Follicle stimulating hormone (FSH) and luteinizing hormone (LH) gene expression during larval development in Senegalese sole (*Solea senegalensis*)

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Abstract

The gonadotropins (GTHs), follicle stimulating hormone (FSH) and luteinizing hormone (LH), determine the reproductive competence of adult breeders, but also participate in the early establishment of the reproductive axis. The present study aimed at studying, by real-time qPCR, the gene expression levels of GTH subunits (FSH β , LH β and the common GP α) during the early development of a flatfish, the Senegalese sole, from 1 to 100 days post hatching (dph). The mRNAs were first detected at 1, 5 and 3 dph for FSH β , LH β and GP α , respectively. Gene expression of FSH β , and also GP α , increased continuously to peak levels at mid-metamorphosis (15 dph) and after a sharp post-metamorphic decrease, mRNA levels were maintained low until a second increment at 90 dph. Contrarily, LH β mRNA levels were

low throughout the experiment and only detectable around metamorphosis. All three subunits were highly expressed in 1-year old soles, with FSH β and GP α mRNA levels 10-fold higher than those of LH β . These results suggest, i) activity of the reproductive axis early after hatching (1 dph), which was highest during the metamorphosis climax and, ii) a predominant role of the FSH, rather than LH, in the early establishment of the reproductive axis in Senegalese sole.

Keywords: Early development; FSH; gonadotropin; larvae, LH, ontogeny; Senegalese sole; *Solea senegalensis*.

Introduction

The gonadotropins (GTHs), follicle stimulating hormone (FSH) and luteinizing hormone (LH), are critical hormones in the regulation of reproduction in vertebrates, including fish (Yaron et al., 2003). Both FSH and LH are glycoproteins synthesised in the pituitary, consisting in a common α subunit (GP α) and a hormone specific β subunit (FSH β and LH β), which provides biological specificity (Pierce and Parson, 1981; Yoshiura et al., 1999).

The role of FSH and LH in the regulation of the reproductive cycle of fish has been studied in many species, mainly in salmonids (Tyler et al., 1991; Gómez et al., 1999; Swanson et al., 2003). These studies have shown that FSH predominates during early gonadal recrudescence, including vitellogenesis and spermatogenesis, while LH is more related to final gonadal maturation, ovulation and spermiation. However, developmental studies conducted during fish ontogeny have reported GTH activity since few days after hatching, suggesting that FSH and LH may regulate not only gametogenesis and steroidogenesis in adult fish, but also earlier processes during the establishment of the reproductive axis (Swanson et al., 1991; Saga et al., 1993; Parhar et al., 2003). These studies have described, through immunohistochemical (IHC) techniques, a marked species-specific variation in the chronological first appearance of each pituitary GTH cell population. For example, in the rainbow trout (*Oncorhynchus mykiss*), FSH cells first appeared 15 days post hatching (dph), 2 weeks before gonadal differentiation (Saga et al., 1993), while LH cells were not detected until vitellogenesis or spermatogenesis occurred (Nozaki et al., 1990). In the cichlid fish (*Cichlasoma dimerus*), FSH cells first appeared at 21 dph, prior to sex differentiation (60 dph), whereas LH cells were detected later, during the sex differentiation period (Pandolfi et al., 2006). In the gilthead seabream (*Sparus aurata*), FSH and LH

immunoreactive cells appeared at 22 and 82 dph, respectively, when the gonads were still undifferentiated (García-Ayala et al., 2003). Contrarily, in the pejerrey (*Odonthestes bonariensis*) LH cells appeared at 2-3 weeks after hatching, whereas FSH immunoreactive cells appeared 1-2 weeks later, in both cases before gonadal differentiation (Miranda et al., 2001). This marked species-specific first immunodetection of pituitary LH and FSH proteins has complicated recognizing the specific roles of FSH and LH on the early development of the reproductive axis in fish. The results suggest, in general, an earlier expression of FSH than LH during development, but information is scarce at early stages of larval development and limited by the sensitivity of the IHC techniques.

The quantitative real-time PCR (qPCR) has become one of the most widespread techniques for gene expression analysis, due to its accuracy, broad dynamic range, sensitivity and reproducibility (Bustin et al., 2005). As far as we know, a single study has described the evolution of FSH and LH mRNA levels during the early development of fish (Wong et al., 2004). This study showed detectable transcripts of FSH β and its specific receptor, but not LH β , in embryos of gilthead sea bream as early as 1 day after fertilization and suggested a predominant role of FSH in the very early establishment of the reproductive axis in fish. This was also supported by a brief communication which reported that FSH β subunit was expressed in the Nile tilapia (*Oreochromis niloticus*) pituitary as early as 0 dph (Fan et al., 2003). Both studies highlighted the higher sensitivity of PCR-based methods compared to IHC techniques in the early detection of GTH activity, a valuable parameter for developmental studies.

Pleuronectiformes (flatfishes) are a broad taxonomic group comprising 11 families and about 500 species worldwide, some of them of high commercial interest (Helfman et al., 1997). Among them, the Senegalese sole (*Solea senegalensis*), has become a priority species for aquaculture diversification in Europe and Mediterranean countries (Dinis et al., 1999; Imsland et al., 2003; Howell et al., 2006), and the focus of intensive research in the last years. The molecular cloning of Senegalese sole GTHs and description of GTH gene expression levels in adult breeders, by qPCR, have been recently reported (Guzmán et al., 2007, Cerdá et al., 2008). To date, there is no information on GTH synthesis and release during the early stages of development Senegalese sole.

The aim of the present study was to analyze GTH gene expression in Senegalese sole during early development, by using specific real-time quantitative PCRs for the FSH β , LH β and GP α subunits. Information on the ontogenesis of the GTH system in Senegalese sole will contribute to understand the early development and establishment of the reproductive axis in

this species, which is a critical determinant of the further reproductive competence of the adult breeders.

Materials and Methods

Experimental fish holding and sample collection

Fertilized eggs from wild-caught Senegalese sole broodstocks were obtained on May 25th 2007 at the Oceanographic Institute (IEO) of Vigo (42°N, 8°W) and transported to our facilities at the Institute of Aquaculture of Torre la Sal (40°N, 0°E) the same day. Eggs, as well as larvae and juveniles, were maintained in 500 l circular tanks (0.8 m² surface), in an open-flow through sea water system (salinity ~37 psu) filtered through 10 and 20 µm cartridge filters (Cuno Incorporated, Meriden, CT) and exposed to natural photoperiod and temperature (see fig. 1). Air diffusers were installed in the centre and perimeter of the tank to ensure gentle and continuous aeration. Dissolved oxygen (6.7 ± 0.2 ppm), pH (7.5 – 8.2), and nitrites and ammonium water levels (< 0.5 ppm) were checked regularly.

Eggs were incubated at a density of 40 eggs l⁻¹ in a single 500 l tank; larvae hatched at 3 d after fertilization (May 28th 2007). Tank density and larval growth and morphology were checked regularly. At 5 days post hatching (dph), tank density was estimated at ~12 larvae l⁻¹. Metamorphosis took place from 12 to 18 dph (see fig. 2). At completion of metamorphosis all larvae were settled over the tank surface and density estimated at 21 dph as ~3000 specimen m². At 28 dph, soles were redistributed in two 500 l tanks to reduce density and fish culture maintained for 1 year in similar conditions. During this period the number of fish decreased due to mortalities associated to the weaning period and the incidence of bacterial infections. The density at 365 dph was ~100 specimen m² in both rearing tanks. Cleaning of the tanks was carefully performed throughout the experimental period by siphoning the bottom of the tank every other day.

From 2 to 12 dph, larvae were fed on rotifers (*Brachionus plicatili*) (5 individuals ml⁻¹); during the rotifer feeding period, microalgae (*Isochrysis galbana*) (3 10⁵ cells ml⁻¹) was added to the larval rearing tanks. From 7 to 90 dph, specimens were fed with newly hatched *Artemia salina* nauplii (4 individual ml⁻¹), and from 10 to 90 dph co-fed with an increasing ratio of commercial dried pellets (Proaqua, Spain). From 90 dph onward, juveniles were exclusively fed with commercial dried pellets; ratio was adjusted daily depending on remaining food observed in the bottom of the tank.

Animals were sampled at 1, 3, 5, 9, 12, 15, 18, 21, 30, 40, 50, 60, 70, 80, 90, 100 and 365 dph. Due to the low amount of RNA obtained from a single larva, pools of larvae were

used in the first sampling points. Samplings from 1 to 18 dph, consisted of pools (n=6) of five specimens; sampling of 21 dph consisted of pools (n=6) of three specimens. Samplings of 30 and 40 dph, consisted of whole individual fish (n=6). Samplings from 50 to 100 dph, consisted of individual heads (n=6). At 365 dph, the skull containing brain and pituitary was collected from individual fish (n=6). Samples (whole larvae, heads or skulls) were immediately frozen in liquid nitrogen and stored at -80° C until used. To follow fish growth during the experimental period (see fig. 1), specimens (n=12) were carefully paper-dried and weighted (Mettler Toledo, XS105), from 15 dph onward.

Fish management and sampling procedures were carried out according to national and institutional regulations (Spanish Council for Scientific Research (CSIC) and Institute of Aquaculture of Torre la Sal Review Board) and the current European Union legislation on handling experimental animals. All fish to be handled were anaesthetised by immersion in 0.3 ml l⁻¹ of 2-phenoxyethanol.

Real-time quantitative PCR (qPCR)

Based on the full cDNA sequences of the Senegalese sole FSH β , LH β and GP α subunits (available at GenBank under [EF617341](#), [EF617342](#) and [EF617342](#) access numbers) (Guzmán et al., 2007), specific qPCRs were developed. Briefly, plasmids containing the specific subunit insert were linearized and used as templates for gene-specific RNA standard synthesis (Wong et al., 2004). Total RNA isolated from a pool of Senegalese sole pituitaries, using Tri-Reagent (Sigma) and treated with DNase (RQ1 RNase-free DNase, Promega), served as the standard for 18s RNA. The amount of each RNA standard was determined using RiboGreen RNA quantification kit (Molecular Probes, Eugene, OR).

For sample analysis, total RNA was isolated from each sample using Tri-Reagent, according to the manufacture's instructions, treated with DNase and quantified (GeneQuant, Pharmacia Biotech). RNA standards (target and reference) and RNA from each sample were reverse-transcribed simultaneously and used as template in qPCR, using the SYBR Green PCR core reagent (Applied Biosystem), containing 200 nM gene-specific primers (Table 1). Amplification reactions were carried out at 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 60 s, using an ABI Prism 7700 Sequence Detection System. After the amplification phase, a dissociation step was carried out at 95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s. Reaction efficiencies were calculated based on the second derivative maximum ($E=10^{-(1/\text{slope})} - 1$). After the qPCR reaction, copy number for unknown samples was

determined by comparing C_T (threshold cycles) values to the specific standard (run in every plate) and normalized to the amount of 18s RNA in each sample.

Amplification efficiencies (%) for the qPCRs, obtained from validation assays using serially diluted RNA, were 102.3 ± 2.1 , 102.5 ± 2.0 and 96.8 ± 3.5 for FSH β , LH β and GP α , respectively. The lowest standard point was 100 copies/reaction for all three transcripts (FSH β , LH β and GP α).

Data analysis

The transcript levels of each gene were expressed as mean $\pm$ SEM values. For statistical analysis, log transformation was applied to correct problems of unequal variances among data. The results were analyzed over the time of each developmental stage (categorized as dph) using one-way ANOVA, with a significance level of $P < 0.05$.

Results

The growth rate of the Senegalese sole specimens was followed through the experimental period and showed a continuous increase from 15 dph onwards (Fig. 1). At a sampling performed over 1 year-old specimens, fish weighted 16.2 ± 1.6 g ($n=15$). The morphology and external features of Senegalese sole specimens was also followed through the experimental period at the binocular and representative developmental stages are shown in Fig. 2. Metamorphic stages were recognized according to Fernández-Díaz et al. (2001). From 1 to 9 dph, symmetric larvae showed a vertical swimming plane. First signs of metamorphic changes were observed at 12 dph, when the left eye started to migrate towards a dorsal position. At 15 dph, individuals changed their swimming plane and the left eye migrated within the ocular side. Complete metamorphosis was observed at 18 dph, when the eye translocation was completed, the orbital arch was completely visible and a final flat body shape acquired.

Steady-state levels of FSH β , LH β and GP α transcripts were quantified by qPCR from 1 to 100 dph (Fig. 3). Both GTH- β mRNAs were detected early during larval development, at 1 and 5 dph for FSH β and LH β , respectively. Nevertheless, the levels and expression profiles of each GTH- β subunit were highly different during development. Transcripts of FSH β showed a steadily increase from 1 to 15 dph, when the highest FSH β mRNA levels were detected (15 dph; metamorphic climax, see Fig. 2). The FSH β mRNA levels decreased after metamorphosis and were maintained constant and low for two months with a second rise observed at 90 dph. By contrast, transcription levels of LH β were only detected from 5 to 18

dph (before and during metamorphosis) and at very low levels. The transcripts of the GP α were first detected at 3 dph and showed a similar profile than those of FSH β , increasing initially to peak levels at 15 dph (metamorphosis) and showing a second later rise to highest levels at 80-90 dph.

Juveniles were further cultured and GTH subunit mRNA levels measured in 1 year-old fish (Fig. 4). At 365 dph, FSH β and GP α mRNA levels increased 40-fold with respect to the previous sampling at 100 dph ($p < 0.001$). The LH β subunit was also highly expressed at 365 dph, compared to previous undetectable levels at 100 dph. Among subunits, the expression levels of both FSH β and GP α were 10-fold higher than those of LH β ($p < 0.001$).

Discussion

This study described, for the first time, the gene expression pattern of GTH subunits during early development in Senegalese sole. The results showed that, i) all GTH subunits were expressed early during larval development and their mRNA levels peaked simultaneously at 15 dph, coinciding with the metamorphosis climax and, ii) the FSH β was expressed earlier and at higher levels throughout larval and juvenile development than the LH β subunit, suggesting a predominant role of FSH in the early establishment of the reproductive axis in Senegalese sole.

The FSH β transcripts were detected at the first sampling point, on day 1 after hatching, and then increased continuously during the first two weeks of larval development. This observation is in agreement with the two previous studies that have analysed FSH β mRNA levels during early development in fish (Nile tilapia: Fan et al., 2003; gilthead seabream: Wong et al., 2004). In Nile tilapia, FSH β mRNA was analyzed by RT-PCR and showed a detectable expression of the FSH β subunit at the day of hatching (Fan et al., 2003). In gilthead seabream, transcripts of FSH β were first detected before hatching, at day 1 after egg fertilization, by qPCR, and showed a synchronized fluctuation with that of their specific receptor during early larval development (Wong et al., 2004). These findings have suggested a primarily role of FSH in the early establishment of the reproductive axis in these species. The available information on GTH synthesis during ontogeny in other fish species becomes from analysis and detection of the GTH proteins, mostly through IHC techniques (Saga et al., 1993; Miranda et al., 2001; Pandolfi et al., 2006; Shimuzu et al., 2008). It has to be considered that synthesis of the protein is a delayed event with respect to the expression of the corresponding gene. Indeed, in the gilthead sea bream, FSH β immunoreactive cells were first detected by IHC at 22 dph (García-Ayala et al., 2003), while FSH β gene expression was

detected by qPCR in the embryos, since the first day after egg fertilization (Wong et al., 2004). Similarly, in the Nile tilapia, first immunoreactive FSH cells were detected at 14 dph (Parhar et al., 2003), while FSH β mRNA levels were detectable since 0 dph (Fan et al., 2003). Early detection of the FSH β protein has only been reported in the neonatal platyfish (*Xiphophorus maculatus*), which showed immunoreactive FSH β cells in the pars intermedia of the pituitary at 2 dph (Mauglio-Cepriano et al., 1994). In Senegalese sole, there is no information on the FSH protein at any developmental stage. Anatomical studies have shown an early development of the Senegalese sole pituitary, which is visible at 1dph (Piñuela et al., 2004). This information, together with the present finding of an early parallel expression of both FSH β and GP α subunit genes, from 1-3 dph onwards, would indicate a relevant function of the FSH protein during the first stages of larval development in Senegalese sole.

The LH β transcripts were first detected in Senegalese sole larvae at 5 dph and although maintained at very low levels, they were detectable until the end of the metamorphic period. This expression pattern is somehow different to that observed previously in the gilthead sea bream, which did not show detectable LH β transcripts during the first 34 d after hatching (Wong et al., 2004). Interestingly, in the gilthead sea bream, although no LH β transcripts were detected during the first month of larval development, mRNA from its specific receptor was detected since 1 dph. These authors pointed out to the possibility that LH β was in fact expressed but at a very few levels, below the detection limit of the qPCR assay (Wong et al., 2004). This information would be in agreement with the present results in Senegalese sole, in which very low expression levels of the LH β subunit were found throughout larval development. Regarding the available information on LH protein synthesis during fish ontogeny, previous studies have shown by IHC that LH β immunoreactive cells were first detected in fish larvae at 14 dph in tilapia (Parhar et al., 2003), 45 dph in the platyfish (Mauglio-Cepriano et al., 1994), 60 dph in the cichlid fish (Pandolfi et al., 2006) and 82 dph in gilthead seabream (García-Ayala et al., 2003). The available information on gene expression and protein analysis show that during fish larval development the synthesis of LH seems to be delayed with respect to that of FSH. The present results in Senegalese sole, showing higher FSH β than LH β mRNA levels throughout larval development, may suggest that FSH, rather than LH, would participate in the early development of the reproductive axis in this species.

The transcript levels of all three GTH subunits peaked simultaneously at 15 dph, coinciding with the period of mid-metamorphosis. As far as we know, there is no previous information on GTH gene expression or protein synthesis in flatfishes during metamorphosis.

Some information is available on the endocrinology of the reproductive axis in other species undergoing metamorphosis. This is the case of the lamprey (*Petromyzon marinus*), which showed an striking gonadotropin-releasing hormone (GnRH) immunoreactivity during the process of metamorphosis, indicating a possible role of the GnRH system in this process (Tobet et al., 1995). In the amphibious bullfrog (*Rana esculenta*), a species which undergoes metamorphosis few days after hatching, a significant increment in plasma FSH and LH was detected at the onset of metamorphosis, coinciding with a rise in brain GnRH and suggesting an activation of the reproductive axis as the metamorphic climax sets in (Fiorentino et al., 1999). This finding is somehow in agreement with the present results in Senegalese sole, where maximum expression of all three GTH subunits was detected at 15 dph, coinciding with the metamorphosis climax. Nevertheless, it should be noted that in the gilthead sea bream, a fish which do not undergo a drastic metamorphic process, a concomitant increase in FSH β , FSH receptor and GnRH (sbGnRH and cII-GnRH) transcripts was observed at 12 dph, indicating a high activation of the reproductive axis on the first two weeks of larval development (Wong et al., 2004). Further studies will be necessary to determine the role of FSH and LH in the establishment of the reproductive axis and its relationship with the process of metamorphosis in Senegalese sole, as well as in other flatfish species.

At completion of metamorphosis, GTH mRNA levels dropped sharply and were maintained low (FSH β) or undetectable (LH β) in post-metamorphic larvae and juvenile Senegalese sole. Interestingly, there was a second rise of FSH β gene expression, and also GP α , at the end of the experimental period, at around 90-100 dph. This increment on GTH expression was also detected in a later sampling performed over 1 year-old Senegalese sole, mainly on FSH β and GP α , but also on LH β transcripts. Although this is out of the scope of the present study, the results suggested the initiation of a GTH-dependent process in 3 month old juvenile Senegalese sole, which could be related to a period of gonad differentiation. There is no information on the process of gonad differentiation in Senegalese sole, but in this species puberty takes place at the age of 2-3 years (Dinis et al., 1999; Guzmán et al, 2008). Contrarily to the scarce information on GTH function during larval development, a number of studies have addressed the involvement of GTHs during the period of sexual differentiation. For example, in the European sea bass (*Dicentrarchus labrax*), GTH mRNAs were undetectable at 100 dph and increased highly at 200 dph, mainly the FSH β and GP α mRNAs, and suggested a core role of FSH in the control of sex differentiation in this species (Molés et al., 2006). A predominant role of FSH in the regulation of sex differentiation have been also suggested in salmonids, in which pituitary FSH β mRNA levels predominated over those of

LH β throughout juvenile development (Naito et al., 1991; Weil et al., 1995). A different situation has been observed in other fish species. For example, in juvenile striped bass (*Morone saxatilis*) LH β mRNA levels were about one order of magnitude higher than those of FSH β (Hassim et al., 1999), while in 1-year old red sea bream (*Pagrus major*) LH β mRNA levels were ~3-fold higher than those of FSH β (Kumakura et al., 2004). These studies showed that the specific roles of FSH and LH in juvenile fish may differ highly between species. The preliminary data obtained in this study might suggest a predominant role of FSH in the control of pre-pubertal processes in juvenile Senegalese sole.

In conclusion, the present study provided first data on GTH subunit gene expression in Senegalese sole larvae and juveniles. Active transcription of FSH β , LH β and GP α was detected since few days after hatching, indicating an early activation of the reproductive axis. The higher transcript levels of FSH β and GP α compared to those of LH β might suggest that FSH, rather than LH, would play a major role in this early establishment of the reproductive axis with a possible participation in the process of metamorphosis.

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Table 1. Gene-specific primers and amplicon size (bp) for each transcript in the qPCR assays.

Transcript	Primer	Nucleotide sequence	Amplicon size (bp)
FSH β	ssFSHf_q	5' GGACCCAAACTACATCCATGAAC 3'	60
	ssFSHr_q	5' CAGTCCCCGTTACAGATCACCTGTCT 3'	
LH β	ssLHf_q	5' CGGTGGAGACGACCATCTG 3'	61
	ssLHr_q	5' GGTATCTTGATGACGGGATCCTT 3'	
GP α	ssGPf_q	5' ACGGGCTGTGAGAAATGCA 3'	56
	ssGPr_q	5' GGATGCTCCCTGGAGAACAA 3'	
18s ^a	18Sf_q	5' GGTACTTTCTGTGCCTACCATGGT 3'	61
	18Sr_q	5' CCGGAATCGAACCCTGATT 3'	

^aGene-specific primer designed from the Senegalese sole 18s complete gene sequence, available at Gene Bank ([EF126042.1](#)).

Figure legends

Figure 1. Evolution of the body weight of Senegalese sole larvae and juveniles (symbols) and fluctuation of water temperatures (dotted line), during the experimental period. Body weight data are expressed as mean $\pm$ SEM (n=15).

Figure 2. Photographs of Senegalese sole at different developmental stages including, pelagic larvae (3 to 9 dph), larvae undergoing metamorphosis (12 to 18 dph) and post-metamorphic individuals (from 21 dph onward). See the migration of the eye during metamorphosis (from 12 to 18 dph) and achievement of the complete flat body shape (18 dph, onward).

Figure 3. Profiles of FSH β , LH β and GP α mRNA levels during the first 100 days after hatching, by qPCR. Levels were normalized to Senegalese sole 18s and represented as mean $\pm$ SEM (n=6). Asterisks “*” indicate significant differences (P<0.05) with respect to the maximum transcript levels, detected at 15 dph. In the upper graph (FSH β transcript levels), hashes “#” indicate significant differences (P<0.05) with respect to 9 and 12 dph. In the lower graph (GP α transcript levels), crosses “+” indicate significant differences (P<0.05) with respect to 18 dph. Non detectable levels are represented by ND. The interval for the metamorphosis process is shown by a grey vertical bar.

Figure 4. Levels of FSH β , LH β and GP α mRNAs in 1-year old Senegalese sole, by qPCR. Levels were normalized to Senegalese sole 18s and represented as mean $\pm$ SEM (n=6). Levels of FSH β and GP α mRNAs were significantly higher than those of LH β (P<0.003 and P<0.007, respectively), represented by different letters.

Fig. 1

Fig. 2

Fig. 3

Fig. 4