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Enzyme activities and pH variations in the digestive tract of gilthead sea bream

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Two investigations were carried out with 150 g gilthead sea bream *Sparus aurata* to determine the relative activity of six digestive enzymes (pepsin, trypsin, chymotrypsin, carboxypeptidase A, carboxypeptidase B and amylase) and the pH variation in the lumen of different parts of the gut of fish fed one or two meals per day. Pepsin activity was found exclusively in the stomach, whereas activities of the other enzymes studied were found in all regions of the gut, including the stomach. The lack of localization of enzyme production in the digestive tract of *S. aurata* is similar to many other species as reported in the literature. The pH variations found in the different regions of the gut could be explained by general digestive physiology following the flow of digesta along the digestive tract. The range of pHs recorded in the various regions of the gut were generally outside the cited optima for many digestive proteases in this species.

Key words: digestion; enzymes; intestine; pH; *Sparus aurata*.

INTRODUCTION

Gilthead sea bream *Sparus aurata* L. is of commercial interest in the Mediterranean region with a production of 67 000 t in 1999 (FAO, 2001). On a commercial scale, fish are fed formulated diets, but in the wild this species has a diverse diet comprising numerous organisms including copepods, polychaetes, amphipods, macrophagous detritus, gastropods, bivalves, barnacles, crabs and prawns (Ferrari & Chieregato, 1981; Wassef & Eisawy, 1985; Andrade *et al.*, 1996).

Fish meal has been a major ingredient in the formulated feeds fed to *S. aurata* in captivity but alternative sources of protein are increasingly being used as fish meal availability decreases and prices increase. The vast majority of these alternative ingredients are plant-based, notably soyabean products. Use of such ingredients introduces components which are known to affect digestive physiology along the digestive tract and which reduce the digestibility and utilization of the nutritional components in the diet.

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Research on the digestive enzymes of *S. aurata* has been very limited and even though a number of enzymes have been identified (Gutierrez *et al.*, 1985; Moyano & Sarasquete, 1993; Sarasquete *et al.*, 1993; Moyano *et al.*, 1996; Munilla-Moran & Saborido-Rey, 1996*a*, *b*), no investigations have been carried out to determine their relative activities in the different sections of the digestive tract or the pH variations prevalent in the gut after feeding. Even less is known about how ingredient composition can affect enzyme activities and thereby digestibility in this species.

In this study, two basic aspects of digestive physiology were studied. First, the activities of six enzymes: pepsin, trypsin, chymotrypsin, carboxypeptidase A, carboxypeptidase B and amylase along the intestine were quantified. Second, the variation of pH along the digestive tract after feeding a formulated diet was studied.

MATERIALS AND METHODS

FISH AND CULTURE CONDITIONS

Sparus aurata of 150 g average mass were used in these investigations. The fish were kept in 270 l fibreglass tanks in the experimental facilities of the Malta Centre for Fisheries Sciences in Malta and fed a formulated extruded diet in two feeds at 1·3% body mass day^{-1}. Sea water at a temperature of 21° C was supplied to the tanks at the rate of 6 l min^{-1}. The photoperiod was 12L : 12D.

DETERMINATION OF ENZYME ACTIVITIES ALONG THE GUT

Fish and sample preparation

Fish used in this investigation were starved for 40 h prior to sampling. At each sampling, 12 fish were killed in ice and dissected immediately. The stomachs, pyloric caecae and the three equally divided parts of the intestine were separated, blotted, weighed and pooled for three groups of four fish each before homogenizing in an aqueous suspension (1 : 10, wet mass : volume ice-cold distilled water) and the resulting solution was then centrifuged in a Hermle (Gosheim, Germany) ZK510 centrifuge at 3750*g* at 4° C for 30 min (Das *et al.*, 1987; Fagbenro, 1990; Sabapathy & Teo, 1995). The supernatants were separated and kept at 4° C until they were analysed the same day. All enzymatic analyses were carried out using standard methods.

Preliminary tests were performed for each of the enzymatic analyses during which various dilutions of the supernatants were tested to determine which gave the greatest change in absorbance in a given time within the absorbance range of the spectrophotometer. These dilutions were used for the rest of the analyses. All determinations were carried out at 25° C.

Determination of pepsin activity

Determination of pepsin activity was carried out using casein as substrate (Rick & Fritsch, 1974; Hsu & Wu, 1979). The reaction was initiated by adding 0·5 ml of properly diluted enzyme solution to 2·5 ml of 2% casein [Sigma-Aldrich (Munich, Germany) C0376] in 0·06 M HCl (pH 1·8). After exactly 10 min incubation, the reaction was stopped by the addition of 5·0 ml 5% trichloroacetic acid. The solutions were left to stand for 1 h and then centrifuged at 2100*g* for 30 min. The absorbance of the supernatant was measured at 280 nm [Perkin Elmer (Uberlingen, Germany) UV/VIS Spectrophotometer Lambda 2]. Activity was expressed as the change in absorbance min^{-1} g $tissue^{-1}$.

Determination of trypsin and chymotrypsin activities

Trypsin activity was measured using N_{α}-*p*-toluenesulphonyl-L-arginine methyl ester (TAME) as substrate (Hummel, 1959). 0·2 ml properly diluted sample solution was added to 6 ml of 0·00104 M TAME (Sigma-Aldrich T4626) in Tris buffer [1·47 g $CaCl_2{\cdot}2H_2O$ dissolved in 200 ml 0·2 M Tris(hydroxymethyl)aminomethane diluted to 1 l, pH 8·1]. The absorbance at 247 nm was recorded and taken again after exactly 10 min. Trypsin activity was expressed as the change in absorbance $\text{min}^{-1}\,\text{g tissue}^{-1}$. Chymotrypsin activity was carried out using the method of Hummel (1959) with *N*-benzoyl-L-tyrosine ethyl ester (BTEE) as substrate. 0·2 ml properly diluted sample solution was added to 6 ml of 0·0005 M BTEE (Sigma-Aldrich B6125) in Tris buffer [10·55 g $CaCl_2{\cdot}2H_2O$ dissolved in 250 ml 0·2 M Tris(hydroxymethyl)aminomethane, adjusted to pH 7·8, diluted to 1 l, and 432 ml methanol added] (Hummel, 1959). The absorbance at 254 nm was recorded and taken again after exactly 10 min. Chymotrypsin activity was expressed as the change in absorbance $\text{min}^{-1}\,\text{g tissue}^{-1}$.

Determination of carboxypeptidase A and carboxypeptidase B activities

The activities of carboxypeptidases A and B were carried out according to the methods of Folk & Schirmer (1963) and Folk *et al.* (1960), respectively, using hippuryl-L-phenylalanine for carboxypeptidase A and hippuryl-L-arginine carboxypeptidase B. In the analysis for carboxypeptidase A activity, 0·2 ml properly diluted sample solution was added to 6 ml of 0·001 M hippuryl-L-phenylalanine (Sigma-Aldrich H6875) in Tris buffer [3·025 g Tris(hydroxymethyl)aminomethane and 29·25 g NaCl in 1 l, pH 7·5]. For carboxypeptidase B determination, 0·2 ml properly diluted sample solution was added to 6 ml of 0·001 M hippuryl-L-arginine (Sigma-Aldrich H6625) in Tris buffer [3·025 g Tris(hydroxymethyl)aminomethane and 5·85 g NaCl in 1 l, pH 7·6]. For both these enzymes, the absorbance at 254 nm was recorded and taken again after exactly 10 min. Their activity was expressed as the change in absorbance $\text{min}^{-1}\,\text{g tissue}^{-1}$.

Determination of amylase activity

Starch was used as the substrate in the determination of amylase activity (Bernfeld, 1951) where 1 ml of properly diluted enzyme was incubated for 3 min with 1 ml 1% starch [1 g soluble starch (Sigma-Aldrich S2630) and 0·035 g NaCl in 100 ml 0·02 M Na_3PO_4, pH 6·9]. The reaction was stopped by the addition of 2 ml 3,5-dinitrosalicylic acid reagent. The solution was then heated for 5 min in boiling water, cooled and 20 ml distilled water added. The absorbance at 540 nm was read and a standard curve was established with maltose (Sigma-Aldrich M5885), to convert readings into mg of maltose. Amylase activity was expressed as mg maltose liberated $\text{min}^{-1}\,\text{g tissue}^{-1}$.

DETERMINATION OF pH VARIATION ALONG THE GUT

Fish and sample preparation

Fish were starved for 64 h prior to the start of the investigation. The fish were divided into two groups, the first group of six fish were killed after feeding at 0830 hours in lethal anaesthetic (2-phenoxyethanol) and then at 2 h intervals until 2030 hours. The second group were fed first at 0830 hours and again at 1430 hours, after which six fish were killed at 2 h intervals until 2030 hours. Sampled fish were immediately dissected and the intestine was divided into three equal parts, not including the rectum, using pieces of thin thread. An additional tie was made between the stomach and its point of attachment to the intestine. pH readings were taken from the stomach and the three intestinal regions. pH was measured using a Reagecon (Shannon, Ireland) Series GC Glass pH Micro Combination Electrode (GCMF 11-100, 4 mm tip) on a Jenway (Essex, U.K.) pH meter at 21° C. The pH probe was inserted into the digesta in the middle of the section being studied and the stable reading recorded.

STATISTICAL ANALYSIS

Data from the replicates for each treatment were combined to provide the data for the statistical analysis which was carried out using STATISTICA Version 5.5 (StatSoft, Tulsa, U.S.A.). Multiple comparisons between means were made using the Student–Newman–Keuls test. The significance level was taken as 0·05.

RESULTS

ENZYME ACTIVITIES

The results of the various enzyme determinations are presented in Table I. Pepsin activity was only detected in the stomach. Trypsin activity was significantly lower in the stomach than in all other regions. Chymotrypsin activity did not vary significantly between the different parts of the gut. The carboxypeptidase A activity of the upper intestine was significantly higher than the activity in the pyloric caecae, which in turn was significantly higher than in any other region studied. Carboxypeptidase B activity recorded in the pyloric caecae was significantly higher than that seen in the lower intestine, but not significantly different to all other regions of the gut. Amylase activities were similar in all regions except the stomach which showed a significantly lower activity.

pH VARIATION WITH FEEDING

When fish were given only one feed, the stomach pH decreased significantly to a value of 2·6 after 8 h, before rising again to 5·1 after 12 h [Fig. 1(a)]. When fish were given two feeds, there was a similar significant decrease in pH immediately upon feeding to a pH of 2·5 at 12 h. In the upper and middle intestine no significant differences were obtained among pH values after feeding [Fig. 1(b), (c)]. In the lower intestine, pH increased significantly from the minimum pH value of 6·9 after feeding to 7·8 at 6 h followed by a period where the pH was unchanged [Fig. 1(d)]. The lower intestine of fish fed twice showed a slight but not significant increase after feeding to reach the maximum recorded value of 7·9 at 12 h.

DISCUSSION

ENZYME ACTIVITIES

The results obtained here clearly demonstrated that, except for pepsin, the activities of all the enzymes were not restricted to one particular section of the gut of *S. aurata*. Rather than being an exception, these results agree with what has been seen in many other fishes. In *S. aurata*, this is probably related to the fact that the pancreas is found as small masses spread along the upper intestine and, in adults, as pancreatic infiltrations of the liver (Elbal & Agulleiro, 1986; Cataldi *et al.*, 1987).

As would be expected from general digestive physiology, the pepsin activity observed in *S. aurata* was restricted to the stomach. The lack of pepsin activity in all other parts of the digestive tract other than the stomach was also found by

TABLE I. Activities of the various enzymes along the digestive tract of gilthead sea bream. Data are presented as mean ± S.E. Means in a row with different superscripts are significantly different ($P < 0{\cdot}05$)

	Stomach	Pyloric caeca	Upper intestine	Middle intestine	Lower intestine
Pepsin (ΔAbs min^{-1} g tissue^{-1})	$5{\cdot}27 \pm 1{\cdot}02^{b}$	$0{\cdot}00 \pm 0{\cdot}00^{a}$	$0{\cdot}00 \pm 0{\cdot}00^{a}$	$0{\cdot}00 \pm 0{\cdot}00^{a}$	$0{\cdot}00 \pm 0{\cdot}00^{a}$
Trypsin (ΔAbs min^{-1} g tissue^{-1})	$0{\cdot}53 \pm 0{\cdot}06^{a}$	$3{\cdot}35 \pm 0{\cdot}57^{b}$	$3{\cdot}97 \pm 0{\cdot}35^{b}$	$1{\cdot}61 \pm 0{\cdot}09^{b}$	$1{\cdot}08 \pm 0{\cdot}08^{b}$
Chymotrypsin (ΔAbs min^{-1} g tissue^{-1})	$2{\cdot}00 \pm 0{\cdot}20$	$4{\cdot}81 \pm 1{\cdot}43$	$2{\cdot}27 \pm 0{\cdot}25$	$2{\cdot}31 \pm 0{\cdot}20$	$1{\cdot}97 \pm 0{\cdot}07$
Carboxypeptidase A (ΔAbs min^{-1} g tissue^{-1})	$0{\cdot}49 \pm 0{\cdot}12^{a}$	$1{\cdot}42 \pm 0{\cdot}34^{b}$	$1{\cdot}98 \pm 0{\cdot}09^{c}$	$0{\cdot}75 \pm 0{\cdot}24^{a}$	$0{\cdot}51 \pm 0{\cdot}13^{a}$
Carboxypeptidase B (ΔAbs min^{-1} g tissue^{-1})	$0{\cdot}70 \pm 0{\cdot}25^{ab}$	$1{\cdot}21 \pm 0{\cdot}09^{b}$	$0{\cdot}74 \pm 0{\cdot}21^{ab}$	$0{\cdot}51 \pm 0{\cdot}07^{ab}$	$0{\cdot}35 \pm 0{\cdot}16^{a}$
Amylase (mg maltose liberated min^{-1} g tissue^{-1})	$0{\cdot}74 \pm 0{\cdot}27^{a}$	$3{\cdot}58 \pm 0{\cdot}28^{b}$	$2{\cdot}67 \pm 0{\cdot}98^{b}$	$3{\cdot}38 \pm 1{\cdot}03^{b}$	$3{\cdot}65 \pm 0{\cdot}83^{b}$

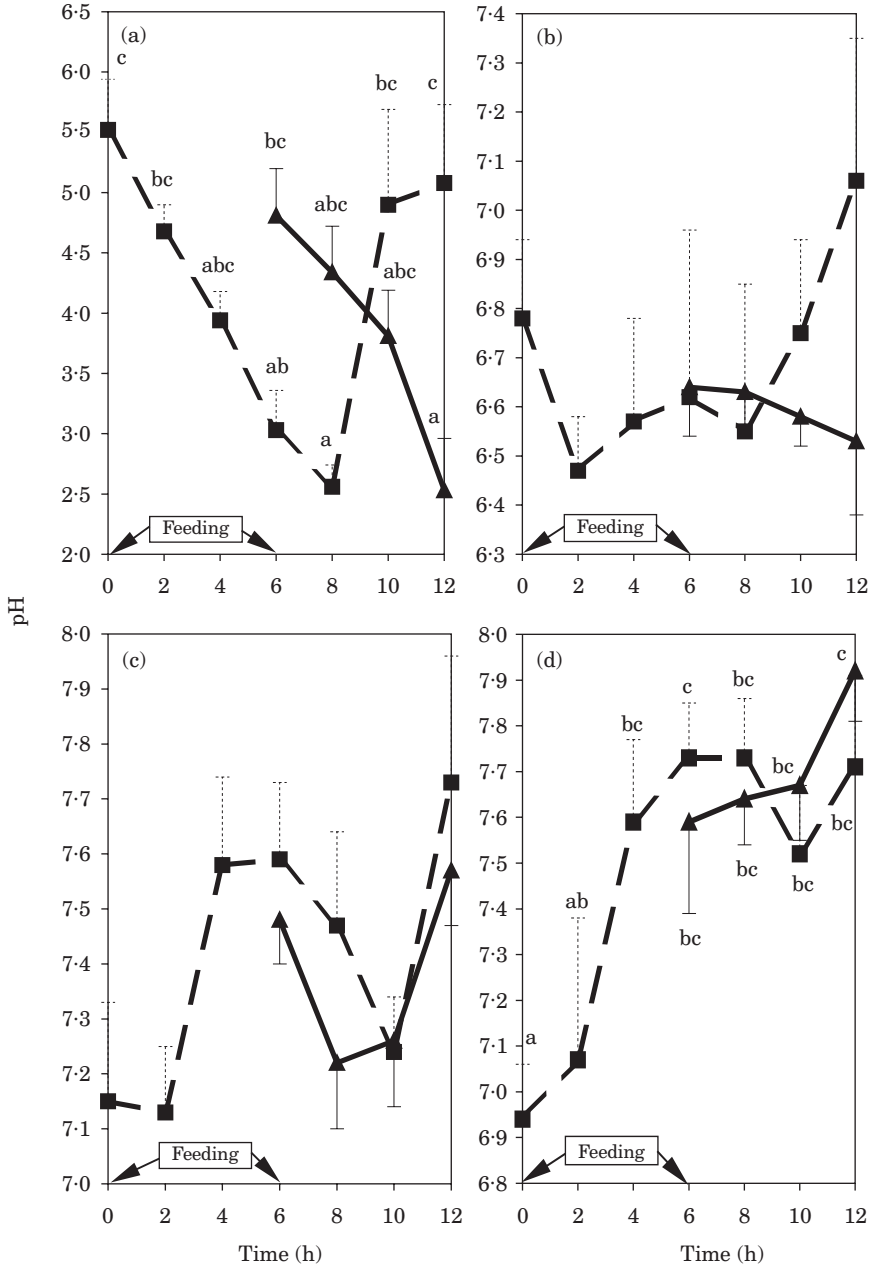

FIG. 1. pH variation (mean ± S.E.) in the intestine of gilthead sea bream after a single feed (— —) and after a second feed (——). (a) stomach, (b) upper intestine, (c) middle intestine and (d) lower intestine. Values with different letters denote significant differences ($P < 0{\cdot}05$).

Buddington & Doroshov (1986) in the white sturgeon *Acipenser transmontanus* Richardson and Chakrabarti *et al.* (1995) in striped snakehead *Channa striata* (Bloch). On the other hand, a number of authors have found that other parts of

the intestine showed a comparable or even higher pepsin activity to that found in the stomach. Such was the case in the dwarf African mud catfish *Clarias isheriensis* Sydenham (Fagbenro, 1990), the Asian sea bass *Lates calcarifer* (Bloch) and the rabbitfish *Siganus canaliculatus* (Park) (Sabapathy & Teo, 1993).

Although all enzymes except pepsin were detected in every region of the gut of *S. aurata*, the relative activities between regions differed from one enzyme to another. Thus, while trypsin and amylase activities were significantly lower in the stomach than all other regions this was not the case with chymotrypsin, carboxypeptidase A or carboxypeptidase B activities. Contrary to expectation, in the latter group of enzymes the average activities in the stomach were very similar, and in some cases higher, than the activities observed in the other parts of the intestine. Considering the pHs prevalent in the stomach of *S. aurata*, and the pH optima of the individual enzymes, it would seem that although the enzymes were detected in the stomach, in reality their actual contribution to digestion would probably be very low.

Many authors have reported the presence of trypsin, chymotrypsin, carboxypeptidases A and B and amylase in the stomach of fishes (Hsu & Wu, 1979; Uys & Hecht, 1987; Sabapathy & Teo, 1993). The variations in the relative activities of these enzymes of *S. aurata* observed in the present study have been reported in species occupying a wide range of habitats and even in species occupying similar habitats. For example, while Fagbenro (1990) found a higher trypsin activity in the upper part of the intestine of *C. isheriensis*, Uys & Hecht (1987) found a higher activity in the more posterior parts of the intestine of African catfish *Clarias gariepinus* (Burchell). In their investigations on amylase activity, Chakrabarti *et al.* (1995) reported that in *C. striata* the highest activity was detected in the lower part of the intestine, in *Notopterus notopterus* (Pallas) and *Puntius javanicus* (Bleeker) the highest activity was found in the middle intestine, in *Oreochromis niloticus* L. and *Labeo rohita* Hamilton the highest activity was found in the oesophagus and only in the *Hypophthalmichthys molitrix* (Valenciennes) and *Cyprinus carpio* L. was the highest activity found in the upper intestine. Indeed, in a number of these fishes the activity in the stomach was even higher than that found in some of the other regions of the intestine.

As apparent from the discussion above, the distribution of digestive enzymes within the intestine of most fishes, including *S. aurata*, does not appear specific to any one part of the gut although a particular region may exhibit the highest activity for a particular enzyme. Many authors have attributed this lack of localization in fishes to being an adaptation to the varied diet a fish may consume in the wild, allowing for an adaptable, to an extent, digestive process whereby the different nutritional components in food items are made available as they pass through the intestine. Chakrabarti *et al.* (1995) put forward the opinion that the intestinal tract of fishes is still at the evolutionary stage where most regions can produce all the principal enzymes before the evolution of site-specific enzyme production seen in higher vertebrates.

Sparus aurata has a varied diet in the wild (Ferrari & Chieregato, 1981; Wassef & Eisawy, 1985; Andrade *et al.*, 1996), but in culture is provided with diets that have a more or less constant composition. These formulated diets

typically contain a wide variety of ingredients, of which plant proteins are becoming more and more important. A better understanding of how enzyme activities affect feed utilization in *S. aurata* and thereby growth performance would be beneficial to the industry. This was the objective of Lemeiux *et al.* (1999) who studied the activities of a number of enzymes in Atlantic cod *Gadus morhua* L. and reported that low trypsin activity could be limiting the growth of this fish. If such a relationship is established also for *S. aurata*, research could then be conducted to determine if this could be changed or manipulated in some way. Also important is knowledge of how different feed ingredients may affect enzyme activity; this would provide information on if and how the choice of ingredients in feed formulations could be changed to allow for a better efficiency of activity by digestive enzymes.

pH VARIATION ALONG THE GUT

Like all enzymes, the activities of digestive enzymes is greatly affected by pH. Different enzymes have different pH optima where activity is maximum; either side of this optimum activity drops off rapidly and significantly such that with some enzymes a pH change of 1 can cause a 50% decrease in activity. This obviously has an important effect on the rate and extent of digestion.

The changes in pH seen in the stomach of *S. aurata* can be explained by normal digestive processes where the pH decreased as acid was secreted in response to feed entering the stomach, followed by increases in pH as acid secretion was stopped and digesta was evacuated. Rapid decreases of pH in the stomach were also seen by Norris *et al.* (1973) in bluegill *Lepomis macrochirus* Rafinesque, Moriarty (1973) in *O. niloticus* and Maier & Tullis (1984) in Mozambique tilapia *Oreochromis mossambicus* Peters. Munilla-Moran & Saborido-Rey (1996*a*) determined that the pH optima for pepsin activity in the stomach of *S. aurata* was 2·0. This agrees with the pH range of 2·0–3·0, given as the optimum pH for pepsin by other authors in various species (Moriarty, 1973; Uys & Hecht, 1987; Munilla-Moran & Saborido-Rey, 1996*a*). The average pH in the stomach of the fish sampled in this investigation ranged from 2·5 to 5·5, the latter being recorded immediately after feeding indicating that, for most of the time, the pH was not optimum for pepsin activity.

The variations in pH of the three parts of the intestine studied over the 12 h period can also be explained by following the flow of digesta along the intestine. As the acidic digesta entered the upper intestine the pH dropped slightly, to increase as bicarbonate ions were secreted into the lumen of the gut. The effect of bicarbonate secretion was evident in that going down the length of the intestine there was a continuous increase in the average pH from 6·8 in the upper intestine to 7·9 measured in the lower intestine. Similar sequences of changes were found by Maier & Tullis (1984) in *O. mossambicus*.

The average pH measured in the upper intestine ranged between 6·5 and 7·1, in the middle intestine between 7·1 and 7·7 and the lower intestine between 6·9 and 7·9. Munilla-Moran & Saborido-Rey (1996*a*, *b*) determined the pH optima for the proteases in the intestine of *S. aurata* to be between 9·5 and 10·0 while that of amylase was between 7·0 and 7·5. These figures agree with those

determined by other authors for amylase (6·4–8·0) (Falge & Schpannkhof, 1976; Ni *et al*., 1992; Tang *et al*., 1994), but are slightly higher than the optimum pH ranges found for trypsin and chymotrypsin (7·8–9·0) (Alliot *et al*., 1974; Jany, 1976; Clark *et al*., 1985; Simpson *et al*., 1989; Joakimsson & Nagayama, 1990) and leucine aminopeptidase (7·0–9·0) (Khablyuk & Proskuryakov, 1983; Clark *et al*., 1987; Joakimsson & Nagayama, 1990).

According to the work of Munilla-Moran & Saborido-Rey (1996*a*) the pH optima of the intestinal proteases were not being reached in the gut of the *S. aurata* studied here, or possibly only being reached for a short period of time. This would mean that the maximum digestive capacities of the proteases were not being achieved. The effect this could have on the overall digestion process is difficult to estimate but it could be very important in culture conditions using expensive formulated feed. It is reasonable to question whether a more optimum pH in the different regions of the gut would really have an effect on the protein digestibility of some of the dietary ingredients and, if so, would lead to a better feed utilization and growth performance of *S. aurata*. This is an important question, especially considering the increasing use of complex plant proteins in place of fish meal in current formulated diets, and one that should be investigated further for this species.

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