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# Gene and protein expression for prolactin, growth hormone and somatolactin in *Sparus aurata*: Seasonal variations

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ABSTRACT

The seasonal variation of PRL, GH and SL gene and protein expression has been analyzed in gilthead sea bream (*Sparus aurata*) pituitaries using Real-Time Q-PCR and Western Blots, respectively. Animals were cultured in earthen ponds under natural photoperiod, temperature and salinity conditions. Samples were taken during winter 2005 (January), spring 2005 (April), summer 2005 (July) and autumn 2005 (October). β-actin, used as the housekeeping gene both for Q-RT-PCR and Western analysis, did not present significant differences among seasons. Higher expression was observed during spring and autumn for PRL, summer and winter for GH, and spring for SL. Expression of PRI, GH and SL, presented seasonal variation, suggesting that these hormones could play a role in the molecular signal transduction of environmental factors (especially of photoperiod and temperature) in eurythermal fish.



## 1. Introduction

Gilthead sea bream (*Sparus aurata*) is a euryhaline species, which lives in environments with different salinities: coastal water, estuaries (brackish water) and lagoons (hypersaline water) (Arias, 1976). Several studies have focussed on the influence of environmental salinity in several aspects of sea bream biology such as limits of salinity tolerance in juveniles (Chervinski, 1984), osmoregulatory, metabolic modifications (Laiz-Carrión et al., 2005; Sangiao-Alvarellos et al., 2005) and optimal salinity for larval development and juvenile growth (Tandler et al., 1995; Laiz-Carrión et al., 2005). In addition, sea bream is also a eurythermal species which normally lives in temperatures ranging from 11 °C (winter) to 24 °C (summer). Due to physiological problems during winter, induced by low temperatures, most of the studies have focused in the phenomena known as "winter syndrome" (Gallardo et al., 2003; Ibarz et al., 2003). Changes in water temperature and salinity during seasons induce adjustments of fish internal processes (i.e. growth, osmoregulation, etc.). These processes are under control of adenohypophyseal hormones, and modifications in adenohypophyseal hormone producing cells can be expected. Prolactin (PRL), growth hormone (GH) and somatolactin (SL) are considered as belonging to the same hormone family due to their structural similarities (Rand-Weaver et al., 1992; Vega-Rubín de Celis et al., 2004). However, they showed different functions related to physiological processes modified by environmental changes in temperature and salinity: i) PRL is a hormone involved in acclimation to hyposmotic media (McCormick, 1995, 2001; Manzon, 2002; Sakamoto and McCormick, 2006; Mancera and McCormick, 2007); ii) GH regulates growth, intermediary metabolism and in some species present osmoregulatory effects (Nishioka et al., 1988; McLean and Donalson, 1993; Sakamoto and McCormick, 2006; Mancera and McCormick, 2007); iii) while the role for SL is not well defined yet, being related to different physiological processes depending on the studied species (Kaneko, 1996; Vega-Rubín de Celis et al., 2004).

In order to determine seasonal variation in PRL, GH, and SL gene and protein expressions, pituitary samples from *S. aurata* specimens, cultured in earthen ponds under natural photoperiod, temperature and salinity, were seasonally collected. Quantitative Real-Time Polymerase Chain Reaction (Q-RT PCR) and Western Blots were used to measure the expression of genes and proteins, respectively. Results will be discussed in relation to the physiological role of these hormones in *S. aurata*.

## 2. Materials and methods

### 2.1. Animals

Immature male gilthead sea bream (*S. aurata*, Sparidae, Perciformes) (200–300 g body mass; one year old; Table 1) were supplied by a local

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**Table 1**
Length and mass of sea bream specimens sampled and photoperiod "PH" (L "hours of light":D "hours of dark") regime of each season.

| Seasons | Length (cm) | Mass (g) | PH (L:D) |
|---|---|---|---|
| Winter (January 2005) | 22.27 ± 0.27 | 233.55 ± 11.27 | 10:14 |
| Spring (April 2005) | 25.06 ± 0.75 | 279.12 ± 21.24 | 13:11 |
| Summer (July 2005) | 23.78 ± 0.70 | 257.40 ± 21.85 | 14:10 |
| Autumn (October 2005) | 24.43 ± 0.51 | 300.15 ± 21.61 | 11:13 |

Data are shown as mean ± SEM.

**Table 2**
Sea bream specific RT-PCR primers for prolactin (PRL), growth hormone (GH), somatolactin (SL) and β-actin.

| Name | Sequence |
|---|---|
| GH-F1 | 5′-GAG CTG AAG ACG GGC ATC CAT C-3′ |
| GH-R1 | 5′-CAG GCA AGT AGT TCG TAG GTT CGT CT-3′ |
| PRL-F1 | 5′-GAC ATC GGC GAG GAC AAC ATT-3′ |
| PRL-R1 | 5′-CGG CAG CGG AGG ACT TTC AG-3′ |
| SL-F1 | 5′-CTG ATG AGT CTG GAG CAA GGT GTG-3′ |
| SL-R1 | 5′-TGA AGC AGC TGA GCA AGG TGT AGT C-3′ |
| β-actin-F1 | 5′-TGA ACC CCA AAG CCA ACA GG-3′ |
| β-actin-R1 | 5′-ATC ACG GGA GTC CAT AAC AAT ACC AG-3′ |

fish farm (CUPIMAR, S.A., San Fernando, Spain). According to the fish farm procedure, specimens ($n = 75{,}000$, 5 g mass) were placed in natural ponds (200 m long × 20 m width × 1.5 m depth, total volume: 6000 $m^3$) at initial density of 0.0625 kg/$m^3$. The mortality was 10%. At sample point the estimated number of fish was 67,500, with final mass of 250 g and density of 2.81 kg/$m^3$. This experiment was done for two years. Fish were maintained in an earthen pond under natural photoperiod, temperature and salinity (Fig. 1). Daily salinity (PSU) and temperature (°C) were measured at the same time of the day (12:00) at the middle of the water column with an ATAGO S/MILL refractometer and thermometer, respectively. Specimens were sampled, and pituitaries ($n = 5$ for Q-RT-PCR and $n = 5$ for Western Blots) were collected during winter 2005 (January), spring 2005 (April), summer 2005 (July) and autumn 2005 (October). Samples were taken only from one natural pond each season. Fish were over-anaesthetized with a lethal dose of 2-phenoxyethanol (1 mL $L^{-1}$), the heads were separated from trunk and pituitaries were rapidly removed and flash-frozen in liquid nitrogen in individual 1.5 mL microcentrifuge tubes, which were stored at −80 °C. The described experiments complied with the Guidelines of the European Union Council (86/609/EU), the Spanish Government (RD 1201/2005) and of the University of Cádiz (Spain) for the use of laboratory animals.

### 2.2. Experimental protocols

#### 2.2.1. Total RNA extraction and Real-Time Q-PCR

RNA from individual pituitary glands was extracted using the QIAGEN RNeasy Mini Kit and the protocol for isolation of total RNA from animal tissues, including the on column RNase-free DNase treatment. The amount of RNA and the quality was spectrophotometrically measured at 260 nm and using the ratio $Abs_{260/280}$, respectively. Gene expression was analyzed by Real-Time Q-PCR using the IQ5 DNA Engine model from Bio-Rad and the procedure from the One Step QuantiTect SYBR Green RT-PCR kit from QIAGEN, species-specific primers, and β-actin as the normalisation gene (Table 2). The total volume used was 25 μL and the amount of template RNA was established at 8 ng/reaction (after different a priori tests) The PCR conditions were: reverse transcription during 30 min at 50 °C; polymerase initial activation during 15 min at 95 °C; 40 cycles of denaturing during 15 s at 95 °C, annealing during 30 s at 56 °C and extension during 30 s at 72 °C; and a final melting curve from 70 °C increasing 0.5 °C every 5 s up to 95 °C to check for primer dimers (Table 2). The ΔΔCt method (Livak and Schmittgen, 2001) was used to determine the relative mRNA expression.

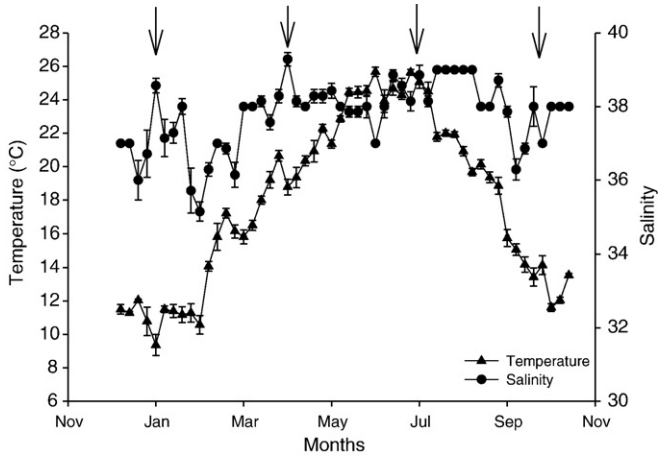


**Fig. 1.** Averaged weekly values for temperature and salinity during the experimental period. Data are shown as mean ± SEM. Arrows show the sampling points.

For species-specific primer design nucleotide GenBank database was searched for the different hormones in the genus *Sparus*. Only one sequence for PRL, with accession no. AF060541 (Santos et al., 1999) was found. For GH three sequences were present (S54890, U01301 and AY038038) with differences in length and in 5 out of 615 nucleotides from the shortest sequence (AY038038); primers were designed based on U01301 (Martínez-Barberá et al., 1994). Two different isoforms, L49205 and Y11144, were found for SL; oligo primers were designed from L49205 (Astola et al., 1996), although in regions where the identity is 95% for F1 primer (one mismatch for the first nucleotide in the 5′ end) and 100% for R1 primer with respect to Y11144. The size of the amplified product is the same for both isoforms. Last, beta-actin primers were designed from a clone isolated from our own cDNA libraries, constructed for other experimental purposes.

#### 2.2.2. Total protein purification and Western Blot analysis

Individual hypophyses were homogenized in 125 μL of homogenizing buffer at 4 °C (20 mM Tris–HCl, 5% glycerol, 5% SDS, 2% 2-mercaptoethanol, pH 6.8). Minced tissue was homogenized with a T 10 basic ULTRA-TURRAX®, three times at high speed, each for 40 s, at 4 °C. This homogenate was heated at 95 °C for 10 min and then centrifuged at 12,000 g for 10 min. After this, samples of the supernatant were collected and protein content was measured with the Lowry method (Lowry et al., 1951).

For electrophoresis, 25 μg of protein from each sample from the supernatants was loaded on polyacrilamide (37.5:1) gels (mini-gels). A 15% resolving gel, with 4% stacking gel, was used through Mini Protean II system (Bio-Rad). Protein standards used were prestained SDS-PAGE Standards Broad Range (Bio-Rad). Gels were run at 120 V for approximately 2 h. Proteins in the gels were transferred at 250 mA to Hybond-N membranes (GE Healthcare Life Sciences) in buffer containing 25 mM Tris–HCl, pH 8.3, 192 mM glycine and 20% methanol, for 2 h at room temperature, using a wet-transfer apparatus. Membranes were first blocked at RT with 5% dry non-fat milk in PBS (phosphate saline buffer) for 1 h, washed once in PBS, 0.1% Tween 20, and then incubated for 1 h with the following primary rabbit antibodies: antiPRL 1:5000, antiGH 1:5000, antiSL 1:10,000 and antiβ-actin 1:200. Antibodies against pituitary hormones were kindly provided by Dr. M. Valdivia, Cádiz, Spain

(Martínez-Barberá et al., 1994; Astola et al., 1996), while heterologous anti-β-actin was obtained from AnaSpec, San José, CA, USA. After washing three times for 15 min with PBS–Tween 20, blots were incubated with secondary antibody antirabbit IgG POD (Santa Cruz), diluted 1:3000, for one hour at room temperature, and followed by three washes in PBS–Tween 20. Antibody binding was visualized by addition of ECL plus (GE Healthcare Life Sciences) and exposure to autoradiographic film Curix RP2 (Agfa) between 2 and 30 s. Bands were captured using a digital video camera and the image was processed using Scion Image 3.0 software to estimate band intensity and surface area.

### 2.3. Statistics

Differences between seasons were tested by one-way ANOVA using the season as factor of variance. *A posteriori* Tukey's test was used to identify significantly different groups. Statistical significance was accepted at $P<0.05$.

## 3. Results

### 3.1. Temperature and salinity

Water temperature was different among seasons, with the highest value at summer (24 °C) and lowest at winter (12 °C). However, water salinity showed a smaller range of variation (36 in winter and 38 in summer) (Fig. 1).

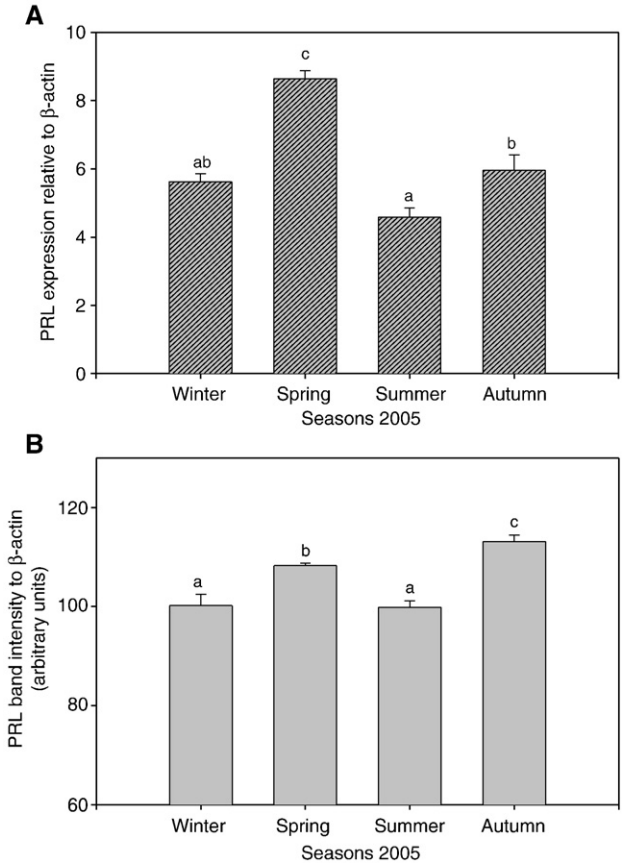


**Fig. 2.** Q-Real-Time PCR (A) and Western Blot (B) expression levels of PRL in pituitary of gilthead sea bream sampled seasonally. Different letters indicate significant differences among seasons (One way ANOVA, $P<0.05$).

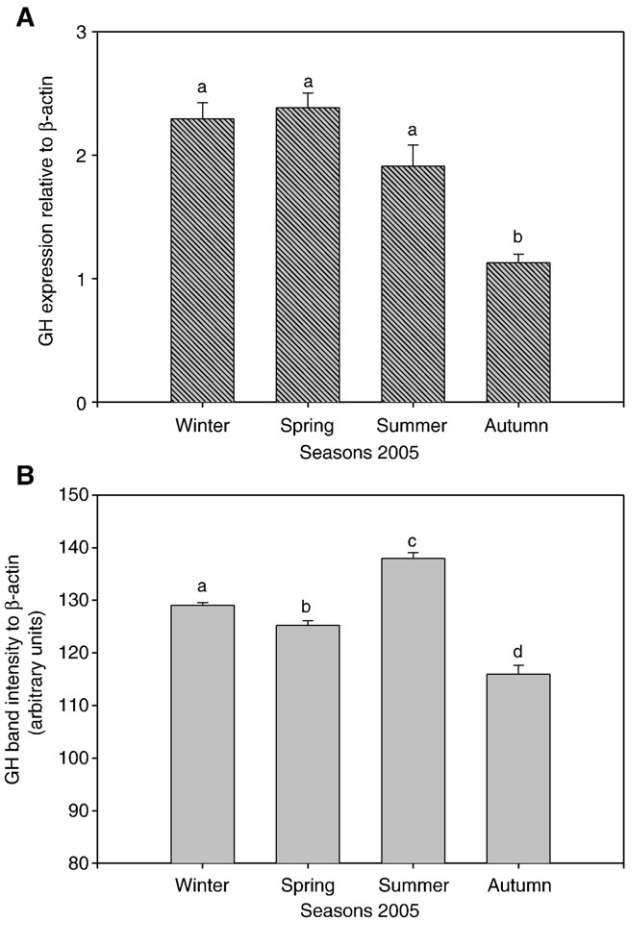


**Fig. 3.** Q-Real-Time PCR (A) and Western Blot (B) expression levels of GH in pituitary of gilthead sea bream sampled seasonally. Different letters indicate significant differences among seasons (One way ANOVA, $P<0.05$).

### 3.2. mRNA expression by Quantitative Real-Time PCR and protein expression by Western Blots

#### 3.2.1. PRL

Gene and protein expressions had similar patterns of variation (Fig. 2A and B), presenting significant differences ($F_{3,16}=31.16$; $P<0.05$ for gene expression; and $F_{3,16}=29.32$; $P<0.05$ for protein expression) among seasons, with the lowest value in summer and the highest values in spring and autumn.

#### 3.2.2. GH

Gene expression for GH was similar among seasons except for autumn, that showed the lowest value ($F_{3,16}=20.52$; $P<0.05$) (Fig. 3A). Pattern of GH protein expression was totally different among seasons and presented statistical differences ($F_{3,16}=98.70$; $P<0.05$), with the highest values during summer and the lowest value during autumn (Fig. 3B).

#### 3.2.3. SL

SL gene and protein expressions showed significant differences among seasons ($F_{3,16}=57.21$; $P<0.05$ and $F_{3,16}=101.89$; $P<0.05$ respectively) and similar pattern of variation, with the highest values during spring and summer and lower during winter and autumn (Fig. 4A and B). SL gene and protein expressions presented a linear and positive relationship with environmental temperature and photoperiod, showing protein expression the best correlation. This correlation is described adequately by the following linear

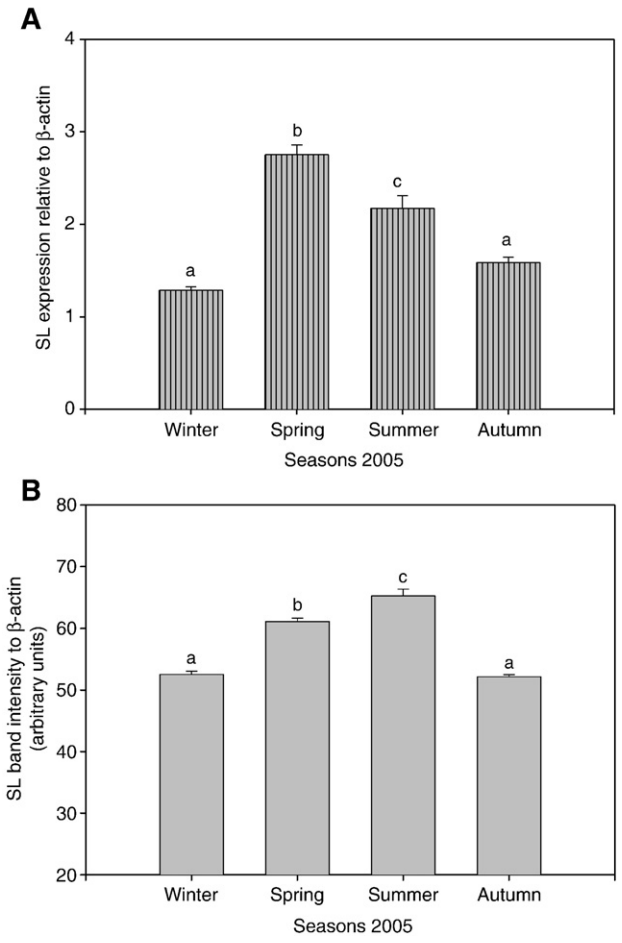


**Fig. 4.** Q-Real-Time PCR (A) and Western Blot (B) expression levels of SL in pituitary of gilthead sea bream sampled seasonally. Different letters indicate significant differences among seasons (One way ANOVA, $P<0.05$).

regression equations: SL gene = 1.1988Temp + 36.321 ($R^2 = 0.8692$) and SL protein = 184.155PH + 14.971 ($R^2 = 0.9373$).

## 4. Discussion

In this work seasonal changes of PRL, GH and SL gene and protein expression were studied in gilthead sea bream pituitaries using Real-Time Q-PCR and Western Blots, respectively. These techniques have become one of the most appropriate ways to detect and quantify mRNAs and proteins at pituitary level. β-Actin was used, both for Q-RT-PCR and Western analysis, as the housekeeping gene (Bustin, 2000, 2002); and its expression did not show significant differences among seasons (data not shown).

Several studies established that PRL is an important hormone for freshwater acclimation in euryhaline teleosts, with increased gene expression, synthesis, secretion and plasma levels after exposure to hypoosmotics environments (McCormick, 2001; Manzon, 2002; Sakamoto and McCormick, 2006; Mancera and McCormick, 2007). Our results indicated changes for PRL gene and hormone expressions between different seasons. However, these differences can not be ascribed to salinity fluctuations because of the scarce variation of this parameter among seasons of the year (Fig. 1). Higher expression at gene and protein levels was found during spring and autumn, when important differences in temperature and photoperiod, up to 8 °C and 3 h of light respectively, were observed with respect to winter. Pituitary PRL gene expression in *Cyprinus carpio* males is deeply influenced by seasonal acclimatization, suggesting that cyclic seasonal shifts are particularly relevant to eurythermal fish (Figueroa et al., 1994). In the same species, it has been shown that photoperiod constitutes a particularly relevant modulator in the neuroendocrine cascade that activates PRL transcription (Figueroa et al., 1997). In this way Falcon et al. (2003) showed that melatonin, strongly related to photoperiod, inhibits pituitary PRL secretion in *Oncorhynchus mykiss*. However, in *Mugil cephalus* it has been found that PRL rhythms do not have a relationship with any aspect of the photoperiod (Spieler et al., 1976). Our results suggested that PRL gene and protein expressions could play a role in the molecular signal transduction that environmental factors trigger in eurythermal fish, depending especially on temperature and photoperiod. Because the main break point for both variables occurs in spring and autumn, coinciding with the major PRL expression, both environmental variables could act, together or independently, as the "main factor". Further studies with a specific experimental design will be necessary to answer this question.

Several studies have analyzed pituitary GH expression and plasma levels in teleostean species. These studies focussed on seasonal variations (*S. aurata*: Pérez-Sánchez et al., 1994; *Oncorhynchus masou*: Bhandari et al., 2003; *C. carpio*: Figueroa et al., 2005), temperature influence (*O. mykiss*: Gabillard et al., 2003), relationship with growth (*S. aurata*: Pérez-Sánchez, 2000; Mingarro et al., 2002) and environmental salinity (*Oreochromis mossambicus*: Riley et al., 2003; *O. mykiss*: Shepherd et al., 2005). Our results showed a decrease in GH gene and protein expression during autumn. In *S. aurata*, the highest plasma GH concentration has been found from spring to early summer (Mingarro et al., 2002), while lowest values were observed in winter (Pérez-Sánchez et al., 1994). This species presents a significant and progressive decrease on plasma GH values along summer related to a concurrent increase in growth rates and plasma IGF-I-like immunoreactivity, in addition to an increase in hepatic GH-binding (Pérez-Sánchez and Le Bail, 1999). This ascent trend of GH plasmatic levels in summer and descent in winter, due to feeding behaviour and changes in photoperiod, has been also reported in other teleost species (Marchant and Peter, 1986; McCormick, 2001). It is interesting to remark that our results were obtained in fish maintained under natural environmental conditions where winter water temperature was around 12 °C. At that temperature a decrease in feeding behaviour could be expected, but not a complete food deprivation (Sala-Rabanal et al., 2003).

In *C. carpio*, GH pituitary gene and protein expressions were affected by seasonal variation, with the greatest increase during summer (Figueroa et al., 2005). Falcon et al. (2003) showed that in *O. mykiss* melatonin contribute to control the increase of GH secretion. In *O. masou* it has been observed seasonal changes for mRNA GH expression, with one peak in summer and another in winter (Bhandari et al., 2003). Our results on mRNA GH expression agree with the results previously reported for other species (see above), but not with the pattern of seasonal variation observed for plasma GH levels in sea bream, although the protein expression values were higher in summer and similar to plasma levels (Pérez-Sánchez et al., 1994; Pérez-Sánchez and Le Bail, 1999; Mingarro et al., 2002). The reason for these apparently contradictory results is unclear and the lack of plasma GH values in our study makes the data difficult to interpret. Nonetheless, the results indicate, as it has previously been suggested, that evaluation of a single GH related parameter is not sufficient to substantiate the activation of the GH axis (see Ágústsson et al., 2003).

The physiological action of SL in teleosts still remains to be unequivocally established, depending on the species studied (see Kaneko, 1996). It is considered that SL is involved in sexual maturation, because plasma levels and pituitary expression increased with sexual maturation more than with environmental factors (*Oncorhynchus kisutch*: Rand-Weaver et al., 1992; Rand-Weaver and Swanson, 1993; *O. masou*: Bhandari et al., 2003; *Oncorhynchus keta*: Onuma et al., 2003). Ágústsson et al. (2003), analyzing pituitary expression, found that SL appeared to be stable during smoltification of *Salmo salar*, ruling out the involvement of this hormone in the endocrine regulation

of the parr–smolt transformation process. Our results suggested that photoperiod (and/or temperature) could control SL expression in *S. aurata*. This agrees with the results reported for *Sciaenops ocellatus*, where one effect on plasma SL levels was especially influenced by photoperiod (early dark phase) and little by salinity (Zhu and Thomas, 1997, 1998). Ayson and Takemura (2006) found also a similar effect of light on SL expression in *Siganus guttatus*. In our study SL mRNA expression presented a peak in spring, when the changes in photoperiod and temperature were more marked, while pituitary protein expression increased at summer (where both abiotic parameters present the maximums values). However in *S. aurata* other study found a seasonal response of SL plasma levels during late autumn (Mingarro et al., 2002). This agrees with the stimulatory effect of SL on lipogenic enzymes and the lipid mobilisation observed in sea bream during winter time (Vega-Rubín de Celis et al., 2004). However, it has been demonstrated in the same species that long-term food deprivation, a situation typical of winter when environmental temperature decreases, failed to increase plasma levels of SL (Company et al., 2001). In this way, present results on SL mRNA and protein expressions related to seasonal variations and data reported for other studies on plasma SL seasonal changes are not conclusive. Further studies, including data on SL mRNA and protein expressions as well as plasma SL concentrations will be necessary to determine physiological action of SL in *S. aurata*. Another reason for these not conclusive results could be due to our incapacity to differentiate between both SL isoforms present in *S. aurata*, given primers were designed in the sequences common to both and with the same amplification size. This is a real limitation, because different isoforms can have different actions and play alternative roles according to environmental and physiological conditions.

In summary, expression of PRL, GH and SL in gilthead sea bream presented seasonal variation, suggesting that these hormones could play a role in the molecular signal transduction of environmental factor (especially photoperiod and temperature) in this species.

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