Food-deprivation induces chronic stress and affects thyroid hormone metabolism in Senegalese sole (*Solea senegalensis*) post-larvae

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Abstract

In vertebrates, stress and thyroid systems interact closely, most likely because of the involvement of both systems in energy metabolism. However, studies on these interactions, especially during larval development, are scarce. Recently, cDNAs coding for corticotropin-releasing hormone (CRH) and CRH-binding protein (CRH-BP), two key players in the regulation of the neuroendocrine stress response, were characterized for the flatfish Senegalese sole (*Solea senegalensis*). To investigate the interactions between stress and thyroid systems in this species, the effects of food deprivation during early development of *S. senegalensis* were assessed. Growth was arrested in food-deprived post-larvae, which was also reflected by decreased carbon and nitrogen contents, indicating increased catabolism. Food deprivation induces chronic stress, as illustrated by enhanced whole-body cortisol levels, as well as an upregulation of *crh* and a decrease of *crh-bp* expression levels. Furthermore, whole-body total T3 concentrations of food-deprived post-larvae were reduced, although *tsh β* subunit expression levels remained unaffected. Our results suggest that food deprivation is a chronic stressor that induces energy-releasing catabolic processes that compensate for the reduced energy intake, and inhibits anabolic processes via the peripheral thyroid system.

Keywords

cortisol, CRH, CRH-BP, starvation, thyroid hormones, TSH, *S. senegalensis*

1. Introduction

It is an obvious fact that the only way in which heterotrophic organisms can take in energy is via food intake. This process, therefore, is extensively regulated, and fasting and starvation induce a number of physiological processes that have evolved to safeguard energy homeostasis in animals (Gorissen et al., 2006; McCue, 2010). Two endocrine systems that are intimately involved in energy metabolism and energy expenditure are the hypothalamo-pituitary-interrenal (HPI) axis and the hypothalamo-pituitary-thyroid (HPT) axis, producing and secreting cortisol and thyroid hormones, respectively. Cortisol is an important glucocorticoid hormone and is involved in the redistribution of energy during a stress response (Aluru and Vijayan, 2007; de Boeck et al., 2001; Wendelaar Bonga, 1997). The potently bioactive thyroid hormone 3,5,3'-triiodothyronine (T3) greatly affects energy expenditure, as its genomic actions generally increase basal metabolic rate in vertebrates (Danforth Jr. and Burger, 1984; Eales, 2006; Yen, 2001). It is very well conceivable that an adequate response of an animal to food deprivation involves concerted, not isolated actions of cortisol and thyroid hormone, being two endocrines basically involved in energy metabolism.

In fishes, depending on the species, the hypothalamic factors thyrotropin-releasing hormone (TRH) and corticotropin-releasing hormone (CRH) can modulate both the HPI- and the HPT axis by stimulating the release of thyroid stimulating hormone (TSH) and/or adrenocorticotrophic hormone (ACTH) from the pituitary, which in turn stimulate their respective target glands to release cortisol and thyroid hormone (Bernier et al., 2009). The biological activity of CRH is further regulated by a soluble binding protein, corticotropin-releasing hormone-binding protein (CRH-BP), which is considered to be a potent modulator of CRH bioactivity (Huising et al., 2004; Huising et al., 2008; Sutton et al., 1995). The dual role of hypothalamic factors in stimulating two endocrine axes confers a central role for the hypothalamus in the orchestration of the response to fasting or starvation. Indeed, in the common carp, *Cyprinus carpio*, central and peripheral interactions between the HPI and HPT axes have already been demonstrated (Geven et al., 2009; Geven et al., 2006). It has been suggested that the TRH neurons in the mammalian hypothalamic paraventricular area (analogous to the teleostean preoptic area) are key in integrating and relaying central and peripheral related to energy homeostasis (Hollenberg, 2008; Lechan and Fekete, 2006), and this could well be the case in teleosts. A similar role can be proposed for CRH (Bernier, 2006; Flik et al., 2006).

In aquaculture, it is important to reach optimal production conditions. This generally implies that a distress-free environment is ideal, since exposure to stressors can lead to allostatic overload (or distress), which negatively affects growth, development and immune functions leading to diseases and reduced animal welfare (Ashley, 2007; Conte, 2004; Ellis et al., 2002). In teleosts, the larval phase is defined by a rapid increase in biomass and changes in morphology, behaviour, and physiology. These aspects depend on the vigour of the individual, abiotic factors such as light, temperature and salinity, and feeding conditions. Moreover, larvae in aquaculture have to adjust to changes in feeding regime such as the transition of live to inert food. In some species, the HPI-axis is already active in early life stages (Stouthart et al., 1998). Therefore, exposure to stressors during early development can induce chronic stress and disrupt thyroid function which can have profound effects on development and later life stages (Flik et al., 2006).

The Senegalese sole (*Solea senegalensis* Kaup) is a flatfish with high economic value in North Africa and the Iberian Peninsula, and is cultured at a commercial scale (Dinis et al., 1999; Imsland et al., 2003). In the last few years, several studies on *S. senegalensis* juveniles have focused on changes in thyroid and stress systems related to various physiological processes of this species (Arjona et al., 2008; Costas et al., 2011b; Ponce et al., 2010; Salas-Leiton et al., 2010), including metabolic and thyroidal changes imposed by fasting in juveniles (Arjona et al., 2010; Costas et al., 2011a). In addition, changes in metabolism and endocrine function during larval development have been assessed in this species (Fernández-Díaz et al., 2001; Gamboa-Delgado et al., 2011; Klaren et al., 2008; Yúfera et al., 1999).

Recently, we characterized the cDNAs coding for *S. senegalensis* CRH and CRH-BP peptides, obtaining new molecular tools to study the endocrine stress response and interaction with the thyroid system (Wunderink et al., 2011). We here describe the effects of food deprivation on the stress and thyroid axes during early development of *S. senegalensis*. Understanding the genetic and physiological mechanisms involved in the interactions between these axes could help to improve larval resistance and growth in this species.

2. Material and Methods

2.1. Animals and experimental design

S. senegalensis larvae were obtained from the Centro Oceanográfico de Santander, Instituto Español de Oceanografía (Santander, Spain) on the 9th of May 2008 and were transported to the laboratories of the Instituto de Ciencias Marinas de Andalucía, CSIC (Puerto Real, Cádiz, Spain). Larvae were transferred to two 300-L tanks containing seawater (33 ppt salinity) and kept at an initial density of 60-70 larvae per litre. Larval culture was similar as previously described (Klaren et al., 2008) and was as following: a continuous water flow of 0.3-0.6 L/min and a 12 h light : 12 h dark photoperiod were maintained. At an age of 3 days post hatching (dph), larvae were fed on rotifers (*Brachionus plicatilis* O.F. Müller 1786), strain S-1 (size range: 120-300 µm; (Yúfera, 1982), at a prey density of 5-10 individuals/mL; and on freshly hatched *Artemia* nauplii from 6 dph onwards. Microalgae (*Nannochloropsis gaditana*) at a final concentration of $3 \cdot 10^5$ cells/mL, were also added to the rearing tanks from first feeding.

Post-larvae of 50 dph (June-July 2008) were divided in two groups, one fed (control) and the other food-deprived during 12 days, that were kept in triplicate 300-L tanks, under the same conditions as described above. Samples were taken from each tank at 3, 6, 9 and 12 days after starting the food deprivation period. To determine dry weight, and carbon and nitrogen contents of tissues, post-larvae ($n = 20$ per tank) were anaesthetized, rinsed with distilled water and stored at -20 °C until further analysis. Post-larvae were rinsed with distilled water, adhering water was removed with a tissue, and post-larvae were pooled to obtain a total sample weight of 0.25 – 0.50 g for whole-body total thyroid hormone analysis, and 0.1 – 0.5 g for cortisol analysis, respectively. All samples were immediately frozen in liquid nitrogen and stored at -80 °C until further analysis. For the purpose of RNA isolation, post-larvae ($n = 9$) were rinsed with distilled water and put in a five-fold volume of RNeasyTM RNA stabilization solution (Ambion®) for 24 h at 4 °C and then stored at -20 °C until further analysis. All experimental procedures complied with the Guidelines of the European Union (2010/63/EU) and Spanish legislation (RD 1201/2005 and law 32/2007) for the use of laboratory animals.

2.2. Dry weight and chemical analyses

Dry weight of post-larvae was determined on freeze-dried individuals ($n = 7-9$ per sample per tank) using a microbalance Mettler Toledo XP2U with 0.1 µg precision. Carbon (C, reflecting the content of lipids and carbohydrates) and nitrogen (N, reflecting protein content) contents were determined with an elemental analyzer (Thermoquest, mod. Flash 1112), using sulphanilamide as

a standard. Samples were lyophilized and homogenized. Each sample, consisting of a pool of 7 post-larvae comprising 1 mg of dry biomass per measurement, was measured in triplicate. Before measurement, samples were pre-incubated during 30 min at 50 °C to remove residual humidity.

2.3. Cortisol extraction and analysis

Cortisol was extracted as previously described (Hiroi et al., 1997). Samples (0.1 – 0.5 g wet weight) were homogenized in five-fold volume of ice-cold phosphate-buffered saline (PBS, 0.01 M, pH 7.3). The homogenate (300 µL) was extracted twice by mixing thoroughly with 3 mL diethyl ether for 2 min and put on dry ice afterwards. The unfrozen diether layer was collected, pooled and evaporated at room temperature. The residue was resuspended in 300 µL tetrachloromethane and mixed for 4 min. Then, 300 µL PBS containing 0.1% gelatine was added, mixed for 2 min and centrifuged at 1550 g (3000 rpm in a Heraeus type 3360 rotor) for 10 min at 4 °C. The aqueous layer was aspirated and aliquots of 10 µL were taken for use in radioimmunoassay (RIA) as previously described (Metz et al., 2005).

2.4. Thyroid hormone extraction and analysis

Thyroid hormones were extracted as previously described (Klaren et al., 2008; Tagawa and Hirano, 1987). Samples (0.25 – 0.50 g wet weight) were homogenized in 2.5 mL ice-cold 99:1 (v/v) methanol:ammonia containing 1 mM of 6-*n*-propyl-2-thiouracil (PTU). Homogenate and extraction media were thoroughly mixed for 10 min on ice, and then centrifuged at 2000 g (15 min, 4 °C). This procedure was repeated twice, supernatants were pooled and lyophilized. The residue was resuspended in 875 µL of a 6:1 (v/v) mixture of chloroform and 99:1 methanol:ammonia containing 1 mM PTU, and 125 µL barbital buffer (50 mM sodium barbital in distilled water, pH 8.6). Samples were mixed for 10 min, the upper phase was aspirated and lyophilized. Residues were redissolved in 175 µL barbital buffer containing 0.1 % bovine serum albumin. Aliquots of 25 and 50 µL were taken for T4 and T3 analysis, respectively. Total T4 (tT4) and T3 (tT3) concentrations were measured in duplicate with a competitive ELISA (Human Gesellschaft für Biochemica und Diagnostica GmbH, Wiesbaden, Germany) previously validated (Klaren et al., 2008).

2.5. RNA extraction and cDNA synthesis

Total RNA was extracted using the commercial kit RNeasy® (Qiagen) according to the manufacturer's instructions. Incubation with RNase-free DNase I (Qiagen) eliminated potential genomic DNA contamination. RNA concentrations were measured by spectrophotometry and the quality of RNA was assessed using the Agilent RNA 6000 Pico or Nano Assay Kits on an Agilent 2100 Bioanalyzer (Agilent Technologies). First-strand cDNA synthesis was carried out using the qScript™ cDNA Synthesis kit (Quanta BioSciences).

2.6. Real-time quantitative PCR

Primers for use in real-time quantitative PCR (RQ-PCR) were designed for *S. senegalensis crh*, *crh-bp* and *tshβ* subunit (accession numbers: FR745427, FR745428 and AB297482, respectively) by use of the Primer3 software (v. 0.4.0.) available at <http://frodo.wi.mit.edu/primer3/>, accessed in May 2011 (Rozen and Skaletsky, 2000). Primer oligonucleotide sequences are shown in Table 1. To perform RQ-PCR reactions, 8 μL cDNA, forward and reverse primers (200nM each) and 10 μL PerfeCta™ SYBR® Green Fastmix™ (Quanta BioSciences) were used. RQ-PCR (10 min at 95 °C, 40 cycles of 30 s at 95 °C and 45 s at 60 °C) was performed on a Mastercycler® EPgradient S RealPlex2 (Eppendorf). *S. senegalensis β-actin* (accession number: DQ485686) was used as an internal control amplicon and a calibrator sample was measured on every RQ-PCR plate to correct for inter-assay differences. PCR amplification efficiencies were similar for all genes. Expression levels *R* were calculated with the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001).

2.7. Statistical analyses

Data were statistically analyzed by one-way ANOVA or Kruskal-Wallis one-way ANOVA on ranks followed by a Bonferroni post-hoc test where appropriate. Differences between two treatments at same sample point were tested using Student's *t*-test for unpaired data. Statistical significance was accepted at $P < 0.05$.

3. Results

Post-larvae from the control group increased in weight over time, whereas food-deprived postlarvae did not grow and showed a significantly lower body weight (Fig. 1). Biomass quality, assessed by the dry mass content of carbon and nitrogen, changed upon food deprivation (Table 2). C content significantly increased in fed post-larvae, whereas that of food-deprived post-larvae decreased. N content also diminished in food-deprived post-larvae, though only significantly at 12 days of food deprivation. Consequently, the C to N ratio (C/N) was lower in food-deprived post-larvae compared to control group (Table 2).

Whole-body cortisol values were significantly elevated in post-larvae that did not receive food for 12 days (Fig. 2). Whole-body levels of $tT4$ were not affected (Fig. 3A), whereas those of $tT3$ were significantly lower in food-deprived post-larvae (Fig. 3B). Food deprivation significantly upregulated *crh* expression (Fig. 4A), downregulated *crh-bp* expression (Fig. 4B), but did not affect *tsh β* subunit expression (Fig. 4C).

4. Discussion

Our study shows that food deprivation has pronounced effects on stress and thyroid axis activities in *S. senegalensis* post-larvae. The increases in body weight and C content in fed postlarvae suggest that during early growth fed post-larvae accumulated reserves in the form of lipids and/or carbohydrates. These are necessary to fuel developmental processes, *e.g.* the body transformation that starts approximately occurring between 10 to 20 dph (Fernández-Díaz et al., 2001; Yúfera et al., 1999). Post-larvae stopped growing when deprived of food, and their poor nutritional status was reflected by a decrease in the body content of C and N, indicating a direct start of catabolism of carbohydrates and proteins, from tissues to release energy. A C/N ratio between 3.76 and 4.00 seems to be common during larval development in marine species (Tucker Jr, 1989; Yúfera et al., 1993), though larger values have been reported for *S. senegalensis*, indicating a higher energy content in the biomass of this species (Gamboa-Delgado et al., 2011; Yúfera et al., 1999). However, the low C/N ratio in food-deprived post-larvae compared to control group suggests that the biomass of food-deprived post-larvae has a lower energy content.

Reports on the effects of food deprivation on stress in adult fish and their interaction with thyroid hormone are scarce. Elevated whole-body cortisol concentrations were found in zebrafish as a result of crowding and food deprivation (Ramsay et al., 2006), and reduced stress resistance was demonstrated in food-deprived Atlantic cod (*Gadus morhua*) (Olsen et al., 2008). Similarly, food-deprived *S. senegalensis* juveniles significantly enhanced plasma cortisol levels (Costas et al., 2011a). Thus far, the effects of food deprivation on the HPI-axis have not been established during early development. In food-deprived *S. senegalensis* post-larvae *crh* expression is upregulated but *crh-bp* expression is downregulated, resulting in a net increase in whole-body cortisol levels. These changes of the HPI-axis are typical of a chronic stress situation as it has been shown in a previous study on *S. senegalensis* juveniles (Wunderink et al., 2011). We can thus assume that food deprivation already for a few days is a chronic stressor for *S. senegalensis* postlarvae.

Compared to control group, food-deprived post-larvae showed lower whole-body total T3 concentrations. A decrease in thyroid hormone levels has also been shown for mammals, birds, and fish species like tilapia (*Oreochromis niloticus*), rainbow trout (*Oncorhynchus mykiss*) and sea bream (*Sparus aurata*) submitted to starvation (Darras, 1995; Leatherland and Farbridge, 1992; Power et al., 2000; Van der Geyten et al., 1998). The observations that *crh* expression increased and that of *crh-bp* decreased, whereas after food deprivation T3 levels decreased,

indicates that CRH most likely does not function as a TSH-releasing factor in Senegalese sole. It is interesting to note that the expression of *tsh β* subunit was not affected, suggesting that the drop in T3 levels is strongly regulated extrathyroidally, probably by changes in peripheral deiodinase activities. These enzymes catalyze the removal of iodine atoms from the T4 prohormone resulting in the bioactive hormone T3 by outer-ring deiodination (ORD), or producing the inactive metabolite reverse T3 (rT3) by inner-ring deiodination (IRD). Furthermore, the IRD pathway can also inactivate T3 by catalyzing the conversion of T3 to diiodothyronine (3,3'-T2). In teleosts, three types of deiodinases have been characterized, *dio1* (coding for Dio1, catalyzing both ORD and IRD), *dio2* (coding for Dio2, catalyzing ORD only) and *dio3* (coding for Dio3, catalyzing IRD only) (Isorna et al., 2009; Orozco and Valverde, 2005). Indeed, a decrease in plasma T4 and T3 levels in fasted tilapia was caused by decreased hepatic Dio2 activity, and thus implying a decrease in T4-to-T3 conversion (Van der Geyten et al., 1998), and likely Dio2 activity had decreased in the food deprived *S. senegalensis* post-larvae of this study as well.

The interaction between stress and thyroid systems during larval development is poorly understood. In juveniles, it is known that thyroid hormones can inhibit the HPI-axis as has been shown for common carp (*Cyprinus carpio*), in which T4 induced an upregulation of *crh-bp* expression in the hypothalamus (Geven et al., 2006). On the other hand, contradictory results are found on the effects of cortisol on the thyroid system: stimulation of hepatic *in vitro* conversion of T4 to T3 in brook charr (*Salvelinus fontinalis*) (Vijayan et al., 1988), enhancement of T4 release by renal tissues in common carp (*C. carpio*) (Geven et al., 2009; Vijayan et al., 1988), reduction in plasma T3 levels in coho salmon (*Oncorhynchus kisutch*), rainbow trout (*O. mykiss*), and in Nile tilapia (*Oreochromis niloticus*) (Brown et al., 1991; Redding et al., 1984), and a reduction of plasma fT4 levels in Senegalese sole (Arjona et al., 2011). In addition, the administration of the synthetic glucocorticoid dexamethasone, that mimics cortisol action, decreased hepatic Dio1 and Dio2 activities in Nile tilapia (Walpita et al., 2007). The overall function of a stress response is to prepare the organism to cope with the stressor, and to this end energy is redistributed by mobilizing glucose and fatty acids, and the catabolism of proteins and fat to increase gluconeogenesis (Kühn et al., 1998; Wendelaar Bonga, 1997). Thyroid hormones, on the other hand, are more involved in anabolic processes, like growth, and react strongly on changes in nutritional status (Eales, 2006; Van der Geyten et al., 1998). Therefore, it is plausible that the stress system inhibits these anabolic processes through inhibiting the thyroid system,

which could explain the responsiveness of thyroid hormones to stress caused by food deprivation.

In conclusion, food deprivation induces chronic stress, as indicated by elevated cortisol levels, enhanced *crh* expression and down-regulation of *crh-bp* expression in *S. senegalensis* post-larvae. In addition, total T3 concentrations were reduced in food-deprived post-larvae. Our results confirm the hypothesis that chronic stress, *viz.* food deprivation, induces catabolic processes to produce energy to cope with a stressor, and inhibits anabolic processes through inhibiting the thyroid system.

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References

- Aluru, N., Vijayan, M.M., 2007. Hepatic transcriptome response to glucocorticoid receptor activation in rainbow trout. *Physiol. Gen.* 31, 483-491.
- Arjona, F.J., Ruiz-Jarabo, I., Vargas-Chacoff, L., Martín del Río, M.P., Flik, G., Mancera, J.M., Klaren, P.H.M., 2010. Acclimation of *Solea senegalensis* to different ambient temperatures: Implications for thyroidal status and osmoregulation. *Mar. Biol.* 157, 1325-1335.
- Arjona, F.J., Vargas-Chacoff, L., Martín del Río, M.P., Flik, G., Mancera, J.M., Klaren, P.H.M., 2008. The involvement of thyroid hormones and cortisol in the osmotic acclimation of *Solea senegalensis*. *Gen. Comp. Endocrinol.* 155, 796-803.
- Arjona, F.J., Vargas-Chacoff, L., Martín Del Río, M.P., Flik, G., Mancera, J.M., Klaren, P.H.M., 2011. Effects of cortisol and thyroid hormone on peripheral outer ring deiodination and osmoregulatory parameters in the Senegalese sole (*Solea senegalensis*). *J. Endocrinol.* 208,
- Ashley, P.J., 2007. Fish welfare: Current issues in aquaculture. *Appl. Anim. Behav. Sci.* 104, 199-235.
- Bernier, N.J., 2006. The corticotropin-releasing factor system as a mediator of the appetite suppressing effects of stress in fish. *Gen. Comp. Endocrinol.* 146, 45-55.
- Bernier, N.J., Flik, G., Klaren, P.H.M., 2009. Regulation and contribution of the corticotropic, melanotropic and thyrotropic axes to the stress response in fishes, in: N.J. Bernier, G. van der Kraak, A.P. Farrell, C.J. Brauner (Eds.), *Fish Physiology*. NY: Academic Press, New York, 235-311.
- Brown, S.B., MacLatchy, D.L., Hara, T.J., Eales, J.G., 1991. Effects of cortisol on aspects of 3,5,3'-triiodo-L-thyronine metabolism in rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.* 81, 207-216.
- Conte, F.S., 2004. Stress and the welfare of cultured fish. *Appl. Anim. Behav. Sci.* 86, 205-223.
- Costas, B., Aragão, C., Ruiz-Jarabo, I., Vargas-Chacoff, L., Arjona, F.J., Dinis, M.T., Mancera, J.M., Conceição, L.E.C., 2011a. Feed deprivation in Senegalese sole (*Solea senegalensis* Kaup, 1858) juveniles: Effects on blood plasma metabolites and free amino acid levels. *Fish Physiol. Biochem.* 37, 495-504.
- Costas, B., Conceição, L.E.C., Aragão, C., Martos, J.A., Ruiz-Jarabo, I., Mancera, J.M., Afonso, A., 2011b. Physiological responses of Senegalese sole (*Solea senegalensis* Kaup, 1858) after stress challenge: Effects on non-specific immune parameters, plasma free amino acids and energy metabolism. *Aquaculture* 316, 68-76.
- Danforth Jr., E., Burger, A., 1984. The role of thyroid hormones in the control of energy expenditure. *Clin. Endocrinol. Metabol.* 13, 581-595.
- Darras, V.M., 1995. Partial food restriction increases hepatic inner ring deiodinating activity in the chicken and the rat. *Gen. Comp. Endocrinol.* 100, 334-338.

341 de Boeck, G., Alsop, D., Wood, C., 2001. Cortisol effects on aerobic and anaerobic metabolism,
 342 nitrogen excretion, and whole-body composition in juvenile rainbow trout. *Physiol. Biochem. Zool.*
 343 74, 858-868.

344 Dinis, M.T., Ribeiro, L., Soares, F., Sarasquete, C., 1999. A review on the cultivation potential of
 345 *Solea senegalensis* in Spain and in Portugal. *Aquaculture* 176, 27-38.

346 Eales, J.G., 2006. Modes of action and physiological effects of thyroid hormones in fish, in: M.
 347 Reinecke, G. Zaccone, B.G. Kapoor (Eds.), *Fish Endocrinology*. Science Publishers, Enfield (NH),
 348 767-808.

349 Ellis, T., North, B., Scott, A.P., Bromage, N.R., Porter, M., Gadd, D., 2002. The relationships
 350 between stocking density and welfare in farmed rainbow trout. *J. Fish Biol.* 61, 493-531.

351 Fernández-Díaz, C., Yúfera, M., Cañavate, J.P., Moyano, F.J., Alarcón, F.J., Díaz, M., 2001. Growth
 352 and physiological changes during metamorphosis of Senegal sole reared in the laboratory. *J. Fish Biol.*
 353 58, 1086-1097.

354 Flik, G., Klaren, P.H.M., van den Burg, E.H., Metz, J.R., Huising, M.O., 2006. CRF and stress in fish.
 355 *Gen. Comp. Endocrinol.* 146, 36-44.

356 Gamboa-Delgado, J., Le Vay, L., Fernández-Díaz, C., Cañavate, P., Ponce, M., Zerolo, R., Manchado,
 357 M., 2011. Effect of different diets on proteolytic enzyme activity, trypsinogen gene expression and
 358 dietary carbon assimilation in Senegalese sole (*Solea senegalensis*) larvae. *Comp. Biochem. Physiol.*
 359 B 158, 251-258.

360 Geven, E.J.W., Flik, G., Klaren, P.H.M., 2009. Central and peripheral integration of interrenal and
 361 thyroid axes signals in common carp (*Cyprinus carpio* L.). *J. Endocrinol.* 200, 117-123.

362 Geven, E.J.W., Verkaar, F., Flik, G., Klaren, P.H.M., 2006. Experimental hyperthyroidism and central
 363 mediators of stress axis and thyroid axis activity in common carp (*Cyprinus carpio* L.). *J. Mol.*
 364 *Endocrinol.* 37, 443-452.

365 Gorissen, M.H.A.G., Flik, G., Huising, M.O., 2006. Peptides and proteins regulating food intake: a
 366 comparative view. *Anim. Biol.* 56, 447-473.

367 Hiroi, J., Sakakura, Y., Tagawa, M., Seikai, T., Tanaka, M., 1997. Developmental changes in low
 368 salinity tolerance and responses of prolactin, cortisol and thyroid hormones to low-salinity
 369 environment in larvae and juveniles of Japanese flounder, *Paralichthys olivaceus*. *Zool. Sci.* 14, 987-
 370 992.

371 Hollenberg, A.N., 2008. The role of the thyrotropin-releasing hormone (TRH) neuron as a metabolic
 372 sensor. *Thyroid* 18, 131-139.

373 Huising, M.O., Metz, J.R., van Schooten, C., Taverne-Thiele, A.J., Hermesen, T., Verburg-van
 374 Kemenade, B.M., Flik, G., 2004. Structural characterisation of a cyprinid (*Cyprinus carpio* L.) CRH,
 375 CRH-BP and CRH-R1, and the role of these proteins in the acute stress response. *J. Mol. Endocrinol.*
 376 32, 627-648.

377 Huising, M.O., Vaughan, J.M., Shah, S.H., Grillot, K.L., Donaldson, C.J., Rivier, J., Flik, G., Vale,
 378 W.W., 2008. Residues of corticotropin releasing factor-binding protein (CRF-BP) that selectively
 379 abrogate binding to CRF but not to urocortin 1. *J. Biol. Chem.* 283, 8902-8912.

380 Imsland, A.K., Foss, A., Conceição, L.E.C., Dinis, M.T., Delbare, D., Schram, E., Kamstra, A., Rema,
 381 P., White, P., 2003. A review of the culture potential of *Solea solea* and *S. senegalensis* *Rev. Fish*
 382 *Biol. Fisher.* 13, 379-407.

383 Isorna, E., Obregon, M.J., Calvo, R.M., Vázquez, R., Pendón, C., Falcón, J., Muñoz-Cueto, J., 2009.
 384 Iodothyronine deiodinases and thyroid hormone receptors regulation during flatfish (*Solea*
 385 *senegalensis*) metamorphosis. *J. Exp. Zool. B* 312, 231-246.

386 Klaren, P.H.M., Wunderink, Y.S., Yúfera, M., Mancera, J.M., Flik, G., 2008. The thyroid gland and
 387 thyroid hormones in Senegalese sole (*Solea senegalensis*) during early development and
 388 metamorphosis. *Gen. Comp. Endocrinol.* 155, 686-694.

389 Kühn, E.R., Geris, K.L., Van Der Geyten, S., Mol, K.A., Darras, V.M., 1998. Inhibition and
 390 activation of the thyroidal axis by the adrenal axis in vertebrates. *Comp. Biochem. Physiol. - A* 120,
 391 169-174.

392 Leatherland, J.F., Farbridge, K.J., 1992. Chronic fasting reduces the response of the thyroid to growth
 393 hormone and TSH, and alters the growth hormone-related changes in hepatic 5'- monodeiodinase
 394 activity in rainbow trout, *Oncorhynchus mykiss*. *Gen. Comp. Endocrinol.* 87, 342-353.

395 Lechan, R.M., Fekete, C., 2006. The TRH neuron: a hypothalamic integrator of metabolism. *Prog.*
 396 *Brain Res.* 153, 209-235.

397 Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using Real-Time
 398 Quantitative PCR and the 2- $\Delta\Delta C_t$ method. *Methods* 25, 402-408.

399 McCue, M.D., 2010. Starvation physiology: Reviewing the different strategies animals use to survive a
 400 common challenge. *Comp. Biochem. Physiol. A* 156, 1-18.

401 Metz, J.R., Geven, E.J.W., van den Burg, E.H., Flik, G., 2005. ACTH, α -MSH, and control of cortisol
 402 release: Cloning, sequencing, and functional expression of the melanocortin-2 and melanocortin-5
 403 receptor in *Cyprinus carpio*. *Am. J. Physiol.* 289, 814-826.

404 Olsen, R.E., Sundell, K., Ringø, E., Myklebust, R., Hemre, G.I., Hansen, T., Karlsen, Ø., 2008. The
 405 acute stress response in fed and food deprived Atlantic cod, *Gadus morhua* L. *Aquaculture* 280, 232-
 406 241.

407 Orozco, A., Valverde, R., 2005. Thyroid hormone deiodination in fish. *Thyroid* 15, 799-813. Ponce,
 408 M., Infante, C., Manchado, M., 2010. Molecular characterization and gene expression of thyrotropin
 409 receptor (TSHR) and a truncated TSHR-like in Senegalese sole. *Gen. Comp. Endocrinol.* 168, 431-
 410 439.

411 Power, D.M., Melo, J., Santos, C.R.A., 2000. The effect of food deprivation and refeeding on the
 412 liver, thyroid hormones and transthyretin in sea bream. *J. Fish Biol.* 56, 374-387.

413 Ramsay, J.M., Feist, G.W., Varga, Z.M., Westerfield, M., Kent, M.L., Schreck, C.B., 2006. Wholebody
414 cortisol is an indicator of crowding stress in adult zebrafish, *Danio rerio*. *Aquaculture* 258, 565-574.

415 Redding, J.M., Schreck, C.B., Birks, E.K., Ewing, R.D., 1984. Cortisol and its effects on plasma
416 thyroid hormone and electrolyte concentrations in fresh water and during seawater acclimation in
417 yearling coho salmon, *Oncorhynchus kisutch*. *Gen. Comp. Endocrinol.* 56, 146-155.

418 Rozen, S., Skaletsky, H., 2000. Primer3 on the WWW for general users and for biologist
419 programmers. *Method. Mol. Biol.* (Clifton, N.J.) 132, 365-386.

420 Salas-Leiton, E., Anguis, V., Martín-Antonio, B., Crespo, D., Planas, J.V., Infante, C., Cañavate, J.P.,
421 Machado, M., 2010. Effects of stocking density and feed ration on growth and gene expression in
422 the Senegalese sole (*Solea senegalensis*): Potential effects on the immune response. *Fish Shellfish*
423 *Immun.* 28, 296-302.

424 Stouthart, A.J.H.X., Lucassen, E.C.H.E.T., van Strien, F.J.C., Balm, P.H.M., Lock, R.A.C., Wendelaar
425 Bonga, S.E., 1998. Stress responsiveness of the pituitary-interrenal axis during early life stages of
426 common carp (*Cyprinus carpio*). *J. Endocrinol.* 157, 127-137.

427 Sutton, S.W., Behan, D.P., Lahrichi, S.L., Kaiser, R., Corrigan, A., Lowry, P., Potter, E., Perrin, M.H.,
428 Rivier, J., Vale, W.W., 1995. Ligand requirements of the human corticotropin-releasing factor-binding
429 protein. *Endocrinology* 136, 1097-1102.

430 Tagawa, M., Hirano, T., 1987. Presence of thyroxine in eggs and changes in its content during early
431 development of chum salmon, *Oncorhynchus keta*. *Gen. Comp. Endocrinol.* 68, 129-135.

432 Tucker Jr, J.W., 1989. Energy utilization in bay anchovy, *Anchoa mitchilli*, and black sea bass,
433 *Centropristis striata striata*, eggs and larvae. *Fish. Bul.* 87, 279-293.

434 Van der Geyten, S., Mol, K.A., Pluymers, W., Kühn, E.R., Darras, V.M., 1998. Changes in plasma T3
435 during fasting/refeeding in tilapia (*Oreochromis niloticus*) are mainly regulated through changes in
436 hepatic type II iodothyronine deiodinase. *Fish Physiol. Biochem.* 19, 135-143.

437 Vijayan, M.M., Flett, P.A., Leatherland, J.F., 1988. Effect of cortisol on the in vitro hepatic
438 conversion of thyroxine to triiodothyronine in brook charr (*Salvelinus fontinalis mitchill*). *Gen.*
439 *Comp. Endocrinol.* 70, 312-318.

440 Walpita, C.N., Grommen, S.V.H., Darras, V.M., Van der Geyten, S., 2007. The influence of stress on
441 thyroid hormone production and peripheral deiodination in the Nile tilapia (*Oreochromis niloticus*).
442 *Gen. Comp. Endocrinol.* 150, 18-25.

443 Wendelaar Bonga, S.E., 1997. The stress response in fish. *Physiol. Rev.* 77, 591-625.

444 Wunderink, Y.S., Engels, S., Halm, S., Yúfera, M., Martínez-Rodríguez, G., Flik, G., Klaren, P.H.M.,
445 Mancera, J.M., 2011. Chronic and acute stress responses in Senegalese sole (*Solea senegalensis*): The
446 involvement of cortisol, CRH and CRH-BP. *Gen. Comp. Endocrinol.* 171, 203-210.

Yen, P.M., 2001. Physiological and molecular basis of thyroid hormone action. *Physiol. Rev.* 81, 1097-1142.

Yúfera, M., 1982. Morphometric characterization of a small-sized strain of *Brachionus plicatilis* in culture. *Aquaculture* 27, 55-61.

Yúfera, M., Parra, G., Santiago, R., Carrascosa, M., 1999. Growth, carbon, nitrogen and caloric content of *Solea senegalensis* (Pisces: Soleidae) from egg fertilization to metamorphosis. *Mar. Biol.* 134, 43-49.

Yúfera, M., Polo, A., Pascual, E., 1993. Changes in chemical composition and biomass during the transition from endogenous to exogenous feeding of *Sparus aurata* L. (Pisces, Sparidae) larvae. *J. Exp. Mar. Biol. Ecol.* 167, 149-161.

Legends to Figures

Figure 1. Dry weight of fed (control, ●) and food -deprived post-larvae for 12 days (□). Statistically significant differences compared to the control group at the same time point are indicated by asterisks (** $P < 0.001$). Different letters indicate significant differences between time points within each treatment ($P < 0.05$). Data are expressed as mean $\pm$ SEM, $n = 21-27$.

Figure 2. Whole-body cortisol levels in fed (control, ●) and food -deprived post-larvae for 12 days (□). Each point represents the average of three pooled samples compromising 0.1-0.5 g wet biomass per sample. Statistically significant differences compared to the control group at the same time point are indicated by asterisks (** $P < 0.01$). The letter b indicates significant differences between time points within each treatment ($P < 0.05$). Data are expressed as mean $\pm$ SEM.

Figure 3. Whole-body total T4 (A) and T3 (B) concentrations in fed (control, ●) and food deprived post-larvae for 9 days (□). Each point represents the average of three pooled samples compromising 0.25-0.50 g wet biomass per sample. Statistically significant differences compared to the control group at the same time point are indicated by asterisks ($P < 0.05$). Data are expressed as mean $\pm$ SEM.

Figure 4. Expression values of *crh* (A), *crh-bp* (B) and *tsh β* subunit (C) in fed (control, ●) and food-deprived post-larvae for 12 days (□). Statistically significant differences compared to the

481 control group at the same time point are indicated by asterisks (** $P < 0.01$; *** $P < 0.001$). Data
482 are expressed as mean $\pm$ SEM, $n = 9$.
483

Tables

Table 1. Oligonucleotide sequences used for RQ-PCR analysis.

Gene	Primer	Sequence (5' → 3')
<i>crb</i>	qssCRH_Fw	CCTGACCTTCCACCTGCTAC
	qssCRH_Rv	GAGATCTTTGGCGGAGTGAA
<i>crb-bp</i>	qssCRHBP_Fw	GGCAATGGCATAGACACCTC
	qssCRHBP_Rv	CACTGGACACCAGCCTCAC
<i>tsbβ subunit</i>	qssTSH_Fw	CATCGACTCCAACCCTGTCT
	qssTSH_Rv	ACTCTGTCCGCACTGGTTCT
<i>β-actin</i>	qssBact_Fw	TCTTCCAGCCATCCTTCCTCG
	qssBact_Rv	TGTTGGCATAACAGGTCCTTACGG

Table 2. Percentages of carbon (C) and nitrogen (N) content, and their ratio (C/N) of normally fed post-larvae (control) and in post-larvae deprived of food for 12 days. Each value represents the average of three pooled samples ($n = 7$) comprising 1 mg larval biomass per measurement. Statistically significant differences compared to control group at same time point are indicated by asterisks ($***P < 0.001$). Different letters indicate significant differences between time points within each treatment ($P < 0.05$). Data are expressed as mean $\pm$ SD.

Time (days)	Control			Food deprived		
	C (%)	N (%)	C/N	C (%)	N (%)	C/N
3	41.25 $\pm$ 0.31 ^a	10.54 $\pm$ 0.25 ^a	3.91	41.23 $\pm$ 0.68 ^a	10.90 $\pm$ 0.28 ^a	3.78
6	44.12 $\pm$ 0.70 ^b	10.60 $\pm$ 0.20 ^a	4.16	39.33 $\pm$ 0.60 ^{ab,***}	10.60 $\pm$ 0.26 ^{ab}	3.72
9	44.46 $\pm$ 0.20 ^b	10.59 $\pm$ 0.24 ^a	4.20	37.93 $\pm$ 0.72 ^{b,***}	10.08 $\pm$ 0.36 ^{ab}	3.76
12	43.50 $\pm$ 0.58 ^b	10.54 $\pm$ 0.25 ^a	4.13	37.01 $\pm$ 1.02 ^{bc,***}	9.78 $\pm$ 0.42 ^b	3.79

Figure 1.

Figure 2.

Figure 3.

502 Figure 4.