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Effects of Parasitism and Envenomation by *Pimpla turionellae* (Hymenoptera: Ichneumonidae) on Hemolymph Free Amino Acids of *Galleria mellonella* (Lepidoptera: Pyralidae)

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ABSTRACT

The effects of dose-dependent envenomation by and parasitization of *Pimpla turionellae* Linnaeus (Hymenoptera: Ichneumonidae) on the ratio of hemolymph free amino acids of the host species *Galleria mellonella* Linnaeus (Lepidoptera: Pyralidae) pupae and larvae were investigated. Of the seventeen different free amino acids detected in the hemolymph of host pupae and larvae by high performance liquid chromatography, the ratio of free amino acids from parasitized and envenomated host pupae did not differ much when compared with those of unparasitized, null- or PBS-injected controls at different time points post-treatments. The exceptions to this trend were an increase in parasitized host pupae for glutamic acid with regard to other experimental groups at 4 and 8 h and a decrease in parasitized host pupae for leucine with regard to 0.01 and 0.05 VRE at 24 h post-treatments. In contrast to pupae, hemolypmh free amino acids of *G. mellonella* larvae differed upon venom injection among treatments and at different time points post-treatments. The ratios of alanine and leucine at 8 h and glutamic acid, serine, glycine+glutamine, valine, methionine, and phenylalanine at 24 h post-treatments differed from those of controls in treatment groups. However, there appeared no changes in the ratio of hemolypmh free amino acids in host larvae at 4 h post-treatments. Our study indicated that parasitism and experimental envenomation of *G. mellonella* by wasps resulted in different effects in the quantity of free amino acids depending on host developmental stage.

Key words: Wasp venom, parasitization, hemolymph, HPLC, amino acids.

INTRODUCTION

One of the most characteristic features of insect hemolymph is the high level of free amino acids. Free amino acid pattern directly influences osmotic phenomena, reflects the cationic and anionic state of hemolymph, and regulates the synthesis of proteins and peptide hormones (Florkin and Jeuniaux, 1974; Chen, 1985; Atmowidjojo *et al*., 1999). High level of free amino acids is also related with many biological mechanisms such as neural transmission, detoxification, synthesis of phosphoplipids, energy production, and morphogenetic processes (Chen, 1985; Assar *et al*., 2010). In addition, it has been suggested that hemolymph composition can be used to assess

phylogenetic relationships between various arthropod taxa (Punzo, 1990). It was shown that hemolymph free amino acid composition is highly variable according to the quality of diet, and presence of toxic materials and xenobiotics in body (Florkin and Jeuniaux, 1974; Hanzal and Jegorov, 1991; Nath *et al*., 1997). The changes in amino acid composition also reflect the metabolic state of an organism; i.e. the main metabolic pathways as well as the developmental state (Hanzal and Jegorov, 1991).

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 (Thompson and Lee, 1994; Richards and Edwards, 1999). In several instances, the effects of parasitism and venom on the host hemolymph protein profile and amino acid content are species-specific (Kunkel *et al*., 1990; Thompson and Lee, 1993; Bischof and Ortel, 1996; Nakamatsu *et al*., 2001). It is concluded by Sak *et al*. (2011) 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.

The solitary endoparasitic wasp, *Pimpla turionellae* Linnaeus (Hymenoptera: Ichneumonidae) envenomates and oviposits into prepupae and pupae of a number of lepidopteran species including the greater wax moth, *Galleria mellonella* Linnaeus (Lepidoptera: Pyralidae). The pupal endoparasitoid *P. turionellae* lacks polydnaviruses (PDVs) and virus-like particles (VLPs) (Ergin *et al*., 2006) so that the wasp venom is likely to play a major role in host regulation. Venom from this wasp contains a number of biologically active components including the proteins; melittin, apamin, the biogenic amines; histamine and serotonin, and the catecholamine; noradrenaline. Additionally, venom displays a mixture of several mid to high range molecular weight proteins (Uçkan *et al*., 2004; Uçkan *et al*., 2006; Ergin *et al*., 2007) and has potent paralytic, cytotoxic, and cytolytic effects toward lepidopteran and dipteran hosts (Ergin *et al*., 2006). Besides, the role of venom from and/or parasitism by *P. turionellae* in suppressing host immune defence (Er *et al*., 2010; Uçkan *et al*., 2010; Er *et al*., 2011) or on hemolymph protein ratio (Sak *et al*., 2011) and profile (Ergin *et al*., 2013) of host has also been studied in vivo and in vitro. However, almost nothing is known about the role of idiobiont endoparasitoid venoms in altering the hemolymph amino acid profile of their hosts. The current study was designed to determine to which extent the ratio of individual free amino acids in the hemolymph of host pupae and larvae is dependent on different venom doses from and parasitism by *P. turionellae*. Venom-induced changes between host pupae and larvae in free amino acid content and ratio were also examined.

MATERIAL AND METHODS

Parasitoid and host rearing

P. turionellae were reared on pupae (1- or 2-d-old) of *G. mellonella* at 25 ± 1°C, 60 ± 5% RH, and 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 three days). Host colony was maintained by feeding the insects with natural blackened comb (Uçkan and Ergin, 2002) 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-d-old females by dissecting out the venom sacs as described previously (Uçkan *et al*., 2004). A venom reservoir equivalent (VRE) was defined as the reservoir material obtained from one wasp. A total number of 42 and 12 adults were fed for larval and pupal assays for three sets of replication. Following centrifugation (3,000 g for 10 min at 25 ± 1°C) to remove cell debris (Ergin *et al*., 2006), venom reservoirs obtained from 1, 2, and 10 females were placed separately in microcentrifuge tubes (1 ml) each containing 100 µl of physiological saline (PBS; 0.138 M NaCl and 0.0027 M KCl in 0.01 M PBS, pH 7.4) (Er *et al*., 2010) to examine the dose-dependent effect of venom on host larvae and pupae. The final concentration in each tube was adjusted to venom reservoir equivalents (VRE) of 0.05, 0.1, and 0.5 in 5 µl of PBS, respectively. Venom samples of 0.02, 0.01, and 0.005 VRE/5 µl used additionally in host larva and pupa assays were adjusted by placing one female reservoir content in microcentrifuge tubes (1 ml) containing 250, 500, and 1,000 µl PBS. These venom concentrations represent doses previously determined to yield host responses yet fall below the calculated LD_{99} for pupae and larvae (Ergin *et al*., 2006), respectively. A 5 µl solution of the venom preparation was injected between the last two lateral abdominal segments of 1-2-d-old pupae (140 ± 20 mg) and on the first hind leg of last instars (260 ± 10 mg) of the host, previously chilled on ice for 10 min, by using a 10 µl Hamilton microsyringe (Hamilton, Reno, NV) (Ergin *et al*., 2006; Er *et al*., 2010). 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 PBS (Er *et al*., 2010).

Parasitization of *G. mellonella* pupae

Parasitization was performed on 1- or 2-d-old host pupae by exposing an individual host pupa (140 ± 20 mg) to an individual 15 to 20-d-old wasp female (Er *et al*., 2010). Parasitized pupae were held at 25 ± 2°C, 60 ± 5% RH, and a photoperiod of 12: 12 h (L: D), as were the controls and venom-treated pupae until hemolymph collection. *P. turionellae* females normally parasitize host prepupae and pupae in nature (Kansu and Uğur, 1984), therefore parasitization was not used as an experimental assay for larvae of *G. mellonella* (Er *et al*., 2010).

Collection and preparation of hemolymph samples

Hemolymph collection was performed at 4, 8, and 24 h post-treatments from venom-injected, parasitized, and control host pupae and larvae. A total number of 63 and 72 hemolymph samples were respectively collected for larval and pupal assays in three sets of replications comprising of 5 larvae or pupae in each. 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) and ejected into an ice cold eppendorf tube containing 1 mg phenylthiourea (Sigma Chemical, St. Louis, MO, USA) (Sak *et al*., 2011) to prevent melanization (Zupko *et al*., 1993). Hemolymph was spun at 3,000 rpm for 10 min at 4°C to remove hemocytes. The supernatant was transferred to a clean eppendorf tube, vortexed with a pipette, and hemolymph suspension was kept at -20˚C until analyses (Sak *et al*., 2011).

Determination of amino acids

Hemolymph free amino acids of host pupae and larvae were analyzed by high performance liquid chromatography (HPLC). Amino acid standards (L-Aspartic acid, L-Glutamic acid, L-Asparagine, L-Serine, L-Glycine, L-Glutamine, L-Histidine, L-Threonine, L-Arginine, L-Alanine, L-Proline, L-Tyrosine, L-Valine, L-Methionine, L-Isoleucine, L-Leucine, L-Phenylalanine, L-Tryptophan, L-Lysine) (100 mM/0.1 M HCl) and all chemicals used (Sigma) were of HPLC grade.

Preparation of hemolymph samples and 19 different amino acid standards was a modification of the method used by Crailsheim and Leonhard (1997) and Pennacchio *et al*. (1999). After thawing, 4 µl of each hemolymph sample and amino acid standard was placed in a sterile eppendorf tube, 10 µl distilled water and 87 µl acetonitrile were added, and vortexed with pipette tip. Samples centrifuged at 8,000 g for 1 min at room temprature (22°C). Of the supernatant, 95 µl were transfered in a new eppendorf tube and centrifuged at 8,000 g for 3 min at room temprature (22°C). 80 µl of the supernatant were then placed in a new eppendorf tube and used in derivatization procedure.

Derivatization with PITC

The derivatization procedure with phenylisothiocyanate (PITC) was a modification of the method used by Vilasoa-Martínez *et al*. (2007). Each hemolymph sample and amino acid standard was dried in a vacuum oven for 2 h at 65°C. Then, 6 µl of methanol-water-triethylamine (TEA) (2:2:1) was added to the residue and the resulting solution was vacuum-dried for a further 10 min at 65°C. Next, 6 µl of the derivatizing reagent methanol-water-TEA-PITC (7:1:1:1 [v/v]) were added and the eppendorf tubes were vortexed for 30 s and left to stand at room temperature for 20 min. Finally, the resulting solution was vacuum-dried for 15 min at 65°C. The solutions were maintained at room temperature if immediately analyzed otherwise stored at +4°C until a day before analyses. Before an hour prior to injection; 150 µl of diluent, consisting of 5 mM sodium phosphate (Na_2HPO_4) with 5% acetonitrile brought to pH 7.43 with phosphoric acid, was added to each eppendorf tube with vortex-mixing for 15 s.

HPLC equipment and conditions

HPLC was performed with a LC-10 AT vp Shimadzu chromatograph equipped with four quaternary pump (FCV-10AL vp Shimadzu), automatic injection system (SIL-10AD

vp Shimadzu), and a Diode Array Detector (SPD-M10A vp). Data processing was carried out with the software programme, Shimadzu LC solution (versiyon 1.2). Amino acids were seperated on a reverse-phase HPLC column (Ace 3 C_{18}, 125 mm × 4.0 mm i.d., 5 µm particle size, MAC-MOD Analytical, Inc., USA). The column temparature was 27 ± 2 °C. The flow rate was 0.9 ml min^{-1} and the detection wavelength was 254 nm. The mobile phase was a gradient prepared from two solutions; A and B. Solution A was 0.14 M sodium acetate buffer containing 0.05% (v/v) TEA (pH adjusted to 6.2 with glacial acetic acid). Solution B was 60: 40 acetonitrile: water (Vilasoa-Martínez *et al*., 2007). Both solvents were carefully filtrated with ultramembrane (30 mm, 0.45 µm pore size, Spartan-Orange Scientific) and degassed for 15 min prior to use. Nineteen amino acids and hemolymph samples were analyzed with an injection time of 35 min, preceded by derivatization step lasting about 180 min, of which 20 min were for reaction between the samples and PITC. The gradient programme was the same method used by Vilasoa-Martínez *et al*. (2007). Gradient elution programme from 90 to 52% A for 22 min was carried out, followed by washing the column with 100% B for 5 min and 10% B for 7 min. Envenomation and parasitization related changes in the percent peak area of each free amino acids were estimated. Each set of experiments was replicated three times for pupae and larvae.

Statistical analysis

Means were compared using one way analysis of variances (ANOVA). Subsequently, means were subjected to Tukey's Honestly Significant Difference (HSD) *post hoc* tests when variances (tested in SPSS by Levene statistics for homogeneity of variances) are homogenous, but Tamhane tests otherwise to assess the significance of the effects of envenomation and parasitization in the percent peak area of each free amino acids. Percentage data was normalized by arcsine transformation prior to analyses. A SPSS software program (version 15.0 for Windows, SPSS Science, Chicago, IL) was used for data analysis. Results were considered statistically significant when $P < 0.05$.

RESULTS

Nineteen amino acids were identified by comparing retention times with those of obtained from individual standard amino acids. Only one of the 19 amino acids tested, aspartic acid, was undetected. High performance liquid chromatograms of hemolymp free amino acids from *G. mellonella* pupae and larvae are shown in Figs. 1 and 2. Changes in the percent peak area of each free amino acid following envenomation and parasitization were shown in Table 1 and 2 for pupae and larvae, respectively. The percentage of free amino acids from parasitized and envenomated host pupae did not differ much when compared with those of unparasitized, null- or PBS-injected controls at different time points post-treatments (Table 1). The exceptions to this trend were a significant increase in parasitized host pupae for glutamic acid with regard to control and other experimental groups at 4 and 8 h ($P<0.05$), and a significant decrease in parasitized host pupae for leucine with regard to 0.01 and 0.05 VRE at

24 h post-treatments (P<0.05). Besides, a significant increasing rate at 8 and 24 h post-treatments with respect to 4 h was only detected in the percentage of proline for PBS-injected pupae (Table 1).

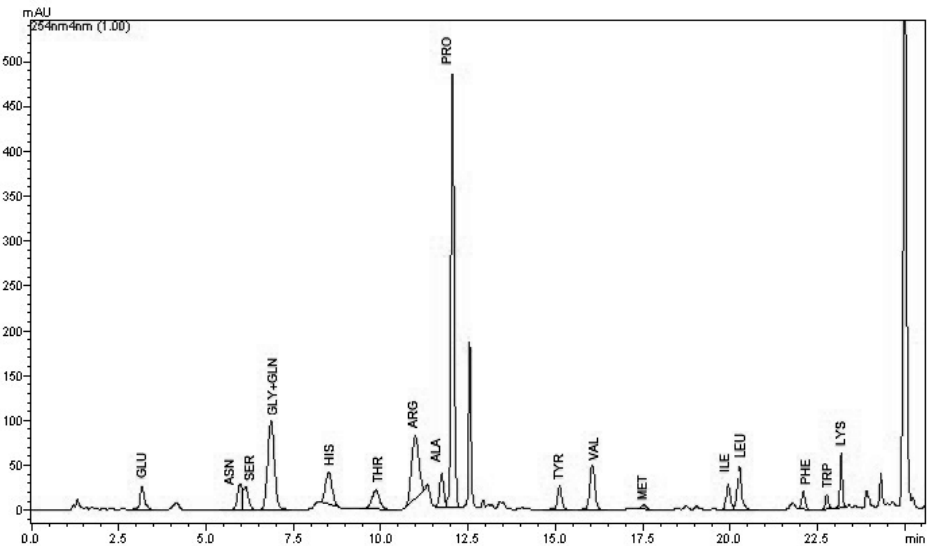

Fig. 1. A typical chromatogram showing amino acids detected in hemolymph of *G. mellonella* pupae.

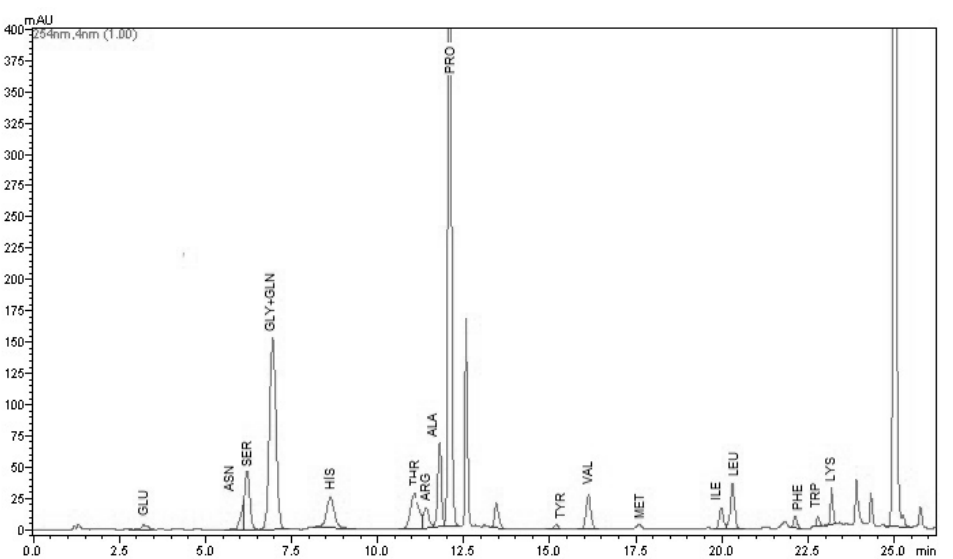

Fig. 2. A typical chromatogram showing amino acids detected in hemolymph of *G. mellonella* larvae.

Table 1. Percent peak area (%) of hemolymph free amino acids of *G. mellonella* pupae experimentally envenomated and parasitized by *P. turionellae* at different times.

Treatment[#]	Peak Area (%) (Mean ± SE)*														
	Glutamic acid (GLU)			Asparagine (ASN)			Serine (SER)			Glycine (GLY) + Glutamine (GLN)			Histidine (HİS)		
	4 h	8 h	24 h	4 h	8 h	24 h	4 h	8 h	24 h	4 h	8 h	24 h	4 h	8 h	24 h
Untreated	1.24± 0.16ax	1.54± 0.32ax	1.78± 0.58ax	1.47± 0.23ax	1.94± 0.67ax	2.19± 0.31ax	1.55± 0.14ax	1.91± 0.67ax	2.09± 0.39ax	6.53± 0.99ax	6.35± 1.48ax	6.98± 0.72ax	4.73± 0.49ax	3.55± 1.19ax	3.80± 1.04ax
Null	0.80± 0.08ax	1.42± 0.17ax	1.82± 0.8a x	1.81± 0.08ax	1.81± 0.60ax	2.01± 0.59ax	1.82± 0.15ax	1.88± 0.21ax	1.87± 0.24ax	6.02± 0.54ax	6.36± 0.28ax	7.18± 0.88ax	5.07± 0.25ax	4.47± 0.14ax	4.64± 0.70ax
PBS	1.6± 0.19ax	1.61± 0.09ax	0.85± 0.32ax	1.65± 0.32ax	1.59± 0.40ax	1.54± 0.23ax	1.70± 0.14ax	1.73± 0.10ax	2.09± 0.23ax	6.93± 0.51ax	7.03± 0.50ax	6.79± 0.53ax	5.17± 0.61ax	4.82± 0.15ax	4.27± 0.36ax
0.005 VRE	1.64± 0.22ax	1.74± 0.27ax	1.18± 0.08ax	1.27± 0.10ax	1.36± 0.13ax	1.44± 0.21ax	1.67± 0.06ax	1.75± 0.15ax	2.15± 0.33ax	6.67± 0.63ax	6.07± 0.39ax	6.24± 0.68ax	5.59± 0.48ax	6.12± 0.64ax	5.52± 0.99ax
0.01 VRE	1.26± 0.31ax	1.74± 0.13ax	1.03± 0.26ax	1.55± 0.16ax	1.40± 0.18ax	1.45± 0.16ax	1.78± 0.04ax	1.86± 0.27ax	2.03± 0.30ax	5.94± 0.85ax	6.29± 0.61ax	6.59± 0.78ax	5.46± 0.18ax	6.12± 1.08ax	5.76± 0.59ax
0.02 VRE	1.88± 0.74ax	1.66± 0.40ax	1.30± 0.48ax	1.53± 0.14ax	1.61± 0.44ax	1.53± 0.13ax	1.86± 0.10ax	2.15± 0.67ax	1.96± 0.26ax	5.42± 0.99ax	6.04± 2.01ax	5.63± 0.56ax	5.77± 1.23ax	3.48± 0.61ax	4.61± 0.69ax
0.05 VRE	1.68± 0.57ax	1.93± 0.65ax	1.73± 0.72ax	2.13± 0.79ax	1.36± 0.31ax	1.34± 0.25ax	1.46± 0.26ax	1.84± 0.28ax	2.26± 0.33ax	3.70± 1.69ax	4.75± 0.99ax	5.61± 1.40ax	5.32± 1.41ax	4.32± 0.88ax	4.39± 0.94ax
Parasitized	5.17± 0.69bx	4.62± 0.46bx	4.02± 1.15ax	1.40± 0.18ax	1.36± 0.09ax	1.01± 0.17ax	2.10± 0.26ax	1.73± 0.11ax	1.87± 0.19ax	4.12± 1.58ax	2.83± 0.43ax	3.93± 2.07ax	5.41± 0.71ax	4.72± 0.41ax	4.08± 0.67ax

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 columns (a-b) and rows (x) followed by the same letter are not significantly different (P > 0.05).

Table 1. Percent peak area (%) of hemolymph free amino acids of *G. mellonella* pupae experimentally envenomated and parasitized by *P. turionellae* at different times.

Treatment#	Peak Area (%) (Mean ± SE)*														
	Threonine (THR)			Arginine (ARG)			Alanine (ALA)			Proline (PRO)			Tyrosine (TYR)		
	4 h	8 h	24 h	4 h	8 h	24 h	4 h	8 h	24 h	4 h	8 h	24 h	4 h	8 h	24 h
Untreated	3.44± 0.22ax	2.76± 1.17ax	2.03± 0.75ax	24.67± 5.94ax	18.57± 5.91ax	21.51± 7.26ax	3.64± 0.64ax	5.23± 2.36ax	3.12± 0.96ax	19.61± 3.59ax	16.48± 4.25ax	19.75± 1.67ax	1.44± 0.48ax	2.15± 0.92ax	6.08± 3.32ax
Null	3.34± 0.37ax	4.17± 0.66ax	4.27± 0.47ax	27.71± 5.11ax	25.24± 3.71ax	21.54± 4.09ax	4.14± 0.15ax	4.78± 0.69ax	4.98± 0.27ax	21.99± 0.11ax	21.41± 2.47ax	22.18± 2.52ax	2.14± 0.23ax	1.81± 0.72ax	1.19± 0.37ax
PBS	4.50± 0.11ax	4.33± 0.34ax	4.08± 0.20ax	24.52± 1.09ax	21.56± 3.22ax	17.72± 5.15ax	4.70± 0.36ax	4.17± 0.22ax	4.37± 0.27ax	15.11± 0.75ax	22.78± 1.06ay	21.69± 1.49ay	2.70± 1.05ax	1.80± 0.35ax	1.7± 0.57ax
0.005 VRE	3.91± 0.35ax	4.64± 0.52ax	3.95± 0.53ax	22.61± 1.95ax	22.90± 0.08ax	20.35± 2.67ax	5.12± 0.27ax	5.30± 0.69ax	5.24± 0.37ax	19.52± 3.45ax	19.09± 1.33ax	22.92± 3.66ax	2.33± 0.25ax	1.87± 0.94ax	1.34± 0.42ax
0.01 VRE	4.54± 0.61ax	4.80± 0.90ax	4.27± 0.84ax	22.28± 3.53ax	23.20± 1.17ax	18.84± 1.85ax	4.57± 0.36ax	4.88± 0.26ax	4.98± 0.37ax	15.48± 3.30ax	19.62± 1.93ax	22.41± 2.28ax	3.51± 1.53ax	1.90± 0.87ax	1.29± 0.51ax
0.02 VRE	4.42± 0.75ax	2.34± 0.48ax	5.29± 1.07ax	22.53± 2.34ax	22.82± 5.43ax	23.91± 3.58ax	4.95± 0.16ax	5.78± 0.94ax	6.45± 0.58ax	20.74± 3.64ax	21.77± 4.44ax	20.11± 5.58ax	4.95± 3.02ax	1.82± 0.78ax	1.65± 0.81ax
0.05 VRE	3.31± 0.91ax	3.61± 0.88ax	3.94± 0.82ax	25.38± 13.42ax	18.07± 2.27ax	15.28± 3.06ax	13.83± 10.54ax	4.52± 0.60ax	5.76± 0.57ax	19.23± 4.23ax	19.27± 2.73ax	20.54± 5.96ax	1.68± 0.70ax	3.83± 2.08ax	5.08± 3.72ax
Parasitized	2.98± 0.76ax	4.32± 0.16ax	3.21± 0.02ax	22.37± 3.33ax	26.54± 2.13ax	22.54± 0.26ax	5.12± 0.76ax	4.30± 0.38ax	5.76± 1.04ax	19.19± 1.62ax	20.34± 0.74ax	25.84± 3.17ax	2.53± 0.37ax	2.62± 0.18ax	2.32± 0.62ax

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 columns (a) and rows (x-y) followed by the same letter are not significantly different (P > 0.05).

Table 1. Percent peak area (%) of hemolymph free amino acids of *G. mellonella* pupae experimentally envenomated and parasitized by *turionellae* at different times.

Treatment[#]	Peak Area (%) (Mean ± SE)*														
	Valine (VAL)			Methionine (MET)			Isoleucine (ILE)			Leucine (LEU)			Phenylalanine (PHE)		
	4 h	8 h	24 h	4 h	8 h	24 h	4 h	8 h	24 h	4 h	8 h	24 h	4 h	8 h	24 h
Untreated	3.33± 0.92ax	8.31± 3.55ax	6.40± 2.44ax	2.91± 2.14ax	2.27± 0.90ax	4.23± 3.56ax	2.91± 1.15ax	3.63± 1.15ax	2.07± 0.72ax	9.84± 5.67ax	12.76± 5.61ax	3.79± 0.28abx	10.99± 2.09ax	10.58± 1.96ax	13.35± 0.40ax
Null	4.55± 0.95ax	4.16± 0.46ax	4.39± 0.82ax	1.83± 0.65ax	1.29± 0.62ax	1.81± 0.86ax	1.54± 0.02ax	1.58± 0.38ax	1.74± 0.22ax	3.83± 0.05ax	4.60± 0.91ax	4.61± 0.33abx	12.10± 1.49ax	13.84± 1.45ax	14.21± 0.97ax
PBS	4.75± 0.98ax	3.87± 0.22ax	6.47± 1.28ax	1.03± 0.32ax	1.36± 0.24ax	2.72± 1.65ax	2.50± 0.49ax	1.57± 0.23ax	1.96± 0.38ax	5.39± 1.47ax	4.73± 0.51ax	5.24± 1.02abx	15.38± 1.88ax	15.71± 1.44ax	15.03± 1.61ax
0.005 VRE	4.12± 0.14ax	4.69± 0.95ax	5.00± 0.96ax	1.47± 0.32ax	1.36± 0.38ax	1.40± 0.73ax	1.76± 0.46ax	2.16± 0.47ax	2.28± 0.45ax	5.57± 0.85ax	6.20± 0.89ax	6.73± 0.89abx	15.17± 1.20ax	13.13± 1.25ax	12.99± 1.80ax
0.01 VRE	5.13± 1.04ax	4.87± 1.15ax	4.76± 1.24ax	5.01± 3.47ax	1.36± 0.43ax	3.53± 1.91ax	2.36± 0.57ax	2.08± 0.41ax	2.51± 0.44ax	7.65± 1.60ax	6.06± 0.68ax	7.45± 0.97bx	11.55± 2.10ax	12.12± 2.08ax	11.70± 2.25ax
0.02 VRE	4.87± 0.90ax	6.08± 2.40ax	5.60± 1.18ax	3.05± 2.22ax	1.34± 0.41ax	1.34± 0.76ax	1.73± 0.31ax	2.46± 0.46ax	2.11± 0.22ax	5.18± 0.61ax	7.25± 1.48ax	6.28± 0.60abx	10.00± 1.50ax	10.42± 0.96ax	9.46± 0.98ax
0.05 VRE	3.04± 0.58ax	4.53± 0.53ax	5.34± 0.90ax	1.95± 0.99ax	2.39± 0.91ax	3.58± 2.03ax	1.48± 0.34ax	2.73± 1.28ax	2.72± 0.64ax	5.17± 0.61ax	7.09± 2.40ax	7.62± 1.62bx	9.15± 0.83ax	13.64± 2.71ax	10.75± 3.38ax
Parasitized	4.82± 0.50ax	4.23± 0.60ax	3.60± 0.88ax	1.99± 0.47ax	1.69± 0.35ax	1.30± 0.42ax	1.67± 0.30ax	1.52± 0.36ax	1.32± 0.32ax	5.45± 0.90ax	3.82± 0.09ax	3.51± 0.45ax	14.35± 0.92ax	14.49± 0.87ax	14.12± 0.97ax

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 columns (a-b) and rows (x) followed by the same letter are not significantly different (P > 0.05).

Table 1. Percent peak area (%) of hemolymph free amino acids of *G. mellonella* pupae experimentally envenomated and parasitized by *P. turionellae* at different times.

Treatment#	Peak Area (%) (Mean ± SE)*					
	Tryptophan (TRP)			Lysine (LYS)		
	4 h	8 h	24 h	4 h	8 h	24 h
Untreated	1.21± 0.30ax	1.42± 0.75ax	0.62± 0.11ax	0.49± 0.21ax	0.55± 0.17ax	0.22± 0.06ax
Null	0.69± 0.07ax	0.77± 0.08ax	0.74± 0.07ax	0.63± 0.08ax	0.42± 0.16ax	0.81± 0.17ax
PBS	0.92± 0.21ax	0.66± 0.17ax	1.00± 0.26ax	1.42± 0.94ax	0.71± 0.31ax	2.51± 1.78ax
0.005 VRE	1.08± 0.39ax	0.97± 0.48ax	0.91± 0.39ax	0.51± 0.05ax	0.66± 0.11ax	0.40± 0.04ax
0.01 VRE	1.34± 0.39ax	1.24± 0.61ax	1.04± 0.52ax	0.60± 0.05ax	0.48± 0.14ax	0.36± 0.11ax
0.02 VRE	0.67± 0.20ax	1.33± 0.38ax	0.98± 0.43ax	0.46± 0.10ax	1.66± 1.25ax	1.79± 1.38ax
0.05 VRE	0.89± 0.24ax	5.61± 3.28ax	1.91± 0.89ax	0.62± 0.21ax	0.52± 0.21ax	2.16± 1.57ax
Parasitized	0.93± 0.32ax	0.56± 0.14ax	1.18± 0.53ax	0.42± 0.07ax	0.32± 0.08ax	0.40± 0.14ax

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 columns (a) and rows (x) followed by the same letter are not significantly different (P > 0.05).

Unlike pupae, hemolypmh free amino acids of *G. mellonella* larvae differed upon venom injection among treatments and at different time points post-treatments (Table 2). The percentages of alanine and leucine at 8 h considerably declined at the highest dose of 0.5 VRE with respect to all other experimental groups (P<0.05). Besides, 0.5 VRE of venom injection also caused remarkable increases and decreases in the percentages of six free amino acids for larvae at 24 h. An increase in glutamic acid, serine, and glycine+glutamine with regard to untreated and null-injected groups for larvae injected with higher doses of venom (0.05, 0.1, and 0.5 VRE) was determined at 24 h (especially for 0.5 VRE) (P<0.05). On the other hand; valine, methionine, and phenylalanine levels at 24 h post-treatments decreased remarkably at all treatment groups with regard to untreated larvae (the most important decreasing rates at 24 h were detected for 0.5 VRE with the percent of 2.748, 0.372 and 1.029, respectively). However, there appeared no changes in the ratio of hemolypmh free amino acids in host larvae at 4 h post-treatments (Table 2). In contrast to pupae, significant quantitative changes among time points after treatments were observed at all doses of venom injected larvae except for 0.05 VRE but not at all control groups in general (Table 2).

Table 2. Percent peak area (%) of hemolymph free amino acids of *G. mellonella* larvae experimentally envenomated by *P. turionellae* at different times.

Treatment#	Peak Area (%) (Mean ± SE)*														
	Glutamic acid (GLU)			Asparagine (ASN)			Serine (SER)			Glycine (GLY) + Glutamine (GLN)			Histidine (HİS)		
	4 h	8 h	24 h	4 h	8 h	24 h	4 h	8 h	24 h	4 h	8 h	24 h	4 h	8 h	24 h
Untreated	0.16± 0.08ax	0.26± 0.13ax	0.23± 0.07ax	0.84± 0.15ax	0.80± 0.33ax	0.68± 0.27ax	2.18± 0.34ax	3.39± 0.81ax	1.67± 0.35ax	14.02± 1.80ax	16.16± 2.65ax	10.62± 1.39abx	4.68± 0.74ax	3.96± 0.40ax	7.05± 1.80ax
Null	0.22± 0.09ax	0.30± 0.14ax	0.24± 0.08ax	0.59± 0.18ax	0.61± 0.09ax	0.58± 0.23ax	2.56± 0.72ax	2.73± 0.43ax	1.60± 0.07ax	12.04± 0.24ax	12.73± 3.08ax	9.72± 0.32ax	5.47± 0.98ax	6.10± 1.53ax	5.09± 0.88ax
PBS	0.26± 0.10ax	0.33± 0.11ax	0.46± 0.21abx	0.75± 0.09ax	1.05± 0.55ax	0.63± 0.21ax	3.29± 0.70ax	2.75± 0.38ax	2.68± 0.38abx	12.50± 1.61ax	10.58± 0.64ax	9.61± 0.89abx	5.16± 0.48ax	5.45± 0.94ax	4.87± 1.63ax
0.02 VRE	0.12± 0.01ax	0.07± 0.01ay	0.06± 0.01ay	0.85± 0.00ax	0.49± 0.00ay	0.37± 0.00az	3.10± 0.00ax	2.06± 0.00ay	1.86± 0.00az	15.01± 0.00ax	10.24± 0.01ay	9.59± 0.05abz	3.79± 0.00ax	3.76± 0.00ax	3.86± 0.05ax
0.05 VRE	0.24± 0.10ax	0.29± 0.08ax	0.36± 0.07abx	0.78± 0.10ax	1.00± 0.50ax	0.89± 0.29ax	3.18± 0.49ax	2.73± 0.37ax	3.23± 0.66abx	13.56± 1.49ax	10.05± 0.84ax	14.34± 1.14abx	5.05± 0.63ax	5.25 0.65ax	5.80± 1.30ax
0.1 VRE	0.25± 0.08ax	0.34± 0.12ax	0.31± 0.10ax	0.69± 0.10ax	1.05± 0.39ax	1.36± 0.81ax	3.04± 0.49ax	2.93± 0.39ax	2.64± 0.52abx	14.84± 1.69ax	13.73± 1.36ax	14.92± 2.06abx	6.22± 0.37ax	3.74± 0.27ay	4.27± 0.53ay
0.5 VRE	0.48± 0.17ax	0.75± 0.14ax	1.13± 0.26bx	0.71± 0.04ax	0.67± 0.07ax	0.72± 0.05ax	3.16± 0.44ax	3.00± 0.20ax	4.28± 0.40bx	14.28± 2.17ax	12.37± 0.52ax	14.44± 0.49bx	5.39± 0.78ax	4.71± 0.07ax	3.89± 0.19ax

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 columns (a-b) and rows (x-z) followed by the same letter are not significantly different (P > 0.05).

Table 2. Percent peak area (%) of hemolymph free amino acids of *G. mellonella* larvae experimentally envenomated by *P. turionellae* at different times.

Treatment#	Peak Area (%) (Mean ± SE)*														
	Threonine (THR)			Arginine (ARG)			Alanine (ALA)			Proline (PRO)			Tyrosine (TYR)		
	4 h	8 h	24 h	4 h	8 h	24 h	4 h	8 h	24 h	4 h	8 h	24 h	4 h	8 h	24 h
Untreated	1.15± 0.20ax	2.73± 1.05ax	3.03± 1.71ax	5.67± 1.20ax	5.41± 1.97ax	5.86± 2.92ax	3.12± 0.43ax	4.86± 0.36ax	4.31± 1.58ax	53.36± 3.17ax	50.33± 3.85ax	51.02± 1.89ax	0.54± 0.07ax	0.89± 0.40ax	0.69± 0.20ax
Null	1.13± 0.10ax	4.56± 2.99ax	3.59± 2.60ax	9.25± 1.67ax	5.02± 2.02ax	8.29± 3.89ax	4.12± 0.67ax	4.24± 1.03abx	2.86± 0.77ax	51.78± 5.34ax	52.00± 6.90ax	58.28± 2.16ax	1.20± 0.29ax	0.83± 0.15ax	0.70± 0.18ax
PBS	1.09± 0.15ax	3.06± 1.79ax	3.66± 2.59ax	8.07± 0.74ax	2.84± 1.20ax	5.23± 1.45ax	3.31± 0.48ax	4.48± 0.46ax	3.82± 0.90ax	52.96± 1.56ax	56.12± 2.59ax	58.08± 4.19ax	1.11± 0.34ax	1.01± 0.44ax	0.85± 0.06ax
0.02 VRE	0.74± 0.00ax	0.88± 0.00ay	1.13± 0.01az	4.68± 0.00ax	7.61± 0.04ay	6.50± 0.03az	3.31± 0.01ax	3.78± 0.02aby	2.30± 0.00az	53.98± 0.02ax	57.74± 0.04ay	60.03± 0.03az	0.63± 0.00ax	1.18± 0.00ay	1.01± 0.00az
0.05 VRE	4.16± 3.37ax	3.48± 2.31ax	3.75± 2.71ax	5.26± 1.23ax	5.16± 2.79ax	4.40± 1.43ax	3.91± 0.88ax	3.89± 0.35abx	4.84± 0.37ax	37.22± 14.44ax	54.06± 0.14ax	50.31± 3.39ax	1.39± 0.42ax	1.48± 0.20ax	1.05± 0.19ax
0.1 VRE	1.09± 0.09ax	2.27± 1.05ax	2.01± 0.85ax	7.55± 1.19ax	6.59± 1.84ax	4.29± 1.33ax	3.75± 0.11ax	4.98± 0.77ax	6.12± 1.18ax	49.15± 3.22ax	50.89± 1.26ax	50.27± 2.21ax	0.86± 0.27ax	1.08± 0.17ax	0.59± 0.12ax
0.5 VRE	1.35± 0.23ax	0.98± 0.05ax	1.03± 0.13ax	8.74± 2.78ax	5.83± 0.79ax	7.04± 0.42ax	3.12± 0.85ax	2.05± 0.37bx	3.95± 0.00ax	49.56± 4.14ax	58.25± 0.37ax	52.25± 1.11ax	0.88± 0.08ax	1.05± 0.19ax	1.32± 0.20ax

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 columns (a-b) and rows (x-z) followed by the same letter are not significantly different (P > 0.05).

Table 2. Percent peak area (%) of hemolymph free amino acids of *G. mellonella* larvae experimentally envenomated by *P. turionellae* at different times.

Treatment[#]	Peak Area (%) (Mean ± SE)*														
	Valine (VAL)			Methionine (MET)			Isoleucine (ILE)			Leucine (LEU)			Phenylalanine (PHE)		
	4 h	8 h	24 h	4 h	8 h	24 h	4 h	8 h	24 h	4 h	8 h	24 h	4 h	8 h	24 h
Untreated	4.15± 0.23ax	2.42± 1.14ax	4.41± 0.38ax	0.80± 0.11ax	1.40± 0.75ax	0.78± 0.09ax	1.91± 0.20ax	1.53± 0.15ax	1.52± 0.22abx	3.59± 0.44ax	2.88± 0.16abx	2.97± 0.47ax	1.92± 0.15axy	1.33± 0.34ax	2.63± 0.09ay
Null	3.03± 0.38ax	3.16± 0.28ax	2.82± 0.16bx	0.75± 0.14ax	0.75± 0.14ax	0.50± 0.11abx	1.40± 0.06ax	1.19± 0.19ax	0.93± 0.09bx	2.47± 0.40ax	2.14± 0.15bcx	2.10± 0.13ax	1.88± 0.20ax	1.30± 0.26ax	1.09± 0.21bx
PBS	3.00± 0.31ax	3.46± 0.11ax	3.27± 0.11abx	0.90± 0.20ax	0.80± 0.12ax	0.53± 0.08abx	1.44± 0.17ax	1.33± 0.09ax	1.16± 0.17abx	2.78± 0.32ax	2.89± 0.11abx	2.42± 0.63ax	1.82± 0.27ax	1.64± 0.52ax	1.06± 0.22bx
0.02 VRE	4.23± 0.01ax	3.79± 0.01ay	4.16± 0.01az	0.44± 0.01ax	0.53± 0.01ay	0.57± 0.03aby	2.15± 0.00ax	1.77± 0.00ay	2.05± 0.00az	3.92± 0.01ax	3.27± 0.00ay	3.33± 0.00az	1.87± 0.02ax	1.62± 0.02ay	2.02± 0.02abz
0.05 VRE	2.63± 0.73ax	3.88± 0.52ax	2.96± 0.17bx	0.58± 0.13ax	0.76± 0.03ax	0.59± 0.08abx	1.38± 0.22ax	1.43± 0.12ax	0.99± 0.09bx	2.24± 0.28ax	2.84± 0.28abx	2.62± 0.22ax	1.68± 0.41ax	1.37± 0.30ax	1.60± 0.25abx
0.1 VRE	3.36± 0.91ax	3.34± 0.28ax	3.68± 0.38abx	1.30± 0.36ax	0.92± 0.10ax	0.56± 0.02abx	1.56± 0.53ax	1.55± 0.28ax	1.59± 0.36abx	3.25± 0.83ax	3.00± 0.33abx	3.54± 0.44ax	1.48± 0.20ax	1.56± 0.39ax	2.04± 0.58abx
0.5 VRE	3.36± 0.35ax	3.23± 0.25ax	2.75± 0.23bx	0.78± 0.09ax	0.54± 0.10axy	0.37± 0.04by	1.65± 0.31ax	1.33± 0.15ax	1.36± 0.19abx	3.04± 0.31ax	1.95± 0.13cy	3.11± 0.01ax	1.51± 0.28ax	1.78± 0.45ax	1.03± 0.10bx

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 columns (a-b) and rows (x-z) followed by the same letter are not significantly different (P> 0.05).

Table 2. Percent peak area (%) of hemolymph free amino acids of *G. mellonella* larvae experimentally envenomated by *P. turionellae* at different times.

Treatment#	Peak Area (%) (Mean ± SE)*					
	Tryptophan (TRP)			Lysine (LYS)		
	4 h	8 h	24 h	4 h	8 h	24 h
Untreated	0.75± 0.04axy	0.49± 0.09ax	0.80± 0.07ay	1.17± 0.44ax	1.16± 0.33ax	1.76± 0.67ax
Null	0.53± 0.09ax	0.58± 0.05ax	0.57± 0.08ax	1.59± 0.61ax	1.79± 0.66ax	1.05± 0.40ax
PBS	0.53± 0.04ax	0.57± 0.11ax	0.56± 0.15ax	1.05± 0.30ax	1.65± 0.51ax	1.11± 0.49ax
0.02 VRE	0.61± 0.00ax	0.78± 0.01ay	0.68± 0.00az	0.57± 0.00ax	0.43± 0.00ay	0.49± 0.00az
0.05 VRE	0.70± 0.09ax	0.74± 0.08ax	1.02± 0.16ax	1.01± 0.23ax	1.60± 0.56ax	1.26± 0.44ax
0.1 VRE	0.61± 0.14ax	0.81± 0.21ax	0.82± 0.15ax	1.00± 0.58ax	1.22± 0.35ax	0.98± 0.40ax
0.5 VRE	0.72± 0.10ax	0.91± 0.25ax	0.60± 0.07ax	1.27± 0.62ax	0.61± 0.15ax	0.75± 0.25ax

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 columns (a) and rows (x-z) followed by the same letter are not significantly different (P>0.05).

DISCUSSION AND CONCLUSIONS

Numerous reports have documented parasitoid venom mediated changes in the hemolymph content of lipids, proteins, and carbohydrates of host insects (Chen, 1985; Thompson and Lee, 1993; Thompson and Lee, 1994; Bischof and Ortel, 1996; Sak *et al*., 2011; Ergin *et al*., 2013). In spite of all the research work done to date on the dependence of amino acid content in host hemolymph upon parasitism, results are different and even contradictory. It is unclear whether these differences may be related to a high variability in free amino acids, nutritional differences, and developmental stage of insect or lack of sufficiently sensitive methods (Florkin and Jeuniaux, 1974; Hanzal and Jegorov, 1991; Crailsheim and Leonhard, 1997; Nath *et al*., 1997).

L-arginine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-tryptophan, L-threonine, and L-valine are the essential amino acids for most of the insects (Chang, 2004). In general, glutamic acid (mainly in the form of glutamine) and proline take the most important quantitative place in the amino acid pool in endopterygotes (Florkin and Jeuniaux, 1974). Hanzal and Jegerov (1991) detected 14 primary amino acids among which glutamine, alanine, gamaaminobutyric acid (GABA), and glycine predominated in the hemolymph of *G. mellonella* larvae. Prolin could not be detected in the same study since it was unable

to react with OPA-thiol derivatization reagent. On the other hand, proline was found as the predominant amino acid and comprised 50 to 80% of total amino acids of the newly emerged bees from the third day on (Crailsheim and Leonhard, 1997). Consistent with these results are the observations in this study that predominant free amino acids in untreated controls were proline (%51.9), glycine + glutamine (%13.4), arginine (%5.7), and histidine (%5.6) in larvae and arginine (%21.8), proline (%18.8), phenylalanine (%12), leucine (%8.3), and glycine + glutamine (%6.7) in pupae after 24 h observations. Our results once more support the concept that 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 parasitoid species like *P. turionellae* (Sak *et al*., 2011). Consistent with this prediction are the observations in this study that the ratio of free amino acids from parasitized and envenomated host pupae did not differ much when compared with those of controls at different time points post-treatments. Moreover, hemolymph total protein concentration remained relatively steady at all doses and at all time points tested in parasitized and venom-injected host pupae (Sak *et al*., 2011). Other consistent results with this prediction are the observations that electrophoretic pattern and O.D. values of proteins of hemolymph from *G. mellonella* pupae and larvae did not differ much among controls, parasitized or those injected with isolated venom (Ergin *et al*., 2013). Neither parasitism nor envenomation caused a complex array of changes in the hemolymph protein profile; there were only a few changes in the amount of some proteins at certain time points (Ergin *et al*., 2013).

Unlike pupae, some hemolypmh free amino acids of *G. mellonella* larvae differed upon venom injection among treatments and at different time points post-treatments. The ratios of alanine and leucine at 8 h and glutamic acid, serine, glycine+glutamine, valine, methionine, and phenylalanine at 24 h post-treatments differed from those of controls in treatment groups. Similarly, unlike the hemolymph total protein of *G. mellonella* pupae that remained steady at all doses and time points tested, protein concentration of hemolymph from host 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, which was almost two times higher than the amount of protein detected for untreated samples (Sak *et al*., 2011). Venom injection especially at 0.5 VRE also caused remarkable increases and decreases in the ratio of six free amino acids at 24 h for larvae. An increase in glutamic acid, serine, and glycine+glutamine with regard to untreated and null-injected groups for larvae injected with the highest doses of 0.5 VRE was determined at 24 h ($P<0.05$). On the other hand; the most important decreasing rates at 24 h were also detected for 0.5 VRE with valine, methionine, and phenylalanine levels at all treatment groups with regard to untreated larvae. Valine, methionine, and phenylalanine are essential amino acids which cannot be synthesized by interconversion of other amino acids and must be ingested as dietary components (Klowden 2007). Therefore, the significant decreases at 24 h in valine, methionine, and phenylalanine levels for larvae injected with 0.5 VRE might be related to the increased metabolization of these amino acids which join citric acid cycle via succinyl

CoA and fumarat (Klowden 2007) to compensate for the increasing energy demand due to venom toxicity. In conclusion, 0.5 VRE had the most important effects on *G. mellonella* larval hemolymph milieu in terms of protein and amino acid content. In a previous study, 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 and it was concluded that stress proteins might have played a role in that event (Sak *et al*., 2011). Our current results showing that 0.5 VRE of venom causing remarkable alterations in the ratio of six free amino acids at 24 h for larvae are parallel to the results of our previous study (Sak *et al*., 2011) emphasizing the prediction of the possibility that stress proteins may take place in this situation.

It is a known fact that amino acids have important role in basic steps of glycolysis in insect metabolism as in other organisms (Kilby and Neville 1957; Klowden 2007; Chen 1985). The fate of two molecules of pyruvate generated in glycolysis vary depending on the organism and the circumstances. For instance, pyruvate is converted to acetyl CoA and enters the citric acid cycle in the presence of sufficient oxygen. In some insects, pyruvate may be transaminated by glutamate to α-ketoglutarate which enters the citric acid cycle and glutamate may play a central role in amino acid metabolism (Kilby and Neville 1957; Klowden 2007). As a result, the significant increase in glutamic acid levels in parasitized host pupae at 4 and 8 h and in venom-injected (0.5 VRE) host larvae at 24 h might have affected the citric acid cycle and metabolic pathways via α-ketoglutarate. Therefore, the likely source of hemolymph glutamic acid and glutamine may be the result of protein catabolism and the dynamics of protein turnover appear to correspond to increases in glutamic acid.

Parasitization of host pupae caused changes only in the levels of glutamic acid (increased at 4 and 8 h) and leucine (decreased at 24 h) with regard to control and other experimental groups. In contrast to pupae, there appeared no change in the ratio of hemolypmh free amino acids in host larvae at 4 h post-treatments. However, the ratio of alanine and leucine at 8 h considerably reduced at the highest dose of 0.5 VRE with respect to all other experimental groups. Increases in glutamic acid, serine, and glycine+glutamine and decreases in valine, methionine, and phenylalanine levels for larvae injected with higher doses of venom (0.05, 0.1, 0.5 VRE) with regard to untreated larvae were determined at 24 h. It is likely that host pupae have different susceptibility and are affected earlier than larvae to parasitoid venom taking results into account that the effects of venom observed for pupae at 4 h post-treatments are observed at 8 h post-treatments for larvae. The earlier presence of high levels of amino acids in treated pupae with respect to larvae displaying a disturbance in biochemical activities also confirms the susceptibility of host puape as target host stage. When injected with parasitoid venom, *G. mellonella* pupae were also far more susceptible than larvae in terms of abdominal mobility and adult emergence (Ergin *et al*., 2006). We also found that the cellular defence reactions occured more rapidly in *G. mellonella* larvae when compared with pupae in terms of hemocyte-mediated encapsulation and melanization, indicating the higher susceptibility of pupal hemocytes to parasitism and

venom injection by *P. turionellae* (Uçkan *et al*., 2010). We believe that the changes in the levels of the analyzed amino acids were severe enough to explain at least the partial adverse effects of venom injection to host larvae.

Consoli and Vinson (2004) evaluated the changes in amino acid and protein composition of the host hemolymph from the tobocco budworm, *Heliothis virescens* (Fabricius) parasitized by *Toxoneuron nigriceps* (Viereck). The protein profile of parasitized larvae was similar to controls throughout the embryonic development, but there appeared increases or decreases in the total amino acid concentration. Besides, single amino acid comparisons during the whole embryonic development of the parasitoid indicated higher concentrations of glycine, serine, histidine, and asparagine in the hemolymph of parasitized host during the first 10-12 h after parasitization, while threonine was found in lower levels. They also found that the level of proline inreased while that of tyrosine decreased at 16-28 h after parasitization (Consoli and Vinson, 2004). The spectra of free amino acids detected in the hemolymph of gypsy moth, *Lymantria dispar* (Linnaeus) larvae did not change qualitatively due to parasitization by gregarious endoparasitiod, *Glyptapanteles liparidis* (Bouché), but levels of some single amino acids were reduced and those of others were elevated (Bischof and Ortel, 1996). Consistent with these results are the observations in this study that the same 17 free amino acids were detected in both pupae and larvae, but the ratio of some single amino acids decreased or increased after venom injection and parasitization. However, the concentration of the most abundant amino acid, arginine in pupae and proline in larvae, more or less remained at the same level. Bischof and Ortel (1996) also found similar result for the most abundant amino acid, histidine did not change after parasitization in host larvae.

P. turionellae venom displays potent paralytic, cytotoxic, and cytolytic effects toward lepidopteran and dipteran hosts (Ergin *et al*., 2006). The biological activity of venom (Ergin *et al*., 2006) and the effects of *P. turionellae* venom injection and parasitism on hemocyte numbers, morphology, viability (Er *et al*., 2010), encapsulation and melanization responses of the hemocytes (Uçkan *et al*., 2010), apoptotic and mitotic indices in the circulating hemocytes (Er *et al*., 2011), and hemolymph total protein content (Sak *et al*., 2011) and protein profile (Ergin *et al*., 2013) of the host, *G. mellonella* larvae and pupae have been previously investigated. Total hemocyte counts declined sharply in pupae and larvae of *G. mellonella* exposed to *P. turionellae*. Besides, parasitism reduced the number of granular cells while increasing the number of plasmatocytes at 4 h post treatments (Er *et al*., 2010). Parasitization by *P. turionellae* suppressed hemocyte-mediated encapsulation and melanization in *G. mellonella* (Uçkan *et al*., 2010). Moreover, the ratio of early and late apoptotic hemocytes increased in *G. mellonella* pupae and larvae upon parasitization and at high doses of venom injection (Er *et al*., 2011). However, an increase in necrotic hemocytes was only observed in parasitized pupae at 24 h and no difference was observed in larvae (Er *et al*., 2011). The mitotic ratio of hemocytes decreased upon parasitization and at high doses of venom in host pupae and larvae (Er *et al*., 2011). Hemolymph total proprotein 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 but not in pupae (Sak *et al*., 2011). The quantities of proteins detected at 4, 8, and 24 h post-treatments in hemolymph of parasitized and envenomated host pupae did not differ much when compared with those of controls. The electrophoretic pattern of hemolymph proteins from venom injected and control groups of larvae did not differ much from that of pupae except for new protein bands detected at 33.823 and 41.553 kDa. However, three bands with 45.385, 99.000, and 126.850 kDa were not detected in larvae (Ergin *et al*., 2013). Of the seventeen different protein bands detected at a range of 19.6-181.12 kDa in the hemolymph, there were only changes in OD values of bands at 23.418, 24.714, 32.434, 34.811, and 45.385 kDa following envenomation and parasitism (Ergin *et al*., 2013). Handling all these results together; both venom from and parasitization by *P. turionellae* showed potent effects in suppressing host immune defence (Er *et al*., 2010; Uçkan *et al*., 2010; Er *et al*., 2011) and some deleterious effects on the protein and free amino acids of *G. mellonella* pupae and larvae (Sak *et al*., 2011; Ergin *et al*., 2013), arguing that the wasp's venom has a broader spectrum of activity than parasitoids that target a single host developmental stage.

Whatever the reasons are, many authors concluded that parasitoids alter their host's metabolism for their own benefit to ensure successful nourishment and maturation (Lawrence, 1990). The changes in the ratio of free amino acids of host, *G. mellonella* hemolymph may constitute a defence mechanism to compensate for osmoregulatory and physiological problems during the stress conditions due to parasitization and envenomation. The decreases or increases in some amino acids ratio of parasitized pupae or larvae envenomated with high venom doses may indicate uses these amino acids in protein synthesis or led to protein catabolism, respectively.

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