**DIETARY EFFECTS ON INSULIN AND GLUCAGON PLASMA LEVELS IN
RAINBOW TROUT (*Oncorhynchus mykiss*) AND GILTHEAD SEABREAM
(*Sparus aurata*)**

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1 **Abstract**

2 The effects of dietary amino acid profile (based on muscle (M) or whole body
3 composition (WB) and the balance between indispensable (IAA) and dispensable amino
4 acids (DAA) in the diet on plasma levels of insulin and glucagon were analyzed in
5 rainbow trout and gilthead sea bream.

6 Plasma insulin values (baseline and 6 h post-feeding) were higher in trout than in
7 sea bream, but the relative post-feeding increase was more pronounced in sea bream.
8 Within the same dietary amino acid profile, diets with lower IAA/DAA, had a lower
9 effect on the post-feeding secretion of insulin in both species. Circulating levels of
10 glucagon (baseline and post-feeding relative increases) were higher in sea bream. In
11 trout, diets with WB amino acid profile had a greater secretory effect on post-feeding
12 glucagon than did diets with M profile, while gilthead sea bream showed an inverse
13 response to circulating glucagon with respect to diet. Muscle insulin and insulin growth
14 factor-I binding parameters were not affected by the dietary regimen.

15 The postfeeding glucagon response depends on both the dietary AA profile and
16 the fish species, while that of insulin seems to be more uniform, and is affected in a
17 similar way regardless of the species.

18

1. Introduction

Partial replacement of dietary fish meal protein with plant protein has been successfully accomplished in a number of teleostean fishes (Burel et al., 2000; Kaushik et al. 1995; 2004; Watanabe et al. 1998). However, fish meal and plant protein differ in a number of ways, including protein and energy content, amino acid profile and mineral composition. Another limitation of the use of vegetable compounds is that plant ingredients contain a certain proportion of anti-nutritional factors (Francis et al. 2001). Furthermore, with respect to indispensable amino acids (IAA) requirements of animals, plant proteins are often limited in one or more amino acids (Sauvant et al. 2004), and therefore in some cases, diets have to be supplemented with amino acids to avoid deficiencies and maintain adequate amino acid profile for correct growth.

The pancreatic hormones, insulin and glucagon, are known to play a key role in regulating the uptake of nutrients by tissues during the postprandial period (Navarro et al., 1993). Some studies reveal that levels of these hormones increase a few hours after food ingestion and contribute to post-feeding amino acid and glucose clearance (Sundby et al., 1991; Navarro et al., 1993).

Insulin is an anabolic hormone that stimulates the uptake of nutrients and incorporation by tissues (Duncan et al., 1998; Pérez-Sánchez and Le Bail, 1999; Peter and Marchant, 1995). In mammals, glucose is the main insulin secretagogue, along with some amino acids, whereas in fish, amino acids are more potent than glucose in stimulating insulin release (Mommensen and Plisetskaya, 1991). However, the response of insulin to dietary protein source (vegetable versus fish meal) and specially to the dietary amino acid profile has been poorly studied. Only the insulintropic activities of various injected amino acids has been compared in the flounder (Andoh, 2007). Insulin and other peptides of its family, such as insulin growth factor-I (IGF-I), act through specific

1 tyrosine kinase membrane receptors (Le Roith et al., 1995). In fish, IGF-I is not only a
2 growth factor, but also acts as a metabolic regulator both *in vivo* and *in vitro* (Castillo et
3 al., 2004; Wood et al., 2005).

4 In mammals, glucagon also increases after a rich protein meal and it has been
5 considered a pivotal hormone in amino acid disposal during an amino acid load. A
6 pattern of biphasic increases in circulating post-feeding glucagon has been observed in
7 both rainbow trout and European sea bass (Navarro et al., 2002), and the second
8 glucagon peak has been suggested to be related to the increase in postprandial amino
9 acids (Navarro et al., 2002). Furthermore, glucagon is known to enhance the uptake of
10 amino acids in fish liver and stimulate the activities of aminotransferases (Inui and
11 Ishioka, 1983). Information regarding the structure and function of fish glucagon and
12 related peptides has increased in the last years (reviewed by Plisetskaya and Mommsen,
13 1996; Moon, 1998; Mommsen, 2000; Mommsen and Busby, 2006). Numerous *in vitro*
14 studies revealed that glucagon activates hepatic gluconeogenic pathway and glucose
15 output. However, the role of glucagon in relation to dietary factors still remains poorly
16 studied.

17 The present studies were part of a multidisciplinary project on the effects of diets
18 with plant protein and with different dietary amino acid profiles on metabolism and the
19 somatotrophic axis in sea bream (Gómez-Requeni et al., 2003) and trout. Here we
20 analyze the response of pancreatic hormones, insulin and glucagon, after adaptation to
21 diets with low content of plant protein and different amino acid profiles and
22 indispensable/dispensable amino acid ratios (IAA/DAA ratio) in relation to nutrient
23 utilization and growth in gilthead sea bream and rainbow trout.

24 25 **2. Material and methods**

2.1. Experimental diets

Four experimental diets based on fish meal and plant ingredients (33-35% replacement) supplemented with free amino acids were developed for rainbow trout (T) and gilthead sea bream (SB) (Tables 1 and 3). For each species, two of the diets (M and WB) were based on the IAA profile and DAA content of muscle (M) and whole body (WB), respectively. In M2 and WB2 diets, DAA content and the IAA/DAA ratio was changed through the incorporation of glutamic acid. For dietary amino acid composition of diets for sea bream, see reference Gómez-Requeni et al., 2003. Dietary amino acid composition of diets for trout is shown in table 2.

2.2. Animals and growth trials

Growth trials with rainbow trout (*Oncorhynchus mykiss*) were conducted in the INRA experimental fish farm (Donzacq, France) at a constant water temperature of 17 ± 1 °C. Studies with gilthead sea bream (*Sparus aurata*) were carried out at the Instituto de Acuicultura, CSIC, de Torre la Sal (Castellón, Spain), where water temperature ranged naturally from 17 to 25 °C. For both species, each experimental diet was hand distributed to triplicate groups of fish for each diet in tanks of 500-l capacity, twice a day (9 h and 16 h) to near satiation (visual observation of the first refusal of feed); the quantity of food was recorded daily over the whole trial which lasted 12 weeks.

2.2.1. Postprandial experiment

Blood samples were taken at the end of growth trials. Twelve fish from each treatment and for each sampling time (4 fish of each tank) were anaesthetised in 100 ppm of 3-aminobenzoic acid ethyl ester (MS222) and blood was taken by caudal

puncture, 6 and 24 h after feeding. The two sampling times were done in two different consecutive days in different individuals. White lateral muscle was sampled 24 h after feeding and frozen in liquid nitrogen for insulin and IGF-I binding studies.

2.2.2. Force-feeding experiment

After 12 weeks of adaptation to the diets, (17°C for rainbow trout; 21°C for seabream) we examined the response of plasma glucose, insulin and glucagon after force feeding. Fifty fish (either rainbow trout or gilthead sea bream) adapted to each of the TM1, TM2, SBM1 and SBM2 diets were divided into five groups of ten fish each and distributed in tanks of 90-l capacity. After 48 h of fasting, fish were force fed with the respective diet by means of stomach intubation at a rate of 1% body weight. Blood samples were taken under anaesthesia with MS222 from a group of ten fish at one of the following time periods: 1, 3, 6, 12 and 24 h after force feeding.

2.5. Analytical methods

Plasma was collected after blood centrifugation (3000 x g, 10 min) and was split into three fractions: for glucose, glucagon and insulin analysis, respectively. A protease inhibitor, Trasylol (Bayer) was added to the plasma fractions (1000 IU/ml plasma) for glucagon analysis. All the plasma aliquots were kept frozen (-20°C).

Plasma glucose levels were analyzed by the glucose-oxidase colorimetric method (GLUCOFIX; Menarini Diagnostics, Firenze, Italy) (Huggett and Nixon, 1957; Sala-Rabanal et al., 2003). Insulin levels were measured by radioimmunoassay (RIA) using bonito (*Thunnus thynnus*) insulin as standard and a rabbit anti-bonito insulin as antiserum (Gutiérrez et al., 1984). It has been probed that this RIA is valid to measure trout and sea bream plasma samples and the antibody cross react with other fish species

1 studied (Navarro et al., 2002). Plasma glucagon levels were quantified by a
2 heterologous radioimmunoassay method validated for fish plasma (Gutiérrez et al.,
3 1984; Navarro et al., 1995).

4 Partial purification of solubilized insulin and IGF-I receptors from white muscle
5 was performed at 4°C, as described by Párrizas et al. (1995) by affinity chromatography
6 on wheat-germ agglutinin (WGA) bound to agarose (Vector Laboratories, Burlingame,
7 USA). The glycoproteins obtained were measured following the method described by
8 Bradford (1976). Binding assays were performed as in Párrizas et al. (1994). A volume
9 of 30–40 µl of the WGA eluate (approximately 30 µg of glycoproteins) was incubated
10 for 14–16 h at 4 °C with increasing concentrations of unlabelled hormone (from 0 to
11 100 nM, final dilution) and radio-labelled ligand as tracer (25 pM, 50µCi/l). Semi-
12 purified receptors were precipitated by addition of 0.08% bovine γ-globulin and 10.4%
13 polyethylene glycol (final concentrations), followed by centrifugation at 14,000×g for 7
14 min at 4 °C. Binding data were analysed in Scatchard plots and only the high-affinity,
15 low-capacity binding sites were considered in the analysis. Porcine insulin was obtained
16 from Lilly (Indianapolis, USA) and human recombinant IGF-I from Chiron
17 (Emeryville, CA, USA). Human Tyr A14 ¹²⁵I-monoiodoinsulin and human recombinant
18 3-¹²⁵I-IGF-I, both with 2000 Ci/mmol specific activity, were purchased from Amersham
19 Life Sci. (Arlington Heights, IL). All other chemicals used were purchased from Sigma
20 (St. Louis, MO, USA).

22 2.6. Statistical analysis

23 Values are given as means with standard errors. Statistical analysis was
24 performed using SPSS 11.5.1. The effect of the dietary adaptation was analysed for the
25 different parameters using the Student's *t* test and Student Newman-Keuls test (p<0.05).

1 Data from force-feeding experiments were subjected to ANOVA and means were
2 compared using the HSD Tukey test or Games-Howell test ($p < 0.05$).

3. Results

3.1. Growth performance and nutritional parameters in rainbow trout and sea bream

3 Data on growth performance in juvenile rainbow trout are shown in Table 4.
4 Final body weight and growth rates did not vary between the groups. Similarly, in
5 juvenile gilthead sea bream, there was no effect of diet on specific growth rates and
6 FGR of SBWB2 group was higher (Table 5).

7 Trout fed TM2 (based on muscle profile and supplemented with Glu) showed a
8 lower hepatosomatic (HSI) index, as well as a lower feed gain ratio (FGR) and protein
9 efficiency (PER).

10 Similarly, in sea bream, the lowest value for HSI was found in those fed the diet
11 SBM2. In fact, HSI values from sea bream fed diets with the muscle IAA profile were
12 lower than those fed the diet reflecting the whole body AA profile. No differences were
13 observed between diets in terms of the protein efficiency ratio, and feed gain ratio
14 values were similar across the different groups.

3.2. Plasma glucose, insulin and glucagon levels after growth trial in rainbow trout and gilthead sea bream

15 In trout, plasma glucose levels 6 h after feeding were higher than baseline
16 plasma values at 24 h (Figure 1A) in all groups. Glycaemia was not different between
17 diets at either of the two sampling times. In gilthead sea bream, plasma glucose values
18 showed some variations between experimental groups only 6 hours after feeding (Figure
19 1B). Baseline and post-feeding glucose values were lower than in rainbow trout.

1 Plasma insulin levels in trout at 24 h were similar among the different groups
2 (range from $19,3 \pm 2,3$ to $21,4 \pm 1,5$ ng/mL). Insulin levels increased 6 h after feeding to
3 approximately 30% above baseline values (range $27,5 \pm 1,5$ to $31,9 \pm 0,7$ ng/mL.
4 Postprandial insulin levels from TM2 (lower IAA/DAA ratio) was lower than those fed
5 TM1 (Figure 1C). Baseline plasma insulin levels (24 h) in gilthead sea bream were not
6 different between diets, with values from $5,6 \pm 0,3$ to $6,2 \pm 0,6$ ng/mL. The relative post-
7 feeding increase in insulin levels was higher than in trout, with plasma insulin values at
8 6 h being approximately twice those observed at 24 h after feeding (Figure 1D). Insulin
9 levels 6 h after feeding in SBM2 and SBWB2 were lower than in SBM1 and SBWB1
10 groups, respectively.

11 Circulating glucagon levels in trout at the end of the growth trial (at 24 h post-
12 feeding) were similar between groups, ranging from $0,6 \pm 0,1$ to $0,7 \pm 0,1$ ng/mL.
13 Glucagon levels were higher 6 h after feeding (in relation to baseline values) only in
14 TWB and TWB2 groups (Figure 1E). Circulating glucagon levels of sea bream at 24 h
15 were higher than in trout (between $2,1 \pm 0,4$ and $2,6 \pm 0,4$ ng/mL). The relative increase in
16 hormone levels after 6 h was also more pronounced than in trout, with glucagon levels
17 three-fold higher than baseline values. Postprandial plasma glucagon levels in the
18 different sea bream groups showed an inverse profile compared to that in rainbow trout.
19 Seabream fed diets WB and WB2 had slightly lower values than those of M groups, the
20 maximum values being observed in the SBM1 group (Figure 1F).

21

22 *3.3. Characterization of muscle insulin and IGF-I receptors*

23 Insulin and IGF-I binding parameters from muscle preparations of trout and sea
24 bream 24 h after feeding are shown in Tables 4 and 5. In both species, IGF-I specific
25 binding was higher than that of insulin, but in sea bream the difference was very

pronounced (three fold). Neither affinity (Kd) nor number of insulin or IGF receptors were affected by diet in sea bream. In trout, the number of IGF-I receptors changed between groups been higher in TM1..

3.4. Force-feeding experiments in rainbow trout and gilthead sea bream

In both species, plasma glucose levels reached maximum levels 1 h after force feeding in trout (TM1 $13,4 \pm 1,2$ and TM2 $13,3 \pm 0,7$ mM) and sea bream (SBM1 $13,2 \pm 0,6$ and SBM2 $10,7 \pm 0,5$ mM). Glycaemia decreased progressively across the post-feeding period, but fell more rapidly in gilthead sea bream (Figures 2A and 2B).

In rainbow trout, circulating insulin showed a similar pattern in both groups during the postprandial period, with increasing values at 3 h and maximum levels at 6 h after feeding. However, hormone levels in the TM1 group were higher than in TM2, at 12 and 24 h after food administration (Figure 2C). Circulating glucagon levels in the TM1 group did not change over time (between $1,4 \pm 0,2$ and $1,6 \pm 0,1$ ng/mL), while in TM2 fish, levels reached their maximum at 1 h and decreased progressively to the minimum at 24 h. Hormone levels were similar between diets except at 24 h, when TM1 levels were higher than TM2 values.

In gilthead sea bream, force feeding did not induce changes in plasma levels of pancreatic hormones. Plasma insulin and glucagon levels were not different between diets (Figures 2D and 2F).

4. Discussion

Great efforts have been made to reduce the level of fish meal in fish feeds by replacing fish meal with plant protein ingredients (Kaushik et al., 1995; Watanabe et al. 1998; Vielma et al., 2000); the effects of fish meal replacement are not well known in

1 sea bream (Gómez-Requeni et al., 2004). Problems related to the presence of anti-
2 nutritional factors or certain amino acid imbalances (Francis et al., 2001; Kaushik et al.,
3 1995), are common to most species. Hormonal control of plant protein utilisation has
4 been poorly studied and, as far as we know, there is no information on the dietary
5 effects of the amino acid profile and IAA/DAA ratio on circulating insulin and glucagon
6 in fish.

7 The results obtained in both species show that increasing the amount of DAA
8 amino acids (glutamic acid) did not affect growth, irrespective of the IAA profile.
9 However, growth was slightly impaired in groups fed diets with lower IAA/DAA. In
10 rainbow trout, the negative impact of TM2 in FCR and PER appears not to be related to
11 the DAA content because these effects would then have been found in the TWB2 diet
12 group with the lowest IAA/DAA ratio, too. It is possible that this is related to the high
13 soy bean meal level in diet TM2. However, it should be noted that the SBM2 diet, with
14 similar composition, did not have such negative effects suggesting that trout are more
15 sensitive to soybean meal than gilthead sea bream. Nevertheless, M2 diets did induce
16 the lowest hepatosomatic indices in both species.

17 Diets with high carbohydrate content have been reported to induce a
18 proportional increase in postprandial glucose in trout (Novoa et al., 2004). In the present
19 study, the absence of notable changes in glycaemia between the groups is consistent
20 with the composition of the diets, which were designed to present different amino acid
21 profiles but with similar carbohydrate content. Furthermore, circulating insulin is not
22 always well correlated with glycaemia levels and it appears to be more related to dietary
23 protein level and composition (Navarro et al., 2002).

24 Baseline levels of insulin (24 h) were higher in trout than in sea bream, in line
25 with previous reports (Navarro et al., 2002). These differences may be related to the

1 variations in the rates of basal hormone secretion or degradation. But no data on these
2 possible differences between fish species are available. However, the postprandial
3 increase relative to baseline values, irrespective of the dietary treatment, was much
4 higher in sea bream, thus suggesting that the response of insulin to food ingestion is
5 relatively higher in this species. The reasons for such differences are not known,
6 because the only data regarding possible insulin secretagogues in sea bream indicate
7 that administration of arginine induces lower increases in plasma insulin levels in sea
8 bream than in salmonids (Vega-Rubin de Celis et al., 2004). Nevertheless, the
9 sensitivity of insulin to many other factors (carbohydrates, gastrointestinal hormones or
10 neural stimulus) might be enhanced in sea bream during the postprandial period.

11 The secretagogue effect of the different diets on circulating plasma insulin levels
12 seems to present a similar pattern in both species. For a given dietary AA profile
13 (muscle or whole body), groups fed with a higher IAA/DAA ratio exhibit higher plasma
14 insulin levels 6 h after feeding. The presence of different proportions of some amino
15 acid components of the different diets could exert a differential stimulation of insulin
16 secretion. In fact, some amino acids such as arginine, lysine, leucine and phenylalanine
17 increase insulin in fish both in vivo and in vitro (Ince and Thorpe, 1977; Matty and
18 Lone, 1985; Navarro et al., 2002; Plisetskaya et al., 1991). Among these amino acids,
19 arginine has been demonstrated to have a strong stimulatory effect on insulin secretion,
20 especially in salmonids (Mommensen and Plisetskaya, 1991), although a dietary excess
21 does not seem to induce such an insulintropic action in the Atlantic salmon (Lall et al.
22 1994). In general, all these amino acids with an insulintropic action are considered
23 essential, this being consistent with the higher insulin values observed in fish fed diets
24 with higher IAA/DAA. Although in salmonids some studies suggest a correlation
25 between circulating insulin levels and fish size (Plisetskaya, 1989; Sundby et al., 1991)

1 the role of insulin stimulating growth appears to be permissive. In mammals, normal
2 portal insulin levels are required to maintain liver insulin growth factor-I (IGF-I)
3 production and normal growth, and a post-feeding increase in plasma insulin is needed
4 to ensure homeostasis. Insulin administration increased plasma IGF-I levels in trout
5 (Baños et al, 1999), and both circulating insulin and IGF-I oscillate in parallel in
6 response to some physiological situations (Baños et al., 1999; Larsen et al., 2001). The
7 profile of differences in plasma IGF-I between dietary treatments, reported in sea bream
8 fed the same diets (Gomez-Requeni et al., 2003), are similar to those found in insulin
9 values in the present study, the minimum circulating IGF-I level being found in the
10 WB2 group with the lowest growth. Thus, it appears that increased post-feeding insulin
11 levels might contribute to a “good hormonal scenario” which permits an optimal
12 nutritional and growth process. However, baseline insulin plasma levels did not exhibit
13 changes associated with dietary adaptation, which is in agreement with the maintenance
14 of insulin binding parameters. Indeed, only large changes in hormone levels can induce
15 variations in the number of receptors available in the membrane (Navarro et al., 1999).

16 In contrast to insulin, baseline circulating levels of glucagon were lower in trout
17 than in sea bream, and were in the range of those reported in previous studies (Gutiérrez
18 et al., 1986; Navarro et al., 1991; Navarro et al., 1992). For many years the only
19 reference for glucagon levels in sea bream has been the report by Gutiérrez et al. (1986).
20 In that study, glucagon levels were analyzed in different species of fish and were found
21 to be highest in sea bream, with levels 2- to 8-fold higher than those of other teleost
22 species studied. More recently, it has been demonstrated that arginine increases
23 circulating glucagon levels in sea bream even more than insulin (Navarro et al., 2002);
24 this contrasts with what happens in salmonids, where the insulinotropic action often
25 predominates over glucagon stimulation (Carneiro et al., 1993). However, no data are

1 available on the effects of other amino acids on glucagon secretion in fish, and even in
2 mammals the information is limited and contradictory (Gannon et al., 2002; Nuttall et
3 al., 2006). Nevertheless, the blood amino acid concentration after a protein meal is
4 known to stimulate glucagon release in the dog (Pek et al., 1969), and the same is true
5 for amino acid mixtures reproducing the physiological pattern in vitro. Nevertheless,
6 differences exist between carnivorous and omnivorous mammals. Although some amino
7 acids stimulate both insulin and glucagon secretion, it is also reported that the main
8 gluconeogenic amino acids are among the most potent alpha cell stimulators, a feature
9 which may be of physiological significance (Rocha et al., 1972). In fish, the dispensable
10 amino acids alanine, aspartate and glutamate are potentially important as gluconeogenic
11 substrates (Moon and Foster, 1995). In agreement with these observations the present
12 study in trout showed that within a profile (muscle or whole body), groups fed a lower
13 IAA/DAA ratio, with a higher proportion of some of those gluconeogenic amino acids,
14 presented slightly higher glucagon levels. It is believed that in mammals the aminogenic
15 glucagon release serves to stimulate hepatic glucose production and to avoid
16 hypoglycaemia resulting from the concomitant insulin secretion (Nuttall et al., 2006).
17 This has not been confirmed in fish, in which the control of glycaemia is not a relevant
18 feature and where baseline levels of gluconeogenesis maintain a modest glucose
19 turnover (Hemre et al., 2001). Nevertheless, glucagon does stimulate gluconeogenesis
20 from amino acids in hepatocytes of eel and trout (Inui and Ishioka, 1983; Inui and
21 Yokote, 1977), and post-feeding circulating glucagon is inhibited by dietary glucose
22 (Novoa et al., 2004). In contrast, it has been described in trout that partial substitution of
23 dietary protein by a single gluconeogenic dispensable amino acid can lead to an
24 inhibition of gluconeogenic liver enzymes, but interestingly, without affecting
25 glycaemia levels (Kirchner et al., 2003).

1 A different profile of glucagon secretion was found between diets in sea bream,
2 and the pattern of secretion was more similar to that observed for insulin. Differences
3 between species in the sensitivity of glucagon to various amino acids cannot be ruled
4 out as a possible explanation. In this regard, the secretion of glucagon in sea bream
5 appears to be especially sensitive to the essential amino acid arginine (Vega-Rubín de
6 Celis et al., 2004). Furthermore, and in contrast to mammals, parallel changes in
7 circulating insulin and glucagon values are commonly found in fish (Mommensen and
8 Plisetskaya, 1991). The post-feeding increase in glucagon irrespective of the diet
9 administered was also higher in sea bream than in trout. In mammals, a balanced
10 response between insulin and glucagon after a protein-rich meal is needed for an
11 optimal utilisation of nutrients. However, we cannot deduce from these experiments that
12 the specific response of pancreatic hormones contributes directly to a better adaptation
13 to diets in sea bream.

14 The force-feeding experiments enabled us to be sure that all animals sampled
15 had eaten the same ratio of food and at the same time, thus eliminating variability in the
16 hormonal response. In this way, these experiments permit us to check the effects of the
17 same levels of ingested food for the different diets. In addition, they were designed to
18 provide a complete hormone profile across the post-feeding period. In force-fed trout,
19 the insulin profile across the various sampling times was similar with both diets, but
20 with higher levels in the TM1 diet, this being consistent with the data from normal
21 administration of feed. This suggests that differences between diets as regards insulin
22 levels 6 h after normal feeding are really due to the intrinsic insulinitropic capacity of
23 dietary components, rather than being a possible consequence of differences in intestinal
24 transit rate or quantity of ingested food, parameters which are controlled and are the
25 same for both diets in forced feed experiment. Nevertheless, the observed profile with

1 maximum levels 6 h after feeding differs from that described previously for trout during
2 standard feeding administration experiments, where very high insulin levels were
3 already reached 1-2 h after ingestion of food (Pérez et al., 1988; Navarro et al., 1993;
4 Novoa et al., 2004). High levels of insulin are actually found even earlier, around the
5 time of food administration, when a reflex pre-absorptive release of insulin is produced;
6 this is related to the visual appeal of food, the time feeding schedule, progressive
7 stomach distension and other stimulating factors (Papatriphon et al., 2001). It appears
8 that with the method of force feeding, which avoids all these external stimulating
9 factors, the early secretion of insulin is reduced. However, force feeding would appear
10 not to be the best method to study glucagon response in trout. Plasma glucose and
11 glucagon levels were higher than in normal feeding conditions and remained high
12 throughout the experimental period, thus suggesting a stress effect due to fish handling.
13 Indeed, glucagon levels have been previously related to stress conditions, such as
14 netting in tanks or handling (Navarro et al., 1992). It is also quite possible that under
15 force-fed conditions, dietary nutrient supply is perhaps higher than that under voluntary
16 feeding conditions.

17 In sea bream, force feeding did not induce changes in plasma levels of
18 pancreatic hormones. In this species, the cephalic phase may be even more necessary
19 than in trout, considering that the increase in insulin after force feeding was much more
20 evident in trout than in sea bream. The fact that no differences were found in insulin
21 levels between diets could suggest that maybe other factors than the composition of
22 diets are also affecting the postprandial hormonal response during normal
23 administration of food. Nevertheless, we cannot be sure because the insulin profile over
24 time does not fit with the postprandial increases expected (Pérez et al., 1988). It is
25 possible that the response to force feeding in this species may not represent the real

1 physiological situation. The levels of glucagon found, which were lower than in a
2 normal feeding situation, suggest a different response of this hormone to a possible
3 stress situation in sea bream as compared to trout. Furthermore, although glucose levels
4 were also high the recovery to baseline values was more rapid in this species.

5 In conclusion, the dietary amino acid profile did not modify insulin levels, but
6 within a profile the decrease in the IAA/DAA ratio induced a lower stimulation of
7 postprandial insulin in both species, thus suggesting a β -cell sensitivity to small changes
8 in dietary amino acid proportions. These changes in postprandial insulin secretion did
9 not affect growth, but appear to contribute to an optimal nutritional and growth process.
10 Although the amino acid profile modulates postprandial glucagon secretion, individual
11 amino acids probably have different tropic activities depending on the fish species, thus
12 illustrating the complexity of glucagon secretion control. A greater emphasis must now
13 be placed on combined insulin and glucagon responses to diet so as to determine the
14 metabolic hormonal status that promotes optimal food use and growth.

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1 **Table legends**

2

3 **Table 1.** Ingredients and analytical composition of the experimental diets for rainbow

4 trout.

5 **Table 2..** Analysed amino acid composition of the four experimental diets for rainbow

6 trout

7 **Table 3..** Ingredients and analytical composition of the experimental diets for gilthead

8 sea bream.

9 **Table 4.** Growth performance, feed efficiency parameters and insulin and IGF-I binding

10 parameters in skeletal muscle of rainbow trout fed the experimental diets for

11 12 weeks.

12 **Table 5..** Growth performance, feed efficiency parameters and insulin and IGF-I

13 binding parameters in skeletal muscle of gilthead sea bream fed the

14 experimental diets for 12 weeks.

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Figure legends

Figure 1. Plasma glucose, insulin and glucagon levels of rainbow trout (A, C, E) and gilthead sea bream (B, D, F). Different letters indicate significant differences between diet groups for the same time sampling (6 or 24 h) at $p<0.05$. Asterisk indicates significant differences between sampling time for each diet at $p<0.05$.

Figure 2. Effect of force feeding on plasma glucose, insulin and glucagon levels of rainbow trout (A, C, E) and gilthead sea bream (B, D, F). Different letters indicate significant differences between sampling times (1, 3, 6, 12 and 24 h) Asterisk indicates significant differences between diets for the same sampling time of force feeding at $p<0.05$.

Table 1.-

	TM1	TM2	TWB1	TWB2
<i>Ingredients (g/Kg)</i>				
Fish meal (CP 70%)	389,9	316,4	460	363,3
Wheat gluten	71,4	0	50	100
Extruded whole wheat	135,7	71,8	200	200
Extruded peas (Aquatex)	215,1	56,8	166,3	158,5
Soybean meal (CP 42%)	25,3	331,3	0	0
Fish oil	101,6	109,5	93,7	104,1
Binder	10	10	10	10
Mineral premix ^a	10	10	10	10
Vitamin premix ^a	10	10	10	10
CaHPO ₄ ·2H ₂ O	10,9	16,1	0	0
IAA mix	20,1	18,2	0	0
L-Glu	0	50	0	44,2
<i>Analytical composition (g/Kg)</i>				
Dry matter	922	905	935	921
Protein	451	463	437	441
Lipid	156	164	157	158
NEF ^b	245	200	276	267
Energy (kJ/Kg DM)	224	221	222	224
IAA ^c	209	209	211	179
DAA ^d	202	255	209	256
IAA/DAA	1,03	0,82	1,01	0,70

^a Mineral and vitamin premix (NRC, 1993).

^b Nitrogen free extract.

^c Indispensable amino acids.

^d Dispensable amino acids.

1 **Table 2**

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Amino acids (g/Kg DM)	TM1	TM2	TWB1	TWB2
ARGININE	26,0	25,8	27,0	22,8
CYSTEINE	4,9	4,7	4,9	5,0
HISTIDINE	11,0	11,2	10,1	8,6
ISOLEUCINE	19,8	18,5	17,2	14,3
LEUCINE	30,5	29,6	33,3	29,2
LYSINE	32,1	35,4	31,4	24,3
METHIONINE	10,1	10,2	10,4	8,7
PHENYLALANINE	17,1	16,3	18,4	16,6
THREONINE	18,1	18,9	18,1	15,3
TRYPTOPHANE	4,3	5,3	4,5	4,1
TYROSINE	13,2	13,0	14,5	12,7
VALINE	21,9	19,7	21,4	17,8
Alanine	22,1	21,8	24,8	20,5
Aspartic acid	34,0	39,7	37,9	30,9
Glutamic acid	80,9	132,7	76,4	139,5
Glycine	23,7	22,9	26,6	22,3
Proline	22,5	18,7	23,6	24,6
Serine	18,9	19,4	19,7	18,2

Indispensable amino acids (in upper case letters).

Dispensable amino acids (in lower case letters).

1 **Table 3.-**

	SBM1	SBM2	SBWB1	SBWB2
<i>Ingredients (g/Kg)</i>				
Fish meal (CP 70%)	499,9	478,1	529,3	484,1
CPSPG ^a	50	0	0	0
Wheat gluten	0	0	100	100
Extruded whole wheat	0	56,1	147,9	138,5
Extruded peas (Aquatex)	199,2	0	100	100
Soybean meal (CP 42%)	121,9	300	0	0
Fish oil	83,6	94,5	89,2	94,3
Binder	10	10	10	10
Mineral premix ^b	10	10	10	10
Vitamin premix ^b	10	10	10	10
IAA mix	15,3	16,7	3,5	0
L-Glu	0	24,6	0	53,1
<i>Analytical composition (g/Kg)</i>				
Dry matter	921	937	893	927
Protein	527	530	521	509
Lipid	157	164	159	152
NEF ^c	154	159	137	196
Energy (kJ/Kg DM)	225	225	225	225
IAA ^d	259	264	261	225
DAA ^e	228	256	263	280
IAA/DAA	1,13	1,03	0,99	0,80

^a Fish soluble protein concentrate from Sopropêche (Boulogne sur Mer, France).

^b Mineral and vitamin premix (NRC, 1993).

^c Nitrogen free extract

^d Indispensable amino acids.

^e Dispensable amino acids.

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1 **Table 4.-**

	TM1	TM2	TWB1	TWB2
IBW (g)	14,1 ± 0,0	14,1 ± 0,0	14,1 ± 0,1	14,1 ± 0,1
FBW (g)	110,0 ± 2,0	100,1 ± 3,9	110,1 ± 8,1	103,6 ± 2,5
HSI (%)	1,9 ± 0,2 ^a	1,4 ± 0,1 ^b	1,9 ± 0,1 ^a	1,8 ± 0,1 ^{ab}
SGR	2,5 ± 0,0	2,4 ± 0,1	2,5 ± 0,1	2,5 ± 0,0
FGR	1,0 ± 0,0 ^a	0,9 ± 0,0 ^b	1,0 ± 0,1 ^a	1,0 ± 0,0 ^a
PER	2,2 ± 0,0 ^a	2,0 ± 0,1 ^b	2,3 ± 0,1 ^a	2,3 ± 0,1 ^a
Kd INS	0,34 ± 0,1	0,11 ± 0,02	0,12 ± 0,07	0,15 ± 0,09
Ro INS	59,1 ± 17,6	25,3 ± 2,5	22,3 ± 10,1	38,8 ± 5,5
% Bsp INS	1,2 ± 0,34	1,23 ± 0,14	1,93 ± 0,2	2,27 ± 0,5
Kd IGF-1	0,24 ± 0,08	0,06 ± 0,02	0,1 ± 0,04	0,05 ± 0,02
Ro IGF-1	72,0 ± 4,6 ^a	29,9 ± 10,7 ^b	32,0 ± 12,8 ^{ab}	41,5 ± 5,5 ^b
% Bsp IGF-1	2,8 ± 0,9	2,5 ± 0,6	3,3 ± 0,8	3,5 ± 1,2

IBW: initial body mass; FBW: final body mass; HIS: hepatosomatic index; SGR: specific growth rate ([100 ln (final body weight)-ln (initial body weight)]/days); FGR: feed gain ratio (dry feed intake/wet weight gain); PER: Protein efficiency ratio (wet weight gain/protein intake) (n=15). Values of number of receptor (Ro) are expressed in fmol of receptors per mg of eluted protein, specific binding (%Bsp) in percentage per 20µg of eluted protein and dissociation constant (Kd) in nM for insulin (INS) and IGF-1(n=8). Values are means ± SE. Different superscript letters indicate significant differences between groups, for each parameter studied at $p < 0,05$.

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1 **Table 5.-**

	SBM1	SBM2	SBWB1	SBWB2
IBW (g)	14,7 ± 0,2	14,7 ± 0,0	14,6 ± 0,1	14,9 ± 0,1
FBW (g)	74,9 ± 0,7	70,9 ± 1,4	72,9 ± 1,4	70,9 ± 1,2
HSI (%)	1,36 ± 0,0 ^{bc}	1,2 ± 0,1 ^c	1,54 ± 0,0 ^a	1,5 ± 0,1 ^{ab}
SGR	2,0 ± 0,0	1,9 ± 0,0	2,0 ± 0,0	1,9 ± 0,0
FGR	1,2 ± 0,0 ^a	1,3 ± 0,1 ^{ab}	1,2 ± 0,0 ^{ab}	1,4 ± 0,0 ^b
PER	1,6 ± 0,0	1,5 ± 0,1	1,6 ± 0,0	1,5 ± 0,1
Kd INS	0,08 ± 0,03	0,19 ± 0,07	0,11 ± 0,03	0,38 ± 0,1
Ro INS	34,8 ± 6,8	101,1 ± 32,9	96,2 ± 34,6	124,5 ± 36,1
% Bsp INS	3,1 ± 0,2	4,3 ± 0,5	6,1 ± 0,0	3,0 ± 0,8
Kd IGF-1	0,07 ± 0,01	0,01 ± 0,0	0,03 ± 0,01	0,07 ± 0,02
Ro IGF-1	115,3 ± 12,5	49,0 ± 9,3	54,6 ± 7,3	138,0 ± 34,3
% Bsp IGF-1	11,3 ± 1,4	9,6 ± 1,3	12,6 ± 1,6	13,6 ± 2,2

IBW: initial body mass; FBW: final body mass; HIS: hepatosomatic index; SGR: specific growth rate ([100 ln (final body weight)-ln (initial body weight)]/days); FGR: feed gain ratio (dry feed intake/wet weight gain); PER: Protein efficiency ratio (wet weight gain/protein intake) (n=15). Values of number of receptor (Ro) are expressed in fmol of receptors per mg of eluted protein, specific binding (%Bsp) in percentage per 20µg of eluted protein and dissociation constant (Kd) in nM for insulin (INS) and IGF-1(n=8). Values are means ± SE. Different superscript letters indicate significant differences between groups, for each parameter studied at $p < 0,05$.

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