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ISSN 0792 - 156X

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PUBLISHER:

Israeli Journal of Aquaculture - BAMIGDEH -  
Kibbutz Ein Hamifratz, Mobile Post 25210,  
ISRAEL

Phone: + 972 52 3965809

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## Activity of the Digestive Protease, Chymotrypsin, in Larvae of the Cultured Sharpsnout Sea Bream (*Diplodus puntazzo*)

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(Received 20.10.07, Accepted 25.11.07)

Key words: sharpsnout sea bream larvae, *Diplodus puntazzo*, chymotrypsin, digestive enzyme, pH

### Abstract

Specific and total activity of the digestive protease, chymotrypsin, was studied in cultured sharpsnout sea bream larvae (*Diplodus puntazzo*) for 35 days from hatching and in a variety of pH levels. Activity was detected upon hatching (2.8±0.34 mm total length), before the onset of exogenous feeding. Specific chymotrypsin activity exponentially increased from mouth opening on day 3 until day 25, then dropped until the end of the experiment. Total chymotrypsin activity sharply increased to day 10, then continued to increase, but slightly, until the end of the experiment. As expected, pH strongly affected both specific and total chymotryptic activity in the digestive tracts of larvae. Both were significantly lower when pH was acidic (1.5, 3.0, 4.0) than when it was alkaline (8.0, 9.0, 10.0) although there were no significant differences within either the acidic or the alkaline range. Our results indicate that chymotrypsin activity is capable of digesting protein before mouth opening and may be a valuable tool for better understanding the nutritional capabilities of young larvae.

### Introduction

A growing number of papers are devoted to the onset and development of digestive enzyme activity in cultured fish larvae. Increasing our knowledge of the nutritional requirements and digestive capacity of larvae

will lead to the development of optimal feeding protocols and improved hatchery production. The digestive functions of marine finfish larvae undergo major developmental changes during the first month of life (Zambonino

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Infante and Cahu, 2001). In at least some species, there may be sufficient levels of main digestive enzymes to allow digestion of prey or artificial diet from the first feeding (Cahu and Zambonino Infante, 1994; Kolkovski, 2001). Digestive enzyme activity can be used as an indicator of food acceptance and, to some extent, of digestive capacity in relation to type of feed. Although structurally similar, trypsin and chymotrypsin recognize different substrates and are specific to pancreatic protein hydrolysis (Nolting et al., 1999; Zambonino Infante and Cahu, 2001). Protease activity is an indicator of the ability of larvae to utilize live food, especially artificial diets. The digestive protease, chymotrypsin, is a convenient short-term indicator reflecting the nutritional condition of larvae and a useful tool for studying nutritional requirements in marine fish larvae (Ueberschar, 1993, 1995; Nolting et al., 1999; Applebaum et al., 2001; Applebaum and Holt, 2003). Recent results indicate the increasing importance of chymotrypsin relative to trypsin in fish larvae; at times, chymotryptic activity may exceed tryptic activity (Lazo et al., 2007).

The sharpshout sea bream (*Diplodus puntazzo*) is a demersal Sparid distributed on rocky bottoms and sea grass beds at depths up to 150 m along the Mediterranean Sea, the Black Sea, the Canary Islands, Cape Verde, and the European and African coasts of the Atlantic Ocean from the Bay of Biscay to Sierra Leone. Generally, it feeds on seaweeds, worms, mollusks, and shrimps (Bauchot and Hureau, 1990). In the Mediterranean, spawning takes place from September to November when the water temperature ranges 20-23°C (Marangos, 1995; Micale et al., 1996). The species is characterized by rudimentary hermaphroditism and is well known as one of the most promising new candidates in Mediterranean aquaculture (Francicevic, 1989; Favaloro et al., 2002; Boglione et al., 2003; Papandroulakis et al., 2004).

The aim of this study was to characterize the pattern of chymotrypsin activities of *D. puntazzo* larvae fed live prey until the beginning of weaning 35 days after hatching.

## Materials and Methods

**Larvae rearing.** Larvae were reared in 1-m<sup>3</sup> conical tanks in a closed seawater system from November 2004 to January 2005 at Teknomar Sea Fish Broodstock Center (Akuvatur Mediterranean Sea Foods, Izmir) for 35 days. During days 0-7, the water temperature was gradually increased from 19.0 to 20°C, during days 8-20 it was further increased to 21°C, by day 30 it reached 22°C, and by day 35 it reached 23°C. Oxygen, salinity, and pH were maintained at >85%, 38.2‰, and 7.7, respectively. Ammonia and nitrite were kept constant at less than 0.01 mg/l. The water in the tanks was static during the first two days. On days 3-12, it was replaced at a rate of 5-7% per day by draining through a 200 µm mesh; the rate was gradually increased with the age of the larvae. The photoperiod was 24 h light per day until algae were no longer added to the tanks, then it was adjusted to 16 h light:8 h dark until the end of the experiment.

After mouth opening on day 3 and until day 25, the larvae were fed rotifers (70% *Brachionus rotundiformis*, 30% *B. plicatilis*) that were cultured with algae and enriched with DHA Protein Selco (Artemia Systems SA, Ghent, Belgium) at a density of 10-15 individuals/ml, plus green water containing *Nannochloropsis* sp., *Chorella* sp., and *Isochrysis* sp. at a density of 2-3 x 10<sup>5</sup> cells/ml. From day 15 to day 30, larvae also received *Artemia* nauplii (AF480, INVE Aquaculture) at 4-6 individuals/ml. From day 25 until end of the experiment, they received *Artemia* metanauplii at 2-4 individuals/ml. Both nauplii and metanauplii were enriched with Protein Selco.

**Sampling.** The growth rate was monitored weekly and at the end of the experiment by sampling 30 larvae from each tank. The specific growth rate (SGR) was calculated as 100(ln final body wt - ln initial body wt)/days. At the end of the experiment, survival was determined by counting the larvae remaining in the tanks.

Larvae (50-250 individuals, depending on age and size) were sampled and pooled for enzyme analysis before food distribution on

days 2, 3, 5, 7, 8, 10, 15, 20, 25, 30, and 35. After the experiment, digestive tracts were isolated (50-100 individuals, depending on age and size) on a glass maintained at 0°C to examine the influence of pH variations from 1.5 to 10.0 on enzymatic activity. The pH tolerance profiles of chymotrypsin were determined by adjusting the pH of substrate buffers.

**Analytical procedure of chymotrypsin.** Whole body homogenates were used for enzymatic assays. Samples were homogenized in 5 volumes (v/w) of ice-cold distilled water. Extracts utilized for enzyme assays were obtained after homogenization of larvae (35 mg/ml) in cold 50 mM Tris-HCl buffer, pH 8.0, followed by centrifugation (13,500 x g, 30 min at 4°C). Chymotrypsin activity was assayed spectrophotometrically using benzoyl-L-tyrosine ethyl ester (BTEE) as the substrate (Worthington, 1982). Absorbance was measured at 256 nm for 5 min. One unit of chymotrypsin activity was defined as 1  $\mu$ mol

of BTEE hydrolyzed per min at 25°C. Enzymatic activities were expressed as specific activity (mU/mg protein) and total activity (mU/larva). Protein was determined by the Bradford method (Bradford, 1976).

**Statistical analysis.** All measurements were carried out in triplicate. Results are given as means $\pm$ SD. The variance homogeneity of the data was performed using Levene's test. Survival was compared by Fischer's chi-square test and enzymatic activity data were compared by one-way ANOVA, followed by Newman-Keul's multiple range test. Differences were significant at 0.05 level. Statistical analyses were performed by SPSS 11.0 software.

## Results

Total length slowly increased until day 25 and then suddenly increased until day 35 (Fig. 1). Weight gradually rose with more rapid increases on days 15 and 30. The weight increase on day 30 coincided with gastric

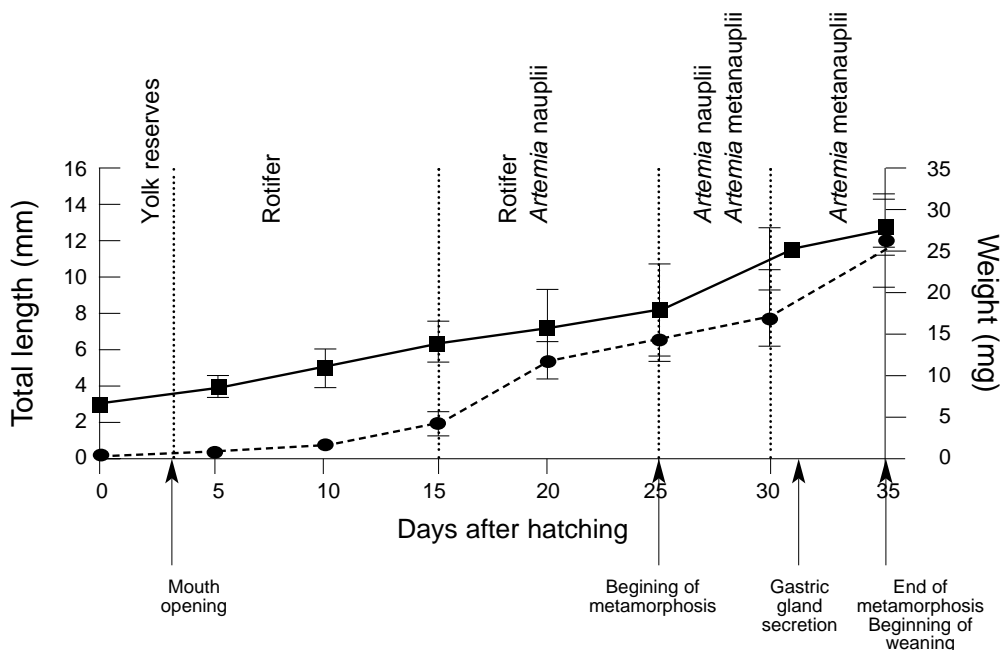


Fig. 1. Growth of sharpsnout sea bream (*Diplodus puntazzo*) larvae in total length (■) and weight (●). Each data point is the means $\pm$ SD of 30 larvae.

gland secretion. Larvae grew more than 20-fold from day 5 to day 35. The SGR averaged 6.9% per day and survival was 27.3%.

Specific chymotrypsin activity was detected immediately after hatching ( $216.38 \pm 46.3$  mU/mg protein) when larvae were  $2.8 \pm 0.34$  mm total length (Fig. 2). It peaked at day 25 ( $1267.53 \pm 188.42$  mU/mg protein) after which it dropped 40% by day 30 ( $p < 0.05$ ) and further declined until the end of the experiment ( $p > 0.05$ ). Total chymotrypsin activity was also detected at hatching. It sharply increased until day 10 ( $0.302 \pm 0.04$  mU/larva;  $p < 0.05$ ), then rose gradually until the end of the experiment ( $p > 0.05$ ). As expected from general characteristics of digestive enzymes, both specific and total chymotryptic activity were affected by pH with the highest activity occurring in pH 8.0 (Fig. 3).

### Discussion

Growth of *D. puntazzo* larvae was satisfactory and similar to that described by Boglione et al. (2003) and Papandroulakis et al. (2004). Survival averaged 27.3% as compared to

$53.7 \pm 7.5\%$  (Papandroulakis et al., 2004) and 18–22% (Franicevic, 1989).

First measured at hatching, chymotryptic activity sharply increased after onset of exogenous feeding on day 3, similar to larvae of other marine fish such as gilthead sea bream (Moyano et al., 1996), sea bass (Zambonino Infante and Cahu, 1994), Senegal sole (Martinez et al., 1999), and red drum (Lazo et al., 2000). Specific activity increased approximately 6-fold from the first detection of activity to the beginning of metamorphosis at day 25. Afterward, it declined about 50% to the end of the experiment. In larva, the secretion of trypsin occurs in response to food ingestion and in pancreatic tissue where most of the trypsin is present as an enzymatically inactive trypsinogen. On the other hand, most trypsin in the intestinal tract is enzymatically active (Ueberschar, 1993, 1995). Therefore, the pattern of specific chymotryptic activity in the current study closely agrees with the pattern of specific trypsin activity in *D. puntazzo* larvae in an earlier study (Suzer et al., 2007a).

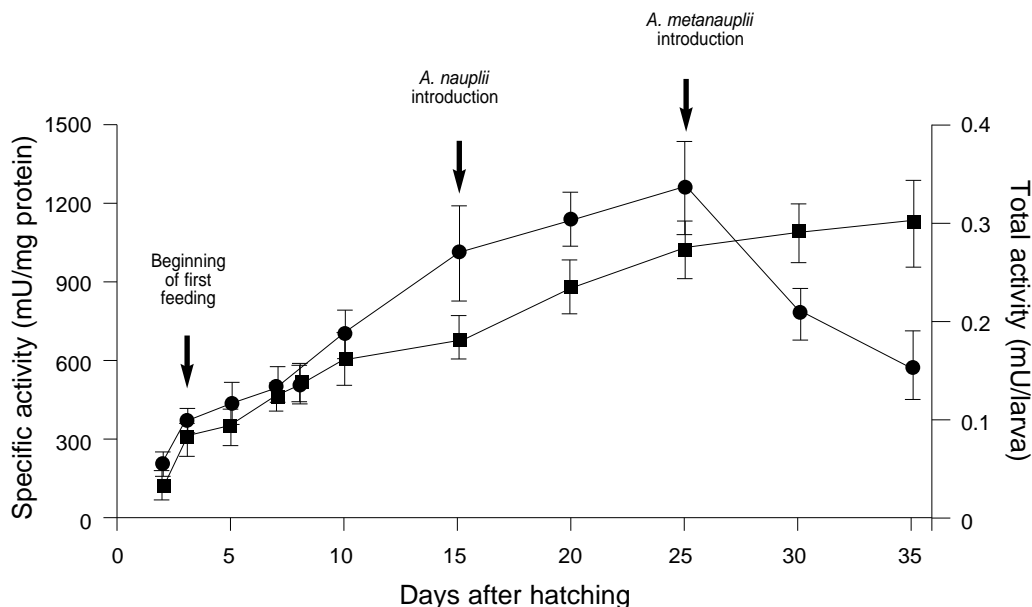


Fig. 2. Specific (●) and total (■) chymotrypsin activity assayed in homogenates of whole sharpsnout sea bream larvae. Results are expressed as means  $\pm$  SD ( $n = 5$ ).

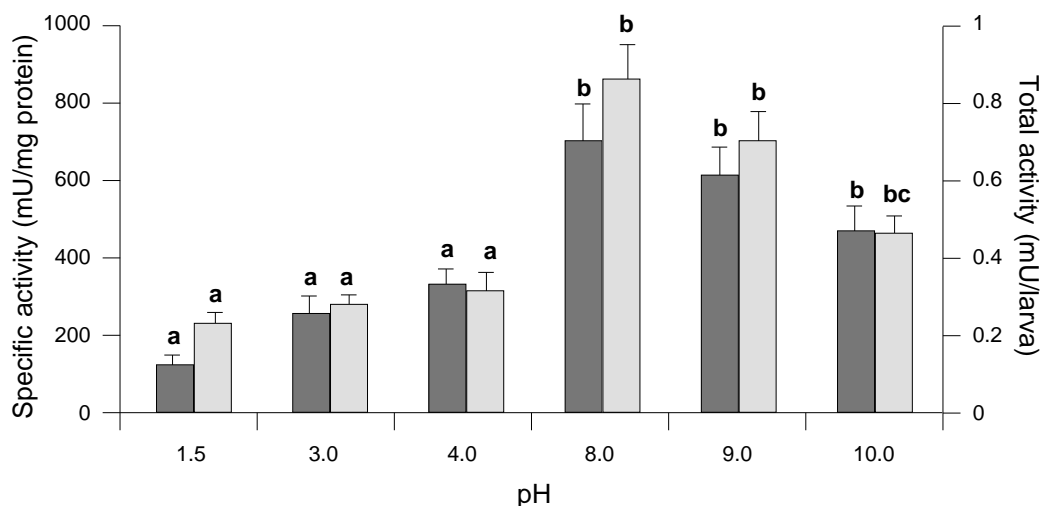


Fig. 3. Specific (■) and total (□) chymotrypsin activity assayed in homogenates of whole sharpsnout sea bream larvae in different pH levels. Results are expressed as means $\pm$ SD ( $n = 5$ ). Different letters above the bars indicate significant differences ( $p < 0.05$ ) between pH levels.

A similar pattern of early increase and later reduction was reported for larvae of Sparids including the common pandora (Suzer et al., 2006) and the red porgy (Suzer et al., 2007b) as well as other marine fish including the herring (Pedersen and Andersen, 1992) and tilapia (Drossou et al., 2006). The onset and changes in trypsin and chymotrypsin patterns in fish larvae may be genetically controlled (Zambonino Infante and Cahu, 2001). The decline in specific enzyme activity of these proteases during larval ontogeny could be explained by the normal increase of tissue proteins in growing larvae, which reflects anatomical and physiological changes and does not correspond to a lowering in the amount of digestive enzymes or dietary shifts (Zambonino Infante and Cahu 2001). On the other hand, total activity increased 10-fold from the first detection of activity to the end of the experiment, i.e., it increased with age and length, in agreement with Zambonino Infante and Cahu (2001).

Like all enzymes, the activity of digestive enzymes is greatly affected by pH. Each enzyme has an optimum pH level at which its activity is maximum. Above or below this

level, activity drops off rapidly and significantly. With some enzymes, a change of 1.0 pH can cause a 50% decrease in activity. Thus, pH has an important effect on the rate and extent of digestion (Deguara et al., 2003; Yufera et al., 2004). In the current study, specific and total activity of chymotrypsin demonstrated similar developmental patterns under different pH values. As expected, both activities were higher in alkaline (pH 8.0-10.0) than in acidic (pH 1.5-4.0) environments, in agreement with Munilla-Moran and Saborido-Rey (1996) who studied the effects of pH and temperature variations on protease activities in redfish, seabream, and turbot.

In conclusion, chymotrypsin was present immediately upon hatching and continuously increased during the larval period, suggesting that chymotrypsin contributes to protein digestion in *D. puntazzo* larvae by synchronously compensating with trypsin for the absence of pepsin until the formation of a functional stomach. Results from this study can contribute to the establishment of feeding strategies and protocol for early stages of marine fish culture, and in studies of early weaning and substitution of live food by extruded microdiet in

marine fish larvae (Cahu and Zambonino Infante, 1994; Zambonino Infante and Cahu, 1994, 2001; Suzer et al., 2007c).

#### Acknowledgements

The authors would like to express our sincere gratitude to the staff of the Teknomar Sea Fish Broodstock Centre where the experiments were conducted (Akuvatur Mediterranean Sea Foods, Izmir) for their most efficient technical assistance.

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