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# 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 involvement of 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 up regulation of *crh* and a decrease of *crh-bp* expression levels. Furthermore, whole-body total T3 concentrations of food-deprived post-larvae were reduced, although *tshβ* subunit expression levels remained unaffected. Our results show 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.



## 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 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 (Wendelaar Bonga, 1997; de Boeck et al., 2001; Aluru and Vijayan, 2007). 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 and Burger, 1984; Yen, 2001; Eales, 2006). 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 adrenocorticotropic 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 (Sutton et al., 1995; Huising et al., 2004; Huising et al., 2008). 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., 2006, 2009). It has been suggested that the TRH neurons in the mammalian hypothalamic paraventricular area

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(analogous to the teleostean preoptic area) are key in integrating and relaying central and peripheral signals related to energy homeostasis (Lechan and Fekete, 2006; Hollenberg, 2008), 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. 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 (Ellis et al., 2002; Conte, 2004; Ashley, 2007). In teleosts, the larval phase is defined by a rapid increase in biomass and changes in morphology, behavior, and physiology. These aspects depend on the vigor 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 focussed on changes in thyroid and stress systems related to various physiological processes of this species (Arjona et al., 2008; Ponce et al., 2010; Salas-Leiton et al., 2010; Costas et al., 2011b), 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 (Yúfera et al., 1999; Fernández-Díaz et al., 2001; Klaren et al., 2008; Gamboa-Delgado et al., 2011).

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 liter. Larval culture was similar as previously described (Klaren et al., 2008) and was as follows: 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 anesthetized, 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 mass 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 RNA*later*™ RNA stabilization solution (Ambion®) for 24 h at 4 °C and then stored at −20 °C. 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 mass and chemical analyses

Dry mass 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 larval energy content) and nitrogen (N, reflecting protein content) contents were determined with an elemental analyzer (Thermoquest, mod. Flash 1112), using sulfanilamide 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 mass) 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 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 (Tagawa and Hirano, 1987; Klaren et al., 2008). Samples (0.25–0.50 g wet mass) 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 barbitone 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