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Combined replacement of fish meal and oil in practical diets for fast growing juveniles of gilthead sea bream (*Sparus aurata* L.): Networking of systemic and local components of GH/IGF axis

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Abstract:

Growth performance and growth regulatory pathways were examined in juvenile gilthead sea bream fed diets containing largely plant-based ingredients. Four isonitrogenous and isolipidic extruded diets with a low level (20%) of fish meal inclusion were formulated with graded levels of a vegetable oil mixture (17:58:25 of rapeseed: linseed: palm oils) replacing fish oil at 33, 66 and 100% (33VO, 66VO and VO diets). All diets were supplemented with lysine (0.55%) and contained soy lecithin (1%). Daily growth coefficients and feed efficiency over the course of an 11-week trial were almost identical in fish fed the FO, 33VO and 66VO diets. The VO diet reduced feed intake and growth without significant effects in proximate whole body composition, nitrogen or energy retentions. The highest concentration of plasma levels of insulin-like growth factor-I (IGF-I) was found in fish fed the 33VO diet. The lowest concentration was attained in fish fed the VO diet, whereas intermediate values were found in fish fed FO and 66VO diets. An opposite trend was found for circulating levels of growth hormone (GH), probably as a result of a reduced negative feedback inhibition from circulating IGF-I. Hepatic expression of IGF-I and GH receptor type I (GHR-I) was regulated in concert and mRNA levels paralleled plasma levels of IGF-I. Hepatic IGF-II and GHR-II were expressed in a more constitutive manner and no changes at the mRNA level were detected. In the skeletal muscle, IGF-I and GHR-I mRNAs did not vary significantly among groups. By contrast, IGF-II mRNA was up-regulated in fish fed the control diet, whereas the highest amount of GHR-II mRNA was attained in fish fed the 66VO diet. All together, these results suggest different growth compensatory mechanisms mediated by IGF-II and GHR-II at the local tissue level. These new insights prompted us to propose that practical diets low in marine ingredients can be used over the productive cycle of gilthead sea bream when essential fatty acids are supplied above the requirement levels.

Keywords: Sparidae; Fish oil; Vegetable oil; Plant proteins; Growth hormone; Growth hormone receptors; Insulin-like growth factors; Endocrine disrupters; Contaminants

50 **1. Introduction**

51

52 Currently, aquaculture is the major consumer of fish meal, a protein-dense feedstuff
53 that approximates the ideal amino acid profile of most cultured livestock. However, fish
54 meal is a limited resource whose availability has remained stable from the late 1980s at
55 approximately 6 million metric tonnes per annum, which limits the continuous growth of
56 aquaculture production (FAO, 2004). Furthermore, inherent variability in fish meal
57 composition due to species, season, geographic origin and processing leads to variation in
58 quality (Opstvedt et al., 2003; Bragadóttir et al., 2004), and most of the future changes in
59 developing novel aquafeeds should be focused on alternative protein sources.

60 The n-3 long-chain highly unsaturated fatty acids (n-3 HUFA) are naturally
61 abundant in the marine environment, and fish oil is the major source of eicosapentaenoic
62 acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 20:6n-3) for aquafeeds. Besides the
63 scarcity of fish oil, which is of great concern for marine fish, these animals have a limited
64 capacity to biosynthesize n-3 HUFA from the shorter chain linolenic acid (18:3n-3), and
65 both EPA and DHA become critical dietary constituents to ensure successful survival,
66 growth, and development of these fish (Sargent et al., 1999, 2002). At this standpoint, it
67 must be noted that fish meal also contains certain amounts of oil rich in n-3 HUFA, and the
68 fish oil added to energized diets can be totally replaced by vegetable oils when fish meal is
69 included at a high level in diets for Atlantic salmon (Bell et al., 2003; Bransden et al., 2003;
70 Torstensen et al., 2004), rainbow trout (Richard et al., 2006a), and the freshwater African
71 catfish (Ng et al., 2004). Similar results have been achieved in a typically marine fish such
72 as turbot (Regost et al., 2003). A high fish oil replacement is also feasible in the Murray
73 cod using casein-based diets (Francis et al., 2006). Likewise, up to 60% of fish oil added to

74 diets has been replaced successfully in juvenile European sea bass (Montero et al., 2005;
75 Mourente et al., 2005) and gilthead sea bream (Izquierdo et al., 2005), but the diets used in
76 these studies also contained 35 to 40% fish meal.

77 Marine derived feedstuffs are also possible vectors of contaminants, such as PCBs,
78 dioxins and other harmful chemicals affecting the safety of farm-raised fish (Jacobs et al.,
79 2002). It is clear that reduction in fish oil levels can lead to a decrease in the contaminant
80 levels of feed and consequently on fish filets (Berntssen et al., 2005; Bethune et al., 2006).
81 Thus, the general consensus is that alternative protein and oil sources are needed to
82 supplement or replace fish meal and fish oil in aquafeeds, contributing to long-term
83 sustainability of the aquaculture industry (Hardy, 2004). In the present study, our objective
84 was hence to maximize the combined replacement of fish meal and fish oil in practical diets
85 for fast growing juveniles of gilthead sea bream. In earlier studies, we had shown that a
86 good proportion of fish meal can be replaced by a mixture of plant protein sources in
87 gilthead sea bream diets (Gómez-Requeni et al., 2003, 2004; Sitjà-Bobadilla et al., 2005).
88 Based on these results, we attempted here to replace fish oil by a blend of vegetable oils,
89 which have been already shown to be very effective in other fish species (Torstensen et al.,
90 2005; Mourente and Bell, 2006; Richard et al., 2006a,b). To address this issue, growth and
91 nutrient retention were analyzed in a conventional manner. Circulating levels of growth
92 hormone (GH) and insulin-like growth factor-I (IGF-I) were used as markers of growth and
93 nutrient status (see Pérez-Sánchez and Le Bail, 1999; Dyer et al., 2004). Also, transcripts of
94 IGFs and GH receptors (GHR) were measured in liver and skeletal muscle by means of
95 real-time PCR assays.

96 **2. Materials and methods**

97

98 *2.1. Diets*

99

100 As shown in Table 1, three diets (33VO, 66VO and VO) with relatively low fish meal
101 inclusion (20%) levels were formulated with practical plant protein ingredients for the graded
102 replacement (33, 66 and 100%) of the added fish oil by a blend of vegetable oils (rapeseed oil:
103 linseed oil: palm oil). A fish oil-based diet (FO diet) equal in lipid content (220 g kg^{-1}) was
104 used as the reference diet. Diets were supplemented with lysine (0.55%) and contained soy
105 lecithin (1%). EPA plus DHA content varied on a dry matter basis between 2.3% (FO diet) and
106 0.3% (VO diet), and the DHA/EPA ratio (1.1-1.2) remained constant. All diets were
107 manufactured using a twin-screw extruder (Clextral, BC 45) in the INRA experimental research
108 station of Donzacq (Landes, France), dried under hot air, sealed and kept in air-tight bags until
109 use.

110 Diet samples were hydrolysed (6N HCl, 110 °C) and amino acid analysis was performed
111 using high-performance liquid chromatography. Tryptophan was determined by the
112 colorimetric method of Basha and Roberts (1977) after alkaline hydrolysis of each sample (see
113 Table 2). Fatty acid methyl esters (FAME) were prepared from aliquots of total lipid by acid-
114 catalysed transmethylation for 16 h at 50 °C (Christie, 1982) after the addition of
115 nonadecaenoic fatty acid (19:0) as an internal standard. FAMEs were extracted and separated in
116 a Fisons Instruments GC 8000 Series (Thermo Electron Co., Rodano, Italy) gas chromatograph,
117 equipped with a fused silica 30 m x 0.25 mm open tubular column (Tracer TR-WAX, film
118 thickness: 0.25 μm-Teknockroma, Spain) and a cold column injection system, using helium as
119 carrier and 50 to 220 °C thermal gradient. Peaks were recorded with Chrom-Card for Windows

120 software (Fisons CE Instruments, Milan, Italy) and identified by comparison with known
121 standards (see Table 3).
122
123 *2.2. Growth trial and fish sampling*
124
125 Gilthead sea bream (*Sparus aurata* L.) fingerlings of Atlantic origin (Ferme Marine
126 de Douhet, Ile d'Oléron, France) were acclimated to laboratory conditions for 20 days
127 before the start of the growth study. Fish of 16 g initial mean body weight were distributed
128 into 12 fibreglass tanks (500-l capacity) in groups of 60 fish each. Water (37.5 ‰ salinity)
129 flow was 20 l/min, and oxygen content of outlet water remained higher than 85%
130 saturation. Day length increased over the course of the trial (May-August) following natural
131 changes at our latitude (40º 5' N; 0º 10' E). Water temperature also varied naturally
132 increasing from 17 to 25 ºC.
133 The growth study was undertaken over 11 weeks (74 days) and each diet was
134 randomly allocated to triplicate groups of fish. Feed was offered by hand to apparent visual
135 satiety in two meals per day (0900 and 1400 h), and feed consumption was recorded daily.
136 Every 3 weeks, fish were counted and group-weighed under moderate anaesthesia (3-
137 aminobenzoic acid ethyl ester, MS 222; 100 μg/ml). Blood and tissue sampling was done at
138 the end of the growth trial from randomly selected fish killed by a blow to the head. Five h
139 after the morning meal (12 animals per diet; 4 animals per tank), blood samples were taken
140 from caudal vessels with heparinised syringes. Following overnight fasting (20 h after the
141 second daily meal), 12 additional fish per dietary treatment were taken for sampling of
142 blood, liver and white skeletal muscle. Plasma was drawn after centrifugation at 3000 x *g*
143 for 20 min at 4 °C, and stored at -30 °C until further hormone analyses. Liver and white

144 muscle were rapidly excised, frozen in liquid nitrogen and stored at -80 °C for RNA
145 extraction.
146
147 *2.3. Chemical composition analyses*
148
149 Proximate analysis of diets was made by the following procedures: dry matter by
150 drying at 105 °C for 24 h, ash by combustion at 550 °C for 12 h, protein (N x 6.25) by the
151 Kjeldahl method, fat after dichloromethane extraction by the Soxhlet method and gross
152 energy in an adiabatic bomb calorimeter (IKA). Specimens for whole body analyses (a
153 pooled sample of 10 fish at the beginning and pools of 5 fish per tank at the end of trial)
154 were ground, and small aliquots were dried to estimate moisture content. The remaining
155 samples were freeze-dried and chemical analyses were performed as indicated for
156 experimental diets.
157
158 *2.4. GH and IGF-I radioimmunoassay*
159
160 Plasma GH levels were assayed by a homologous gilthead sea bream
161 radioimmunoassay (RIA), using recombinant GH as tracer and standard (Martínez-
162 Barberá et al., 1995). Sensitivity and midrange of the assay were 0.1 ng/ml and 2.1 to 2.3
163 ng/ml, respectively.
164 After acid-ethanol precipitation, circulating levels of IGF-I were measured with a
165 generic fish IGF-I RIA (Vega-Rubín de Celis et al., 2004). The assay was based on the use
166 of recombinant red sea bream IGF-I (GroPep, Adelaide, Australia) as tracer and standard,
167 and anti-barramundi (Asian sea bass) IGF-I serum (GroPep, Adelaide, Australia) (1:8000)

168 as first antibody. A goat anti-rabbit IgG (1:20) (Biogenesis, Poole, UK) was used as
169 precipitating antibody. The sensitivity and midrange of the assay were 0.05 and 0.7 to 0.8
170 ng/ml, respectively.

171

172 *2.5. RNA extraction and RT procedure*

173

174 Total RNA extraction was performed with the ABI PRISM™ 6100 Nucleic Acid
175 PrepStation (Applied Biosystems, CA, USA). Briefly, liver and white skeletal muscle were
176 homogenized at a ratio of 25 mg/ml with a guanidine-detergent lysis reagent. The reaction
177 mixture was treated with protease K, and RNA purification was achieved by passing the
178 tissue lysate (0.5 ml) through a purification tray containing an application-specific
179 membrane. Wash solutions containing DNase were applied, and total RNA was eluted into
180 a 96-well PCR plate. The RNA yield was 40-50 µg with absorbance measures ($A_{260/280}$) of
181 1.9 to 2.1.

182 Reverse transcription (RT) with random decamers was performed with the High-
183 Capacity cDNA Archive Kit (Applied Biosystems). For this purpose, 500 ng total RNA
184 were reverse transcribed in a final volume of 100 µl. RT reactions were incubated 10 min at
185 25 ºC and 2 h at 37 ºC. Control reactions were run without reverse transcriptase and were
186 used as negative real-time PCR controls.

187

188 *2.6. Real-time PCR*

189

190 Real-time PCR was performed using an iCycler IQ Real-time Detection System
191 (Bio-Rad, Hercules, CA, USA) as previously described (Calduch-Giner et al., 2003).

192 Diluted RT reactions were used for PCR reactions in 25 μl volume. Each PCR-well
193 contained SYBR Green Master Mix (Bio-Rad) with specific primers for target and
194 reference genes at a final concentration of 0.9 μM (see Table 4).

195 The efficiency of PCR reactions for target and reference genes varied between 87
196 and 97%. The dynamic range of standard curves (serial dilutions of RT-PCR reactions)
197 spanned five orders of magnitude, and the amount of product in a particular sample was
198 determined by interpolation of the cycle threshold (Ct) value. The specificity of reaction
199 was verified by analysis of melting curves and by electrophoresis and sequencing of PCR
200 amplified products. Reactions were performed in triplicate and fluorescence data acquired
201 during the extension phase were normalized to β-actin, using the delta-delta method (Livak
202 and Schmittgen, 2001).

203

204 *2.7. Statistics*

205

206 Tank average values of growth, feed intake and nutrient retention were used as
207 experimental units in one way analysis of variance followed by Student-Newman-Keuls
208 test at a significance level of $P<0.05$. Plasma levels of GH and IGF-I were analysed by one
209 and two-way analysis of variance, followed by Student-Newman-Keuls test. Correlation
210 analyses between hepatic transcripts and plasma hormone levels were made by Pearson
211 Product Moment correlations ($P<0.05$).

212

3. Results

Diets 33VO and 66VO were well accepted by fish, and animals grew rapidly from 16 to 91-92 g over the course of the 11-week growth study (Table 5). No differences in feed intake (69 to 67.5 g/fish), daily growth indices (2.66 to 2.68%), and feed (1.09 to 1.11) or protein (2.21 to 2.25) efficiencies were found among control fish (FO) and fish fed 33VO and 66VO diets. Total replacement of fish oil by the vegetable oil blend (diet VO) reduced feed intake (61 g/fish) and daily growth indices (2.43%) without any significant effect on whole body composition. Nitrogen (35 to 37%) and energy (50 to 52%) retentions were not altered by dietary treatments, remaining high in all experimental groups. Lipid deposition in mesenteric and liver depots was not affected significantly by dietary treatments, although there was a trend for liver fat to increase with fish oil replacement.

At the end of the growth study, plasma levels of IGF-I were decreased over the course of the post-pandrial period ($P<0.05$) (Fig. 1). The highest IGF-I concentration was found in fish fed the 33VO diet and the lowest in fish fed the VO diet irrespective of sampling time (5 to 20 h postfeeding). Intermediate values were found in control fish and fish fed the 66VO diet.

There was no significant effect of dietary treatment on plasma GH levels (Fig. 2). However, the trend was opposite to that of plasma IGF-I levels. First, the overall plasma GH concentration increased over the course of post-pandrial period ($P<0.05$). Secondly, the lowest GH concentration was found in fish fed the 33VO diet whereas increased values were observed in fish fed the VO diet.

Hepatic IGF-I mRNA and plasma levels of IGF-I (20 h postfeeding) were positively correlated ($P< 0.05$). The highest amount of IGF-I mRNA was found in fish fed the 33VO

237 diet with a progressive and significant decrease with additional fish oil replacement,
238 whereas control fish remained at intermediate values (Fig. 3A). IGF-II was expressed at a
239 reduced level and no significant changes were found with dietary treatments, although the
240 trend for IGF-II mRNA was similar to that reported for IGF-I mRNA (Fig. 3B).

241 Hepatic levels of GHR-I mRNA correlated positively with hepatic transcripts of
242 IGF-I and plasma levels of IGF-I (Fig. 4A). Thus, GHR-I mRNA decreased progressively
243 and significantly with the graded replacement of fish oil in fish fed 33VO, 66VO and VO
244 diets. Intermediate values were found in fish fed diet FO. The overall expression of GHR-II
245 was of the same order of magnitude, but no significant changes in GHR transcripts were
246 detected with dietary treatments (Fig. 4B).

247 Muscle expression of IGF-I was lower in comparison to that of IGF-II, and no
248 significant effect of dietary treatments on IGF-I mRNA levels were detected (Fig. 5A). By
249 contrast, IGF-II mRNA was down-regulated in fish fed vegetable oils irrespective of the
250 degree of replacement (Fig. 5B).

251 The overall muscle expression of GHR-I and II was of the same order of magnitude.
252 There was no consistent change on GHR-I mRNAs with dietary treatment (Fig. 6A). By
253 contrast, transcripts of GHR-II were progressively up-regulated in fish fed 33VO and 66VO
254 diets, decreasing thereafter with the 100% of replacement of fish oil (VO diet) (Fig. 6B).
255

256 **4. Discussion**

257

258 The overall growth indices attained in the current work by juvenile gilthead sea
259 bream are higher than those reported for fish of the same age under similar light and
260 temperature conditions (Gómez-Requeni et al., 2003, 2004). This excellent growth
261 performance in all experimental groups could be attributed to improved diet formulation,
262 fish management and culture conditions. However, fish fed the VO diet showed a reduced
263 feed intake and increased liver fat deposition, which is characteristic of a wide range of
264 dietary and hormonal imbalances (see McClain et al., 2004; Avramoglu et al., 2006).
265 Indeed, in juvenile gilthead sea bream fed diets with amino acid imbalances, peripheral
266 lipolysis and tissue expression of lipoprotein lipase are regulated in concert to increase the
267 flux of dietary fatty acids through the liver (Albalat et al., 2005; Saera-Vila et al., 2005a).
268 This can be of special relevance during fasting and over-wintering, and extensive work is
269 now underway for this risk assessment.

270 Quantitative requirements of essential fatty acids (EFA) appear to vary depending
271 on fish species and growth stage (Sargent et al., 2002). Thus, the biological demand for n-3
272 HUFA was at least 1.3% for flatfish larvae (Le Milinaire et al., 1983), whereas
273 requirements for juvenile and grower fish were reduced to 0.8% (Gatesoupe et al., 1977;
274 Lee et al., 2003; Kim and Lee, 2004) and 0.6% (Lèger et al., 1979), respectively. Similar
275 requirements have been reported for juveniles of European sea bass (Skalli and Robin,
276 2004) and gilthead sea bream (Kalogeropoulos et al., 1992) fed defatted fish meal and
277 casein-based diets, respectively. Likewise, no detrimental growth effects were found in the
278 present study in fish fed the 66VO diet (0.9% EPA + DHA, see Table 3), which indicates
279 that fish oil replacement by alternative vegetable oils is feasible at a high level when EFA

280 requirements are covered. Partial fish oil replacement has been conducted successfully in a
281 wide variety of fish species, but this is the first report that maximizes the simultaneous
282 replacement of fish meal and fish oil in practical aquafeeds for fast growing juvenile marine
283 fish.

284 Fish growth rates vary with season, age and nutritional status and most of these
285 regulatory events are mediated by the GH/IGF axis (Company et al., 2001; Pérez-Sánchez
286 et al., 2002). The wide tissue distribution of GHRs supports the pleiotropic action of GH,
287 although the liver is the most important target tissue of GH and the primary source of
288 systemic IGF-I (endocrine form). In this scenario, changes on the plasma binding capacity
289 of the 33-47 kDa IGF-binding protein represents in rainbow trout an effective mechanism
290 to limit biologically active IGFs (free IGF fraction), keeping growth and GH secretion
291 under control (Gómez-Requeni et al., 2005). Likewise, circulating levels of IGF-I are
292 positively correlated with growth rates and dietary protein levels in Atlantic salmon and
293 Asian sea bass (Dyer et al., 2004). Plasma IGF-I levels are also a good indicator of growth
294 in channel catfish (Silverstein et al., 2000; Li et al., 2006). Similarly, in gilthead sea bream,
295 circulating GH and IGF-I are good markers of nutritional disorders arising from changes in
296 ration size (Pérez-Sánchez et al., 1995, 2002), dietary energy/ratio (Martí-Palanca et al.,
297 1996; Company et al., 1999) and dietary protein source (Gómez-Requeni et al., 2003,
298 2004). In the current work, the decreased growth of fish fed the VO diet were accordingly
299 paralleled by decreased plasma levels of IGF-I. Since IGF-I mRNA and GHR-I mRNA
300 were also reduced, the reduction in growth could be attributed to a transcriptional defect in
301 the signal transduction of GHR in spite of increased plasma levels of GH. This metabolic
302 feature leads to liver GH resistance as is now widely accepted in several fish species

303 (Pérez-Sánchez et al., 1995; Beckman et al., 2004; Pierce et al., 2005; Wilkinson et al.,
304 2006).

305 Growth in fish fed the VO diet (0.3% EPA + DHA; see Table 3) was only 90% of
306 the maximum observed, and there was no mortality in this group over the course of the
307 study. Similar results were reported for juvenile European sea bass fed defatted fish meal
308 diets (Skalli and Robin, 2004), which suggests that marine fish are relatively tolerant to
309 dietary fish oil restriction despite of the recognized essentiality of n-3 HUFA. As stated
310 very early by Watanabe (1982), the triacyglycerol and polar lipid fractions of lipids, both
311 containing adequate amounts of EPA and DHA, have the same EFA value. Takeuchi and
312 Watanabe (1979) have shown that a level of EFA exceeding four times the requirement of
313 rainbow trout leads to poor growth and feed utilisation. Detrimental growth effects have
314 also been reported in juvenile flounder when dietary n-3 HUFA becomes excessive (Kim
315 and Lee, 2004), and 25% replacement of fish oil by palm oil fatty acid distillate improved
316 weight gain of African catfish (Ng et al., 2004). In the present study, growth performance
317 of fish fed FO, 33VO and 66VO diets was almost identical, but the balance between
318 endocrine and locally produced IGFs differed depending on dietary treatment. Thus, in fish
319 fed the FO diet, the reduced gene expression and protein production of hepatic IGF-I was
320 apparently compensated by the increased expression of IGF-II at the local tissue level.

321 In mammals, IGF-II mRNA is detected in many fetal tissues but decreases quickly
322 during postnatal development (Daughaday and Rotwein, 1989). Accordingly, IGF-II null
323 mice are small at birth but continue to growth postnatally at a rate similar to wild-type. By
324 contrast, IGF-I null mice born were small and most died in the early neonatal stages. All
325 this strongly supports the key role of IGF-I during prenatal and postnatal growth. However,
326 hepatic IGF-I is not crucial for postnatal growth in mammals, and liver-specific IGF-I

327 knockout mice show normal growth due to the compensatory action of autocrine/paracrine
328 IGF-I (see Le Roith et al., 2001a,b). As postulated above in the present study,
329 compensatory increases of systemic IGF-I also occur in fish but, in this case, most of these
330 effects are dependent on local IGF-II. Indeed, substantial amounts of IGF-II are expressed
331 later in life in a wide range of fish species, including common carp (Vong et al., 2003),
332 rainbow trout (Chauvigné et al., 2003), Nile tilapia (Caelers et al., 2004), channel catfish
333 (Peterson et al., 2004), and gilthead sea bream as already evidenced in previous studies
334 (Duguay et al., 1996; Radaelli et al., 2003) and confirmed here. Furthermore, as found for
335 IGF-I, the main site for fish IGF-II expression is the liver, but in contrast to IGF-I, other
336 organs such as skeletal muscle also express quite high levels of IGF-II mRNA. Thus, fast
337 growing families of channel catfish express hepatic and muscle IGF-II at a high rate
338 (Peterson et al., 2004), and the growth spurt of juvenile rainbow trout during refeeding
339 could be mediated by muscle IGF-II (Chauvigné et al., 2003). Accordingly, it is reasonable
340 to assume that IGF-II acts in fish as an important growth-promoting factor through all the
341 life cycle, although most of these regulatory capabilities might have been lost during the
342 evolution of higher vertebrates.

343 To our knowledge, the precise mechanism(s) regulating the relative contribution of
344 systemic and local IGFs on fish growth remains unexplored. However, we suspect that
345 some results of the current study could be mediated by factors other than dietary fatty acids.
346 One of these factors might be the reduction in unwanted feed-borne lipid soluble
347 contaminants with the reduction in fish oil level. This assumption is based on our
348 complementary data (unpublished results) showing that dioxin-like PCBs in the FO diet
349 were markedly reduced with the graded fish oil replacement, as shown in previous studies
350 (Berntssen et al., 2005; Bethune et al., 2006). Experimental evidence also indicates that the

351 wasting syndrome caused in mice by 3-methylcholanthrene is mediated by aromatic
352 hydrocarbon receptors (AHRs) that interact with xenobiotic responsive elements (XREs) in
353 the GHR promoter, disrupting the liver GH signalling pathway (IGF production) (Nukaya
354 et al., 2004). Likewise, several XREs have been identified in the 5′-flanking region of
355 gilthead sea bream GHR-I (unpublished results), although further studies are needed to
356 determine whether these cis-regulatory elements are functional in fish. Besides, it has been
357 proven that expression levels of GH and prolactin (PRL) are regulated in rainbow trout by
358 persistent xenoestrogens and antiestrogenic pollutants (Elango et al., 2006), and both
359 estradiol and 4-nonylphenol suppress growth and plasma levels of IGF-I in juvenile
360 Atlantic salmon (Arsenault et al., 2004). It is not surprising, therefore, that gonadal steroids
361 modulate hepatic production of IGF-I and IGFBPs in tilapia (Riley et al., 2004) and coho
362 salmon (Larsen et al., 2004). All this provides regulatory mechanisms for dimorphic growth
363 patterns in fish, but at the same time makes the GH/IGF axis more vulnerable to potential
364 anthropogenic feed-borne contaminants.

365 Transgenic models in mice also indicate that major effects of GH on growth are
366 dependent on IGF-I expression, which requires intact insulin and IGF-I receptor signalling
367 in skeletal muscle (Kim et al., 2005). However, GH regulates other mitogenic factors, and
368 there is now experimental evidence supporting the up-regulated expression of GHRs during
369 muscle repair and maintenance (Casse et al., 2003). In fish, it is believed that genetic
370 duplication and divergence of two GHR subtypes (GHR type I and II) would take place on
371 an early ancestor of fish lineage (Saera-Vila et al., 2005b; Jiao et al., 2006). GHR-I was
372 first described in non-salmonid fish (Calduch-Giner et al., 2001), conserving most of the
373 structural features of mammalian GHRs. By contrast, GHR-II (also named somatolactin
374 receptor by Fukada et al., 2005) is unique to teleosts and encompasses most of the

375 published GHR sequences of salmonid fish. These two GHR subtypes are conserved in a
376 wide range of fish species, although apparent silencing and/or genomic loss of GHR-II was
377 reported in the flatfish lineage with the occurrence of truncated variants of GHR-I (Pérez-
378 Sánchez et al., 2002; Saera-Vila et al., 2005b). In this scenario, the current study confirms
379 and extends the notion that major GH effects on growth and hepatic IGF expression are
380 mediated by GHR-I in gilthead sea bream. By contrast, GHR-II emerges as a more
381 constitutive gene that does not necessarily require intact IGF-pathways to exert a protective
382 and/or growth promoting action. This is consistent with the up-regulated expression of
383 GHR-II in skeletal muscle of fish fed the 66VO diet. In this way, we previously reported
384 that GHR-II is up-regulated in the skeletal muscle of fasted juvenile gilthead sea bream
385 (Calduch-Giner et al., 2003). Similarly, Fukada et al. (2004) indicated that transcript levels
386 of GHR-II are not related in masu salmon to decreased expression of hepatic IGF-I during
387 fasting.

388 In summary, our data strongly support that combined replacement at a high level of
389 fish meal and oil is possible in diets of gilthead sea bream, contributing to the development
390 of sustainable aquafeeds. Data also bring new insights on the compensatory regulation of
391 systemic and local components of the GH/IGF axis (see Fig. 7 for a comprehensive survey)
392 in growing fish. Additional studies are underway to further explore the potential of
393 practical diets low in marine ingredients over the full cycle of gilthead sea bream farming,
394 addressing also issues related to potential feed-borne endocrine disruption of GH/IGF axis.

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396

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656 **Table 1**. Ingredients and chemical composition of experimental diets.

Ingredient (g/kg)	FO	33VO	66VO	VO
Fish meal (CP 70%) [1]	15	15	15	15
CPSP 90 [2]	5	5	5	5
Corn gluten meal	40	40	40	40
Soybean meal	14.3	14.3	14.3	14.3
Extruded wheat	4	4	4	4
Fish oil [3]	15.1	10.1	5.1	0
Rapeseed oil	0	0.85	1.7	2.58
Linseed oil	0	2.9	5.8	8.8
Palm oil	0	1.25	2.5	3.8
Soya lecithin	1	1	1	1
Binder (sodium alginate)	1	1	1	1
Mineral premix [4]	1	1	1	1
Vitamin premix [5]	1	1	1	1
$CaHPO_4.2H_2O$ (18%P)	2	2	2	2
L-Lysine	0.55	0.55	0.55	0.55
Proximate composition				
Dry matter (DM, %)	93.4	94.2	94.8	95.4
Crude protein (% DM)	48.9	48.7	49.0	48.6
Crude fat (% DM)	22.2	22.3	22.1	22.3
Ash (% DM)	6.5	6.6	6.6	6.4
EPA + DHA (% DM)	2.3	1.6	0.9	0.3
Gross energy (kJ/g DM)	24.7	24.7	24.6	24.5

657
658 [1]Fish meal (Scandinavian LT)
659 [2]Fish soluble protein concentrate (Sopropêche, France)
660 [3]Fish oil (Sopropêche, France)
661 [4]Supplied the following (mg / kg diet, except as noted): calcium carbonate (40% Ca) 2.15 g,
662 magnesium hydroxide (60% Mg) 1.24 g, potassium chloride 0.9 g, ferric citrate 0.2 g,
663 potassium iodine 4 mg, sodium chloride 0.4 g, calcium hydrogen phosphate 50 g, copper
664 sulphate 0.3, zinc sulphate 40, cobalt sulphate 2, manganese sulphate 30, sodium selenite
665 0.3
666 [5]Supplied the following (mg / kg diet): retinyl acetate 2.58, DL-cholecalciferol 0.037, DL-α
667 tocopheryl acetate 30, menadione sodium bisulphite 2.5, thiamin 7.5, riboflavin 15,
668 pyridoxine 7.5, nicotinic acid 87.5, folic acid 2.5, calcium pantothenate 2.5, vitamin B_{12}
669 0.025, ascorbic acid 250, inositol 500, biotin 1.25 and choline chloride 500

Table 2. Amino acid, mineral and trace element composition of the diets along with data on amino acid needs (Kaushik 1998).

Amino acid (%)	FO/VO diets	Needs
Arg	2.18	1.86
His	0.93	0.86
Ile	1.92	1.11
Leu	5.58	1.73
Lys	2.42	2.23
Met	1.05	
Cys	0.61	
Cys + Met	1.66	1.24
Phe	2.47	
Tyr	2	
Phe + Tyr	4.47	2.23
Thr	1.69	0.99
Trp	0.39	0.25
Val	2.15	1.49
Ser	2.23	
Ala	3.36	
Asp	3.69	
Glu	8.37	
Gly	2.01	
Pro	3.02	
Minerals (%, µg/g)		
Phosphorous (%)	1.08	
Magnesium (%)	0.18	
Potassium (%)	0.74	
Iron (µg/g)	216	
Copper (µg/g)	15	
Manganese (µg/g)	19	
Zinc (µg/g)	50	
Selenium (µg/g)	0.9	

717 **Table 3**. Fatty acid composition of experimental diets (% total FAME). Values
718 are means of two determinations; tr = trace value < 0.05

Fatty acid	FO	33VO	66VO	VO
14:0	5.02	3.70	1.89	0.59
15:0	0.35	0.22	0.13	0.12
16:0	16.7	16.9	16.9	16.7
16:1n-7	4.63	2.97	1.96	0.76
16:1n-9	0.22	0.15	tr	tr
16:3	0.49	0.35	0.26	0.14
16:3n-3	0.19	0.13	0.08	tr
16:4	0.40	0.29	0.17	tr
17:0	0.41	0.29	0.23	0.10
18:0	2.55	2.92	3.43	3.73
18:1n-9	12.5	17.5	21.9	25.9
18:1n-7	1.92	1.69	1.49	1.21
18:2n-6	12.1	15.7	19.2	21.3
18:3n-3	1.58	8.94	16.3	23.2
18:4n-3	2.16	1.47	0.82	0.20
20:0	0.30	0.30	0.31	0.29
20:1n-9	7.24	5.12	3.05	1.06
20:1n-7	0.21	0.16	0.09	tr
20:2n-6	0.17	0.12	0.11	tr
20:3n-3	0.08	0.07	tr	tr
20:4n-6	0.31	0.22	0.13	tr
20:4n-3	0.43	0.28	0.15	tr
20:5n-3 (EPA)	6.86	4.68	2.75	0.94
22:0	tr	0.16	0.16	0.17
22:1n-11	10.19	6.74	3.68	0.74
22:1n-9	0.56	0.43	0.29	0.16
22:2n-6	0.24	0.17	tr	tr
22:5n-3	0.64	0.40	0.18	tr
22:6n-3 (DHA)	8.34	5.68	3.38	1.06
Total	96.9	97.7	98.9	98.4
Saturates	25.3	24.5	22.9	21.7
Monoenes	37.6	34.8	32.4	29.8
n-6 HUFA[1]	0.31	0.22	0.12	0.7
n-3 HUFA[1]	16.3	11.9	6.5	2

719 [1]Fatty acids with more than 20 carbon atoms and more than 3 double bonds.

720 **Table 4**. Primers for real-time PCR. Forward primer, f; reverse primer, r.

Gene	Accession			Primer sequence	Position
β-actin	X89920	f	5'-	TCC TGC GGA ATC CAT GAG A	811-829
		r	5'-	GAC GTC GCA CTT CAT GAT GCT	861-841
GHR-I	AF438176	f	5'-	ACC TGT CAG CCA CCA CAT GA	1275-1294
		r	5'-	TCG TGC AGA TCT GGG TCG TA	1373-1354
GHR-II	AY573601	f	5'-	GAG TGA ACC CGG CCT GAC AG	1690-1709
		r	5'-	GCG GTG GTA TCT GAT TCA TGG T	1764-1743
IGF-I	AY996779	f	5'-	TGT CTA GCG CTC TTT CCT TTC A	112-133
		r	5'-	AGA GGG TGT GGC TAC AGG AGA TAC	195-172
IGF-II	AY996778	f	5'-	TGG GAT CGT AGA GGA GTG TTG T	406-427
		r	5'-	CTG TAG AGA GGT GGC CGA CA	514-495

721
722

723 **Table 5.** Data on growth performance, whole body composition, and nutrient gain and retention of
724 gilthead sea bream fed the four experimental diets for 11 weeks. Each value is the mean ± SEM of
725 data from triplicate groups. Data on viscera, liver and mesenteric fat indices were calculated from
726 16 fish.

	FO	33VO	66VO	VO	*P* [1]
Initial body weight (g)	16.1 ± 0.09	16.3 ± 0.01	16.3 ± 0.03	16.1 ± 0.09	*0.31*
Final body weight (g)	91.7 ± 0.45[b]	91.3 ± 0.90[b]	91.1 ± 1.20[b]	80.9± 0.28[a]	*<0.001*
Viscera (g)	8.28 ± 0.45	8.50 ± 0.33	8.37 ± 0.31	8.35 ± 0.46	*0.77*
Mesenteric fat (g)	1.72 ± 0.24	1.66 ± 0.11	1.79 ± 0.15	1.52 ± 0.14	*0.43*
Liver (g)	1.78 ± 0.12	1.82 ± 0.09	1.92 ± 0.08	1.72 ± 0.13	*0.68*
VSI (%) [2]	9.36 ± 0.30	9.12 ± 0.23	9.10 ± 0.24	9.89 ± 0.55	*0.28*
MFI (%) [3]	1.78 ± 0.23	1.73 ± 0.39	1.95 ± 0.17	1.78 ± 0.15	*0.45*
HSI (%) [4]	1.85 ± 0.07	1.93 ± 0.11	2.09 ± 0.09	2.02 ± 0.16	*0.38*
Liver fat (%)	15.9 ± 0.83	17.7 ± 0.94	18.7 ± 1.05	19.3 ± 0.51	*0.06*
DM intake (g/fish)	68.8 ± 0.60[b]	68.9 ± 0.66[b]	67.6 ± 0.25[b]	61.3 ± 0.77[a]	*<0.001*
Weight gain (%)	467.6 ± 6.2[b]	460.3 ± 5.2[b]	460.1 ± 7.1[b]	401.9 ± 3.1[a]	*<0.001*
DGI (%) [5]	2.68 ± 0.03[b]	2.66 ± 0.03[b]	2.66 ± 0.05[b]	2.43 ± 0.02[a]	*<0.001*
FE [6]	1.10 ± 0.01	1.09 ± 0.01	1.11 ± 0.02	1.06 ± 0.01	*0.07*
PER [7]	2.21 ± 0.01	2.23 ± 0.01	2.25 ± 0.04	2.14 ± 0.02	*0.06*
Whole body composition (% wet matter)					
Moisture	64.3 ± 0.29	64.1 ± 0.48	64.1± 0.28	63.9 ± 0.32	*0.70*
Crude protein	15.9 ± 0.46	16.0 ± 0.52	16.7 ± 0.43	16.9 ± 0.12	*0.10*
Crude fat	14.1 ± 0.67	14.3 ± 0.34	14.4 ± 0.42	14.4 ± 0.10	*0.95*
Ash	2.88 ± 0.11	3.44 ± 0.27	3.38 ± 0.14	3.61 ± 0.16	*0.09*
Retention (% intake)					
Nitrogen	35.4 ± 1.27	35.1 ± 1.12	36.9 ± 1.92	37.3 ± 0.63	*0.56*
Energy	50.1 ± 2.01	50.3 ± 1.06	52.5 ± 0.55	51.9 ± 0.76	*0.58*

727
728 Initial body composition: water, 70.9%; protein, 15.1%; lipid, 9.3%; ash, 3.4%
729 [1]P values result from analysis of variance. Different superscript letters in each row indicate significant
730 differences among dietary treatments (Student Newman-Keuls test, P<0.05).
731 [2]Viscerosomatix index = (100 × viscera wt) / fish wt
732 [3]Mesenteric fat index = (100 × mesenteric fat wt) / fish wt
733 [4]Hepatosomatic index = (100 × liver wt) / fish wt
734 [5]Daily growth index = $[100 \times (\text{final fish wt}^{1/3} - \text{initial fish wt}^{1/3})]$ / days
735 [6]Feed efficiency = wet weight gain / dry feed intake
736 [7]Protein efficiency ratio = wet weight gain / protein intake

737 **Legends of figures**

738

739 Figure 1. Plasma levels of insulin-like growth factor-I (IGF-I) in fish fed experimental diets
740 5 h after the meal (A) and following overnight fasting (B). Each value is the mean ± SEM
741 of 10 to 12 animals. Values with different letters are significantly different ($P<0.05$).

742

743 Figure 2. Plasma growth hormone (GH) levels in fish fed experimental diets 5 h after the
744 meal (A) and following overnight fasting (B). Each value is the mean ± SEM of 10 to 12
745 animals.

746

747 Figure 3. Normalized mRNA levels of IGF-I (A) and IGF-II (B) in the liver of fish fed
748 experimental diets (20 h postfeeding). Each value is the mean ± SEM of 6 to 8 animals.
749 Values with different letters are significantly different ($P<0.05$).

750

751 Figure 4. Normalized mRNA levels of GHR-I (A) and GHR-II (B) in the liver of fish fed
752 experimental diets (20 h postfeeding). Each value is the mean ± SEM of 6 to 8 animals.
753 Values with different letters are significantly different ($P<0.05$).

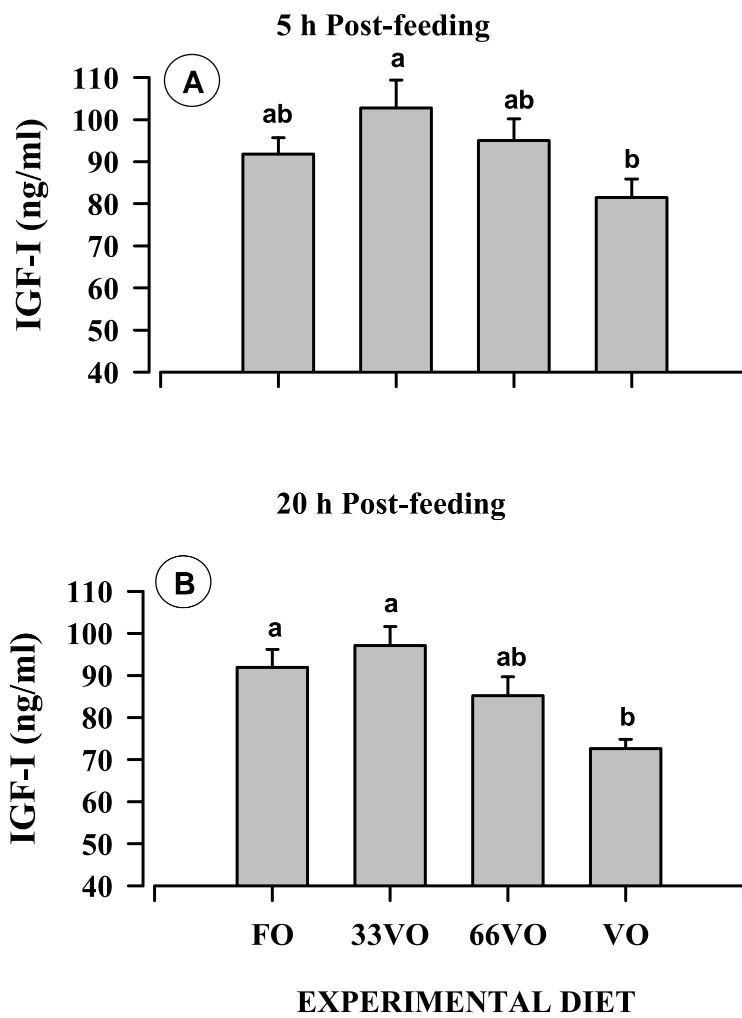
754

755 Figure 5. Normalized mRNA levels of IGF-I (A) and IGF-II (B) in the skeletal muscle of
756 fish fed experimental diets (20 h postfeeding). Each value is the mean ± SEM of 6 to 8
757 animals. Values with different letters are significantly different ($P<0.05$).

758

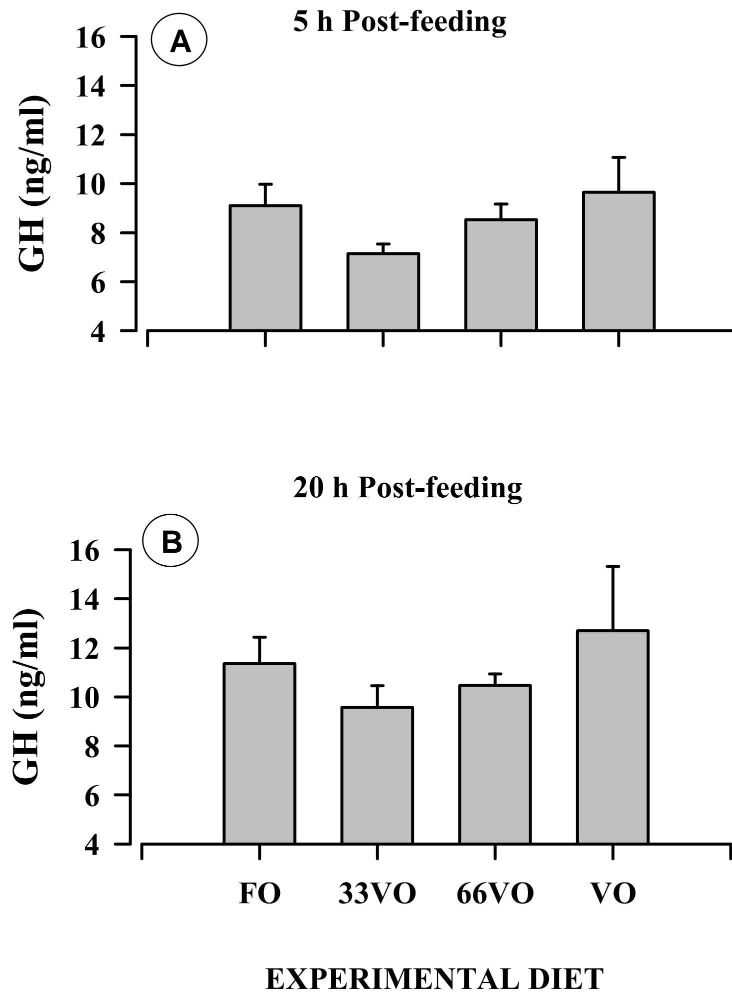
759 Figure 6. Normalized mRNA levels of GHR-I (A) and GHR-II (B) in the skeletal muscle of
760 fish fed experimental diets (20 h postfeeding). Each value is the mean ± SEM of 6 to 8
761 animals. Values with different letters are significantly different ($P<0.05$).
762
763 Figure 7. Proposed model for the balanced regulation of systemic and local components of
764 GH/IGF axis. Growth dysfunction occurs when the reduced production of systemic IGF-I is
765 not compensated at the local tissue level (fish fed VO diet). Compensatory IGF-II
766 production occurs at the local tissue level in fish fed FO diet. Alternatively, other
767 compensatory mechanisms of GH/IGF axis could be mediated at the local tissue level by
768 GHR-II via unknown factors, X, (66VO diet).

769 Figure 1



770

771 Figure 2



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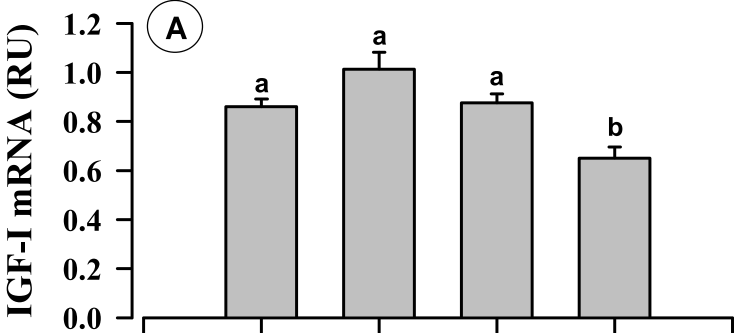

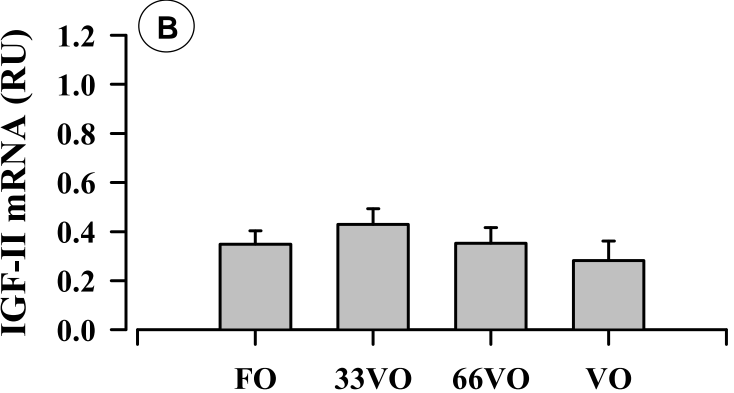

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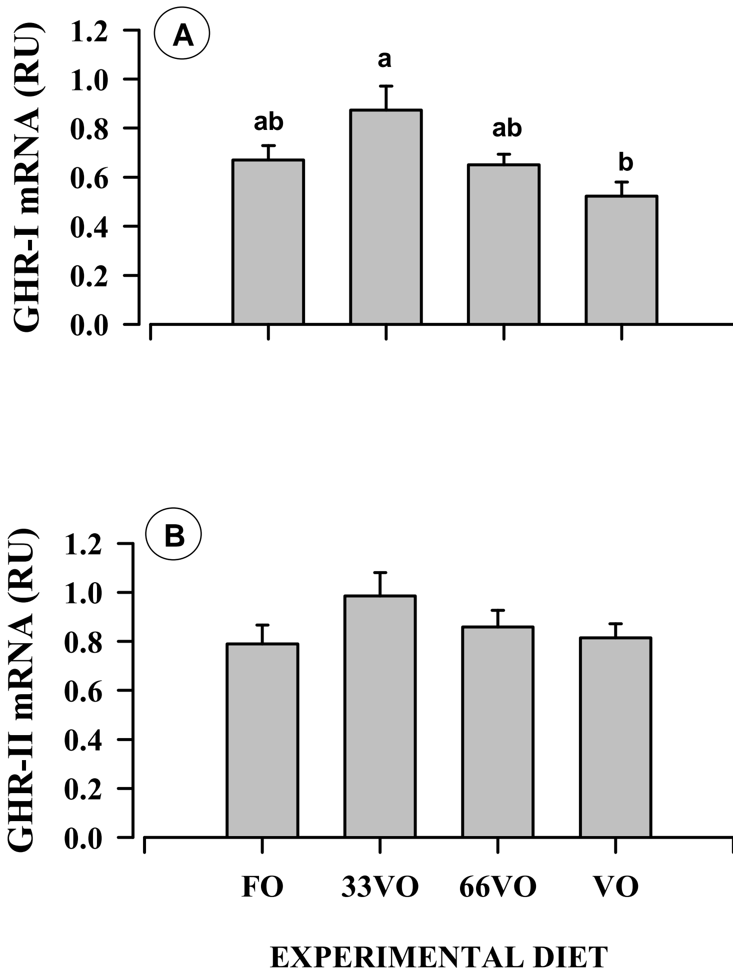
775 Figure 4



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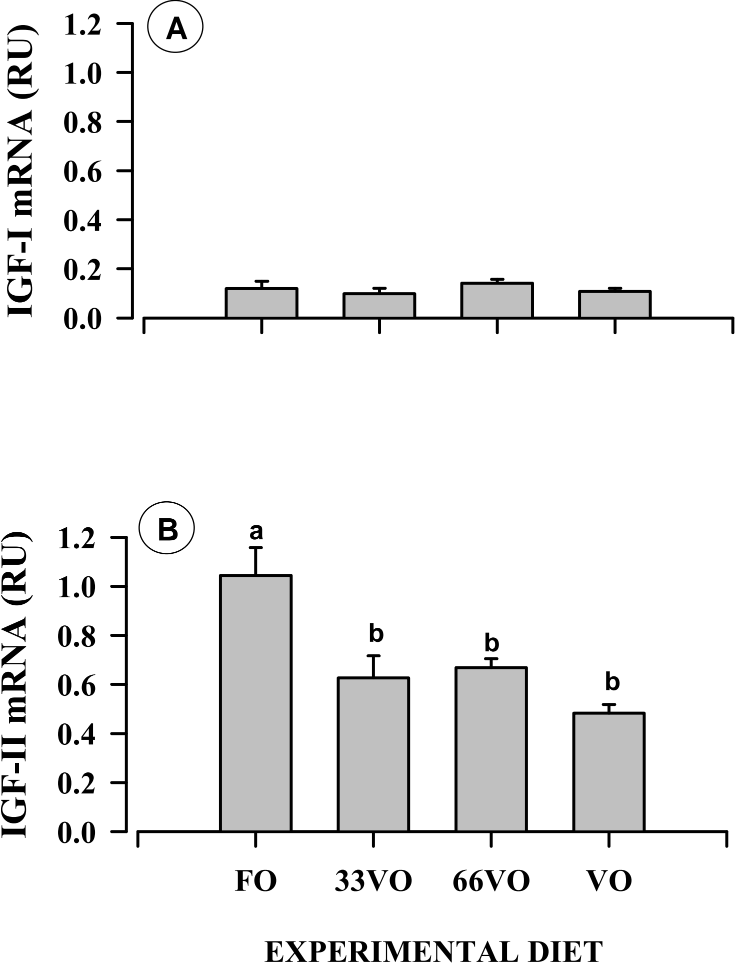
777 Figure 5



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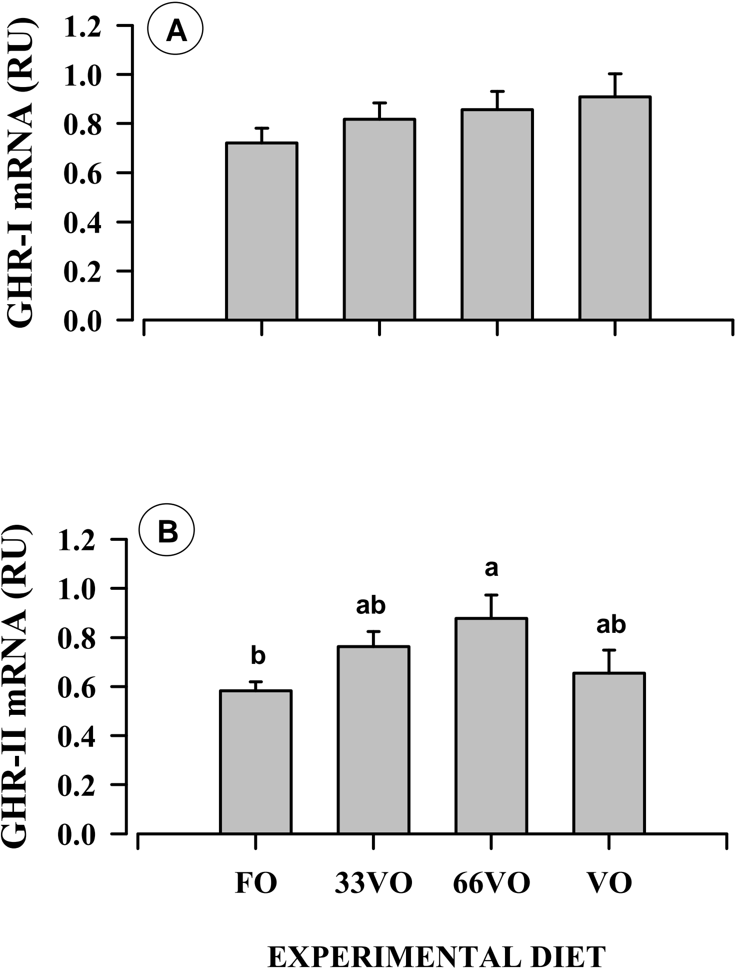
779 Figure 6

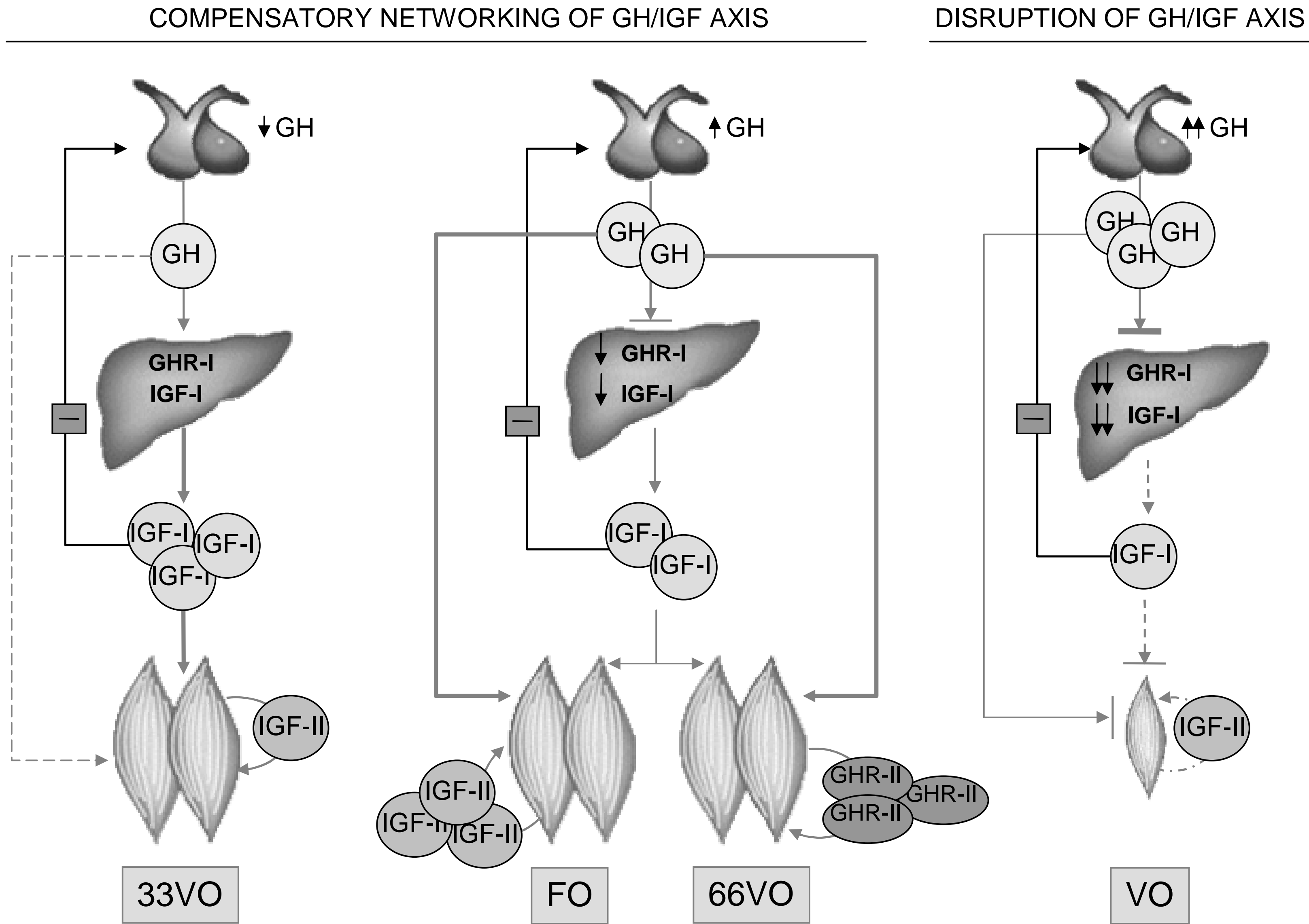


780 EXPERIMENTAL DIET

781 Figure 7

782

783



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