

**Orijinal araştırma (Original article)**

**Age dependent DNase activity in larvae, pupae  
and adult stages of Mediterranean Flour Moth  
*Ephestia kuehniella* Zeller (Lepidoptera:  
Pyralidae)**

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**Summary**

Programmed cell death is an important process in normal embryonic development, adult tissue maintenance, maintaining animal homeostasis by controlling cell numbers, removing abnormal cells, and sculpting developmental structures in normal morphogenesis. DNA fragmentation, carried out by DNase activity, is one of the important events during morphogenesis of insect development. In this study, DNase activities of holometabolic *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) were evaluated during its life stages. The tissues of *E. kuehniella* degenerate during larval-pupal metamorphosis. The DNase activities for the 27 day old larvae, 1 day old pupae and adult (youngest individuals) were found to be 0.270, 0.428, and 0.310 kunitz unit (Ku), respectively. The DNase activities of larvae, pupae and adult were found to be 0.376, 0.759 and 0.436 Ku for middle; and 0.522, 1.535 and 1.156 Ku for the oldest age groups, respectively. There was increasing DNase activity of larvae depending on the ages. Age dependent increase in DNase activity was also observed for pupae and adults. Our findings indicated that DNase activity is the leading force for programmed cell death during metamorphosis. When different developmental stages were considered, the highest DNase activity was observed in pupae and the lowest in the larvae.

**Key words:** DNase activity, *Ephestia kuehniella*, programmed cell death

**Anahtar sözcükler:** DNaz aktivitesi, *Ephestia kuehniella*, programlanmış hücre ölümü

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## Introduction

Deoxyribonucleases (DNases), the enzymes that break down DNA molecules, have over time been classified into many different groups based upon the various biochemical properties they exhibit (Laskowski, 1967). DNase enzymes were first identified in mammals; however, proteins with high homology have recently been identified in invertebrates (Evans & Aguilera, 2003). Cleavage of genomic DNA into nucleosomal fragments (DNA ladder formation) is a well known phenomenon (Wyllie, 1980) and is often used as the biochemical marker of apoptosis (Wyllie et al., 1984). The occurrence of nucleosomal DNA fragmentation has been recognized as an important feature of apoptosis. Apoptotic DNA fragmentation has been shown to be catalyzed by multiple DNases (Shiokawa et al., 2006)

It is essential to understand the molecular basis of endonucleases catalyzing the DNA fragmentation in order to elucidate the mechanism of Programmed cell death (PCD). PCD is an important process in normal embryonic development and in adult tissue maintenance of multicellular organisms. It is crucial for plant and animal development, insect and amphibian metamorphosis, organ morphogenesis, tissue homeostasis, ageing, and the removal of infected or damaged cells (Jacobson et al., 1997). The term apoptosis has been used to describe the special features of programmed cell death (Wyllie, 1981). PCD usually proceeds through a stereotypical series of distinct morphological stages that include cellular condensation, DNA fragmentation and the formation of apoptotic bodies (Kerr et al., 1972; Wyllie, 1980). Developmental processes are regulated by many events, including cell proliferation, differentiation, migration and cell death. Cells undergo one or more of these processes. Programmed cell death is an essential event in animal development and is important for maintaining animal homeostasis by controlling cell numbers, removing abnormal cells, and sculpting developmental structures in normal morphogenesis (Takemoto et al., 2007). The decision to live or die is determined by each cell based on a critical balance between evolutionarily conserved death activators and death inhibitors (Martin, 2002; Danial & Korsmeyer, 2004). Cells initiating their own destruction are a common phenomenon in both invertebrate and vertebrate development (Bowen & Lockshin, 1981). Selective cell death is seen as a fundamental process during the embryogenesis (Saunders, 1966) of insects, which involves massive and coordinated cell destruction of the transient tissues (Bowen & Lockshin, 1981).

One of the first studies of PCD characterized insect intersegmental muscle degeneration (Lockshin & Williams, 1964, 1965) in response to the ecdysone pulse that signals the onset of metamorphosis. In *Drosophila* sp. (Diptera: Drosophilidae) the salivary gland is sculpted by caspase-mediated PCD initiated by the steroid hormone 20-hydroxyecdysone (ecdysone)

(Takemoto et al., 2007). During insect metamorphosis, the steroid 20-hydroxyecdysone (ecdysone) activates PCD to eliminate unneeded larval cells (Robinow et al., 1993). *Drosophila* larval salivary glands are an excellent system for studying the genetic hierarchy that is activated by steroids during programmed cell death. A pulse of ecdysone 10-12 hours after puparium formation triggers caspase-mediated programmed cell death of *Drosophila* larval salivary glands (Jiang et al., 1997).

Insect metamorphosis entails a radical reconstruction of the body plan, from a crawling larva to a highly motile and reproductively active adult fly. The massive destruction of obsolete larval tissues is an integral aspect of this transformation, allowing for their replacement by adult tissues and structures. Apoptotic cells fragment their DNA into units of approximately 200 base pairs or integral multiples thereof well before they die and lyse (Kerr et al., 1972; Wyllie, 1980). These fragments result from the action of an endogenous endonuclease.

It might be expected that the pattern of enzyme activity in rapidly growing, undifferentiated tissue and adult differentiated tissues would differ. For these reasons it was decided to investigate the type of DNase activity found in a developing immature stages as compared to the activity of pupae and adult of the same species. In the current study we investigated the DNase activity in larvae, pupae and adult stages and our goal is to clarify the involvement of DNase in apoptosis associated with *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) metamorphosis.

## **Material and Methods**

### ***Ephestia kuehniella* culture**

*Ephestia kuehniella* were reared on a mixture consisting of 1 kg wheat flour, 55 g yeast and 30 g of wheat germs (Marec et al., 1999). All the insects tested in this experiment were from laboratory stock cultures reared at  $27 \pm 1$  °C,  $70 \pm 5\%$  r.h., and alternating 14 h light: 10 h dark cycles (Yılmaz et al., 2007).

### **Enzyme extraction**

*Ephestia kuehniella* larvae, pupa and adults were used as enzyme source for determination of DNase activity. Approximately fifteen insects were selected uniformly from each stage and ages, and three replicates were used for each treatment. Insects from different stages were put into tubes containing 1ml of 0.1 M Sodium acetate buffer (pH=5) and homogenized on ice. The homogenate were transferred to fresh tubes and centrifuged at 5000 rpm for 10 minutes. After centrifugation, 0.2 ml supernatant was used as enzyme source.

### Enzyme assay

The specific activity of a given DNase preparation reflects the potency of the enzyme per unit mass in degrading double-stranded DNA. Historically, this activity has been expressed in Kunitz units (Kunitz, 1950), where 1 Kunitz unit is the amount of DNase added to 1 mg/ml salmon sperm DNA that causes an increase of 0.001 absorbance units per min when assayed in a 0.1 M NaOAc (pH 5.0) buffer (Kunitz, 1950).

0,2 ml Calf thymus DNA solution (1 mg/ ml) and 0.2 ml supernatant containing the enzyme obtained from each stage were added into tubes containing 1 ml sodium acetate buffer. In control, 0.2 ml Bovine Serum Albumin (BSA) was added instead of enzyme source. The samples were incubated at 37 °C for 30 minutes and 1.6 ml perchloric acid (HClO<sub>4</sub>) were added to each tube to stop the enzymatic reaction and kept at 4 °C for 15 minutes.

Following incubation at 4 °C for 15 minutes, the samples were centrifuged at 5000 rpm for 10 minutes and the spectrophotometric measurement of DNAase activity was carried out at 260 nm.

### Results

In the current study we investigated the DNase activity of different life stages of *E. kuehniella*. DNAase activity of different age groups (27, 30, 34 day old larvae; 1, 3, 7 day old pupae and adult) from each stages were determined.

Mean DNase activity of larvae was found to be 0.270, 0.376 and 0.522 for the age groups of 27, 30 and 34, respectively. Significant differences among DNase activity for different age groups of larval stages were obtained ( $F = 74.781$ ; d.f. =2;  $P < 0.0001$ ) (Fig. 1).

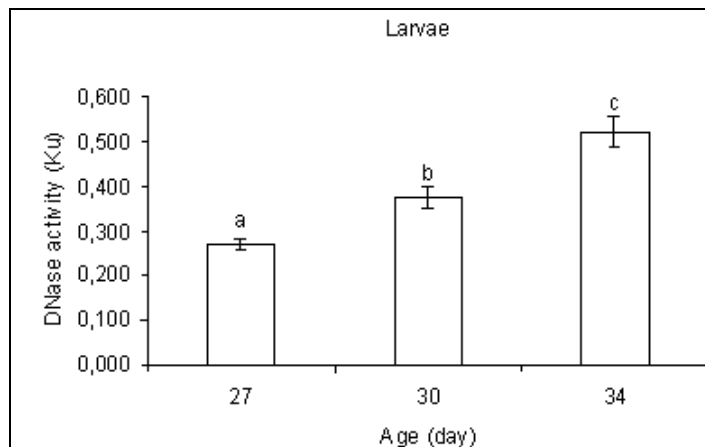


Figure 1. DNase activity of larvae at different ages.

Mean DNase activity of pupae were 0.428, 0.759 and 1.535 for the age groups of 1, 3 and 7, respectively. Age dependent activity increase (0.310, 0.436 and 1.156) was also observed for adult stages. The DNase activity values were statistically significant for different age groups of pupae and adults. (For pupae, F: 84.545, df: 2, P<.0001; for adult, F: 369,295, df: 2, P< 0.0001; Figs. 2 and 3).

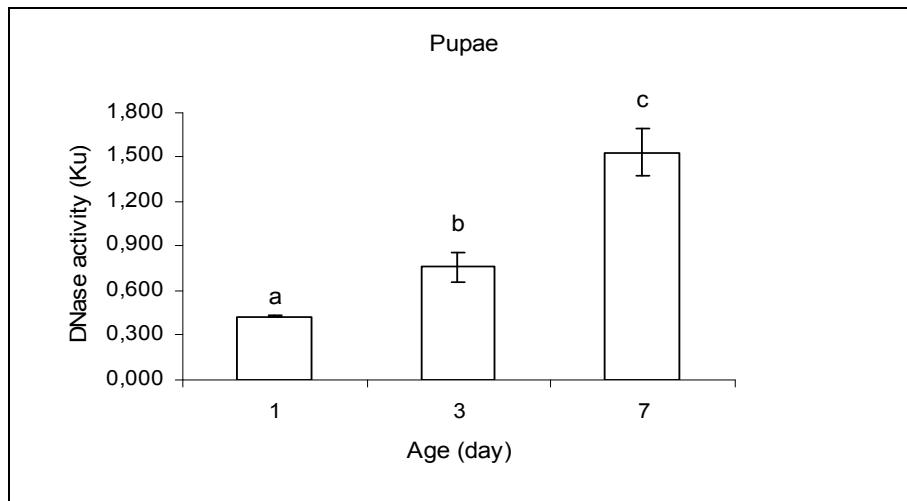


Figure 2. DNase activity of pupae at different ages.

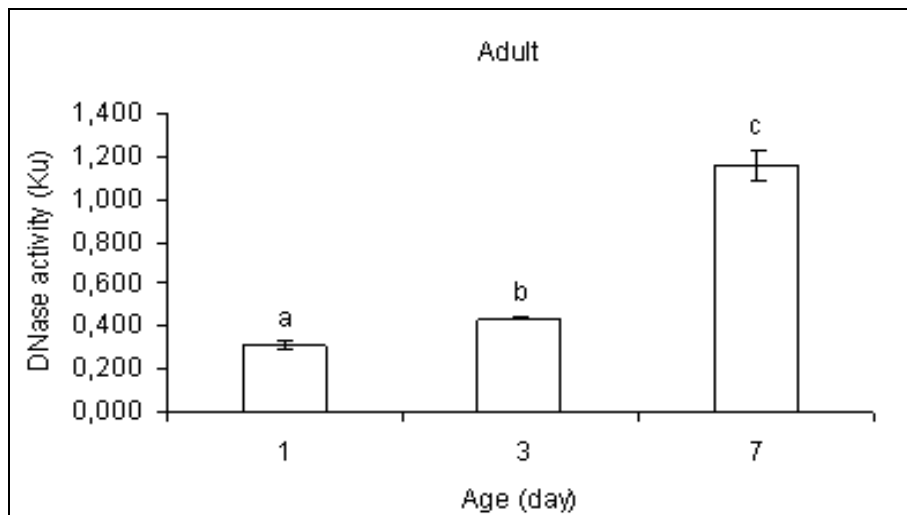


Figure 3. DNase activity of adults at different ages.

The highest DNase activity was obtained in all age groups of the pupal stages. The DNase activities for the 27 day old larvae, 1 day old pupae and youngest adults were found to be 0.270, 0.428, and 0.310 (Ku), respectively. The difference between DNase activity among life stages were also found to be statistically significant ( $F=98.061$ ;  $d.f.=2$ ;  $P<0.0001$ ) and the highest activity observed in pupae for all age groups (Figs. 4-6). That is to say, the DNase activities of larvae, pupae and adult were found to be 0.376, 0.759, and 0.436 Ku for middle; and 0.522, 1.535, and 1,156 Ku for oldest groups, respectively.

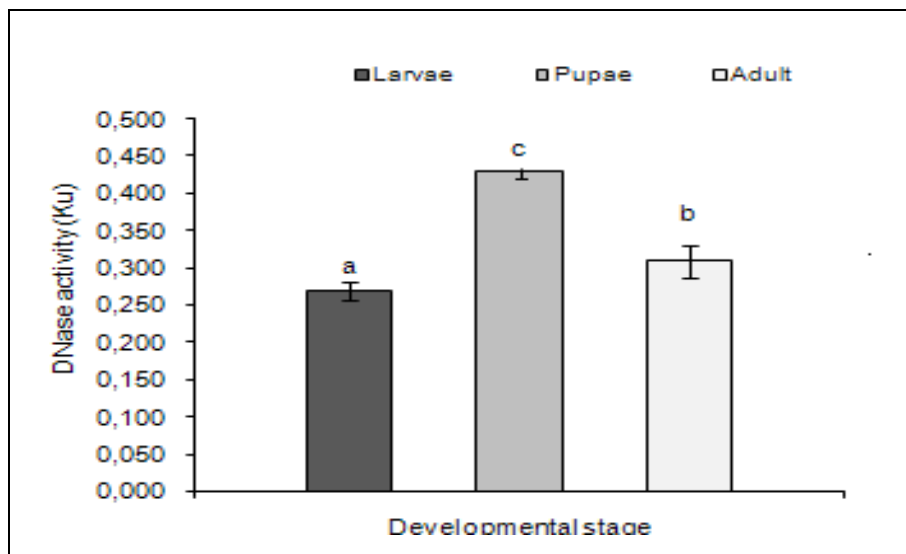


Figure 4. DNase activity of the youngest groups of each stage (27 day old larvae, 1 day old pupae and adult).

In the middle age group, DNase activity of pupae were significantly different from the DNase activity of larvae and adult ( $F=40.451$ ;  $d.f.=2$ ;  $P<0.0001$ ; Fig. 5).

The difference among the DNase activity of larvae, pupae and adult in the oldest age groups were also statistically significant ( $F=75.253$ ;  $d.f.=2$ ;  $P<0.0001$ ; Fig. 6)

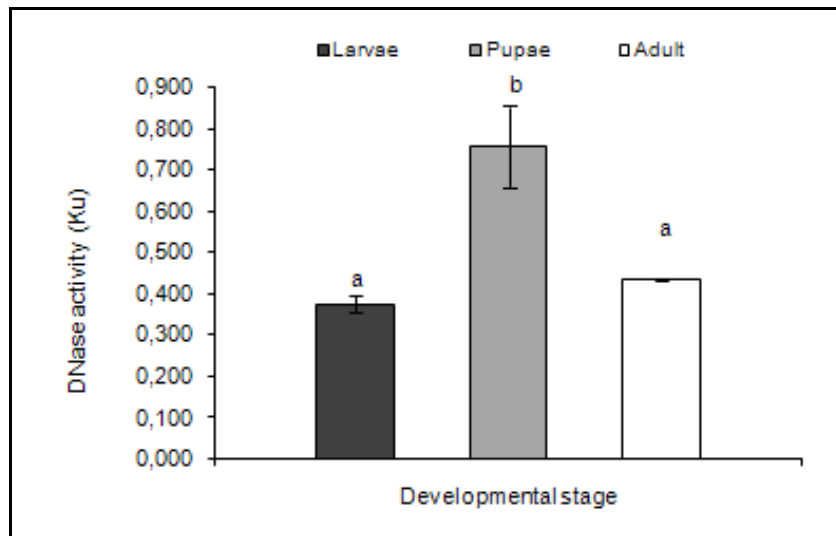


Figure 5. DNase activity of the middle age groups of each stage (30 day old larvae, 3 day old pupae and adult).

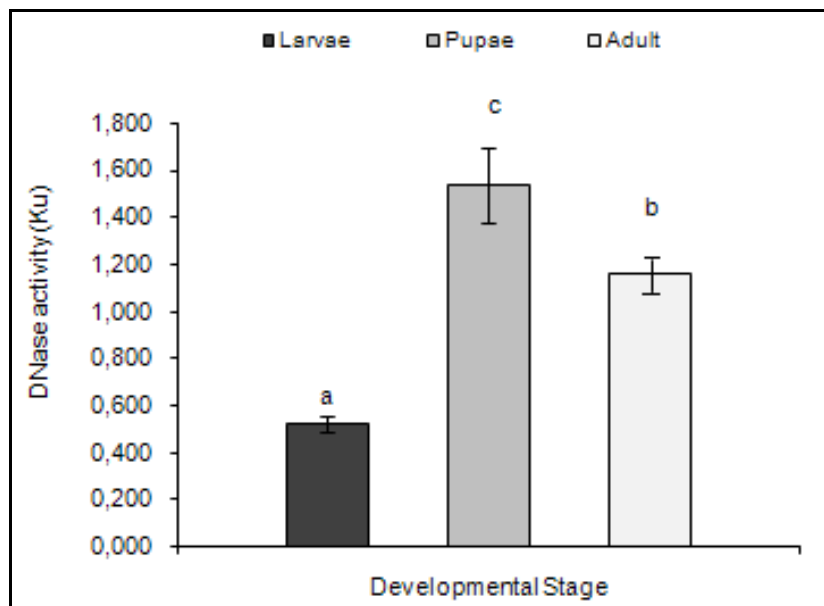


Figure 6. DNase activity of the oldest groups of each stage (34 day old larvae, 7 day old pupae and adult).

## Discussion

In this study, DNAse activities of holometabolic *E. kuehniella* were evaluated in its larval, pupal and adult stages. The tissues of *E. kuehniella*

degenerate during larval-pupal metamorphosis. There was difference among DNase activity of larvae at the ages of 27, 30 and 34 days. Age dependent activity increase was also evident for pupae and adult stages. This result indicates that DNase activity increases at the old individuals of the each stage. Our findings suggest that DNase activity is the leading force of programmed cell death during metamorphosis processes. It is worth to mention that, when the different developmental stages are considered, the lowest and the highest DNase activity observed in larval and pupal stages, respectively. The increased DNase activity in the end of larval life suggests an increased enzyme activity that is restricted to a particular time in development, the beginning of metamorphosis. Detwiler & MacIntyre (1978) reported that DNase activity was found to be developmentally controlled, reaching a maximum during metamorphosis. It was reported that maximal levels of enzyme activity are attained in the blow-fly larvae after the feeding phase has ceased (Bowen et al. 1996). The similar results were also reported in a study carried out by Laufer & Nakase (1965) at the salivary gland development in *Chironomus thummi* Kieffer (Diptera: Chironomidae) during metamorphosis. A class of activities, which is probably of lysosomal origin, is more prevalent in the prepupal tissues. The data suggest that an increased synthesis of lysosomes is a general reaction of most larval tissues at the onset of metamorphosis irrespective of whether a tissue undergoes total histolysis (Boyd & Boyd, 1970)

The reason for the highest DNase activity in pupal stage may originate from the physiological apoptotic cell death. Dai & Gilbert (1997) showed that, cell death of the prothoracic glands of *Manduca sexta* Linnaeus (Lepidoptera: Sphingidae) were related to DNA and nuclear fragmentation, apoptotic body formation, heterophagocytosis, numerous autophagic vacuoles, and intense membrane endocytosis during pupal-adult metamorphosis. Goncu & Parlak (2008) showed that DNA fragmentation is the obvious biochemical marker of apoptosis. In the pupal stage all the tissues are lysed and new tissues are formed, and the activity of nucleases (DNase, RNase), polymerases and ligases involved in nucleic acid metabolism increases. It is reported that the changes in enzyme activity during development reflect changes in concentration of protein rather than the more indirect action of interfering substances that lead to changes which alter enzyme activity (Laufer & Nakase, 1965).

As it is expected, the DNase activity was found to be lowest at the larval period compared to other life stages, since larvae feeds extensively and shows developmental changes in this particular stage. DNase activity was higher in adult stage than that of the larvae. This may be due to the consequence of aging and subsequent metabolic process-dependent tissue destruction. Jiang et al. (1997), indicated that the cessation of feeding at the end of larval development is coupled to the subsequent histolysis of the larval midgut of *M.*



*sexta*. Likewise our result showed that the DNase activity increased following the cessation of feeding.

This study provides a clue for understanding the role of DNase activity in discrete stage and tissue-specific programmed cell death responses of *E. kuehniella* during development. Lee & Baehrecke (2001) demonstrated that the morphological changes in *Drosophila* may be attributed in part to the activity of enzymes typically associated with apoptosis. Our study also indicates that activity of DNase in metamorphic processes of *E. kuehniella* may be evaluated as a response to classic apoptotic cell death. Also these morphological changes may be attributed in part to the activity of enzymes typically associated with apoptosis.

## Özet

### Un güvesi *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae)'nın larva, pupa ve ergin dönemlerindeki yaşa bağlı DNaz aktivitesi

Programlanmış hücre ölümü normal embriyonik gelişimde, ergin dokularının korunmasında, hücre sayısının kontrol edilmesi suretiyle hayvan homeostazisinin sürdürülmesinde anormal hücrelerin uzaklaştırılmasında, normal gelişim sırasında gelişim dönemlerinin şekillenmesinde önemli bir süreçtir. DNaz etkisiyle oluşturulan DNA parçalanması, böcek gelişimi ve morfogenezindeki önemli olaylardan biridir. Bu çalışmada holometabol başkalaşım gösteren *Ephestia kuehniella* Zeller (Pyralidae: Lepidoptera)'nın gelişim dönemlerindeki DNaz aktivitesi incelenmiştir. Larvadan pupaya geçiş sırasında *E. kuehniella*'nın dokuları yıkıma uğrar. 27 günlük larva, 1 günlük pupa ve genç erginlerin (1 günlük) DNaz aktivitesi sırasıyla 0.270, 0.428 ve 0.310 kunitz unit (Ku) olarak bulunmuştur. Larva, pupa ve erginlerin orta yaş grupları için DNaz aktivitesi sırasıyla 0.376, 0.759 ve 0.436 Ku olarak bulunmuşken ileri yaş dönemleri için 0.522, 1.535 ve 1,156. Ku olarak hesaplanmıştır. Larva, pupa ve ergin dönemlerinde yaşa bağlı olarak DNaz aktivitesinin arttığı gözlenmiştir. Elde edilen bulgular başkalaşım sırasında DNaz aktivitesinin programlanmış hücre ölümüne neden olan başlıca etkenlerden biri olduğunu göstermiştir. DNaz aktivitesinin en fazla pupa döneminde, en az ise larva döneminde olduğu belirlenmiştir.

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