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Research Article

Impact of four diets on pupal and larval total protein and digestive α-amylase activity in *Ephestia kuehniella* (Lepidoptera: Pyralidae)

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Abstract: Mediterranean flour moth, *Ephestia kuehniella* Zeller, is important for mass rearing of parasitoid insects such as *Bracon* wasps and it's feeding on cereals leads to economic losses in flour mills. In current research, the effects of four different diets: wheat, barley, oat, and maize flours, on protein content of larval and pupal whole-body, gut and fat bodies of the last instars and the digestive α-amylase activity were evaluated. In addition, their protein patterns were compared using polyacrylamide gel electrophoresis (SDS-PAGE). The protein contents of fifth instars whole-body, fat bodies, gut, and also pupa varied in different diets. On the whole, the lowest protein levels were found in all experiments in oat and the highest in barley and wheat. Other biological parameters such as insect weight and digestive α-amylase activity were also significantly affected by the decrease in protein content. The results obtained from the estimation of protein content by a colorimetric method and SDS-PAGE were consistent with each other; the low or high protein contents were also clearly visible in the gels. According to the results of enzyme activity and protein contents of insects reared on different diets, barley and wheat are reported to be appropriate diets for this pest, while oat and maize are not suitable diets.

Keywords: Mediterranean flour moth, protein, fat bodies, α-amylase activity, SDS-PAGE

Introduction

The Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), is an important and a common stored-product moth with worldwide distribution. Cereals with high level of carbohydrates, especially starch, are the most important food sources of this species. The feces and webbings of larvae cause additional contamination of stored-products (Rees, 2003).

Nutritional needs of an individual or group of insects are shaped by their physiology, behavior, and performance (Lihoreau *et al*., 2015). The size to which an individual insect grows is affected by both genetic and environmental factors that operate through complex molecular and physiological mechanisms. Moreover, food quality affects many life-history traits such as larval and adult performance (Moreau *et al*., 2006). High food quality enhances the rate of development and increases survival in some insect species (De Haas *et al*., 2006).

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Fat bodies play a number of important roles during insect life e.g., synthesis, storage and release of proteins. These proteins composed of amino acids that are used in the morphogenesis process (Tojo, 1971; Chapman, 2013). In addition, the fat bodies monitor and respond to the physiological needs of insects during different life stages and under different biological conditions, thereby coordinate insect growth, metamorphosis and reproduction (Keeley, 1985). Generally, 90% of the hemolymph proteins are made by fat bodies. During transition from larva to pupa, the secretion of proteins into the hemolymph stops and stores granularly in the fat bodies (Chapman, 2013).

Proteins are found in gut, hemolymph and fat bodies of insects and play different physiological roles at different life stages. The amount and type of proteins in different developmental stages and impact of diet on them as well as the activity of digestive enzymes of different insects have been studied. Chippendale and Beck (1967) examined the fat bodies protein concentration of the European corn-borer, *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae), during diapause and pre-pupal period. Also, Chippendale and Kilby (1969) examined the relationship between hemolymph and fat bodies protein content during development of the large white, *Pieris brassicae* L. (Lepidoptera: Pieridae). Turunen (1978) investigated hemolymph soluble proteins of the large white and showed that the amount and type of hemolymph proteins and lipoproteins varied significantly during developmental period. Bouayad *et al.* (2008) investigated the effects of four different diets including wheat flour, dates, sorghum, and barley on protein and glycogen synthesis, α-amylase activity and post-embryonic development of the Indian meal moth, *Plodia interpunctella* Hübner (Lepidoptera: Pyralidae) and resulted that protein content and α-amylase activity were lower in larvae reared on dates compared to the other diets. Kumar *et al.* (2011) reported different protein patterns related to developmental stages of insects, in different tissues including: hemolymph, fat bodies, midgut and reproductive organs of the tasar silkworm, *Antheraea mylitta* Drury (Lepidoptera: Saturniidae). Also, in their experiments, the amount of protein in eggs laid by newly emerged adults was significantly different from that laid by three-days-old adults. Wang *et al.* (2010) found that the fat bodies protein content of the common fruit fly, *Drosophila melanogaster* Meigen (Diptera: Drosophilidae), gradually increased during larval development and increased more rapidly during early pupal period. It seems that sudden increase in amount of fat bodies proteins in early pupal period is due to the change in fat bodies function from producer to the storage organ. In another study on the amount of hemolymph protein of the Confused flour beetle, *Tribolium confusum* du Val (Coleoptera: Tenebrionidae), it was found that hemolymph protein content increased gradually during larval development and peaked in the last instar, but decreased at the pupal and adult stages (Lingampally *et al.*, 2013). Karol *et al.* (2018) studied the black solider fly, *Hermetia illucens* L. (Diptera: Stratiomyidae), and showed that the amount of larval crude protein content was affected by concentration of diet nutrients and insect density.

Maintaining the integrity of the insect's digestive system is very important because of its important role in obtaining food, otherwise the insect life cycle will be disrupted. Therefore, digestive enzymes in insects are an important target point in pest management (Becker-Ritt and Carlini, 2012). Amylases are the most common polysaccharidases that digest starch and glycogen. Insect digestive amylases are α-amylases (α-1,4-glucan-4-glucanohydrolases, EC 3.2.1.1). Alpha-amylases are endo-amylases that hydrolyze α-1,4 glycosidic bonds in polysaccharides

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(Carlini and Grossi-de-Sa, 2002; Franco *et al*., 2002).

Due to the importance of the Mediterranean flour moth as a substitute host for the laboratory rearing of parasitoids and predators aimed for biological control and its irreparable damage as a stored pest, we considered it necessary to investigate the impact of different diets on protein content in whole body of larvae and pupae, fat bodies and gut of the last instars of this insect were evaluated. The effect of diet on α-amylase activity of last instars was also investigated. The results were evaluated by analyzing the protein pattern of the samples in polyacrylamide gel electrophoresis.

Materials and Methods

Insect rearing

The Mediterranean flour moth larvae were reared in the plant protection laboratory of University of Tabriz. Insect colony was maintained at 28 ± 2 °C, 50 ± 5% R. H. and photoperiod of 16:8 h (L: D). In this study, four diets including wheat, oat, barley and maize flours (70%), plus bran (15%) and yeast (15%) were used.

Preparation of protein and enzymatic samples

For whole-body samples, 10 fifth instars reared on different diets were weighed and placed in a microtube containing 400 µl distilled water. Moreover, 7 pupae obtained from larvae reared on different diets, were weighed and placed in 300 µl distilled water. For preparation of gut and fat bodies samples, again 10 fifth instars were cold-immobilized on ice for 10 minutes and carefully dissected in 50 µl distilled water under stereomicroscope. Guts of larvae were separated and ground up in a microtube containing 100 µl of distilled water. Also, parietal and perivisceral fat bodies of larvae were collected with brush. Then, preparations were homogenized and centrifuged at 10,000 rpm for 30 min at 4 °C. The supernatants were stored at -20 °C for further use as protein and enzyme source (Ashouri *et al*., 2017).

Protein concentration determination

The protein concentration of the samples was measured according to the Bradford (1976) method, using bovine serum albumin as a standard. 10 µl of 50 × diluted samples of gut, fat bodies, and larval and pupal whole-body samples were mixed with 190 µl of staining solutions. Optical densities were measured at 595 nm using ELISA reader, BioTek® ELx800 (Winooski, Vermont, USA). The samples protein content was estimated by using the measured optical density and standard protein curve equation.

Polyacrylamide gel electrophoresis

Protein pattern of prepared samples was detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed according to Laemmli (1970) using 12% stacking and 4% resolving gels. Samples were denatured by heating at 95 °C for 5 min and β-mercaptoethanol was used to break disulphide bonds. Electrophoresis was performed at 140 V until the color band of the sample buffer reached to the bottom of the gel. The gels were transferred to coomassie brilliant blue R-250 staining (0.05% w:v in methanol, water, and acetic acid 50:40:10) overnight and destaining (methanol, water, and acetic acid 40:50:10) up to forming of clear bands. Then gels were imaged on the scanner. The apparent molecular mass was measured in the presence of standard protein ladder (11-180 kDa, Sinaclon®, Cat.No. SL7011).

α- amylase activity assay

The α-amylase activity was measured by the dinitrosalicylic acid (DNS) procedure (Bernfeld, 1955), using 1% soluble starch as substrate. The enzyme source (10 µl) was

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incubated for 30 min at 37 °C with 65 µl glycine buffer (0.05 M; pH: 10), and 25 µl soluble starch. The reaction was stopped by addition of 100 µl DNS and heated in a boiling water for 10 min. After cooling for 5 min, absorbance was read at 540 nm. Appropriate blanks (reaction without enzyme extract as control) were run for all investigations. Tests were performed in three biological and technical replications. The enzymatic activity unit is expressed as a percentage of the α-amylase activity related to the wheat diet.

Statistical analysis

Data was analyzed using SPSS Statistics 20 by one-way ANOVA followed by mean comparison with Duncan's multiple range test at 5% probability level. Charts were drawn with Microsoft Excel 2016.

Results

Total protein content

A comparison of means showed significant differences in total protein content of the 5th instars whole-body on different diets ($F_{3,11}$ = 3.751, $p < 0.05$). The lowest protein content was in larvae reared on oat and the other three diets did not differ significantly (Fig. 1A).

The results showed a significant difference between the amount of the 5th instars gut protein in different diets ($F_{3,11}$ = 16.19, $p < 0.001$). The highest amount of protein was in the gut of larvae reared on maize and then in those reared on barley, wheat and oat, respectively (Fig. 1B).

There was a significant difference in protein content of the 5th instars fat bodies in different diets ($F_{3,11}$ = 23.93, $p < 0.000$). The fat bodies of larvae reared on barley had the highest protein and the least amount was related to oat. No significant difference was observed between the protein content of the 5th instars fat bodies in wheat and maize diets (Fig. 1C).

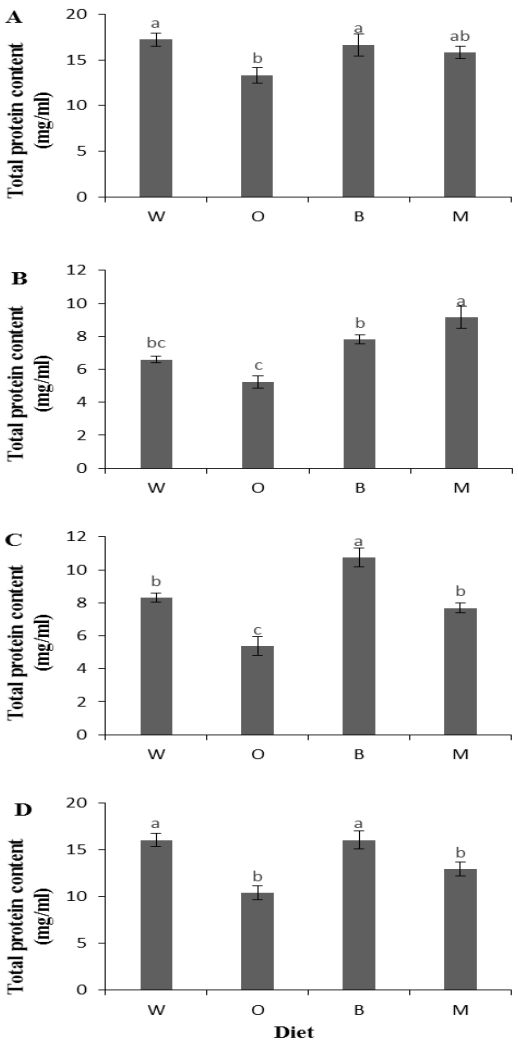

Figure 1 Total protein content of A; larval whole-body, B; gut of last instars, C; fat bodies of last instars, D; pupal whole-body of *Ephestia kuehniella* on different diets. The letters M, O, B and W represent maize, oat, barley and wheat diets, respectively. Means marked with different letters on each column indicate significant differences ($p < 0.05$) based on Duncan's multiple range test.

There was a significant difference between the protein content of pupa in different diets ($F_{3,11}$ = 11.8, $p < 0.003$). The highest amount of pupal protein belonged to the pupa that developed on wheat and barley, respectively, which did not differ significantly. The least protein content was seen on maize and oat diet, with no significant difference (Fig. 1D).

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Effect of different diets on digestive α-amylase activity

The α-amylase activity of the 5th instars gut in different diets showed a significant difference ($F_{3,11}$ = 14.434, p < 0.001). The comparison of means showed that larvae reared on oat and maize diet had the least and highest enzyme activity, respectively. There was no significant difference in the enzyme activity between wheat and barley diets (Fig. 2). The α-amylase activity in the wheat diet was considered as 100% and the other data was calculated based on it.

Effect of different diets on the larval and pupal weight

Weight of the 5th instars reared on different diets showed a significant difference ($F_{3,11}$ = 71.27, p < 0.000). The highest larval weights were respectively related to maize, barley and wheat, and the least amount was related to oat diet (Fig. 3A).

Pupal weight of the Mediterranean flour moth reared on different diets showed a significant difference ($F_{3,11}$ = 8.779, p < 0.007). The highest pupal weight was related to maize and barley and the lowest one was related to wheat and oat diets (Fig. 3B).

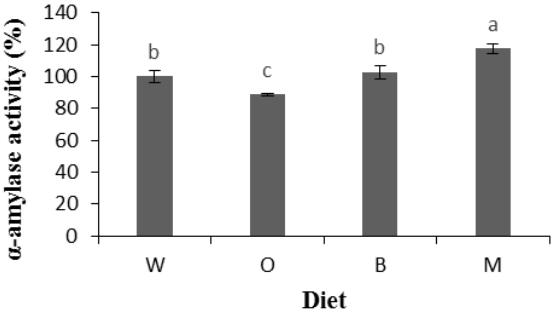

Figure 2 Digestive α-amylase activity of the 5th instars of *Ephestia kuehniella* reared on different diets. The letters M, O, B and W represent maize, oat, barley and wheat diets, respectively. Means marked with different letters on each column indicate significant differences (p < 0.05) based on Duncan's multiple range test.

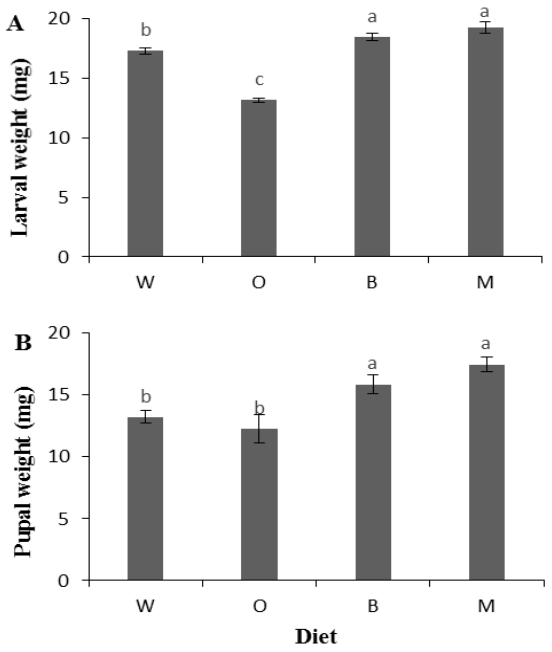

Figure 3 A) Weight of the 5th instars and B) pupal weight of *Ephestia kuehniella* reared on different diets. The letters M, O, B and W represent maize, oat, barley and wheat diets, respectively. Means marked with different letters on each column indicate significant differences (p < 0.05) based on Duncan's multiple range test.

Polyacrylamide gel electrophoresis

Protein pattern of the whole body, gut and fat bodies of the last instars and pupae which fed on different diets were compared using polyacrylamide gel electrophoresis (SDS-PAGE) and are presented in Fig. 4. As shown in Fig. 4A, the intensities of whole-body last instars protein bands in barley, wheat and maize diets were more than that of the oat. Gut protein bands of larvae reared on different diets generally had low intensity (Fig. 4B). As shown in Fig. 4C, protein pattern of the last instars fat bodies fed on oat showed the lowest intensity and barley represented the highest one. Similar results were observed in the case of pupal protein (Fig. 4D). Generally, results of estimating protein content from colorimetric studies according to the Bradford's method and protein patterns of polyacrylamide gels, were in accordance. Low or high protein contents in

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experiments were also clearly evident in gels. Comparing figures 4A and 4D the protein patterns of the whole-body of last instars and pupae had some differences and some of the bands that were observed in pupae did not exist in the larvae and *vice versa*. Due to the existence of numerous bands, explanation of individual bands has been ignored.

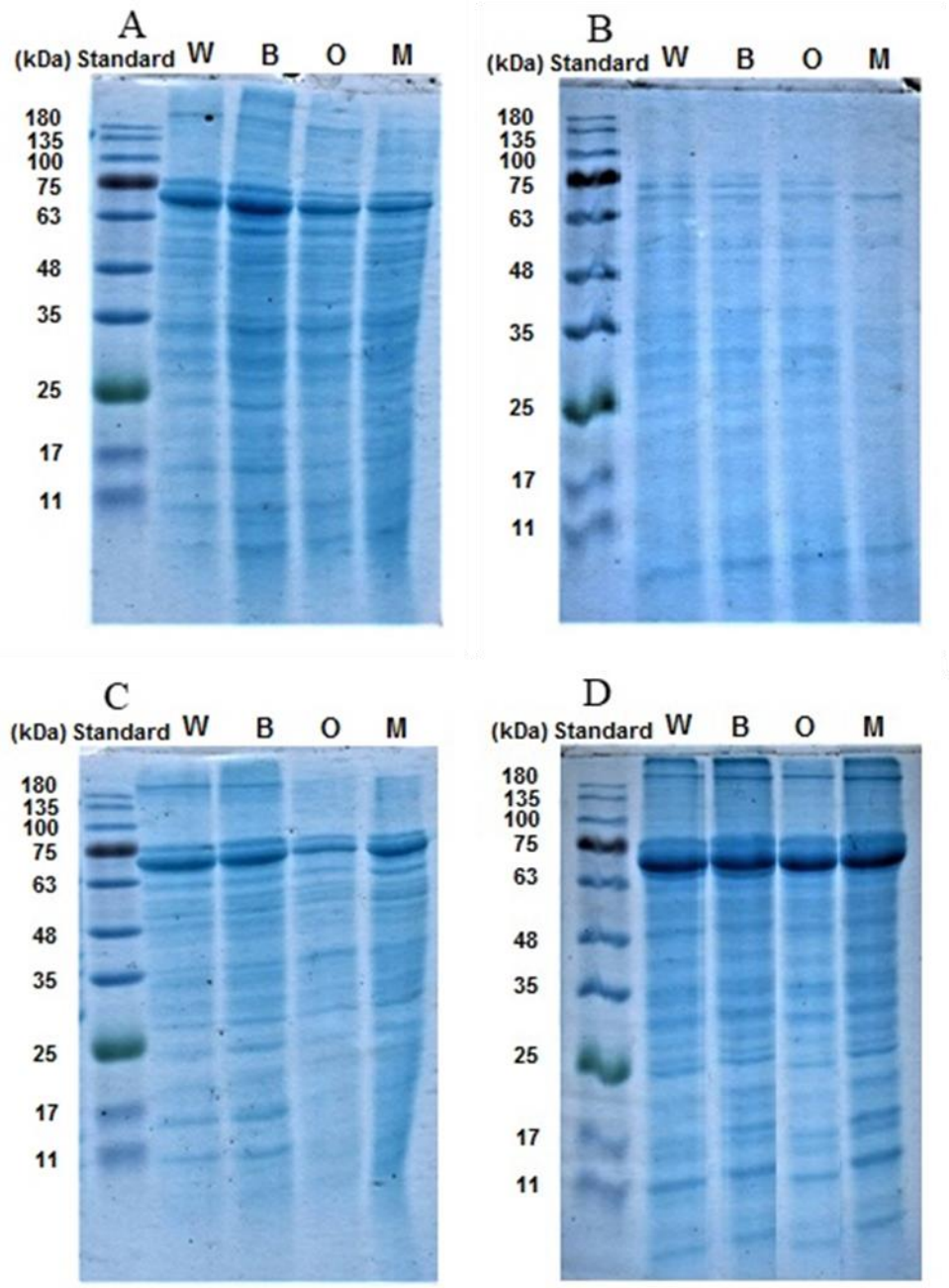

Figure 4 SDS-PAGE for A; larval whole-body, B; 5th instars gut, C; 5th instars fat bodies, D; pupal protein content of *Ephestia kuehniella* fed on different diets. W: wheat, B: barley, O: oat, M: maize. Coomassie brilliant blue was used for staining proteins. The left columns correspond to standard protein ladder (11-180 kDa).

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Discussion

Large amounts of relevant proteins, such as storage proteins used as an amino acid reservoir for morphogenesis, lipophorins responsible for the lipid transport in circulation, or vitellogenins for egg maturation, are secreted by the fat bodies and immature stages of holometabolous species are known to store nutrients needed for adult development in the fat bodies (Arrese and Soulages, 2010). Considering the importance of the Mediterranean flour moth in the breeding of parasitoid insects such as *Bracon* wasps and the economic losses of this pest in flour mills, this insect was chosen for studying diet effects. Performance and body composition of insect larvae depend on quality and quantity of their diet (Barragan Fonseca *et al*., 2018). According to the results of this study, it can be stated that protein content of the 5th instars whole-body, gut, fat bodies and pupae of the Mediterranean flour moth, varies in different diets. In almost all experiments the lowest amount of protein was observed in oat and the highest was in barley and wheat. Barragan Fonseca *et al*. (2018) investigated the effect of dietary nutrient concentration and larval rearing density on protein and fat contents of larvae of the black soldier fly, *Hermetia illucens* L. (Diptera: Stratiomyidae). Also, in their study there were significant differences in larval crude protein content among the three diets. But according to the study of De Grandi-Hoffman *et al.* (2010) on the effect of diet on protein concentration and hypopharyngeal gland development in worker honey bees, *Apis mellifera* L. (Hymenoptera: Apidae) protein amounts and size of hypopharyngeal gland acini did not differ between the two feeding treatments. In the current research, total body protein contents of last instars and pupa, were similar in amount but differed in the kind of proteins. Chippendale and Kilby (1969) showed that between pre-pupation and newly emerged pupae of the large white (*Pieris brassicae*), 4.9 mg of insect haemolymph protein content was reduced and the protein content of fat bodies was increased by 5.5 mg. In fact, during the pupation process, haemolymph-depleted proteins are stored in the fat bodies. Protein analysis results by Chippendale (1970) also showed that the protein content of the fat bodies of southwestern corn borer, *Diatraea grandiosella* Dyar (Lepidoptera: Crambidae), gradually increased during the last days of larval period and peaked in newly emerged pupae.

The activity of digestive enzymes must be effectively controlled in pests. When enzyme activity is inhibited, insect digestion is impaired and their energy production is reduced (Carlini and Grossi-de-Sa, 2002). In this research, biological parameters such as insect weight and digestive enzyme activity were also affected by a decrease in protein content and the highest amounts were recorded in maize and barley diets. Baker (1988) reported that the maize weevil, *Sitophilus zeamais* Motschuls (Coleoptera: Curculionidae), and the rice weevil, *S. oryzae* L., had the lowest α-amylase activities when fed on wheat compared to barley. Wool *et al.* (1986) also showed that the protein content of wheat grains can affect the α-amylase activity of feeding the flour beetles. According to Bouayad *et al.* (2008), protein content and α-amylase activity were lower in Indian meal moth larvae reared on dates compared to other diets and the 4th instars weight was also affected by diet. In this study, larval and pupal weight was also affected by different diets. However, Toews *et al.* (2000) showed that the adult weight of the lesser grain borer, *Rhyzopertha dominica* Fabricius (Coleoptera: Bostrichidae), reared on different wheat cultivars with different protein percentages had no significant differences. However, despite not examining the effect of diets on larval period and adult fertility, the larval period in maize diet was significantly increased and they did not reach to adult stage in the objective observations. In addition to greater increase in quantity and quality of protein in barley and wheat diets compared to maize and oat, an increase in female fertility was also observed (data not shown). Consequently, barley along with wheat is

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introduced as a suitable diet, while oat and maize diets are not favorable for rearing this insect.

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Statement of Conflicting Interests
The Authors state that there is no conflict of interest.

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بررسی اثر چهار رژیم غذایی بر میزان پروتئین کل لارو و شفیره و فعالیت آلفا-آمیلاز شب‌پره مدیترانه‌ای آرد *Ephestia kuehniella* (Lepidoptera: Pyralidae)

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چکیده: با توجه به اهمیت شب‌پره مدیترانه‌ای آرد *Ephestia kuehniella* Zeller، در پرورش حشرات مفیدی مانند زنبورهای براکون و از طرفی زیان اقتصادی این آفت در کارخانه‌های آرد، در این پژوهش تأثیر چهار رژیم غذایی آردهای گندم، جو، یولاف و ذرت بر تغییرات میزان پروتئین کل بدن لاروهای سن آخر و شفیره‌ها، میزان پروتئین روده و اجسام چربی لاروهای سن آخر، و فعالیت آنزیم آلفا-آمیلاز گوارشی روده لاروهای سن آخر مورد ارزیابی قرار گرفت. الگوی پروتئینی نمونه‌های تهیه شده با استفاده از الکتروفورز ژل پلی‌آکریل‌آمید واسرشت‌گر (SDS-PAGE) نیز مقایسه گردید. میزان پروتئین کل بدن لاروهای سن پنجم، اجسام چربی، روده و شفیره در رژیم‌های غذایی مختلف متفاوت بود و کم‌ترین میزان آن تقریباً در تمامی آزمایش‌ها در یولاف و بیش‌ترین آن در جو و گندم مشاهده شد. سایر فراسنجه‌های زیستی نظیر وزن حشره و فعالیت آنزیم گوارشی نیز به‌همراه کاهش میزان پروتئین بدن حشره، به‌صورت معنی‌داری تحت تأثیر قرار گرفتند. نتایج به‌دست‌آمده برای تخمین مقدار پروتئین از مطالعات رنگ‌سنجی و هم‌چنین تصاویر مربوط به ژل‌های پلی‌آکریل‌آمید واسرشت‌گر با هم هم‌خوانی داشتند و کم و یا زیاد بودن میزان پروتئین بدن حشره در آزمایش‌ها به‌طور واضحی در ژل‌ها نیز نمایان بود. براساس نتایج به‌دست آمده از میزان پروتئین و فعالیت آنزیم گوارشی، دو رژیم غذائی آرد جو و آرد گندم رژیم‌های غذایی مناسبی برای این آفت گزارش می‌شوند، این درحالی است که آرد یولاف و ذرت رژیم‌های غذایی مطلوبی برای این حشره نبودند.

واژگان کلیدی: شب‌پره مدیترانه‌ای آرد، پروتئین، اجسام چربی، فعالیت آلفا-آمیلاز، ژل پلی‌آکریل‌آمید

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