Unraveling vasotocinergic, isotocinergic and stress pathways after food deprivation and high stocking density in the gilthead sea bream

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Abstract

The influence of chronic stress, induced by food deprivation (FD) and/or high stocking density (HSD), was assessed on stress, vasotocinergic and isotocinergic pathways of the gilthead sea bream (*Sparus aurata*). Fish were randomly assigned to one of the following treatments: (1) fed at low stocking density (LSD-F; 5 $kg \cdot m^{-3}$); (2) fed at high stocking density (HSD-F, 40 $kg \cdot m^{-3}$); (3) food-deprived at LSD (LSD-FD); and (4) food-deprived at HSD (HSD-FD). After 21 days, samples from plasma, liver, hypothalamus, pituitary and head-

kidney were collected. Both stressors (FD and HSD) induced a chronic stress situation, as indicated by the elevated cortisol levels, the enhancement in corticotrophin releasing hormone (*crh*) expression and the down-regulation in corticotrophin releasing hormone binding protein (*crhbp*) expression. Changes in plasma and liver metabolites confirmed a metabolic adjustment to cope with energy demand imposed by stressors. Changes in *avt* and *it* gene expression, as well as in their specific receptors (*avtrv1a*, *avtrv2* and *itr*) at central (hypothalamus and pituitary) and peripheral (liver and head-kidney) levels, showed that vasotocinergic and isotocinergic pathways are involved in physiological changes induced by FD or HSD, suggesting that different stressors are handled through different stress pathways in *S. aurata*.

Introduction

Fish, not only in the nature but also when farmed (i.e. in aquaculture), must cope with different stressors, like lack of food availability, high densities, transport, air exposure during handling, or water biochemical quality (Wendelaar Bonga, 1997, 2011; Barton, 2002). Magnitude and duration of the stress response is dependent on the severity and extension of the stressor, but always the stress stimuli launch an endocrine stress response through activation of the hypothalamic-pituitary-interrenal (HPI) axis with the release of cortisol (Gorissen and Flik, 2016; Schreck and Tort, 2016). This stimulation is mediated by several neurotransmitters and neuroendocrine factors. Thus, the HPI axis starts its endocrine cascade by secreting the Corticotrophin releasing hormone (Crh), further regulated by a soluble binding protein (Crhbp), but also by other neuropeptides as Arginine vasotocin (Avt) and/or Isotocin (It) (Bernier et al., 2009). It is known that Crh and Avt, both together and separated, stimulate the release of the Adrenocorticotropic hormone (Acth), activating HPI axis (Bernier et al., 2009; Jerez-Cepa et al., 2016; Winberg et al., 2016). Acth derives from the precursor protein Proopiomelanocortin (Pomc) and stimulates the interrenal cells of the head-kidney to produce and release cortisol. Finally, cortisol hormone, produced as the final step of the HPI axis, is the main steroid in fish involved in several physiological processes (Wendelaar Bonga, 1997, 2011; Mommsen et al., 1999; Mancera and McCormick, 2007, Schreck and Tort, 2016). The pleiotropic characteristics of neuropeptides belonging to the Avt family, together with a direct regulation of cortisol production by their specific receptors in the interrenal tissue (Cádiz et al., 2015), give light to understand their physiological function in

several biological processes where cortisol presents a role (i.e. metabolic regulation and stress endocrine response) (Bernier et al., 2009; Mancera et al., 2017). Moreover, Avt and It have been proposed as welfare indicators of fish under different stress situations (confinement, disturbance, high density or food deprivation) (see Martins et al., 2012 for review).

Among stress factors, food availability and rearing conditions are very important issues that must be controlled in cultured fish. Indeed, food restriction mainly produced by inadequate environmental conditions, as depletions in water temperature or overwintering in the aquaculture sector, or the temporal and spatial inconsistency of food in wild fish, could generate adverse situations activating the stress axis with the subsequent reorganization in metabolism as well as inhibition in growth processes (Bauer and Schlott, 2004; Pérez-Jiménez et al., 2007; Eroldoğan et al., 2008; Furné et al., 2012). In addition, different studies have evaluated the effects of inadequate stocking densities as a source of chronic stress in aquaculture, also demonstrating internal disorders that negatively compromise physiological processes as growth capacity, mobilization of energy sources, alterations in behaviour, and poor feed utilization (Montero et al., 1999; Sangiao-Alvarellos et al., 2005; Herrera et al., 2009; Li et al., 2012; de las Heras et al., 2015; Millán-Cubillo et al., 2016). In this sense, the reduction in the occurrence and the severity of stressors is increasingly necessary to provide an improvement in animal welfare.

In the last years, our Research Group has focused his research activities on the study of the vasotocinergic and isotocinergic pathways and their interaction with the stress axis in the gilthead sea bream (*Sparus aurata*) (Kleszczyńska et al., 2006; Sangiao-Alvarellos et al., 2006; Mancera et al., 2008, 2017; Martos-Sitcha et al., 2013, 2014a,b; Cádiz et al., 2015; Skrzynska et al., 2017). *S. aurata* is a highly cultured fish in all the Mediterranean area, and is considered as a model species not only for the aquaculture industry but also at physiological level, given the great knowledge reported in terms of growth, nutrition or energy metabolism, among others (Pavlidis and Mylonas, 2011). In the present study, we assessed the involvement of the vasotocinergic, isotocinergic and stress pathways in defining the physiological response of *S. aurata* after exposition to food deprivation and high density in a factorial design (2 x 2), evaluating different players not addressed in previous studies. In addition, an integrative view of alterations produced by these factors on blood biochemistry and hormonal parameters as markers of metabolic response was also provided.

Materials and methods

Animal care and experimental conditions

Juvenile gilthead sea breams (*Sparus aurata* 200 ± 20 g body mass) males 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 the wet laboratories at Faculty of Marine and Environmental Sciences (Puerto Real, Cádiz). During the experiment fish were maintained under natural photoperiod (July) for our latitude (36º 31' 44" N) and constant temperature (18-19 ºC). Animals were then distributed in eight different tanks and acclimated to these conditions for 15 days previous to the experimental use, showing a normal feeding pattern during this acclimation period. These eight tanks constituted the four experimental groups (in duplicate): 1) fed at low stocking density (control, LSD-F; 5 $kg \cdot m^{-3}$); 2) fed at high stocking density (HSD-F, 40 $kg \cdot m^{-3}$); 3) food-deprived at LSD (LSD-FD); and 4) food-deprived at HSD (HSD-FD). These experimental conditions have previously been tested by our Research Group and clearly demonstrated an activation of the stress axis in *S. aurata* (Arends et al., 1999, 2000; Sangiao-Alvarellos et al., 2005, Mancera et al., 2008; Skrzynska et al., 2017). Fish from LSD-F and HSD-F groups were fed once a day with commercial dry pellets at a ratio of 1 % of body mass, whereas animals from food-deprived groups (LSD-FD and HSD-FD) were not fed during the 21 days of the experimental time. The described experiment complied with the National (RD53/2013) and the current EU legislation (2010/63/EU) on the handling of experimental fish, and was authorized by the board of Experimentation on Animals of the University of Cádiz (UCA), and approved from the Ethical Committee Competent Authority (Junta de Andalucía Autonomous Government) under the reference number: 28-04-15-241.

Sampling

21 days after the beginning of the experiment, 12 fish from each experimental condition (n = 6 per tank) were anaesthetized with 2-phenoxyethanol (1 mL/L seawater) (Sigma, Cat. # P-1126) and sampled. The anesthesia process was completed in less than 3 min. Body length and body mass were measured and blood was collected from the caudal peduncle using ammonium-heparinized syringes (Sigma, 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 (3 min, 10,000 g, 4 ºC), was stored at -80 ºC until analysis of cortisol

and metabolites. Livers were 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 turn, representative liver and head-kidney biopsies, as well as both hypothalamic lobes and complete pituitary glands were placed in Eppendorf tubes containing an appropriate volume (1/10 w/v) of RNA*later*® (Applied Biosystems). These samples were kept for 24 h at 4 °C and then stored at -20 °C until total RNA isolation was performed.

Analytical methods

Plasma metabolites and cortisol level

Glucose, lactate and triglycerides (TAG) concentrations were measured using commercial kits from Spinreact (Barcelona, Spain) (Glucose-HK: Ref. 1001200; Lactate: ref. 1001330; TAG: ref. 1001311) adapted to 96-well microplates. All assays were run on an Automated Microplate Reader (PowerWave 340, BioTek Instrument Inc., Winooski, VT, USA) controlled by KCjunior^{TM} software. Standards and samples were measured in quadruplicate and duplicate, respectively.

Plasma cortisol levels were measured by Enzyme-Linked Immunosorbent Assay (ELISA) as previously described by Martos-Sitcha et al. (2014b) in this fish species. In short, steroids were extracted from 5 µL of plasma in 100 µL RB (PPB (potassium phosphate buffer) 100 mM, NaN_3 1.54 mM, NaCl 400 mM, $EDTANa_2$ 1 mM, BSA (bovine serum albumin) 15 mM) and 1.2 mL methanol (Panreac), then evaporated during 48–72 h at 37 °C. Cortisol EIA standard (Cat. #10005273), goat anti-mouse IgG monoclonal antibody (Cat. #400002), specific cortisol express EIA monoclonal antibody (Cat. #400372) and specific cortisol express AChE tracer (Cat. #400370) were obtained from Cayman Chemical Company (Michigan, USA). Standards and extracted plasma samples were run in duplicate. The percentage of recovery was determined as 95 % (Martos-Sitcha et al., 2014b). The inter- and intra-assay coefficients of variation (calculated from the sample duplicates) were 2.93 ± 0.34 % and 4.32 ± 0.59 %, respectively.

Hepatosomatic index and liver metabolites

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

Hepatic glucose, lactate and TAG concentrations were measured as described above for plasma determinations. Liver glycogen levels were assessed using the method from Keppler and Decker (1974), in which glucose obtained via glycogen breakdown (after subtracting free glucose levels) was determined using the previously described commercial glucose kit. All the assays were run on an Automated Microplate Reader as described above.

Total RNA isolation

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

Quantification of mRNA expression levels

According to the previous works described by Martos-Sitcha et al. (2013, 2014a,b), 50 ng of total RNA from the pituitary, or 500 ng of total RNA from the hypothalamus, liver and head-kidney, were used for reverse transcription in a final volume of 20 µL using 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 ng or 10 ng of cDNA, from the pituitary or from hypothalamus, head-kidney and liver, respectively.

Primers for *crh* (acc. no. **KC195964**)*, crhbp* (acc. no. **KC195965**)*, avt* (acc. no. **FR851924**), *it* (acc. no. **FR851925**), *avtrv1a* (acc. no. **KC195974**), *avtrv2* (acc. no. **KC960488**), *itr* (acc. no. **KC195973**) and *beta actin* (*actb*, acc. no. **X89920**) from *S. aurata* (at the final concentration provided in Table 1) were used as previously described by Martos-Sitcha et al. (2013, 2014a,b). Several calibration plots with different template concentrations in serial dilutions (from 10 ng (1 ng in pituitaries) to 100 fg of input total RNA from their corresponding target tissue) had amplification efficiencies and r^2 of 0.96-1.02 and 0.991-0.998, respectively, for all primer pairs used. The PCR profile was as follows: 95 ºC, 10 min; [95 ºC, 20 s; 60 ºC, 30 s] X 40 cycles; melting curve [60 ºC to 95 ºC, 20 min], 95 ºC, 15 s. The melting curve was used to ensure that a single product was amplified and to verify the absence of primer-dimer artifacts. Results were normalized to *actb* owing its low variability (less than 0.15 C_T in pituitary, and less than 0.20 C_T in hypothalamus, head-kidney or liver) under our experimental conditions. Relative gene quantification was performed using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001).

This manuscript follows the ZFIN Zebrafish Nomenclature Guidelines for gene and protein names and symbols
(https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines).

Statistical analysis

The results are presented as mean ± standard error of the mean (SEM). After the normality of the data and the homogeneity of variance were checked, comparison between groups was evaluated using two-way ANOVA with density (low and high) and feeding conditions (fed and food-deprived) as main factors. 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 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 disturbances or any alterations in fish behavior were observed in any experimental group.

Plasma biochemistry

Values of plasma parameters are shown in Table 2. Cortisol levels significantly increased in fish kept at HSD under both fed and food-deprived conditions. In addition, a significant increase in this hormone was observed in LSD-FD fish when compared with LSD-F group. No variation was observed in this parameter between different food regimens (F *vs* FD) at HSD. Glucose significantly decreased in HSD-F fish respect to both LSD-F and HSD-FD groups. Lactate showed a significant decrease in both LSD-FD and HSD-F groups compared with fish kept at LSD-F conditions. Triglycerides were affected by high stocking density, decreasing significantly in fish maintained under both feeding conditions (HSD-F and HSD-FD) conditions compared to LSD groups. Moreover, this metabolite also presented a significant decrease after food deprivation under both stocking densities. Proteins values decreased significantly after food deprivation (LSD-FD and HSD-FD) compared to fed groups.

Hepatic metabolic indicators

Changes in hepatic metabolites and hepatosomatic index (HSI) in gilthead sea breams exposed to different food regimens and stocking densities are also showed in Table 2. HSI significantly decreased in fish maintained under food deprivation in both LSD and HSD conditions. In addition, a significant decrease in this index was also observed in HSD-F fish when compared with LSD-F group. Lactate diminished significantly in HSD-FD group compared to fish kept under HSD-F conditions. Triglycerides were affected by food deprivation, with a significant decrease in fish maintained under both LDS-FD and HSD-FD conditions when compared with LSD-F and HSD-F, respectively. Moreover, this metabolite also showed a significant decrease in fish fed and maintained under HSD conditions. Finally, free hepatic glucose did not have variations among groups, whereas glycogen significantly decreased after food deprivation in both LSD and HSD groups.

Hypothalamic expression of *crh* and *crhbp*

Expression for *crh* mRNA significantly increased in fish kept at HSD in both fed and food-deprived conditions. In addition, a significant enhancement in *crh* gene expression was observed in LSD-FD fish when compared with LSD-F group. However, no variation was observed between different food regimens (F *vs* FD) at HSD (Figure 1A). Moreover, *crhbp* was affected by both stocking density and food regimen, showing its lowest mRNA expression values in the HSD-FD group, and being significantly different when compared with both LSD-FD and HSD-F groups (Figure 1B). In addition, a significant correlation between hypothalamic *crh*, *crhbp* and plasma cortisol levels (*crh vs* cortisol: $y = 0.864 \cdot x + 1.502$, $R = 0.937$, $p<0.0001$; *crh vs crhbp*: $y = -0.039 \cdot x + 1.532$, $R = 0.625$, $p<0.01$; *crhbp vs* cortisol: $y = -8.601 \cdot x + 23.84$, $R = 0.602$, $p<0.01$) was also observed.

Hypothalamic expression of *avt* and *it* precursors

Fish kept under FD did not show variations in *avt* gene expression under different stocking densities, although a significant decrease in fed fish maintained at the highest stocking density (HSD-F) was noted when compared with the LSD-F group (Figure 2A). However, both food deprivation (LSD-FD) and high stocking density (HSD-F) significantly decreased *it* mRNA values when compared with LSD-F group (Figure 2B).

Expression of *avtrv1a*, *avtrv2* and *itr*

Hypothalamic expression

The expression of *avtrv1a* mRNA enhanced significantly in fish maintained under both fed (HDS-F) and food deprivation (HSD-FD) conditions when compared with LSD-F and LSD-FD, respectively (Figure 3A). In addition, a significant up-regulation of *avtrv1a* expression was also observed in HSD-FD compared with HSD-F group. No variations were produced by food regimen and stocking density in the *avtrv2* gene expression (Figure 3B). However, the *itr* mRNA expression enhanced significantly in food-deprived fish maintained at the highest stocking density (HSD-FD) when compared with the LSD-FD group (Figure 3C).

Pituitary expression

Food regimen (LSD-FD) and stocking density (HSD-F) significantly decreased hypophyseal mRNA expression of *avtrv1a* and *itr* genes when compared with LDS-F fish (Figures 4A and 4C, respectively). However, *avtrv2* expression was not affected along the experimental time by any of the combinations studied (Figure 4B).

Head-kidney expression

mRNA expression of head-kidney *avtrv1a* and *itr* genes did not show significant variations produced by different combinations of food regimens and stocking densities (Figures 5A and 5C, respectively). However, a significant decrease in *avtrv2* expression was observed in LSD-FD and HSD-F when compared with LSD-F group (Figure 5B).

Hepatic expression

Hepatic mRNA levels of the *avtrv1a* gene were up-regulated in food-deprived fish maintained at the highest stocking density (HSD-FD) when compared with the LSD-FD group (Figure 6A). On the other hand, food deprivation significantly decreased *avtrv2* gene expression in fish maintained under low stocking density (LSD-FD) when compared with fed fish (LSD-F). In addition, *avtrv2* gene expression increased by stocking density under food deprivation (Figure 6B). Finally, a significant up-regulation of *itr* gene expression was observed in HSD-FD fish when compared with both the LSD-FD and HSD-F groups (Figure 6C).

Discussion

The activation of stress pathways due to food deprivation (FD) and/or high stocking density (HSD) has been demonstrated to alter the endocrine system, negatively affecting energy metabolism and growth performance (Barton, 2002; Simó-Mirabet et al., 2017). A similar situation has previously been reported in *S. aurata* individuals under both stressors (Mancera et al., 2008).

Endocrine regulation of stress pathways involves several players (Wendelaar Bonga, 2011; Gorissen and Flik, 2016). In fact, our Research Group has assessed the role of different endocrine systems in the gilthead sea bream after different challenges (Arends et al., 1999, 2000; Martos-Sitcha et al., 2013, 2014a,b; Skrzynska et al., 2017; Ruiz-Jarabo et al., 2017,

among others). Concerning vasotocinergic and isotocinergic pathways, previous results suggested a role of Avt and It hormones in the stress response (Mancera et al., 2008; Cádiz et al., 2015; Skrzynska et al., 2017) together with a stimulatory metabolic role (Sangiao-Alvarellos et al., 2006). However, the complex action of the Avt/It endocrine cascade after FD and HSD conditions in *S. aurata* in particular, and in teleost fish in general, remains unclear due to the scarce information available.

Metabolic adjustments produced by different stress sources

Both FD and HSD induced metabolic alterations in plasma, concomitantly with decreases in hepatic energy reserves of challenged fish, as it has been reported in several fish species (Vijayan et al., 1996; Rueda et al., 1998; Menezes et al., 2015) including in *S. aurata* (Arends et al., 1999, 2000; Mancera et al., 2008; Gil-Solsona et al., 2017; Skrzynska et al., 2017). Thus, metabolic results indicated that the experimental model used here was effective to trigger a stress response in *S. aurata* (Arends et al., 2000; Mancera et al., 2008), also highlighting a clear mobilization of energy reserves in response to both stress situations, not only in order to cope with an increased energy demand but also to recover the homeostatic status (Montero et al., 1999; Vargas-Chacoff et al., 2009; Costas et al., 2011). In a general way, our results showed that the combination of both stressors affects carbohydrate metabolism as denoted by a decrease in hepatic glycogen levels, together with plasma lactate and proteins values, probably related to the increased use of these metabolites as substrate for hepatic gluconeogenesis. In fact, an enhancement in both glycogenolytic and gluconeogenic potentials in the liver, together with a decline in the HSI, has ben previously demonstrated in several teleosts, including *S. aurata* (Vijayan et al., 1990; Soengas et al., 1996; Power et al., 2000; Pottinger et al. 2003; Sangiao-Alvarellos et al., 2005; among others). Interestingly, it is worthy to note a decrease in triglyceride and protein levels in liver and/or plasma, pointing out to an important role of lipid and protein catabolism, in addition to the carbohydrate utilization, to face periods of nutrient deficiency (Mommsen, 2004; Miller et al., 2009). Moreover, the metabolic action observed herein must be controlled by a complex network of physiological processes, in which several hormones from stress, vasotocinergic and isotocinergic pathways could be involved (see below).

Plasma cortisol and hypothalamic *crh* and *crhbp* expression

In teleosts, HPI axis starts with the synthesis and release of Crh at hypothalamic level to finally produce the cortisol hormone in the interrenal cells of the head-kidney (Wendelaar Bonga, 1997, 2011; Flik et al., 2006; Bernier et al., 2009). In this process, the soluble Crhbp can bind with higher affinity for Crh than Crh receptor can, acting as a strong modulator of the required and complex endocrine cascade through a feedback system (Huising et al., 2004; Wunderink et al., 2011). Plasma cortisol levels increased in individuals under both experimental conditions (FD and HSD), confirming an activation of the stress axis by both stressors, in agreement to previous results reported in challenged sea breams under a similar experimental design (Mancera et al., 2008). However, present results showed that HSD was more effective than FD to enhance plasma cortisol levels, since the HSD in fed fish produced a 6-fold increase in this hormone in contrast to the 2-fold increase induced by FD under LSD condition. Moreover, our results also indicated a significant correlation between hypothalamic *crh*, *crhbp* and plasma cortisol levels, demonstrating an important role and interconnection of these endocrine factors in the stress pathway activation. Interestingly, combination of both stressors did not induce an extra enhancement in plasma cortisol levels and *crh* gene expression respect to values observed in the group submitted to HSD, suggesting the existence of a saturation of the stress axis and the activation of feedback mechanisms to avoid a saturation of glucocorticoids in the organism, or even that Crh-producing cells are activated differently by crowding and food restriction as demonstrated in other players of the HPI axis in this species after the combination of two different stressors (Rotllant et al., 2001). This idea can also be understood as a trade-off effect of the HPI action, to counteract the putative synergism with other endocrine and/or metabolic pathways involved against the cumulative stress process produced. This interesting feature has been previously reported in fish, where crowding stress increased cortisol values with a concomitant decrease in growth hormone (Gh) levels (Pérez-Sánchez and Le Bail, 1999), helping to avoid the production of mitochondrial reactive oxygen species (ROS) in excess (Calduch-Giner et al., 2010). Thus, *crhbp* was down-regulated under FD and HSD, although it only was significantly different when both stressors were applied at the same time. According to both maximum *crh* gene expression and cortisol plasma values registered, these synergic results makes us to hypothesize that bioactive Crh could be more available due to the decrease in its binding protein regulator, although its modulation along the combination of the stressors studied could also suggest that Crhbp acts as a factor that increases the half-life of the active Crh in the

organism (Westphal and Seasholtz, 2006). Taken together, our results agree with the idea that *crhbp* can be considered as a strong modulator of the stress response induced by FD or HSD (Huising et al., 2004; Wunderink et al., 2011; Martos-Sitcha et al., 2014b). Even so, the orchestration in the upper levels of the triggered endocrine cascade has also others players involved in the regulation and secretion of the corticotrophin (Acth) cells under chronic stress at hypophyseal level (see above).

Up-stream regulation of the vasotocinergic and isotocinergic pathways

In *Dicentrarchus labrax*, it is confirmed that Avt binding sites are located in the areas occupied by corticotrophin (Acth) cells that controlled cortisol secretion in the interrenal tissue (Moons et al., 1989). Thus, Acth secretion has a greater plasticity due to its synergic regulation by at least two hormones (Avt and Crh) whose expression can occur independently, hypothesizing a cooperation between both endocrine systems (Rivier and Vale, 1983; Bernier et al., 2009; Jerez-Cepa et al., 2016). Although the influence of FD (and re-feeding) on the regulation of hypothalamic Avt and It pathways has been previously assessed in *S. aurata* (Skrzynska et al., 2017), this is the first report on the effects of HSD or the combination of both stressors in these endocrine systems. Our results indicated that FD enhanced plasma cortisol levels (Mancera et al., 2008; Martos-Sitcha et al., 2014b; present results), although an inhibitory effect has also been reported here for *avt* and *it* expression, as well as in previous studies (see Skrzynska et al., 2017). However, plasma Avt and It levels were not changed by FD during 14 days if not accompanied by higher stocking densities (Mancera et al., 2008), whereas 21 days of FD alone did not change plasma Avt or decreased It values (Skrzynska et al., 2017). In addition, previous studies have also showed an increase in hypothalamic *avt* and *it* mRNAs in *S. aurata* after the administration of exogenous cortisol (Cádiz et al., 2015). This reinforces the idea that the stress answer is under control of a complex network of diverse endocrine system (Winberg et al. 2016), where fine interactions depending on the stress source, together with the presence and origin of cortisol (administered or synthesized by the animal itself), are needed to counteract the hyper-saturation of the stress response. In fact, hypothalamic gene expression levels of *avt* and *it* showed a general depletion in stressed fish, which made us to think about the negative feedback produced by high values of plasma Avt, It or cortisol hormones (Mancera et al., 2008; Skrzynska et al., 2017; present results).

In teleosts, including *S. aurata*, Avt and/or It can be involved in metabolic and endocrine changes associated to stressful situations (Sangiao-Alvarellos et al., 2004, 2006; Martos-Sitcha et al., 2013, 2014a; Cadiz et al., 2015), and its control in the first steps of the hormonal cascade at central level must be orchestrated by specific receptors to guarantee a homeostatic load of the endocrine players involved. Our results highlighted that hypothalamic *avtrv1a* expression was up-regulated in fish submitted to HSD, and exacerbated when HSD and FD stressors are combined. These results are reinforcing our idea that Avt can mediate metabolic enhancement or stimulates/modulates the stress axis through the *avtrv1a* receptor (see Cádiz et al., 2015, for review). However, *avtrv1a* expression is influenced by the stressor applied, since animals under salinity challenges did not alter its expression (Martos-Sitcha et al., 2014a), while the administration of exogenous cortisol down-regulated it (Cadiz et al., 20015). Under food deprivation, contradictory results have been obtained in *S. aurata*, since this stressor did not affect (present results at LSD) or increase (present results at HSD; Skrzynska et al., 2017: ~8 $kg \cdot m^{-3}$) its expression, where those differences seem to be greatly influenced by stocking conditions. The same fact could be also plausible for *itr* expression, inasmuch as its levels are clearly influenced depending on the stocking density (Skrzynska et al., 2017; present results). In addition to that, the expression of this type of receptor has been identified in hypophyseal cells (Martos-Sitcha et al., 2014a) where Acth is also produced (Antoni, 1984; Moons et al., 1989). Hypophyseal *avtrv1a* and *itr* expression were found to be down-regulated in stressed fish, which may indicate that the synthesis activation of both neuropeptides in hypothalamic neurons and their storage/release in the pituitary are also finely controlled by these receptors. Thus, a possible role of these receptors in regulating negative feedback on Avt/It secretion, or possibly as a modulator of vasotocin-induced Acth release and activation of the HPI axis, cannot be ruled out (Baker et al., 1996; Engelmann et al., 2004). On the other hand, the absence of changes observed in hypothalamic and hypophyseal mRNA expression levels for *avtrv2* would suggest that this receptor is lesser involved (if any) in metabolic changes and storage/release processes induced by these stress situations, which perfectly agree with previous results obtained in *S. aurata* individuals under food deprivation (Skrzynska et al., 2017).

Local action of Avt and It mediated by its specific receptors in head-kidney and liver

Although little is known about the putative regulation of cortisol production by Avt and It in the interrenal tissue of the head-kidney, recent studies suggest that these hormones could exercise a local control of cortisol dynamics at local level mediated by their specific receptors (Cádiz et al., 2015; Jerez-Cepa et al., 2016). Our results showed non-significant differences in the head-kidney expression of both *avtrv1a* and *itr* genes, suggesting that these receptors are not associated with chronic stress situation due to FD and HSD in this organ. However, decreases in *avtrv2* induced by both stressors (FD and HSD) suggest the existence of some functional relationship of this receptor during the chronic stress situation, possibly reflecting a feedback process mediated by Avt or cortisol hormones in challenged fish (Mancera et al., 2008; Cádiz et al., 2015). However, it is necessary to remember that, in addition to different cell types responsible for several physiological actions (e.g. osmoregulation, immune function), two different stress-related hormone-secreting cells can be found in the interrenal tissue of the head-kidney: i) chromaphin cells, responsible for catecholamines synthesis, and ii) steroidogenic cells, where cortisol is produced (Fierro-Castro et al., 2015). For this reason, specific isolation of different renal cell types and the evaluation of each Avt/It receptor under both FD and HSD conditions, or even under other acute or chronic challenging models, will be necessary in order to establish the role of Avt/It pathways in the stress response.

In particular, the liver is considered the main organ responsible for metabolic supply and energy storage in vertebrates, and its activation during stress situation is well established (Polakof et al., 2012). In *S. aurata*, it has previously been demonstrated that different stress situations (FD, HSD, salinity challenge, exogenous cortisol administration) induced changes in hepatic metabolism to cope the required metabolic demand (Sangiao-Alvarellos et al., 2005; Mancera et al., 2008; Benedito-Palos et al., 2014; Cádiz et al., 2015; Martos-Sitcha et al., 2016). Changes in gene expression for the three studied receptors (*avtrv1a*, *avtrv2* and *itr*) under FD and HSD suggest that vasotocinergic and isotocinergic pathways are involved in metabolic activation to orchestrate the energy needed to survive (see Table 2) (Mommsen et al., 1999; Laiz-Carrión et al., 2012). However, the different pattern of changes observed also suggest specific roles of each Avt and It receptors. In fact, for *avtrv1a*, and possibly also *itr*, their observed changes could be attributed to the partial regulation of hepatic carbohydrate metabolism by the action of both Avt and It (Janssens and Lowrey, 1987; Moon and Mommsen, 1990), playing a role as a reinforcement of the cortisol action against the combination of both stressors, as previously suggested in this fish species (Martos-Sitcha et al., 2014a; Cádiz et al., 2015; Skrzynska et al., 2017). Moreover, the down-regulation of

avtrv2 in fish challenged only with FD (LSD-FD), and its concomitant increase when fish were also kept at higher stocking densities (HSD-FD) makes us to hypothesize a minor role of this receptor in food-deprived fish (Skrzynska et al., 2017), if FD is not accompanied by another stressor. All together it could be interpreted as a compensatory regulator in combination with the stimulated metabolic effect exerted by the cortisol hormone (Laiz-Carrión et al., 2012; Sangiao Alvarellos, 2005) in agreement with the response observed in round goby (*Neogobius melanostomus*) after overcrowding or social stress (Sokołowska et al., 2013).

Conclusions

Our experimental approach demonstrates that both stressors (FD and HSD) induced a chronic stress situation, as indicated by the elevated cortisol levels, the increase in *crh* and the down-regulation in *crhbp* gene expression. Some of the changes in energy metabolism are confirmatory of previous studies (Mancera et al., 2008), although present results also provide new information related to stress orchestration and metabolic adjustments in order to cope with FD, HSD or their combination in different tissues, from those involved upstream in the endocrine cascade till others related to peripheral actions. In addition, results presented herein, together with those previously reported for *S. aurata* (Sangiao-Alvarellos et al., 2006; Mancera et al., 2008; Martos-Sitcha et al., 2013; Cádiz et al., 2015; Skrzynska et al., 2017), further support a complex network of endocrine pathways involved in the stress response, where the contribution of the vasotocinergic and isotocinergic pathways is clearly evidenced. Previous and current studies are focused to underline the physiological action of several players of both vasotocinergic and isotocinergic endocrine systems under different stress models, supporting the potential use of them in a tissue-specific manner as valuable biomarkers.

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Figure legends

Figure 1. Effects on hypothalamic *crh* (A) and *crhbp* (B) mRNA expression levels in *S. aurata* specimens maintained for 21 days under different stocking densities and feeding conditions: (1) fed at low stocking density (LSD-F; 5 $kg \cdot m^{-3}$); (2) fed at high stocking density (HSD-F, 40 $kg \cdot m^{-3}$); (3) food-deprived at LSD (LSD-FD); and (4) food-deprived at HSD (HSD-FD). Values are represented as means ± S.E.M. * represents significant differences at the same stocking density, whereas # represents significant differences at the same feeding condition ($p < 0.05$, two-way ANOVA followed by Tukey's test).

Figure 2. Effects on hypothalamic *avt* (A) and *it* (B) mRNA expression levels in *S. aurata* specimens maintained for 21 days under different stocking densities and feeding conditions. For further details, see the legend in Figure 1.

Figure 3. Effects on hypothalamic *avtrv1a* (A), *avtrv2* (B) and *itr* (C) mRNA expression levels in *S. aurata* specimens maintained for 21 days under different stocking densities and feeding conditions. For further details, see the legend inFigure 1.

Figure 4. Effects on pituitary *avtrv1a* (A), *avtrv2* (B) and *itr* (C) mRNA expression levels in *S. aurata* specimens maintained for 21 days under different stocking densities and feeding conditions. For further details, see the legend in Figure 1.

Figure 5. Effects on head-kidney *avtrv1a* (A), *avtrv2* (B) and *itr* (C) mRNA expression levels in *S. aurata* specimens maintained for 21 days under different stocking densities and feeding conditions. For further details, see the legend in Figure 1.

Figure 6. Effects on hepatic *avtrv1a* (A), *avtrv2* (B) and *itr* (C) mRNA expression levels in *S. aurata* specimens maintained for 21 days under different stocking densities and feeding conditions. For further details, see the legend in Figure 1.

Table legends

Table 1. Specific primers used for the semi-quantitative qPCR expression analysis and sizes of the amplified products.

Table 2. Effects on plasma and liver parameters, as well as on hepatosomatic index (HSI), in gilthead sea breams maintained for 21 days under different stocking densities and feeding conditions. Values are represented as means ± S.E.M., n = 12 fish per group. * Significantly different ($p < 0.05$) at the same stocking density. #, Significantly different ($p < 0.05$) at the same feeding condition. ($p < 0.05$, two-way ANOVA followed by Tukey's test).

Table 1.

Primers	*Nucleotide sequence*	*Primer concentration*	*Amplicon size*
qPCR-*crh*$_{F}$	5'-ATGGAGAGGGGAAGGAGGT-3'	200 nM	176 bp
qPCR-*crh*$_{R}$	5'-ATCTTTGGCGGACTGGAAA-3'		
qPCR-*crhbp*$_{F}$	5'-GCAGCTTCTCCATCATCTACC-3'	200 nM	147 BP
qPCR-*crhbp*$_{R}$	5'-ACGTGTCGATACCGCTTCC-3'		
qPCR-*avt*$_{F}$	5'-AGAGGCTGGGATCAGACAGTGC-3'	200 nM	129 bp
qPCR-*avt*$_{R}$	5'-TCCACACAGTGAGCTGTTTCCG-3'		
qPCR-*it*$_{F}$	5'-GGAGATGACCAAAGCAGCCA-3'	200 nM	151 bp
qPCR- *it*$_{R}$	5'-CAACCATGTGAACTACGACT-3'		
qPCR-a*vtrv1a*$_{F}$	5'-GACAGCCGCAAGTGATCAAG-3'	400 nM	203 bp
qPCR-*avtrv1a*$_{R}$	5'-CCCGACCGCACACCCCCTGGCT-3'		
qPCR-*avtrv2*$_{F}$	5'-ATCACAGTCCTTGCATTGGTG-3'	600 nM	120 bp
qPCR-*avtrv2*$_{R}$	5'-GCACAGGTTGACCATGAACAC-3'		
qPCR-*itr*$_{F}$	5'-GGAGGATCGTTTTAAAGACATGG-3'	400 nM	120 bp
qPCR-*itr*$_{R}$	5'-TGTTGTCTCCCTGTCAGATTTTC-3'		
qPCR-*actb*$_{Fw}$	5'-TCTTCCAGCCATCCTTCCTCG-3'	200 nM	108 bp
qPCR-*actb*$_{Rv}$	5'-TGTTGGCATACAGGTCCTTACGG-3'		

Table 2.

		Plasma		*Liver*	
Parameter	***Density***	***Fed***	***Food-deprived***	***Fed***	***Food-deprived***
Cortisol	*Low*	4.36 ± 1.67	10.06 ± 2.86*	-	-
(ng·mL^{-1})	*High*	25.36 ± 4.32$^{\#}$	25.88 ± 5.16$^{\#}$	-	-
Glucose	*Low*	3.24 ± 0.20	3.21 ± 0.09	6.39 ± 0.38	6.37 ± 0.91
(mM)	*High*	2.89 ± 0.09$^{\#}$	3.25 ± 0.08*	7.19 ± 0.58	5.83 ± 0.46
Lactate	*Low*	2.43 ± 0.18	1.58 ± 0.18*	0.033 ± 0.003	0.032 ± 0.001
(mM)	*High*	1.53 ± 0.13$^{\#}$	1.17 ± 0.12	0.040 ± 0.005	0.027 ± 0.003*
Triglycerides	*Low*	2.74 ± 0.46	1.56 ±0.12*	4.27 ± 0.17	1.88 ± 0.18*
(mM)	*High*	2.20 ± 0.20$^{\#}$	0.95 ± 0.07*$^{\#}$	2.80 ± 0.36	1.92 ± 0.24*$^{\#}$
Proteins	*Low*	36.51 ± 1.49	25.72 ± 1.49*	-	-
(mg·dL^{-1})	*High*	35.29 ± 1.82	27.63± 2.73*	-	-
Glycogen	*Low*	-	-	36.58 ± 1.84	7.16 ± 0.37*
(µmol g^{-1} wet mass)	*High*	-	-	35.00 ± 2.23	5.96 ±2.08*
HSI	*Low*	-	-	1.47 ± 0.06	0.76 ± 0.03*
	High	-	-	1.25 ± 0.05#	0.76 ± 0.04*

Figure 1.

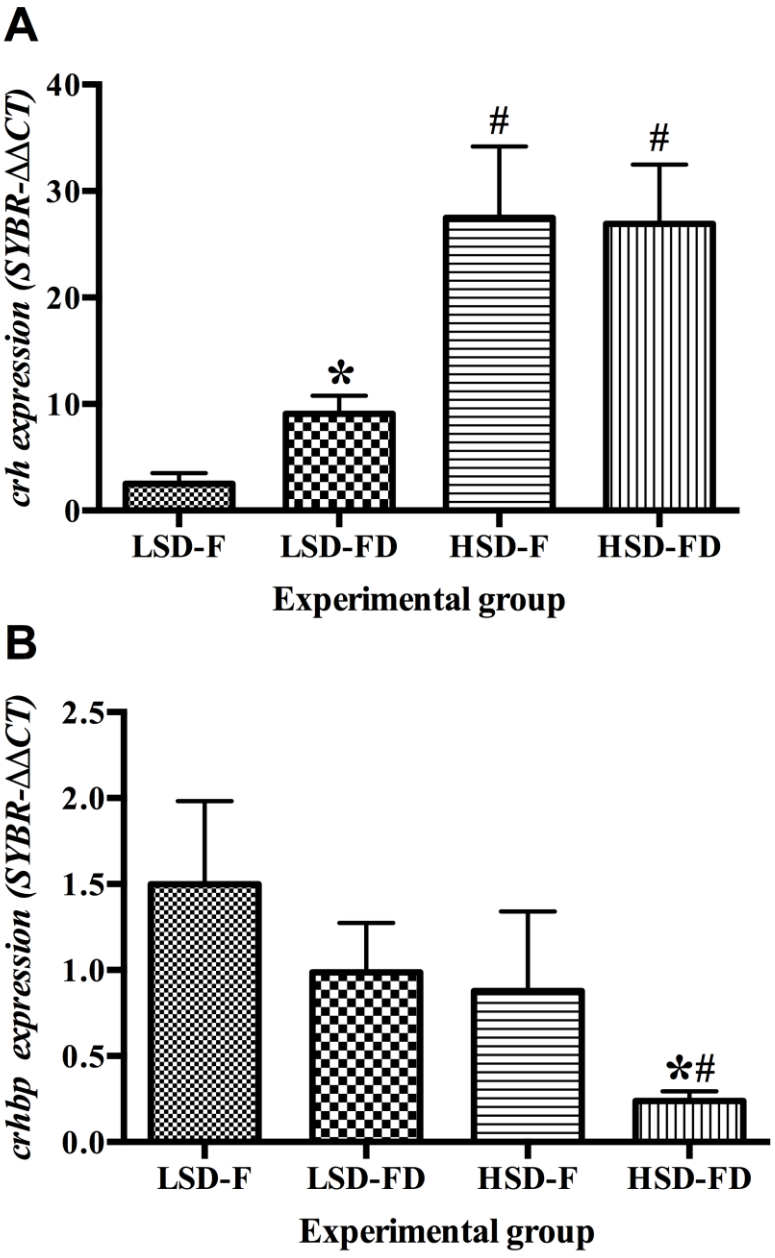

Figure 2.

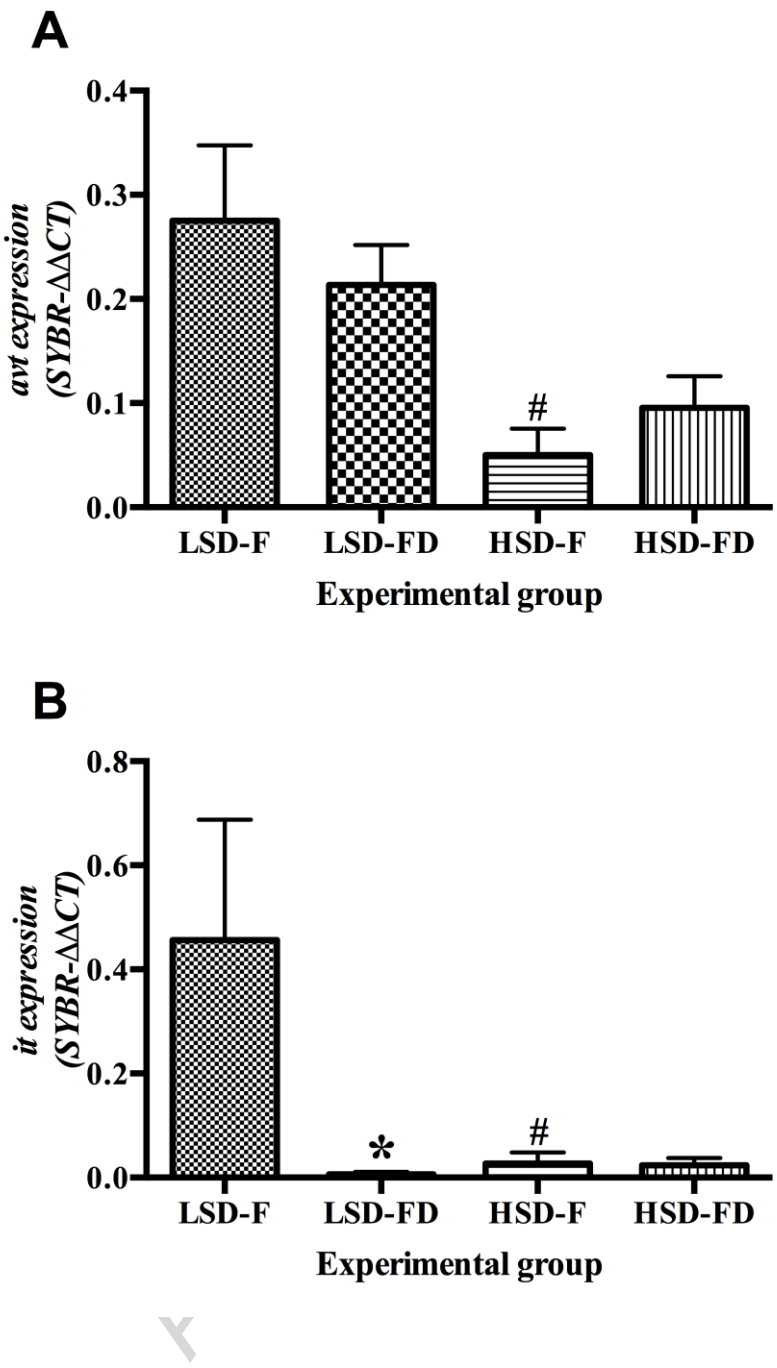

Figure 3.

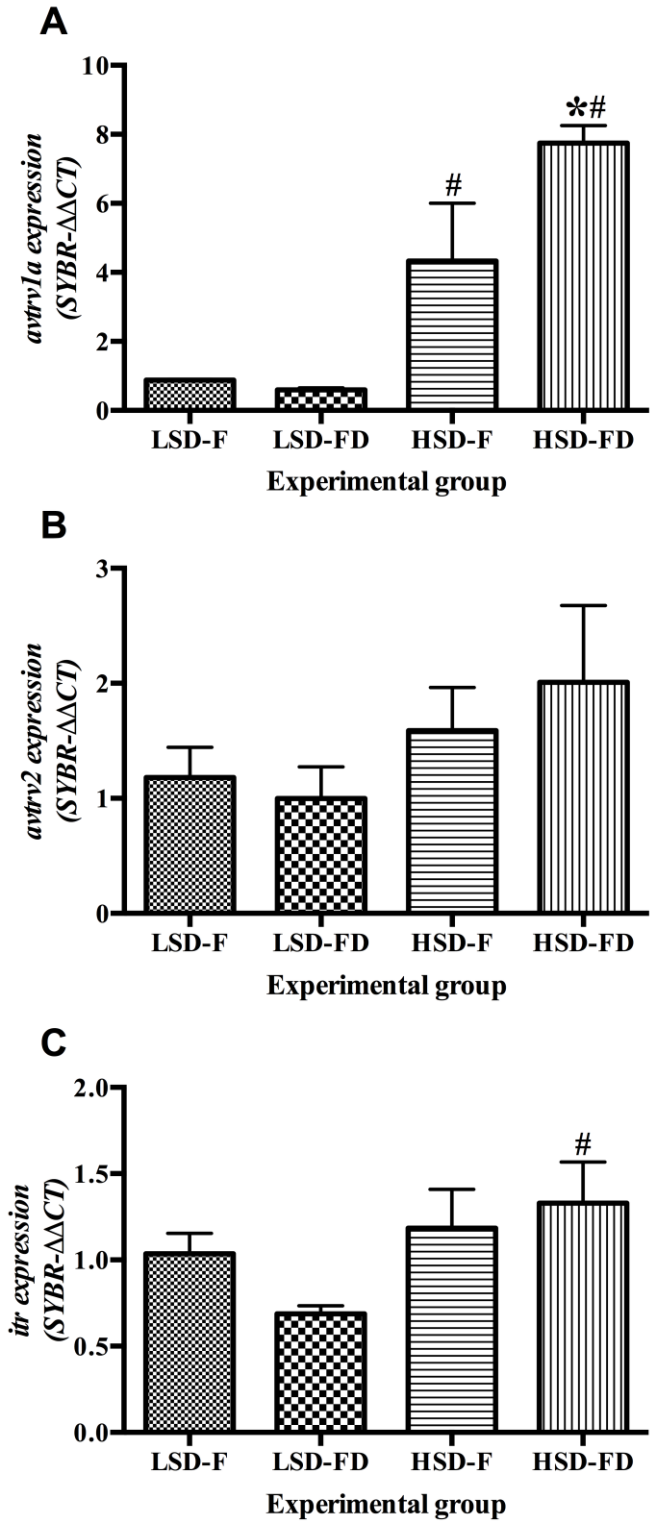

Figure 4.

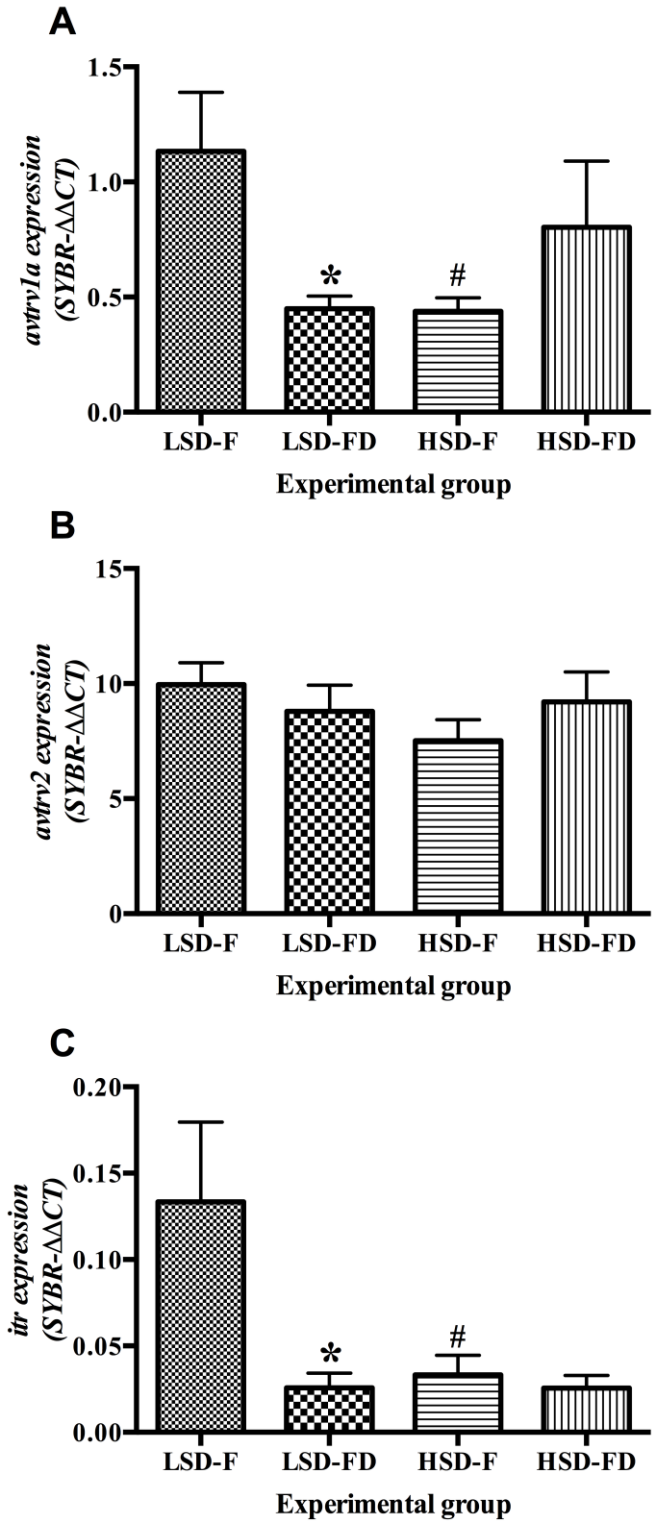

Figure 5.

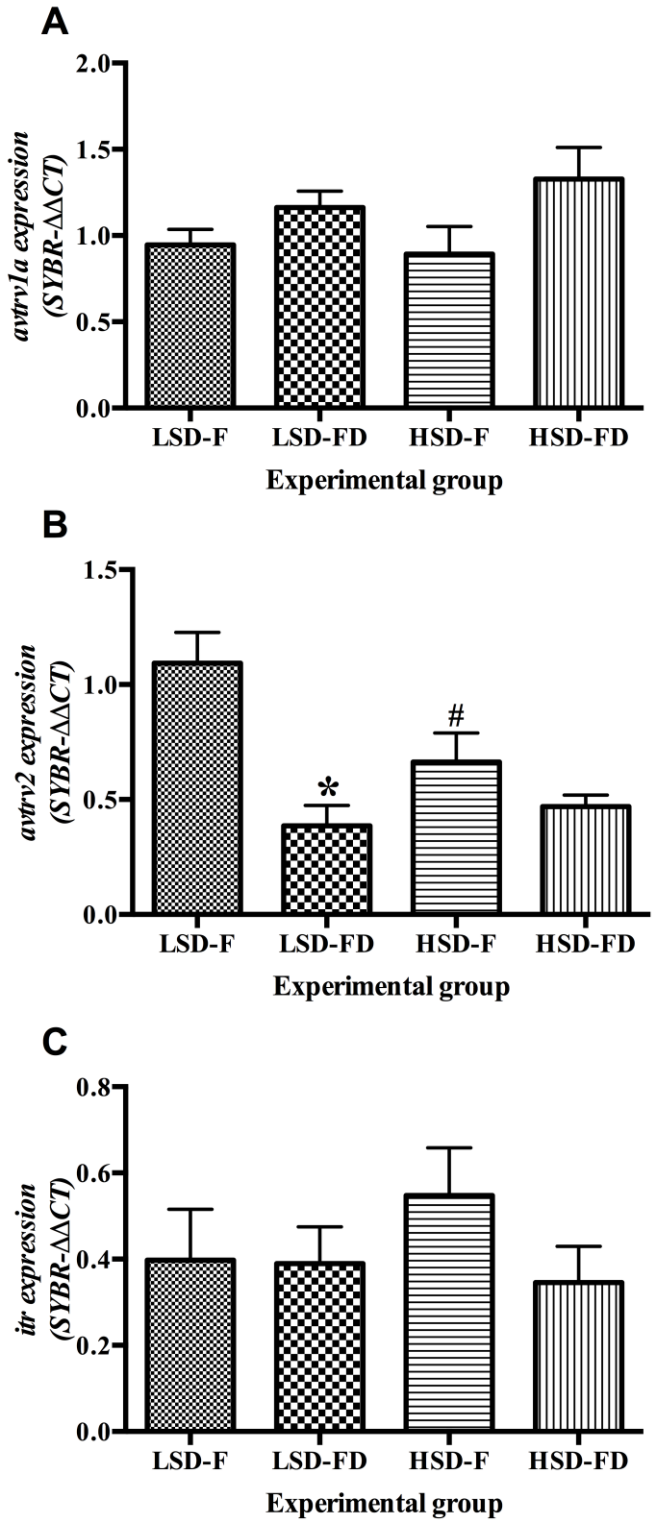

Figure 6.