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The Effects of Rate-restricted Feeding Regimes in Cycles on Digestive Enzymes of Gilthead Sea-bream, *Sparus aurata*

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Abstract

In order to understand enzymatic alteration during the restricted-to-fed feeding schedules in cycles, the modulation of key enzyme activities in digestion was studied in different part of gastrointestinal track of juvenile gilthead sea bream (*Sparus aurata*). Juveniles (6.4 g) were stocked into 12 tanks at a density of 16 fish per tank. Four different feeding schedules were tested on triplicate groups of juvenile fish: (1) fish were fed to apparent satiation twice a day throughout 48 days, (2) starvation for 1 day and then re-fed for 2 days (S1), (3) 50% satiation for 2 days and then re-fed to apparent satiation for 2 days (R2), (4) 50% satiation for 6 days and then re-fed to apparent satiation for another 6 days (R6). The activity of total protease, amylase and lipase in the control group was higher than those S1 and R6 in whole gastrointestinal track. The restricted feeding in R2 enhanced the activities of lipase, amylase, and, to some extent, total protease in fish, with the mostly pronounced effect exhibited in the pyloric caeca. Generally, total protease and lipase activity of fish in R2 exhibited no significant difference compared to the control, except amylase activity in mid-intestine. Thus, because of preserved digestive enzyme activities, some food restriction (R2) cycles may be recommended as a routine procedure in commercial production of juvenile gilthead sea bream.

Key words: *Sparus aurata*, restricted feeding, total protease, lipase, amylase.

Introduction

The costs of formulated feed and labour associated with feeding are the major components of the expenditure of carnivore fish production (Love, 1970; Jobling, 1994; De Silva and Anderson 1998). Optimizing the feeding strategy is a prime consideration in intensive carnivorous fish production management due to high protein content in their diet. Protein (as fish meal) is an expensive ingredient in carnivorous marine fish diet. Thus, research in aquaculture nutrition is being directed towards the improvement of feed and protein utilization (Gómez-Requeni *et al*., 2004; Eroldoğan *et al*., 2004; Eroldoğan *et al*., 2006a; 2006b).

Theoretically the maximum feed and protein utilization of fish occurs at a feeding rate above the maintenance feeding level but below the maximum or satiation feeding level (Eroldoğan *et al*., 2004; Rowland *et al*., 2005; Eroldoğan *et al*., 2008). Feed restriction schedules up to 25-50% of satiation without growth suppression are suggested to provide many advantages such as easy feed management, high feed utilization and low water pollution (Pirhonen and Forsman 1998; Einen *et al*., 1999).

The relationship with growth rate related with feed utilization may be the result of specific digestive enzymes' capacity (i.e. total protease, α-amylase, and lipase) which could be caused by changes in protein metabolism and appetite mechanism. In this sense, study of digestive enzymes is an essential step towards understanding the mechanism of digestion and how organisms adapt to changes in the nutritional environment (Sunde *et al*., 2004). Analysis of digestive enzymes activities is an easy and reliable methodology that can be used as indicator of digestive processes and nutritional condition of fish. Changes in digestive enzyme activity in response to periods of fasting may indicate the most critical nutrient and energy reserves, and those metabolized or conserved in the face of increasing food deprivation or restriction (Harms *et al*., 1991; Johnston *et al*., 2004).

There are a few studies focused on the effects of starvation on digestive capacity in fish (Mommsen *et al*., 2003; Krogdahl and Bakke-McKellep, 2005). Some approaches were taken in some cold water species i.e. Atlantic cod, *Gadus morhua*, (Bélanger *et al*., 2002); Japanese flounder, *Paralichthys olivaceus*, (Bolasina *et al*., 2006); and Atlantic salmon, *Salmo salar* (Krogdahl and Bakke-McKellep, 2005). Cyclic restricted feeding regimes in relation to animal digestive enzyme activity have not been studied in any of marine fish species so far. Although the activity of the main digestive enzymes in gilthead sea bream has been recently assessed (Alarcón *et al*., 2001; Munilla-Morán and Saborido-Rey, 1996), little attention has been paid to the changes in digestive enzyme activity of different sections of the gastrointestinal track of this species when restricted feeding schedules were exposed in cycles. Such information is interesting for culturists and feed manufacturers striving to improve the feed utilization.

Therefore, the objectives of the current study were, (i) to examine the effects of the restricted feeding regimes on digestive enzyme activity, (ii) to compare the gastrointestinal sections so as to assess if there are any regional differences in intestinal digestive capacities by measuring activities of total protease, amylase and lipase; (iii) to assess possible digestion of carbohydrate rather than proteins in juvenile gilthead sea bream under restricted feeding condition.

Materials and Methods

Fish, Experimental Design and Feeding

Gilthead sea bream (*Sparus aurata*) juveniles weighing 6.35±0.05 g (mean±SD) were obtained from a local commercial farm and transported to an indoor system where they were held in two 1000-L fiberglass tanks for a period of three weeks (acclimation period) prior to the start of the experiment. Feed (3 mm in diameter) was in the form of sinking extruded pellets manufactured for gilthead sea bream by Çamlı Feed Ltd., Turkey. Proximate composition of the feed was 45% crude protein, 12% crude fat, 12% ash and 12% moisture.

The fish were randomly distributed among 12 circular plastic tanks (155-L), giving 16 fish per tank. Each tank was continuously supplied with flow-through seawater (40 ppt) filtered by 80 µm sand filter at a flow rate of approximately 2 L min^{-1}. Throughout the 48-day experimental period, the rearing water in each tank was permanently saturated with oxygen by supplying air continuously through air-stones from an air-blower. The dissolved oxygen and pH were 6.7-7.5 mg L^{-1} and 7.0-7.7, respectively. Average water temperature ranged from 23.5°C to 24.5°C. The photoperiod was held at 12 dark: 12 light during the experiment.

The treatments implemented in the present study were:

(A) Control: fish were fed to apparent satiation twice a day throughout the experimental period,

(B) S1: starvation for 1 day and then re-fed for 2 days to apparent satiation level (16 cycles throughout 48 days),

(C) R2: 50% restricted feeding for 2 days and then re-fed for 2 days to apparent satiation (12 cycles throughout 48 days),

(D) R6: 50% restricted feeding for 6 days and then re-fed for 6 days to apparent satiation (4 cycles throughout 48 days).

Feeding ratio (% body weight/day) was calculated as follow: 100 x [(dry food fed per day) / ((final weight + initial weight) / 2) / number of day)]. Throughout the experiment, except the satiation feeding periods, the restricted groups always received 50% of the amount of feed consumed by the control group. For example, if the calculated feeding rate for the control was calculated as 5% body weight/day, the amount of feed given to the groups during the next restricted feeding period was adjusted as 2.5% body weight/day.

According to feeding schedules, diet was given twice a day (between 09:00-10:00 and 18:00-19:00) and was achieved by presenting a small quantity of feed every few minutes until the fish ceased to show interest.

Enzymatic Assays

At the end of the experiment, fish were starved for one day and three fish from each tank were sacrificed using a scalpel to sever the spine. Sampling of the digestive tracks was done within the first meal (09:00-10:00). Fish were dissected on a glass cutting board kept on ice. The digestive track and associated organs were immediately removed from the carcass after the fish were killed. The intestines were freed from the other organs and all visible fat removed. The digestive system was then divided into the following sections: stomach (ST), pyloric intestine with caecae (PC) and mid-intestine without distal chamber (IN). Three 0.5 cm^2 pieces from each region were cut, blotted and pooled for four groups of three fish each before homogenizing in an aqueous suspension (5 volumes v/w of ice-cold distilled water). Extracks utilized for enzyme assays were obtained after homogenization of samples (~35 mg ml^{-1}) in cold 50 mM Tris–HCl buffer, pH 8.0, followed by centrifugation (13,500 × g; 30 min at 4°C). The supernatants were transferred to marked test tubes immediately and stored at -20°C freezer until they were analyzed the following day.

The total protease activity was assessed using the casein-hydrolysis method of Walter (1984) as described by Furné *et al*. (2005). The buffers used were KCl–HCl 0.1 M (pH 1.5), glycine–HCl 0.2 M (pH 3.0), citrate 0.1 M-phosphate 0.2 M (pHs 4.0 and 7.0), Tris–HCl 0.1 M (pH 8.5 and 9.0) and glycine–NaOH 0.1 M (pH 10.0). We chose a none-specific technique due to the fact this method enables the quantification of different photolytic activities as a function on pH: the activity of pepsin (acidic pH), chymotrypsin and trypsin activity (neutral or slightly basic pH) and other enzymes such as carboxypeptidases, elastases, and collagenases (basic pH) (Furné *et al*., 2005; De Almeida *et al*., 2006).

The α-amylase (E.C. 3.2.1.1) activity was determined by a starch-hydrolysis method, according to Robyt and Whelan (1968). Maltose was used as standard and the activity unit α-amylase was defined as the quality of enzyme that produced one mmol of maltose ml^{-1} min^{-1} at pH 7.5 and 25°C. Absorbance was determined at 600 nm. This method was previously used to evaluate amylase activity in gilthead sea bream (Moyano *et al*., 1996), rainbow trout and Adriatic sturgeon (Furné *et al*., 2005).

The lipase (E.C. 3.1.1.3) activity was assayed by

the evaluation of the degradation of triacylglycerols, diacylglycerols, and monoacylglycerols to free fatty acids, following the method of Bier (1955) as described in Furné *et al.* (2005). The enzyme activities measured in the intestine are given as specific activities in homogenates calculated as follows: specific activity= enzyme activity (U ml^{-1}) / protein (mg ml^{-1}). Protein was assayed according to Bradford procedure (1976).

Statistical analysis of data was performed with the J.M.P. version 3.2.1 statistical software (SAS Institute, 1996). Followed by a Tukey Kramer HSD test, mean results per tank were subjected to one-way analyses of variance (ANOVA) with feeding schedules as the independent variable. The level of significance was chosen at $P<0.05$, and the results are presented as groups means (n=3).

Results

Throughout the experiment, survival ranged from 98% to 100%. Over the course of 48 days, average weight gains (g) per tank (±S.D.) were 37.8±0.10, 26.9±0.75, 28.7±0.61 and 27.3±0.70 g in control, S1, R2 and R6, respectively. A significant difference in activities is that all three digestive enzymes were found among the tested groups whereas there was no tank variation within the replication of the tanks. Results of total protease activity measured in the stomach (ST), pyloric (PC) and intestine (IN) showed a different distribution of this enzyme along the alimentary track (Figure 1). However, the activity of this enzyme in control and R2 was significantly higher than that of S1 and R6 throughout the digestive track (Figure 1).

Amylase activity was the most important activity identified along the gastrointestinal track, being mainly observed in the PC and the IN extracks. As shown in Figure 2, amylase was detected throughout the gastrointestinal track (ST, PC and IN), but highest amylase activity was found in PC, regardless of treatment (Figure 2). Tukey Kramer HSD test showed that amylase activity in ST was significantly lower in R6 compared to the control, S1 and R2. Specific activity of this enzyme in IN was higher in control compare to all other feeding regimes (Figure 2). As expected, there was no significant difference in lipase activity in ST for all tested groups (Figure 3). On the other hand, it was clear that activity of lipase in PC (14-fold) and IN (5-fold) was higher than in ST, irrespective of treatment. Lipase activity in PC of the control, S1 and R2 was significantly higher than that in R6. Specific activity of this enzyme in IN was higher in control and R2 compare to S1 and R6 (Figure 3).

Discussion

The increasing feed intake and rapid weight gain in fish during the recovery period are often accompanied by improved food conversion (Russell and Wootton, 1992; Jobling, 1994). However, in

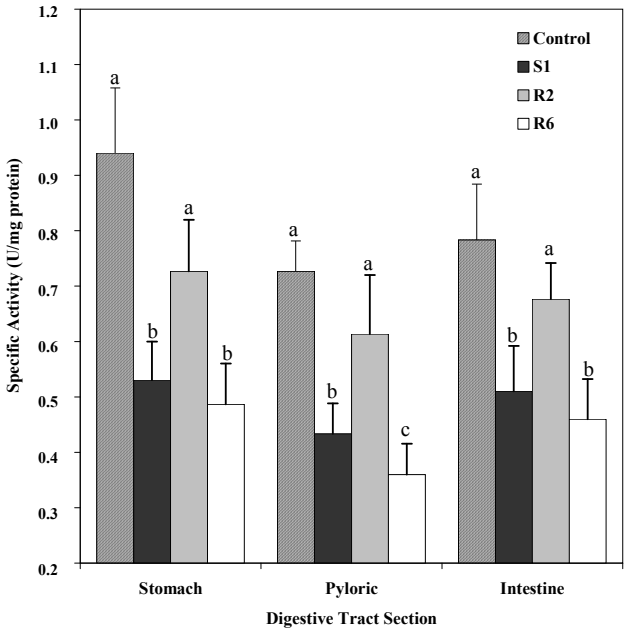

Figure 1. Activity of total protease measured in the different sections of the digestive tract of gilthead sea bream subjected to four feeding regimes during 48 days of rearing. Values are means ± S.D. (n=3, each n consist of measurements of triplicate analysis).

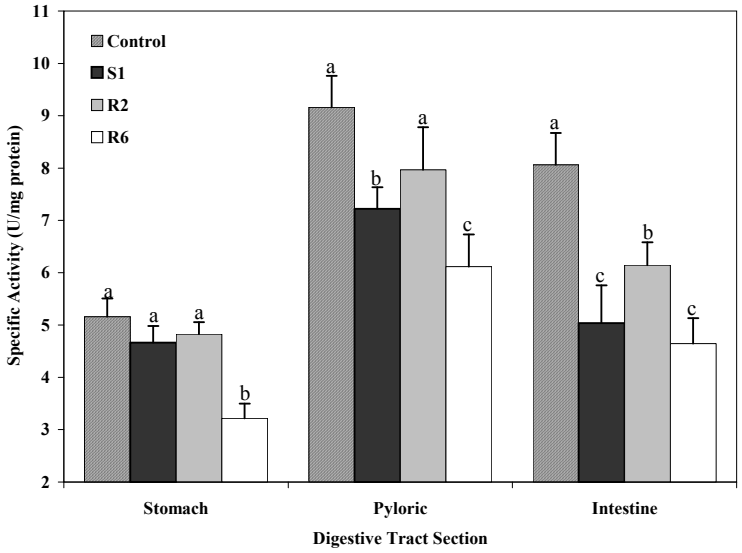

Figure 2. Activity of amylase measured in the different sections of the digestive tract of gilthead sea bream subjected to four feeding regimes during 48 days of rearing. Values are means ± S.D. (n=3, each n consist of measurements of triplicate analysis).

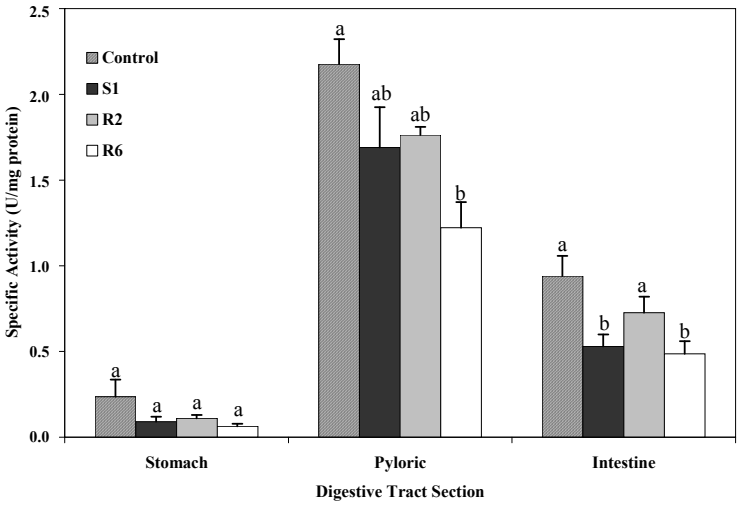

Figure 3. Activity of lipase measured in the different sections of the digestive tract of gilthead sea bream subjected to four feeding regimes during 48 days of rearing. Values are means ± S.D. (n=3, each n consist of measurements of triplicate analysis).

some cases, fish fed restricted ratio have been reported to show improved feed efficiency without increasing feed intake (Russell and Wootton, 1992; Wang *et al*., 2000; Eroldoğan *et al*., 2004). This is thought to be due to high digestive enzyme activities induced by restricted feeding regimes as also suggested by Barrington (1957) and Jobling (1994). Thus it would be of interest to study correlation between restricted feeding ration and digestive enzymes in juvenile gilthead sea bream.

As commonly known protein digestion is a complex process in fish and occurred not only in stomach but also other parts of digestive systems such as pyloric caeca and intestine. Hence, acidic protease, pepsin, and peptidases are mainly appointed in protein digestion in digestive system. Within this context, in the present study, the acidic total protease activity in ST was found to be higher compared to that in PC and IN. Regarding treatments, restricted feeding in R2 and control showed higher total protease activity than in other tested groups, suggesting that restricted feeding increases the total protease activity in digestive track which is also in agreement with results obtained as in other carnivorous fish species i.e. Atlantic cod (Bélanger *et al*., 2002) and Asian sea bass (Harpaz *et al.*, 2005). Furthermore, the significant higher activity of total protease in whole part of the digestive track in R2 compared with its activity in S1 and R6 may be

the results of an increased effort of fish to digest proteins in order to maximize protein utilization in R2 groups.

Low amylase activity in carnivorous fish is the general assumption (Hidalgo *et al.*, 1999; Krogdahl *et al.*, 2005). As a carnivorous species, gilthead sea bream has low amylase activities in the liver and intestine compared to carp (*Cyprinus carpio*) and gold fish (*Carassius carassius*) (Hidalgo *et al.*, 1999). However, under stress condition (i.e. starvation, restricted feeding) fish can change carbohydrate metabolism. Sangiao-Alvarellos *et al*. (2005) found an increasing capacity to export glucose which is mobilized from liver glycogen stores in gilthead sea bream exposed to food deprivation for 2 weeks, indicating possible carbohydrate metabolism. Indeed, amylase is stimulated by glycolytic chains, glycogen, and starch in fish larvae and juveniles (Péres *et al*., 1998; Krogdahl *et al*., 2005). Thus, expecting an increase amylase activity in digestive track is not surprising in fish subjected to starvation. In the present study, amylase activity was the main activity identified along the digestive track, being mainly observed in the PC and IN. Interestingly, activity of this enzyme in ST of sea bream was detected in the present study. This is consistent with the findings of Alarcón *et al*. (2001) who also observed amylase of activity in stomach of 50-g gilthead sea bream. In fact, it should be difficult to explain the presence and activity of these enzymes, which have an optimum pH neutral to alkaline, considering the acid environment existing in the stomach. However, activity of amylase in ST in the recent study was considered an artefact resulting from contamination of stomach extracts with pancreatic tissue during manipulation for dissection. On the other hand, in the present study, the ratio of amylase: protease activity in control, S1, R2 and R6 was 12.6, 16.7, 13.0, 17.0 in PC, respectively (un-presented data). Hidalgo *et al*. (1999) postulated that the high amylase: protease activity ratio in gilthead sea bream and eel could possibly be due to the digestion of carbohydrate rather than proteins. It would appear that a similar phenomenon may have occurred in our tested fish, although it was not possible to analyze this in detail as only a single point in time was sampled.

It is commonly known that lipase showed relatively higher activity at an alkaline region between pH 7.0-9.0 (Iijima *et al*., 1998) and activity of this enzyme was found in extracts of the pancreas, pyloric caecae and upper intestine (De Silva and Anderson, 1998). Similarly, present findings in our study supported that lipase activity in PC and IN were relatively higher than those in ST where lipase activity was almost non-existent. Most fish species rely on body lipid and protein stores during period of food deprivation and diverging results are obtained with respect to the importance of glycogen as an energy reserve (Ojaveer *et al*., 1996; Hemre *et al*., 2002). In general, increase in lipase activity is indicative of lipid use in fish. However, in the present study, there was no significant difference in lipase activity in the tested groups, suggesting that lipid is actively catabolised in control, S1 and R2, with the exception of fish in R6. These data also suggest that the duration of food restriction (R2) and starvation (S1) in cycles was too short to cause potential decrease in lipase activity and catabolised lipid was used for energy during short-term restricted ration and starvation.

In conclusion, the significant increase in total protease, lipase and amylase activity in digestive track in fish fed with restricted feeding regime (R2) suggests that protein utilization is occurring and that protein is an important energy source under conditions of food deprivation. Taking the observed enzymatic activity into account, some food restriction cycles may be recommended as a routine procedure in commercial production of gilthead sea bream to increase feed efficiency due to increasing digestive enzyme activity in gastrointestinal track.

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