

# The effects of parasitism by the ectoparasitoid *Bracon hebetor* Say (Hymenoptera: Braconidae) on host hemolymph proteins in the Mediterranean flour moth *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae)

Hülya ALTUNTAŞ\*, Ali Yavuz KILIÇ, Hülya SİVAS ZEYTİNOĞLU

Anadolu University, Faculty of Science, Department of Biology, 26470 Eskişehir - TURKEY

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**Abstract:** Parasitism of lepidopteran larvae by braconid wasps has significant effects on host hemolymph proteins and the physiology of the host insect. In this study the post-parasitism effects of a gregarious idiobiont ectoparasitoid (*Bracon hebetor*) on hemolymph plasma proteins in the final instar larvae of the host (*Ephestia kuehniella*) were investigated. Hemolymph plasma proteins were analyzed using spectrophotometry and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Total quantity of plasma proteins in the host's hemolymph decreased slightly 24 and 48 h after parasitization; however, SDS-PAGE analysis showed that the quantity of 12 proteins decreased, whereas that of 5 proteins increased in concentration in the hemolymph of parasitized host larvae 24 and 48 h post-parasitism. Therefore, we conclude that host regulation of *E. kuehniella* by *B. hebetor* involves only quantitative changes in host plasma proteins and does not lead to upregulation of novel proteins.

**Key words:** Host regulation, hemolymph proteins, ectoparasitoid

## Konak un güvesi *Ephestia kuehniella* Zeller, 1879 (Lepidoptera: Pyralidae) larvalarının hemolenf proteinleri üzerine ektoparazitoid *Bracon hebetor* Say, 1836 (Hymenoptera: Braconidae)'un parazitik etkileri

**Özet:** Braconid ve Ichneumonid parazitoidler tarafından konak böcek türlerin parazitlenmesi sonucunda konak hemolenf proteinlerinde ve konak fizyolojisinde önemli değişiklikler meydana gelmektedir. Bu çalışmada ise gregar, idiobiont ve ektoparazitoid *Bracon hebetor* Say (Hymenoptera: Braconidae)'un, konak *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) olgun larvalarının hemolenf plazma proteinleri üzerindeki parazitik etkileri araştırılmıştır. Hemolenf plazma proteinleri spektrofotometrik ve sodyum dodesil sülfat-poliakrilamid jel elektroforez yöntemleri ile araştırılmıştır ve protein bantları densitometrik olarak analiz edilmiştir. Hemolenfteki total plazma protein miktarı parazitlenmeden 24 ve 48 saat sonra giderek azalmıştır. Ancak, SDS- PAGE analizine göre, 24 ve 48 saatlik parazitlenmeye bağlı olarak konak hemolenfindeki 12 proteinin miktarı azalırken, beş proteinin miktarı artmıştır. Böylece, *B. hebetor*'un *E. kuehniella*'nın hemolenf proteinleri üzerindeki parazitik etkisinin sadece kantitatif yönden olduğu ve yeni proteinlerin sentezine neden olmadığını düşünmekteyiz.

**Anahtar sözcükler:** Konak düzenlemesi, hemolenf proteinleri, ektoparazitoid

\* E-mail: hyalcitas@anadolu.edu.tr

## Introduction

The braconid *Bracon hebetor* Say is a gregarious, idiobiont arrhenotokous ectoparasitoid that parasitizes lepidopteran larvae and is an important biological control agent for several stored product moth pests (Gül and Gülel, 1995; Heimpel et al., 1997; Darwish et al., 2003). *B. hebetor* has been widely used in various studies related to host-parasitoid interactions because of its high reproductive rate, short generation time, and considerable range of host species (Yu et al., 2002; Gündüz and Gülel, 2005).

The success of biological control programs is highly dependent on the nature of the host-parasitoid interaction, which has led to many host-parasitoid systems being investigated in great detail (Vinson and Iwantsch, 1980; Rolle and Lawrence, 1994; Baker and Fabrick, 2000; Salvador and Consoli, 2008). Knowledge of the physiological and biochemical interactions in host-parasitoid systems is important for the efficient production of biological control agents (Hentz et al., 1998; Vinson et al., 2001; Nakamatsu and Tanaka, 2003). In vitro rearing techniques have been used to determine the quantitative and qualitative needs, as well as the nutritional biochemistry of natural enemies. However, improvement of these techniques depends on knowledge and understanding of the physiology, nutrition, genetics, and behavior of parasitoids (Magro and Para, 2003).

Parasitism of arthropods by nematodes, trematodes, insects, and pathogens frequently elicits both qualitative and quantitative changes in hemolymph proteins and detectable amino acids in the host (Vinson and Iwantsch, 1980; Beckage et al., 1987). It is well known that parasitism by ichneumon and braconid endoparasitoids causes multiple alterations in the physiology of their insect hosts, such as endocrine and developmental programming (Lawrence and Lanzrein, 1993), metabolism (Thompson, 1985), immunocompetence (Stoltz et al., 1988), behavior (Horton and Moore, 1993), and pigmentation (Beckage et al., 1990). When endoparasitoids parasitize their hosts, especially lepidopteran species, qualitative and quantitative changes occur in the profile of host plasma proteins (Rolle and Lawrence, 1994; Vinson et al., 2001; Consoli and Vinson, 2002; Rahbe et al., 2002). In the

latter case, proteins may be negatively regulated or suppressed in the host larvae by the developing parasitoid (Beckage and Kanost 1993). For example, the concentration of arylphorin in *Manduca sexta* hemolymph decreased after parasitism by *Cotesia congregata* (Beckage et al., 1987). On the other hand, parasitism-specific proteins (Soldevila and Jones, 1994) or polydnviruses (Beckage et al., 1987) may be derived from parasitoid larvae, co-injected into the host together with the eggs, or derived from serosal cells (teratocytes) that dissociate after eggs hatch (Vinson and Iwantsch, 1980). In many instances these components provide protection pathways and host regulation for the developing endoparasite by interfering with various defenses (Asgari et al., 1998). Recently, Salvador and Consoli (2008) reported that 2 parasitism-specific host hemolymph proteins (125 and 48 kDa) were upregulated by the endoparasitoid *Cotesia flavipes*.

In contrast, only a few studies on the effects of parasitism by ectoparasitoids on the hemolymph proteins of their insect hosts have been published (Coudron et al., 1997; Richards and Edwards, 1999; Baker and Fabrick, 2000). Like endoparasitoids, adult ectoparasitoids introduce their venom and/or other fluids into their host at oviposition; however, ectoparasitoid venom often causes paralysis of the host and alters development of the host by arresting the larval molting cycle. Unlike endoparasitoids, their eggs are deposited on the outside of their host, where they develop into larvae (Shaw, 1981). Additionally, little is known about the effect of ectoparasitoid secretions on the plasma protein profiles in their hosts (Quistad et al., 1994; Doury et al., 1997; Asgari et al., 2003; Nakamatsu and Tanaka, 2003; Parkinson et al., 2003). The present study aimed to investigate if parasitization by *B. hebetor* affects the concentration and profile of plasma proteins in the host *E. kuehniella*.

## Materials and Methods

### Insect rearing

The Mediterranean flour moth *E. kuehniella*, used as the host, and the ectoparasitoid *B. hebetor* were provided as gifts by Dr. Eylem Akman Gündüz (Ondokuz Mayıs University, Samsun, Turkey).

A laboratory culture of *E. kuehniella* was reared in 500-mL glass jars in constant darkness at  $25 \pm 2$  °C and relative humidity of  $55 \pm 2\%$ . The jars contained whole-wheat flour (2:1) that was previously heated in order to ensure the absence of any infestation. Every week female moths were allowed to oviposit by transferring them into new oviposition jars for 24 h, so that host larvae would be constantly available during the experimental period.

A stock culture of *B. hebetor* was reared on 25- to 30-day-old final larval stadium hosts in cylindrical 250-mL glass jars under the same environmental conditions described for the host culture. In addition, adult wasps were fed a 50% aqueous honey solution absorbed onto a cotton pad and were allowed to mate in a glass tube (16 × 100 mm) for 24 h. Female wasps were then separated from males and used to parasitize hosts.

#### Host parasitization

Host parasitization was performed under the same environmental conditions described above. Newly ecdysed *E. kuehniella* final instars were selected from the culture and parasitized individually by exposure to a single mated *B. hebetor* female in a glass tube for at least 24 h. Larvae of the same age and weight as those exposed to parasitoids were selected as controls. Wasps were removed from the tube immediately after laying eggs.

#### Haemolymph collection

Hemolymph samples were obtained from parasitized larvae 24 h and 48 h after parasitization, and from the unparasitized control insects of the same age. Hemolymph was collected from 10 insects at each time point and 5 µL of hemolymph was obtained from each larva. Unparasitized larvae were cleaned of adhering food material and anesthetized on ice for 10 min. The parasitized larvae were not anesthetized due to the relaxing effect of the wasp's paralyzing venom. Parasitized and unparasitized larvae were dropped into 70% ethanol for 2-3 min for surface sterilization. Hemolymph was collected by cutting the third proleg with micro-scissors while lightly massaging the larval body with forceps. Care was taken to avoid rupturing the larval gut or dislodging the fat body. Hemolymph was drawn into a 10-µL micropipette and transferred to a microcentrifuge tube placed on ice. Subsequently, a few crystals of 1-phenyl-2-thiourea were added to each

sample to prevent melanization of the hemolymph. When a sufficient quantity of hemolymph was collected the samples were centrifuged at 10,000 ×g for 5 min at 4 °C to pellet cell debris. Plasma samples were stored at -20 °C until analysis.

#### Measurement of the hemolymph protein concentration

Plasma samples were diluted 1:20 with distilled water and protein concentrations were measured according to Bradford (Bradford, 1976), using Coomassie blue G250 protein-assay reagent at 595 nm (Eppendorf BioPhotometer). A standard curve was prepared using bovine serum albumin as the standard. Each sample was analyzed in triplicate and the experiment was repeated at least 3 times.

#### SDS-PAGE analysis

The protein profiles of hemolymph obtained from parasitized and unparasitized *E. kuehniella* larvae were analyzed using 11% SDS-PAGE, according to Laemmli (1970). Briefly, 20 µg of protein from the each sample was mixed with sample buffer (0.125 M Tris, 4% SDS (w/v), 20% glycerol (v/v), 10% 2-mercaptoethanol (v/v), 0.2% bromophenol blue (w/v, pH 6.8) at a ratio of 1:3, and then was boiled for 4 min and kept on ice until loading onto the gel. Wide- and low-range molecular weight markers (Sigma) were also loaded into parallel lanes on the gel. After electrophoresis at 120 V, the gel was stained with 0.04% Coomassie blue R 250 in 50% methanol/10% acetic acid overnight and destained in 10% methanol/10% acetic acid solution. The gel was subsequently scanned using a UVitec gel documentation (BioLab) system and the optical density (OD) of the bands was analyzed using a Gel-Pro analyzer (software v.4.0, United Bio., NJ, USA). Each experiment was repeated at least 3 times. The percentage of OD values of the protein bands from the parasitized host were determined as follows:

$$\% \text{ OD of protein} = (\text{OD value of parasitized protein}) / (\text{OD value of unparasitized protein}) \times 100.$$

#### Statistical Analysis

The quantity of hemolymph total protein was inferred using one-way analysis of variance (ANOVA). Subsequently, means were separated using Tukey's honestly significant difference (HSD) post hoc test (SPSS Inc., 1999).

### Results

Spectrophotometric analysis of total plasma protein present in the hemolymph of *E. kuehniella* 24 and 48 h after parasitization showed that there was a slight decrease in total protein, as seen in Table 1; the difference in total plasma protein between parasitized and unparasitized hosts was not statistically significant ( $P > 0.05$ ).

Table 1. Total plasma protein values of the unparasitized and the parasitized *E. kuehniella* larvae.

Hour	Total Hemolymph Protein Mean $\pm$ SE ( $\mu\text{g}/\mu\text{L}$ )	
	Control	Post-parasitism
24	228.00 $\pm$ 35.56	235.67 $\pm$ 56.05
48	257.67 $\pm$ 34.82	210.00 $\pm$ 73.75

The mean  $\pm$  S.E. of data is from 3 independent experiments ( $P > 0.05$ ).

After spectrophotometric analysis total plasma protein was further analyzed by SDS-PAGE. As shown in Figure 1, 25 bands were observed in the hemolymph of parasitized host larvae, while 26 bands were produced by the blood of unparasitized larvae. The quantity of protein in the parasitized host larvae was generally quantitatively different than that in unparasitized larvae (Table 2). On the other hand, there were no qualitative differences in terms of novel protein bands, the one exception being a 14.5-kDa protein, which disappeared from the hemolymph of parasitized larvae.

Each protein band from 3 independent experiments was analyzed using a Gel-Pro analyzer, and then was evaluated by comparison with controls (Table 2.). Following parasitization, the quantity of 12 protein bands (55.3, 41.2, 39.4, 37.2, 35.6, 34.7, 33.9, 32.1, 29.6, 23.1, 15.1, and 14.5 kDa) significantly decreased ( $>40\%$ ). On the other hand, the quantity of

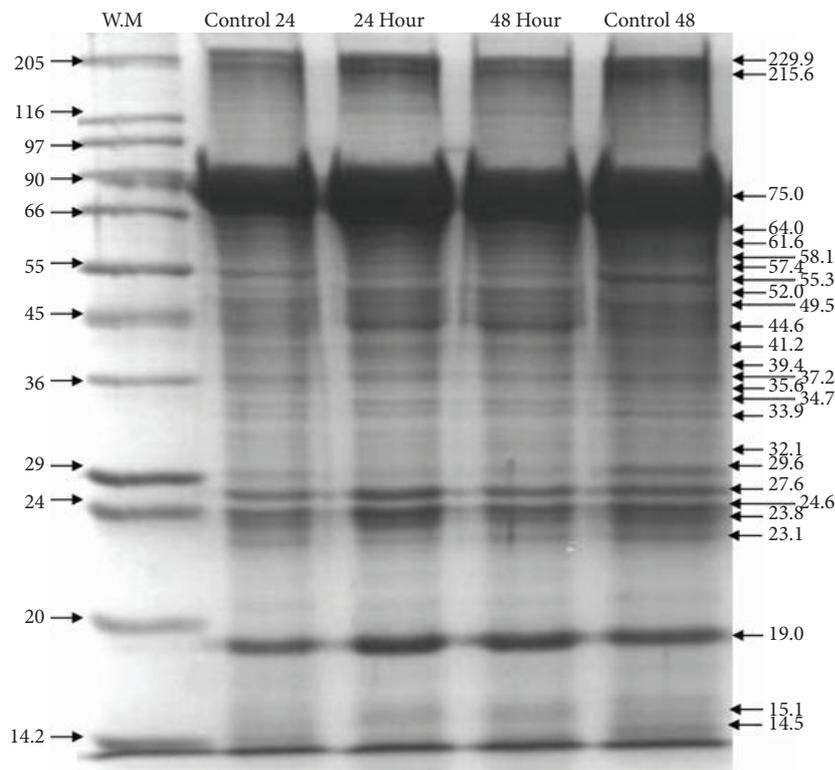


Figure 1. Protein profiles of hemolymph from unparasitized as control 24 and 48 h and post-parasitized *E. kuehniella* larvae after 24 and 48 h incubation were identified by SDS/PAGE using 11% gel. W.M: Wide range molecular weight marker (205-14.2 kDa). Equal protein amounts of 20  $\mu\text{g}$  per lane were loaded. A representative result from at least 3 independent experiments is shown. Arrows indicate the molecular weight of protein bands.

Table 2. Changes in the level of hemolymph proteins from *E. kuehniella* parasitized with *B. hebetor* at different hours analyzed by Gel-Pro Analyzer system.

Proteins (kDa)	(% ) OD values of proteins post-parasitism	
	24 h	48 h
229.9	16.62 <sup>+</sup>	3.98
215.6	12.20 <sup>+</sup>	4.81 <sup>-</sup>
55.3	40.60 <sup>-</sup>	41.58 <sup>-</sup>
41.2	40.63 <sup>-</sup>	41.48 <sup>-</sup>
39.4	54.79 <sup>-</sup>	60.74 <sup>-</sup>
37.2	52.62 <sup>-</sup>	57.37 <sup>-</sup>
35.6	80.13 <sup>-</sup>	76.44 <sup>-</sup>
34.7	63.13 <sup>-</sup>	68.19 <sup>-</sup>
33.9	72.16 <sup>-</sup>	72.89 <sup>-</sup>
32.1	80.83 <sup>-</sup>	80.83 <sup>-</sup>
29.6	72.07 <sup>-</sup>	70.03 <sup>-</sup>
27.6	12.84 <sup>+</sup>	14.22 <sup>-</sup>
24.6	28.91 <sup>+</sup>	1.46 <sup>-</sup>
23.1	60.49 <sup>-</sup>	46.85 <sup>-</sup>
19.0	35.60 <sup>+</sup>	34.65 <sup>+</sup>
15.1	61.35 <sup>-</sup>	64.13 <sup>-</sup>
14.5	100.00 <sup>-</sup>	100.00 <sup>-</sup>

Proteins increased (+) and decreased (-) in the parasitized host as compared to the control after 24 and 48 h.

5 protein bands (229.9, 215.6, 27.6, 24.6, and 19.0 kDa) increased in the hemolymph of parasitized larvae. These important differences in the quantity of proteins post-parasitism are illustrated in graph form in Figure 2; however, the level of some other proteins remained constant, regardless of parasitism.

**Discussion**

Physiological and biochemical interactions within host-parasitoid systems are important to the success of biological control processes (Hentz et al., 1998; Uçkan and Gülel, 2002; Salvador and Consoli, 2008). Therefore, the effects of parasitization by hymenopteran species on the protein concentration and profile in lepidopteran hosts have been investigated extensively, especially in the case of endoparasitoids (Rolle and Lawrence, 1994; Bischof and Ortel, 1996; Vinson et al., 2001; Consoli and Vinson, 2002; Rahbe et al., 2002; Park et al., 2005; Kaleli et al., 2007; Salvador and Consoli, 2008). On the other hand, the effects of parasitism by ectoparasitoids on hemolymph proteins in their hosts have received comparatively less attention (Kanost et al., 1990; Coudron et al., 1997; Richards and Edwards, 1999; Nakamatsu and Tanaka, 2003).

Insect hemolymph contains a number of proteins involved in diverse developmental, physiological, and immunological processes. Some of these proteins have

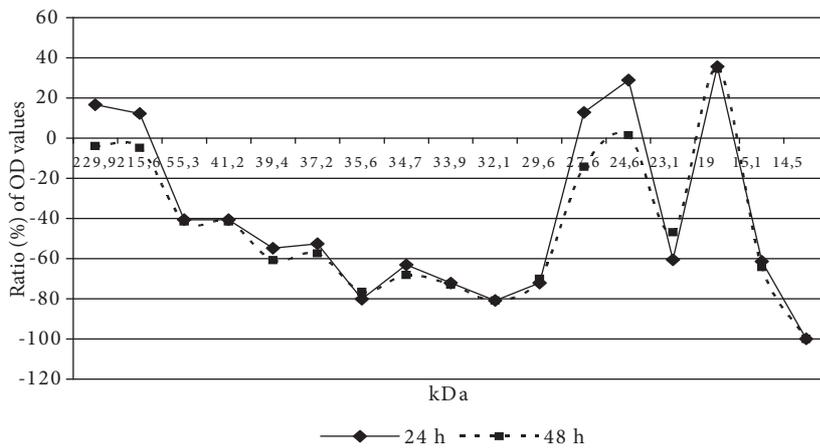


Figure 2. Significant changes in the level of some proteins post-parasitism given in Table 2.

been isolated and their dual functions in hemolymph were confirmed (Riddiford, 1983; Rosell and Coons, 1991). Previous studies reported that the total quantity of hemolymph protein is reduced during ectoparasitism (Coudron et al., 1997) and endoparasitism (Beckage and Kanost, 1993); however, according to the present results, the total quantity of hemolymph protein decreased slightly following parasitism.

Baker and Fabrick (2000) reported that parasitization of *Plodia interpunctella* last instar larvae by *Habrobracon hebetor* did not qualitatively change plasma proteins when analyzed by SDS-PAGE, indicating that parasitization did not induce any novel hemolymph proteins. The fact that we did not observe any novel hemolymph proteins in the present study supports the results of this earlier study. On the other hand, we observed for the first time that a 14.5-kDa protein disappeared in host hemolymph after parasitization; this protein might have been broken down or immunosuppressed by the feeding parasitoid larvae to ensure its normal development

Proteins between 75 and 55.3 kDa may include storage proteins, such as hexamerins (Kanost et al., 1990; Nakamatsu and Tanaka, 2003). Only one of the proteins in this size category, a 55.3-kDa protein, significantly decreased in concentration in the hemolymph of our parasitized hosts. Hexamerins are synthesized in the fat body, secreted into the larval hemolymph, and taken up again by the fat body shortly before pupation. Within the pupal fat body, these proteins are initially stored in protein granules, and are later proteolytically broken down to supply the amino acids necessary for the completion of adult development (Martins et al., 2008; Pick et al., 2008). Consequently, the 55.3-kDa protein may have been consumed by parasitoid larvae as an amino acid resource for development.

Arylphorin is another storage protein with several subunits that was affected by developing parasitoid larvae as a result of nutrient usage (Dong et al., 1996). According to Beckage and Kanost (1993), in the hemolymph of parasitized *Manduca sexta* larvae the level of some subunits of arylphorin, such as apolipophorin I (approximately 240 kDa), apolipophorin II (approximately 78 kDa), apolipophorin III (approximately 22 kDa), and serpin (approximately 75 kDa), were changed slightly.

Furthermore, insecticyanin (approximately 19 kDa) was detected at higher levels in the hemolymph of parasitized host larvae. The results of the present study are similar in terms of the differences observed in the relative abundance of the 229-, 75-, 41.2-, 39.4-, 37.2-, 35.6-, 34.7-, 33.9-, 32.1-, 29.6-, 23.1-, and 19.0-kDa proteins. Additionally, the level of the 24.6- and 19-kDa protein bands increased significantly, which confirms the results of a previous study (Nakamatsu and Tanaka, 2003). These bands may be defensive proteins, such as insecticyanins, which are synthesized by the parasitized host as an immune reaction in response to ectoparasitoid larvae. An alternative explanation has been suggested by Nakamatsu and Tanaka (2003), who suggest that these proteins may be related to ectoparasitoid venom that contains a diverse range of proteins and peptides. A previous study demonstrated that the venom of the ectoparasitoids *Euplectrus comstockii* and *E. plathypenae* affected the level of hemolymph storage proteins in *Heliothis virescens* larvae (Coudron et al., 1997). Quistad et al. (1994) identified 6 paralytic toxins in the venom of the ectoparasitoid *B. hebetor*, with at least 2 of the isolated proteins demonstrating high insecticidal activity toward 6 species of lepidopteran larvae. In addition, 9 major reacting proteins (47.5-9.2 kDa) detected in the female ectoparasitoid *Eupelmus orientalis* were also found in the hemolymph proteins of the host larvae stung by *E. orientalis*. The quantity of protein synthesized in host larvae stung by female *E. orientalis* was maximal 12 h after envenomation (Doury et al., 1997). In other studies venom proteins are reported to have an immunosuppressive role (Asgari et al., 2003; Parkinson et al., 2003) or even induce host castration (Digilio et al., 2000). Similarly, in the present study elevation in the quantity of protein may have been the result of the effects of paralytic toxins in the venom.

The present study shows that parasitism had several effects on the host hemolymph. These could be related to parasitoid nutrition, development, and immunosuppression in the host. A decrease in some hemolymph proteins in host larvae following parasitism is predictable, while an increase in other proteins could be related to decomposition of the fat body and tissue proteins, or these proteins could also be affected by parasitoid venom for host regulation. Further investigations are required to identify these proteins and their activity using various assays.

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