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ORIGINAL PAPER

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

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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: (1) normal feeding (control), (2) food deprivation for 21 days; and (3) 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 (1) the integration and control of different physiological pathways related to stress and food intake (i.e., the hypothalamus), (2) hormonal release into the bloodstream (i.e., the pituitary), and (3) 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

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Introduction

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

requires special attention (Wendelaar Bonga 1997; Barton 2002).

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

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

Materials and methods

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 1000 L tanks constituting the three different experimental groups in duplicate: the (1) control (food-supplied), (2) food-deprived, and (3) 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 53/2013 and law 32/2007) regarding the use of laboratory animals. The experimental procedure was authorized by the board of Experimentation on Animals of the University of Cádiz (UCA) and approved by the Ethical Committee Competent Authority (Junta de Andalucía Autonomous Government) under the reference number 28-04-15-241.

Sampling

At the start of the experiment, 12 fish were anaesthetized with a lethal dose of 2-phenoxyethanol (1 mL/L water) (SIGMA-ALDRICH, Cat. # P-1126) and sampled (control day 0). The anesthesia process in this and subsequent procedures was completed in less than 3 min. The remaining experimental fish were subjected to one of the following three treatments: (1) fed, (2) food-deprived, and (3) re-fed from day 14. Twelve fish (6 per tank) from each experimental group were sampled at 7, 14 and/or 21 days from the beginning of the trial. The body length and body

mass were measured. Blood was collected from the caudal peduncle with ammonium-heparinized syringes (SIGMA-ALDRICH, Cat. # H-6279, 25,000 units/3 mL of saline 0.6% NaCl), and the fish were subsequently killed by spinal sectioning. Plasma, obtained after the whole blood was centrifuged, and was stored in 1 mL aliquots at −80 °C until the AVT/IT analysis. The liver was weighed separately to calculate the hepatosomatic index (HSI), divided into multiple portions, immediately frozen in liquid nitrogen, and finally stored at −80 °C for subsequent analyses. In addition, a representative liver biopsy and both hypothalamic lobes and six pituitary glands (three of each experimental duplicate) were placed in Eppendorf tubes containing an appropriate volume (1/10 w/v) of RNA*later*® (Applied Biosystems). Those samples were kept for 24 h at 4 °C and then stored at −20 °C until total RNA isolation was performed. Furthermore, the six remaining pituitary glands were immediately snap-frozen in liquid nitrogen and stored at −80 °C for an AVT/IT storage analyses.

Analytical methods

HSI and liver metabolites

The HSI was determined as follows: HSI = 100 × (liver weight/body weight). For the assessment of metabolite levels, the livers were finely minced in an ice-cold petri dish, subsequently homogenized by mechanical disruption (Ultra-Turrax, T25 basic, IKA®-WERKE) with 7.5 vol. (w/v) of ice-cold 0.6 N perchloric acid and neutralized after the addition of the same volume of 1 M $KHCO_3$. Prior to centrifugation, an aliquot of each homogenate was taken for a triglyceride (TAG) determination. The homogenate was subsequently centrifuged (30 min, 13,000*g*, 4 °C) and the supernatant was recovered, aliquoted, and stored at −80 °C until used in the metabolite assays.

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

Total RNA isolation

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

Quantification of mRNA expression level

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

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

AVT and IT content in the plasma and the pituitary gland

AVT and IT in the plasma and the pituitary gland were determined through high-performance liquid chromatography (HPLC) with fluorescence detection preceded by solid-phase extraction (SPE) based on Gozdowska et al. (2006) and Martos-Sitcha et al. (2013b). Plasma samples (1 mL each) were acidified with 1 M HCl (100 µL) and centrifuged at 6000*g* for 20 min at 4 °C, and frozen

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

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

pituitaries were weighed and sonicated in 0.5 mL Milli-Q water (Microson™ XL, Misonix, USA), acidified with glacial acetic acid (1.25 µL) and then placed in a boiling water bath for 3.5 min. The pituitary extracts were cooled and centrifuged at 6000*g* for 15 min at 4 °C. After that, the supernatants were loaded onto a previously conditioned (1 mL MeOH, 1 mL water) SPE column (30 mg/mL, STRATA-X, Phenomenex). Water (600 µL) and 0.1% TFA (trifluoroacetic acid) in 5% acetonitrile (600 µL) were passed through the column to wash away impurities. The peptides were eluted with 2 × 600 µL of 80% acetonitrile. The resultant eluate was evaporated to dryness using a Turbo Vap LV Evaporator (Caliper Life Science, USA), and the samples were stored at −80 °C until HPLC analysis. Before quantitative analysis, the samples were resuspended in 40 µL 0.1% trifluoroacetic acid (TFA), then divided into two aliquots to provide duplicates for analysis. The pre-column derivatization of AVT and IT in each of the 20 µL samples was performed using 3 µL of a 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) solution (30 mg NBDF in 1 mL of acetonitrile) in a mixture of 20 µL phosphoric buffer (0.2 M, pH 9.0) and 20 µL acetonitrile. The solution was heated to 60 °C for 3 min in a dry heating block and cooled on ice. Next, 4 µL of 1 M HCl was added. The derivatized samples were assayed using an Agilent 1200 Series Quaternary HPLC System (Agilent Technologies, USA). Chromatographic separation was achieved using an Agilent ZORBAX Eclipse XDB-C18 column (150 mm × 4.6 mm I.D., 5 µm particle size). A gradient elution system was used to separate the derivatized peptides. The mobile phase consisted of solvent A (0.1% TFA in H_2O) and solvent B [0.1% TFA in acetonitrile: H_2O (3:1)]. The linear gradient was 45–70% eluent B in 20 min. The flow rate was set at 1 mL/min, and the column temperature at 20 °C. The injection volume was 67 µL. Fluorescence detection was conducted at 530 nm with excitation at 470 nm.

Statistical analysis

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

Results

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

Table 2 Biometric parameters of the *S. aurata* groups maintained under feeding (control), food deprivation and re-feeding conditions

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}

The results are expressed as the mean ± SEM ($n = 12$/group). Significant differences between the different conditions are identified with different letters ($p < 0.05$, one-way ANOVA followed by Tukey's test)

Liver metabolites and the HSI

A time course of the changes of the hepatic metabolites and the HSI are shown in Table 3. No differences between any of the analyzed parameters were detected in the control group. The hepatic glucose was stable in all groups for the entire experimental duration. However, the 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.

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 (Fig. 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 (Fig. 1a). The *pro-it* mRNA expression progressively decreased in the food-deprived fish over 21 days, with the lowest values observed on day 21 (Fig. 1b). However, the expression of both propeptides did not reach the control level after 7 days of re-feeding.

Hypothalamic mRNA expression of AVT and IT receptors

No differences were observed in the *avtr*s or *itr* mRNA expression in the control group (Fig. 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 (Fig. 2a). Food deprivation and re-feeding did not change *avtr v2* gene expression (Fig. 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 (Fig. 2c).

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)

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 ± 2.08^{A}*	49.07 ± 3.53^{A}*
	Food-deprived		34.51 ± 4.50^{a}	18.18 ± 2.16$^{b\#}$	14.86 ± 1.24$^{b\#}$
	Re-fed				32.81 ± 3.60‡
Triglycerides (μmol/g ww)	Control	4.89 ± 0.47^{Aa}	4.86 ± 0.66^{A}	5.05 ± 0.16^{A}*	4.89 ± 0.67^{A}*
	Food-deprived		3.89 ± 0.56^{a}	3.29 ± 0.26$^{a\#}$	3.20 ± 0.26$^{a\#}$
	Re-fed				4.94 ± 0.46*
HSI	Control	1.18 ± 0.09^{Aa}	1.23 ± 0.04^{A}*	1.18 ± 0.05^{A}*	1.19 ± 0.04^{A}*
	Food-deprived		0.99 ± 0.05$^{a\#}$	0.86 ± 0.05$^{ab\#}$	0.80 ± 0.03$^{b\#}$
	Re-fed				1.08 ± 0.04*

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)

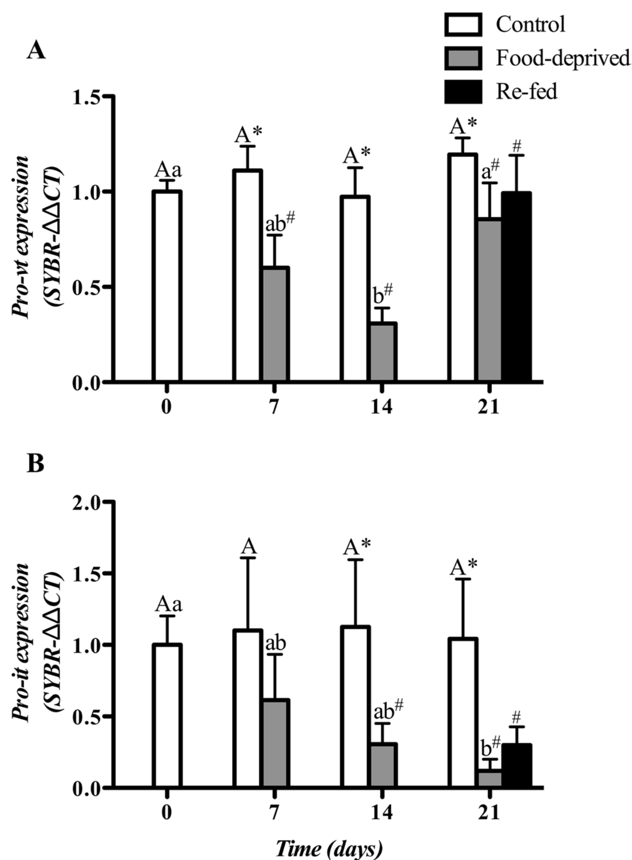

Fig. 1 Time course of the changes in the hypothalamic *pro-vasotocin* (*pro-vt*; A) and *pro-isotocin* (*pro-it*; B) mRNA expression levels in *S. aurata* maintained under different feeding conditions (fed, food-deprived and re-fed). Values are expressed as the mean ± SEM ($n = 10$–12 fish per group). 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)

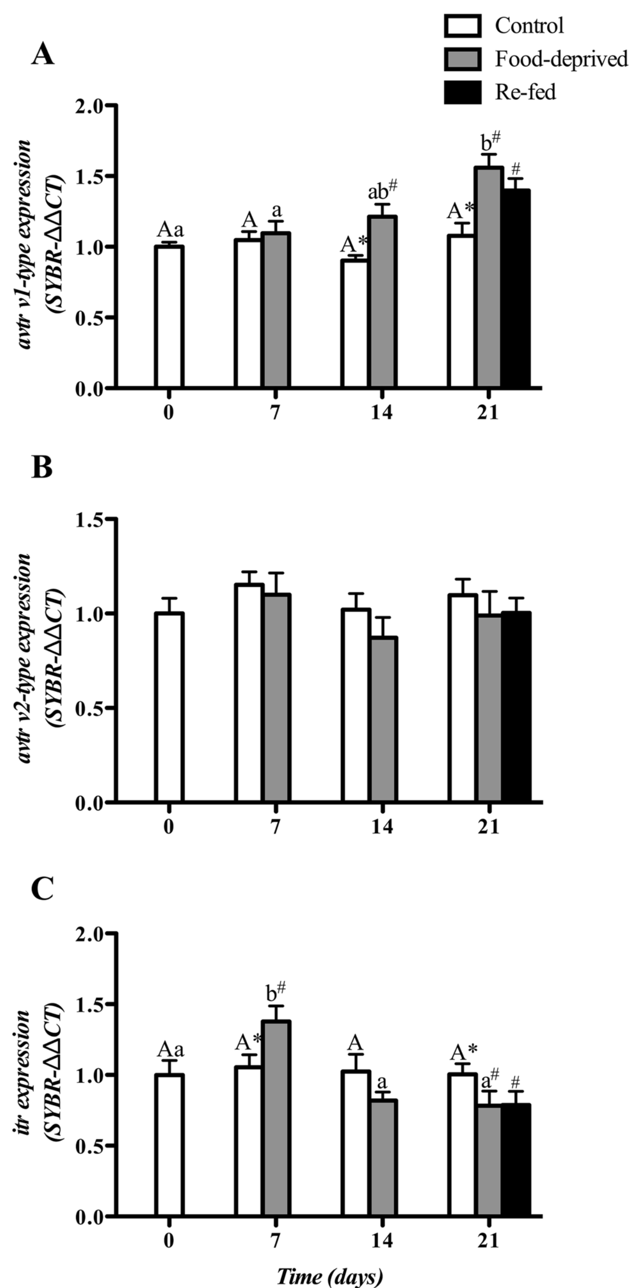

Fig. 2 Time course of the changes in the hypothalamic *avtr v1* (**a**), *avtr v2* (**b**) and *itr* (**c**) mRNA levels in *S. aurata* maintained under different feeding conditions (fed, food-deprived and re-fed). For further details, see the legend in Fig. 1

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 Fig. 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.

Hypophyseal AVT and IT storage

The control group did not show any variation in the pituitary AVT level, whereas the pituitary AVT level in the food-deprived fish significantly increased from day 14 onwards (Fig. 4a). In addition, the pituitary AVT level also increased in the re-fed group relative to the control group, but the level was not significantly different from that

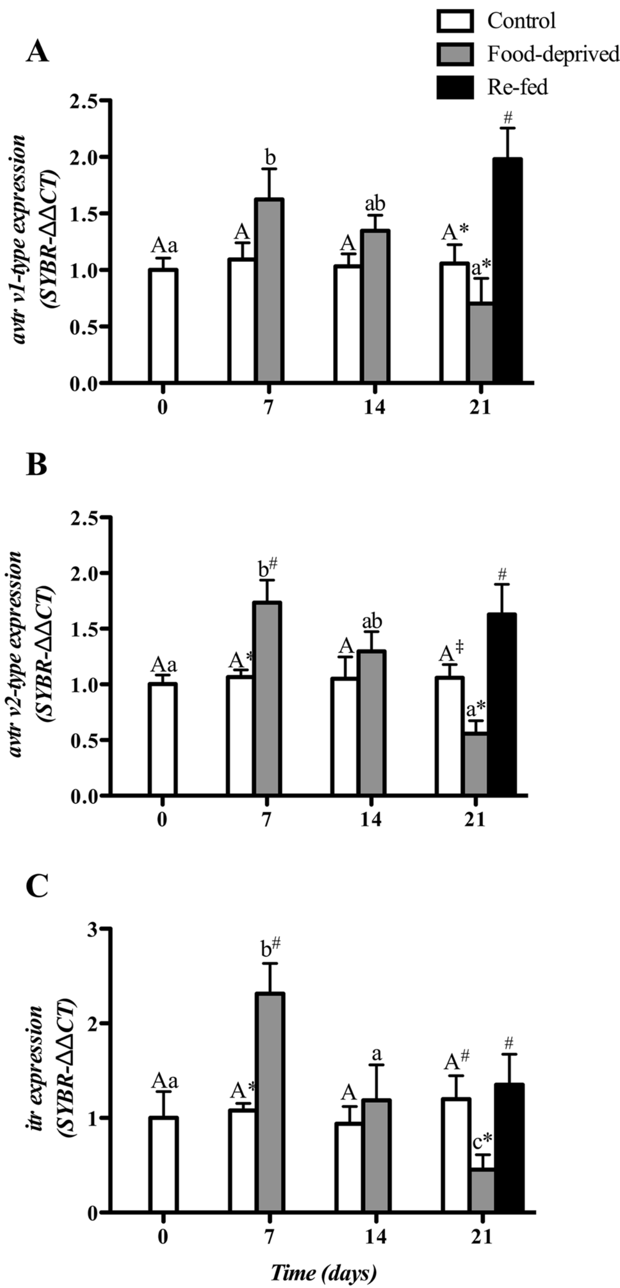

Fig. 3 Time course of the changes in the pituitary *avtr v1* (**a**), *avtr v2* (**b**) and *itr* (**c**) mRNA levels in *S. aurata* maintained under different feeding conditions (fed, food-deprived and re-fed). For further details, see the legend in Fig. 1

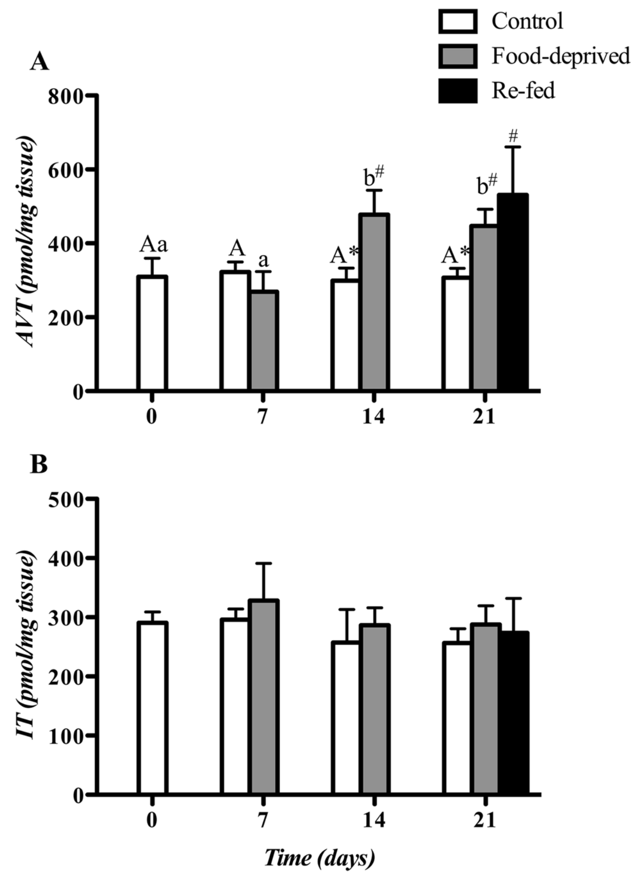

Fig. 4 Time course of the changes in the AVT (**a**) and IT (**b**) pituitary storage levels in *S. aurata* maintained under different feeding conditions (fed, food-deprived and re-fed). For further details, see the legend in Fig. 1

in the food-deprived fish. In contrast, the IT storage was unchanged for the entire experimental duration in all three groups (Fig. 4b).

Plasma AVT and IT

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

Hepatic mRNA expression of AVT and IT receptors

In the liver, no changes were observed in the *avtr* or *itr* mRNA expression in the control group (Fig. 6). However, *avtr v1* expression significantly increased in both the food-deprived and re-fed fish relative to the control at day 21 (Fig. 6a). In addition, *avtr v2* mRNA expression significantly decreased in the food-deprived fish over the

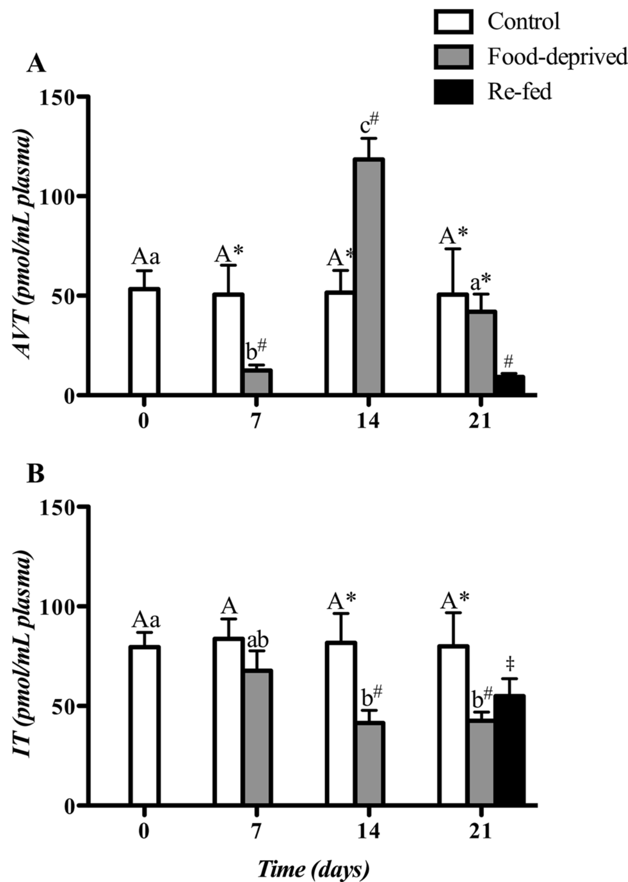

Fig. 5 Time course of the changes in the AVT (**a**) and IT (**b**) plasma levels in *S. aurata* maintained under different feeding conditions (fed, food-deprived and re-fed). For further details, see the legend in Fig. 1

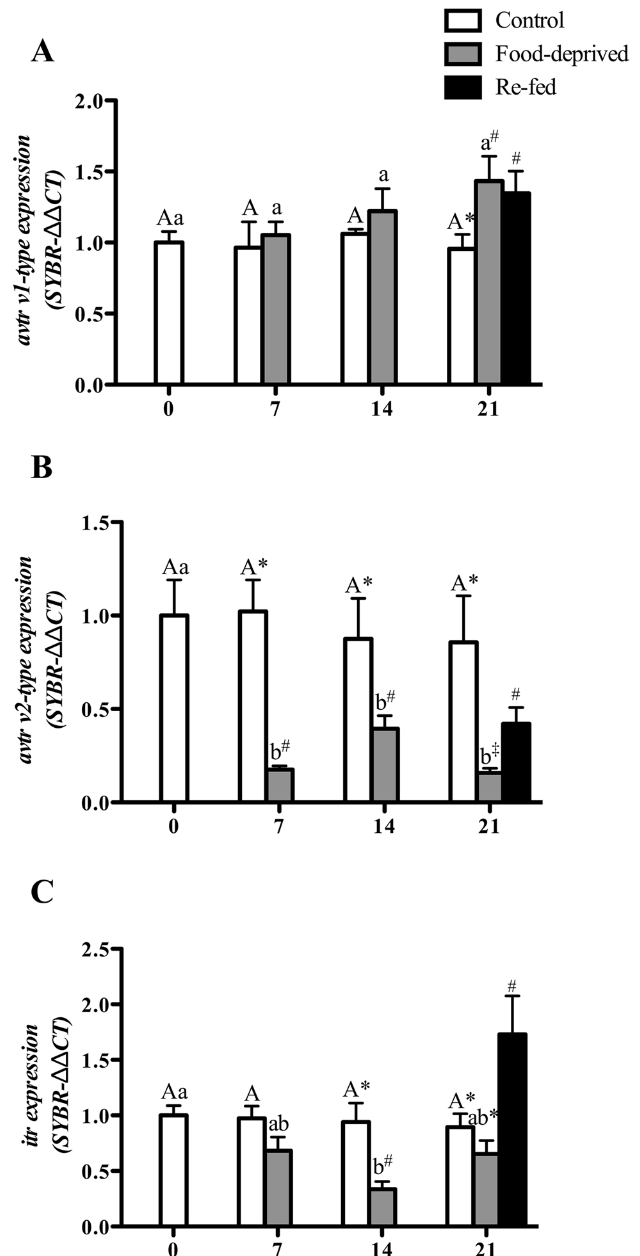

Fig. 6 Time course of the changes in the hepatic *avtr v1* (**a**), *avtr v2* (**b**) and *itr* (**c**) mRNA levels in *S. aurata* maintained under different feeding conditions (fed, food-deprived and re-fed). For further details, see the legend in Fig. 1

experimental duration. The *avtr v2* mRNA expression partially recovered after re-feeding, but did not reach control levels (Fig. 6b). The *itr* mRNA expression progressively decreased and was significantly lower than the control level at day 14. The *itr* mRNA expression in the re-fed group was significantly higher than that of the control and the food-deprived fish at day 21 (Fig. 6c).

Discussion

The involvement of vasotocinergic and isotocinergic systems in osmoregulation (Kleszczyńska et al. 2006; Martos-Sitcha et al. 2013a, b, 2014a, 2015a), metabolism (Sangiao-Alvarellos et al. 2006), the stress response related to a high stocking density (Mancera et al. 2008) and cortisol administration (Cádiz et al. 2015) have been previously studied in *S. aurata*. To the best of our knowledge, this study is a more complete and integrated view of the crosstalk/interactions between the starvation/re-feeding processes associated with the activation of the vasotocinergic and isotocinergic pathways in gilthead sea bream as previously reported by Mancera et al. (2008).

Metabolic indicators and HSI

Starvation is well tolerated by many fish species in nature. To survive periods of unfavorable feeding conditions, fish mobilize their energy reserves to adjust their metabolism in a species-specific manner (Navarro and Gutiérrez 1995).

Plasma metabolic indicators have been previously studied after starvation and re-feeding in the same fish sampled in this study (Martos-Sitcha et al. 2014b), suggesting that the energy requirements are supplied by glucose during the first days of FD and later by lactate. In addition, reduced hepatic glycogen and triglycerides, which are reflected by a reduction of the body and liver mass (i.e., the HSI), indicated that these metabolites were serving as an energy supply due to the high response and extent to the lipolytic machinery of the hepatic tissue during FD (Benedito-Palos et al. 2014), which has also been reported in different teleost species (Shimeno et al. 1990; Machado et al. 1988; Farbridge and Leatherland 1992; Mehner and Wieser 1994; Soengas et al. 1996; Pascual et al. 2003; Polakof et al. 2006). In the re-fed group, the glycogen level partially recovered after 1 week of feeding, as has also been described in mammals (Carmean et al. 2013) and other fish species (Black and Love 1986; Collins and Anderson 1995; Pujante et al. 2015). Moreover, a full recovery of TAG and HSI occurred after re-feeding. Given that hepatic lipids are the first reserves used after liver glycogen, as has been previously described in other teleosts [*Gadus morhua* (Guderley et al. 2003), *Rhamdia hilarii* (Machado et al. 1988), *Leuciscus idus* (Segner and Braunbeck 1988) and *Cyprinus carpio* (Shimeno et al. 1990)], the recovery observed in metabolite storage during re-feeding could be a strategy for rapid uptake and energy redistribution in the body, with the basal storage levels being partially restored. This finding suggests that *S. aurata* require more time to reach carbohydrate homeostasis, but not hepatic lipid storage homeostasis after FD and re-feeding.

AVT and IT systems

Some authors consider long-term FD as a serious stress factor in fish that increases the cortisol level and affects metabolic processes (Vijayan et al. 1993; Sangiao-Alvarellos et al. 2005; Mancera et al. 2008). Our results regarding the circulating levels of cortisol hormone after starvation have been previously reported for the same fish used in this study, clearly suggesting that the stress system had been activated (Martos-Sitcha et al. 2014b).

In teleosts, corticotrope cells from the anterior pituitary gland synthesize ACTH, which in turn stimulates the secretion of cortisol by the interrenal tissue (Wendelaar Bonga 1997; Bernier et al. 2009). Moreover, the corticotrope cells are innervated by hypothalamic neurons that produce AVT (Batten et al. 1990), indicating a role for AVT in the activation of the stress response system in fish (Bernier et al. 2009). Vasotocinergic and isotocinergic systems have a wide range of physiological functions that depend on many internal and external factors that control various processes at the neuronal level, such as *pro-vt* and *pro-it* mRNA expression, peptide synthesis, transport, maturation and storage, and the release of mature nonapeptides into the circulation (Mancera et al. 2017). The plasma levels of active AVT and IT could be reduced by: (1) inhibition during the production of the pro-peptides at the level of the hypothalamus and/or during processes related to peptide maturation, (2) inhibition of the release of mature nonapeptides in the neurohypophysis, (3) renal clearance, and (4) inactivation of nonapeptides by plasma or tissue peptidases. However, nonapeptide binding to specific receptors in target organs triggers their physiological action (Ward et al. 1990; Agirregoitia et al. 2005; Martos-Sitcha et al. 2013b, 2014a). Therefore, any investigation of the response of the vasotocinergic and isotocinergic systems requires a comprehensive analysis at the level of peptide synthesis, storage and release, and specific receptors in the target tissues of interest. Starvation stress can affect the vasotocinergic and isotocinergic pathways in the sea bream. To our knowledge, little data about a link between starvation and the activity of AVT/AVP and IT/OXY systems exist for vertebrates (Flynn et al. 2002; Tachibana et al. 2004; Gesto et al. 2014). Our study is the first extensive analysis of the response of both the vasotocinergic and isotocinergic systems after food deprivation and re-feeding in *S. aurata*.

Regulation of AVT and IT synthesis and release under starvation conditions

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

During the first week of starvation, a decrease in hypothalamic *pro-vt* expression and plasma AVT levels was observed, but there were no changes in either AVT storage in the pituitary or in *avtr* expression in the hypothalamus. Thus, the increased plasma cortisol values previously demonstrated in these same specimens after FD conditions (Martos-Sitcha et al. 2014b) could suggest an inhibitory effect of this hormone on several elements of the vasotocinergic system. Therefore, cortisol can be considered one of the key players in the orchestration in response to FD (Chang et al. 2002; Pujante et al. 2015), possibly as a state of alert in response to FD during the first week. This effect could also be an important issue during re-feeding (Uchida et al. 2003; Martos-Sitcha et al. 2014b). Moreover, an increase in the mRNA level of both *avtr v1* and *avtr v2* in the pituitary indicated the activation of both receptors, which could be linked to feedback mechanisms both controlling AVT release and, for instance, indirectly regulating

cortisol production. Notably, AVT storage in the pituitary is maintained at the same level in spite of the lower hypothalamic *pro-vt* mRNA and plasma AVT levels, guaranteeing the hormonal homeostasis (neutral balance among synthesis and release) in the pituitary gland. However, in a previous study, the administration of exogenous cortisol in gilthead sea bream increased hypothalamic *pro-vt* mRNA without a change in either the pituitary or plasma AVT level (Cádiz et al. 2015), suggesting that other endocrine factors, such as Urotensin I, melanin-concentrating hormone, neuropeptide Y, or thyrotrophic-releasing hormone could be involved in the differential response in addition to cortisol (Winberg et al. 2016). This fact highlights that complex interconnections between different endocrine axes can be made for the correct regulation of the stress system. This response has also been demonstrated *in vitro* in this fish species, in which cortisol administration induced AVT secretion from pituitary cells (Kalamarz-Kubiak et al. 2014), or even that AVT hormone potentiate cortisol release when co-administrated with ACTH in *Cyprinus carpio* (Jerez-Cepa et al. 2016).

However, during the second week of FD, the hypothalamic *pro-vt* mRNA levels further decreased, but the AVT level in the pituitary and the plasma significantly increased, suggesting that the AVT system changed in response to FD at this time. This apparent controversial result is interesting, although changes in the dynamic of AVT storage at pituitary level as well as hormonal release and plasma clearance (see Agirregoitia et al. 2005) induced by food-deprivation need to be assessed to understand our data and to establish the possible interconnection with several orexigenic and anorexigenic factors. Hormones belonging to the AVT/AVP family have been demonstrated to mediate anorexigenic effects in mammals (Flynn et al. 2002), birds (Tachibana et al. 2004) and fishes (Gesto et al. 2014). For example, the intracerebroventricular (i.c.v.) administration of AVT drastically decreased the food intake in juvenile rainbow trout (*Oncorhynchus mykiss*) (Gesto et al. 2014). In addition, studies have shown that AVP induced anorexigenic effects via a V1a-type receptor, but the blockade of this receptor produced orexigenic effects in neuropeptide-Y-induced mice (Aoyagi et al. 2009). Our results, in agreement with previous studies, indicate a progressive increase in the hypothalamic expression of *avtr v1* after 3 weeks of FD, suggesting that this AVT receptor could have a time-dependent modulatory role on the neuropeptide-mediated control of food intake.

Moreover, the increase observed in the AVT content, both at plasma and hypophyseal levels after 14 days of starvation, suggests that these key components of the pathway are regulated through long-term adaptation. This could be attributed to both types of AVT receptors in the pituitary, regulating the retention and release of the mature peptide into the blood stream. This phenomenon could be attributed to both intracellular and/or paracellular signaling in pituitary cells, where *avtr*s could organize its release and promote integrated physiological changes down-stream. Even so, further studies will be necessary in order to clarify the proposed regulatory mechanism operating in the pituitary. For that, all changes produced in *pro-vt* and *avtr*s up-stream should be taken into account. This singularity highlights the clear orchestration of the complete axis from the beginning of this pathway. This fact has even been determined when the homeostatic level of AVT via *pro-vt* production has been reached after different processes, such as (1) appetite (this work), (2) cortisol production as one of the primary stress response (Cádiz et al. 2015), as well as its interconnection with AVT demonstrated *in vitro* as a component of the HPI axis (Kalamarz-Kubiak et al. 2014), and (3) metabolic organization of the hypothalamic neurons induced by AVT (Sangiao-Alvarellos et al. 2006). Nevertheless, the negative feedback of any of these pathways cannot be ruled out as being associated with the lower levels of *pro-vt* mRNA that were observed.

To our knowledge, only partial data regarding FD and isotocinergic pathways have been reported in fish (Mancera et al. 2008). The activation of the isotocinergic system has been demonstrated in *S. aurata* under different stress conditions, such as a high stocking density (Mancera et al. 2008), osmotic challenge (Kleszczyńska et al. 2006; Martos-Sitcha et al. 2013b), or even by chronic stress simulation mimicked by cortisol treatment (Cádiz et al. 2015). Moreover, the oxytocinergic system has been proposed to be involved in the regulation of feeding behavior in mammals. For instance, OXY and its agonists administered by i.c.v. injection inhibit feed intake, whereas these effects are prevented by administration of OXY antagonists (Arletti et al. 1990; Lokrantz et al. 1997).

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

Hepatic expression of avtr and itr genes under starvation

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

Re-feeding process

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

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

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

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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