

Differential Regulation of Luteinizing Hormone and Follicle Stimulating Hormone Expression during Ovarian Development and under Sexual Steroid Feedback in the European Eel

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ABSTRACT

Pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are, in teleost as in mammals, under the control of hypothalamic factors and steroid feedbacks. In teleosts, feedback regulations largely vary depending on species and physiological stage. In the present study the regulation of FSH and LH expression was investigated in the European eel, a fish of biological and phylogenetical interest (as a representative of a primitive group of teleosts). The eel FSH β subunit was cloned, sequenced and together with earlier isolated eel LH β and glycoprotein hormone α (GP α) subunits used to study the differential regulation of LH and FSH. *In situ* hybridization indicated that FSH β and LH β are expressed by separate cells of the proximal pars distalis of the adenohypophysis, differently from the situation in mammals. The profiles of LH β and FSH β subunit expression were compared during experimental ovarian maturation, using dot-blot assays. Expression levels for LH β and GP α increased throughout ovarian development with a positive correlation between these two subunits. Conversely, FSH β mRNA levels decreased. To understand the role of sex steroids in these opposite variations, immature eels were treated with estradiol, (E₂) and testosterone (T), both steroids being produced in eel ovaries during gonadal development. E₂ treatment induced increases in both LH β and GP α mRNA levels, without any significant effect on FSH β . In contrast, T treatment induced a decrease in FSH β mRNA levels, without any significant effect on the other subunits. These data demonstrate that steroids exert a differential feedback on eel gonadotropin expression, with an E₂-specific positive feedback on LH and a T-specific negative feedback on FSH, leading to an opposite regulation of LH and FSH during ovarian development.

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INTRODUCTION

Pituitary gonadotropins (LH and FSH) as well as thyrotropin hormone (TSH) are members of the pituitary glycoprotein hormone family. In all vertebrates where they have been characterised, glycoprotein hormones consist of two subunits: a common glycoprotein hormone α -subunit (GP α) and a β -subunit (LH β , FSH β or TSH β) which confers to the hormone its biological specificity [for review: 1, 2].

In fish, reproduction is, as in mammals, under the control of the brain-pituitary-gonadal axis (B-P-G axis). However, this axis shows some phylogenetical variations. For instance in teleosts, production of gonadotropins is stimulated by gonadotropin-releasing hormone (GnRH) as in mammals, but is also repressed by dopamine [for review: 3]. In mammals, studies show that gonadal steroids play a negative feedback on pituitary hormones, but at certain points in the reproductive cycle such as the preovulatory surge, this effect can be reversed and become a positive feedback. In teleosts, data on steroid feedback indicated large variations according to the species, physiological stage, and methods of investigation [4-6].

A negative feedback was shown by the early work of Billard et al. [7], which demonstrated an increase in plasma LH levels after castration in rainbow trout. Negative feedback on LH was further evidenced in various teleosts [for review: 4, 8]. In contrast, other studies showed a positive feedback on LH, particularly when fish were treated at a juvenile stage: in European eel, injections of estradiol (E₂) induced an increase in pituitary LH content [9] and mRNA levels of GP α [10] and LH β subunits [11]. This effect was also demonstrated not only in other juvenile teleosts but also in adult fish with variations according to the stage of the reproductive cycle [for review: 4-6].

1 So far, data on FSH regulation are limited in fish and immunological or molecular
2 tools for FSH have been obtained only recently in a few teleost species. Recent studies
3 indicated the occurrence of a negative feedback on FSH, treatment with E₂ inducing a
4 decrease in mRNA levels of FSH β subunit in coho salmon [12]. In another salmonid,
5 Atlantic salmon, inhibitory or stimulatory effects of testosterone (T) on FSH were
6 demonstrated according to the period of the seasonal reproductive cycle [13].

7 The teleostean group includes a wide number of species with important evolutionary
8 diversity, leading to species specific variations in the regulation of the B-P-G axis.
9 Understanding the gonadotropin regulation in European eel, a representative of a
10 phylogenetical old primitive group of teleosts (elopomorphes), is of interest because it may
11 help to elucidate ancestral regulatory mechanisms [14]. Furthermore, it is of biological
12 interest since different aspects of the eel life cycle, such as sexual maturation and spawning
13 under natural conditions are still unknown. Indeed, eel gonadal development remains blocked
14 at a prepubertal stage as long as the oceanic reproductive migration does not occur, due to a
15 deficient production of pituitary gonadotropins, [for review: 15]. Gonadal development can
16 be induced in eel by long-term gonadotropic treatments, such as injections of carp or salmon
17 pituitary extracts in females European eel [16], and Japanese eel [17], or of human chorionic
18 gonadotropin in males of both species[17, 18]; however, artificial reproduction in eel is still
19 very challenging. Recently, the first Japanese glass eel have been produced in captivity, more
20 than 20 years after the first leptocephalus larvae were obtained, however egg quality is still
21 highly variable and the survival rate of the larvae very low [19]. Induction of sexual
22 maturation in female European eel and obtaining fertilised eggs appears even more difficult
23 than in the Japanese eel [20,21]. Requirement for further investigations on eel reproductive
24 endocrinology has become a critical issue in the context of the dramatic decline of eel
25 populations [22].

1 Until now, only LH had been investigated in the European eel, due to the lack of
2 molecular tools for FSH. In the present study, we cloned and sequenced the FSH β subunit.
3 This new tool, together with other previously developed molecular probes for the European
4 eel [23, 24], allowed us to characterize the localisation of FSH and LH expressing cells in the
5 pituitary and to investigate the variations of LH and FSH subunits expression under
6 hormonal treatments. We compared the expression of the two gonadotropins during the
7 experimental ovarian development induced by gonadotropic treatment, as well as under the
8 effect of steroid treatments (E₂ or T). This let us demonstrate that opposite feedback
9 mechanisms are involved in the regulation of LH and FSH expression during ovarian
10 development.

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MATERIALS AND METHODS

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Experimental fish

16 Female European eels, *Anguilla anguilla*, were caught by professional fishermen in
17 the Loire river (St Florent le Vieil, France), during their downstream migration (at the silver
18 stage, corresponding to early vitellogenesis). For gonadotropic treatment experiments, eels
19 were transferred to the Aquaculture station of Cemagref-Bordeaux (St Seurin sur Isles,
20 France). For steroid treatment experiments, and *in situ* hybridization studies, eels were
21 transferred to the laboratory at MNHN (Paris, France). The animals were handled in
22 accordance with European Union regulations concerning the protection of experimental
23 animals.

24

Gonadotropic treatment

25

1 *Preparation of Carp pituitary extract:* Carp pituitaries were extracted according to the
2 method described by Burzawa-Gérard [25]. In brief, one g of lyophilized carp pituitary
3 powder ('Catfish' company, Den Bosch, The Netherlands) was homogenized in 15 ml of
4 extraction buffer: 0.15M NaCl, 0.01M phosphate buffer pH 7.4, containing 0.04 mg/ml
5 protease inhibitor Aprotinin (Sigma Chemical Co., St Louis, MO, USA). The homogenate
6 was left overnight at 4°C. After centrifugation (4000g) for 10 min, the supernatant was
7 removed and stored at 4°C. 5 ml of extraction buffer was added to the pellet. The solution
8 was frozen, defrosted, centrifugated and the supernatant was removed and stored at 4°C. This
9 manipulation was repeated twice.

10 *Eel treatment:* 42 eels (846±50g) were used. A group of 7 eels was kept in freshwater
11 (FW controls) while the others were acclimatized to natural seawater (SW) tanks under
12 natural temperature. Treatment was started after 3 weeks of SW acclimatization. Two groups
13 of 7 eels (FW controls and SW controls) were sacrificed at the onset of treatment. The other
14 eels (28) were kept in SW and received one perivisceral injection a week of carp pituitary
15 extract at a dose equivalent to 20 µg pituitary powder / g body weight, according to the
16 method previously developed [16,26]. Groups of 7 eels were sacrificed at different times up
17 to 16 weeks of treatment.

18

19 *Steroid treatments*

20 24 eels (423 ± 7g) were acclimatized to artificial sea water (Aquarium systems,
21 Strasbourg, France) at 16 +/- 2°C, and distributed in three experimental groups of 8 eels. Eels
22 received one perivisceral injection a week of 2 µg / g E₂, or T (Sigma) suspended in saline
23 (0.15 M NaCl) according to [23], or of saline alone (control eels), during 7 weeks.

24

25 *Sampling procedure*

1 Sample collections were performed one week after the last injections. Eels were
2 sacrificed by decapitation. The pituitary glands were quickly removed, frozen in liquid
3 nitrogen and stored at -80°C until extraction. Ovaries were dissected and weighed for
4 gonadosomatic index determination ($\text{GSI} = (\text{gonad weight} / \text{body weight}) \times 100$).

5

6 *Full-length cDNA sequence of FSH β subunit*

7 A European eel pituitary cDNA library constructed into $\lambda\text{gt}10$ vector [23, 24] was
8 used to amplify a partial FSH β cDNA. Gene specific primers (primer 2 and primer 4, Table
9 1) were designed based on the nucleotide sequences of the Japanese eel (*Anguilla japonica*)
10 FSH β cDNA (GenBank Accession No. AB016169, [28]). The conditions for PCR were as
11 follows: 2 μl of amplified library lysate was mixed with 0.4 μM of each primer, 200 μM
12 deoxynucleotriphosphate, 10 mM Tris-HCl, 1.5 mM MgCl_2 , 50 mM KCl, pH 8.3 and 1 U
13 Taq DNA polymerase in a final volume of 50 μl . Cycling parameters were 3 min
14 denaturation at 94°C followed by 30 cycles of 1 min denaturation at 94°C , 1 min annealing
15 at 53°C and 1 min elongation at 72°C , the final elongation step was extended to 10 min.

16 To obtain a full-length sequence, FSH β cDNAs were amplified from pituitary total
17 RNA using the RACE (Rapid Amplification of the cDNA Ends) method.

18 - 3'RACE. First strand cDNA was synthesized from 1 μg of total RNA, using a modified
19 oligo (dT) primer (3'-RACE CDS Primer A, Table 1) according to the SMARTTM RACE
20 cDNA Amplification Kit (Clontech Laboratories Inc, Palo Alto, CA, USA) and used as a
21 template for PCR amplification with a gene specific primer 2 and a Universal Primer A mix
22 (Table 1), containing the complementary sequence to the oligo(dT) primer.

23 - 5'RACE. First strand cDNA was synthesized from 1 μg of total RNA, using a modified
24 oligo (dT) primer (5'-RACE CDS Primer, Table 1) and the SMART II A oligo which is
25 incorporated at the 5' end (Clontech Smart Technology). This first strand cDNA is used as a

1 template for PCR amplification with a gene specific primer 3 and a Universal Primer A mix
2 (Table 1), containing the complementary sequence to the SMART oligo.

3 Both 3' and 5' RACE PCR reactions were performed in 50 μ l using Clontech
4 Advantage 2 Polymerase Mix (0.2 μ M of each primer, 200 μ M deoxynucleotriphosphate, 1x
5 Clontech Advantage 2 polymerase buffer, 1x Clontech Advantage 2 polymerase mix).
6 Thermal cycling was performed on a MWG Thermalcycler (MWG Biotech, Ebersberg,
7 Germany) using the following programme: 5 cycles 94° C - 30 sec, 72° C - 2 min, 5 cycles
8 94° C - 30 sec, 70° C - 30 sec, 72° C - 2 min, 30 cycles 94° C - 30 sec, 66° C - 30 sec, 72° C -
9 2 min.

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11 *Subcloning and sequencing*

12 The PCR products were separated by agarose gel electrophoresis, and purified using a
13 gel purification kit (BioRad Laboratories, Hercules, CA, USA). The PCR products were
14 subcloned into pGEM T Easy plasmids (Promega, Madison, WI, USA). PCR products of 5
15 independent PCR amplifications were cloned to avoid PCR errors. Both strands were
16 sequenced with the CEQ™ 8000 sequencer (Beckman Coulter Inc, Fullerton, CA, USA)
17 using a dye terminator cycle sequencing kit (Beckman Coulter).

18

19 *Sequence analysis*

20 Nucleotide sequence identity was performed using the BLAST program (Genbank,
21 NCBI). The signal peptide and putative cleavage site were predicted using the Signal P V2.0
22 (Center of Biological Sequence Analysis, Danmark <http://www.cbs.dtu.dk>). Alignment of the
23 amino acid sequences and analysis of sequence similarities were performed using Clustal W
24 multiple sequences alignment program (Clustal W, WWW Service at the European
25 Bioinformatics Institute).

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Northern blot analysis

The specificity of the isolated FSH β subunit probe was assessed by Northern Blot analysis. Pituitaries from female European eels were collected and RNA was extracted using Trizol reagent (Invitrogen AB, Sweden), following the manufacturer's instructions. Five micrograms of total RNA was denatured, subjected to electrophoresis, blotted on Hybond-N+, Nylon membrane (Amersham Biosciences, Uppsala, Sweden) and UV- crosslinked. A 426 bp long FSH β cDNA fragment, obtained by PCR using the primer 1 and primer 2 (including the full coding region) and subcloned into pGEM T Easy, was used as probe for hybridizations. An antisense FSH β probe was synthesized by *in vitro* transcription using T7 RNA polymerase and labelled with [α -³²P] dUTP according to the Strip-EZTM RNA kit (Ambion Inc, Austin, Texas, USA). The blot was prehybridized for 1 hour at 65° C, with ULTRAhyb hybridization solution (Ambion Inc, Texas, USA) and then incubated over night with the radioactive probe (1x10⁶ cpm/ml hybridization solution). After hybridization the blot was washed twice in 1 X SSC, 0.1% SDS at 65° C for 15 minutes each and then 15 min in 0.1 X SSC, 0.1% SDS at 65°C and 30 min at 68° C. The hybridization signals were analyzed using a phosphorimager (Molecular imager, Bio-Rad laboratories Inc).

Dot blot assay of LH β , FSH β and GP α mRNAs

Individual pituitaries were homogenized in 200 μ l of Trizol reagent and sonicated a few seconds. Total RNA was extracted and dot blot were performed according to the method previously described [29] with a few modifications. RNA was denatured in 50% formamide, 7% formaldehyde and 1xSSC at 65°C for 15 min and blotted onto Hybond-N+ Nylon membrane (Amersham Biosciences, Uppsala, Sweden) through a Hybri-blot manifold

1 (Invitrogen AB, Sweden). The membrane was air-dried and RNAs were immobilized by
2 baking at 80°C for 2h.

3 The following probes were used in dot blot analysis: FSH β probe (426 bp, as
4 described above), LH β probe (404 bp, [23]) and GP α subunit probe (371 bp, [24]). All
5 probes revealed single bands after northern hybridization, which made them suitable to use in
6 dot-blot assays. RNA probes were synthesized by *in vitro* transcription and labeled with [α -
7 ³²P]UTP according to the Strip-EZTM RNA kit (Ambion Inc). The membrane was
8 prehybridized for 1h at 65°C with a hybridization solution (ULTRAhyb, Ambion Inc) and
9 then hybridized with the labelled RNA probe (1 x 10⁶ cpm/ml of hybridization solution)
10 overnight at 65°C. After high stringency washings (see above) the membranes were scanned
11 and analyzed with a phosphor imager. Membranes were stripped according to the protocol of
12 the Strip-EZTM RNA kit (Ambion Inc) and hybridized under the same conditions with a
13 different probe. To correct for unequal RNA loadings the blots were subsequently
14 hybridized with a 18s rRNA eel cDNA probe labelled with [α -³²P]dATP according to the
15 Strip-EZTM DNA kit (0.5x10⁵ cpm/ml of hybridization solution) as internal standard.

16

17 *In situ hybridization*

18 *Animals and tissue preparation:* Six female silver eels (250-300 g) were
19 anaesthetized by immersion in 0.2% 3-aminobenzoic acid ethyl ester solution and perfused
20 intracardially with saline phosphate buffer (PBS = 0.6% NaCl in 0.1 M phosphate buffer, pH
21 7.4) and then with fixation solution (4% paraformaldehyde in PBS), according to our
22 previous procedure [30,31]. The skull was removed; the brain and attached pituitary were
23 carefully dissected and then stored overnight in fresh fixation solution at 4°C. On the
24 following day, the brain and attached pituitary were immersed for 8 h in 15% sucrose in PBS

1 at 4°C. For cryostat sectioning, the tissues were frozen in cold iso-pentane and stored at -
2 80°C.

3 *cRNA probes:* cDNAs encoding the eel FSH β fragment (426 bp) and eel LH β
4 fragment (404 bp) inserted in pGEM T Easy Vector (Promega) were used as matrix for FSH β
5 and LH β probes. The plasmids were linearized and antisense probes were synthesized by *in*
6 *vitro* transcription using T7 RNA polymerase (FSH β), SP6 RNA polymerase (LH β) and
7 labeled with digoxigenin-UTP (Roche Diagnostics, Meylan, France).

8 *In situ hybridization procedure:* The hybridization was carried out on 26 micrometers
9 transversal pituitary sections cut with a cryostat (Leica, Germany), thaw-mounted on
10 Superfrost plus slides (Gassalem, France), and stored at -80°C until use. On the day of
11 hybridization, the sections were quickly brought to room temperature, rinsed in 2 X standard
12 saline citrate (SSC), acetylated, treated with Tris 0.1 M glycine (pH 7), rinsed in 2 X SSC,
13 dehydrated in a series of concentrations of ethanol and air-dried. Hybridization was carried
14 out as previously described [31]. In brief, the probes were diluted (1 μ g/ml) in a mixture
15 containing 50% formamide, 10% dextran sulfate, 1 mg/ml baker yeast tRNA, 1 X Denhardt's
16 solution. Alternate sections were hybridized respectively with FSH β and LH β probes.
17 Hybridization was carried out in humid chambers overnight at 60°C and stopped by three
18 washes (15 min and 2 x 30 min) in 50% formamide, 1 X SSC, 0.1% Tween-20 at 60°C,
19 followed by two 30 min washes in MABT (0.1 M maleic acid, 0.15 M NaCl, 0.1% Tween-
20 20, pH 7.4).

21 *Detection:* For immunodetection of the digoxigenin-labelled probes, the sections were
22 saturated in MABT containing 20% normal goat serum (Sigma) and 2% blocking reagent
23 (Roche) for 1 hour, then incubated overnight at room temperature with antidigoxigenin Fab-
24 fragments conjugated with alkaline phosphatase (Roche) diluted 1:2000 in MABT. After four
25 20 min washes in MABT, the alkaline phosphatase activity was visualized with a freshly

1 prepared colour solution containing 1.65 mg 5-bromo-4-chloro-3-indolylphosphate and 3.3
2 mg nitro blue tetrazolium substrate (Boehringer Mannheim, Germany) according to the
3 manufacturer's instructions. Sections were analyzed with a Leica-DMRB microscope and
4 recorded with a Leica digital camera.

5

6 *Statistical analysis*

7 Results are given as mean +/- SEM. Comparisons of means were analysed with one-
8 way ANOVA followed by Tukey's HSD multiple comparison test. Data were log
9 transformed to meet homogeneity of variances. Correlations were tested using Pearson's test.
10 P values of less than 0.05 were considered to indicate statistically significant differences.

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RESULTS

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15 *Full-length cDNA encoding FSH β subunit*

16 The full-length cDNA of the European eel FSH β subunit was compiled from the data
17 obtained by the 5' and 3' RACE. The cDNA was 1069 bp long and had an open reading
18 frame of 381 bp, beginning with the first ATG codon at position 35 bp and ending with the
19 stop codon at position 416 bp. A putative polyadenylation signal ATTAAA was recognized
20 17 bp upstream from the poly-A tail. The position of the signal peptide cleavage site was
21 determined using the Signal P V2.0 program, predicting a signal peptide of 19 amino acids
22 and a mature peptide of 108 amino acids. Two potential N-linked glycosylation sites were
23 found at position 8 and 25 asparagine residues from the N-terminus of the putative mature
24 peptide. The cloned FSH β sequence showed 97 % sequence identities with the Japanese eel

1 FSH β at both the amino acid and the nucleotide levels. The sequence was submitted to the
2 GenBank on Oct 28, 2002 (accession no. AY169722, Schmitz et al.).

3 The specificity of the 426 bp FSH β probe was validated by Northern blot. The
4 analysis of total pituitary RNA hybridized with labelled FSH β revealed a single band of
5 about 1280bp (Fig 1).

6

7 *Distribution of LH and FSH expressing cells in the eel pituitary*

8 Expression of LH β - and FSH β mRNA was detectable by *in situ* hybridization in the
9 pituitary of the six female silver eel investigated (Fig 2.). In all cases, LH β and FSH β
10 hybridization signals were observed only in the proximal pars distalis (PPD) of the pituitary
11 (median part of the adenohypophysis, where the gonadotrophs are known to be located [32]).
12 No signal was observed in the anterior part of the pituitary (rostral pars distalis, RPD) nor in
13 the posterior part (pars intermedia, PI). The photos (Fig. 2) show alternate transverse sections
14 of eel pituitary, hybridized with FSH β probe (Fig. 2A) and LH β probe (Fig. 2B),
15 respectively. Hybridization signals of LH β and FSH β were not observed in the same area of
16 the PPD on adjacent sections (Fig 2A and 2B) indicating that both subunits are expressed by
17 different cells. No hybridization signals were found with sense LH β or FSH β probes.

18

19 *Effect of gonadotropic treatment*

20 In control eels, no variation in any parameter was observed after transfer from fresh
21 water (FW) to sea water (SW) (Fig 3 A, B, C, D). Long-term treatment with carp pituitary
22 extract induced a gradual ovarian development (Fig. 3A). After 16 weeks, GSI reached 33 +/-
23 3%, a much higher value ($P < 0.001$) than in controls (GSI = 1.8 +/- 0.1%).

24 Treatment with carp pituitary extract also induced a strong increase in LH β subunit
25 mRNA levels which reached, after 16 weeks, a value 120 times higher than that seen in

1 control eels ($P < 0.001$) (Fig. 3C). We also observed in these animals an increase in GP α
2 subunit mRNA levels (x 8 as compared to control eels after 16 weeks, $P < 0.001$) (Fig. 3D).
3 In contrast, FSH β mRNA levels significantly decreased after 16 weeks (x 0.2 as compared to
4 controls, $P < 0.001$) (Fig. 3B).

5 Correlation studies between individual variations in mRNA levels of gonadotropin
6 subunits and GSI (Fig. 4) showed a highly significant positive correlation between GSI and
7 LH β ($r = 0.78$, $n = 42$, $P < 0.001$) (Fig. 4B) and between GSI and GP α ($r = 0.76$, $n = 42$, $P <$
8 0.001) (Fig. 4C). Inversely, mRNA levels of FSH β were negatively correlated with GSI ($r = -$
9 0.54 , $n = 42$, $P < 0.01$) (Fig. 4A). Comparison between subunit mRNA levels (Fig. 5) showed
10 a strong positive correlation between LH β and GP α ($r = 0.92$, $n = 42$, $P < 0.001$) (Fig. 5B).
11 Conversely, we observed negative correlations between mRNA levels of FSH β and GP α ($r =$
12 -0.45 , $n = 42$, $P < 0.01$) (Fig. 5A) and between FSH β and LH β ($r = -0.52$, $n = 42$, $P < 0.01$)
13 (Fig. 5C).

14

15 *Effect of sex steroid treatments*

16 Neither E₂ nor T treatment induced, after 7 weeks, any significant variation in GSI as
17 compared to control eels (data not shown). E₂ treatment had no effect on FSH β mRNA levels
18 (Fig. 6A) but significantly increased LH β mRNA levels (x 6.5 as compared to control eels,
19 $P < 0.01$) (Fig. 6B) as well as GP α mRNA levels (x 2.4 as compared to control eels, $P <$
20 0.001) (Fig. 6C). In contrast, T, which had no effect on LH β nor GP α mRNA levels (Fig. 6B
21 and C), induced a significant decrease in FSH β mRNA levels (x 0.3 as compared to controls,
22 $P < 0.001$) (Fig. 6A). Correlation studies between individual variations in mRNA levels of
23 gonadotropin subunits in eels from the three groups showed a positive correlation between

1 LH β and GP α ($r = 0.73$, $n = 24$, $P < 0.01$), but no significant correlations between LH β and
2 FSH β nor FSH β and GP α .

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DISCUSSION

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7 In this study, the full-length cDNA encoding European eel FSH β subunit was cloned
8 and its nucleotide sequence determined. The cDNA was 1069 bp long, encoding a 127 amino
9 acid peptide. A signal peptide of 19 amino acids and a mature peptide of 108 amino acids
10 were predicted. The sequence was submitted in October 2002 to the GenBank (accession no.
11 AY169722) and later confirmed by Degani et al. [33]. The amino acid sequence encoding the
12 mature FSH peptide shared 98 % amino acid identities with the Japanese eel and 71%
13 identities with other anguilliformes (*Conger conger* and *Conger myriaster*). Sequence
14 similarities between the European eel, a phylogenetically old teleost and relatively modern
15 teleosts (cyprinodontiformes, salmoniformes and perciformes) were comparatively low and
16 ranged from 38% to 45% supporting the idea that FSH β rapidly diversified during the
17 evolution of teleost species [2].

18 *In situ* hybridization of eel pituitary with homologous LH β and FSH β probes provided
19 the first data on the distribution of the pituitary cells expressing messenger RNAs for LH β
20 and FSH β in the eel. LH β and FSH β hybridization signals were both detected in the pituitary
21 of European female silver eel and were observed only in the proximal pars distalis (PPD) of
22 the pituitary, while no signal was observed in the anterior part or in the posterior part of the
23 pituitary. In the PPD itself, hybridization signals for LH β and FSH β were not observed in the
24 same area on adjacent sections, suggesting that LH and FSH are expressed by separate cells in

1 the eel. Further studies, using co-hybridization with both probes on the same section, are
2 currently developed to firmly address this question. In mammals, it was demonstrated that
3 FSH and LH are expressed by the same pituitary gonadotropic cells [34,35]. In contrast,
4 studies in teleosts by immunocytochemistry and *in situ* hybridization suggested, as in our
5 study, that LH β and FSH β are expressed by separate cells: in salmonids [36-38], tuna [39],
6 tilapia [6,40], gilthead sea bream [41] and halibut [42]. The expression of LH β and FSH β by
7 the same cells, as in mammals, or by separate cells, as in teleosts, is an important evolutionary
8 divergence, with potential implications for the regulatory mechanisms of the two
9 gonadotropins. Our study in a representative of an early group of teleosts indicates that the
10 expression of LH β and FSH β by separate gonadotropic cells, is an original and ancestral
11 character among teleosts.

12 Using specific molecular probes, we investigated the differential regulation of the
13 expression of LH β and FSH β , under experimental hormonal treatments. In agreement with
14 previous studies [16,43], long-term treatment with carp pituitary extract stimulated ovarian
15 vitellogenesis, leading to a gradual increase in GSI which reached 33% after 16 weeks, an
16 index much higher than in control eels (1.8 %). The gonadotropic treatment also induced
17 strong increases in LH β and GP α mRNA levels. This rise in LH β and GP α subunits mRNA
18 levels appeared to be concomitant with the increase in GSI as indicated by strong positive
19 correlations between GSI and mRNA levels of these subunits. These results are in agreement
20 with our previous studies, where an increase in pituitary content of LH hormone, as measured
21 by RIA, was observed in experimentally matured eels [43]. In addition, our results are in
22 agreement with those obtained in the Japanese eel, showing an increase in LH β and GP α
23 mRNA levels during the induction of ovarian development [44,45]. These data indicate that

1 the increase in pituitary LH synthesis during induced ovarian development in the eel, is
2 mediated by increases in LH β and GP α mRNA levels.

3 A stimulation in LH synthesis has currently been observed in various teleost species,
4 both during experimentally induced gonadal development in juvenile fish (rainbow trout
5 [46]), as well as during natural sexual maturation in adult fish (rainbow trout [47]; goldfish
6 [48]; tilapia [49]; striped bass [50]). The increase in pituitary content of LH hormone
7 probably serves as a “reserve” which will permit the LH surge triggering ovulation or
8 spermiation, at the end of gametogenesis. The strong positive correlation between mRNA
9 levels of GP α and LH β , observed in our study, indicates a possible common regulation of the
10 two subunits during gonadal development. However, the rate of stimulation of GP α was
11 lesser than that of LH β . This could be explained by the fact that the GP α subunit is common
12 to LH, FSH and TSH hormones, and only the fraction of α subunit expressed in LH cells may
13 be affected by the positive regulation.

14 Concerning the regulation of FSH β mRNA, these results are the first obtained in the
15 European eel. In the silver female European eel, the abundance of mRNA transcripts coding
16 for the FSH β was low, but detectable in individual pituitaries. In contrast with the observation
17 for LH β and GP α , there was no increase in expression levels of FSH β after gonadotropic
18 treatment. Inversely, during the experimental gonadal development, a significant decrease in
19 the mRNA levels of FSH β was observed. The correlation analysis indicates that this decrease
20 was gradual and concomitant with the increase in GSI.

21 Thus, our data show an opposite regulation of LH β and FSH β during experimental
22 maturation, with an increase in LH β and a decrease in FSH β mRNA levels. This opposite
23 regulation was substantiated by a significant negative correlation between individual LH β and

1 FSH β levels. In the Japanese eel, a dramatic decrease in FSH β mRNA levels was reported in
2 experimentally matured males and females, and FSH β mRNA levels were undetectable after
3 14 weeks of gonadotropic treatment [28]. So, in both Japanese and European eels, an opposite
4 regulation in FSH and LH is observed during induced maturation. Supposing that FSH
5 hormone synthesis and release follow the same profile as its mRNA expression, it is likely
6 that FSH does not play a crucial role at the final stages of eel gonadal development, in
7 contrast with LH. However, we must be aware that these results were obtained under
8 experimentally induced maturation and hormonal patterns might be different under natural
9 conditions, as suggested by recent results obtained in New Zealand long finned eels, *Anguilla*
10 *diffenbachii* [45].

11 Regarding the first stages of ovarian development, the comparison between Japanese
12 and European eels shows some discrepancies: a strong expression of FSH β was observed in
13 untreated Japanese eel while LH β was undetectable [28]. This led the authors to suggest that
14 only FSH would be involved in the control of the early stages of vitellogenesis, while LH
15 would trigger the final steps. In contrast, our results from *in situ* hybridization as well from
16 dot blot analysis indicate that, in untreated female silver eels, both FSH β and LH β were
17 detectable and expressed at a low level, suggesting potential roles of the two gonadotropins
18 during the early stages of vitellogenesis. Similarly, a low expression of FSH β at the early
19 vitellogenic stage was also observed in the New Zealand long finned eel [45] contrasting a
20 high expression of FSH β found in the Japanese eel [45]. These studies suggest some common
21 and some different features in the expression and potential roles of LH and FSH during
22 gonadal development among different eel species,

23 Large variations in gonadotropin expression during gametogenesis have been observed
24 among different teleost species [51]. In salmonids, such as coho salmon (*Onchorynchus*

1 *kisutch*), plasma levels of FSH are high in the first stage of vitellogenesis, while LH is
2 essentially undetectable. This situation reverses in the late stage of vitellogenesis when LH
3 predominates [52]. Similar expression profiles of FSH β and LH β were obtained in rainbow
4 trout (*Onchorynchus mykiss*) [53], suggesting that two gonadotropins play separate roles
5 during gonadal development in salmonids: FSH would control the first stages of
6 gametogenesis, whereas LH would act during the final steps [54]. In contrast, in a cyprinid,
7 the goldfish (*Carassius auratus*), both LH β and FSH β subunits were expressed early in
8 immature fish and remained elevated throughout the ovarian development [55]. Among
9 perciforms, various profiles have been observed: in juvenile striped bass, *Morone saxatilis*,
10 FSH β mRNA levels were lower than LH β mRNA levels, but increased quickly at the onset of
11 gonadal development, suggesting a role for FSH in the onset of vitellogenesis [56]. In the sea
12 bass, *Dicentrarchus labrax*, mRNA levels for the three subunits (GP α , LH β and FSH β)
13 increased simultaneously throughout the sexual maturation [57, 58], providing no indication
14 for differential roles of LH and FSH. All these results indicate a strong heterogeneity in the
15 regulation and potential physiological roles of the two gonadotropins between teleost species,
16 even among the same sub-group of teleosts.

17 In the female European eel, experimental maturation induces an increase in production
18 of sexual steroids, E₂ and T [59,60]. Our previous studies demonstrated that sexual steroids
19 were responsible for the positive feedback on LH. First, we could show that castration
20 prevents the increase in LH content (as measured by RIA) induced by the gonadotropic
21 treatment [43]. Second, E₂ treatment was able to induce an increase in LH synthesis [9,27]),
22 an effect mediated by increases in mRNA levels for its subunits, GP α and LH β [10,11]. In the
23 present study, we compared the effect of sex steroids on both LH β and FSH β subunit mRNA
24 levels. E₂ -, but not T- treatment, induced a significant increase in LH β and GP α mRNA

1 levels, in agreement with our previous data. This indicates that E₂ is likely to be responsible
2 for the positive regulation of LHβ and GPα subunits occurring during induced gonadal
3 development. In contrast, the present first data on FSHβ indicate no effect of E₂ on FSHβ
4 mRNA levels. Conversely, T treatment had a significant negative effect on FSHβ mRNA
5 levels. This suggests that T could account for the negative regulation of FSHβ during
6 experimental gonadal development. The combination of T and E₂, which are both secreted
7 during ovarian development in the eel, is therefore likely responsible for the opposite
8 regulation of LH and FSH expression during induced maturation. Furthermore, these two
9 sexual steroids could also play a synergic role in these positive and negative feedbacks.
10 Indeed, we previously demonstrated a potentiating effect of T on E₂-stimulation of LH and
11 mammalian type-GnRH (mGnRH) levels in the eel [27]. Further studies are needed to
12 investigate their combined effect on FSH.

13 These are the first results concerning the differential regulation of LH and FSH by
14 sexual steroids in the eel. Opposite feedbacks by sexual steroids on LH and FSH have also
15 been observed in some other teleosts. In a cyprinid, the goldfish, both T and E₂ had an
16 inhibitory effect on FSHβ mRNA levels, while they had a stimulatory action on LHβ mRNA
17 levels [48]. Similar results were found in a perciform, the sea bass, where treatment with T
18 and E₂ suppressed basal FSHβ expression, but slightly increased LHβ expression [57,58]. In
19 contrast, such regulations were not evidenced in salmonids. In female coho salmon, neither T
20 nor E₂ had any effect on FSHβ mRNA [12]. In male Atlantic salmon, the effect of T on FSH
21 synthesis was shown to be inhibitory or stimulatory according to the season [13]. This
22 suggests important variations in steroid regulation of the gonadotropin subunits in teleosts,
23 depending on the species and the physiological stage.

1 In the European eel, we demonstrated a differential feedback by steroids on
2 gonadotropins. It is suggested that T and E₂, which are both secreted by the eel ovary, could
3 therefore be responsible for the opposite variations of LHβ and GPα on one side, and FSHβ
4 on the other side observed during experimental maturation. However, this does not exclude
5 that additional factors are involved in the differential regulation of LH and FSH during
6 maturation. A few investigations in teleosts have been performed on gonadal peptides (inhibin
7 /activin), which play a crucial role in the regulation of FSH in mammals [for review: 61].
8 Data in goldfish indicate an opposite effect of recombinant goldfish activin B on FSHβ and
9 LHβ, with a stimulatory effect on FSHβ and an inhibitory action on LHβ [62]. In other teleost
10 species, contradictory results were obtained after treatments with human or porcine
11 recombinant activin or inhibin and more investigation are clearly required [for review: 6].

12 The effect of sexual steroids, as observed in the present study, may be exerted directly
13 on pituitary cells, or indirectly, at the brain level. Our previous data indicated that the up
14 regulation of LH induced by E₂ *in vivo*, was not a direct action on pituitary cells. Indeed, in
15 vitro studies with primary culture of eel pituitary cells, demonstrated that E₂ had no
16 stimulatory effect on LHβ and GPα mRNA levels, or on the LH protein [29]. In contrast, E₂
17 action was likely mediated through GnRH stimulatory control of LH and GPα expression [for
18 review: 6], as suggested by the significant increase in eel brain and pituitary mGnRH levels
19 under E₂ treatment [27], as well as during induced maturation [26,43]. Similarly, preliminary
20 experiments on the regulation of FSH expression in vitro also suggest that the effect of
21 testosterone is not directly exerted at the pituitary level.

22 In conclusion, cloning of the eel FSHβ subunit allowed us to investigate the
23 differential regulation of the expression of LH and FSH in the European eel. LH and FSH
24 were expressed by different cells in the PPD of the pituitary as suggested by *in situ*

1 hybridization. During experimental maturation, LH and FSH undergo an opposite regulation:
2 mRNA levels of LH β and GP α increased, and inversely FSH β mRNA levels decreased.
3 Differential regulations were also observed after *in vivo* treatment with sexual steroids: E₂
4 induced an increase in LH β and GP α mRNA levels, while T induced a decrease in FSH β
5 mRNA levels. The differential feedbacks by T and E₂ could therefore account for the opposite
6 regulation of LH and FSH during gonadal development.

7

8

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FIGURE LEGENDS

FIG. 1. Northern blot analysis of pituitary mRNA for FSH β . Five micrograms of total RNA extracted from pools of female silver eel pituitaries were electrophoresed. Hybridization with eel FSH β probe revealed a single transcript at about 1280bp. The position of RNA molecular weight markers are shown on the right.

FIG. 2. Localization by *in situ* hybridization of FSH β (Fig. 2A) and LH β (Fig. 2B) expressing cells in the eel pituitary. Adjacent transversal sections of female silver eel pituitary were hybridized with digoxigenin-labelled RNA probes for eel FSH β and LH β . Arrows indicate cells positive for FSH β or LH β antisense probes. Hybridization signals were only observed in the proximal pars distalis (PPD) of the adenohypophysis. No labelling was shown in the pars intermedia (PI). Labelling of FSH β and LH β were not observed in the same areas of the PPD suggesting the expression of each hormone by different cells. Mediobasal hypothalamus (MBH). Bar = 100 μ m.

FIG. 3. Effects of gonadotropic treatment on ovarian development and expression of gonadotropin subunits. Female silver eels were transferred to sea water and treated for up to 16 weeks (w) with carp pituitary extract which induced a progressive increase in gonadosomatic index (Fig. 3A). Pituitary mRNA levels for gonadotropin subunits were measured by dot blot using RNA probes for eel FSH β (Fig. 3B), LH β (Fig. 3C) and GP α (Fig. 3D) and normalized using 18s RNA. FW = freshwater control eels, SW = seawater control eels. Means are given \pm SEM (n = 7 eels/group). ***, $P < 0.001$, as compared to FW or SW controls, ANOVA.

FIG. 4. Correlations between individual mRNA levels for gonadotropin subunits and gonadosomatic index (GSI) from control eels and eels treated with carp pituitary extract. For details, see legend of Fig 3. Negative correlation was observed between GSI and FSH β ($P < 0.01$, Fig. 4A), while positive correlations were observed between GSI and LH β ($P < 0.001$, Fig. 4B), and between GSI and GP α ($P < 0.001$, Fig. 4C).

FIG. 5. Correlations between individual mRNA levels for various gonadotropin subunits from control eels and eels treated with carp pituitary extract. For details, see legend of Fig 3. Negative correlations were observed between FSH β and GP α ($P < 0.01$, Fig. 5A) and between FSH β and LH β ($P < 0.01$, Fig. 5C); in contrast, a highly significant positive correlation was found between LH β and GP α ($P < 0.001$, Fig. 5B).

FIG. 6. Effect of steroid treatments on the expression of gonadotropin subunits. Female silver eels were transferred to seawater and treated for 7 weeks with estradiol (E₂), testosterone (T) or vehicle (controls). Pituitary mRNA levels for gonadotropin subunits were measured by dot blot using RNA probes for eel FSH β (Fig. 6A), LH β (Fig. 6B) and GP α (Fig. 6C) and normalized using 18s RNA. Means are given \pm SEM (n = 8 eels/group). **, $P < 0.01$; ***, $P < 0.001$ as compared to controls, ANOVA.

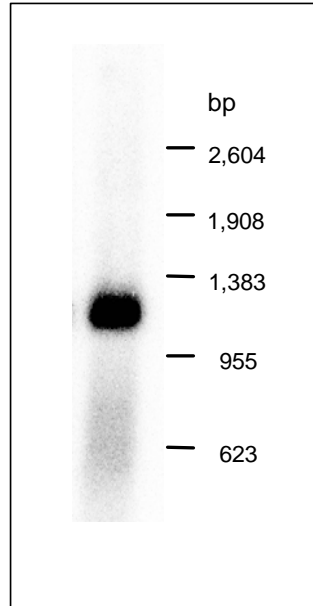


Fig 1

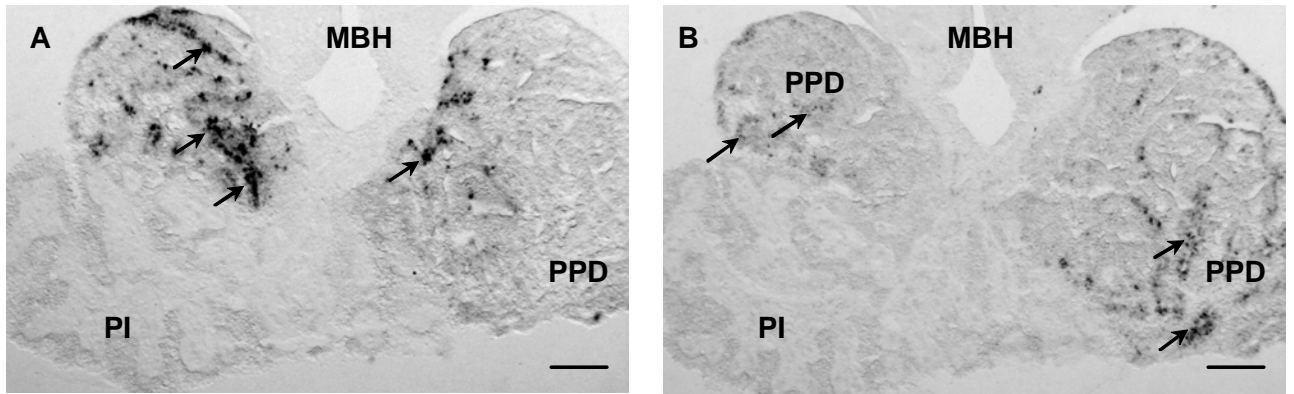


Fig 2

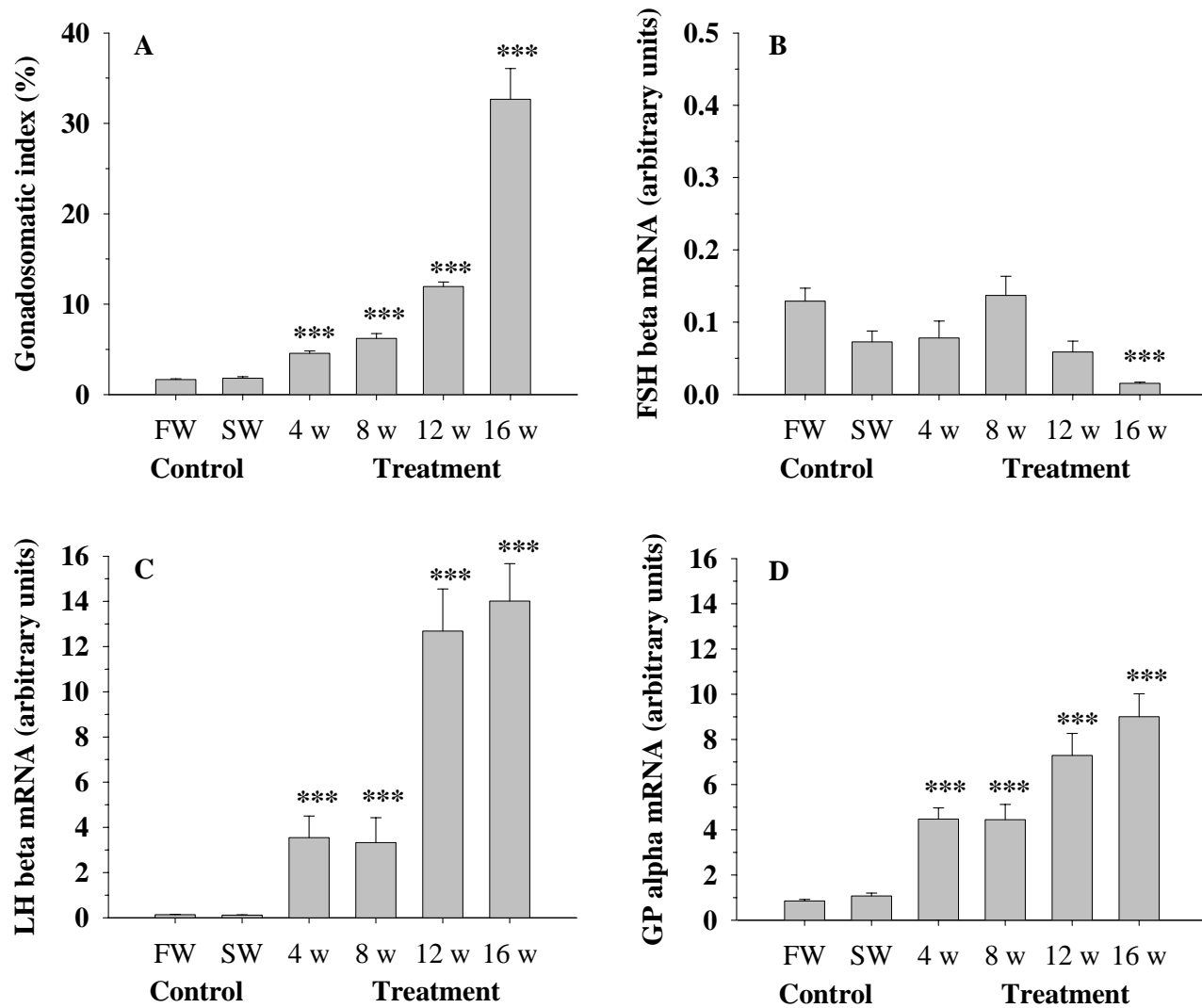


Fig 3

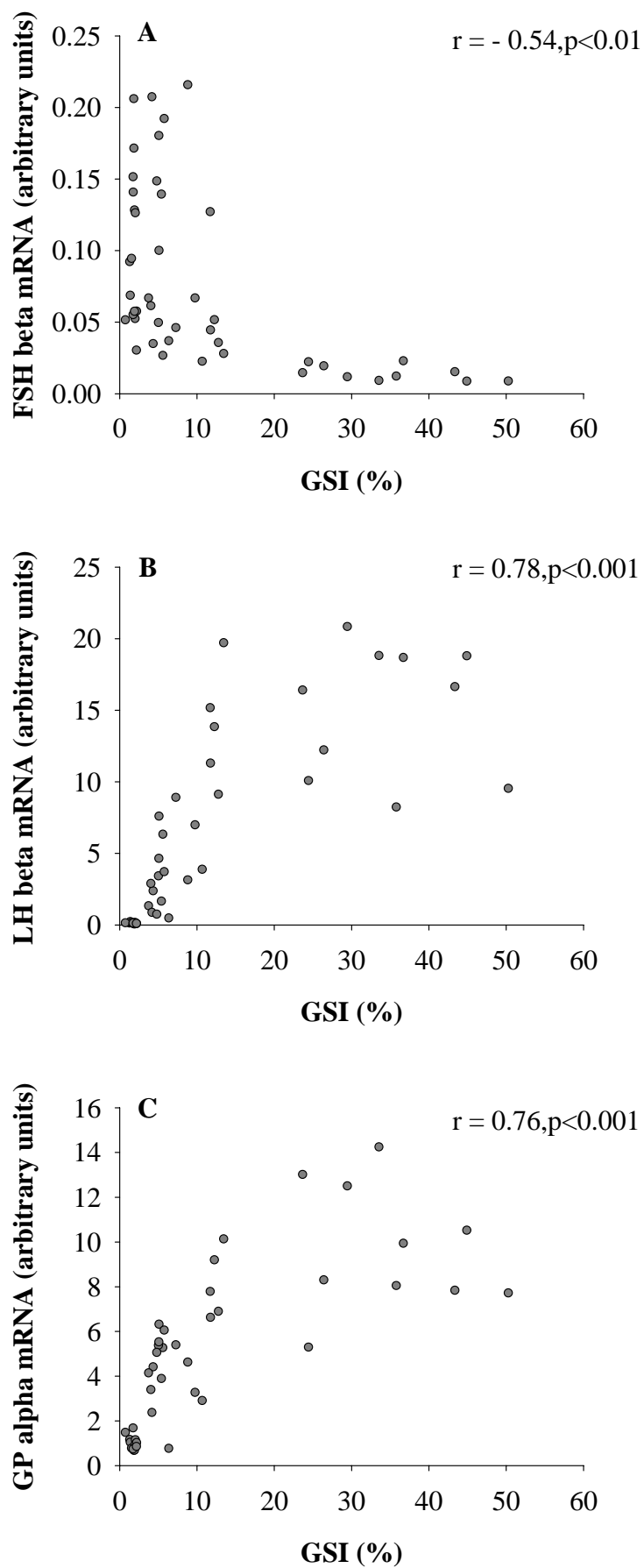


Fig 4

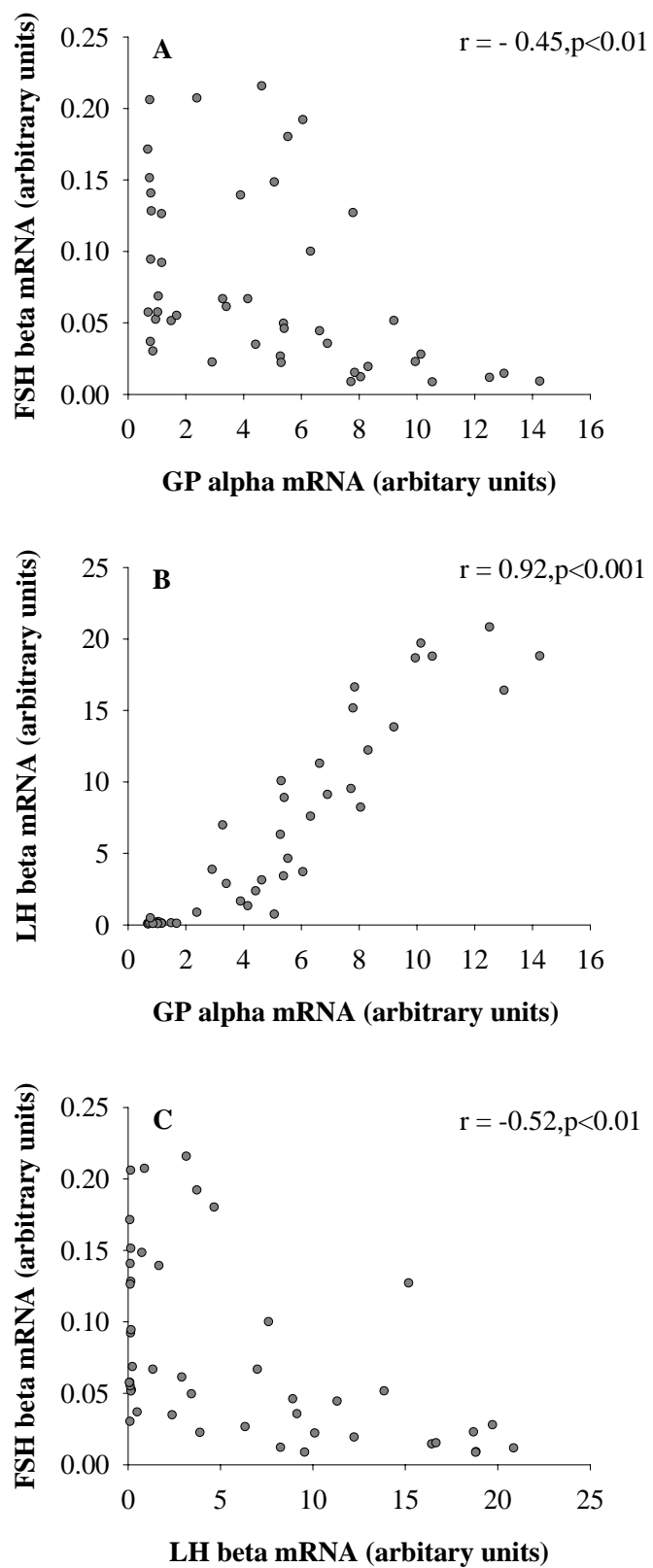


Fig 5

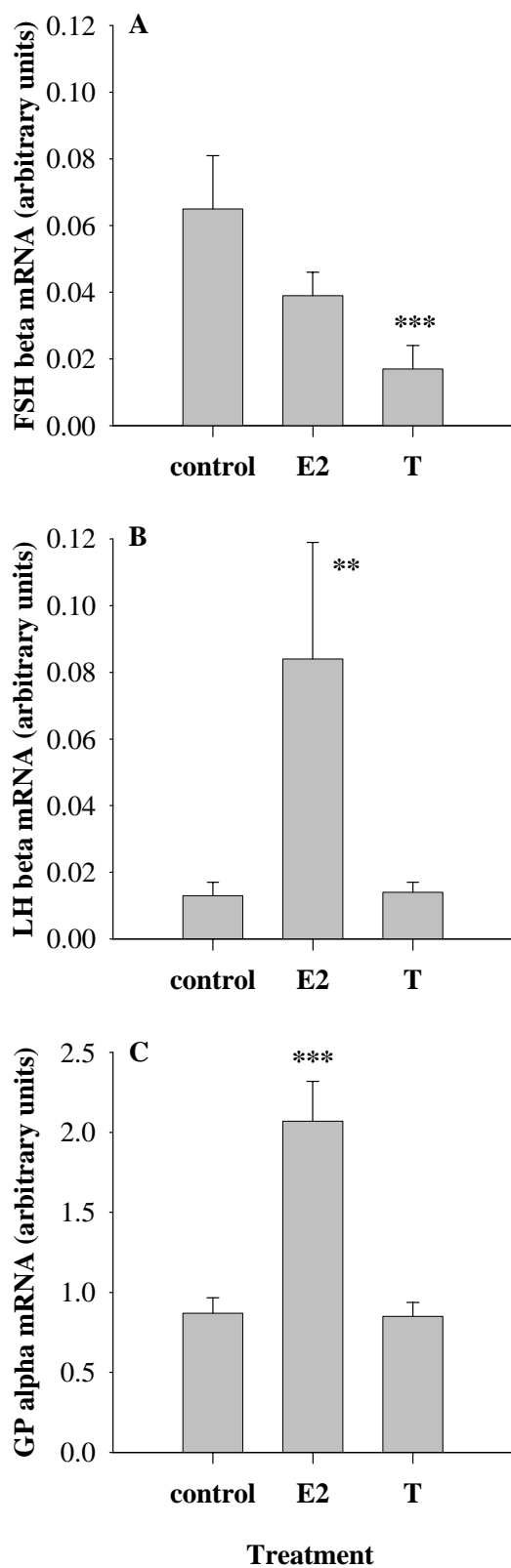


Fig 6