The effect of starvation and re-feeding on vasotocinergic and isotocinergic pathways in immature gilthead sea bream (*Sparus aurata*)

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Control
Food-deprived
Re-fed

A

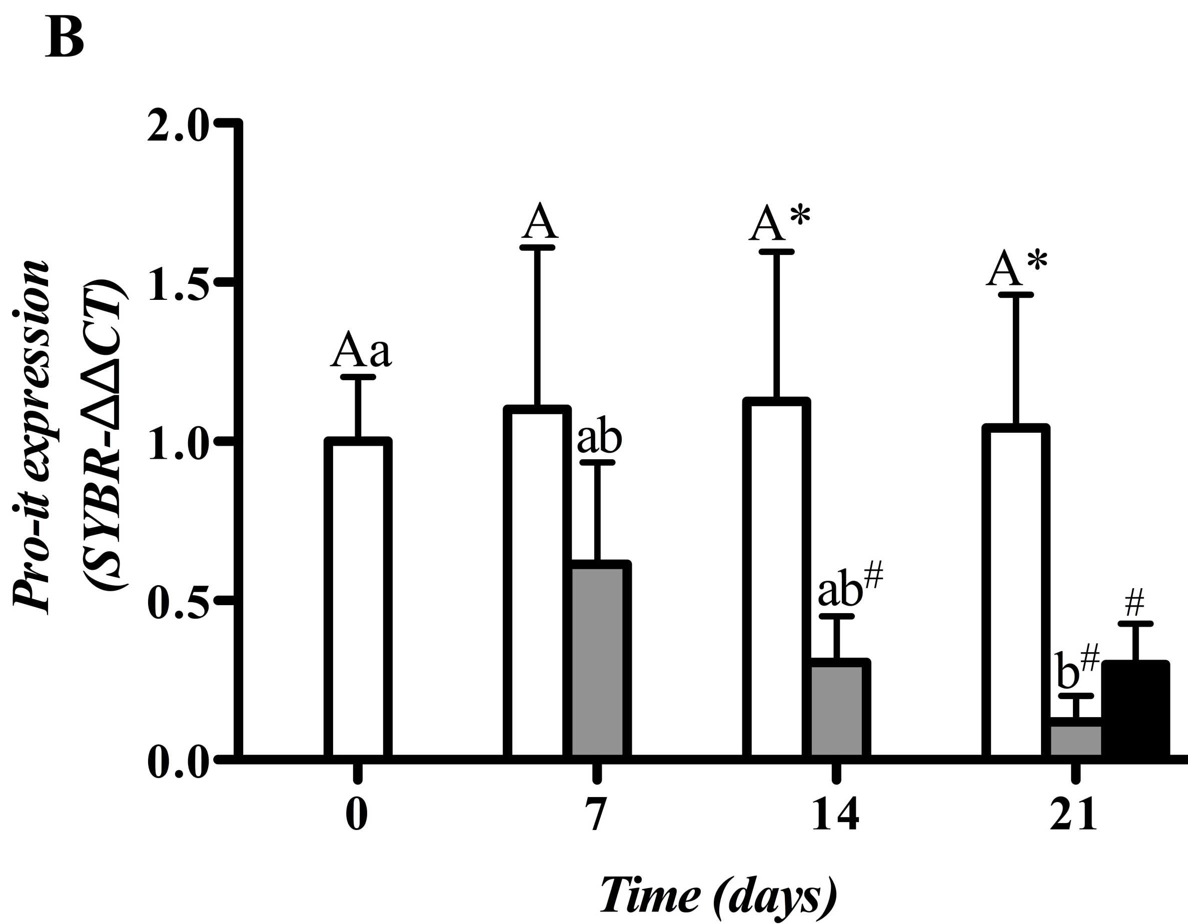
Pro-vt expression (SYBR-ΔΔCT)

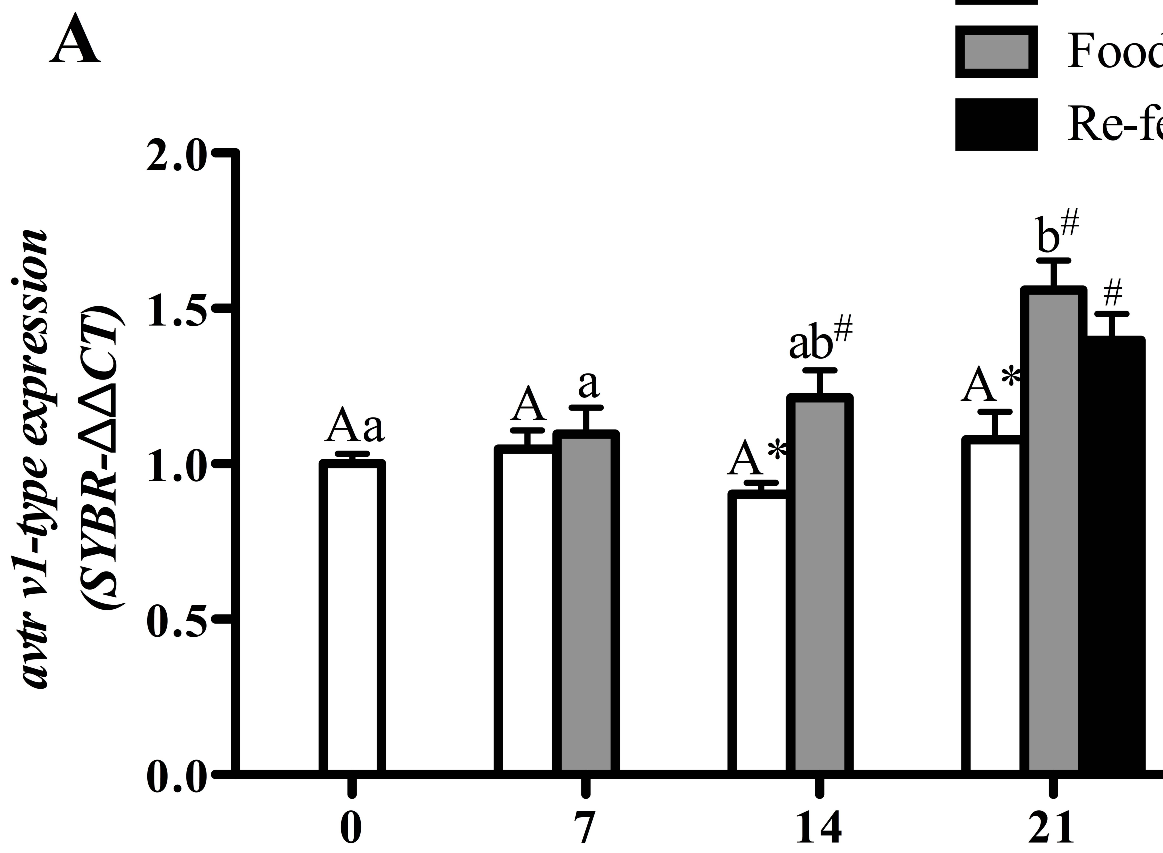
1.5
1.0
0.5
0.0

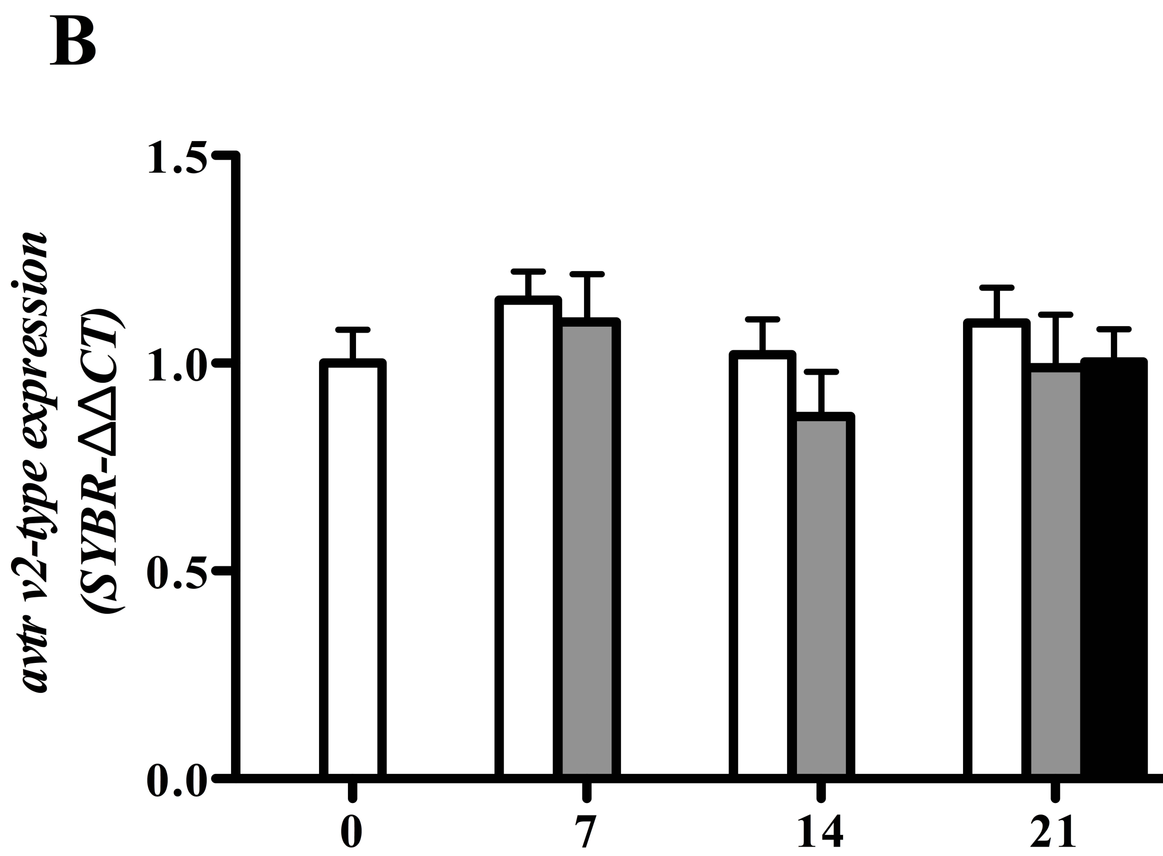
Aa
A*
ab#
A*
b#
A*
a#
#

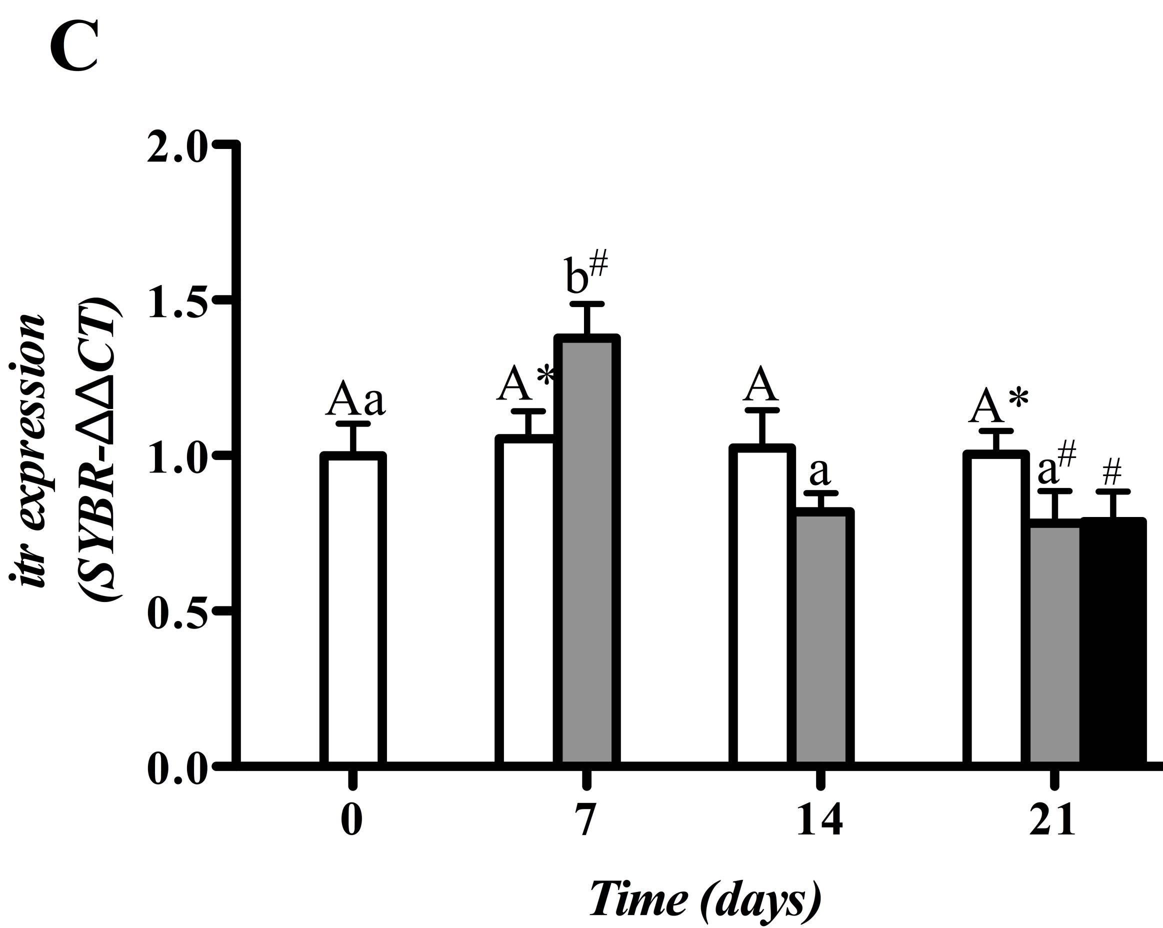
0
7
14
21

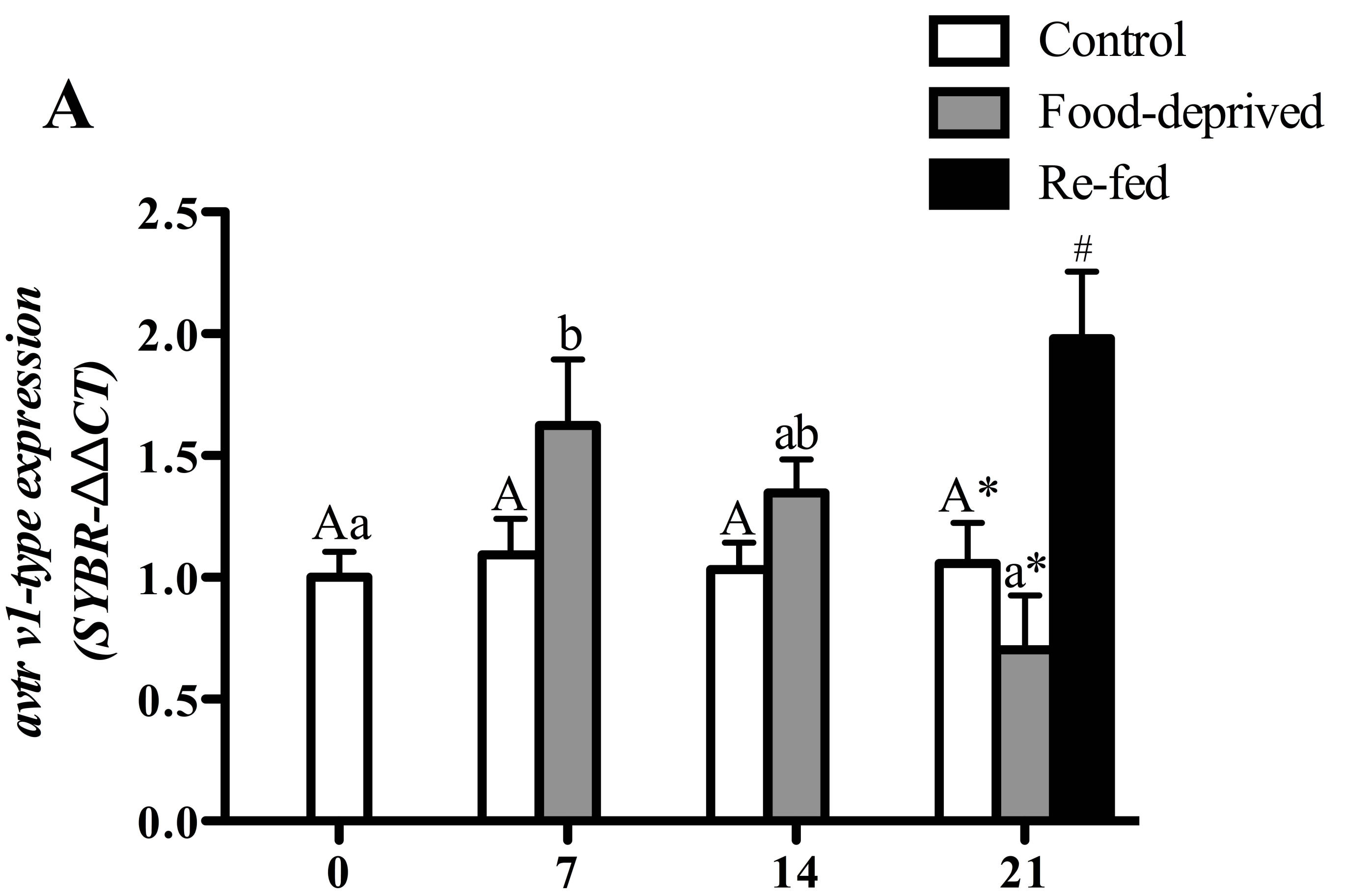
B

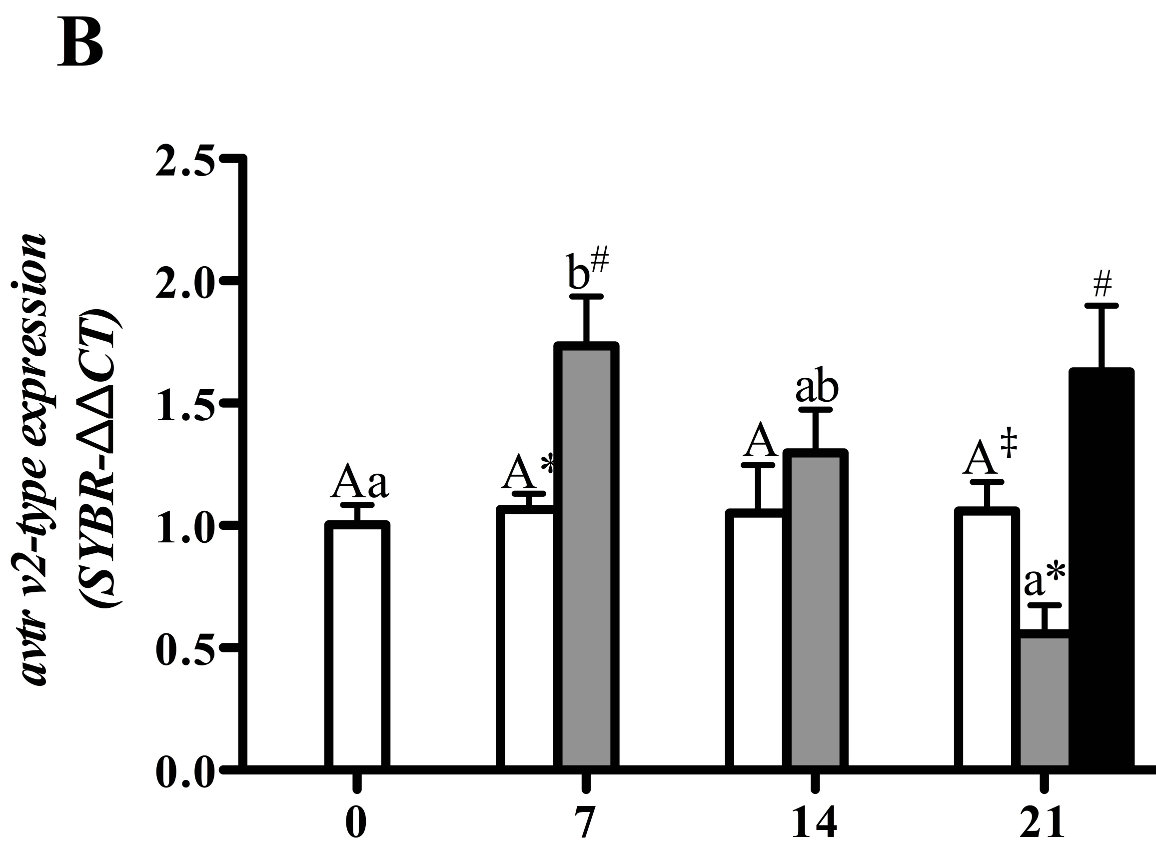


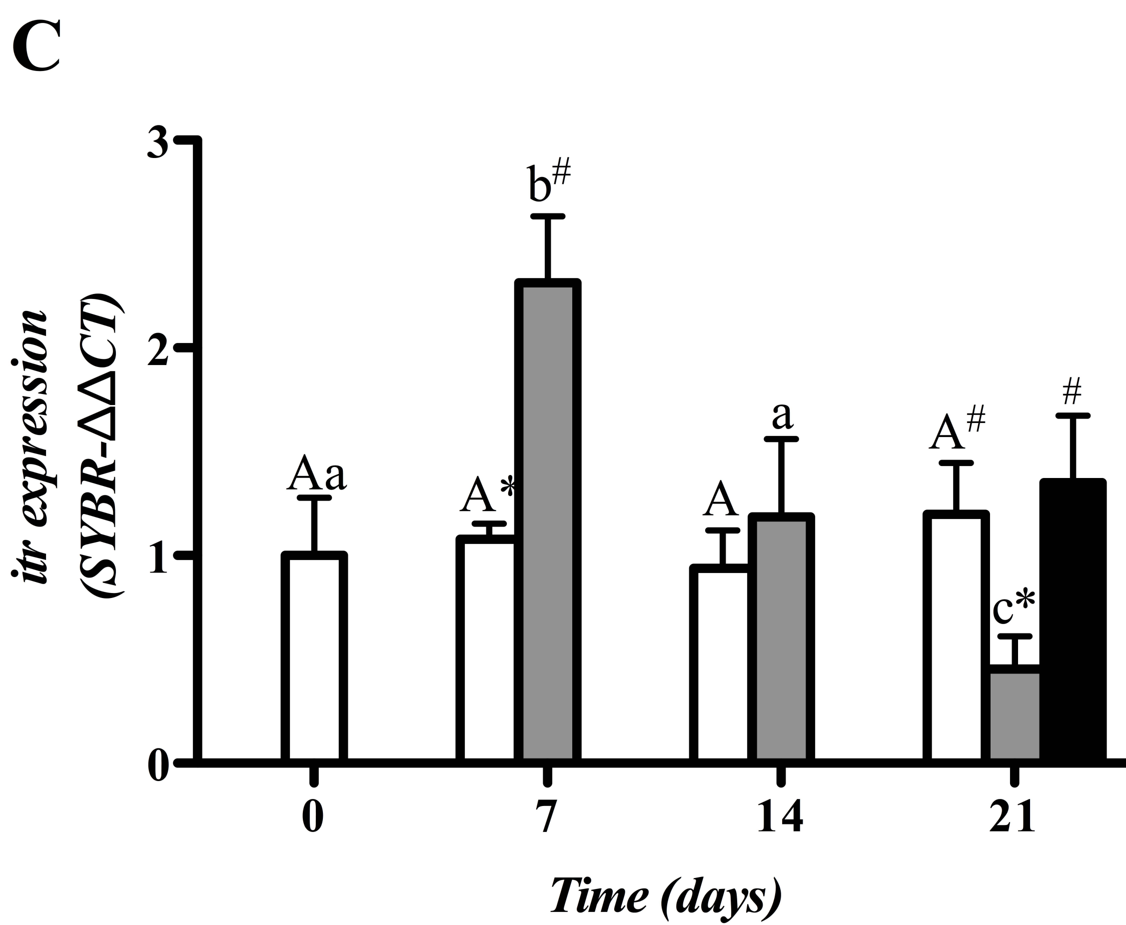

A
Control
Food-deprived
Re-fed
avtr v1-type expression (SYBR-ΔΔCT)
2.0
1.5
1.0
0.5
0.0
Aa
A
a
A*
ab#
A*
b#
#
0
7
14
21


B
avtr v2-type expression (SYBR-ΔΔCT)
1.5
1.0
0.5
0.0
0
7
14
21


C
itr expression (SYBR-ΔΔCT)
2.0
1.5
1.0
0.5
0.0
Aa
A*
b#
A
a
A*
a#
#
0
7
14
21
Time (days)


A
Control
Food-deprived
Re-fed
avtr v1-type expression
(SYBR-ΔΔCT)
2.5
2.0
1.5
1.0
0.5
0.0
Aa
A
b
A
ab
A*
a*
#
0
7
14
21


B
avtr v2-type expression
(SYBR-ΔΔCT)
2.5
2.0
1.5
1.0
0.5
0.0
Aa
A*
b#
A
ab
A‡
a*
#
0
7
14
21


C
itr expression
(SYBR-ΔΔCT)
3
2
1
0
Aa
A*
b#
A
a
A#
c*
#
0
7
14
21
Time (days)

Control
Food-deprived
Re-fed

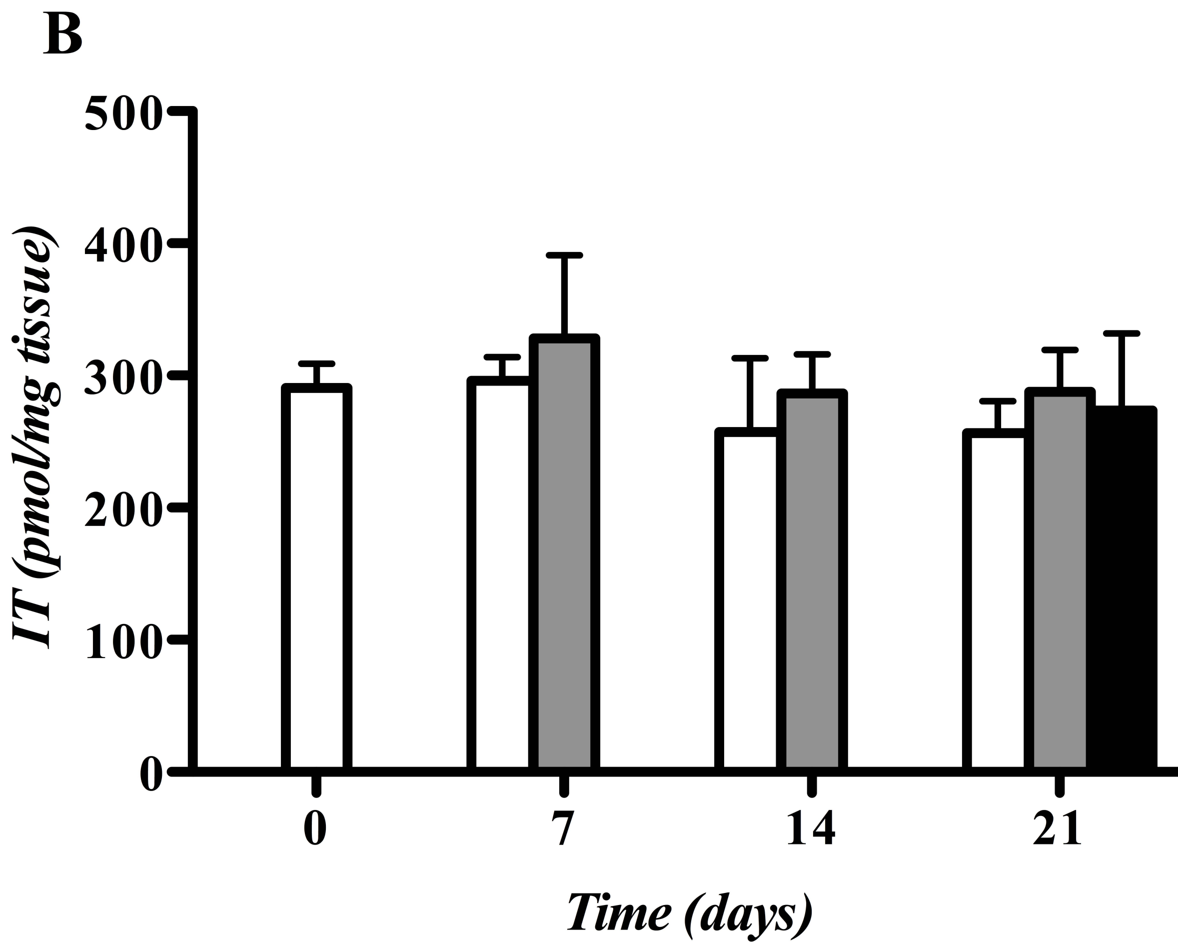
A

800
600
400
200
0

AVT (pmol/mg tissue)

Aa
A
a
b#
A*
b#
A*
#

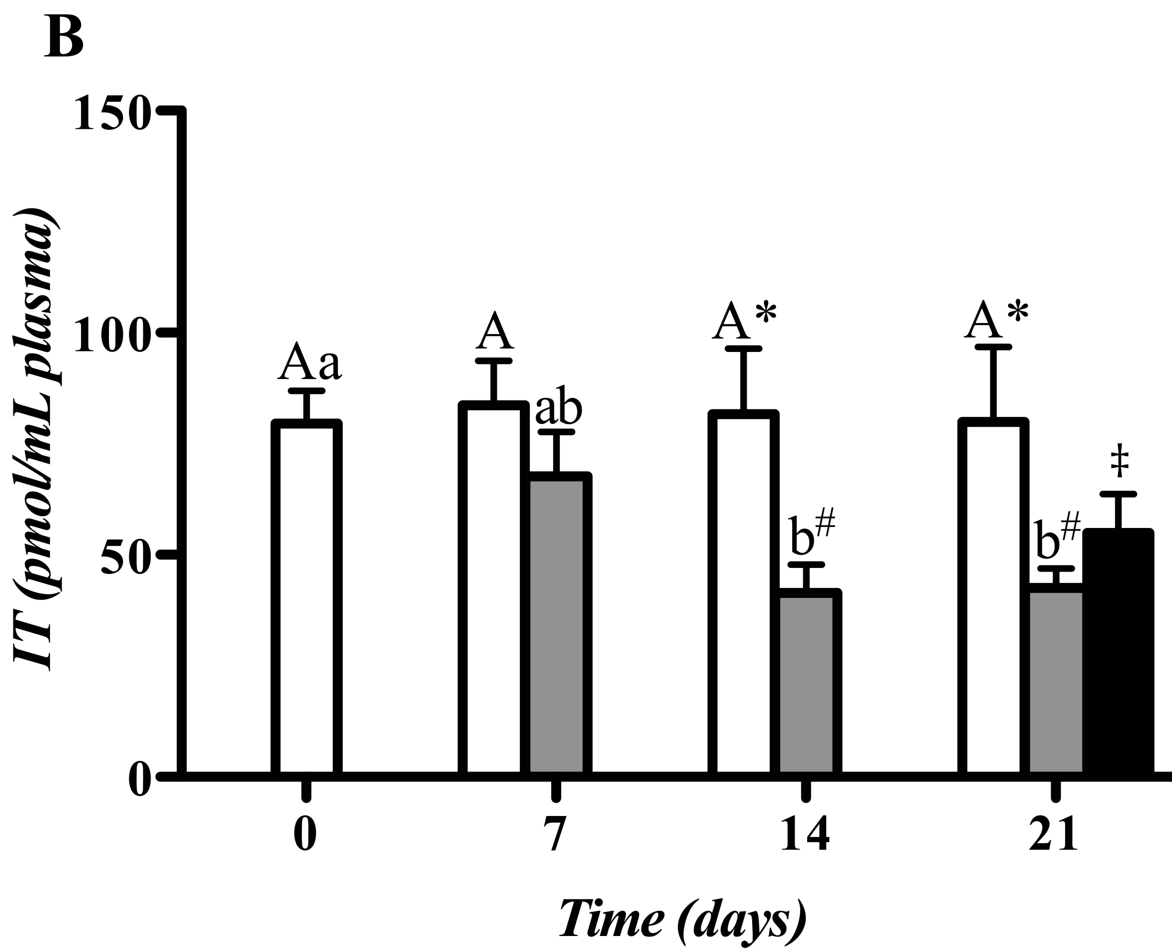
0
7
14
21

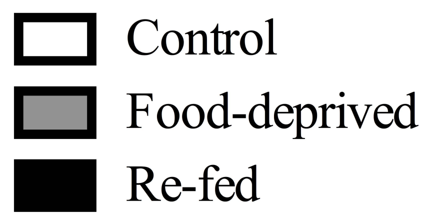


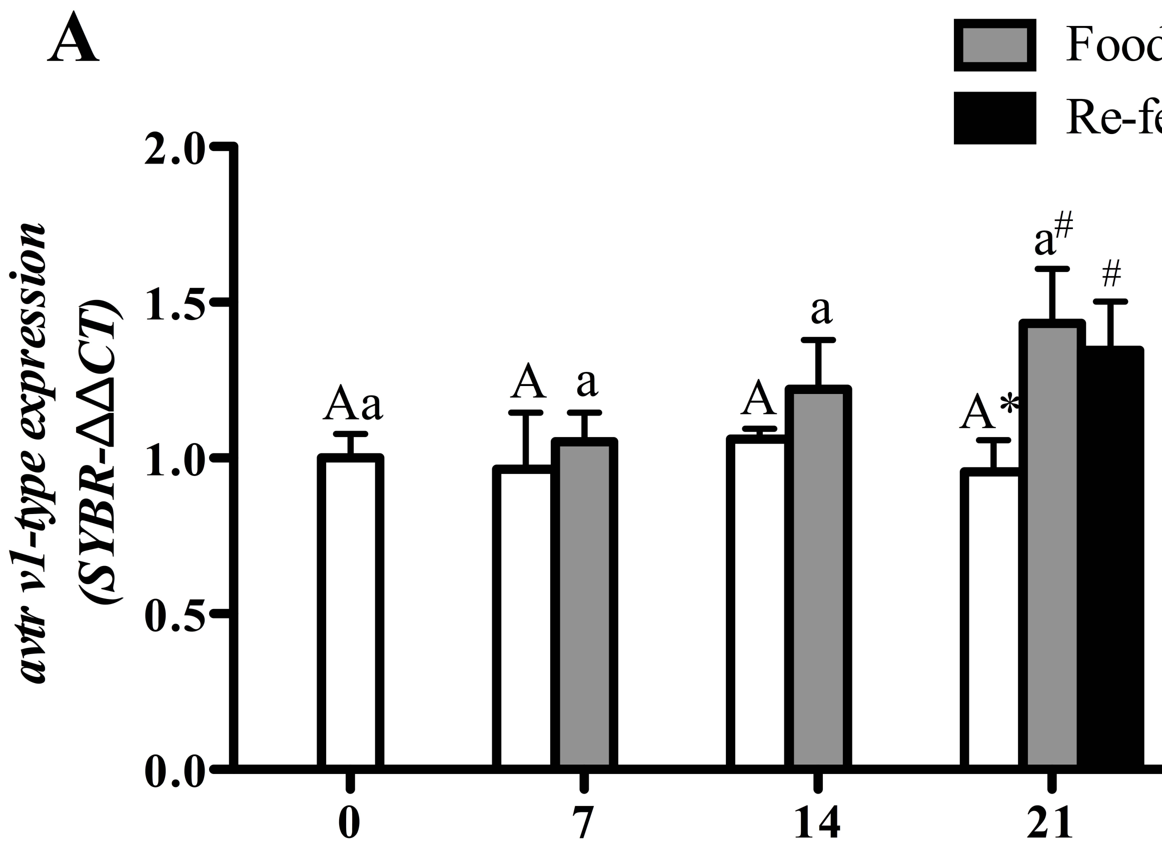
Control
Food-deprived
Re-fed

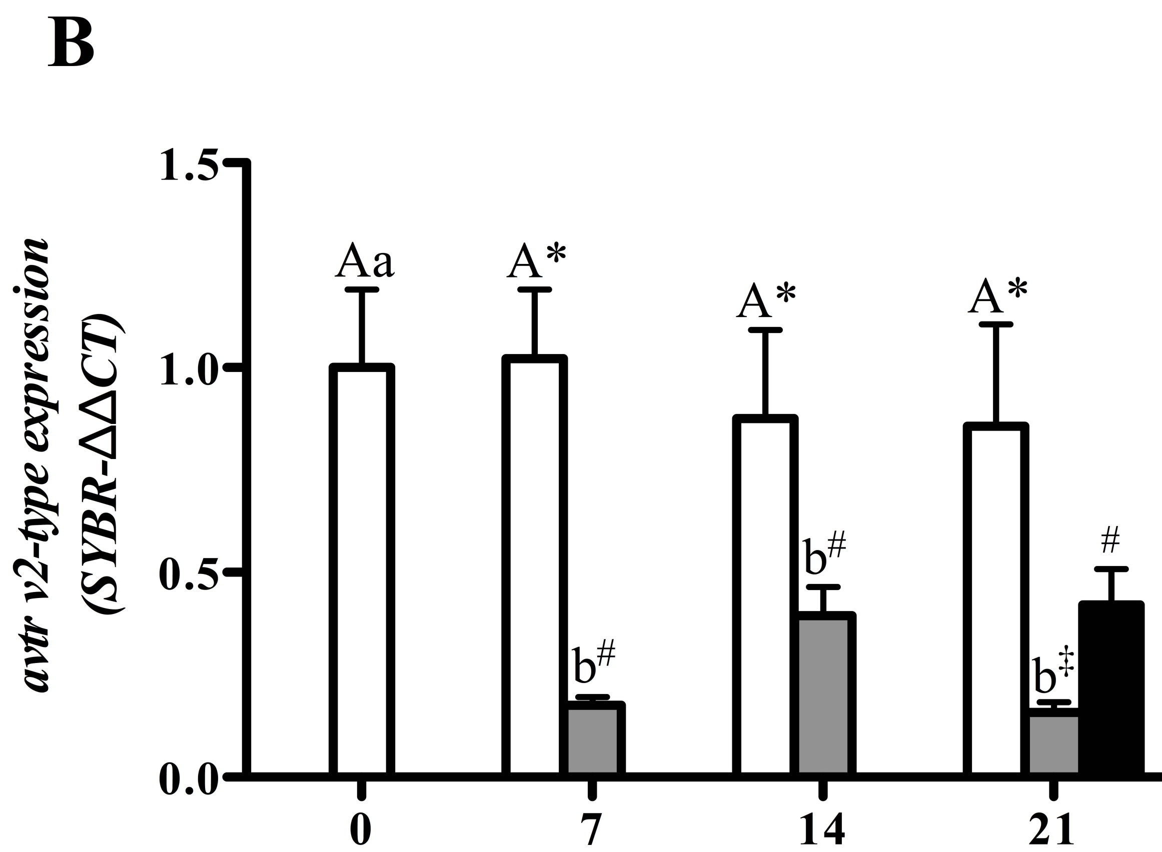
A

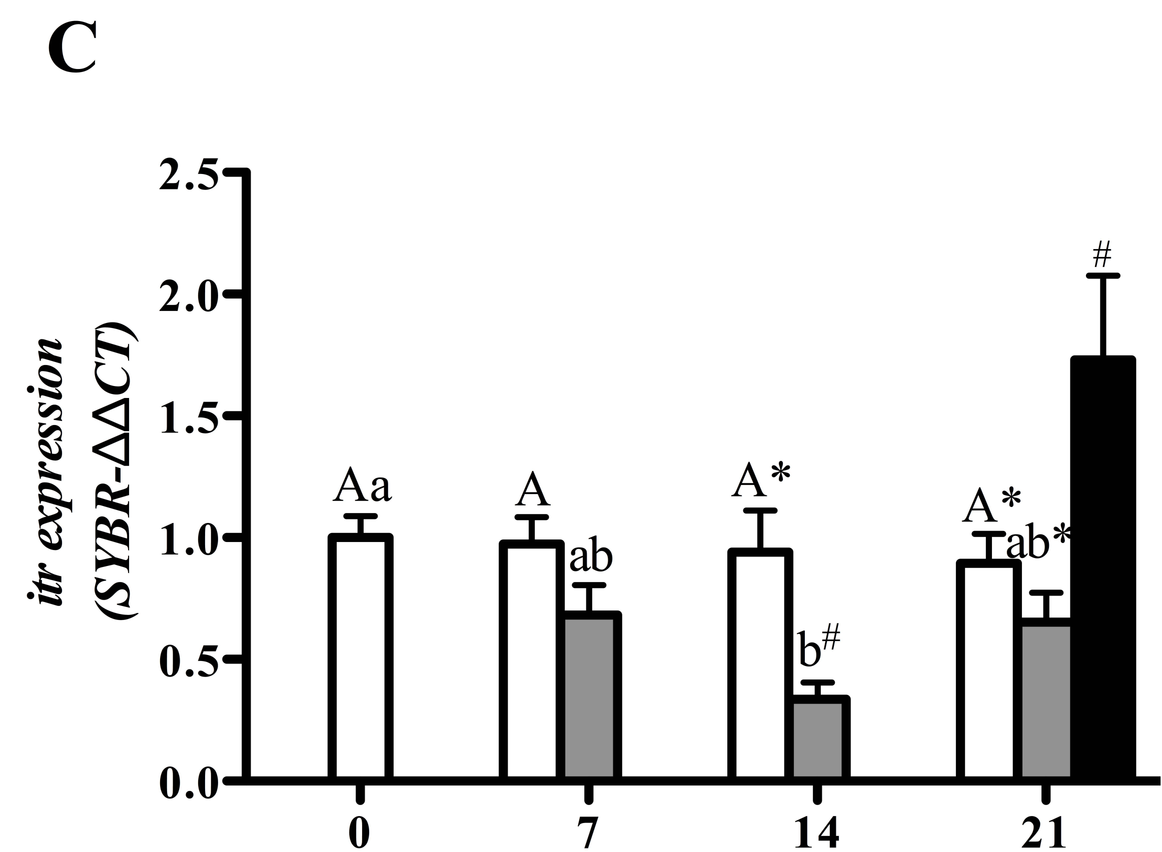
AVT (pmol/mL plasma)
150
100
50
0
Aa
A*
b#
c#
A*
A*
a*
#
0
7
14
21




Control
Food-deprived
Re-fed


A
avtr v1-type expression
(SYBR-ΔΔCT)
2.0
1.5
1.0
0.5
0.0
0
7
14
21
Aa
A
a
A
a
A*
a#
#


B
avtr v2-type expression
(SYBR-ΔΔCT)
1.5
1.0
0.5
0.0
0
7
14
21
Aa
A*
b#
A*
b#
A*
b‡
#


C
itr expression
(SYBR-ΔΔCT)
2.5
2.0
1.5
1.0
0.5
0.0
0
7
14
21
Aa
A
ab
A*
b#
A*
ab*
#

ABSTRACT

This study describes the responses of the vasotocinergic and isotocinergic systems to food deprivation and re-feeding processes in immature gilthead sea bream (*Sparus aurata*). The animals were subjected to the following experimental treatments: i) normal feeding (control), ii) food deprivation for 21 days; and iii) re-feeding for 7 days, beginning 14 days after starvation. The animals were sampled at 0, 7, 14 and 21 days from the beginning of the trial. The pituitary and plasma arginine vasotocin (AVT) and isotocin (IT) levels and the hypothalamic *pro-vasotocin* and *pro-isotocin* mRNA expression levels were measured. In addition, the mRNA levels of three receptors, *avtr v1*, *avtr v2* and *itr*, were analyzed in target organs associated with i) the integration and control of different physiological pathways related to stress and food intake (i.e., the hypothalamus), ii) hormonal release into the bloodstream (i.e., the pituitary), and iii) metabolism and its control (i.e., the liver). The metabolic parameters in the liver were also determined. The hepatosomatic index decreased, and hepatic metabolites were mobilized beginning in the early stages of starvation. Moreover, an over-compensation of these parameters occurred when the fish were re-fed after starvation. In terms of the vasotocinergic and isotocinergic systems, feed restriction induced a clear time-dependent regulation among metabolic organization, stress regulation and orexigenic processes in the mature hormone concentration and pro-peptide and receptor mRNA expression. Our results reveal the important role of the AVT/IT endocrine systems in the orchestration of fish physiology during starvation and re-feeding and indicate their involvement in both central and peripheral organs.

Keywords: *arginine vasotocin, food deprivation, isotocin, receptors,* Sparus aurata, *stress.*

51 **1. INTRODUCTION**

52 The gilthead sea bream *(Sparus aurata*) has become a common species for farming along the
53 Mediterranean coastline in the past 20 years (**FAO, 2014**). Their fast growth and high survival
54 rate are characteristics essential for successful fish farming. Nevertheless, fish growth is
55 affected not only by the feeding rate, which can be standardized, but also by biotic and abiotic
56 factors, such as feeding conditions, which are difficult to standardize (**Brett, 1979**). Fish in
57 aquaculture are subjected to many unfavorable conditions, such as crowding, disturbances and
58 handling, that strongly affect their feeding behavior **(Kulczykowska and Sánchez-Vázquez,
59 2010**). Therefore, the occurrence of stress has considerable significance regarding energy
60 metabolism and fish growth, and it requires special attention (**Wendelaar Bonga, 1997;
61 Barton, 2002**).

62 Periods of food deprivation (FD) are common for wild fish due to the temporal and spatial
63 inconsistency of food availability (**Pérez-Jiménez et al., 2007; Bayir et al., 2011; Furné et
64 al., 2012; Pujante et al., 2015**). However, FD beyond a certain critical level (starvation)
65 decreases the metabolic activity of an animal. Similarly, fish in aquaculture may feed
66 intermittently with periods of fasting, during which they are subjected to stress. Once food
67 becomes available again (for wild fish) or the stress disappears (for farmed fish), both the feed
68 intake and the normal metabolic rate are restored (**Méndez and Wieser, 1993; Metón et al.,
69 2003; Morales et al., 2004**). Although several studies in teleost fish have shown that stress
70 affects the feed intake (see **Kulczykowska and Sánchez-Vázquez, 2010,** for a review), to our
71 knowledge, the effects of starvation and subsequent re-feeding on stress-related hormones in
72 fish other than cortisol are still unknown.

73 Both the endocrine and the nervous systems control and coordinate different physiological
74 processes to maintain homeostasis during short- or long-term environmental changes. In fish,
75 two neuropeptides, arginine vasotocin (AVT) and isotocin (IT), which are, respectively,
76 related to mammalian arginine vasopressin (AVP) and oxytocin (OXY), play several roles,
77 including mediating stress response. Changes in the hypothalamic, pituitary and plasma
78 AVT/IT concentrations have been found in many fish species subjected to different types of
79 stress, e.g., confinement, disturbance, high density (HD), FD or rapid osmotic challenge
80 (**Kulczykowska, 2001; Kleszczyńska et al., 2006; Mancera et al., 2008; Kulczykowska et
81 al., 2009**). In teleosts, including *S. aurata*, these pleiotropic hormones interact with
82 adrenocorticotropic hormone (ACTH)/corticotropin-releasing hormone (CRH), (**Fryer et al.,
83 1985; Bernier et al., 2009**) to control cortisol release in the interrenal cells (**Mancera et al.,**

2008; Sanguiao-Alvarellos et al., 2006; Cádiz et al., 2015). Recently, AVT and IT nonapeptides have been nominated as welfare indicators of the internal state of an individual after confinement, disturbance, HD or FD (see **Kulczykowska et al., 2009, 2010; Martins et al., 2012** for a review).

Many of our recent studies have been focused on the regulation of the vasotocinergic, isotocinergic and stress pathways in *S. aurata* under different experimental conditions, such as changes in the environmental salinity and the administration of AVT or cortisol (**Kleszczyńska et al., 2006; Sanguiao-Alvarellos et al., 2006; Martos-Sitcha et al., 2013b, 2014a, 2014b; Cádiz et al., 2015**). In this study, we investigated the impact of FD and re-feeding on the AVT and IT systems and their implications on metabolism. Our familiarity with the metabolism and the endocrine regulation of the stress response in gilthead sea bream indicated that this species was an appropriate model for this study.

2. MATERIALS AND METHODS

2.1. Animals and experimental conditions

Immature gilthead sea bream (*Sparus aurata*, ~200 g body mass) were provided by *Servicios Centrales de Investigación en Cultivos Marinos* (SCI-CM, CASEM, University of Cádiz, Puerto Real, Cádiz, Spain; Operational Code REGA ES11028000312) and transferred to wet laboratories at the Faculty of Marine and Environmental Sciences (Puerto Real, Cádiz). During the experiment, the fish were maintained under a natural photoperiod (February-March) for our latitude (36° 31' 44" N) and a constant temperature (18-19 °C).

The animals were acclimated to laboratory conditions for at least 15 days before experiments were initiated and had normal feeding and behavioral patterns during this period. Subsequently, the fish were randomly distributed into 1,000 L tanks constituting the 3 different experimental groups in duplicate: the i) control (food-supplied), ii) food-deprived, and iii) re-fed groups. The control fish were fed once a day with commercial dry pellets at a ratio of 1 % of body mass, while animals from the food-deprived group were not fed during the 21 days of the experiment. However, two out of four tanks of the food-deprived fish were fed with a similar feed ration as the control group beginning at day 14 until the end of the experiment. This group constituted the re-fed group. The experiment was performed according to the Guidelines of the European Union (2010/63/UE) and Spanish legislation (RD

115 53/2013 and law 32/2007) regarding the use of laboratory animals. The experimental
116 procedure was authorized by the board of Experimentation on Animals of the University of
117 Cádiz (UCA) and approved by the Ethical Committee Competent Authority (Junta de
118 Andalucía Autonomous Government) under the reference number 28-04-15-241.

119

120 **2.2. Sampling**

121 At the start of the experiment, 12 fish were anaesthetized with a lethal dose of 2-
122 phenoxyethanol (1 mL/L water) (SIGMA-ALDRICH, Cat. # P-1126) and sampled (control
123 day 0). The anesthesia process in this and subsequent procedures was completed in less than 3
124 min. The remaining experimental fish were subjected to one of the following three treatments:
125 i) fed, ii) food-deprived, and iii) re-fed from day 14. Twelve fish (6 per tank) from each
126 experimental group were sampled at 7, 14 and/or 21 days from the beginning of the trial. The
127 body length and body mass were measured. Blood was collected from the caudal peduncle
128 with ammonium-heparinized syringes (SIGMA-ALDRICH, Cat. # H-6279, 25,000 units/3
129 mL of saline 0.6 % NaCl), and the fish were subsequently killed by spinal sectioning. Plasma,
130 obtained after the whole blood was centrifuged, and was stored in 1 mL aliquots at -80 °C
131 until the AVT/IT analysis. The liver was weighed separately to calculate the hepatosomatic
132 index (HSI), divided into multiple portions, immediately frozen in liquid nitrogen, and finally
133 stored at -80 °C for subsequent analyses. In addition, a representative liver biopsy and both
134 hypothalamic lobes and six pituitary glands (three of each experimental duplicate) were
135 placed in Eppendorf tubes containing an appropriate volume (1/10 w/v) of RNA*later*®
136 (Applied Biosystems). Those samples were kept for 24 h at 4 °C and then stored at -20 °C
137 until total RNA isolation was performed. Furthermore, the 6 remaining pituitary glands were
138 immediately snap-frozen in liquid nitrogen and stored at -80 °C for an AVT/IT storage
139 analyses.

140

141 **2.3. Analytical methods**

142 *2.3.1. HSI and liver metabolites*

143 The HSI was determined as follows: HSI = 100 × (liver weight / body weight). For the
144 assessment of metabolite levels, the livers were finely minced in an ice-cold petri dish,
145 subsequently homogenized by mechanical disruption (Ultra-Turrax, T25 basic, IKA®-
146 WERKE) with 7.5 vol. (w/v) of ice-cold 0.6 N perchloric acid and neutralized after the

147 addition of the same volume of 1 M $KHCO_3$. Prior to centrifugation, an aliquot of each
148 homogenate was taken for a triglyceride (TAG) determination. The homogenate was
149 subsequently centrifuged (30 min, 13,000 g, 4 °C) and the supernatant was recovered,
150 aliquoted, and stored at -80 °C until used in the metabolite assays.

151 Glucose and TAG concentrations were measured using commercial kits from Spinreact
152 (Barcelona, Spain) (Glucose-HK ref. 1001200; TAG ref. 1001311) adapted to a 96-well
153 microplate. Liver glycogen levels were assessed using the method of **Keppler and Decker**
154 **(1974)**, in which glucose obtained via glycogen breakdown (after subtracting the free glucose
155 level) is determined using the previously described commercial glucose kit. All the assays
156 were run on an Automated Microplate Reader (PowerWave 340, BioTek Instrument Inc.,
157 Winooski, USA) controlled by KCjunior™ software. Standards and samples were measured
158 in quadruplicate and duplicate, respectively.

159 *2.3.2. Total RNA isolation*

160 Total RNA was isolated from complete pituitaries using a NucleoSpin®RNA XS kit
161 (Macherey-Nagel), and the NucleoSpin®RNA II kit (Macherey-Nagel) was used for total
162 RNA extraction from hypothalamus and liver. An on-column RNase-free DNase digestion
163 was used for gDNA elimination by following the manufacturer's instructions. The amount of
164 RNA was spectrophotometrically measured at 260 nm with the BioPhotometer Plus
165 (Eppendorf) and the quality determined using a 2100 Bioanalyzer using an RNA 6000 Nano
166 Kit (Agilent Technologies). Only samples with an RNA integrity number (RIN) higher than
167 8.5, indicative of intact RNA, were used for real-time PCR (qPCR).

168 *2.3.3. Quantification of mRNA expression level*

169 First, 50 ng of total RNA from the pituitary, or 500 ng of total RNA from the hypothalamus
170 and liver, were used for reverse transcription in a final volume of 20 µL using a qSCRIPT™
171 cDNA Synthesis Kit (Quanta BioSciences). The qPCR was performed with a fluorescent
172 quantitative detection system (Eppendorf Mastercycler ep realplex^2 S). Each reaction mixture,
173 in a final volume of 10 µL, contained 0.5 µL of each specific forward and reverse primers, 5
174 µL of PerfeCTa SYBR® Green FastMix™ 2x (Quanta BioSciences) and 4 µL containing
175 either 1 ng or 10 ng of cDNA from the pituitary or from the hypothalamus and liver,
176 respectively.

177 Primers for *pro-vt*, *pro-it*, *avtr v1*, *avtr v2* and *itr* from *S. aurata* (at the final concentrations
178 provided in Table 1) were used as previously described by **Martos-Sitcha et al. (2013b,
179 2014a)** and designed from the nucleotide sequences available at the NCBI website (acc. no.
180 *pro-vt*: **FR851924**; acc. no. *pro-it*: **FR851924**; acc. no. *avtr v1*: **KC195974**; acc. no. *avtr v2*:
181 **KC960488**; acc. no. *itr*: **KC195973**). The PCR profile was as follows: 95 °C, 10 min; [95 °C,
182 20 s; 60 °C, 30 s] × 40 cycles; melting curve [60 °C to 95 °C, 20 min], 95 °C, 15 s. The
183 melting curve was used to ensure that a single product was amplified and to verify the
184 absence of primer-dimer artifacts. The results were normalized to β-actin (*actb*, acc. no.
185 **X89920**) because of its low variability (less than 0.15 C_T in the pituitary and less than 0.20 C_T
186 in the hypothalamus and liver) under our experimental conditions. Relative gene
187 quantification was performed using the $\Delta\Delta C_T$ method (**Livak and Schmittgen, 2001**).

188 *2.3.4. AVT and IT content in the plasma and the pituitary gland*

189 AVT and IT in the plasma and the pituitary gland were determined through high-performance
190 liquid chromatography (HPLC) with fluorescence detection preceded by solid-phase
191 extraction (SPE) based on **Gozdowska et al. (2006)** and **Martos-Sitcha et al. (2013b)**.
192 Plasma samples (1 mL each) were acidified with 1 M HCl (100 µL) and centrifuged at 6,000
193 g for 20 min at 4 °C, and frozen pituitaries were weighed and sonicated in 0.5 mL Milli-Q
194 water (Microson™ XL, Misonix, USA), acidified with glacial acetic acid (1.25 µL) and then
195 placed in a boiling water bath for 3.5 min. The pituitary extracts were cooled and centrifuged
196 at 6000 g for 15 min at 4 °C. After that, the supernatants were loaded onto a previously
197 conditioned (1 mL MeOH, 1 mL water) SPE column (30 mg/mL, STRATA-X, Phenomenex).
198 Water (600 µL) and 0.1 % TFA (trifluoroacetic acid) in 5 % acetonitrile (600 µL) were passed
199 through the column to wash away impurities. The peptides were eluted with 2 × 600 µL of 80
200 % acetonitrile. The resultant eluate was evaporated to dryness using a Turbo Vap LV
201 Evaporator (Caliper Life Science, USA), and the samples were stored at -80 °C until HPLC
202 analysis. Before quantitative analysis, the samples were resuspended in 40 µL 0.1 %
203 trifluoroacetic acid (TFA), then divided into two aliquots to provide duplicates for analysis.
204 The pre-column derivatization of AVT and IT in each of the 20 µL samples was performed
205 using 3 µL of a 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) solution (30 mg NBD-F in 1
206 mL of acetonitrile) in a mixture of 20 µL phosphoric buffer (0.2 M, pH 9.0) and 20 µL
207 acetonitrile. The solution was heated to 60 °C for 3 min in a dry heating block and cooled on
208 ice. Next, 4 µL of 1 M HCl was added. The derivatized samples were assayed using an
209 Agilent 1200 Series Quaternary HPLC System (Agilent Technologies, USA).

210 Chromatographic separation was achieved using an Agilent ZORBAX Eclipse XDB-C18
211 column (150 mm × 4.6 mm I.D., 5 µm particle size). A gradient elution system was used to
212 separate the derivatized peptides. The mobile phase consisted of solvent A (0.1 % TFA in
213 H_2O) and solvent B (0.1 % TFA in acetonitrile: H_2O (3:1)). The linear gradient was 45-70 %
214 eluent B in 20 min. The flow rate was set at 1 mL/min, and the column temperature at 20 °C.
215 The injection volume was 67 µL. Fluorescence detection was conducted at 530 nm with
216 excitation at 470 nm.

217 **2.4. Statistical analysis**

218 The results are presented as the mean ± SEM. After the normality and the homogeneity of
219 variance were checked, a comparison between the groups was evaluated using two-way
220 ANOVA with treatment (control and food-deprived) and time course (0, 7, 14 and 21 days) as
221 the primary factors, and the differences between the control, food-deprived and re-fed groups
222 on day 21 were assessed with a one-way ANOVA followed by a post hoc comparison with
223 Tukey's test when appropriate. Differences among the biometric parameters were assessed
224 using one-way ANOVA with treatment (control, food-deprived and re-fed) as the primary
225 factor, followed by a post hoc comparison with Tukey's test. A comparison of replicate tanks
226 for all of the parameters was performed with Student's *t*-test. A significance level of $p < 0.05$
227 was adopted. All tests were performed using the GraphPad Prism® (v.5.0b) software for
228 Macintosh.

229

230 **3. RESULTS**

231 Non-significant differences were found for all the parameters assessed between replicate
232 tanks. In addition, no mortality, health disturbance or any alterations in fish behavior were
233 observed in any experimental group. The biometric parameters are shown in Table 2. The
234 final body mass was the only parameter that significantly decreased in food-deprived and re-
235 fed fish relative to the control.

236 **3.1. Liver metabolites and the HSI**

237 A time course of the changes of the hepatic metabolites and the HSI are shown in Table 3. No
238 differences between any of the analyzed parameters were detected in the control group. The
239 hepatic glucose was stable in all groups for the entire experimental duration. However, the
240 glycogen and TAG concentrations significantly decreased in the food-deprived fish relative to

the control from days 14 until 21. In the re-fed group, TAG but not glycogen completely recovered to the control level by the end of the experiment. The HSI showed a similar trend, significantly decreasing in food-deprived fish for the experimental duration, but recovered to a level close to that of the control after one week of re-feeding.

3.2. Expression of hypothalamic *pro-vt* and *pro-it*

Both the *pro-vt* and *pro-it* mRNA expression did not differ in the control group over the experimental duration (Figure 1). The *pro-vt* mRNA expression significantly decreased until day 14 in the food-deprived fish, then increased until day 21 but did not reach the control level (Figure 1A). The *pro-it* mRNA expression progressively decreased in the food-deprived fish over 21 days, with the lowest values observed on day 21 (Figure 1B). However, the expression of both pro-peptides did not reach the control level after 7 days of re-feeding.

3.3. Hypothalamic mRNA expression of AVT and IT receptors

No differences were observed in the *avtr*s or *itr* mRNA expression in the control group (Figure 2). However, the *avtr v1* mRNA level was clearly higher by the end of the experiment in the food-deprived fish and was significantly different from the control level at day 21. The *avtr v1* mRNA level decreased in the re-fed group but did not reach the control level (Figure 2A). Food deprivation and re-feeding did not change *avtr v2* gene expression (Figure 2B). Finally, the *itr* mRNA expression level showed a biphasic response in the food-deprived fish, increasing during the first 7 days, then decreasing to a value significantly lower than that of the control group at the end of the experiment. The re-fed group was not different from the starved fish but had a lower *itr* mRNA level than that of the control (Figure 2C).

3.4. Hypophyseal mRNA expression of AVT and IT receptors

The pituitary expression of the AVT (*v1-* and *v2- types*) and the IT receptors are shown in Figure 3. No changes were observed in the mRNA expression of these three receptors in the control group. However, the food-deprived fish showed the same response pattern for all three receptors—a significant increase during the first 7 days of FD, followed by a progressive decrease in the expression level, which was significant relative to the control level at day 21. In the re-fed group, the mRNA expression level of both AVT receptors and *itr* increased respect to the food-deprived fish, increasing its expression or reaching the control values, respectively.

3.5. Hypophyseal AVT and IT storage

272 The control group did not show any variation in the pituitary AVT level, whereas the pituitary
273 AVT level in the food-deprived fish significantly increased from day 14 onwards (Figure 4A).
274 In addition, the pituitary AVT level also increased in the re-fed group relative to the control
275 group, but the level was not significantly different from that in the food-deprived fish. In
276 contrast, the IT storage was unchanged for the entire experimental duration in all three groups
277 (Figure 4B).

278 **3.6. Plasma AVT and IT**

279 Plasma AVT and IT were unchanged in the control group during the experiment (Figure 5).
280 The food-deprived fish showed biphasic variation in the AVT level, with a lower plasma
281 content during the first 7 days followed by a significant increase after day 14, returning to
282 values close to that of the control at day 21. At days 7 and 14, these values were significantly
283 different from that of the control. The plasma AVT level in the re-fed group significantly
284 decreased on day 21 relative to that of the control and the food-deprived fish (Figure 5A). The
285 plasma IT level significantly decreased in the food-deprived fish from day 14 onward relative
286 to the level in the control. The fish from the re-fed group showed partially restored IT levels,
287 but they were not as high as that of the control (Figure 5B).

288 **3.7. Hepatic mRNA expression of AVT and IT receptors**

289 In the liver, no changes were observed in the *avtr* or *itr* mRNA expression in the control
290 group (Figure 6). However, *avtr v1* expression significantly increased in both the food-
291 deprived and re-fed fish relative to the control at day 21 (Figure 6A). In addition, *avtr v2*
292 mRNA expression significantly decreased in the food-deprived fish over the experimental
293 duration. The *avtr v2* mRNA expression partially recovered after re-feeding but did not reach
294 control levels (Figure 6B). The *itr* mRNA expression progressively decreased and was
295 significantly lower than the control level at day 14. The *itr* mRNA expression in the re-fed
296 group was significantly higher than that of the control and the food-deprived fish at day 21
297 (Figure 6C).

298

299 **4. DISCUSSION**

300 The involvement of vasotocinergic and isotocinergic systems in osmoregulation
301 (**Kleszczyńska et al., 2006; Martos-Sitcha et al., 2013a, 2013b, 2014a, 2015a**), metabolism
302 (**Sangiao-Alvarellos et al., 2006**), the stress response related to a high stocking density

303 (**Mancera et al., 2008**) and cortisol administration (**Cádiz et al., 2015**) have been previously
304 studied in *S. aurata*. To the best of our knowledge, this study is a more complete and
305 integrated view of the crosstalk/interactions between the starvation/re-feeding processes
306 associated with the activation of the vasotocinergic and isotocinergic pathways in gilthead sea
307 bream as previously reported by **Mancera et al. (2008)**.

308 **4.1. Metabolic indicators and HSI**

309 Starvation is well tolerated by many fish species in nature. To survive periods of unfavorable
310 feeding conditions, fish mobilize their energy reserves to adjust their metabolism in a species-
311 specific manner (**Navarro and Gutiérrez, 1995**). Plasma metabolic indicators have been
312 previously studied after starvation and re-feeding in the same fish sampled in this study
313 (**Martos-Sitcha et al., 2014b**), suggesting that the energy requirements are supplied by
314 glucose during the first days of FD and later by lactate. In addition, reduced hepatic glycogen
315 and triglycerides, which are reflected by a reduction of the body and liver mass (i.e., the HSI),
316 indicated that these metabolites were serving as an energy supply due to the high response and
317 extent to the lipolytic machinery of the hepatic tissue during FD (**Benedito-Palos et al.,**
318 **2014**), which has also been reported in different teleost species (**Shimeno et al., 1990;**
319 **Machado et al., 1988; Farbridge and Leatherland, 1992; Mehner and Wieser, 1994;**
320 **Soengas et al., 1996**; **Pascual et al., 2003; Polakof et al., 2006**). In the re-fed group, the
321 glycogen level partially recovered after one week of feeding, as has also been described in
322 mammals (**Carmean et al., 2013**) and other fish species (**Black and Love, 1986; Collins and**
323 **Anderson, 1995; Pujante et al., 2015**). Moreover, a full recovery of TAG and HSI occurred
324 after re-feeding. Given that hepatic lipids are the first reserves used after liver glycogen, as
325 has been previously described in other teleosts [*Gadus morhua* (**Guderley et al., 2003)**,
326 *Rhamdia hilarii* (**Machado et al., 1988)**, *Leuciscus idus* (**Segner and Braunbeck, 1988)** and
327 *Cyprinus carpio* (**Shimeno et al., 1990**)], the recovery observed in metabolite storage during
328 re-feeding could be a strategy for rapid uptake and energy redistribution in the body, with the
329 basal storage levels being partially restored. This finding suggests that *S. aurata* require more
330 time to reach carbohydrate homeostasis but not hepatic lipid storage homeostasis after FD and
331 re-feeding.

332 **4.2. AVT and IT systems**

333 Some authors consider long-term FD as a serious stress factor in fish that increases the
334 cortisol level and affects metabolic processes (**Vijayan et al., 1993; Sangiao-Alvarellos et**

335 **al., 2005; Mancera et al., 2008**). Our results regarding the circulating levels of cortisol
336 hormone after starvation have been previously reported for the same fish used in this study,
337 clearly suggesting that the stress system had been activated (**Martos-Sitcha et al., 2014b**).

338 In teleosts, corticotrope cells from the anterior pituitary gland synthesize ACTH, which in
339 turn stimulates the secretion of cortisol by the interrenal tissue (**Wendelaar Bonga, 1997;**
340 **Bernier et al., 2009**). Moreover, the corticotrope cells are innervated by hypothalamic
341 neurons that produce AVT (**Batten et al., 1990**), indicating a role for AVT in the activation of
342 the stress response system in fish (**Bernier et al., 2009**). Vasotocinergic and isotocinergic
343 systems have a wide range of physiological functions that depend on many internal and
344 external factors that control various processes at the neuronal level, such as *pro-vt* and *pro-it*
345 mRNA expression, peptide synthesis, transport, maturation and storage, and the release of
346 mature nonapeptides into the circulation (**Mancera et al., 2017**). The plasma levels of active
347 AVT and IT could be reduced by: i) inhibition during the production of the pro-peptides at the
348 level of the hypothalamus and/or during processes related to peptide maturation, ii) inhibition
349 of the release of mature nonapeptides in the neurohypophysis, iii) renal clearance, and iv)
350 inactivation of nonapeptides by plasma or tissue peptidases. However, nonapeptide binding to
351 specific receptors in target organs triggers their physiological action (**Ward et al., 1990;**
352 **Agirregoitia et al., 2005, Martos-Sitcha et al., 2013b, 2014a**). Therefore, any investigation
353 of the response of the vasotocinergic and isotocinergic systems requires a comprehensive
354 analysis at the level of peptide synthesis, storage and release, and specific receptors in the
355 target tissues of interest. Starvation stress can affect the vasotocinergic and isotocinergic
356 pathways in the sea bream. To our knowledge, little data about a link between starvation and
357 the activity of AVT/AVP and IT/OXY systems exist for vertebrates (**Flynn et al., 2002;**
358 **Tachibana et al., 2004; Gesto et al., 2014**). Our study is the first extensive analysis of the
359 response of both the vasotocinergic and isotocinergic systems after food deprivation and re-
360 feeding in *S. aurata*.

361 *4.2.1. Regulation of AVT and IT synthesis and release under starvation conditions*

362 Differences observed at the hypothalamic level in both the vasotocinergic and isotocinergic
363 endocrine systems during starvation suggest that the orchestration of the stress pathway is a
364 combination of different elements in which the Hypothalamus-Pituitary-Interrenal (HPI) axis
365 is involved, among other processes (**Bernier et al., 2009; Kulczykowska and Sánchez-**
366 **Vázquez, 2010**).

367 During the first week of starvation, a decrease in hypothalamic *pro-vt* expression and plasma
368 AVT levels was observed, but there were no changes in either AVT storage in the pituitary or
369 in *avtr* expression in the hypothalamus. Thus, the increased plasma cortisol values previously
370 demonstrated in these same specimens after FD conditions (**Martos-Sitcha et al., 2014b**)
371 could suggest an inhibitory effect of this hormone on several elements of the vasotocinergic
372 system. Therefore, cortisol can be considered one of the key players in the orchestration in
373 response to FD (**Chang et al., 2002; Pujante et al., 2015**), possibly as a state of alert in
374 response to FD during the first week. This effect could also be an important issue during re-
375 feeding (**Uchida et al., 2003; Martos-Sitcha et al., 2014b**). Moreover, an increase in the
376 mRNA level of both *avtr v1* and *avtr v2* in the pituitary indicated the activation of both
377 receptors, which could be linked to feedback mechanisms both controlling AVT release and,
378 for instance, indirectly regulating cortisol production. Notably, AVT storage in the pituitary is
379 maintained at the same level in spite of the lower hypothalamic *pro-vt* mRNA and plasma
380 AVT levels, guaranteeing the hormonal homeostasis (neutral balance among synthesis and
381 release) in the pituitary gland. However, in a previous study, the administration of exogenous
382 cortisol in gilthead sea bream increased hypothalamic *pro-vt* mRNA without a change in
383 either the pituitary or plasma AVT level (**Cádiz et al., 2015**), suggesting that other endocrine
384 factors, such as Urotensin I, melanin-concentrating hormone, neuropeptide Y, or thyrotrophic-
385 releasing hormone could be involved in the differential response in addition to cortisol
386 (**Winberg et al., 2016**). This fact highlights that complex interconnections between different
387 endocrine axes can be made for the correct regulation of the stress system. This response has
388 also been demonstrated *in vitro* in this fish species, in which cortisol administration induced
389 AVT secretion from pituitary cells (**Kalamarz-Kubiak et al., 2014**), or even that AVT
390 hormone potentiate cortisol release when co-administrated with ACTH in *Cyprinus carpio*
391 (**Jerez-Cepa et al., 2016**).

392 However, during the second week of FD, the hypothalamic *pro-vt* mRNA levels further
393 decreased, but the AVT level in the pituitary and the plasma significantly increased,
394 suggesting that the AVT system changed in response to FD at this time. This apparent
395 controversial result is interesting, although changes in the dynamic of AVT storage at
396 pituitary level as well as hormonal release and plasma clearance (see **Agirregoitia et al.,**
397 **2005**) induced by food-deprivation need to be assessed to understand our data and to establish
398 the possible interconnection with several orexigenic and anorexigenic factors. Hormones
399 belonging to the AVT/AVP family have been demonstrated to mediate anorexigenic effects in

400 mammals (**Flynn et al., 2002**), birds (**Tachibana et al., 2004**) and fishes (**Gesto et al., 2014**).
401 For example, the intracerebroventricular (i.c.v.) administration of AVT drastically decreased
402 the food intake in juvenile rainbow trout (*Oncorhynchus mykiss*) (**Gesto et al., 2014**). In
403 addition, studies have shown that AVP induced anorexigenic effects via a V1a-type receptor,
404 but the blockade of this receptor produced orexigenic effects in neuropeptide-Y-induced mice
405 (**Aoyagi et al., 2009**). Our results, in agreement with previous studies, indicate a progressive
406 increase in the hypothalamic expression of *avtr v1* after three weeks of FD, suggesting that
407 this AVT receptor could have a time-dependent modulatory role on the neuropeptide-
408 mediated control of food intake.

409 Moreover, the increase observed in the AVT content, both at plasma and hypophyseal levels
410 after 14 days of starvation, suggests that these key components of the pathway are regulated
411 through long-term adaptation. This could be attributed to both types of AVT receptors in the
412 pituitary, regulating the retention and release of the mature peptide into the blood stream. This
413 phenomenon could be attributed to both intracellular and/or paracellular signaling in pituitary
414 cells, where *avtr*s could organize its release and promote integrated physiological changes
415 down-stream. Even so, further studies will be necessary in order to clarify the proposed
416 regulatory mechanism operating in the pituitary. For that, all changes produced in *pro-vt* and
417 *avtr*s up-stream should be taken into account. This singularity highlights the clear
418 orchestration of the complete axis from the beginning of this pathway. This fact has even been
419 determined when the homeostatic level of AVT via *pro-vt* production has been reached after
420 different processes, such as i) appetite (this work), ii) cortisol production as one of the
421 primary stress response (**Cádiz et al., 2015**), as well as its interconnection with AVT
422 demonstrated *in vitro* as a component of the HPI axis (**Kalamarz-Kubiak et al., 2014**), and
423 iii) metabolic organization of the hypothalamic neurons induced by AVT (**Sangiao-**
424 **Alvarellos et al., 2006**). Nevertheless, the negative feedback of any of these pathways cannot
425 be ruled out as being associated with the lower levels of *pro-vt* mRNA that were observed.

426 To our knowledge, only partial data regarding FD and isotocinergic pathways have been
427 reported in fish (**Mancera et al., 2008**). The activation of the isotocinergic system has been
428 demonstrated in *S. aurata* under different stress conditions, such as a high stocking density
429 (**Mancera et al., 2008**), osmotic challenge (**Kleszczyńska et al., 2006; Martos-Sitcha et al.,**
430 **2013b**), or even by chronic stress simulation mimicked by cortisol treatment (**Cádiz et al.,**
431 **2015**). Moreover, the oxytocinergic system has been proposed to be involved in the regulation
432 of feeding behavior in mammals. For instance, OXY and its agonists administered by i.c.v.

433 injection inhibit feed intake, whereas these effects are prevented by administration of OXY
434 antagonists (**Arletti et al., 1990; Lokrantz et al., 1997**).

435 In this study, a significant decrease of the hypothalamic *pro-it* mRNA level was observed
436 during FD, suggesting the inhibition of its synthesis resulting in a lower plasma IT level with
437 no changes in the IT pituitary content. *In vitro* studies in the gilthead sea bream demonstrated
438 that cortisol inhibited the IT secretion from pituitary cells (**Kalamarz-Kubiak et al., 2014**),
439 implying the involvement of cortisol in the regulation of the IT plasma level. During the first
440 week of FD, a transitory increase in hypothalamic *itr* expression was noted, with no changes
441 in the pituitary IT level. Nevertheless, other experimental approaches will be necessary to
442 elucidate the role of IT in the orexigenic/anorexigenic response (if any) as it has been
443 previously described for AVT and OXY (an IT mammalian homolog), in which other proteins
444 involved in appetite regulation and in the control of food intake seem to also be implicated
445 and interconnected (**Volkoff et al., 2005; Gesto et al., 2014**).

446 *4.2.2. Hepatic expression of* avtr *and* itr *genes under starvation*

447 The gene expression of different types of AVT and IT receptors has been demonstrated in
448 many peripheral tissues in several teleost species, including *S. aurata*. The expression levels
449 are modified depending on the physiological challenge (**Moon and Momsen; 1990,**
450 **Hausmann et al., 1995; Guibbolini et al., 2000, Lema 2010; Martos-Sitcha et al., 2013a,**
451 **2014a, Cádiz et al., 2015**). The existence of hepatic AVT/AVP and IT/OXY receptors
452 indicates the direct action of both nonapeptides in this important energy-supplying organ, and
453 in the regulation of different metabolic enzymes. AVT and/or IT treatment induces
454 hyperglycemia in teleosts, presumably by increasing the hepatic glycogenolytic potential and
455 free-glucose production (**Janssen and Lowrey, 1987; Moon and Mommsen, 1990;**
456 **Sangiao-Alvarellos et al., 2006**). In this study, FD gradually increased hepatic *avtr v1*
457 expression, which coincided with a decrease in the glycogen level. Thus, our results confirm
458 that the regulation of carbohydrate metabolic enzymes can be controlled at least in part by this
459 type of receptor, as has been previously reported in *S. aurata* (**Martos-Sitcha et al., 2014a;**
460 **Cádiz et al., 2015**). Interestingly, an inverse situation for both the *avtr v2* and *itr* genes was
461 also found, suggesting a minor role for these receptors (if any) during FD in this species. In
462 this context, their depletion could be understood as an adaptive response in which the cellular
463 machinery is focused to prime the expression of the genes that actually play a feedback role
464 during FD, or even as a self-down-regulation mediated by cortisol and other elements to avoid

465 excessive stimulation of the metabolic rate in the hepatic tissue (**Martos-Sitcha et al.,**
466 **2014b**).

467 *4.2.3. Re-feeding process*

468 Re-feeding after long-term FD usually induces the rapid weight recovery known as
469 compensatory growth. However, other responses can be observed because recovery from FD
470 depends on several factors, such as species, environmental conditions, or even the length of
471 the FD period (**Navarro and Gutiérrez, 1995; McCue, 2010; Pujante et al., 2015**). During
472 re-feeding, the fish exhibited compensatory growth and rapid restoration of their initial
473 metabolic state (**Metón et al., 2003; Morales et al., 2004, Pujante et al., 2015**). The
474 metabolic parameters of the plasma (**Martos-Sitcha et al., 2014b**), hepatic metabolite levels,
475 and HSI assessed in this study indicate the existence of a clear metabolic compensatory
476 process after 7 days of re-feeding.

477 In *S. aurata*, several studies have assessed changes in the HPI axis after acute or chronic
478 stress (**Rotllant and Tort, 1997; Arends et al., 1999; Rotllant et al., 2000, 2001**), focusing
479 on the stimulation of food consumption (**Bernier et al., 2004**) or metabolic reorganization
480 mediated by the endocrine system (**Mommsen et al., 1999**). Nevertheless, a permanent state
481 of alert after a prolonged starvation period (**Uchida et al., 2003**), which is also indicated by
482 the voracity of the animals at feeding time, could better explain the increase in the plasma
483 cortisol and glucose levels (**Martos-Sitcha et al., 2014b**) without ruling out a combination of
484 all of them. No information exists regarding the role of AVT/IT system in the recovery of
485 food administration after a long-period of starvation.

486 In a manner similar to the metabolic compensatory process, compensatory changes in the
487 vasotocinergic and isotocinergic systems while recovering from starvation were also apparent.
488 Re-feeding enhanced the plasma cortisol level in *S. aurata* (**Sangiao-Alvarellos et al., 2005**).
489 The analysis of the results obtained after a week of re-feeding showed the existence of
490 simultaneous stabilization of the pituitary AVT level along with the AVT plasma level, which
491 could be due to cortisol. Similar indicators of metabolic economy (i.e., the plasma metabolite
492 level, the liver metabolite content) were similarly indicative of a depressed stress axis in
493 fasted animals, which prompted recovery to the levels found in the control fish within one
494 week (**Martos-Sitcha et al., 2014b).** AVP has been demonstrated to stimulate hepatic
495 glycogenolysis in mammals (**Smith et al., 2003**), as has AVT in amphibians (**Janssens et al.,**
496 **1983; Ade et al., 1995**) and fishes (**Sangiao-Alvarellos et al., 2006**). A similar effect has

been previously suggested in *S. aurata* under different stress conditions (**Martos-Sitcha et al., 2014a; Cádiz et al., 2015**), and our results are consistent with the proposed metabolic role of AVT in the stimulation of glycogenolysis. Nevertheless, the magnitude of changes depends on the parameters assessed, demonstrating that a week of re-feeding may be not sufficient for a complete endocrine, neurohumoral and metabolic reorganization, and more time might be necessary to orchestrate such reorganization and a return to a normal metabolic state. In this context, the plasma AVT level was lower in the re-fed group, which leads us to hypothesize that it was depleted in the bloodstream, probably by binding to specific receptors, and/or it was eliminated by specific peptidases (**Agirregoitia et al., 2005**), such that physiological functions coupled to these hormones could be recovered. The enhancement of hepatic *avtr v2* and *itr* expression, but not of *avtr v1*, also suggests an important metabolic role for both receptors during re-feeding at the hepatic level. Nevertheless, the inverse pattern of changes of hepatic *avtr v1* with respect to *avtr v2*/*itr* expression during FD (see above) and re-feeding suggests different metabolic roles for these receptors.

5. CONCLUSIONS

This study provides strong evidence that changes in the synthesis, storage and release of AVT and IT are involved in the response of *S. aurata* to starvation and re-feeding. Thus, AVT and IT seem to be part of a complex network of endocrine, metabolic and stress pathways, in which the clear time response and sensitivity of each is clearly observed, possibly involving a greater response to other indirect factors (e.g., appetite or food intake), which has been previously demonstrated for AVT but not as clearly for isotocin. Nevertheless, it is clear that hypothalamic and hypophyseal factors could mediate physiological activity at the level of peripheral tissues/organs (i.e., the liver).

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533

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FIGURE LEGENDS

Figure 1. Time course of the changes in the hypothalamic *pro-vasotocin* (*pro-vt*; A) and *pro-isotocin* (*pro-it*; B) mRNA expression levels in *S. aurata* maintained under different feeding conditions (fed, food-deprived and re-fed). Values are expressed as the mean ± SEM (n = 10-12 fish per group). Significant differences among the samples under the same condition are identified with different letters (capital letters: control group; lower case letters: food-deprived group). Different symbols show differences between groups at the same time ($p < 0.05$, two-way ANOVA followed by Tukey's test).

Figure 2. Time course of the changes in the hypothalamic *avtr v1* (A), *avtr v2* (B) and *itr* (C) mRNA levels in *S. aurata* maintained under different feeding conditions (fed, food-deprived and re-fed). For further details, see the legend in Figure 1.

Figure 3. Time course of the changes in the pituitary *avtr v1* (A), *avtr v2* (B) and *itr* (C) mRNA levels in *S. aurata* maintained under different feeding conditions (fed, food-deprived and re-fed). For further details, see the legend in Figure 1.

Figure 4. Time course of the changes in the AVT (A) and IT (B) pituitary storage levels in *S. aurata* maintained under different feeding conditions (fed, food-deprived and re-fed). For further details, see the legend in Figure 1.

Figure 5. Time course of the changes in the AVT (A) and IT (B) plasma levels in *S. aurata* maintained under different feeding conditions (fed, food-deprived and re-fed). For further details, see the legend in Figure 1.

Figure 6. Time course of the changes in the hepatic *avtr v1* (A), *avtr v2* (B) and *itr* (C) mRNA levels in *S. aurata* maintained under different feeding conditions (fed, food-deprived and re-fed). For further details, see the legend in Figure 1.

789 **Table 1.** Specific primers used for the semi-quantitative qPCR expression analysis.

Primers	*Nucleotide sequence*	*Primer concentration*	*Amplicon size*
qPCR-pro-vt F	5'-AGAGGCTGGGATCAGACAGTGC-3'	200 nM	129 bp
qPCR-pro-vt R	5'-TCCACACAGTGAGCTGTTTCCG-3'		
qPCR-pro-it F	5'-GGAGATGACCAAAGCAGCCA-3'	200 nM	151 bp
qPCR-pro-it R	5'-CAACCATGTGAACTACGACT-3'		
qPCR-avtr v1 F	5'-GACAGCCGCAAGTGATCAAG-3'	400 nM	203 bp
qPCR-avtr v1 R	5'-CCCGACCGCACACCCCCTGGCT-3'		
qPCR-avtr v2 F	5'-ATCACAGTCCTTGCATTGGTG-3'	600 nM	120 bp
qPCR-avtr v2 R	5'-GCACAGGTTGACCATGAACAC-3'		
qPCR-itr F	5'-GGAGGATCGTTTTAAAGACATGG-3'	400 nM	120 bp
qPCR-itr R	5'-TGTTGTCTCCCTGTCAGATTTTC-3'		
qPCR-actb F	5'-TCTTCCAGCCATCCTTCCTCG-3'	200 nM	108 bp
qPCR-actb R	5'-TGTTGGCATACAGGTCCTTACGG-3'		

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793 **Table 2.** Biometric parameters of the *S. aurata* groups maintained under feeding (control),
794 food deprivation and re-feeding conditions. The results are expressed as the mean ± SEM (n =
795 12/group). Significant differences between the different conditions are identified with different
796 letters ($p < 0.05$, one-way ANOVA followed by Tukey's test).

Parameter	Control	Food-deprived	Re-fed
Initial body mass (g)	196.98 ± 8.06[a]	198.71 ± 7.47[a]	196.75 ± 7.60[a]
Initial body length (cm)	21.78 ± 0.28[a]	21.73 ± 0.33[a]	21.75 ± 0.25[a]
Final body mass (g)	221.38 ± 6.65[a]	193.01 ± 6.41[b]	197.01 ± 6.27[b]
Final body length (cm)	22.33 ± 0.26[a]	21.72 ± 0.19[a]	22.08 ± 0.25[a]

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Table 3. Time course of the changes in the hepatic metabolites (*g ww*: grams wet weight) and HSI in *S. aurata* groups maintained under different feeding conditions (fed, food deprived and re-fed). Values are expressed as the mean ± SEM (n = 10-12 fish per group). Significant differences among samples under the same conditions are identified with different letters (capital letters: control group; lower case letters: food-deprived group). Different symbols show differences between groups at the same time ($p < 0.05$, two-way ANOVA followed by Tukey's test).

Parameter	Treatment	Day 0	Day 7	Day 14	Day 21
Glucose (µmol/g ww)	Control	4.23 ± 0.32^{Aa}	3.61 ± 0.64^{A}	2.02 ± 0.47^{A}	2.37 ± 0.38^{A}
	Food-deprived		2.92 ± 0.56^{a}	3.55 ± 0.33^{a}	2.82 ± 0.42^{a}
	Re-fed				3.69 ± 1.02^{a}
Glycogen (µmol/g ww)	Control	46.10 ± 1.99^{Aa}	46.30 ± 3.32^{A}	$44.82 \pm 2.08^{A*}$	$49.07 \pm 3.53^{A*}$
	Food-deprived		34.51 ± 4.50^{a}	$18.18 \pm 2.16^{b\#}$	$14.86 \pm 1.24^{b\#}$
	Re-fed				$32.81 \pm 3.60^{\ddagger}$
Triglycerides (µmol/g ww)	Control	4.89 ± 0.47^{Aa}	4.86 ± 0.66^{A}	$5.05 \pm 0.16^{A*}$	$4.89 \pm 0.67^{A*}$
	Food-deprived		3.89 ± 0.56^{a}	$3.29 \pm 0.26^{a\#}$	$3.20 \pm 0.26^{a\#}$
	Re-fed				4.94 ± 0.46*
HSI	Control	1.18 ± 0.09^{Aa}	$1.23 \pm 0.04^{A*}$	$1.18 \pm 0.05^{A*}$	$1.19 \pm 0.04^{A*}$
	Food-deprived		$0.99 \pm 0.05^{a\#}$	$0.86 \pm 0.05^{ab\#}$	$0.80 \pm 0.03^{b\#}$
	Re-fed				1.08 ± 0.04*