UNIVERSITAT DE BARCELONA
FACULTAT DE BIOLOGIA
DEPARTAMENT DE FISIOLOGIA

PAPER DE LA INSULINA I FACTORS DE CREIXEMENT TIPUS INSULINA EN LA REGULACIÓ DEL CREIXEMENT I METABOLISME EN TRUITA I ORADA

Tesi Doctoral

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El paper de la insulina i de l'IGF-I en el creixement compensatori de l'orada (*Sparus aurata*).

The role of insulin and IGF-I in the compensatory growth of the Gilthead Sea Bream (*Sparus aurata*).

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Resum

Amb el propòsit d'examinar el fenomen del creixement compensatori (CC) en l' orada (*Sparus aurata*), es va utilitzar un protocol experimental en el que després d'1, 3 i 4 setmanes de dejuni, els animals van ser realimentats de manera saciant (*ad libitum*) durant 1 i 2 mesos.

Després d'1 mes de realimentació, els guanys en el pes final i l'acceleració en el creixement dels grups que havien dejunat prèviament durant 1 i 3 setmanes (grups W1 i W3), eren comparables als valors que s'obtenien en el grup control (alimentats continuadament), indicant que s'havia donat una compensació parcial en el creixement.

Els valors plasmàtics d'insulina i d'IGF-I van disminuir durant els diferents períodes de dejuni, mentre que després d'1 i de 2 mesos de realimentació es va observar una recuperació ràpida en els valors en plasma dels dos pèptids. El contingut d'aigua, lípids i glicogen en el fetge i el múscul va disminuir significativament durant el dejuni i es va recuperar totalment després de dos mesos de realimentació. De la mateixa manera, durant el dejuni, els nivells de glucosa circulant van disminuir dràsticament, mentre que els àcids grassos lliures (FFA) van incrementar. Després d'1 i de 2 mesos de realimentació, els valors de glucosa i FFA en plasma van ser comparables als del grups control.

Els estudis de binding d'insulina i d'IGF-I en semipurificacions de proteïnes solubles del múscul blanc, indicaven que el binding d'IGF-I incrementa durant el dejuni, mentre que el binding d'insulina disminueix. Després d'1 i de 2 mesos de realimentació el binding per a tots dos pèptids és similar al que es va observar en el grup control.

Aquest estudi mostra que els valors circulants d'insulina i d'IGF-I després de la realimentació són paral·lels a la recuperació en el creixement, tal i com indiquen els guanys en el pes i les acceleracions en el creixement dels grups que havien dejunat prèviament. Tanmateix, el binding específic per a insulina i per a IGF-I indica que aquests receptors tenen una funció diferent en resposta a la situació nutricional de l'animal.

The role of insulin and IGF-I in the compensatory growth of the Gilthead Sea Bream (*Sparus aurata*).

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Key words: compensatory growth, insulin, insulin like growth factor I (IGF-I), white muscle, hormonal regulation.

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Abstract

In order to examine compensatory growth (CG) induced by re-feeding in gilthead sea bream (*Sparus aurata*), we used an experimental protocol involving 1-, 3- or 4-week of fasting followed by a one- or two-month re-feeding period. After one month of re-feeding, final body weight gain and growth acceleration in groups fasted for 1 or 3 weeks (W1 and W3) were comparable to the control group (C) values, indicating that partial CG was achieved. Plasma insulin and IGF-I values decreased during the fasting trial, while normal levels were restored after 1 month of re-feeding. Fasting significantly reduced lipid and glycogen content in liver and muscle, which were recovered after 2 months of re-feeding. Food restriction also had a marked effect on depressing circulating glucose plasma levels whereas FFA plasmatic levels were clearly elevated, indicating that lipid depot utilization contributes to the energy metabolism of the gilthead sea bream. After re-feeding for 1 or 2 months, glucose and FFA plasmatic levels were comparable to C group values. Insulin and IGF-I binding assays in partial semi-purifications of soluble proteins from white skeletal muscle showed that IGF-I binding increased with fasting while insulin binding decreased. However, the specific binding for these two peptides was comparable to C group values after re-feeding for 1 or 2 months. Our data indicate that circulating insulin and IGF-I peptides after re-feeding are well correlated with growth recovery, as shown by weight gain and specific growth acceleration of groups previously fasted. Furthermore, the specific binding for insulin and IGF-I indicates that these two receptors play distinct roles in response to the nutritional status of the animal.

1. **Introduction**

Gilthead sea bream (*Sparus aurata*) is a widely cultured fish in Southern Europe and responds to changes in dietary composition and ration size (Reviewed in Pérez-Sánchez and LeBail, 1999; Metón et al., 2003). Studies on several fish species have shown that nutritional status provokes profound effects on the growth and development of somatic tissues, particularly skeletal muscle (Navarro and Gutiérrez, 1995; Rescan 1998; Chauvigné et al., 2003; Power et al., 2000). Although information is available for salmonids, few studies have addressed the effects of food deprivation on the metabolism and the endocrine system of *Sparus aurata* (Pérez-Sánchez et al., 1994; Company et al., 2001; Power et al., 2000; Albalat et al., 2005).

The natural life cycle of many fish species includes a fasting period (Reviewed by Navarro and Gutiérrez, 1995). Moreover, compensatory growth (CG) of fish implies a readjustment of growth rate to minimize the discrepancy between achieved and desired growth rate, caused by a period of under-nutrition or unfavourable environmental conditions (Hubbell 1971; Xie et al., 2000; Reviewed by Ali et al., 2002). From a practical point of view, this compensatory strategy is of great interest to the aquaculture industry because feeding programmes can be designed to improve growth rates while minimizing costs (Hayward et al., 1997). However, in terms of biochemical indices, there are few data on the effects of fasting on subsequent re-feeding in gilthead sea bream (Company et al., 1999; Power et al., 2000; Grigorakis and Alexis, 2005).

Fish liver is one of the first organs to be affected by food deprivation (Power et al., 2000), and plays a major role in glucose homeostasis. In *S. aurata*, this organ exhibits a sensitive control to regulate glucose mobilization or production through cycling substrates and products in the glycolytic/gluconeogenic pathway (Metón et al., 2003). The maintenance of glycaemia during food deprivation is directly related to the capacity of hepatic glycogen mobilization, which occurs early in fasting (Lowe et al., 1970). Glucose homeostasis during fasting also depends on the subsequent activation of hepatic gluconeogenesis and the reduction in the rate of glucose utilization (Reviewed by Navarro and Gutiérrez 1995). Information regarding metabolic changes under conditions of food deprivation in gilthead sea bream is also limited to skeletal muscle (Power 2000; Grigorakis and Alexis, 2005; Sangiao-Alvarellos et al., 2005).

Circulating levels of growth hormone (GH) and insulin-like growth factors (IGFs) provide a useful tool to monitor growth performance and nutritional status

(Pérez-Sánchez et al., 1994; Reviewed by Pérez-Sánchez and Le Bail, 1999). In the same manner, it is well established that insulin and thyroid hormone are strongly affected by nutrition (Reviewed by Navarro and Gutiérrez, 1995; Cerdà-Reverter et al., 1996; Power 2000). Extended fasting normally causes a reduction in plasmatic insulin levels (Reviewed by Navarro and Gutiérrez, 1995), and a strong decrease in IGF-I plasma levels (Reviewed by Pérez and Le Bail, 1999), which may be related to high circulating levels of GH and plasma free fatty acids (FFA) (Reviewed by Björsson 1997).

To our knowledge, there is no information available on IGF-I or insulin binding in white skeletal muscle under a range of nutritional conditions in the gilthead sea bream. However, several authors have reported the physiological and endocrine mechanisms used by this species to recover from food deprivation by means of re-feeding (Powell et al., 2000; Grigorakis and Alexis, 2005; Sangiao-Alvarellos et al., 2005). Nevertheless, although salmonids show distinct degrees of CG mechanisms, little is known about those underlying CG in terms of endocrine responses (Miglavs and Jobling, 1989; Quinton and Blake, 1990; Jobling and Koskela, 1996; Bilton and Robins, 1998; Damsgard and Dill, 1998).

Here we studied circulating insulin and IGF-I peptides and receptor binding, mobilization of body energy reserves, together with the variations in circulating concentrations of glucose and FFA, in juvenile gilthead sea bream, a Mediterranean teleost of high commercial value, after a period of food deprivation followed by re-feeding.

2. Materials and Methods

2.1. *Animals and experimental conditions*

Three hundred and fifty sexually immature gilthead sea breams (*Sparus aurata*), were acclimatized to laboratory conditions at the Aquaculture Centre-IRTA (Sant Carles de la Ràpita, Tarragona, Spain). To study the effects of re-feeding on fasting, fish weighing 52, 5±4, 12 grams were randomly distributed in eight rectangular 500-litre indoor tanks supplied with aerated seawater (3.8%). Water temperature ranged from 17 to 20ºC under a light–dark cycle of 12:12 hours. The experiment was performed from October 2003 to January 2004.

In the first stage of the trial, three fasting conditions were induced: 1 week (W1), 3 weeks (W3) and 4 weeks (W4). Controls (C) were fed until satiety with commercial pellets throughout the experiment. In the second stage of the trial the W groups were fed to satiation twice a day for 4 weeks. These groups were deprived of food in a manner whereby all groups finished their fasting period at the same time and were re-fed simultaneously also during the same period of 4 (December) or 8 (January) weeks. Two tanks for each treatment were used.

2.2 *Sample collection.*

Initial weight was calculated at the beginning of the experiment as the average of 24 fish (3 animals per tank). Fish were weighed under moderate anaesthesia (3-aminobenzoic acid ethyl ester, MS-222; 100 ppm). At the end of each experimental period (fasting or re-feeding), four fish per tank (8 fish per treatment) were rapidly anaesthetized and killed by a blow to the head. Blood samples were taken from the caudal vessels with heparinised syringes. Plasma samples were obtained after blood centrifugation (700×*g*, 10 min, 4º C) and then stored at -20 ºC until further glucose, FFA, insulin and IGF-I analyses.

Standard length (cm) and body weight (g) were measured and specific growth rates (SGR) for fish in the different treatments were calculated as follows: 100*ln (W2-W1)/ T2-T1 (W2: weight at time 2; W1: initial weight before running the experiment). The condition factor (an indicator of body shape) was calculated as follows: CF=body

weight X 100/body length ^{3}. The hepatosomatic index (HSI) was calculated as (liver weight (g)/body weight (g))* 100.

2.3 *Partial purification of receptors and ligand binding assays*.

Partial purification of solubilized insulin and IGF-I receptors from white muscle was performed at 4 °C, as described by Párrizas *et al.* (1995), by affinity chromatography on wheat-germ agglutinin (WGA) bound to agarose (Vector Laboratories, Burlingame, USA). The glycoproteins obtained were measured following the method described by Bradford (1976). Binding assays were performed as in Párrizas *et al*. (1994). A volume of 30–40 μl of the WGA eluate (approximately 30 μg of glycoproteins) was incubated for 14–16 hours at 4 °C with increasing concentrations of cold hormone (from 0 to 100 nM, final dilution) and the radiolabelled ligand as tracer (25 pM). Semi-purified receptors were precipitated by addition of 0.08% bovine γ-globulin and 10.4% polyethylene glycol (final concentrations), followed by centrifugation at 14,000×*g* for 7 min at 4 °C. Binding data were analyzed in Scatchard plots and only the high-affinity, low-capacity binding sites were considered in the analysis. Porcine insulin was obtained from Lilly (Indianapolis, USA) and human recombinant IGF-I from Chiron (Emeryville, CA, USA). Human Tyr A14 ^{125}I-monoiodoinsulin and human recombinant 3-^{125}I-IGF-I, both with 2000 Ci/mmol specific activity, were purchased from Amersham Life Sci. (Amersham Pharmacia Biotech Europe GmbH, Barcelona, Spain). All the other chemicals used were from Sigma Aldrich Química (Alcobendas, Madrid, Spain).

2.4 *Biochemical analyses of plasma parameters*.

Plasma glucose concentration was determined by the glucose oxidase colorimetric method (GLUCOFIX; Menarini Diagnostics, Firenze, Italy) (Huggett and Nixon, 1957; Sala-Rabanal et al., 2003), and plasma FFAs were analyzed using a commercial enzymatic method (NEFA-C, Wako Test). Plasma insulin levels were measured by radioimmunoassay (RIA) using bonito insulin as standard and rabbit anti-bonito insulin as an antiserum (Gutiérrez et al., 1984). Plasma was subjected to an acid-ethanol cryoprecipitation, as described by Shimizu *et al.* (2000), and the total amount of IGF-I was determined by RIA, following Vega-Rubín de Celis *et al.* (2004). Recombinant bream (*Pagrus auratus*) IGF-I (100% amino acid similarity with *S. aurata* IGF-I) was purchased from GroPep and used as tracer and standard.

2.5 *Analytical Methods*.

Liver and muscle samples were powdered in liquid nitrogen and separated into two fractions. One fraction was used to determine tissue water content by difference in weight before and after drying the sample at 105 °C for 24 h. In the other fraction, total lipid was extracted gravimetrically after two extractions with chloroform/methanol (2:1, v/v) and purification was performed following Folch *et al.* (1957). Lipid extracts were dried on a Rotavap (Büchi rotavapor 4-114, Switzerland) and weighed. All solvents contained 0.01% butylated hydroxytoluene (BHT) as anti-oxidant. Results are expressed as percentage lipid of wet weight (n=8 for each experimental group). Glycogen concentration was measured colorimetrically by the antrone method described by Fraga *et al*. (1956), with some modifications (Alemany et al., 1973) such as using a glycogen curve as standard. Results are expressed as mg g^{-1} liver wet weight (n= 8 for each experimental group).

2.6 *Statistical analysis*

Data are reported as mean values ± standard error of mean (S.E.M). The fasting/re-feeding effects were analysed with one-way ANOVA followed by Tukey´s test when variances were homogeneous.

3. **Results**

3.1 *Growth performance*.

Table 1 shows final mean weights (w), specific growth rates (SGR) and condition factors (CF) of fish at the end of both experimental periods (fasting and re-feeding). Food deprivation induced a progressive reduction in the parameters studied ($P< 0.05$) when compared to C. After 1 month of re-feeding W1 and W3, but not W4, reached the final body weight of C. Despite the continued availability of food during the second month of re-feeding, there was no significant weight gain for W groups when compared to C group.

SGR decreased in all fasted fish. After one month of re-feeding, rates were restored to C group values, except for W4. After two months of re-feeding, the SGRs of all W groups were similar although slightly lower than C. The trajectory of weight recovery of W1 and W3 after 1 month of re-feeding was comparable to C, indicating that growth was accelerated in these groups.

TABLE 1. Mean weight (W), Specific Growht Rate for Weight (SGRW) and Condition Factor (CF) in gilthead seabream reared at the different regime treatments : control (C), 1 (1W), 3 (3W) or 4 (4W) weeks fasting during fasting or after refeeding trials (1 or 2 months).

	W (g)			SGRW (%)			CF (%)		
Group	Fasted	Fed 1	Fed 2	Fasted	Fed 1	Fed 2	Fasted	Fed 1	Fed 2
C	67,05±4,55^{a}	74,9±1,36^{a}	89,78±4,59^{a}	0,75±0,26^{a}	0,59±0,03^{a}	0,57±0,05^{a}	2,72±0,09^{a}	2,58±0,11^{a}	2,58±0,05^{a}
W1	52,34±3,69^{b}	75,69±1,89^{a}	76,85±4,72^{b}	-0,01±0,24^{b}	0,60±0,07^{a}	0,39±0,07^{b}	2.76±0,11^{a}	2,51±0,04^{a}	2,58±0,06^{a}
W3	51,34±2,58^{b}	72,33±4,41^{a}	76,7±4,25^{b}	-0,09±0,17^{b}	0,51±0,1^{a}	0,40±0,09^{b}	2,65±0,07^{b}	2,56±0,02^{a}	2,48±0,06^{a}
W4	49,63±2,59^{bc}	61,24±4,67^{b}	74,39±4,74^{b}	-0,22±0,17^{c}	0,22±0,14^{b}	0,37±0,06^{bc}	2,52±0,10^{c}	2,42±0,07^{b}	2,64±0,14^{b}

Note. W, SGR and CF means(±S.E.M) correspond to measurement of at least 8 fish at the end of each regime trial. The *P* value corresponds to the results of the one way (fasting/refeeding) analysis of variance (Kruskal-Wallis rank test).Where there is a significant regime effect, different letters indicate differences ($P<0.05$) between means.

3.2 *Liver and muscle lipid, glycogen and water content.*

Table 2 shows the effects of fasting on HSI and glycogen and lipids in liver. Food deprivation clearly decreased HIS values in all W groups and re-feeding tended to restore the values of W groups to those shown by C (Table 2). The decrease in HIS in W groups during food deprivation was caused by the loss of liver weight and final body weight of the animals (Table 1). Hepatic lipid content (%) in W4 was significantly lower (P< 0.001), dropping to almost 50% of C group value, during the fasting trial, although lower effect was observed in W3 group. Glycogen content (mg g^{-1}) dramatically decreased after 3 or 4 weeks fasting. After 1 month of re-feeding, the W1 and W3 groups did not reach the glycogen values shown by C. A seasonal effect was found in liver stores of C, especially for glycogen, which decreased with respect to the first sampling.

TABLE 2. Hepatosomatic index (%) (n=8), liver lipids (n=8) and liver glycogen (n=8) (means ± S.E) in gilthead seabream reared at the different regime treatments : control (C), 1 (1W), 3 (3W) or 4 (4W) weeks fasting during fasting or after refeeding trials (1 or 2).

	HSI (%)			Liver lipids (%)			Liver glycogen (mg g^{-1})		
Group	Fasted	Fed 1	Fed 2	Fasted	Fed 1	Fed 2	Fasted	Fed 1	Fed 2
C	2,610±0,2^{a}	1,93±0,09^{a}	1,56±0,7^{a}	9,68±0,34^{a}	7,77±0,47^{a}	7,93±0,54^{a}	375,23±53,93^{a}	221,47±15,65^{a}	137,86±13,46^{a}
1 W	1,62±0,21^{b}	1,63±0,13^{b}	1,68±0,15b	9,00±0,73^{a}	6,50±0,28^{b}	6,08±0,30^{b}	206,55±33,62^{b}	162,30±20,51^{b}	144,48±22,13^{a}
3W	1,24±0,08^{c}	1,82±0,15^{a}	1,70±0,11c	8,68±0,33^{b}	8,31±0,59c	5,51±0,26^{c}	84,21±10,34^{c}	188,03±18,01^{c}	187,25±21,19^{b}
4W	0,80±0,07^{d}	1,87±0,10a	1,60±0,19^{a}	5,39±0,26^{c}	7,30±0,31^{a}	6,10±0,35^{b}	44,40±1,87^{d}	215,62±27,14^{a}	170,53±44,63^{b}

Note. Stadistical analysis was made at each feeding trial (Fasting /Fed 1/ Fed 2).The P value corresponds to the results of one way analysis of variance followed by Kruskall-Wallis test. Where there is a significant regime effect, different letters indicate differences ($P<0.05$) between means.

The percentage of total lipid in white skeletal muscle decreased in W4 during fasting and glycogen percentage also declined by more than 50% of the C value (Table 3). One month of re-feeding did not restore glycogen content in the W3 and W4 groups to C values, whereas lipid content was recovered for both re-feeding groups. Water content was not affected in either liver (data not shown) or muscle in any of the experimental trials.

TABLE3. Muscle water (%) (n=8), lipids (n=8) and glycogen (n=6) (means ± S.E) in gilthead seabream reared at the different regime treatments : control (C), 1 (1W), 3 (3W) or 4 (4W) weeks fasting during fasting or after refeeding trials (1 or 2).

	Muscle water (%)			Muscle lipids (%)			Muscle glycogen (%)		
Group	Fasted	Fed 1	Fed 2	Fasted	Fed 1	Fed 2	Fasted	Fed 1	Fed 2
C	76,66 ±0,47	77,32 ±0,50	76,66 ±0,47	4,14 ±0,22	4,20 ±0,34	3,30 ±0,28	0,34±0,07	0,27 ±0,07	0,18 ±0,03
1 W	77,03 ± 0,66	76,70 ±0,51	77,03 ±0,66	4,19 ±0,41	4,02 ±0,33	3,75 ±0,22	0,14 ±0,02	0,24 ±0,03	0,17 ±0,03
3W	78,04 ±0,49	77,24 ±0,50	78,04 ±0,49	4,28 ±0,24	4,46±0,08	3,86 ±0,30	0,21 ±0,05	0,18 ±0,02	0,17 ±0,02
4W	77,80 ±0,33	77,59 ±0,65	77,80 ±0,33	3,29 ±0,29	3,14 ± 0,18	3,78 ±0,19	0,10 ±0,03	0,16 ±0,01	0,30 ±0,09

Note. Stadistical analysis was made at each feeding trial (Fasting /Fed 1/ Fed 2).The P value corresponds to the results of one way analysis of variance followed by Kruskall-Wallis test. Where there is a significant regime effect, different letters indicate differences (P<0.05) between means.

3.3 *Effects of fasting and re-feeding on plasma metabolites, insulin and IGF-I levels.*

Plasma insulin (Fig. 1A) and IGF-I (Fig 1.B) levels decreased significantly with fasting (P< 0.05) and re-feeding clearly restored these two peptides to C values.

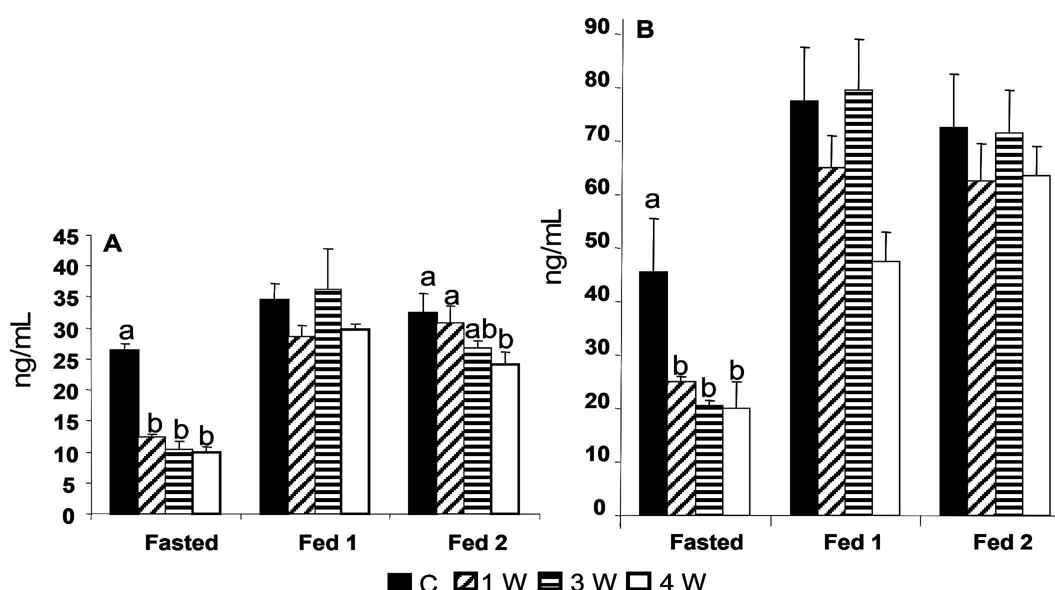

Fig 1. Plasma levels of Insulin (A) and IGF-1(B) in gilthead seabream from control (C), 1 (1W), 3 (3W) or 4 (4W) weeks fasting seabream during fasting or refeeding trials (1 or 2). The means (±S.E..M) of at least five different semipurifications, each performed in duplicated are shown. Differences were determined by the non-parametric Mann-Withney U test. Different letters indicates significantly different means at P<0.05.

Glucose plasma levels (Table 4A) were decreased in W3 and W4 groups compared to C. After 1 and 2 months of re-feeding, glycaemia levels were similar in all the experimental groups. FFA values progressively increased during fasting (Table 4 B). After re-feeding periods (1 or 2 months), W groups showed similar FFAs to those found of C.

TABLE 4. Glucose (mg/dL) and FFA (mEq) plasma levels in gilthead seabream reared at the different regime treatments : control (C), 1 (1W), 3 (3W) or 4 (4W) weeks fasting during fasting or after refeeding trials (Fed1/ Fed 2).

	Glucose (mg/dL)			FFA (mEq)		
Group	Fasted	Fed 1	Fed 2	Fasted	Fed 1	Fed 2
C	87,57±2,79[a]	73,24±3,72[a]	79,18±3,52[a]	0,55±0,02[a]	0,627±0,02	0,634±0,039
1 W	80,89±3,08[a]	88,18±3,72[b]	82,39±3,6[b]	0,66±0,04[b]	0,612±0,065	0,621±0,034
3W	64,97±9,90[b]	74,6±4,5[a]	82,93±3,28[b]	0,77±0,04[c]	0,637±0,035	0,636±0,036
4W	62,92±9,8[b]	83,42±3,5[b]	89,57±5,45[b]	0,83±0,07[d]	0,626±0,014	0,623±0,019

Note. The *P* value corresponds to the results of the one way (fasting/fed1 or fed 2) analysis of variance (Kruskal-Wallis rank test).Where there is a significant regime effect, different letters indicate differences ($P<0.05$) between means.

3.4 *Insulin and IGF-I binding in white muscle.*

Table 5 shows muscle insulin and IGF-I (3A and 3B respectively) receptor number (Ro) and affinity constant (Kd) throughout the experiment. Fasting decreased Ro for insulin whereas Ro for IGF-I in the W4 group was two times higher than in C ($P< 0.05$). Re-feeding trials restored the Ro for insulin and IGF-I in all W groups to C values.

A	Kd			Ro		
	Fasted	Fed 1	Fed 2	Fasted	Fed 1	Fed 2
C	0,28±0,13	0,23±0,06	0,08±0,013	110±0,7[a]	106±0,5	210±0,4
1 W	0,09±0,014	1,8±0,25	0,2±0,04	90±0,19[b]	190±0, 1	180±0,8
3W	0,013±0,05	0,49±0,23	0,2±0,04	60±0,16[c]	160±0,8	170±0,8
4W	0,08±0,07	0,28±0,12	0,7±0,32	60±0,012[c]	160±0,5	168±0, 8

B	Kd			Ro		
	Fasted	Fed 1	Fed 2	Fasted	Fed 1	Fed 2
C	0,04±0,01	0,02±0,05	0,07±0,5	100±0,03[a]	130±0,1	280±0,08
1 W	0,02±0,006	0,02±0,01	0,05±0,013	100±0, 4[a]	80±0,9	140±0,04
3W	0,03±0,004	0,03±0,01	0,02±0,002	130±0,25[b]	76±0,167	140±0,02
4W	0,04±0,03	0,03±0,003	0,03±0,004	230± 1,88[c]	78±0,08	100±0,02

Table 5. Characteristics of insulin (A) and IGF-I (B) binding to semipurified receptor preparations from white skeletal muscle in gilthead seabream from control (C), 1 (1W), 3 (3W) or 4 (4W) weeks fasting trout during fasting or refeeding trials (1 or 2). The means of 5 animals in each group are shown. Bars indicate error of the mean. Different letters indicate values significantly different at
Kd, affinity of the receptors as constant of dissociation (nM)
Ro, receptor number (fmol/mg eluted protein)

During fasting, specific binding (% Bsp) for insulin decreased while an opposite response was observed for IGF-I Bsp % in white skeletal muscle (Fig. 2A and 2B respectively). After re-feeding trials, both insulin and IGF-I Bsp (%) were comparable to C values.

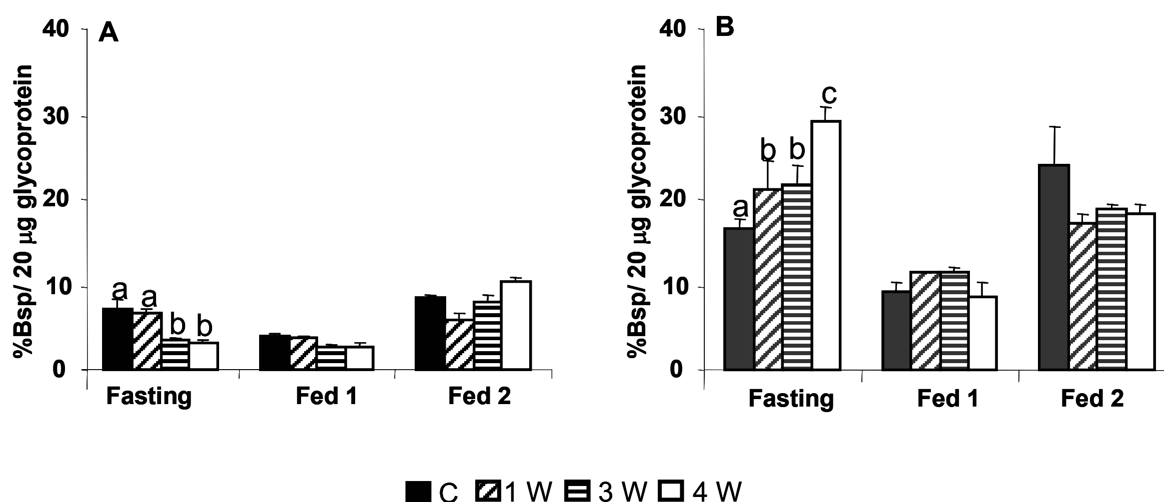

Fig. 2. Changes in percentage binding of insulin (A) and IGF-I (B) in white muscle of seabream from control (C), 1 (1W), 3 (3W) or 4 (4W) weeks fasting seabream during fasting or refeeding trials (1 or 2). The means (±SEM) of at least five different semipurifications, each performed in duplicated are shown. Differences were determined by the non-parametric Mann-Withney U test. Different letters indicates significantly different means at $P<0.05$.

4. Discussion

In the present report we studied whether periods of fasting of distinct lengths followed by single points of re-feeding induce CG in *Sparus aurata*. We demonstrated that partial CG can be induced by 1 and 3 weeks fasting. We examined the response of body energy storage and insulin and IGF-I systems during CG. An inverse relationship between circulating levels of IGF-I and the number of IGF receptors was observed during fasting conditions.

Gurney and Nisbet (2004) reported that CG is a phenomenon shown by many organisms which grow faster during recovery from starvation than during constant exposure to the same food environment. In mammals, CG is characterized by alterations in the secretion of several hormones that promote rapid growth (Reviewed by MacKenzie et al., 1998). Thus, under nutritional stress, insulin levels are reduced and lipolytic and glycogeonolytic signals promote the mobilization of body reserves. On the contrary, during full re-feeding, a rapid activation of protein anabolic hormones promotes somatic growth over deposition of reserves (Reviewed by MacKenzie et al., 1998).

CG has been studied in fresh water fish (Reviewed by Ali et al., 2003), but few data are available on marine species (Kim and Lowell, 1995; Hayward et al., 1997; Qian et al., 2000; Xie et al., 2001; Rueda et al., 1998; Barreto et al., 2003; Eroldoğan et al., 2006). In fact, only one study evaluated the changes in the IGF system through CG in the hybrid sea bass (Picha et al., 2006). Therefore, here we addressed this CG mechanism in the gilthead sea bream, a commercial and widely cultured marine species, and focused on the role of insulin and IGF-I and metabolic markers.

The W1 and W3 groups achieved the same final weight and SGR as the C group, indicating partial CG after one month of re-feeding for the two W groups. These results are comparable to those found by Xie et al. (2001), who reported CG in gibel carp re-fed for 1 month after 2 weeks of food deprivation.

In contrast, the W4 group did not achieve C values in any of the re-feeding trials. However, our results corroborate that, after a first rapid phase of growth, the acceleration of growth declines to the rates shown by the C group (Reviewed by Ali et al., 2003), and that 1 month of re-feeding is a suitable period for inducing CG in gilthead sea bream. Using cyclic deprivation protocols in the gilthead sea bream, Eroldoğan et al. (2006) reported the difficulty of food-restricted animals to achieve the

same size at the same age as their control contemporaries. Our study shows a partial compensation in the growth of the gilthead sea bream fasted for 1 or 3 weeks, suggesting that fixed periods of food deprivation and re-feeding are useful strategies to induce CG in this species. In this regard, partial or complete compensation have been observed in other marine species using fixed periods food deprivation (Rueda et al., 1998; Wang et al., 2000; Tian and Quin 2003).

Generally, in fish, liver glycogen is the first substrate to be used during fasting (Reviewed by Navarro and Gutiérrez, 1995). In our study, fasting provoked a rapid depletion of hepatic glycogen in all W groups. These results are consistent with those reported by Metón et al. (2003) and Sangaiao-Alvarellos et al. (2005) in the same species, where 18 or 14 days fasting, respectively, decreased liver glycogen. As described by Power et al. (2000), we also observed that fasting promoted glycogen depletion together with hypoglycemia. In addition, coinciding with other studies (Power et al., 2000; Metón et al., 2003), re-feeding restored liver glycogen and glucose plasma levels to control values. Power et al. (2000) observed that 3 weeks fasting promoted increases in liver water content and resulted in the appearance of vesicles and hepatocyte atrophy. In contrast, we did not detect changes in water content, which suggests the lack of extensive structural damage.

The percentage of carbohydrate reserves in white muscle is usually low (Bone and Marshall, 1982; Navarro and Gutiérrez, 1995). In agreement with results in *Gadus morhua* and *Brycon cephalus* (Martínez et al., 2002; Figuereido-Garutti et al., 2002), fasting also decreased muscle glycogen content. Lim and Ip (1989) considered that decreases in glycogen in the muscle of fasted *Boleophthalmus boddareti* indicated that this substrate was the first fuel to be utilized for muscle activity. As found in liver, 2 months of re-feeding restored muscle glycogen to control values, which indicates the recovery of metabolic conditions.

In relation to lipid metabolism, hepatic lipid stores decreased in all W groups during fasting, as reported in a similar study (Power et al., 2000). The pattern of hepatic lipid recovery was similar in all groups after 1 and 2 month of re-feeding. Fasting also decreased muscle lipid percentage, as recently described by Grigorakis et al. (2005) in the same species. This parameter was restored after both re-feeding periods. FFA plasma levels increased in an inverse manner to glucose plasmatic levels, as observed during fasting conditions in trout (Pottinger et al., 2003). This observation suggests that the utilization of lipids during fasting contributes to fueling gilthead sea bream, and

facilitates tolerance to the hypoglycemic condition during food deprivation. Studies in gilthead sea bream also report an increase in FFA after 11 days of fasting (Albalat et al., 2005), even when glucose plasma levels are less affected. Furthermore, re-feeding restores FFA to C group values, indicating that lipid mobilization had finished (Pottinger et al., 2003). In the present study, the general pattern of energy utilization was the same for all fasted groups; glycogen and fat muscle percentages declined more slowly than in liver. The liver was depleted of its energy before any other tissue, and re-feeding tended to restore this organ first, which is the most metabolically active organ (Power et al., 2000).

Insulin is an anabolic hormone which induces glucose and amino acid uptake in liver and skeletal muscle and promotes lipogenesis and protein synthesis in both tissues, but mostly in the liver (Machado *et al*., 1988; Wenderlaar Bonga, 1993). Few studies have measured insulin plasmatic levels in gilthead sea bream. We found that fasting decreased insulin plasmatic levels, which is consistent with the results of a recent study in the same species (Albalat et al., 2005). As described by others (Reviewed in Navarro et al., 2005), re-feeding recovered plasmatic insulin levels to C values. This recovery initially favoured growth-promoting processes, while lipid and carbohydrate deposition was achieved after two months of feeding. This observation is consistent with the role of insulin facilitating the direct utilization of ingested nutrients (Reviewed by Mackenzie et al., 1998).

IGF-I peptide is structurally and functionally related to proinsulin (Reviewed by Duan, 1998). This peptide plays a crucial role in muscle somatic growth and development in mammals (Reviewed in Yakar et al., 2005). In fish, IGF-I stimulates proliferation and has metabolic effects on cultured trout myocytes (Castillo et al., 2004). IGF-I plasmatic levels showed a rapid decrease during food deprivation in all fasted groups, which coincides with the findings of previous studies on gilthead sea bream (Pérez-Sánchez et al., 1994 and Gómez-Requeni et al., 2004). The resulting decline in IGF-I production probably explains the decreased growth in W groups during fasting (Reviewed by MacKenzie, 1998). IGF-I plasmatic values were restored during re-feeding periods in all the W groups. In mammals (Ellenberger et al., 1989), re-feeding clearly restores IGF-I plasmatic levels during CG. Our results point to a positive parallelism between SGRs and plasmatic IGF-I values after 1 and 2 month of re-feeding, confirming that IGF is a useful tool by which to monitor the nutritional status and growth performance of *Sparus aurata* (Reviewed by Pérez-Sánchez and Le Bail,

1999). However, the partial CG response in W1 and W 3 groups was characterized by SGRs comparable to the C group, but was not accompanied by overcompensation in plasma IGF-I values. Thus, the accelerated growth in these groups during CG may be facilitated by a relative increase or change in plasma IGF-I rather than by absolute concentrations alone (Picha et al., 2006).

Among vertebrates, the interaction of IGF-I with its receptor stimulates, in a time- and concentration-dependent manner, the differentiation and proliferation of myoblast and satellite cells (Reviewed by Oksbjerg et al., 2004). Our study provides the first analysis of the changes in specific binding for insulin and IGF-I in white skeletal muscle of gilthead sea bream in response to food deprivation and subsequent re-feeding. IGF-I-specific binding was two fold higher than insulin binding during the experiment, as previously reported in *Onchorynkus mykiss* (Párrizas 1994; Baños et al. 1998; Méndez 2000). On the basis of these observations, we hypothesize that IGF-I makes a greater contribution to the regulation of muscle function under nutritional status than insulin. The binding for insulin in white skeletal muscle decreased in all the fasted W groups, as described by Párrizas et al. (1994) in *Cyprinus carpio,* whereas re-feeding tended to restore the values to those shown by the C group in both re-feeding trials (1 or 2 month). In *Onchorynchus mykiss* (Montserrat et al., 2006, submitted), the specific binding and receptor number of IGF I receptor in white skeletal increased with fasting. Previous studies in mammals demonstrate that fasting up-regulates IGF-I-specific binding in a range of tissues (Lowe et al., 1989) as well as its mRNA expression (Hernandez-Sanchez et al. 1997). Increases in hepatic IGF-I mRNA expression have been reported together with depressed levels of plasma IGF-I in the hybrid White Sea bass after fasting (Picha et al., 2006). On the basis of current data, together with the previous findings by Picha et al. (2006), the up-regulation in the specific binding of IGF-I receptor during fasting raises the possibility that target tissues are more sensitive to changes in circulating IGF-I when favourable conditions are restored. In addition, the anti-apoptotic effects of IGF-I have been described in cultured myocytes in rodents (Wu et al., 2004). We therefore propose that increases in IGF-I receptor number prevent protein degradation and muscle damage under catabolic processes such as fasting.

In conclusion, we show that partial CG was promoted by re-feeding after periods of fasting in the gilthead sea bream. Our data indicate that energy stores are clearly mobilized during fasting in order to assume an intermediary metabolism. When fish were deprived of food, insulin and IGF-I receptors displayed a distinct role in white skeletal muscle. Decreases in plasmatic IGF-I along with an up-regulation in its receptor suggest that IGF-I is a crucial mediator of muscle protection. However, insulin and IGF-I peptides promote growth recovery after re-feeding, as reflected by weight gain and specific growth acceleration.

Acknowledgements

We thank Alicia Estévez from the Aquaculture Centre-IRTA (Sant Carles de la Ràpita, Tarragona, Spain) for technical advice and supervision of feeding protocols. This study has been supported by AGL2004-06319-C02/ACU from the *Ministerio de Educación y Ciencia* to I.N., *Centre de Referència en Aqüicultura* (CRA)-2004 303038/ 2.2 to J.G.

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