Levels of encapsulation and melanization in *Galleria mellonella* (Lepidoptera: Pyralidae) parasitized and envenomated by *Pimpla turionellae* (Hymenoptera: Ichneumonidae)

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Abstract

The endoparasitic wasp *Pimpla turionellae* L. (Hymenoptera: Ichneumonidae) injects its pupal host with venom during oviposition. Venom from *P. turionellae* has previously been shown to contain a mixture of biologically active components, which display potent paralytic, cytotoxic and cytolytic effects towards lepidopteran and dipteran hosts. This study was undertaken to investigate if parasitism and/or envenomation by *P. turionellae* affects the encapsulation and melanization responses of its host *Galleria mellonella* L. (Lepidoptera: Pyralidae) in larval and pupal stages. Analysis of the effects of venom on encapsulation and melanization of the Sephadex A-25 beads revealed that the number of beads strongly encapsulated and melanized were reduced by more than 50% at 4 and 24 h post-venom injection into pupae. Injection of a lethal dose of venom (0.5 venom reservoir equivalent) in the last instar larvae was sufficient to reduce the ability of haemocytes to encapsulate the beads by more than 50% at 4 h post-injection. Similar results were also obtained when beads were recovered from parasitized pupae indicating that parasitization by *P. turionellae* suppressed haemocyte-mediated encapsulation in *G. mellonella*. We found that the cellular defence reactions occur more rapidly in larvae compared with pupae of *G. mellonella*, indicating the higher susceptibility of pupal haemocytes to parasitism and venom injection.

Introduction

Insects are known to possess an innate immune system capable of recognizing non-self like parasitic wasp eggs and larvae. Thus, successful parasitism by parasitic wasps requires evasion or circumvention of this immune system. The primary immune response towards internal parasites and other foreign entities that enter the insect's haemocoel is encapsulation (Lackie 1988; Strand and Pech 1995a; Gillespie et al. 1997; Pech and Strand 2000). Encapsulation has been studied in detail in different orders, and in most Lepidoptera granulocytes and plasmatocytes are the key haemocytes involved in encapsulation (Schmit and Ratcliffe 1978; Strand and Pech 1995a). The sequence of how different haemocyte types are engaged in encapsulation, including recognition, opsonization, recruitment of cells and formation of a multilayer sheath has also been described (Ratcliffe 1993; Lavine and Strand 2002, 2003; Nardi et al. 2003). Encapsulation begins when host granulocytes attach to the surface of a foreign target. The attached granulocytes lyse or degranulate, releasing the contents of their granules over the foreign object. This is assumed to attract and allow the plasmatocytes to attach. Termination of capsule formation occurs when a subpopulation of granulocytes adheres in a monolayer around the periphery of the capsule

(Schmit and Ratcliffe 1978; Strand and Pech 1995a; Pech and Strand 1996; Schmidt et al. 2001; Luo and Pang 2006). The process is ultimately accompanied by blackening of the capsule because of melanization and finally the encapsulated organism almost always dies (Schmidt et al. 2001; Lavine and Strand 2002). Several factors, including asphyxiation, the local production of cytotoxic quinones or semiquinones via the proPO activation cascade during melanization, free radicals and antibacterial peptides have been suggested to function as killing agents (Nappi et al. 1995, 2000; Gillespie et al. 1997; Lavine and Strand 2002; Jiravanichpaisal et al. 2006).

Recent observations suggest that different wasp species employ different molecular strategies in overcoming the defence system of the host insect (Lu et al. 2006). In permissive hosts, encapsulation response is circumvented by the maternally derived secretions [polydnaviruses (PDVs), virus-like particles (VLPs) and ovarian fluids] injected by adult female parasitoids around the time of oviposition, which suppress, modify or regulate the host condition (Kitano 1986; Luckhart and Webb 1996; Beckage 1998; Shelby and Webb 1999; Ibrahim and Kim 2006; Suzuki and Tanaka 2006). The role of endoparasitoid venom in suppressing host immune defence has not been clearly determined. In many braconid species, venom may contribute to the inhibition of encapsulation by enhancing the effects of PDV or calyx fluid (Kitano 1986; Tanaka 1987a,b; Wago and Tanaka 1989). However, a limited number of studies suggest that venom from endoparasitoid species devoid of symbiotic viruses may alone perturb host immune defences. For example, venom from *Pimpla hypochondriaca* (Retzius) (Hymenoptera: Ichneumonidae) and *Pteromalus puparum* L. (Hymenoptera: Pteromalidae) suppress encapsulation response in their respective hosts, *Lacanobia oleracea* (L.) (Lepidoptera: Noctuidae) and *Pieris rapae* (L.) (Lepidoptea: Pieridae) (Richards and Parkinson 2000; Cai et al. 2004). Nevertheless, most studies have focused on egg-larval endoparasitoids and little is known about the effects of pupal endoparasitoid venom on haemocytic encapsulation responses (Parkinson et al. 2002; Cai et al. 2004).

The solitary idiobiont pupal endoparasitoid *P. turionellae* L. (Hymenoptera: Ichneumonidae) lacks PDVs and VLPs so that the wasp venom is likely to play a major role in host regulation. The biochemical properties of venom from this wasp were previously investigated (Uçkan et al. 2004, 2006; Ergin et al. 2007). Additionally, *P. turionellae* venom displays potent paralytic, cytotoxic and cytolytic effects towards lepidopteran and dipteran hosts (Ergin et al. 2006; Er et al. 2009). The details of how this wasp venom operates to induce host paralysis and evoke cell death have also been partially determined in previous studies (Keenan et al. 2007; Rivers et al. 2007). Here, we further aimed at determining whether *P. turionellae* parasitism and/or envenomation affect the rate of encapsulation and melanization response of its lepidopteran host *Galleria mellonella* L. (Lepidoptera: Pyralidae).

Materials and Methods

Parasitoid and host rearing

Laboratory colonies of the host species, *G. mellonella*, were established from individuals that were collected from the honeycombs maintained by beekeepers around Balıkesir, Turkey. *Pimpla turionellae* were reared on pupae of the host, *G. mellonella* at $25 \pm 1°C$, $60 \pm 5\%$ RH, and with a photoperiod of 12 : 12 h, L : D. Adult parasitoids were fed a 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 *P. turionellae* venom and injection into *G. 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 the LD_{99} 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 phosphate buffered saline (PBS) (0.138 M NaCl and 0.0027 M KCl in 0.01 M PBS, pH 7.4). Last instar larvae 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 micro syringe (Hamilton, Reno, NV). Petroleum jelly was applied to the injection area to prevent haemolymph loss (Richards and Edwards 1999). These larvae and

pupae were referred to as 'experimentally envenomated' in the text. Controls consisted of untreated pupae and larvae, null-injected (empty injection) and injected with only 5 μl PBS.

Parasitization of *G. mellonella* pupae

Parasitization was performed on day 1 or 2 of the host pupae by exposing an individual host pupa (140 $\pm$ 20 mg) to an individual 15 to 20-day-old wasp female. Parasitized pupae were held at 25 $\pm$ 2°C, 60 $\pm$ 5% RH under a photoperiod of 12 : 12 h, L : D as were the controls and venom-treated pupae. *Pimpla turionellae* females normally parasitize host pre-pupae and pupae in nature (Kansu and Uğur 1984), therefore parasitization was not used as an experimental assay for host larvae.

Injection of chromatography beads

Upon injection into the haemocoel of the last instar larvae and 1- to 2-day-old pupae of *G. mellonella*, the positively charged DEAE Sephadex A-25 beads (40–120 μm in diameter; Sigma Chemical Co., St Louis, MO, USA) evoked a strong encapsulation response. Therefore, these beads were used in all subsequent experiments. To facilitate identification of the beads *in vivo* they were stained with 0.1% Coomassie blue in PBS for 1 h. Following staining, the supernatant was removed and the coloured beads were washed three times in fresh PBS (Richards and Parkinson 2000). The beads were finally resuspended in sterile PBS at a concentration of approximately 15–20 beads/10 μl. For *in vivo* encapsulation assays, insects were injected with venom doses below the LD$_{99}$ calculated for *G. mellonella* pupae and larvae (Ergin et al. 2006) as mentioned above. One hour after venom injection and parasitization, larvae and pupae were chilled on ice, and then 10 μl of PBS was injected into the control insects and 10 μl of PBS containing 15–20 Sephadex A-25 beads was injected into the venom treated and parasitized larvae and pupae by using a 50 μl Hamilton microsyringe with 22-gauge needle (Hamilton). All insects were then maintained at room temperature for 4 and 24 h. Each set of experiments was replicated three times with different and freshly prepared venom solutions and each replicate contained five larvae and pupae.

Encapsulation and melanization assay

At 4 and 24 h post-treatments with Sephadex A-25 bead injection, insects were dissected under a stereomicroscope. All beads that could be found were removed, added to a drop of PBS on a microscopy slide, and overlaid with a cover slip. Beads were then observed by phase contrast microscope (Olympus BX 51; Olympus Corp., Tokyo, Japan) and scored for encapsulation and melanization. The encapsulation response was defined as negative (no, or only a few haemocytes attached to beads), weak (2–10 layers of haemocytes around beads) and strong (more than 10 layers of haemocytes around beads) (fig. 1) (Richards and Dani 2008). At the same time, the extent of the melanization of each capsule was determined by observing the blackening of the capsule and the beads were defined as melanized if the melanized area covering more than the quarter of the capsule.

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. Student's *t*-test was also used to determine significant difference between two time points in melanization responses of pupae and larvae. A statistical software program (SPSS, version 15.0 for Windows; SPSS Science, Chicago, IL) was used for data analysis. Results were considered statistically significant when P < 0.05.

Results

In the haemolymph of *G. mellonella* pupae and larvae, Sephadex A-25 beads elicited a strong encapsulation reaction and the thicknesses of the capsules varied at 4 and 24 h after bead injection. The encapsulating material was less compacted in the pupae than that encapsulating beads implanted into the larval stage. Three-way ANOVAs indicated that the encapsulation rates of host pupae (F = 432.587; d.f. = 2; P = 0.000) and larvae (F = 228.058; d.f. = 2; P = 0.000) were dependent on the extent of encapsulation, but not treatments (pupae; F = 0.175; d.f. = 7; P = 0.990, larvae; F = 0.270; d.f. = 6; P = 0.951) and time (pupae; F = 1.999; d.f. = 1; P = 0.158, larvae; F = 0.590; d.f. = 1; P = 0.443). Envenomation and parasitization–time interactions (pupae; F = 0.181; d.f. = 7; P = 0.999, larvae; F = 0.107; d.f. = 6; P = 0.996) were not significant for encapsulation response of pupae and larvae, indicating that variations as a result of venom doses, parasitization and controls were consistent between two time
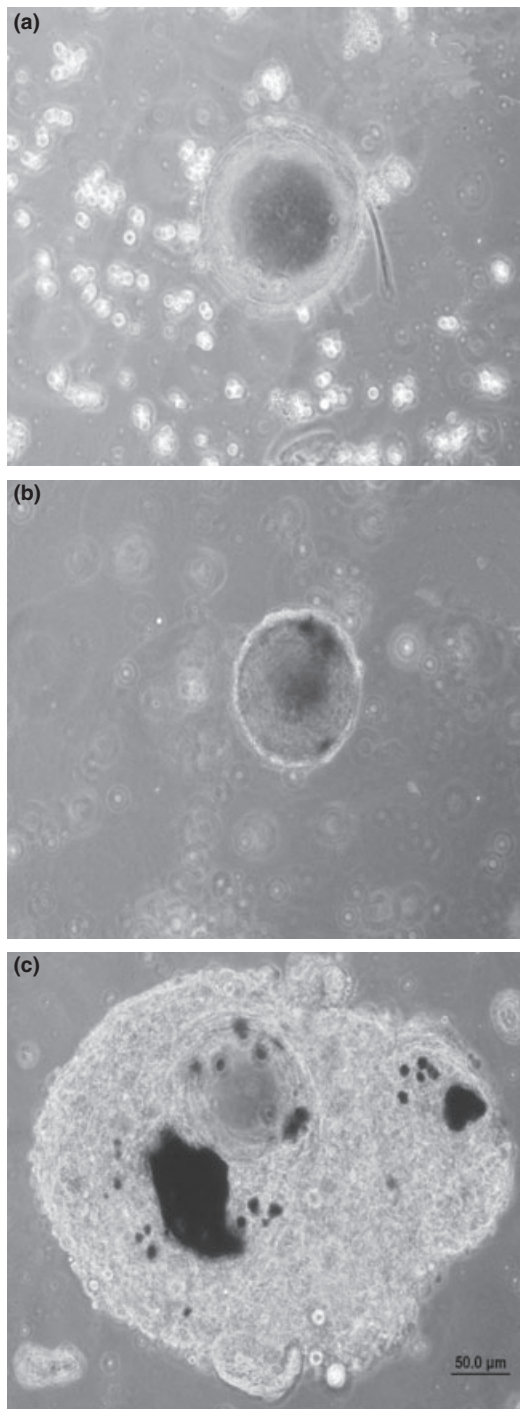
Fig. 1 Encapsulation of Sephadex A-25 beads in *Galleria mellonella* larvae. (a) Negative (no or only a few beads attached to the bead), (b) weak (2–10 layers of hemocytes around the bead), (c) strong (more than 10 layers of hemocytes around the bead).

points. However, the extent of encapsulation was significantly influenced by time (pupae; F = 97.230; d.f. = 2; P = 0.000, larvae; F = 61.348; d.f. = 2; P =

0.000) and treatments (pupae; F = 23.499; d.f. = 14; P = 0.000, larvae; F = 52.695; d.f. = 12; P = 0.000) (table 1).

Effects of parasitization and envenomation on *in vivo* encapsulation responses in *G. mellonella* pupae

In untreated pupae, 10.2% and 48.6% of the injected beads were strongly encapsulated after 4 and 24 h, respectively (table 2). The percentage of strong encapsulation indicated a considerable decline (F = 15.498; d.f. = 7, 112; P = 0.000) in *G. mellonella* pupae exposed to *P. turionellae* parasitization and venom injection (0.05 VRE) at 24 h after bead implantation. Of the 155 beads dissected from 15 parasitized pupae after 24 h, only nine were strongly encapsulated while 76 were weakly encapsulated, and 70 were completely devoid of encapsulating material. At 4 and 24 h after venom injections, there were gradual increases in the percentage of none encapsulated beads with increasing venom doses. The percentage of none encapsulated beads increased 2–6 times at any doses compared with untreated pupae. By 24 h after injection, 11.9% of the beads were strongly encapsulated in pupae injected a venom dose of 0.05 VRE whereas 48.6% of beads were encapsulated in untreated individuals. The effect of null-injection and PBS-injection was more similar to untreated ones than that of parasitized and venom-treated groups.

Effects of envenomation on *in vivo* encapsulation responses in *G. mellonella* larvae

After introduction of the beads into the haemocoel of untreated *G. mellonella* larvae, 46.3% and 84.9% of the beads recovered were surrounded by multiple layers (10 or more) of concentrically arranged haemocytes at 4 and 24 h, respectively (table 3). When Sephadex A-25 beads were injected into the haemocoel of null- and PBS-injected host larvae and dissected out at 4 and 24 h later, there was no difference on the encapsulation response compared with untreated larvae (Tukey's HSD *post hoc* tests). Similar results were obtained using experimentally envenomated larvae (0.02 and 0.05 VRE injections). By contrast, the percentage of strong encapsulation indicated a considerable decline at higher doses of venom (0.1 and 0.5 VRE) injections after 4 (F = 24.149; d.f. = 6, 98; P = 0.000) and 24 h (F = 42.304; d.f. = 6, 98; P = 0.000). (table 3). Almost a quarter and half of the beads were not encapsulated at 24 h post-injections at 0.1 and 0.5 VREs injections

Table 1 ANOVAS of the effects of different treatments, time, encapsulation levels and their interactions on the encapsulation response by *Galleria mellonella*

Stage	Source	d.f.	MS	F	P	r^2
Pupa	Treatment	7	0.008	0.175	0.990	0.69
	Time	1	0.092	1.999	0.158	
	Extent of encapsulation	2	19.861	432.587	0.000	
	Treatment × time	7	0.04	0.081	0.999	
	Treatment × extent of encapsulation	14	1.079	23.499	0.000	
	Time × extent of encapsulation	2	4.464	97.230	0.000	
	Treatment × time × extent of encapsulation	14	0.226	4.923	0.000	
	Error	672	0.046			
Larva	Treatment	6	0.016	0.270	0.951	0.69
	Time	1	0.035	0.590	0.443	
	Extent of encapsulation	2	13.349	228.058	0.000	
	Treatment × time	6	0.006	0.107	0.996	
	Treatment × extent of encapsulation	12	3.084	52.695	0.000	
	Time × extent of encapsulation	2	3.591	61.348	0.000	
	Treatment × time × extent of encapsulation	12	0.423	7.228	0.000	
	Error	588	0.059			

of venom, respectively. Meanwhile, 50.4% and 47.0% of the beads recovered were weakly encapsulated (surrounded by 2–10 layers of haemocytes).

Effects of parasitization and envenomation on melanization of capsules in *G. mellonella* pupae

Untreated host pupae were able to melanize more than 50% of the beads within 24 h after injection and there was a significant difference (t = 8.446; d.f. = 28; P = 0.000) in the melanization response when compared with that observed at 4 h (table 4). The rate of melanization of beads was significantly affected by the time-passed post-treatments at all experimental and control groups except for injection of 0.05 VRE venom (t = 1.868; d.f. = 28; P = 0.072) and parasitized (t = 1.402; d.f. = 28; P = 0.172) ones. In *G. mellonella* pupae, the percentage of melanized capsules differed significantly at 4 (F = 4.07; d.f. = 7, 112; P = 0.001) and 24 h (F = 23.07; d.f. = 7, 112; P = 0.000) post-treatments. More than 10% and 40% of beads were melanized in control groups whereas the ratio further decreased to 3.9% and 10.7% in parasitized pupae at 4 and 24 h, respectively. The ratio of melanized capsules was

Table 2 Encapsulation of Sephadex DEAE A-25 beads in *Galleria mellonella* pupae experimentally envenomated and parasitized by *Pimpla turionellae*

Treatment[#]	Total beads assessed		Extent of encapsulation (% ± SE and ≠ of beads encapsulated)*					
			None		Weak		Strong	
	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h
Untreated	140	167	6.2 ± 1.6 a 9	5.7 ± 1.7 a 10	83.6 ± 3.4 a 118	45.7 ± 5.1 a 77	10.2 ± 3.8 ab 13	48.6 ± 5.3 a 80
Null	168	155	12.9 ± 2.5 abc 22	7.9 ± 2.0 ab 12	66.7 ± 3.3 bcd 114	46.5 ± 3.4 a 71	20.4 ± 4.0 b 32	45.6 ± 4.3 a 72
PBS	156	161	8.3 ± 2.4 ab 13	6.4 ± 1.5 ab 11	79.4 ± 3.9 ab 123	52.0 ± 5.0 a 82	12.3 ± 3.9 ab 20	41.6 ± 5.2 ab 68
0.005	150	159	20.4 ± 2.8 bcd 31	10.9 ± 2.6 ab 17	73.5 ± 2.8 abc 111	56.7 ± 2.7 a 90	6.1 ± 2.6 a 8	32.4 ± 3.4 ab 52
0.01	143	165	27.4 ± 5.5 cd 37	17.3 ± 3.4 b 28	62.8 ± 5.0 cd 93	58.6 ± 3.3 a 97	9.8 ± 3.2 ab 13	24.1 ± 3.5 b 40
0.02	166	164	33.2 ± 3.8 de 57	17.8 ± 2.2 b 28	60.3 ± 3.2 cd 99	51.2 ± 5.0 a 84	6.5 ± 2.3 ab 10	31.0 ± 5.5 ab 52
0.05	165	152	33.7 ± 3.7 de 56	39.4 ± 4.7 c 61	61.8 ± 4.1 cd 102	48.7 ± 5.5 a 76	4.5 ± 1.8 ab 7	11.9 ± 4.2 c 15
Parasitized	161	155	47.1 ± 6.1 e 77	45.3 ± 4.8 c 70	49.8 ± 5.6 d 79	49.3 ± 4.0 a 76	3.1 ± 2.2 a 5	5.4 ± 2.3 c 9

[#]Pupae were untreated, null-injected, injected with 5 µl PBS or different concentrations of venom, or parasitized by *P. turionellae*, then 1 h later injected with 10 µl PBS containing 15–20 beads. Beads were dissected out and assessed at 4 and 24 h post-treatments.
*Numbers in columns (a–e) followed by the same letter are not significantly different (P > 0.05).

Table 3 Encapsulation of Sephadex DEAE A-25 beads in *Galleria mellonella* larvae experimentally envenomated by *Pimpla turionellae*

Treatment[#]	Total beads assessed		Extent of encapsulation (% ± SE and ≠ of beads encapsulated)*					
			None		Weak		Strong	
	4 h	24h	4 h	24 h	4 h	24 h	4 h	24 h
Untreated	160	167	7.9 ± 2.2 a 13	4.0 ± 1.6 a 7	45.8 ± 4.4 a 74	11.1 ± 2.7 a 18	46.3 ± 4.3 b 73	84.9 ± 3.7 a 142
Null	167	174	7.1 ± 1.8 a 12	4.8 ± 1.6 a 8	34.9 ± 3.9 ab 57	14.5 ± 3.0 a 26	58.0 ± 4.1 ab 98	80.7 ± 4.2 a 140
PBS	164	162	7.4 ± 1.9 a 12	2.2 ± 1.2 a 3	37.8 ± 7.1 ab 65	14.6 ± 3.7 a 24	54.8 ± 6.9 b 87	83.2 ± 3.7 a 135
0.02	165	160	7.1 ± 2.9 a 12	4.0 ± 1.4 a 7	20.3 ± 4.0 b 34	18.5 ± 4.9 a 29	72.6 ± 5.6 a 119	77.5 ± 4.9 a 124
0.05	165	158	17.1 ± 3.8 ab 28	4.5 ± 2.0 a 7	46.7 ± 5.7 a 76	11.4 ± 3.4 a 19	36.2 ± 5.2 bc 61	84.1 ± 3.7 a 132
0.1	156	170	30.6 ± 5.8 b 44	23.0 ± 4.0 b 38	45.9 ± 5.4 a 72	50.4 ± 5.2 b 87	23.5 ± 4.2 c 40	26.6 ± 3.7 b 45
0.5	141	157	51.1 ± 6.9 c 72	45.6 ± 5.5 c 71	43.9 ± 6.2) a 61	47.0 ± 5.0 b 74	5.0 ± 2.0 d 8	7.4 ± 2.7 c 12

[#]Larvae were untreated, null-injected, injected with 5 μl PBS or different concentrations of venom, then 1 h later injected with 10 μl PBS containing 15–20 beads. Beads were dissected out and assessed at 4 and 24 h post-treatments.
*Numbers in columns (a–d) followed by the same letter are not significantly different (P > 0.05).

Table 4 Melanization of Sephadex DEAE A-25 beads in *Galleria mellonella* pupae experimentally envenomated and parasitized by *P. turionellae*

Treatment[#]	Melanized (%)*	
	Time after treatment	
	4 h	24 h
Untreated	11.3 ± 2.5 ab x	55.0 ± 3.7 a y
Null-injected	22.2 ± 3.6 b x	53.0 ± 3.1 a y
PBS-injected	10.5 ± 2.2 ab x	43.2 ± 4.4 ab y
0.005 VRE injected	9.8 ± 2.8 ab x	33.1 ± 3.4 b y
0.01 VRE injected	7.6 ± 2.2 a x	27.6 ± 3.0 b y
0.02 VRE injected	9.5 ± 2.4 ab x	35.4 ± 4.5 ab y
0.05 VRE inected	3.9 ± 1.3 a x	11.1 ± 2.6 c x
Parasitized	3.9 ± 1.6 a x	10.7 ± 3.4 c x

[#]Each represents the mean of melanized beads from 15 pupae.
*Numbers in columns (a–c) and rows (x–y) followed by the same letter are not significantly different (P > 0.05).

Table 5 Melanization of Sephadex DEAE A-25 beads in *Galleria mellonella* larvae experimentally envenomated by *P. turionellae*

Treatment[#]	Melanized (%)*	
	Time after treatment	
	4 h	24 h
Untreated	44.6 ± 4.1 ab x	82.3 ± 3.1 a y
Null-injected	42.5 ± 3.2 ab x	72.6 ± 3.9 a y
PBS-injected	44.5 ± 5.6 ab x	82.4 ± 3.5 a y
0.02 VRE injected	48.0 ± 4.9 a x	74.8 ± 3.6 a y
0.05 VRE injected	36.6 ± 2.4 ab x	79.8 ± 2.3 a y
0.1 VRE injected	26.9 ± 3.4 bc x	40.4 ± 3.5 b y
0.5 VRE injected	13.8 ± 3.1 c x	20.9 ± 3.4 c x

[#]Each represents the mean of melanized beads from 15 larvae.
*Numbers in columns (a–c) and rows (x–y) followed by the same letter are not significantly different (P > 0.05).

significantly lower (Tukey's HSD *post hoc* test) in pupae injected with lower doses of venom (0.005 and 0.01 VREs) at 24 h post-injections compared with untreated and null-injected groups (table 4). A significant variation (Tukey's HSD *post hoc* test) in the ratio of melanized beads also appeared at higher dose of venom injection (0.05 VRE) and parasitized groups at 24 h compared with control groups and lower doses of venom.

Effects of envenomation on melanization of capsules in *G. mellonella* larvae

A greater percentage of capsules were melanized at 4 and 24 h post-treatment in untreated *G. mellonella* larvae compared with pupae (table 5). The ratio of the capsules that were melanized differed significantly between 4 and 24 h in all control and experimental groups except for the injection of 0.5 VRE of venom. In larvae, the percentage of melanized

capsules also differed significantly at 4 (F = 10.19; d.f. = 6, 98; P = 0.000) and 24 h (F = 30.13; d.f. = 6, 98; P = 0.000) post-treatments. The ratio of melanized capsules did not indicate a considerable variation among null-, PBS- and lower doses (0.02 and 0.05 VREs) of venom injection at 4 and 24 h post-treatments (Tukey's HSD *post hoc* tests). However, the percentage of melanized beads significantly decreased to 26.9 and 40.4% by injection of a 0.1 VRE of venom at 4 and 24 h, respectively. The lowest ratios of melanization were observed at 4 and 24 h post-treatments as 13.8% and 20.9% upon injection of larvae with the highest dose of venom (0.5 VRE).

Discussion

For many endoparasitoids in families Ichneumonidae and Braconidae, secretions of maternal origin are injected into the host along with the egg(s) during oviposition. These secretions contain endosymbiotic viruses (e.g. polydnavirus, entomopoxvirus) or VLPs that aid in the manipulation or alteration of the host (Luckhart and Webb 1996; Luo and Pang 2006). In hymenopteran parasitoids that are devoid of symbiotic viruses, venom appears to play a major role in host immune suppression and host regulation. Upon locating a suitable host, females of *P. turionellae* always inject venom prior to ovipositing a single egg into the haemocoel of the pyralid hosts (Kansu and Uğur 1984). It was previously shown that venom isolated from adult wasp females of *P. turionellae* displayed paralytic activity and toxicity in multiple life stages of natural hosts, and has been shown to be cytotoxic towards cultured cells from two orders of insects (Ergin et al. 2006) and host haemocytes (Osman 1978). Furthermore, it was suggested that the initial phases of venom intoxication likely involve a change in plasma membrane permeability: susceptible cells retract cytoplasmic extensions, round and eventually swell (Keenan et al. 2007). The present work describes how parasitism and experimental envenomation affect the encapsulation and melanization rates of haemocytes in *G. mellonella* larvae and pupae.

The major immune response used by lepidopteran hosts to defend against endoparasitoids is encapsulation and the efficacy of this response is known to be influenced by a number of parameters, including the number of haemocytes available and their ability to spread (Strand and Pech 1995; Richards and Parkinson 2000). Studies with endoparasitic wasps indicate that in some cases, haemocyte number, morphology

and viability may be affected by VLPs and/or PDV (Beckage 1998). For example, Suzuki and Tanaka (2006) demonstrated that VLPs present in the venom of *Meteoris pulcricornis* (Wesmael) (Hymenoptera: Braconidae) induce host haemocyte apoptosis, and this is associated with a reduction in haemocyte numbers and encapsulation responses. In *Busseola fusca* Fuller (Lepidoptera: Noctuidae) larvae parasitized by *Cotesia sesamiae* Cameron (Hymenoptera: Braconidae) (including PDV), the decrease in the plasmatocyte number is thought to account for a suppressed encapsulation response (Mochiah et al. 2003). However, microscopic analysis of tissues associated with the female reproductive tract of *P. turionellae* has provided no evidence of PDV in this wasp, and thus it appears that some other factor, presumably venom alone, is responsible for the inhibition of the host haemocyte encapsulation *in vivo*. These results are similar to those reported in the systems of *C. glomeratus* Linnaeus (Hymenoptera: Braconidae)/*P. rapae* Linnaeus (Lepidoptera: Pieridae) (Kitano 1986), *P. hypocondriaca/L. oleracea* (Richards and Parkinson 2000; Parkinson et al. 2002), *P. puparum/P. rapae* (Cai et al. 2004) where only venom was shown to prevent encapsulation of host haemocytes. *Pimpla hypocondriaca* venom has been demonstrated to affect the morphology and spreading behaviour of host haemocytes and to suppress encapsulation responses *in vivo* (Richards and Parkinson 2000). It was suggested that venom suppressed the ability of the haemocytes present to encapsulate the beads, possibly by damaging the haemocytes or impairing their spreading behaviour (Richards and Parkinson 2000). Similarly, in *P. puparum/P. rapae* system venom alone could suppress the spreading of plasmatocytes and inhibit the ability of host haemocyte encapsulation *in vitro* (Cai et al. 2004). According to Strand and Pech (1995), the most direct way of preventing encapsulation is to destroy, deplete from circulation, or alter the behaviour of the haemocytes that mediate encapsulation. Hu et al. (2003) reported that parasitism by *Macrocentrus cingulum* Brischke (Hymenoptera: Braconidae) did not cause a significant difference in the percentage of melanized capsules in *Ostrinia furnacalis* Guenee (Lepidoptera: Pyralidae). However, we could not find a report that reveals the role of venom on the melanization of the capsules.

Parasitism and experimental envenomation of *G. mellonella* by *P. turionellae* displayed markedly different effects on the encapsulation response depending on the host developmental stages. The encapsulation of Sephadex beads described in this

work indicates that a strong encapsulation reaction can occur in pupae of *G. mellonella* but the encapsulation material was less compacted compared with larvae. Only after 4 h *in vivo*, 46.3% of the beads recovered from the untreated larvae were surrounded by multiple layers of concentrically arranged haemocytes demonstrating that a strong encapsulation response had occurred. However, in untreated pupae this ratio was only 10.2% (see tables 2 and 3). This trend was also observed between the ratios of beads strongly encapsulated in venom-treated and parasitized host pupae and larvae indicating the higher susceptibility of pupal haemocytes to parasitism and venom injection is consistent with the oviposition preference of adult females, which select pupae over larva when given a choice (Kansu and Uğur 1984).

Analysis of the effects of venom on encapsulation and melanization of the Sephadex A-25 beads revealed that the number of beads strongly encapsulated and melanized were reduced by more than 50% at 4 and 24 h post-injection of venom in pupae (0.05 VRE) and at in larvae (0.5 VRE) (see tables 2–5). Similar results were also obtained when beads were recovered from parasitized pupae indicating that parasitization by *P. turionellae* suppressed haemocyte mediated encapsulation in *G. mellonella*. The rates of encapsulation and melanization observed at 24 h upon injection of a lethal dose of venom (0.05 VRE) and parasitization were identical suggesting that female wasps inject that amount which was also close to the LD_{99} dose of venom (0.06 VRE) calculated for pupae previously (Ergin et al. 2006).

This study indicates that both venom from and parasitization by *P. turionellae* showed potent effects on the encapsulation and melanization responses of haemocytes from *G. mellonella* pupae and larvae, arguing that the wasp's venom has a broader spectrum of activity than parasitoids that target a single host developmental stage. This is also consistent with our previous suggestion (Ergin et al. 2006) that the paralytic activity of venom from *P. turionellae* may be a rapid means to suppress host cellular and/or humoral immune responses to facilitate parasitoid development.

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