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Changes in digestive enzyme activities during different developmental stages of the crayfish *Astacus leptodactylus* Eschscholtz, 1823

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ABSTRACT

The present study determined changes in protease, α -amylase and lipase activities during embryonic and post-embryonic developmental stages of the freshwater crayfish *Astacus leptodactylus* Eschscholtz, 1823. The embryonic stages studied comprised Phase III (blastula formation), Phase X (embryos with anlagen of masticatory) and Phase XIV (embryos with strongly developed posterior hepatopancreas lobes). The post-embryonic stages comprised Stage I (no feeding) and Stage II (prior to the onset of feeding). Protease and α -amylase activities were relatively high as compared with lipase activity. Protease activity values showed a steady increase from Phase III to Stage I. α -amylase and lipase activities were low in Phase XIV. The activities of these two enzymes tended to increase just before hatching and reached the highest levels in Stage II. Digestive enzyme activities increased in accordance with maturation of the hepatopancreas and prior to the onset of external feeding. Protease activity was high in the early stage of embryonic development and continued to increase in later stages. On the other hand, α -amylase and lipase activities were low until maturation of the hepatopancreas and then remained high. The results of the present study provide important information on changes in digestive enzyme levels during different developmental stages of *A. leptodactylus*.

Keywords: *Astacus leptodactylus*, Crayfish, Digestive enzymes, Embryonic development

The narrow-clawed crayfish *Astacus leptodactylus* Eschscholtz, 1823 is a commercially important indigenous freshwater species in Turkey and Europe. The entire crayfish production in Turkey is from wild harvests (Harlioglu *et al.*, 2012). Harvest of this species decreased gradually in Turkey since the occurrence of crayfish plague in 1985. According to data from TUIK (2015), the crayfish harvest dramatically declined from 5,000 t yr⁻¹ in 1984 to 532 t yr⁻¹ in 2014. Holdich *et al.* (2009) indicated that non-indigenous European crayfish species outnumbered the indigenous crayfish species by 2:1 and that non-indigenous crayfish species would become the dominant species unless steps were taken to protect indigenous crayfish. Therefore, studies need to focus on culture of indigenous crayfish species. To understand the nutritional requirements of egg-bearing females and early-stage larvae, it is important to know about the changes in the enzyme activities during early developmental stages (Dai *et al.*, 2009). Hammer *et al.* (2000) indicated that changes in digestive enzymes during early development would reflect changes in digestive capabilities. A number of studies suggested that information on digestive enzyme activities is important to determine the dietary component and to understand the nutritional requirements of an organism (Galgani and Nagayama, 1987; Glass and Stark,

1995; Figueiredo *et al.*, 2001; Figueiredo and Anderson, 2009).

Some studies have reported on the digestive enzyme activity during embryonic development of the crayfish *Procambarus clarkii* and *Cherax quadricarinatus* (Hammer *et al.*, 2000; Luo *et al.*, 2008a; b; Dai *et al.*, 2009). Despite the importance of *A. leptodactylus* in Europe, very little is known about the digestive physiology of this species, particularly during its early life history stages. The aim of the present study was to determine changes in digestive enzymes activities during embryonic and post-embryonic developmental stages of *A. leptodactylus*, in order to gain knowledge on its digestive physiology and nutritional needs during early developmental stages.

Crayfish (*A. leptodactylus*) were collected using traps from Egirdir Lake, Isparta, Turkey, a natural habitat of the species, in February 2015. Egg-bearing females ($n = 6$) of the same embryonic stage were separated from the others. Selected healthy females were then transferred to the aquaculture laboratory of Egirdir Fishery Faculty of Suleyman Demirel University. Broodstocks with a mean total length of 15 ± 1 cm were stocked in 70 l tanks ($n=2$ in each tank). The water temperature was maintained between 9 and 16°C (mean

of 13.5°C). Dissolved oxygen ranged from 8 to 10 mg l⁻¹. Shelters were placed in broodstock tanks and the water was well aerated. During incubation period (142 days), broodstock were fed a commercial shrimp feed which had 38% protein and 8% lipid. Eggs of six females were used for enzymatic analysis. Each sample was assayed in triplicate. The eggs and larvae were photographed using a stereomicroscope (Nikon SMZ-U DIA STAND). Embryonic and post-embryonic developmental stages were: assessed according to Celada *et al.* (1987; 1991) and Holdich (1992) (Fig. 1). The embryonic stages were: Phase III (blastula formation), Phase X (embryos with anlagen of masticatory) and Phase XIV (embryos with strongly developed posterior hepatopancreas lobes). The post-embryonic phases were classified as Stage I (no feeding) and Stage II (prior to the onset of feeding).

and post-embryonic stages of *A. leptodactylus* were estimated as described by Walter (1984), using casein (10 mg ml⁻¹) in 50 mm Tris-HCl buffer at pH 8.5 as the substrate. The mixtures, including extracts of embryonic and post-embryonic stages of *A. leptodactylus* and substrate were incubated and then the reaction was stopped by the addition of 500 µl of trichloro acetic acid (120 g l⁻¹). α-Amylase and lipase activities were estimated spectrophotometrically using a Beckman Coulter biochemical autoanalyser (Beckman Coulter, ABD) and compatible test kits according to the kinetic reaction principle. The soluble protein concentrations of embryonic and post-embryonic stages of *A. leptodactylus* were determined according to Bradford (1976). Enzyme activities were expressed as specific activity (U mg⁻¹ protein⁻¹).

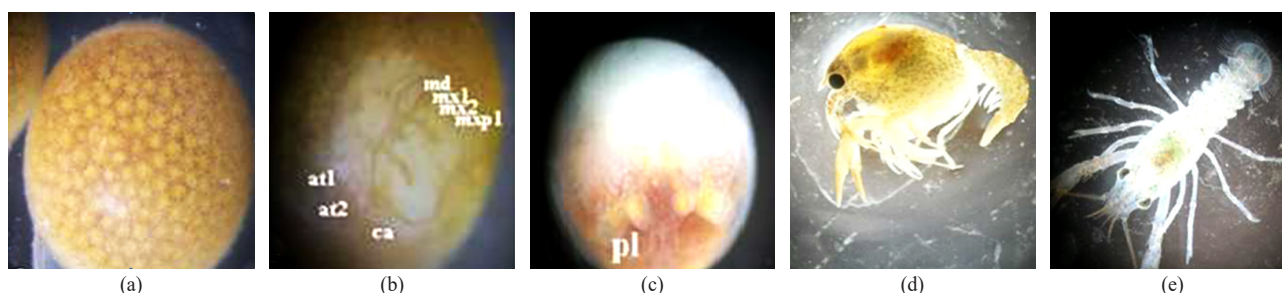


Fig. 1. Embryonic (three stages) and post-embryonic (two stages) development of *A. leptodactylus*

(a) Phase III: blastula; (b) Phase X: embryo with anlagen of masticatory; (c) Phase XIV: embryo with strongly developed posterior hepatopancreas lobes; (d) Stage I: no feeding; (e) Stage II: prior to the onset of feeding (Bahadır Koca, 2013). at1 - first antenna; at2 - second antenna; md - mandible; mx 1 - first maxilla; mx 2 - second maxilla; mxp 1 - first maxilliped; mxp 2 - second maxilliped; mxp 3 - third maxilliped; pl - posterior hepatopancreas lobes; ca - carapace

Egg samples were collected periodically according to the method of Celada *et al.* (1987). Eggs and Stage I samples were removed carefully using a clean forceps and placed in a petridish with a small amount of water. They were blotted dry and weighed to the nearest 0.001 g on a microbalance (Dai *et al.*, 2009) followed by placement in 1.5 ml snap-cap tubes containing ice-cold general enzyme buffer (50 % mM Tris HCl, pH; 8.5). The samples were stored at -80°C until used for analyses (Hammer *et al.*, 2000).

The minimum amount of tissue needed for the analyses was approximately 20 mg. Stage II samples (whole animals) were placed in a volume of ice-cold general enzyme buffer. The samples were then thoroughly homogenised in 1.5 ml snap-cap microfuge tubes using a teflon pestle and immediately placed on ice. The samples were stored at -80°C until analyses (Hammer *et al.*, 2000).

The samples were rinsed in distilled water after thawing and then homogenised and centrifuged (16,000 g, 30 min. at 4°C). Total protease activities of the embryonic

The results were compared by a one-way analysis of variance. Tukey's test was conducted for determining differences of enzyme activities among embryonic stages of the freshwater crayfish *A. leptodactylus*. Differences were regarded as statistically significant at $p < 0.05$.

α-Amylase activity remained constant from Phase III to Phase XIV and tended to increase after Phase XIV ($p < 0.05$) (Fig. 3). α-Amylase activity showed significant increase prior to the onset of feeding ($p < 0.05$) and the highest activity was recorded in Stage II (0.0529 ± 0.015 U mg protein⁻¹) (Fig. 2).

Lipase activity showed significant increase prior to the onset of feeding ($p < 0.05$) (Fig.3). Lipase specific activity increased constantly from Phase III to Phase X and subsequently decreased from Phase X to Phase XIV. The activity increased linearly after Phase XIV ($p < 0.05$) and the highest lipase activity (0.0059 ± 0.0019 U mg protein⁻¹) was recorded at Stage II prior to the onset of feeding ($p < 0.05$).

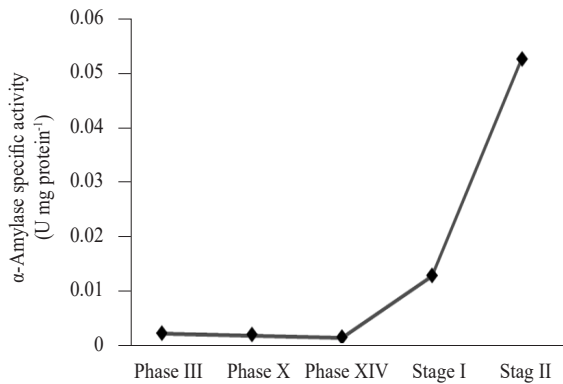


Fig. 2. α -Amylase activity during embryonic and post-embryonic developmental stages of *A. leptodactylus*

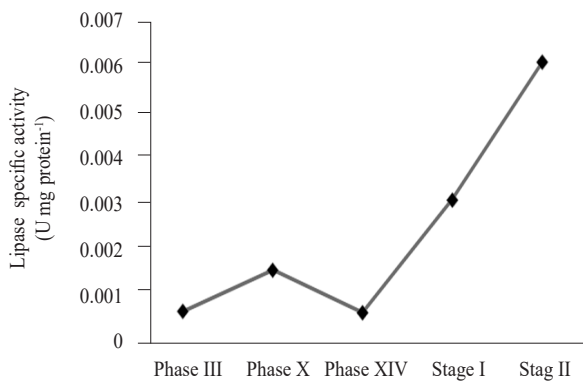


Fig. 3. Lipase activity during embryonic and post-embryonic developmental stages of *A. leptodactylus*

The highest and lowest protease activity values were recorded in Stage I (2.3214 ± 0.0556 U mg protein⁻¹) and Phase III (1.0376 ± 0.1083 U mg protein⁻¹), respectively (Fig. 4) ($p < 0.05$). Protease activity values tended to increase from Phase III to Stage I. The activity was similar for Phase XIV and X as well as for Phase XIV and Stage I. Protease activity of Stage II was not determined due to insufficient sample (Fig. 2).

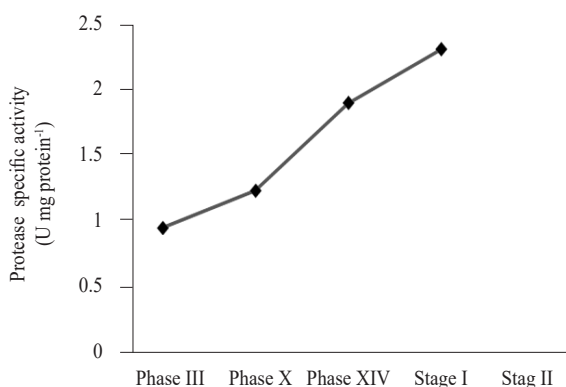


Fig. 4. Protease activities observed during embryonic and post-embryonic developmental stages of *A. leptodactylus*

According to the results of the present study, digestive enzyme activities of *A. leptodactylus* increased prior to external feeding. Similarly, Hammer *et al.* (2000) reported increase in digestive enzyme activities prior to the onset of feeding in *P. clarkii*. The same study revealed that digestive capability was attained prior to feeding and that it could be controlled genetically. Icely and Nott (1992) indicated that the process of digestion aided by digestive enzymes occurred in the foregut and hepatopancreas. Many researchers confirmed that the hepatopancreas is the principle organ for the digestion and absorption of nutrients, as well as the secretion of digestive enzymes (Gibson and Barker, 1979; Dall and Moriarty, 1983; Lovett and Felder, 1989; Icely and Nott, 1992; Hammer *et al.*, 2000). In the present study, the digestive enzyme activities of *A. leptodactylus* increased with development of the hepatopancreas as observed by Hammer *et al.* (2000) in *P. clarkii*.

Discordant findings have been reported with respect to amylase-specific activity in crayfish. Dai *et al.* (2009) reported that amylase-specific activity increased during the middle stages of embryogenesis (stage II, cleavage and blastula; stage IV, egg nauplius) in *P. clarkii*. Hammer *et al.* (2000) indicated that α -amylase-specific activity gradually increased from egg stage to 40 days of embryonic development and further tended to decrease in *P. clarkii*. In *C. quadricarinatus*, highest amylase-specific activity was recorded in the pre-hatching stage (Luo *et al.*, 2008b). In the present study, α -amylase activity was lower than that reported in the above mentioned studies. However, it remained at similar levels before Phase XIV and tended to increase after Phase XIV, *i.e.*, after the development of posterior hepatopancreas lobes. The reason for differences observed in α -amylase activities in *P. clarkii* and *A. leptodactylus* could be attributed to the fact that they are warm and cold water species, respectively.

Low lipase activity observed during embryonic and post-embryonic stages of *A. leptodactylus* was similar to that reported by earlier studies (Luo *et al.* 2008b; Dai *et al.*, 2009). Dai *et al.* (2009) indicated that lipase might not play an important role during embryogenesis of crustaceans. Luo *et al.* (2008b) observed the highest and lowest lipase activity in embryos during Stage V (pigment formation) and Stage III (gastrula formation), respectively. On the other hand, Dai *et al.* (2009) reported decreased lipase activity until the hatching stage. In contrast, in the present study, lipase activity increased gradually after Phase XIV (close to hatching). Results of the present study suggests that lipase activity increase with the formation of hepatopancreas and it gets even higher after the organism starts feeding.

Protease activity was found to increase during embryonic and post-embryonic development of *A. leptodactylus*, in the present study. Luo *et al.* (2008a) also reported that total proteolytic activity increased significantly during embryonic development. The reason for higher protease activity as compared to that of amylase and lipase activities in early stages may be due to the fact that protein forms major structural component of cells (Karl *et al.*, 2014).

Garcia-Guerrero *et al.* (2003) observed that proteins were most abundant (63.2%), followed by lipids (32.3%) and carbohydrates (4.4%) in eggs during embryonic development of the crayfish *C. quadricarinatus*. Zambonino Infante and Cahu (2001) reported that the fluctuations observed in specific activities of enzymes were not due to a diminution in enzyme synthesis but the result of increase in tissue proteins. Protease activity was found to be high in the early stage of embryonic development and continued to increase in later stages. On the other hand, α -amylase and lipase activities were low up to the time of hepatopancreas development, increased subsequently and then remained high. The results of the study provide information on dynamics of important digestive enzyme levels in the different developmental stages of *A. leptodactylus*.

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