towards the host *Drosophila yakuba* Burla (Diptera: Drosophilidae). They reported qualitative differences in venoms explaining the variations of success in encapsulation ability of wasps on hosts as the venom protein profiles of the two parasitoid lines are quite different (Labrosse et al. 2005). Venom from *P. hypocondriaca* that lacks PDV and VLP kills the *Lacanobia oleracea* Linnaeus (Lepidoptera: Noctuidae) hemocytes by apoptosis in a dose–responsive manner (Richards and Dani 2007).

It is known that the maintenance of circulating hemocytes is supplied by the mitosis of circulating hemocytes itself and from hematopoietic organs (Jones 1970; Ratcliffe et al. 1985). In *Galleria mellonella* L. (Lepidoptera: Pyralidae), *Bombyx mori* Linnaeus (Lepidoptera: Bombycidae), and *Euxoa declarata* Walker (Lepidoptera: Noctuidae) mitosis in circulating hemocytes has been observed and it was confirmed that 1–8% of the population of circulating hemocytes are in the mitotic phase (Shapiro 1968; Jones 1970; Arnold and Hinks 1976; Beaulaton 1979). It was revealed that in *P. separata*, mitosis of circulating hemocytes halted after *C. kariyai* PDV plus venom injection (Teramoto and Tanaka 2004). However, the effects of endoparasitoid venom or parasitization on the mitosis of host hemocytes have not been studied in any detail.

In the case of solitary idiobiont pupal endoparasitoid *P. turionellae*, both PDVs and VLPs are absent. Venom produced in venom glands contains a number of biologically active components including melittin, apamin, the biogenic amines; histamine and serotonin, and the catecholamine noradrenaline. Additionally, venom from this endoparasitoid wasp contains several mid to high range molecular weight proteins (Uçkan et al. 2004, 2006; Ergin et al. 2007) and has previously been shown to be cytotoxic with limited cytolysis toward cultured cells established from *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae) (Ergin et al. 2006). The present study was undertaken to investigate further if *P. turionellae* parasitism and/or venom affect the apoptotic and mitotic indices of the circulating hemocytes of its host *G. mellonella*.

Materials and Methods

Insect rearing

Laboratory colonies of the host species, *G. mellonella* were established from individuals that were collected from the honeycombs maintained from beekeepers around Balıkesir, Turkey. *P. turionellae* were reared on the pupae of the host, *G. mellonella* at 25 ± 1°C, 60 ± 5% RH, and with a photoperiod of 12 : 12 h, L : D. Adult parasitoids were fed 30% (v/v) honey

solution and provided with host pupae (four pupae for every 10 female wasps once every 3 days). Host colony was maintained by feeding the insects with honeycomb (Uçkan et al. 2004).

Preparation of *Pimpla turionellae* venom and injection into *Galleria mellonella*

Venom reservoir contents were isolated from honey- and host-fed 15 to 20-day-old females by dissecting out the venom sacs as described previously (Uçkan et al. 2004). The venom sacs were then torn open using thin forceps and the solution spun at 3000 *g* for 10 min at room temperature to remove cellular debris. The isolated crude venom was adjusted to doses below LD_{99} (lethal dose) calculated for *G. mellonella* pupae and larvae (Ergin et al. 2006). Venom was adjusted to 0.05, 0.02, 0.01, and 0.005 venom reservoir equivalents (VREs) for pupae and 0.5, 0.1, 0.05, and 0.02 VREs for larvae with PBS (0.138 M NaCl and 0.0027 M KCl in 0.01 M PBS, pH 7.4). Last instars of *G. mellonella* (260 ± 10 mg) and 1 to 2-day-old pupae (140 ± 20 mg) previously chilled on ice for 10 min, were then injected with a 5 μl solution of the venom preparation between the last two lateral abdominal segments of host pupae and on the first hind leg of larvae by using a 10 μl Hamilton microsyringe (Hamilton, Reno, NV). Petroleum jelly was applied to the injection area to prevent hemolymph loss (Richards and Edwards 1999). These larvae and pupae were referred to as 'experimentally envenomated' in the text. Controls consisted of pupae and larvae untreated, null-injected and injected with only 5 μl PBS.

Parasitization of *Galleria mellonella* pupae

Parasitization was performed on day 1 or 2 of the host pupae by exposing an individual host pupa (140 ± 20 mg) to an individual 15 to 20-day-old wasp female. Parasitized pupae were held at 25 ± 2°C, 60 ± 5% RH (relative humidity) under a photoperiod of 12 : 12 h, L : D as were the controls and venom-treated pupae. Since parasitism of larval stages was not achievable by this parasitoid, larvae were only used in venom injection experiments.

Analysis of cell viability and mitotic indices by fluorescence microscopy – acridine orange/ethidium bromide double staining

The occurrence of apoptosis in venom-treated, parasitized and untreated *G. mellonella* larvae and pupae was detected using acridine orange/ethidium bromide double staining (fig. 1). This method of detecting apoptosis is based on the loss of plasma membrane integrity as cells die (Cendoroglo et al. 1999). Acridine orange penetrates into living and dead cells, emitting green fluorescence as a result of intercalation in double-stranded DNA and red–orange fluorescence after binding with single-stranded RNA and due to its accumulation in lysosomes.

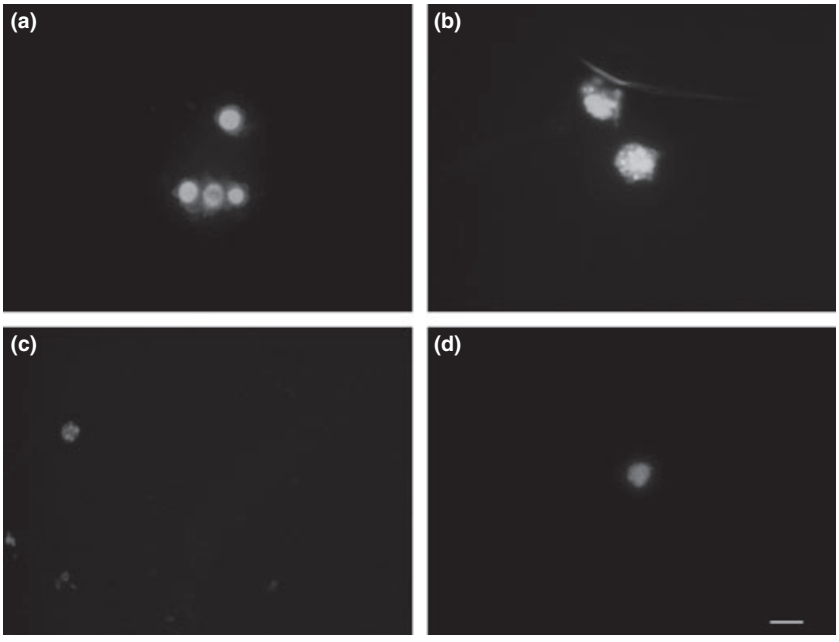

Fig. 1 Acridine orange/ethidium bromide double staining of *G. mellonella* hemocytes with characteristic symptoms of apoptosis. (a) Normal hemocytes from untreated larvae, (b) early apoptosis, (c) late apoptosis, (d) necrosis from parasitized larvae of *G. mellonella*. Scale bar 10 μm.

Ethidium bromide emits red fluorescence after intercalation in DNA of cells with an altered cell membrane (at a late stage of apoptosis and necrosis) (Kosmider et al. 2004).

To investigate the effects of parasitization and venom injection on the percentage of viable, apoptotic, necrotic and mitotic hemocytes 1- to 2-day-old host pupae and last instars of approximately the same sizes were used. Pupae were bled by piercing the cuticle at the abdomen and larvae on the first hind leg with a sterile 19-gauge needle. Five microlitres of hemolymph from each individual pupa and larva was collected with a glass micro capillary tube (Sigma Chemical Co., St. Louis, MO) and poured on a sterile microscope slide. The slides were allowed to stand in room temperature to facilitate the adhesion of hemocytes to the glass. Acridine orange (100 μg/ml; Sigma Chemical Co.) and ethidium bromide (100 μg/ml; Sigma Chemical Co.) stock solutions were prepared in PBS, respectively. A dye cocktail was prepared by adding equal volumes of ethidium bromide and acridine orange. Ten microlitres of the dye cocktail was spread on the glass slide, covered with a cover slip and immediately examined using fluorescence microscope at blue filter (Olympus BX 51, Olympus Corp., Tokyo, Japan). Cells were identified as viable (green nucleus with red–orange cytoplasm with an intact membrane), early apoptotic (cell membrane still continuous but chromatin condensation and an irregular green nucleus are visible), late apoptotic (ethidium bromide penetrates through altered cell membrane and stains the nuclei orange, while fragmentation or condensation of chromatin is still observed) and necrotic (orange nucleus with intact structure) (Cendoroglo et al. 1999; Kosmider et al. 2004). The percentages of apoptotic, necrotic, and viable cells were determined at 4, 8, and 24 h post-treatments and the ratios were referred to as 'cell viability' in the text. The frequency of mitotic indices was also observed at all time points with all treatments for larvae and pupae. Controls consisted of pupae and larvae untreated, null-injected and injected with only 5 μl PBS. Three host pupae and larvae were evaluated for each experimental and control assays at a given time and 500 cells from an individual pupa and larva were counted and differentiated in each of three replicates for hemocyte viability and mitotic indices.

Apoptosis detection

In a parallel set of experiments aimed at detecting venom-induced apoptosis, hemocytes from *G. mellonella* were collected from last instar larvae and seeded into a 96-well plate in TC-100 (Sigma) containing 10% fetal bovine serum (Sigma) (100 μl/well) at a concentration of 2 000 cells/well. Hemocytes were allowed to incubate for 1 h at 27°C prior to the addition of an LC_{99} dose of venom. At 15 min intervals for 1 h, and then at 1-h time points up until 6 h, cells were stained with annexin V-FITC and propidium iodide using an annexin V-FITC apoptosis detection kit (BioVision, Mountain View, CA) following the manufacturers instructions. The kit relies on cells undergoing early apoptosis translocating membrane phosphatidylserine (PS) to the cell surface. Annexin V is a protein with high affinity for PS, and thus early apoptotic cells can be detected when FITC is conjugated to annexin V. Propidium iodide (PI) accumulates in the cell nucleus when membrane integrity is lost. FITC excitation was induced at 478–500 nm and emitted light monitored at 535 nm, while excitation of PI was achieved using a rhodamine excitation filter (excitation at 507 nm) with an emission maximum of 560 nm. Cell-derived fluorescent images were visualized using a 40 × objective and a Spot Insight Firewire color digital camera mounted on a phase contrast inverted microscope (Nikon Eclipse TE-300) and connected to Macintosh Power PC G5 (Apple). Images were captured with Spot software (v. 4.5; Sterling Heights, MI).

Statistical analysis

Means were compared using one- and three-way analysis of variance (ANOVA) and subsequently, means were separated using Tukey's Honestly Significant Difference (HSD) *post hoc* test. Percentage data was normalized by arcsine transformation prior to analyses. A statistical software program (SPSS, version 15.0 for Windows) was used for data analysis. Results were considered statistically significant when $P < 0.05$.

Results

Three-way ANOVAS indicated that the ratios of apoptotic, necrotic, and viable hemocytes in host pupae and larvae are dependent on the treatment ($P < 0.05$) and the extent of cell viability ($P < 0.05$), but not time ($P > 0.05$). Envenomation and parasitization – time and apoptotic, necrotic and viable cell ratios–time interactions were not significant ($P > 0.05$) for cell viability of pupae and larvae, indicating that variations as a result of venom doses, parasitization, and controls and viability indices were

consistent among time points. However, the extent of cell viability was significantly influenced by treatments ($P < 0.05$) in pupae and larvae (table 1).

Effects of parasitization and envenomation on apoptotic and necrotic indices (%) in *Galleria mellonella* pupae

To determine whether experimental envenomation and parasitism of *P. turionellae* alter the ratio of apoptotic and necrotic hemocytes of *G. mellonella*, pupae were bled following parasitism or injections at different time intervals post-treatments. Acridine orange and ethidium bromide double staining of hemocytes indicated that early apoptosis was present in 1.80%, 2.53%, and 2.67% of hemocytes at 4, 8, and 24 h post-treatments, respectively (fig. 2). The effect of null- and PBS-injection was more similar to untreated controls than that of parasitized or envenomated experimental groups. Injection of 0.005, 0.01, and 0.02 VREs did not result in a significant increase in the percentage of early apoptosis with respect to controls at 4, 8, and 24 h. More than 10% of hemocytes were in early apoptosis in treatments with 0.05 VRE at 4 h ($F = 6.872$; d.f = 7, 16; $P = 0.001$) and 8 h ($F = 10.686$; d.f. = 7, 16; $P = 0.000$), and 0.02 VRE at 24 h ($F = 4.246$; d.f. = 7, 16; $P = 0.008$). However, the ratio of late apoptotic hemocytes increased significantly for 0.01 and 0.02 VRE at 24 h ($F = 11.247$; d.f = 7, 16; $P = 0.000$), and for 0.02 VRE at 4 h ($F = 4.355$; d.f. = 7, 16, $P = 0.007$) and 8 h ($F = 8.860$; d.f. = 7, 16; $P = 0.000$) post-treatments when compared to control groups. The percentage of necrosis was highest with 0.05 VRE (4.47%) at 4 h ($F = 3.908$; d.f = 7, 16; $P = 0.011$) and with parasitization at 8 h (4.00%) ($F = 3.857$; d.f = 7, 16; $P = 0.012$) and 24 h (4.53%) ($F = 3.657$; d.f. = 7, 16; $P = 0.015$) post-treatments whereas the rate was always lower than 2% in controls. The highest rate of early and late apoptotic cells was observed when pupae were parasitized. The ratios were 19.13%, 17.20%, and 15.80% for early apoptotic cells and 8.07%, 9.00%, and 7.20% for late apoptotic cells at 4, 8, and 24 h, respectively. At all doses and time points, the percentage of early and late apoptotic cells was higher than that of necrotic cells upon parasitization and experimental envenomation. Cell viability was observed in 95.67%, 94.53%, and 95.13% of hemocytes at 4, 8, and 24 h, respectively in untreated pupae. Dose-dependent reductions in hemocyte viability were observed at 4 h ($F = 13.235$; d.f. = 7, 16; $P = 0.000$), 8 h ($F = 15.721$; d.f. = 7, 16; $P = 0.000$), and 24 h ($F = 14.737$; d.f. = 7, 16; $P = 0.000$) with respect to controls. The lowest viability values were observed at parasitized groups, as 69.87%, 69.80%, and 72.47% of cells were viable at 4, 8, and 24 h, respectively.

Table 1 ANOVAs of the effects of different treatments, time, cell viability and their interactions on the apoptotic and necrotic indices after venom treatment and parasitization by *P. turionellae*

Stage	Source	d.f.	MS	F	P	r^2
Pupa	Treatment	7	0.011	3.832	0.001	0.991
	Time	2	0.000	0.134	0.875	
	Cell viability	3	19.211	6741.480	0.000	
	Treatment × time	14	0.000	0.066	1.000	
	Treatment × cell viability	21	0.090	31.570	0.000	
	Time × cell viability	6	0.002	0.551	0.769	
	Treatment × time × cell viability	42	0.002	0.719	0.897	
	Error	192	0.003			
Larva	Treatment	6	0.008	3.548	0.002	0.995
	Time	2	0.000	0.063	0.939	
	Cell viability	3	25.717	11692.45	0.000	
	Treatment × time	12	0.000	0.100	1.000	
	Treatment × cell viability	18	0.042	19.032	0.000	
	Time × cell viability	6	0.003	1.224	0.297	
	Treatment × time × cell viability	36	0.001	0.591	0.968	
	Error	168	0.002			

Effects of envenomation on apoptotic and necrotic indices (%) in *G*alleria *mellonella* larvae

The apoptotic and necrotic indices of larvae, though less in pupae, also tended to increase in a dose-dependent manner upon injection of venom at all time points. Early apoptosis was observed in 0.47%, 0.67%, and 1.00% of hemocytes at 4, 8, and 24 h, respectively in untreated control larvae (fig. 3). The effect of null-injection, PBS-injection, 0.02 and 0.05 VRE injections on early apoptotic hemocytes were similar to that of untreated controls. However, the indices varied significantly at 4 h ($F = 4.252$; d.f = 6, 14; $P = 0.012$) and 8 h ($F = 14.048$; d.f. = 6, 14; $P = 0.000$), and 24 h ($F = 4.252$; d.f. = 6, 14; $P = 0.012$). Almost 5% of hemocytes were in early apoptotic phase upon injection of 0.5 VRE at all time points. The ratio of late apoptotic cells was significantly higher in larvae envenomated by 0.1 and 0.5 VREs at 8 h ($F = 8.322$; d.f. = 6, 14; $P = 0.001$) and 24 h ($F = 8.649$; d.f. = 6, 14; $P = 0.000$) post-treatments but the increase was not significant at the end of 4 h ($F = 2.391$; d.f. = 6, 14; $P = 0.084$) at all venom doses when compared to controls. In contrast, the ratios of necrotic hemocytes from

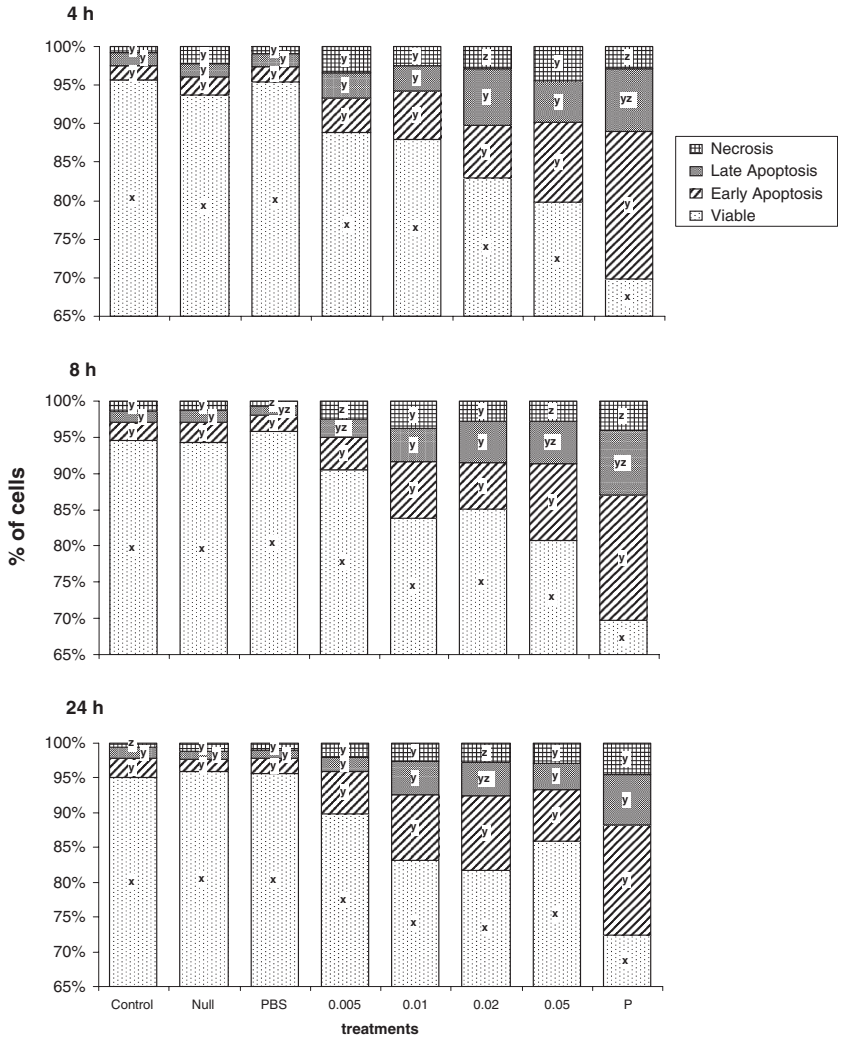

Fig. 2 *Pimpla turionellae* parasitism and venom-induced apoptosis, necrosis and viability of *G. mellonella* pupal hemocytes. Three host pupae were evaluated for each experimental and control assays at a given time and 500 cells from an individual pupa were counted and differentiated in each of three replicates. Columns followed by the same letter (x–y–z) are not significantly different ($P > 0.05$). P = parasitization.

untreated and venom-treated groups were both in the range of 0.20–2.33%, and statistical analysis from three replicates revealed no significant difference between the ratios of necrotic cells in the larvae (4 h; $F = 2.681$; d.f. = 6, 14; $P = 0.059$, 8 h; $F = 1.489$; d.f = 6, 14; $P = 0.252$, 24 h; $F = 2.033$; d.f. = 6, 14; $P = 0.129$). More than 98% of cells were viable at all time points in untreated larvae. Significant decreases in hemocyte viability was observed at 0.1 VRE at 8 and 24 h and 0.5 VRE at 4 h ($F = 5.397$; d.f = 6, 14; $P = 0.004$), 8 h ($F = 18.240$; d.f. = 6, 14; $P = 0.000$) and 24 h ($F = 8.571$; d.f. = 6, 14; $P = 0.000$) compared to controls and lower venom doses.

Effects of parasitization and envenomation on mitotic indices (%) in *Galleria mellonella* pupae and larvae

To determine whether experimental venom injection and parasitism (only for pupae) of *P. turionellae* alter the ratio of mitotic hemocytes in *G. mellonella*, both pupae and larvae were bled following parasitism or injections at different time intervals post-treatments. The mean frequencies of mitosis in hemocytes of untreated, experimentally envenomated and parasitized *G. mellonella* pupae are given in table 2. The microscopic analyses of slides indicated that mitosis was observed in 1.13%, 1.40%, and 1.80% of hemocytes from untreated pupae at 4, 8, and 24 h, respectively. The effect of null- and PBS-injection was similar to untreated controls than that of parasitized or 0.05 VRE injected groups. It was evident that mitotic indices differed significantly among experimental and control groups at 4 h ($F = 10.755$; d.f. = 7, 16; $P = 0.000$), 8 h ($F = 15.873$; d.f. = 7, 16; $P = 0.000$), and 24 h ($F = 5.633$; d.f. = 7, 16; $P = 0.002$) post-treatments. The percentage of mitotic index decreased in a dose-dependent manner with mitosis completely prevented at the highest

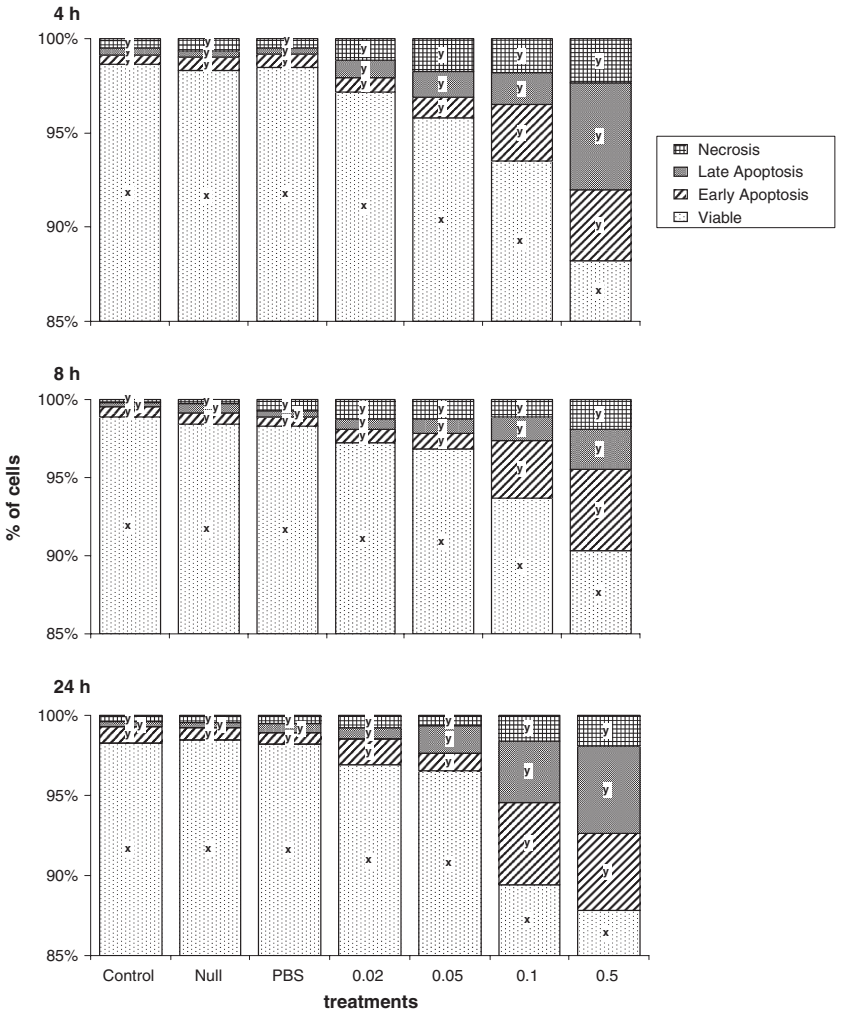

Fig. 3 *Pimpla turionellae* venom-induced apoptosis, necrosis and viability of *G. mellonella* larval hemocytes. Three host larvae were evaluated for each experimental and control assays at a given time and 500 cells from an individual larvae were counted and differentiated in each of three replicates. Columns followed by the same letter (x–y) are not significantly different (P > 0.05).

Table 2 Frequency of mitosis (%) in hemocytes of *G. mellonella* pupae experimentally envenomated and parasitized by *P. turionellae*

	Frequency of mitosis (% ± SE)[1]		
	Time post-treatments[2] (h)		
Treatment	4	8	24
Untreated	1.13 ± 0.30 a x	1.40 ± 0.40 a x	1.80 ± 0.52 a x
Null-injected	1.00 ± 0.72 ab x	0.87 ± 0.30 ab x	1.67 ± 0.80 a x
PBS-injected	1.27 ± 0.61 a x	1.20 ± 0.20 ab x	0.93 ± 0.50 ab x
0.005 VRE-injected	0.27 ± 0.23 bc x	0.47 ± 0.30 abc x	0.33 ± 0.41 ab x
0.01 VRE-injected	0.13 ± 0.23 c x	0.27 c ± 0.30 d x	0.33 ± 0.30 ab x
0.02 VRE-injected	0.07 ± 0.12 c x	0.13 ± 0.12 cd x	0.67 ± 0.61 ab x
0.05 VRE-injected	0.00 ± 0.00c x	0.00 ± 0.00 d x	0.07 ± 0.12 b x
Parasitized	0.00 ± 0.00 c x	0.00 ± 0.00 d x	0.00 ± 0.00 b x

[1]Each represents the mean of three replicates.
[2]Numbers in columns (a–d) and rows (x) followed by the same letter are not significantly different (P > 0.05).

dose (0.5 VRE) of venom and parasitization at all time points. The only exception to this trend were at 24 h with venom doses >0.01 VRE, in which 0.67% and 0.07% of cells were in mitosis at 0.02 and 0.05 VRE, respectively. The effect of venom injection and parasitization on the frequency of mitotic hemocytes

of host pupae was treatment- ($P < 0.05$) but not time- ($P > 0.05$) dependent, and the relationships between treatments and the mitotic indices were not influenced by time ($P > 0.05$) (table 3).

In contrast, the frequency of mitotic hemocytes differed significantly with respect to the type of treatment ($P < 0.05$) and time ($P < 0.05$), but the relationship between treatment and the mitotic indices was not influenced by time ($P > 0.05$) in host larvae (table 3). Untreated larvae normally displayed 1.93%, 2.40%, and 2.12% cells in the mitotic phase at 4, 8, and 24 h post-treatments, respectively (table 4). The percentage of hemocytes in the mitotic phase tended to decrease at 4 h ($F = 7.268$; d.f. = 6, 14; $P = 0.001$), 8 h ($F = 22.981$; d.f. = 6, 14; $P = 0.000$), and 24 h ($F = 4.776$; d.f. = 6, 14; $P = 0.008$) in host larvae envenomated by wasp venom. Mitosis almost halted at the highest dose 0.05 VRE at the end of 8 and 24 h. Significant differences were observed among treatments in the ratio of mitotic hemocytes at 4 and 8 h upon injection of doses >0.05 VRE and at 24 h with 0.5 VRE injection compared to untreated, null- and PBS-injected larvae.

Table 3 ANOVAs of the effects of different treatments, time, and their interactions on the mitotic indices after venom treatment and parasitization by *P. turionellae*

Stage	Source	d.f.	MS	F	P	r^2
Pupa	Treatment	7	0.020	25.201	0.000	0.799
	Time	2	0.002	2.352	0.106	
	Treatment × time	14	0.001	0.665	0.796	
	Error	48	0.001			
Larva	Treatment	6	7.012	15.532	0.000	0.747
	Time	2	2.550	5.650	0.007	
	Treatment × time	12	0.732	1.621	0.122	
	Error	42	0.451			

Apoptosis detection

Hemocytes from *G. mellonella* attached to the surface of the 96-well plates and began to spread during the 1-h incubation period prior to the addition of saline or venom (fig. 4a). Upon addition of saline (PBS) or with untreated cells, the hemocytes remained attached to the 96-well plates and showed little evidence of vacuole formation or blebbing at all time points observed during the 6 h of observations. Staining of these control cells with annexin V-FITC and PI did not reveal any evidence of apoptosis or loss of membrane integrity at 15 min (fig. 4b–c) or 1 h post-treatment (fig. 5b–c) at 27°C. In fact, longer incubation with saline up to 6 h did not result in annexin V binding to the cell surfaces or accumulation of PI in nuclei (data not shown). By contrast, when venom was added to wells containing hemocytes, a large number of cells bound annexin V as evidenced by green fluorescent 'halos' along the plasma membranes (fig. 4e). By 1 h post-venom treatment, nearly all hemocytes appeared labelled with the annexin V-FITC probe (fig. 5e). Consistent with this staining pattern, venom-treated hemocytes also emitted red fluorescence in the majority of cells by 15 min (fig. 4f), with further increases in PI fluorescence detected 45 min later (fig. 5f). Additional incubation with venom up to 6 h did not result in further increases in the fluorescent signals of annexin V-FITC or PI, most likely because the hemocytes were either in late stage apoptosis or dead.

Discussion

Endoparasitoid species that develop inside the hemocoel of their hosts must avoid cell-mediated immune

Table 4 Frequency of mitosis (%) in hemocytes of *G. mellonella* larvae experimentally envenomated by *P. turionellae*

	Frequency of mitosis (% ± SE)[1]		
	Time post-treatments[2] (h)		
Treatment	4	8	24
Untreated	1.93 ± 0.61 a x	2.40 ± 0.60 a x	2.13 ± 0.70 a x
Null-injected	1.00 ± 0.91 abc x	1.87 ± 0.50 a x	2.07 ± 0.12 a x
PBS-injected	1.67 ± 0.50 a x	3.60 ± 1.71 a x	1.53 ± 0.94a x
0.02 VRE-injected	1.40 ± 0.60 ab x	1.93 ± 0.12 a x	1.27 ± 1.13 ab x
0.05 VRE-injected	0.33 ± 0.31 bc x	1.60 ± 0.60 a x	1.33 ± 0.64 a x
0.1 VRE-injected	0.20 ± 0.20 c x	0.07 ± 0.12 b x	0.40 ± 0.69 ab x
0.5 VRE-injected	0.07 ± 0.12 c x	0.00 ± 0.00 b x	0.00 ± 0.00 b x

[1]Each represents the mean of three replicates.
[2]Numbers in columns (a–d) and rows (x) followed by the same letter are not significantly different ($P > 0.05$).

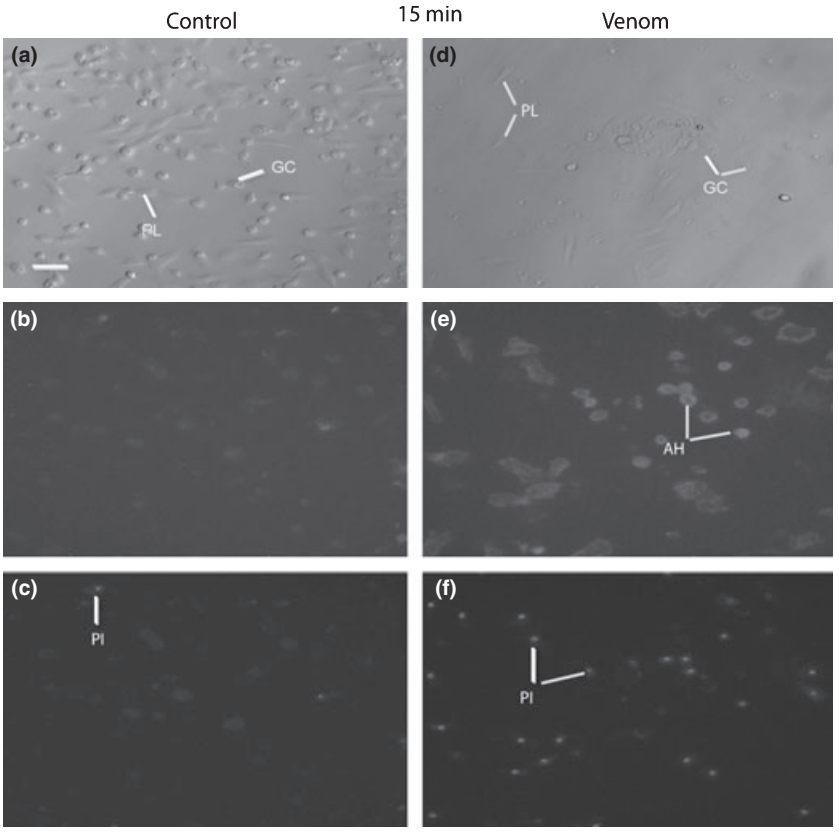

Fig. 4 Fluorescence microscopy of hemocytes collected from *G. mellonella* incubated with crude venom from *P. turionellae* and double stained with an annexin-V sensitive probe (conjugated to FITC) and propidium iodide. Qualitative labeling of annexin V in the plasma membrane (b and e) or cellular uptake of propidium iodide (c and f) were monitored 15 min after exposure to wasp venom. Cells exposed to PBS served as controls (a–c) and a 0.25 VRE dose of *P. turionellae* venom was used for toxicity assays (d–f). PL = plasmatocyte, GC = granular cell, AH = annexin halo, PI = propidium iodide. The bar corresponds to 18 μm.

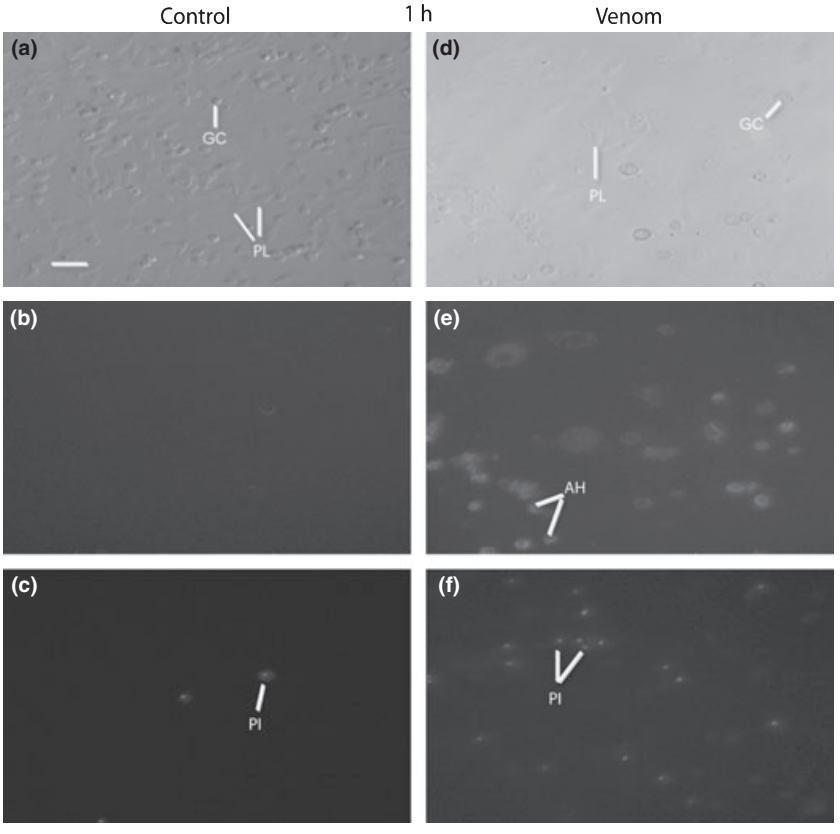

Fig. 5 Fluorescence microscopy of hemocytes collected from *G. mellonella* incubated with crude venom from *P. turionellae* and double stained with an annexin-V sensitive probe (conjugated to FITC) and propidium iodide. Qualitative labeling of annexin V in the plasma membrane (b and e) or cellular uptake of propidium iodide (c and f) were monitored 1 h after exposure to wasp venom. Cells exposed to PBS served as controls (a–c) and a 0.25 VRE dose of *P. turionellae* venom was used for toxicity assays (d–f). PL = plasmatocyte, GC = granular cell, AH = annexin halo, PI = propidium iodide. The bar corresponds to 15 μm.

responses and many species achieve this by suppression of host immunity with maternally derived factors (virulence factors and/or venom) that are injected by adult females at the time of oviposition. Whether the parasitoids produce other immune suppressive factors such as PDV or not, venom may complement or replace the functions of other maternal factors (Wu et al. 2008). *P. turionellae* is one such endoparasitoid that apparently depends only on maternal venom. Venom- mediated paralytic activity, toxicity in multiple life stages of the host, and cytotoxicity to cultured cells from two orders of hosts of *P. turionellae* venom have been demonstrated previously (Ergin et al. 2006). Here, we aimed to determine if *P. turionellae* parasitization or venom is capable of killing insect hemocytes by programmed cell death or apoptosis, and the effects of venom on mitosis events in circulating hemocytes.

Acridine orange/ethidium bromide double staining indicated that parasitism and experimental envenomation of *G. mellonella* by *P. turionellae* resulted in markedly different effects on the ratio of apoptotic hemocytes circulating in hemolymph depending on the host developmental stages. The ratio of early and late apoptotic hemocytes increased more than 100% compared to untreated, null- and PBS injected controls for host pupae and larvae at higher doses of venom and after parasitization for pupae. In contrast, significant increases in necrotic hemocytes was only observed in parasitized groups at 24 h in *G. mellonella* pupae and no significant difference was observed in larvae. *P. turionellae* venom was more effective in eliciting a decrease on hemocyte viability in pupae compared to larvae. In fact, the higher susceptibility of pupal hemocytes to parasitism and venom injection is consistent with the oviposition preference of adult females, which select pupae over larvae when given a choice (Kansu and Uğur 1984). Apoptosis, triggered by symbiotic viruses of parasitoid wasps has already been reported in previous studies. *M. demolitor* PDV's induces apoptosis in host *P. includens* hemocytes (Strand and Pech 1995). In *Diachasmimorpha longicaudata* Ashmead (Hymenoptera: Braconidae)/*Anastrepha suspensa* Loew (Diptera: Tephritidae) system, the entomopoxvirus of the parasitoid caused hemocyte apoptosis (Lawrence 2005). In host *P. separata* parasitized by *C. kariyai*, the hemocytes increased in number and PDVs induced apoptosis in the circulating hemocytes and hematopoietic organs (Teramoto and Tanaka 2004). Suzuki and Tanaka demonstrated that injection of *M. pulchricornis* virus like particles into *P. separata* induced apoptosis in hemocytes particularly granulocytes and reduced the encapsulation ability of host hemocytes (Suzuki and Tanaka 2006). The authors suggested that induction of apoptosis could be triggered directly or indirectly by the viral gene products expressed in host cells (Suzuki and Tanaka 2006). In *Plutella xylostella* Linnaeus (Lepidoptera: Plutellidae) larvae parasitized by *Diadegma semiclausum* Hellen (Hymenoptera: Ichneumonidae) necrosis of prohemocytes and damage of the hematopoietic organs were observed. However, in contrast to our findings, no apoptotic hemocytes were detected and parasitism by *D. semiclausum* did not influence the host's circulating hemocyte viability (Huang et al. 2009).

Venom-induced apoptosis was also observed *in vitro* using hemocytes from last instar larvae of *G. mellonella*. The phosphatidylserine (PS)-sensitive protein annexin V binds to plasma membranes during early stages of apoptosis, yielding an intense green halo around apoptotic cells. Such fluorescent signals were detected within 15 min in hemocytes exposed to a LC_{99} dose of venom, and by 1 h post-treatment, nearly all cells bound annexin V-FITC. Correspondingly, at these same time points, venom-treated hemocytes displayed increasing red fluorescence in nuclei attributed to accumulation of propidium iodide (PI) as a result of a loss in plasma membrane integrity. Preliminary observations have also revealed that when these experiments are performed in media lacking a source of calcium, PI accumulates in the nucleus but annexin V does not bind to the hemocytes. These findings indicate that venom from *P. turionellae* induces apoptosis in hemocytes by a pathway dependent on extracellular calcium influx. However, all forms of cell death induced by this venom do not have this same requirement (Rivers et al. 2007). More work is needed to clarify the importance of extracellular calcium and venom-elicited apoptosis.

In parasitoid species that are devoid of PDVs or other symbiotic viruses such as *P. hypochondriaca* (Parkinson and Weaver 1999; Richards and Parkinson 2000), *P. puparum* (Cai et al. 2004; Wu et al. 2008), *P. turionellae* (Ergin et al. 2006) and *N. vitripennis* (Rivers et al. 2002), venoms alone perturb host immune defences. In the system *P. puparum*/*Pieris rapae* Linnaeus (Lepidoptera: Pieridae) venom alone was shown to prevent spreading and encapsulation of hemocytes however staining of filamentous actin showed that the cytoskeleton of host hemocytes was not visibly affected by venom treatment (Cai et al. 2004). It was demonstrated that venom from the ectoparasitic wasp *N. vitripennis* causes the host

hemocytes to die by oncosis (Rivers et al. 2002). The observed cytotoxic effects triggering apoptosis or oncosis could be attributed to the components such as metalloproteinases, peptidases, serine protease, and calreticulin which were recently identified in wasp venom (De Graaf et al. 2010). Venom from *P. hypocondriaca* that lacks PDV and VLP kills the *L. oleracea* hemocytes by apoptosis in a dose–responsive manner (Richards and Dani 2007). Our results indicate that hemocyte viability declines with increasing concentrations of venom and these findings complement other studies involving *P. hypocondriaca* venom.

Maintenance of circulating hemocytes in Lepidoptera has been attributed to mitosis in hemocytes in circulation, as well as to the release of newly differentiated hemocytes from hematopoietic organs (Gardiner and Strand 2000; Huang et al. 2009). Our results in untreated hosts confirm those of others reporting that normally 1–8% of the population of circulating hemocytes are in the mitotic phase in *G. mellonella*, *B. mori*, and *E. declarata* (Shapiro 1968; Jones 1970; Arnold and Hinks 1976; Beaulaton 1979). However, both parasitized or envenomated *G. mellonella* pupae and at higher doses (>0.05) in larvae the ratio of mitotic hemocytes decreased significantly below 0.5% compared to control groups at all time points (tables 2 and 4). Though there are few studies on the effect of endoparasitoid venom or parasitization on mitosis of host hemocytes, it was revealed that mitosis of circulating hemocytes halted after the injection of *C. kariyai* PDV plus venom into *P. separata* (Teramoto and Tanaka 2004). The authors demonstrated that the PDV plus venom caused the disappearance of the 4C and 8C ploidies, and that PDV alone produced the humoral plasma factors that suppress the cell cycle (Teramoto and Tanaka 2004). Similarly, we suggest that *P. turionellae* parasitization and venom affect hemocyte viability and induce cells to die by apoptosis and decrease the frequency of mitotic hemocytes for their progeny to develop inside the hemocoel of their host *G. mellonella*.

Acknowledgements

We are grateful to Dr Ekrem Ergin for his contribution to the article. We thank Dr Stefan Vidal and two anonymous reviewers for valuable comments on this manuscript. This research was in part supported by grants (2006-106T255) from The Scientific and Technological Research Council of Turkey (TÜBİTAK) and (2007/49) BAU Research Foundation.

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