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Changes in the hemolymph total protein of *Galleria mellonella* (Lepidoptera: Pyralidae) after parasitism and envenomation by *Pimpla turionellae* (Hymenoptera: Ichneumonidae)

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Abstract: Venom from the endoparasitoid *Pimpla turionellae* L. (Hymenoptera: Ichneumonidae) contains a mixture of biologically active components, which display potent paralytic, cytotoxic, and cytolytic effects towards hosts. Here, we further investigate if parasitism or envenomation by *P. turionellae* alters total protein of its host *Galleria mellonella* L. (Lepidoptera: Pyralidae). Various venom concentrations representing doses previously determined to yield host responses yet fall below the calculated LD99 were used for pupae and larvae. Parasitization was only assayed for host pupa since *P. turionellae* females normally parasitize host prepupae and pupae in nature. Hemolymph total protein concentration remained relatively steady at all doses and at all time points tested in parasitized and venom-injected host pupae and larvae. The only exception to this trend was with the highest dose of venom (0.5 VRE) at 24 h for larvae that almost 2 times higher amount of protein were detected with regard to untreated ones. It is likely that the increase in protein concentration in a non-permissive host stage in the present study was induced by venom and/or general injury because the same trend was also observed in null- and PBS-injected larvae. However, neither of the treatments increased the protein concentration of *G. mellonella* larvae to the same extent that 0.5 VRE injection did, indicating that the increase observed in the latter treatment was not simply the result of wounding or injection of fluid. Thus, we favor the possibility that stress proteins may play a role in this event.

Key words: Wasp venom, parasitism, hemolymph, total protein

Pimpla turionellae (Hymenoptera: Ichneumonidae) parazitlemesi ve zehir enjeksiyonu sonrası konak *Galleria mellonella* (Lepidoptera: Pyralidae) hemolenf toplam proteinindeki değişiklikler

Özet: Endoparazitoid *Pimpla turionellae* L. (Hymenoptera: Ichneumonidae) zehiri konak türü üzerinde felç edici, sitotoksik ve sitolitik etkiler gösteren biyolojik olarak aktif bileşenlerin karışımıdır. Bu çalışmada, *P. turionellae* dişilerinden elde edilen zehir salgısının ve doğal parazitlemenin konak tür, *Galleria mellonella* L. (Lepidoptera: Pyralidae) hemolenfi toplam protein miktarına etkileri belirlendi. Konak pupa ve larvaları için daha önce tepki verdikleri hesaplanan LD_{99} dozu altındaki farklı zehir dozları kullanıldı. *P. turionellae* dişileri doğada sadece konak prepupa ve

pupalarını parazitlediğinden doğal parazitleme sadece konak pupalarında çalışıldı. Hemolenf toplam protein miktarı parazitlenen ve zehir enjekte edilen tüm konak pupa ve larvalarında tüm dozlarda ve tüm zamanlarda fazla değişiklik göstermedi. Sadece, larvalarda 24 saat sonunda en yüksek zehir dozunda (0,5 kese eşdeğeri zehir) hiçbir işleme tabi tutulmayan kontrol grubundakilere oranla protein miktarında iki kata varan bir artış görüldü. Protein miktarındaki bu artış eğilimi boş enjeksiyon ve sadece fizyolojik su enjekte edilen larvalarda da görüldüğünden hedef konak evresi olmayan larva evresinde bu artışın zehir ve/veya yaralanmadan kaynaklanmış olabileceği düşünülmektedir. Ancak, diğer hiçbir uygulamada larva protein miktarının 0,5 kese eşdeğeri zehirdekine benzer derecede artış göstermemesi bu artışın sadece yaralanma ve/veya zehir enjeksiyonundan kaynaklanmadığını göstermektedir. Protein artışına konağa enjeksiyon sonucu salgılanan stres proteinlerinin yol açabileceği olasılığı değerlendirilmektedir.

Anahtar sözcükler: Parazitoid zehiri, parazitleme, hemolenf, toplam protein

Introduction

Insect parasitoids are highly efficient at manipulating the physiology, metabolism, and endocrinological state of their hosts. Host conditioning can result from injection of factors of maternal origin derived from ovarian secretions (1-4) or venom glands (5-9), and/or rely on cells and fluids released from eggs and developing parasitoid progeny (10-12). Endoparasitic koinobiont parasitoids regulate the nutritional and physiological states of their hosts to ensure the successful development of eggs and larvae (13-15). The precise mechanisms used to alter the hosts have not been fully revealed, but for many koinobiont species, venom and viruses (e.g., polydnaviruses, entomopox virus) are used alone or synergistically to alter the nutritional content of the host (13,15).

Parasitism-mediated manipulation of the host nutritional condition is frequently manifested through changes in the hemolymph content of the host. More specifically, host plasma commonly displays quantitative and qualitative changes in protein and amino acid profiles when endoparasitic wasps parasitize their insect hosts (16,17). In several instances, the effects of parasitism and venom on the host hemolymph protein profile are species-specific. For example, the concentration of several host hemolymph proteins decreased when *Hyposoter exiguae* (Viereck) (Hymenoptera: Ichneumonidae) parasitized larvae of the cabbage looper, *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae) (18,19). Similarly, parasitism of larvae of *Pieris rapae* (L.) (Lepidoptera: Pieridae) by *Apanteles glomeratus* L. (Hymenoptera: Braconidae) (20) resulted in a decrease in the concentration of host hemolymph storage proteins. When *Cotesia* (= *Apanteles*) *congregata* (Say) (Hymenoptera: Braconidae) parasitizes *Manduca sexta* (L.) (Lepidoptera: Sphingidae), expression of several hemolymph proteins is dramatically altered, including a decrease in the storage protein arylphorin (21). By contrast, arylphorin levels increased in *T. ni* parasitized by *Chelonus* sp. (Hymenoptera: Braconidae) (22,23). Parasitism of *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) by *C. marginiventris* (Cresson) (Hymenoptera: Braconidae) also caused elevations in hemolymph proteins as evidenced by the early production of several high molecular weight proteins (24). In the *C. kariyai* Watanabe (Hymenoptera: Braconidae) – *Pseudaletia separata* Walker (Lepidoptera: Noctuidae) system, the protein concentration in the hemolymph of larvae injected with calyx fluid and venom increased, yet in parasitized hosts, protein levels dropped (25). The free amino acid profile of *Lymantria dispar* L. (Lepidoptera: Lymantriidae) larvae parasitized by *Glyptapanteles liparidis* Bouche (Hymenoptera: Braconidae) did not change qualitatively; however, levels of some single amino acids were reduced and those of others were elevated (26). Thompson and Lee (1993) (27) reported no effect on amino acid concentration in *M. sexta* parasitized by *C. congregata*. It is clear from these observations that, though the wasps induce an array of changes in the host hemolymph content, the alterations in host condition depend on multiple factors being injected or secreted into the host.

Venoms from these koinobiont species are frequently associated with temporary paralysis, and parasitized hosts continue to grow and develop even after parasitization (28). By contrast, most idiobiont parasitoids paralyze their hosts permanently, and

thus preserve the hosts while the parasitoid progeny feed and develop (29). Such differences in the action of venoms from koinobiont and idiobiont wasps argue that changes in the nutritional content of the hosts (i.e. hemolymph proteins and amino acids) are more likely to be associated with hosts that continue to feed and grow during parasitism, and not when the host is paralyzed. Despite this prediction, almost nothing is known about the role of idiobiont endoparasitoid venoms in altering the hemolymph profile of proteins of their hosts. In this study, the solitary idiobiont pupal endoparasitoid *Pimpla turionellae* L. (Hymenoptera: Ichneumonidae) was used to examine the impact of venom on the host nutritional condition. Changes in the hemolymph total protein were examined in pupae and larvae of *Galleria mellonella* L. (Lepidoptera: Pyralidae) following parasitism and envenomation.

Materials and methods

Parasitoid and host rearing

Pimpla turionellae were reared on pupae (1- or 2-day-old) of *G. mellonella* at 25 ± 1 °C, 60 ± 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 (4 pupae for every 10 female wasps once every 3 days). Host colony was maintained by feeding the insects with natural blackened comb (30) to maintain similarity to their natural media in bee hives.

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 as described (31). Following centrifugation (3000 ×*g* for 10 min at 25 ± 1 °C) to remove cell debris, final venom concentrations were 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). These venom concentrations represent doses previously determined to yield host responses yet fall below the calculated LD_{99} for pupae and larvae (32), respectively. A 5 µL solution of the venom preparation was injected between the last 2 lateral abdominal segments of 1- to 2-day-old host pupae (140 ± 20 mg) and on the first hind leg of last instar larvae (260 ± 10 mg) by using a 10 µL Hamilton microsyringe (Hamilton, Reno, NV, USA). Vaseline was applied to the injection area to prevent hemolymph loss. Controls consisted of pupae and larvae untreated, null-injected, and those injected with only 5 µL of PBS.

Parasitization of *G. mellonella* pupae

Parasitization was performed on 1- or 2-day-old 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 under a photoperiod of 12:12 h LD, as were the controls and venom-treated pupae, until hemolymph collection. *P. turionellae* females normally parasitize host prepupae and pupae in nature (33); therefore, parasitization was not used as an experimental assay for larvae of *G. mellonella*.

Hemolymph collection and total protein determination

Hemolymph collection was performed at 4-, 8-, and 24-h post-treatments from venom-injected, parasitized and control host pupae and larvae. Pupae were bled by piercing the cuticle at the abdomen and larvae on the first hind leg with a sterile 19-gauge needle. Five microliters of hemolymph from each individual pupa and larva were collected at each time period and for each treatment with a glass microcapillary tube (Sigma Chemical Co., St. Louis, MO, USA) and ejected into an ice cold Eppendorf tube containing 1 mg of phenylthiourea (Sigma Chemical) to prevent melanization (34). The hemolymph was spun at 3000 rpm for 10 min at 4 °C to remove hemocytes. The supernatant was transferred to a clean Eppendorf tube and vortexed with a pipette. Three microliters of hemolymph suspension was used for total protein analysis and the remaining sample was kept at –20 °C for further analyses. The resultant supernatant containing plasma was diluted 1:500 with distilled water. Total protein concentration in hemolymph was measured according to the Lowry method (35) using an UNICAM Helios-α spectrophotometer (Cambridge, UK) at 750 nm wavelength. A standard curve was prepared by using bovine serum albumin (BSA, Merck). Protein determinations were repeated 3 times for each experimental and control group.

Statistical analysis

Means were compared using one- or two-way analysis of variance (ANOVA) and subsequently, means were separated using Tukey's Honestly Significant Difference (HSD) post hoc test. SPSS (version 15.0 for Windows, SPSS Science, Chicago, IL, USA) was used for data analysis. Results were considered statistically significant when $P < 0.05$.

Results and discussion

Effects of parasitization and venom injection on the protein concentration of pupae

Total protein concentration of *G. mellonella* pupae did not differ significantly among treatments at 4 ($F = 0.539$; $df = 7, 16$; $P = 0.793$), 8 ($F = 0.754$; $df = 7, 16$; $P = 0.632$), and 24 ($F = 1.049$; $df = 7, 16$; $P = 0.437$) h post-treatments (Table 1). Similarly, hemolymph total protein concentration remained relatively steady at all time points tested in parasitized and venom-injected host pupae, regardless of the venom concentration injected into *G. mellonella* (Table 1). Analyses using two-way ANOVA indicated that the effect of venom injection and parasitization on the total protein concentration of host pupae was not treatment ($P = 0.701$) and time ($P = 0.083$) dependent, and the relationship between treatment and the total protein concentration was not influenced by time ($P = 0.737$) (Table 2).

Effects of venom injection on the protein concentration of larvae

Total protein concentration of *G. mellonella* larvae did not differ significantly among treatments at 4 ($F = 1.096$; $df = 6, 14$; $P = 0.411$) and 8 ($F = 1.744$; $df = 6, 14$; $P = 0.183$) h post-treatments, but was different at 24 ($F = 2.883$; $df = 6, 14$; $P = 0.05$) h (Table 3). Hemolymph total protein concentration remained relatively steady at all time points tested in venom-injected host larvae, regardless of the venom concentration injected into *G. mellonella* except for 0.5 VRE at 24 h (Table 3). Analyses using two-way ANOVA indicated that the effect of venom injection on the total protein concentration of host larvae was treatment ($P = 0.037$) but not time ($P = 0.101$) dependent, and the relationship between treatment and the total protein concentration was influenced by time ($P = 0.030$) (Table 2).

Table 1. Hemolymph total protein concentration (mg/mL) of *G. mellonella* pupae experimentally envenomated and parasitized by *P. turionellae*.

Treatment	Total Protein (mg/mL) (Mean ± SEM)*			Statistics (ANOVA)		
	Time post-treatment (h)**					
	4	8	24	F	df	P
Untreated	111.12 ± 5.6a x	148.12 ± 37.3a x	125.59 ± 12.4a x	0.663	2, 6	0.549
Null-injected	110.32 ± 10.4a x	150.25 ± 12.2a x	103.52 ± 14.1a x	4.191	2, 6	0.073
PBS-injected	111.32 ± 2.5a x	117.72 ± 23.4a x	92.52 ± 13.6a x	0.695	2, 6	0.535
0.005 VRE-injected	120.12 ± 3.8a x	100.45 ± 12.6a x	113.45 ± 7.2a x	1.337	2, 6	0.331
0.01 VRE-injected	121.32 ± 18.0a x	133.25 ± 20.4a x	106.72 ± 8.3a x	0.655	2, 6	0.553
0.02 VRE-injected	102.92 ± 18.4a x	123.85 ± 22.3a x	93.19 ± 10.2a x	0.782	2, 6	0.499
0.05 VRE-injected	147.52 ± 43.3a x	113.52 ± 9.2a x	105.59 ± 9.5a x	0.725	2, 6	0.522
Parasitized	114.72 ± 0.9a x	113.92 ± 4.1a x	107.12 ± 3.5a x	1.769	2, 6	0.249

* Each represents the mean and standard error of mean of 3 replicates with 25 µL hemolymph obtained from 5 individuals (140 ± 20 mg).
** Numbers in rows (a) and columns (x) followed by the same letter are not significantly different ($P > 0.05$).

Table 2. ANOVAs of the effects of different treatments, time, and their interactions on the hemolymph protein concentration of *G. mellonella* pupae and larvae.

Stage	Source	df	MS	F	P	r^2
Pupa	Treatment	7	566.729	0.664	0.701	0.30
	Time	2	2234.084	2.618	0.083	
	Treatment × time	14	620.528	0.727	0.737	
	Error	48	853.488			
Larva	Treatment	6	4714.996	2.497	0.037	0.52
	Time	2	4577.291	2.424	0.101	
	Treatment × time	12	4140.198	2.193	0.030	
	Error	42	1888.176			

Table 3. Hemolymph total protein concentration (mg/mL) of *G. mellonella* larvae experimentally envenomated by *P. turionellae*.

Treatment	Total Protein (mg/mL) (Mean ± SEM)*			Statistics (ANOVA)		
	Time post-treatment (h)**					
	4	8	24	F	df	P
Untreated	225.2 ± 19.8a x	187.3 ± 31.7a x	155.1 ± 11.3a xy	2.417	2, 6	0.170
Null-injected	205.9 ± 36.0a x	172.6 ± 17.1a x	202.9 ± 29.8a xy	0.411	2, 6	0.680
PBS-injected	176.1 ± 9.3a x	229.4 ± 17.1a x	199.7 ± 21.7a xy	2.508	2, 6	0.162
0.02 VRE-injected	203.1 ± 5.9a x	203.6 ± 2.0a x	207.3 ± 31.0a xy	0.016	2, 6	0.984
0.05 VRE-injected	207.8 ± 14.4a x	213.9 ± 10.8a x	265.3 ± 37.4a xy	1.741	2, 6	0.253
0.1 VRE-injected	226.9 ± 20.1a x	227.6 ± 17.5a x	257.4 ± 44.1a xy	0.351	2, 6	0.717
0.5 VRE-injected	173.1 ± 21.0a x	239.6 ± 18.6ab x	330.1 ± 47.0b y	6.215	2, 6	0.035

* Each represents the mean and standard error of mean of 3 replicates with 25 µL hemolymph obtained from 5 individuals (260 ± 10 mg).
** Numbers in rows (a-b) and columns (x-y) followed by the same letter are not significantly different (P > 0.05).

Parasitism- and venom-related changes in hemolymph protein profiles and total or specific protein levels have been detected in numerous parasitoid-host model systems. Not surprisingly, the impact of the parasitoids on the host hemolymph has been variable, and in many cases, the hemolymph protein changes seem to be part of the host conditioning necessary for the parasitoid's larvae to successfully complete development (2,5,25,26). Altering the host nutritional condition for the benefit of wasp offspring is generally thought to be most common for koinobionts, and would presumably not be expected for a solitary idiobiont like *P. turionellae* (36). Consistent with this prediction are the observations in this study that protein concentrations of hemolymph from *G. mellonella* pupae did not differ among controls, parasitized, or those injected with isolated venom.

However, the present result contradicts an earlier study (37) describing a dramatic decrease in the total protein concentration of *G. mellonella* pupae parasitized by *P. turionellae* at 1 and 6 h, and a significant decrease at 12 and 36 h. On the other hand, the authors reported considerable increases at 48 and 72 h post-parasitism, whereas no significant changes were observed at 3, 24, and 60 h between total protein levels of parasitized and unparasitized hosts (37). We do not expect differences in protein quantity since idiobiont parasitoids like *P. turionellae* usually paralyze their host, inhibiting continued host growth and lacking the ability to regulate host metabolism. Venom from this idiobiotic wasp contains several mid to high range molecular weight proteins, as well as noradrenalin, apamin, and melittin (31), phospholipase B, histamine, and serotonin (38). The last of these are also consistent with the apparent nonspecific paralytic action of the venom (39).

An alternative explanation for the absence of variation in the hemolymph protein concentration may be associated with the host stage being attacked since the pupae cannot feed and represent a closed nutritional container for the parasitoid progeny. On the other hand, protein concentration of hemolymph from *G. mellonella* larvae showed an extensive increase at all venom doses and was considerably higher at the end of 24 h at the highest dose of 0.5 VRE with respect to untreated larvae. Previous reports have also documented the production of storage proteins and an increase of the titer of those storage proteins in the hemolymph of parasitized lepidopteran larvae (25,40,41). These types of host alterations are perhaps more likely to occur when host larvae are attacked by ectoparasitoids that rely entirely on venom to alter the development of their insect hosts and cause an arrestment of the larval-larval molting process in the host (42). It would seem, however, that *P. turionellae* would be predicted to induce similar host changes, unless those factors other than venom also participate in host conditioning, or the venom operates by a different mode of action than these ectoparasitic species. Considering that the composition of venom from *P. turionellae* appears to be unique from that reported for other parasitic wasps (31,43,44), the latter scenario is a likely explanation for the differential venom effects on host hemolymph protein content.

It is likely that the increase in protein concentration in a non-permissive host stage in the present study was induced by venom and/or general injury since the same trend was also observed in null- and PBS-injected larvae. However, neither of the treatments increased the protein concentration of *G. mellonella* larvae to the same extent that 0.5 VRE injection did, indicating that the increase observed in the latter treatment was not simply the result of wounding or injection of fluid. Although, at present, there are insufficient data to determine which of these scenarios is correct, we favor the possibility that stress proteins may play a role in this event.

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