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Comparative gene expression of gonadotropins (FSH and LH) and peptide levels of gonadotropin-releasing hormones (GnRHs) in the pituitary of wild and cultured Senegalese sole (*Solea senegalensis*) broodstocks

J.M. Guzmán [a], M. Rubio [b], J.B. Ortiz-Delgado [c], U. Klenke [d], K. Kight [d], I. Cross [b], I. Sánchez-Ramos [b], A. Riaza [e], L. Rebordinos [b], C. Sarasquete [c], Y. Zohar [d], E.L. Mañanós [a,*]

[a] Institute of Aquaculture of Torre la Sal, Spanish Council for Scientific Research (CSIC), Torre la Sal s/n, 12595-Cabanes, Castellón, Spain
[b] Laboratorio de Genética, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, Polígono Río San Pedro, 11510-Puerto Real, Cádiz, Spain
[c] Institute of Marine Sciences of Andalucía, Spanish Council for Scientific Research (CSIC), Polígono Río San Pedro, 11510-Puerto Real, Cádiz, Spain
[d] Center of Marine Biotechnology, University of Maryland Biotechnology Institute, 701 E. Pratt Street, Baltimore, MD 21202, USA
[e] Stolt Sea Farm s.a, 15292-Lira, Carnota, A Coruña, Spain

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ABSTRACT

The Senegalese sole (*Solea senegalensis*) is a valuable flatfish for aquaculture, but it presents important reproductive problems in captivity. Spawning is achieved by wild-caught breeders but cultured broodstocks fail to spawn spontaneously and, when they do, eggs are unfertilized. To gain knowledge on the physiological basis underlying this reproductive dysfunction, this study aimed at analyzing comparative hormone levels between wild and cultured broodstocks at the spawning season. The Senegalese sole gonadotropin (GTH) subunits, FSHβ, LHβ and GPα, were cloned and qualitative (*in situ* hybridization) and quantitative (real-time PCR) assays developed to analyze pituitary GTH gene expression. In females, FSHβ and GPα mRNA levels were higher in wild than in cultured broodstocks, whereas in males all three subunits were highest in cultured. By ELISA, three GnRH forms were detected in the pituitary, displaying a relative abundance of GnRH2>GnRH1>GnRH3. All GnRHs were slightly more abundant in wild than cultured females, whereas no differences were observed in males. Plasma levels of vitellogenin and sex steroids were also analyzed. Results showed endocrine differences between wild and cultured broodstocks at the spawning period, which could be related to the endocrine failure of the reproductive axis in cultured breeders.

1. Introduction

The brain–pituitary–gonad (BPG) axis regulates the reproductive function in vertebrates, including fish. External and internal signals are primarily integrated in the brain by the hypothalamic neurons producing the gonadotropin-releasing hormones (GnRHs). The GnRHs stimulate the synthesis and secretion of gonadotropins (GTHs) in the pituitary, which are critical modulators of gametogenesis and gonadal maturation, through the mediation of gonadal steroids and growth factors (Schulz and Miura, 2002; Yaron et al., 2003).

Three GnRH forms, GnRH1 (so-called seabream GnRH or sbGnRH), GnRH2 (so-called chicken-II GnRH or cGnRH-II) and GnRH3 (so-called salmon GnRH or sGnRH) have been found to coexist in the brain of several teleosts, including flatfishes (Lethimonier et al., 2004; Pham et al., 2006). The GnRH1 is found to be the predominant form in the pituitary and considered the most relevant GnRH in the control of GTH synthesis and secretion (Powell et al., 1994; Gothilf et al., 1996), whereas the gonadotropic role of GnRH2 and GnRH3 may vary among species (Holland et al., 1998, 2001; Rodríguez et al., 2000; Andersson et al., 2001).

The GTHs, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are heterodimers which share a common α subunit (GPα) and possess a hormone specific β subunit (FSHβ and LHβ) (Pierce and Parson, 1981; Yaron et al., 2003). In salmonids, FSH predominates during vitellogenesis and spermatogenesis, while secretion of LH peaks at final gonadal maturation, ovulation and spermiation (Gómez et al., 1999; Swanson et al., 2003). In non-salmonid species, information on GTH releasing profiles is mostly restricted to LH because of the limited availability of FSH immunoassays. However, some information on the bioactivity of both GTHs has been obtained through molecular approaches, by analyzing gene expression levels of their subunits. Among non-salmonid species, single spawners have shown a temporal gene expression of FSHβ and LHβ similar to those of salmonids, with a clear prevalence of LHβ gene expression at final stages of gonadal maturation (Yoshiura et al., 1999; Kim et al., 2005). On the other hand, multiple spawners seem to express a progressive and simultaneous increase of both FSHβ and

* Corresponding author. Tel.: +34 964 319500; fax: +34 964 319509.
E-mail address: evaristo@iats.csic.es (E.L. Mañanós).

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LHβ gene expression during gonadal maturation (Elizur et al., 1996; Sohn et al., 1999; Jackson et al., 1999; Mateos et al., 2003; Kajimura et al., 2001; Weltzien et al., 2003; Meiri et al., 2004).

Optimal rates of synthesis and secretion of both GnRHs and GTHs are critical for successful gonadal maturation and spawning (Peter and Yu, 1997; Zohar and Mylonas, 2001). In aquaculture, fish often exhibit some degree of reproductive dysfunction, most commonly, absence or reduced egg quality and quantity in females and diminished milt production in males (Mylonas and Zohar, 2001). Failure to spawn in captivity has been associated to inhibited LH release from the pituitary (Zohar, 1988; Mylonas et al., 1997, 1998). Further studies have suggested a negative effect of confinement directly onto GnRH1 mRNA and pituitary peptide levels (Zohar and Mylonas, 2001). Therefore, the brain-pituitary endocrine system seems to be impaired under culture conditions, affecting adversely the normal functioning of the endogenous GnRH and GTH system and thus the reproductive process.

The Senegalese sole (*Solea senegalensis*) is a highly valuable fish which has become a priority species for European and Mediterranean aquaculture diversification (Imsland et al., 2003; Cañavate, 2005). However, the current production of this flatfish is largely based on wild-caught breeders, which display spontaneous fertilized spawning under rearing conditions (Dinis et al., 1999; Anguis and Cañavate, 2005). Contrarily, cultured breeders (hatched and raised in captivity) fail to spawn spontaneously and when they do, fecundity is reduced and eggs are generally found unfertilized (García-López et al., 2007; Guzmán et al., 2008). The causes of these generational differences, even when wild and cultured broodstocks are reared under similar culture conditions for several years, are unknown.

A number of recent studies have described several aspects of Senegalese sole reproduction in both wild (Anguis and Cañavate, 2005; García-López et al., 2006a) and cultured broodstocks (García-Lopez et al., 2006b, 2007; Guzmán et al., 2008). However, there is no information on GnRHs and GTHs in Senegalese sole, neither endocrine studies aimed directly to compare wild and cultured breeders. Recently, the molecular cloning of Senegalese sole GTHs has been presented (Guzmán et al., 2007; Cerdá et al., 2008), and information on GTH gene expression in cultured males given (Cerdá et al., 2008).

The purpose of the present study was to analyze pituitary GTH gene expression and pituitary GnRH content on paralleled wild and cultured Senegalese sole broodstocks at the spawning time, in an attempt to detect an endocrine failure which might be related to reproductive dysfunctions found in cultured breeders. For this, the Senegalese sole FSHβ, LHβ and GPα subunits were cloned and specific real-time quantitative PCRs (qPCRs) developed. Analysis of GTH subunit mRNA levels and GnRH pituitary content (by ELISA) was performed in both females and males, and correlated with vitellogenin (VTG) and sex steroid plasma levels, and gonadal histology. The distribution of GTH subunit gene expression cells in the pituitary of males and females was studied by *in situ* hybridization.

2. Materials and methods

2.1. Animal housing and sampling

The Senegalese sole (*Solea senegalensis*, Soleidae, Pleuronectiformes) breeders used in this study were obtained from two broodstocks (wild and cultured) reared at the facilities of the fish farm "Stolt Sea Farm s.a." (A Coruña, Spain, 42° N 8° W). Wild broodstocks consisted of fish captured from the wild by fishermen as juveniles, in 2003, transported immediately to the abovementioned fish farm and further grown and reared at the holding facilities. Cultured broodstocks consisted of females (5 year-old) and males (6 year-old) hatched and raised in captivity from spawn obtained from wild-caught breeders at the same fish farm. Both wild and cultured broodstocks were always kept under similar culture conditions, maintaining both broodstocks in paralleled, but separate tanks. Tanks were supplied with an open flow-through sea water system (salinity 36‰, 3 renewals per day) and exposed to natural cycles of photoperiod and temperature. Tank densities were maintained at around 2 kg m^2 and sex ratios at around 1:1. Fish were fed daily *ad libitum* with dry pellets (ProAqua).

All tanks were equipped with egg collectors. Spawning was checked daily (twice) and quantity and quality of eggs recorded. The spawning of wild broodstock took place from March to September, whereas the cultured broodstock produced some spontaneous but unfertilized spawn from April to June. For the present study, sampling was performed on 28th of May 2007, considered as the full spawning period. Female ($n=4$) and male ($n=4$) breeders were sampled from wild and cultured broodstock, identified through passive integrated transponder tags (pit-tags, AVID). For sampling, fish were deeply anaesthetised by immersion in 2-phenoxyethanol (3 mL L^{-1}) and body weight and length recorded. Blood (1 mL) was collected from the caudal vasculature with heparinised syringes and placed in ice-cold heparinised tubes with aprotinin (0.15 IU mL^{-1}) (Sigma, USA). Plasma was obtained by centrifugation (3,000 *g*, 15 min, 4 °C) and stored at −20 °C. Fish were sacrificed by decapitation for dissection of tissues. Pituitary glands were collected, immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Gonads were removed and weighed for calculation of the gonadosomatic index (GSI = gonad weight × 100/body weight). For histology, a portion of the gonad was taken from the middle part and placed in fixative (4% formaldehyde, 1% glutaraldehyde). The gonad was further embedded in glycol methacrylate resin (Technovit 7100, Heraeus Kulzer, Germany), cut into 3 μm sections and stained with methylene blue/azure II/basic fuchsin (Bennett et al., 1976).

All procedures were carried out according to national and institutional regulations (Spanish Council for Scientific Research (CSIC), Institute of Aquaculture of Torre la Sal Review Board) and the current European Union legislation on handling experimental animals.

2.2. Molecular cloning of Senegalese sole GTH subunits

For cloning, four pituitaries were collected from mature male ($n=2$) and female ($n=2$) Senegalese sole. Fish were sacrificed by phenoxyethanol overdose and pituitary glands removed and immediately frozen in liquid nitrogen. Total RNA was extracted from the pooled pituitaries using RNeasy Mini kit (Qiagen). Reverse transcription was performed on 600 ng of total RNA with Powerscript Reverse Transcriptase (Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol for 5′ and 3′ first strand synthesis.

Complete cDNA sequences for FSHβ, LHβ and GPα subunits were obtained by two rounds of RACE-PCR, using the SMART™-RACE-PCR protocol (Clontech). Partial cDNA sequences were first isolated using primers FSH-FW, LH-RV and GP-RV (Table 1). Thermal cycling

Table 1
Primers used to isolate the Senegalese sole FSHβ, LHβ and GPα subunit cDNAs, by Smart RACE RT-PCR.

Transcript	Primer	Direction	5′ to 3′ Primer sequence
FSHβ	FSH-FW[a]	Forward	5′ GATGVAGYTRGTYSTCATGG 3′
	ssFSHr[c]	Reverse	5′ CATAGGTCCAGTCCCCGTT 3′
LHβ	LH-RV[a]	Reverse	5′ GGCTGNAGRCTCTCRAAGGT 3′
	ssLHf[c]	Forward	5′ CTCTGGAGAAGGACGGATGT 3′
GPα	GP-RV[b]	Reverse	5′ GTGGCAGTCTGTGTGGTTCC 3′
	ssGPf[c]	Forward	5′ TGCTGCTTCTCCAGAGCGTA 3′

[a] Degenerated primers previously used in the three-spined stickleback (Hellquist et al., 2004). R = G or A, Y = T or C, S = G or C, N = G, A, T or C.
[b] Primer designed based on a consensus sequence of GPα from Atlantic halibut (GenBank accession no. AJ417770.1), Japanese flounder (AF268692) and European sea bass (AF269157.1).
[c] Senegalese sole gene specific primers used for the second round RACE-PCR.