Development of a homologous enzyme-linked immunosorbent assay for European sea bass FSH. Reproductive cycle plasma levels in both sexes and in yearling precocious and non-precocious males

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Abstract

Since the late 1980s, gonadotropins have been isolated and characterized in several fish species, but specific immunoassays for the follicle-stimulating hormone (FSH) have only been developed for a few. The present study reports the development and use of a specific and homologous competitive ELISA for measuring FSH in European sea bass (*Dicentrarchus labrax*) using a recombinant FSH and its specific antiserum. Recombinant European sea bass FSHβ and FSH heterodimer were produced in the methylotrophic yeast *Pichia pastoris* and a baculovirus expression system, respectively. Specific polyclonal antibodies, generated by

rabbit immunization against recombinant FSHβ, were used at a final dilution of 1:8000. Recombinant FSH heterodimer was used to generate a standard curve and for coating of microplates (166 µg/ml). The sensitivity of the assay was 0.5 ng/ml [B_0-2SD], and the intra- and inter-assay coefficients of variation were 2.12% (n=10) and 5.44% (n=16) (B_i/B_0 ~ 45%), respectively. A high degree of parallelism was observed between the standard curve and serially diluted plasma and pituitary samples of European sea bass.

The ELISA developed was used to study the plasma FSH profiles of mature males and females during the reproductive cycle, and those of immature juvenile males under different light regimes. The analysis showed that FSH increased significantly during the intermediate stages of spermatogenesis and during vitellogenesis. Analyses in immature juvenile males showed that the continuous light photoperiod significantly reduced plasma FSH levels, and consequently, testicular growth and precocious puberty. In conclusion, the immunoassay developed has proven to be sensitive, specific and accurate for measuring European sea bass FSH, and it represents a valuable tool for future studies on the reproductive endocrinology of this species.

Keywords: FSH; recombinant; yeast; ELISA; reproductive cycle; Perciform

1. Introduction

The gonadotropins (GTHs), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are key hormones in the control of reproduction in vertebrates. These heterodimeric glycoproteins are synthesized and secreted by the pituitary gland. GTHs are formed by the non-covalent association of a common α-subunit with distinct β-subunits that confer hormone

specificity [5, 26]. Each subunit is encoded by a single, separate gene [11], while dimerization and glycosylation are prerequisites for GTHs to achieve full biological activity.

As in higher vertebrates, fish GTHs regulate gametogenesis and steroidogenesis. In salmonids, from which most of the information on seasonal GTHs profiles comes, FSH is believed to regulate the early stages of gametogenesis, such as vitellogenesis and spermatogenesis, whereas LH is generally accepted to be the hormone responsible for the final maturation processes, such as oocyte maturation, ovulation and spermiation [15]. However, the functional duality between FSH and LH at critical moments in the reproductive process in other fish species has not yet been fully clarified, mainly due to a lack of appropriate research tools like assays to measure GTH levels. Traditionally, the immunoassays developed to determine GTH levels in fish have been radioimmunoassays (RIA) or enzyme-linked immunosorbent assays (ELISA) based on native GTHs purified from fish pituitaries, as well as their specific antibodies. The purification of native GTHs is a highly demanding process in terms of time, cost and the large number of pituitary glands required [15]. Since the late 1980s, GTHs have been isolated and characterized in several fish species, but homologous immunoassays for FSH have only been developed for three fish species with synchronous ovarian development: chum salmon (*Oncorhynchus keta*) [36], coho salmon (*Oncorhynchus kisutch)* [39, 40] and rainbow trout (*Oncorhynchus mykiss*) [12]. For other fish species, quantitative tools like these have only been available for LH, a fact that has hampered studies. Over the last decade, the isolation and characterization of cDNAs encoding GTH subunits in a wide range of fish species has made it possible to produce species-specific recombinant GTHs. The production of recombinant GTHs represents a good alternative to native hormones, as they can be continuously produced, ensuring their availability and preventing cross-contamination with other related glycoproteins [15]. However, over the last few years, homologous immunoassays for FSH and LH using

recombinant gonadotropins have been successfully developed for only one perciform species with asynchronous ovarian development: the tilapia (*Oreochromis niloticus*) [1].

The European sea bass (*Dicentrarchus labrax*) is a marine perciform fish with a group-synchronous ovarian type development [9], and there is information available on the endocrine control of its reproductive function, particularly regarding the role of LH in adults [23, 24, 29] and juveniles [30, 31]. Nevertheless, there is a lack of information on the role of FSH in either adults or juveniles. The European sea bass is a very important species for intensive aquaculture, and under these conditions, a large number of males undergoes puberty just after sexual differentiation [32]. In light of this, precocity becomes one of the most significant problems to be solved, since these fish experience growth depletion and vulnerability to diseases [42]. Several works have demonstrated the effectiveness of photoperiod in preventing early puberty in male European sea bass [6, 30]. Nevertheless, further studies are needed to understand the underlying mechanisms of this inhibition, in particular with regard to the role of FSH.

Recently, we have produced recombinant European sea bass FSHβ subunit in a baculovirus expression system and developed a dot-blot immunoassay capable of measuring FSH in pituitary samples [21]. However, this assay could not be validated for plasma, because plasma FSH levels were often below the assay detection limit.

The objectives of this study were threefold: (1) to produce a new batch of recombinant European sea bass FSHβ in the methylotrophic yeast *Pichia pastoris* and generate specific antibodies; (2) to develop a more sensitive and versatile immunoassay for European sea bass FSH, capable of determining FSH levels in both plasma and pituitary samples; and (3) to study the plasma FSH profiles in adult males and females during the reproductive cycle, and in juvenile males under different light regimes.

2. Materials and methods

2.1 Experimental fish and sample collection

Male and female European sea bass from the stock raised at the facilities of the Torre de la Sal Aquaculture Institute (40º NL) were used to study seasonal FSH profiles during the reproductive cycle. Plasma samples were from fish pertaining to previously reported experiment [29]. Briefly, fish were sampled monthly throughout the entire period of their first sexual maturation. At each sampling point, five fish of each sex were anesthetized, weighed, measured and sacrificed. Blood was collected via a caudal vein puncture using heparinized syringes, centrifuged at 2500*g* for 25 min at 4ºC and plasma stored at -20ºC until the time of analysis. Pituitaries were removed, immediately frozen in liquid nitrogen and stored at -80°C. For analysis, pituitaries were mechanically homogenized in TBS-T (10 mM Tris-base, 150 mM NaCl and 0.05% Tween-20) using sterile sets of needle and syringe. The extract was centrifuged (3000*g* for 15 min at 4°C) to eliminate debris and stored at -80°C until assayed. Gonads were dissected, fixed, sectioned (3 μm) and stained according to the method described by Bennett et al. [4] for histological analysis. The stages of testicular development were classified by means of light microscopy, following previously established criteria [6]: stage I, immature testes; stage II-IV, testicular growth (early, mid and late recrudescence); stage V, fully spermiating testes and stage VI, post-spawning. The ovarian stages were classified as described by Rocha et al. [29], following previously established criteria [3]: previtellogenesis (prevtg); early vitellogenesis (evtg); late-vitellogenesis and post-vitellogenesis (lvtg-pvtg); maturation–ovulation (mat–ovul) and atresia (atre).

Juvenile male European sea bass (seven months of age) obtained from Aquanord (Gravelines, France) were used to study the effect of a continuous light regime, which is known to impair

precocious puberty in males [6], on plasma FSH levels. Immature juvenile males were subjected to either simulated natural photoperiod (NP) or continuous light (LL) (24h light/day) treatments for ten months (June-March). In October, December, January and March, 13 fish from each group were anesthetized, weighed, measured and sacrificed (Gonadosomatic index (GSI): NP ≥ 0.05% (Oct) and ≤ 2.15% (Mar); LL ≥ 0.03% (Oct) and ≤ 0.11% (Mar)). Blood was collected as described above.

All fish were sacrificed in accordance with Spanish legislation concerning the protection of animals used for experimentation and other scientific purposes.

2.2 Construction of FSHβ expression plasmid

Complementary DNA fragments containing the entire open reading frame (ORF) of mature European sea bass *FSHβ* (480 bp) (GenBank accession number: AF543314) and a C-terminal 6xHis tag were obtained by PCR, using specific primers (Forward: 5'GG *GAATTC* GGG CAG GGC TGC AGC TTC3'; Reverse: 5'AT *GCGGCCGC* TTA ATG ATG ATG ATG ATG ATG AAA GGA CAG ACA GCT GGG TAT3') and the proofreading *PfuTurbo* DNA polymerase (Stratagene; La Jolla, CA, USA). Restriction sites were added at the 5' end of the forward and reverse primers. The fragment was directionally cloned into the donor plasmid pPIC9K (Invitrogen Corp.; Carlsbad, CA, USA) between *Eco*RI / *Not*I restriction sites. The pPIC9K plasmid contains the yeast AOX1 promoter followed by the α-Factor signal sequence, which directs the recombinant protein to the secretory pathway. The pPIC9K-FSHβ construct was sequenced to confirm its identity.

2.3 Recombinant FSHβ production in yeast

148 The pPIC9K-FSHβ construct was linearized with *Bgl*II and used to transform methylotrophic
149 yeast *Pichia pastoris*, strain GS115 (Invitrogen), by electroporation, using an ECM 830
150 Electroporation system (BTX; Holliston, MA, USA). The host strain GS115 has a mutation in
151 the histidinol dehydrogenase gene (*his4*) that prevents it from synthesizing histidine. The
152 pPIC9K expression plasmid complements *his4* in the host, so 85 transformants were selected
153 for their ability to grow on histidine-deficient MD medium (1.5% agar, 2% dextrose, 1.34%
154 yeast nitrogen base, $4x10^{-5}$% biotin). The pPIC9K plasmid contains the bacterial kanamycin
155 gene that confers resistance to the antibiotic G418 (Geneticin; Invitrogen). Due to the fact that
156 multiple plasmid integration events may increase the levels of expressed recombinant protein,
157 the transformants were further screened for high resistance to G418 on YPD medium (2%
158 agar, 1% yeast extract, 2% peptone, 2% dextrose and G418 at various concentrations (0.5-2
159 mg/ml)). Screening of FSHβ production was performed by Western blot analysis. The clones
160 with the highest production levels were cultured to examine the time-course of FSHβ
161 synthesis. Initially they were grown in BMGY medium (1% yeast extract, 2% peptone, 1.34%
162 yeast nitrogen base, 1% glycerol, $4x10^{-5}$ % biotin and 100 mM potassium phosphate, pH 6)
163 under shaking for 21 h at 29ºC. The cells were then harvested by centrifugation at 2000*g* for 5
164 min at room temperature (RT) and afterwards re-suspended and cultured in 1/4 volume of
165 BMMY medium (BMGY with 0.5% methanol instead of 1% glycerol). Incubation continued
166 for another 144 h at 29 ºC, and methanol was added at a concentration of 0.5% every 24 h
167 (induction phase). Samples of the culture supernatant were collected at different times (0, 24,
168 48, 72, 96, and 144 h) by centrifugation at 15000*g* for 3 min at RT and analyzed by FSH dot-
169 blot immunoassay (see below). As a negative control, GS115 cells were transformed using an
170 expression plasmid without the sea bass FSHβ cDNA and treated in the same manner.
171
172 *2.4 FSH dot-blot immunoassay analysis*

The production level of FSHβ during the time course was measured by homologous FSH dot-blot immunoassay according to Molés et al. [21]. Briefly, the sample preparations (250 μl of culture supernatant) were denatured (5% 2-mercaptoethanol (2-ME), 4 min at 95ºC) and immobilized on a PVDF membrane (Immobilon P, Millipore; Billerica, MA, USA) using a Bio-Dot microfiltration apparatus (Bio-Rad Laboratories Inc., CA, USA). The membrane was blocked overnight with 5% skim milk at 4ºC, incubated with 1:2000 anti-European sea bass FSHβ (AbFSHβ-1) [21] for 90 min at room temperature, washed, and further incubated with 1:1000 goat anti-rabbit IgG-horseradish peroxidase conjugate (GAR-HRP, Bio-Rad Laboratories Inc.) for 60 min at room temperature. The immunodetection was performed by chemiluminescence (Western Blotting Luminol Reagent, Santa Cruz Biotechnology, Inc., CA, USA). Immunoreactivity signal (intensity / mm^2) was calculated for each dot and compared to those obtained from serial dilutions of standard (purified native European sea bass FSH [19]). This assay has a sensitivity of 162 ng/ml, with intra- and inter-assay coefficients of variation of 9.8% and 11.5%, respectively.

2.5 Recombinant FSHβ purification

The European sea bass FSHβ was produced and harvested at 72 h after induction by methanol. The supernatant (1L) of a culture of GS115-FSHβ clone 6 was concentrated (5-6 ml) by ultrafiltration using Centricon Plus-70 Biomax 5 centrifugal filter devices (Amicon, Millipore) and later purified by immobilized metal affinity chromatography (IMAC Ni^{2+}) using His GraviTrap prepacked columns (GE Healthcare; Chalfont St. Giles, UK), according to the manufacturer instructions. Briefly, the pH of the supernatant was adjusted to 7-8 with diluted acetic acid and the prepacked columns were equilibrated with PBS (20 mM sodium

198 phosphate, 500 mM NaCl, pH 7.4) containing 20 mM imidazole. After loading the
199 concentrated supernatant, two washings with PBS (20 mM and 70 mM imidazole) were
200 performed. Finally, the bound FSHβ was eluted with PBS containing 250 mM imidazole. The
201 identity of purified protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel
202 electrophoresis (SDS-PAGE) and Western blot analysis.
203
204 *2.6 SDS-PAGE and Western blot analysis*
205
206 Proteins were electrophoresed through 15%-SDS-PAGE gels under reducing conditions (5%
207 2-ME). The separated proteins were stained with Coomassie blue or transferred to PVDF
208 membranes (Immobilon P, Millipore). The membranes were blocked overnight with 5% skim
209 milk at 4ºC, incubated with 1:3000 antibodies against European sea bass FSHβ (AbFSHβ-1)
210 [21] for 90 min at room temperature, washed, and further incubated with 1:2000 GAR-HRP
211 (Bio-Rad Laboratories Inc.) for 60 min at room temperature. The immunodetection was
212 performed by chemiluminescence (Western Blotting Luminol Reagent, Santa Cruz
213 Biotechnology, Inc.).
214
215 *2.7 European sea bass FSHβ antibody production*
216
217 Polyclonal antibodies against the purified recombinant FSHβ (AbFSHβ-2) were produced by
218 a commercial company (Agrisera, Sweden). Two rabbits were immunized with 150 μg of
219 FSHβ in Freund's complete adjuvant by subcutaneous injection. Four subsequent
220 immunizations (II-V) were carried out with 30 μg of antigen in Freund's incomplete adjuvant
221 at 3-week intervals. Rabbits were bled 2 weeks before immunizations (pre-immune serum)

and 2 weeks after immunizations III, IV and V, in order to perform the corresponding titration test. The final bleeding was performed 2 weeks after the fifth immunization.

2.8 European sea bass FSH ELISA

A competitive ELISA for sea bass FSH determination was developed using the specific antiserum generated (AbFSHβ-2) and recombinant FSH heterodimer produced in a baculovirus expression system [20]. The protocol was based on that previously described for ELISAs of striped bass LH [17] and sea bass LH [18], and was adapted as follows:

1) **Coating**. Polystyrene ELISA 96-well microplates (Maxisorp, Nunc; Thermo Fisher Scientific Inc., MA, USA) were coated with 50 μl/well of recombinant European sea bass FSH heterodimer solution (166.5 ng/ml, diluted in sodium carbonate buffer 0.05 M, pH 9.6) overnight at 4ºC. Three wells were coated with the same concentration of bovine serum albumin (BSA, Sigma-Aldrich; St. Louis, MO, USA) to determine the non-specific binding. After coating, the wells were washed (3 x 1 min) with PBST (sodium phosphate buffer 0.01 M, pH 7.2, containing 0.9% NaCl and 0.05% Tween-20). The plate wash was repeated after every step of the assay.
2) **Blocking**. To reduce background, wells were blocked with 100 μl/well of PBST buffer containing 2% BSA for 30 min at 37ºC.
3) **Incubation with primary antibodies**. Before distribution into the wells, 60 μl standard and unknown samples (final dilution 1:4 for plasma and 1:4000 for pituitary) were first preincubated with 60 μl AbFSHβ-2 (final dilution 1/8000) in microcentrifuge tubes (overnight at 4ºC). The standard curve ranged from 0.32 to 162.5 ng/ml of sea bass FSH. All standards, samples and the antiserum solution were

diluted in PBST buffer containing 2% normal goat serum (NGS, Sigma-Aldrich). After preincubation, samples and standards were dispensed in duplicate (50 µl/well) into the coated wells and incubated for 48 h at 4ºC without shaking. The non-specific binding wells and five FSH coated wells (maximum binding, B_0) received only AbFSHβ-2 solution.

4) **Incubation with secondary antibodies**. The antigen-antibody complexes formed were detected by incubation with 50 µl/well of GAR-HRP, diluted 1:1000 in PBST-2% NGS buffer for 1 h at 37ºC.

5) **Color development**. The presence of enzyme complexes was detected by the addition of 100 µl/well of TMB reagent (Bio-Rad Laboratories Inc.). The reaction was carried out in complete darkness at RT for 25 min, and was stopped with 100 µl/well of 1 N sulphuric acid. Absorbances were read after 5 min at 450 nm, using an automatic microplate reader (Bio-Rad Laboratories Inc.).

2.9 Data representation and Statistical analysis

To study the FSH profiles of males and females during the reproductive cycle, the samples were grouped according to gonadal stage of development (see Section 2.1). Data are presented as mean ± SEM. The significance of the differences between group means of hormone levels was determined by one-way analysis of variance (ANOVA) followed by post hoc testing using the Holm-Sidak method, performed with SigmaStat 3.5 software (Systat Software Inc.; Chicago, IL, USA). When the test of equal variance failed, an ANOVA on Ranks (Kruskal-Wallis) was performed, followed by all pairwise multiple comparison procedures (Dunn's method).

For ELISA data calculations, sigmoid curves were linearized using the logit transformation (logit $(B_i/B_0) = \ln (B_i–NSB/B_0–B_i)$), where B_i represents the binding of each point, B_0 is the maximum binding and NSB the non-specific binding.

3. Results

3.1 Production of Recombinant European sea bass FSHβ

After transformation, thirteen colonies GS115 with His^+ Mut^+ phenotype and resistance to 2 mg/ml G418 were selected and used for screening FSHβ production by Western blot analysis. A candidate protein reacted intensely with the AbFSHβ-1 antibody [21] in all the colonies analyzed (Fig. 1). Yeast transformed with the vector alone served as a negative control and yielded no band (Fig. 1). The clones that produced the highest amount of recombinant FSHβ were selected and examined for time-course production and secretion of FSHβ. Analysis by dot-blot immunoassay of samples of culture supernatant collected at different times (0, 24, 48, 72, 96, and 144 h) revealed that the yields of FSHβ increased up to 48 h after induction with methanol and remained constant between 72 and 144 h (Fig. 2). The clone with highest level of production was cultured to large scale for 72 h. After the purification process, the collected fractions were analyzed by SDS-PAGE and Western blot. The Coomassie blue stain showed a production with a high yield. A single band was intensely stained, although proteins of lower molecular weight were also observed (Fig. 3A). A Western blot analysis confirmed that the purified protein and the low molecular weight products were FSHβ (Fig. 3B), indicating that some protein degradation may have taken place. The molecular weight detected for FSHβ produced in yeast turned out to be slightly higher than that of the FSHβ previously produced in the baculovirus system (Fig. 3A). The purified FSHβ was used to generate specific

296 polyclonal antibodies through rabbit immunizations. The antiserum produced was tested in
297 Western blot, and specifically immunoreacted with pituitary extract and recombinant
298 European sea bass FSHβ forms produced in yeast and the baculovirus system, but did not
299 recognize recombinant European sea bass LH (Fig. 4), indicating the FSHβ specificity of the
300 antibodies.
301
302 *3.2 Development and validation of an ELISA for European sea bass FSH*
303
304 A homologous competitive ELISA was developed for FSH determination in pituitary and
305 plasma samples, using recombinant FSH heterodimer for coating and the standard curve, and
306 the specific antiserum generated (AbFSHβ-2) for immunodetection. A series of tests were
307 performed to optimize the ELISA protocol by studying the behavior of the standard curve
308 under different temperatures, incubation times and coating concentrations.
309 Under optimized conditions, described in Materials and methods, the sensitivity of the assay,
310 defined as the lowest dose of FSH capable of reducing the optical density more than the mean
311 plus 2 standard deviations of the zero dose of FSH [B_0 – 2SD], was 0.50 ng/ml (B_i/B_0 >
312 93,9%). The half maximum displacement occurred at 11.32 ng/ml (B_i/B_0 = 50%).
313 The precision of the assay was tested by calculating the intra- and inter-assay coefficients of
314 variation (CV). The intra-assay CV, tested by measuring replicates of the same sample in a
315 single assay plate, was 4.7, 2.1 or 1.4%, according to the calculations at 25, 48 or 74% of
316 binding, respectively (n = 10). The inter-assay CV, calculated by measuring the same sample
317 in different assays was 8.6, 5.4 or 3%, according to the calculations at 23, 44 or 83% of
318 binding, respectively (n=16).

The specificity of the assay was tested by cross-reaction of AbFSHβ-2 with recombinant European sea bass LH produced in a baculovirus expression system [20], which showed no immunoreaction even at concentrations as high as 1000 ng/ml (Fig 5A).

Finally, the assay was validated for European sea bass plasma and pituitary samples by testing the parallelism with the standard curve. Displacement curves obtained with serial dilutions of plasma and pituitary extracts produced sigmoid curves similar to the FSH standard curve (Fig. 5A). In order to test the possibility of using the European sea bass FSH ELISA for FSH measurements in other fish species, displacement curves of serial dilutions of pituitary extracts and plasma from other fish species were compared with the FSH standard curve. Non-parallelism with the FSH standard curve was observed for thicklip grey mullet (*Chelon labrosus*), sea bream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*), which shows the high degree of specificity of AbFSHβ-2 for European sea bass FSH (Fig. 5B).

3.3 Determination of FSH levels

3.3.1 FSH levels during the European sea bass reproductive cycle

Plasma profiles of FSH were examined throughout the first reproductive cycle of both sexes, which occurred during the second and third year of life in males and females, respectively. In males, FSH levels increased significantly during active spermatogenesis (stages II-IV) and fell to baseline levels just before full spermiation (stage V) (Fig. 6A). In females, the FSH levels peaked at vitellogenesis and post-vitellogenesis, while the minimum values were detected at maturation-ovulation (Fig. 6B). In both sexes, the baseline levels of FSH were higher than 15 ng/ml.

344 *3.3.2 FSH levels of juvenile European sea bass under different light regimes*

345

346 Plasma FSH levels of juvenile European sea bass exposed to simulated natural photoperiod
347 (control group) or continuous light were examined from October to March. The analysis
348 showed that continuous light significantly reduced plasma FSH levels as compared to the
349 control group. Under natural photoperiod, plasma FSH levels increased steadily until reaching
350 their maximum level in March (GSI = 2.15%), while FSH levels of fish subjected to
351 continuous light, remained unchanged (GSI = 0.11%) (Fig. 7). The percentage of precocious
352 juvenile males in the control group was about 65%, on the contrary, under a regime of
353 continuous light, the number of premature males was much lower, about 10% (data not
354 shown).

355

356 **4. Discussion**

357

358 This study describes the development and use of a specific and homologous ELISA for
359 European sea bass FSH, using recombinant FSH and its specific antiserum. In teleosts,
360 homologous immunoassays for FSH have only been developed for three salmonid species and
361 one perciform species [1, 12, 34, 36, 39]. Traditionally, the immunoassays developed to
362 determine GTHs levels in fish have been based on native GTHs purified from fish pituitaries
363 and their specific antibodies. Since purification of native FSH is a very demanding process,
364 the production of recombinant forms is a good alternative that yields large quantities of
365 protein, ensuring its availability and no cross-contamination with other related glycoproteins.
366 In the present study, we have developed a sensitive and accurate immunoassay for FSH, an
367 ELISA able to measure FSH in plasma and pituitary samples. For this purpose, we produced
368 recombinant European sea bass FSHβ in the methylotrophic yeast *Pichia pastoris*, which was

used to generate specific antibodies. Prior to this, we had produced recombinant European sea bass FSH heterodimer and FSHβ subunit using a baculovirus expression system [20, 21], as well as its specific antibodies (AbFSHβ-1). However, AbFSHβ-1 reacted very weakly with FSH under native conditions; in spite of which we were still able to develop a dot-blot immunoassay capable of measuring pituitary FSH under denatured conditions. Nevertheless, to develop a more sensitive and accurate ELISA-like immunoassay, it was necessary to produce new antibodies that could recognize nondenatured forms of FSH. Accordingly, a new batch of recombinant FSHβ was produced in a yeast system. The main advantage of this system, compared to the baculovirus system previously used [21], is that the FSHβ was efficiently secreted and easily purified from the culture medium, although the production yield in yeast was somewhat lower than in the baculovirus system. In addition, yeast culture does not require costly sophisticated growing media and is fairly easy to establish and scale up.

The size of recombinant FSHβ generated in yeast was slightly higher than that previously produced in the baculovirus system. This fact might be due to different degrees of glycosylation, since *Pichia pastoris* have mostly a high-mannose-type N-linked glycosylation [13] while insect cells, used in the baculovirus system, assemble insect-specific paucimannose-type N-glycans [14].

To develop the FSH specific ELISA, recombinant FSH heterodimer [20] was used as coating and to generate the standard curve. The ELISA developed for European sea bass FSH showed optimal characteristics in terms of precision, specificity and sensitivity, similar to those reported for other fish GTH immunoassays. The precision was high, ensured by low intra- and inter-assay coefficients of variation, which were both below 5% and 9%, respectively. These variations are lower or similar to those reported for FSH immunoassays in other fish species, which are in the range of 4-8% and 10-12% for intra- and inter-assay CVs, respectively [1,

12, 36]. The specificity was demonstrated by the absence of AbFSHβ-2 immunoreaction with European sea bass LH; no cross-reactivity was detected in either the Western blot or ELISA. Finally, the sensitivity of the European sea bass FSH ELISA (0.50 ng /ml) was much higher than that of the FSH dot-blot immunoassay previously developed (162 ng/ml) [21], and high enough to measure plasma FSH levels. Moreover, the sensitivity was comparable to those reported for fish GTH immunoassays, which ranged from 0.2 to 2.34 ng/ml for RIAs [12, 28, 34, 36, 39, 41] and 0.24 pg/ml to 0.65 ng/ml for ELISAs [1, 17, 18, 33].

The European sea bass FSH ELISA was validated for plasma and pituitary samples by testing the parallelism with the standard curve. The results indicated that native sea bass FSH, both in blood and the pituitary, was parallel to the recombinant FSH heterodimer used in the standard curve, and therefore was immunologically similar. We also tested the usefulness of the developed European sea bass FSH ELISA to measure FSH in the pituitary and plasma of other fish species. Non-parallelism was observed between the FSH standard curve and the pituitaries of Senegalese sole, thicklip grey mullet and sea bream. It can therefore be determined that AbFSHβ-2 has a high degree of specificity for European sea bass FSH, and is therefore not suitable to measure FSH levels in either the pituitary or the plasma of these species.

The ELISA developed was used to analyze, for the first time, immunoreactive plasma FSH levels in a marine perciform with multiple-batch group-synchronous ovarian development, the European sea bass. In adult males, the plasma levels of FSH increase during testicular growth (II-IV stages) and decline just before full spermiogenesis-spermiation (V stage), suggesting that in European sea bass, FSH plays an important role in the regulation of early-mid phases of spermatogenesis, but not in spermiogenesis and spermiation. The plasma levels of 11-Ketotestosterone (11-KT) and LH have been previously analyzed in the same animals [29]. The 11-KT profile coincided with that of FSH in the present study, with levels gradually

419 increasing during testicular growth, and dropping off once full spermiation begins. In eels, it
420 has been demonstrated that recombinant FSH induces complete spermatogenesis by
421 stimulating the production of spermatogenesis-inducing steroids, such as 11-KT [25].
422 Similarly, in European sea bass, we have shown a stimulatory effect of native FSH in the
423 production of 11-KT in testicular tissue cultured *in vitro* [19]. 11-KT is the major androgen of
424 teleost species and is considered to play an important role in the spermatogenesis of several
425 fish species, mainly in the initiation of spermatogonial proliferation toward meiosis [35]. On
426 the other hand, plasma LH levels increased gradually, although not significantly, as
427 spermatogenesis progressed, with a significant elevation in late recrudescence (stage IV) that
428 was maintained in full spermiation [29]. These results are comparable to those observed in
429 male rainbow trout, where maximum levels of plasma FSH appear during mid- to late
430 testicular growth, and maximum plasma LH levels during spermiation [28].
431 In female European sea bass, the maximum plasma FSH levels were detected during
432 vitellogenesis and post-vitellogenesis, whereas a significant decrease was observed in
433 maturation-ovulation. Nevertheless, the pituitary FSH profile presented an opposite trend,
434 showing low FSH levels in previtellogenesis and early vitellogenesis, high in late- and post-
435 vitellogenesis and maturation-ovulation and again low in atresia [21]. These results indicate
436 an active synthesis and secretion of FSH in the course of vitellogenesis, whereas during
437 maturation-ovulation FSH secretion seems to decrease, accumulating in the pituitary. On the
438 other hand, the plasma FSH bioactivity profile, measured in the same samples by *in vitro*
439 bioassay [21], showed a tendency to achieve maximums of bioactivity in late- and post-
440 vitellogenesis although plasma FSH concentration is maintained. Interestingly, the lowest
441 plasma FSH levels obtained in the present study were found in maturation-ovulation
442 coinciding with a moderately high FSH bioactivity [21]. This could be indicating an increase
443 of the FSH potency in these stages. In fact, several studies have consistently demonstrated the

444 occurrence of FSH isoforms with different potencies throughout the human menstrual cycle
445 [43]. Moreover, in our case, estradiol (E2) and LH plasma levels have been previously
446 analyzed in these same animals [29]. The maximum plasma E2 levels were observed during
447 late- and post-vitellogenesis, coinciding with the maximums of plasma FSH levels calculated
448 here and the maximum of plasma FSH bioactivity [21]. In females, E2 is known to stimulate
449 the hepatic synthesis of vitellogenin, which is then progressively incorporated into the
450 growing oocytes during oogenesis [16]. Moreover, several studies have shown that FSH
451 stimulates the *in vitro* production of E2 in ovarian explants or isolated vitellogenic follicles
452 [19, 20, 22, 27, 37]. LH plasma levels peaked at maturation-ovulation, the point when FSH
453 reached minimum values (Rocha et al., [29] and this study, respectively). These profiles for
454 female European sea bass GTHs are concordant with those found in salmonids. In coho
455 salmon, Swanson [38] reported that the plasma FSH concentration increased during
456 vitellogenesis, with the highest levels occurring during mid- to late-vitellogenesis, after which
457 they decreased as ovulation approached. In rainbow trout, plasma FSH levels significantly
458 increased at the onset of vitellogenesis [28], were maintained during vitellogenesis and then
459 decreased prior to maturation [7], while plasma LH levels peaked at maturation-ovulation [7,
460 28]. Previous analysis of European sea bass pituitaries revealed similar FSH and LH profiles
461 during the reproductive cycle of females [21]; nevertheless, the data obtained in plasma
462 suggest a different control of release to the bloodstream (Rocha et al., [29] and this study).
463 Additionally, pharmacokinetic studies of GTHs in European sea bass [20] and in mammals
464 [8] have shown that FSH has a longer half-life in blood than LH, which indicates greater
465 clearance rates for LH. Thus, our results suggest that in the case of European sea bass,
466 constitutive levels of FSH in the bloodstream would be necessary for long-term stimulation of
467 gonadal growth, whereas short-term increases in plasma LH levels might have a more specific

effect during certain phases of the reproductive cycle, and therefore are likely to be cleared more quickly.

The plasma FSH levels in the group of juvenile males under simulated natural photoperiod (control group) increased gradually until reaching a maximum value in March. On the contrary, under continuous light, the levels of plasma FSH were maintained constantly low throughout the experimental period, which probably led to a lower percentage of precocious juvenile males. The FSH content in the pituitary gland showed the same profiles (Carrillo, M., Felip, A., Molés, G., Yilmaz, O., Zanuy, S., unpublished data), indicating that the inhibitory effect of continuous light on FSH was at the level of synthesis, not release. These results are in agreement with previous studies in juvenile male European sea bass and adult Atlantic Cod (*Gadus morhua*), where continuous light produced a significant reduction of gonadotropin subunit gene expression, 11-KT plasma levels and testicular growth [2, 10, 30]. All these results suggest that continuous light reduces precocity by impairing the synthesis of FSH. This results in a decrease of 11-KT levels that very likely reduces spermatogonial proliferation towards meiosis, and consequently, testicular growth.

In conclusion, the production of recombinant FSHβ in yeast has allowed us to generate specific antibodies and develop a homologous ELISA capable of measuring FSH in both plasma and pituitary samples from European sea bass. The results of the analysis performed suggest that FSH plays an important role during active spermatogenesis and vitellogenesis, whereas LH would seem to be involved in the final reproductive events, such as spermiation, oocyte maturation and ovulation. In juvenile males, the continuous light regime significantly reduced FSH synthesis, which is probably the cause of the reduction of precocious puberty rates. Finally, this assay represents a valuable tool for future studies on the reproductive endocrinology of this species.

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Figure legends

Fig. 1. Screening of yeast FSHβ production. Analysis by Western blot with AbFSHβ-1. Lanes 1-8: Cell lysates (15 μl) from different GS115 colonies transformed with pPIC9K-FSHβ. C-: Negative control; Cell lysate (15 μl) from a colony containing pPIC9K. M: Bio-Rad prestained Broad Range molecular weight marker.

Fig. 2. Time-course of yeast FSHβ production. Analysis by FSH dot-blot immunoassay of three GS115 clones expressing FSHβ and one wild type control. Each point is the mean of two replicates (250 µl supernatant/sampling point).

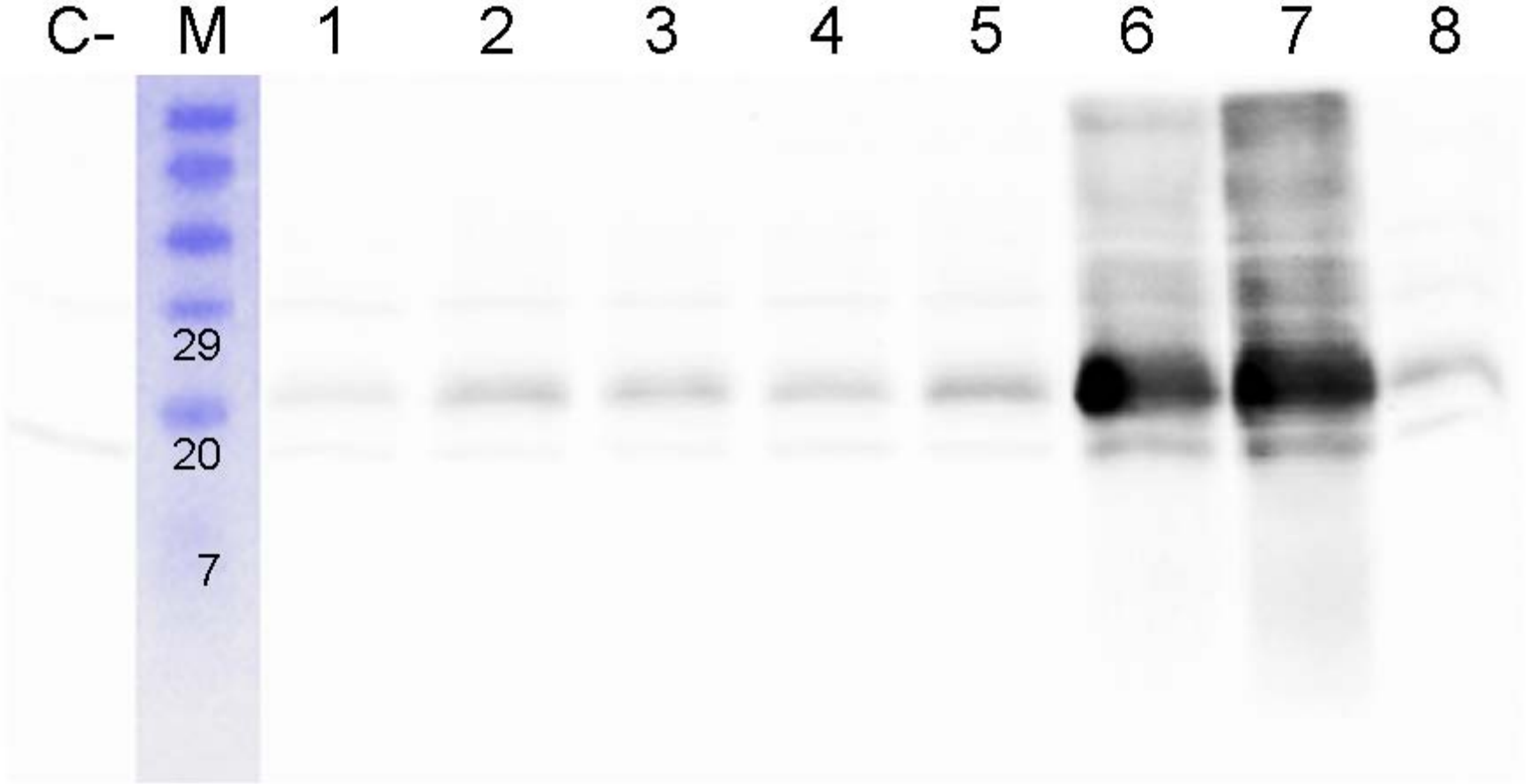
Fig. 3. SDS-PAGE and Western blot analysis of purified recombinant European sea bass FSHβ. **A**) Staining with Coomassie blue and **B**) Western blot with AbFSHβ-1 after affinity chromatography (IMAC Ni^{2+}) of recombinant FSHβ produced in yeast (GS115). Lane 1: Flow-through (10 µl); Lane 2: Wash with PBS containing 70 mM Imidazole (20 µl); Lanes 3 to 6: Elution with PBS containing 250 mM Imidazole (20 µl); Lane 7: Elution with PBS containing 500 mM Imidazole of FSHβ produced in baculovirus system (20 µl). M: Bio-Rad prestained Broad Range molecular weight marker.

Fig. 4. Analysis by Western blot of the polyclonal antibodies against recombinant European sea bass FSHβ produced in yeast (AbFSHβ-2; 1:15000). Lane 1: FSHβ produced in yeast (GS115) (100 ng), Lane 2: Pituitary extract (300 ng), Lane 3: FSHβ of FSH heterodimer produced in baculovirus system (320 ng). Lane 4: LH heterodimer produced in baculovirus system (420 ng). M: Bio-Rad prestained Broad Range molecular weight marker.

Fig. 5. Validation of European sea bass FSH ELISA. **A**) Parallelism between FSH standard curve and displacement curves obtained with serial dilutions of plasma, pituitary extracts (Pit) and recombinant European sea bass LH. **B**) Parallelism between standard curve and displacement curves obtained with serial dilutions of plasma and pituitary extracts from different fish (1 and 2). Thicklip grey mullet (Tgm), sea bream (Sb) and Senegalese sole (Ss). Each point is the mean of two replicates.

Fig. 6. Seasonal profiles of plasma FSH in male and female European sea bass during the reproductive cycle. Values were classified according to stage of gonadal development as determined by histology. **A**) Males: immature (I); early recrudescence (II); mid recrudescence (III); late recrudescence (IV); fully spermiating (V) and post-spawning (VI). **B**) Females: previtellogenesis (prevtg); early vitellogenesis (evtg); late- and post-vitellogenesis (lvtg-pvtg); maturation-ovulation (mat-ovul) and atresia (atre). Points represent mean ± SEM values (n= 5-19, depending on the stage). Different letters indicate significant differences between developmental stages (P=0.05).

Fig. 7. Plasma FSH levels in immature males of European sea bass exposed to simulated natural photoperiod (NP) or continuous light (LL) regimes for ten months (between June and March). Points represent mean ± SEM (n= 5-9, depending on the sampling point). Different letters indicate significant differences between sampling points (P=0.05) (lowercase letters for NP and capital letters for LL). Asterisks indicate significant differences between the two treatments at equivalent sampling points (P<0.05).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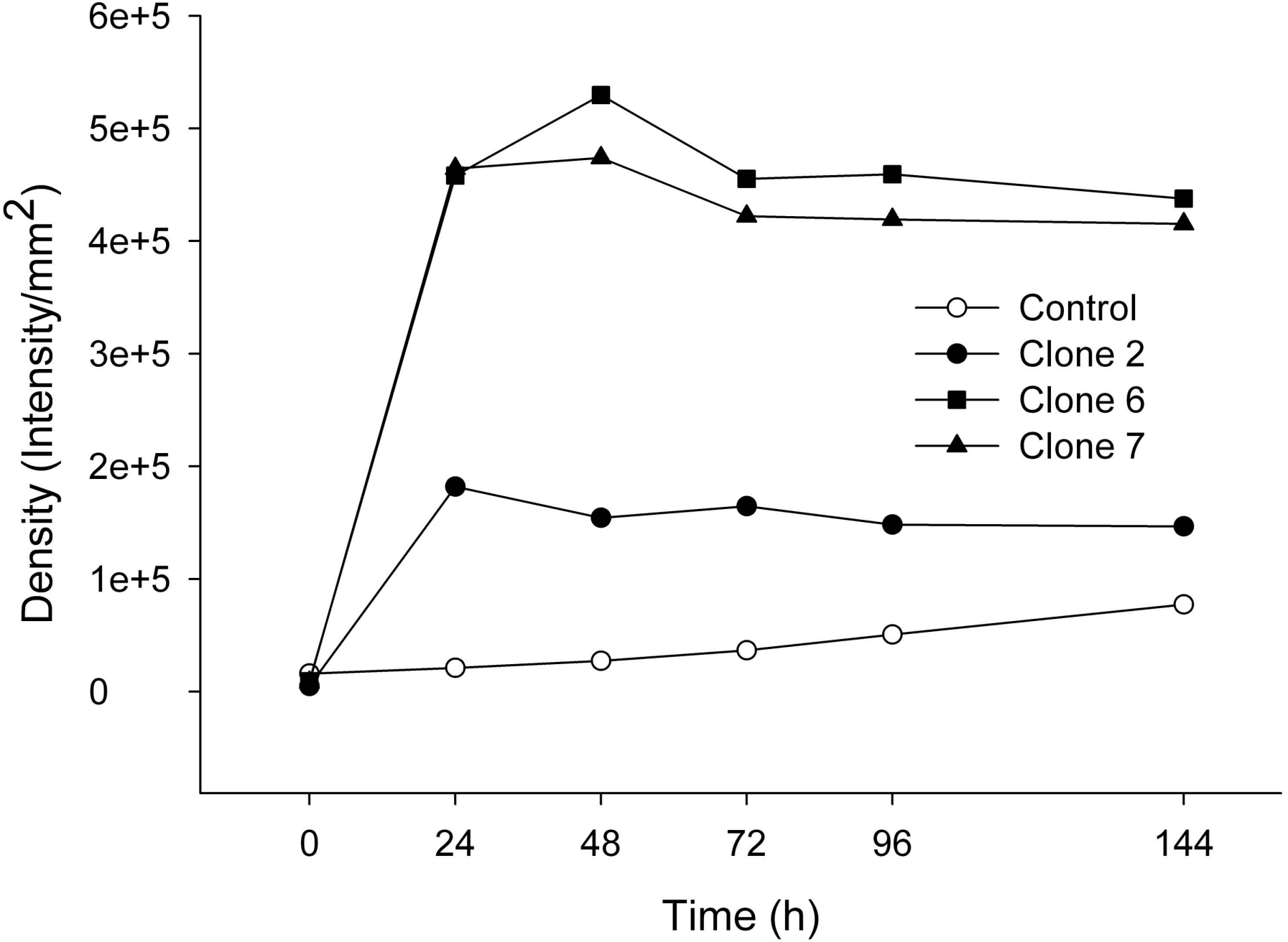
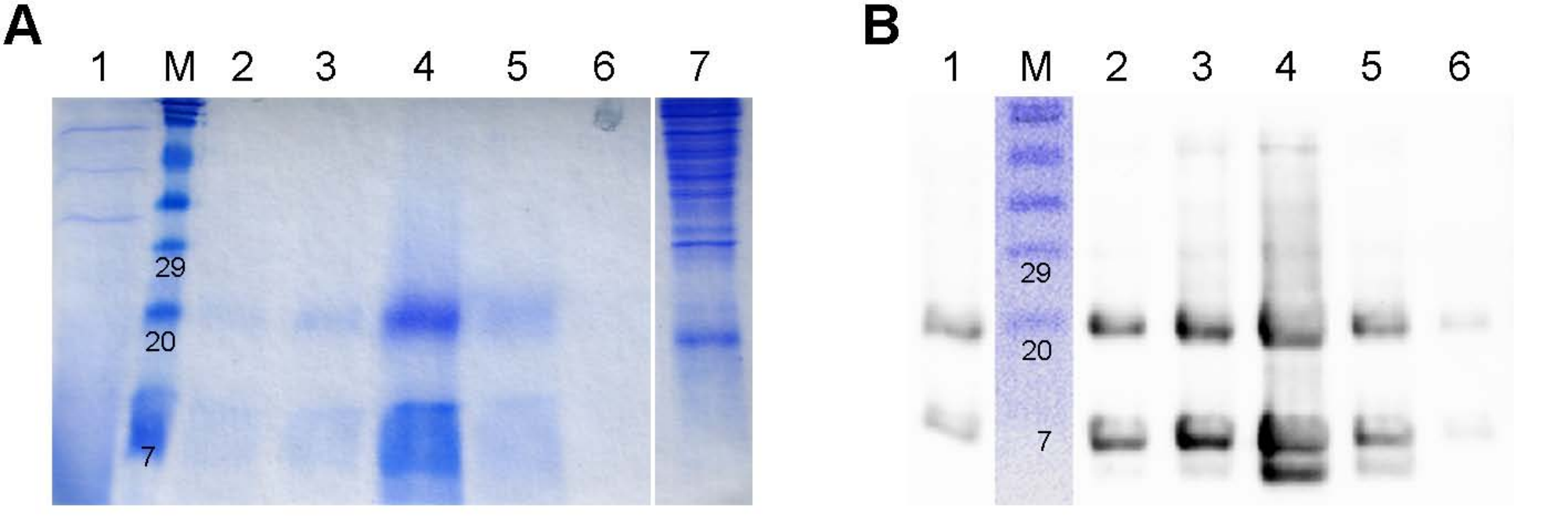

6e+5
5e+5
4e+5
3e+5
2e+5
1e+5
0
Density (Intensity/mm2)
0
24
48
72
96
144
Time (h)
Control
Clone 2
Clone 6
Clone 7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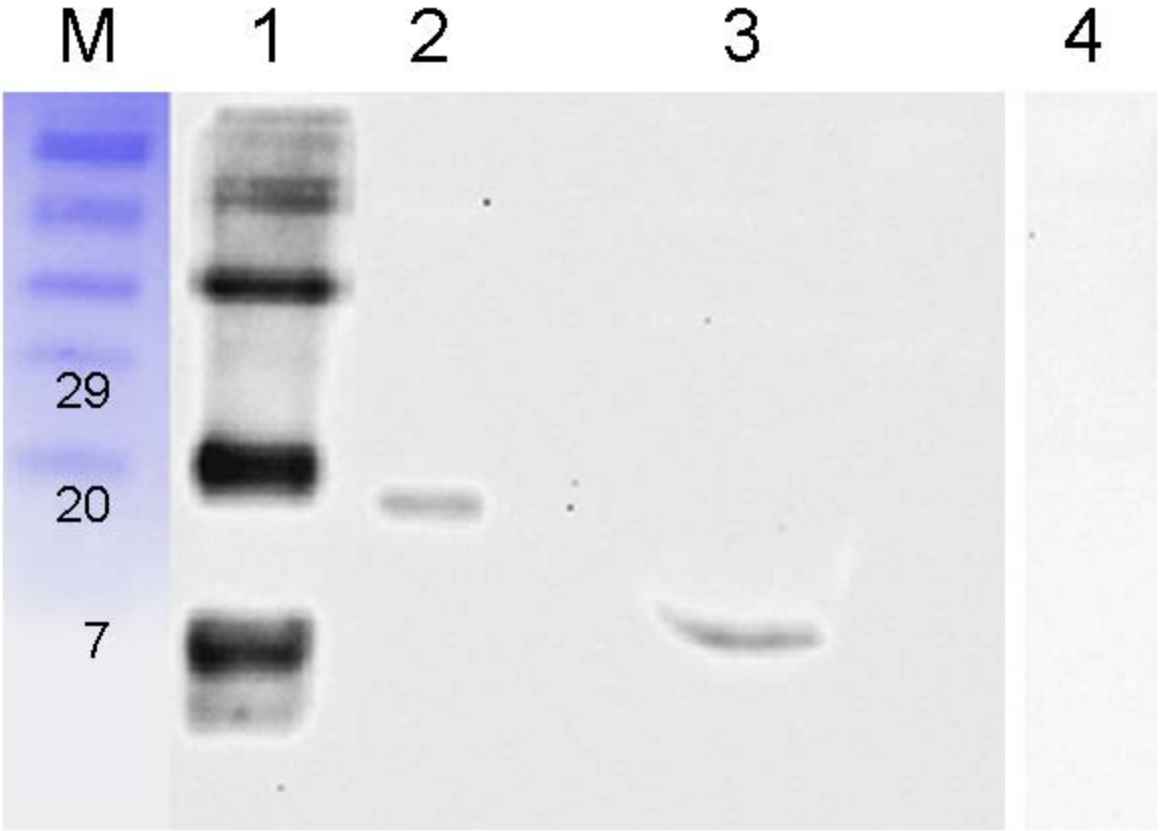
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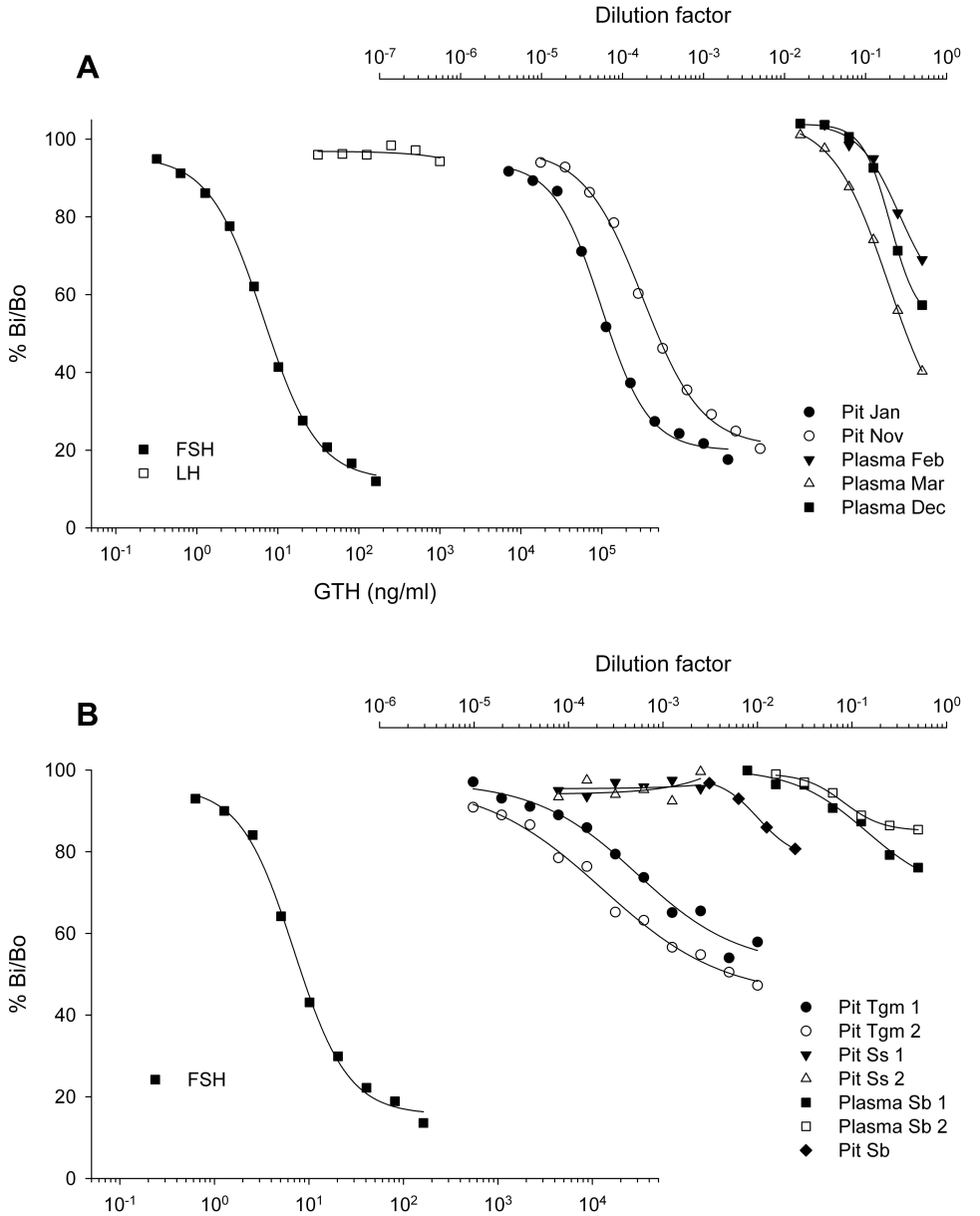
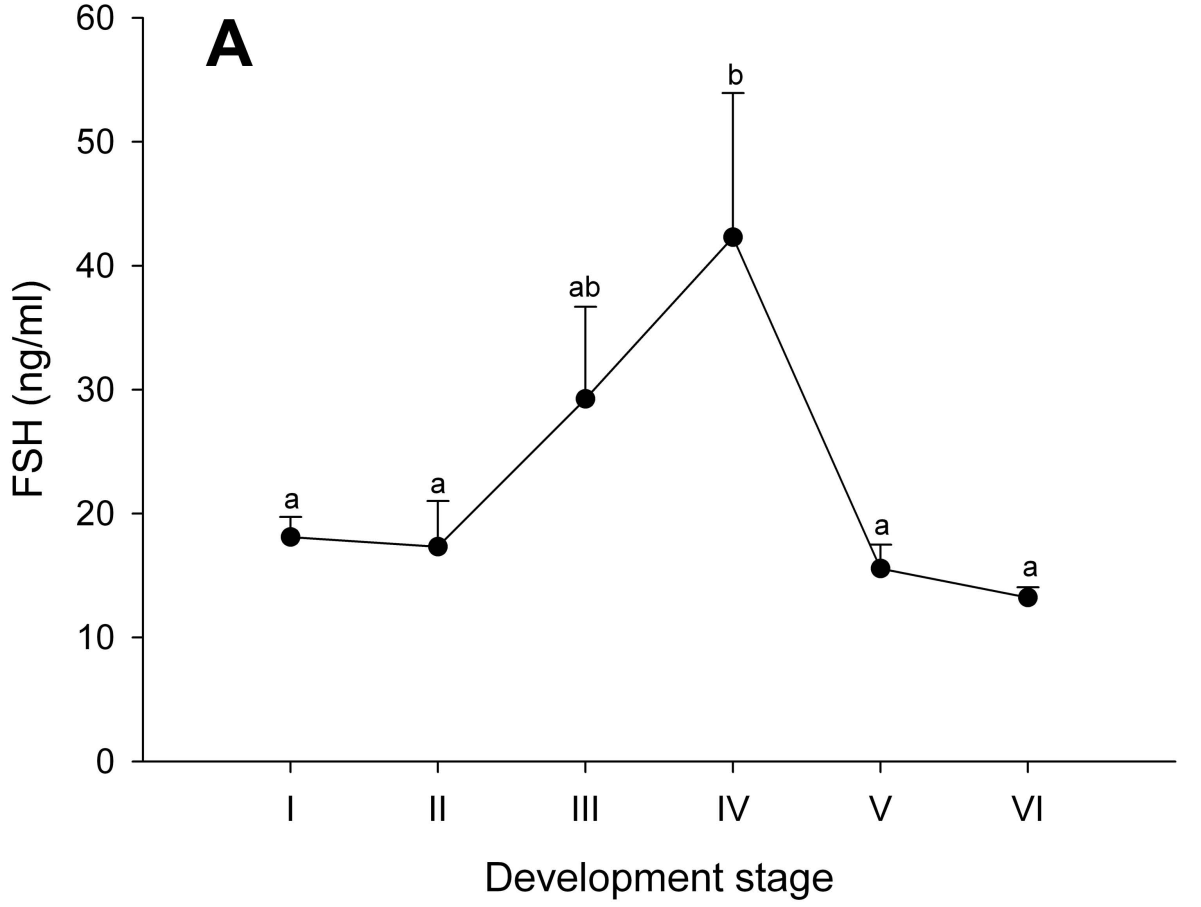

A
Dilution factor
10-7 10-6 10-5 10-4 10-3 10-2 10-1 100
% Bi/Bo
100 80 60 40 20 0
FSH
LH
Pit Jan
Pit Nov
Plasma Feb
Plasma Mar
Plasma Dec
10-1 100 101 102 103 104 105
GTH (ng/ml)
B
Dilution factor
10-6 10-5 10-4 10-3 10-2 10-1 100
% Bi/Bo
100 80 60 40 20 0
FSH
Pit Tgm 1
Pit Tgm 2
Pit Ss 1
Pit Ss 2
Plasma Sb 1
Plasma Sb 2
Pit Sb
10-1 100 101 102 103 104


A
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FSH (ng/ml)
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a
ab
b
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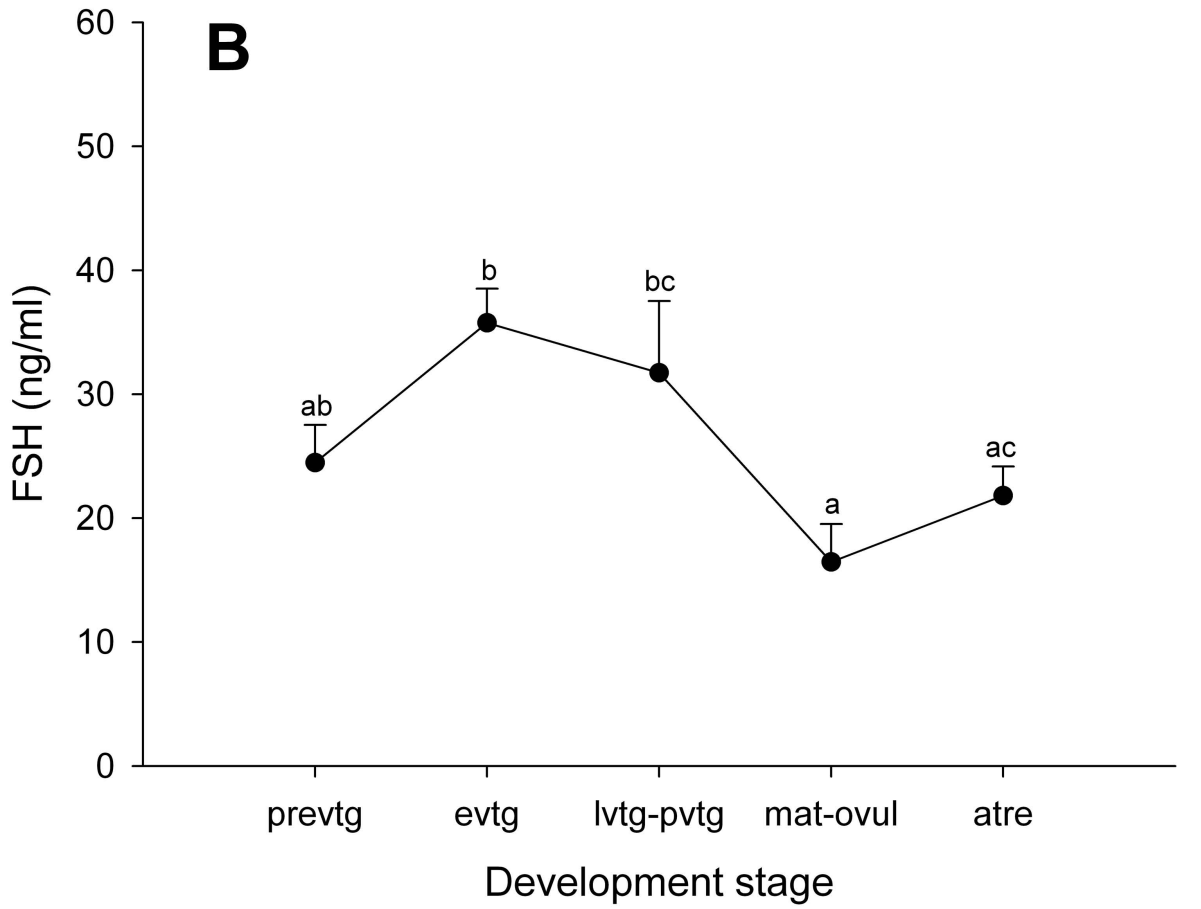
a
I
II
III
IV
V
VI
Development stage


B
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40
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10
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FSH (ng/ml)
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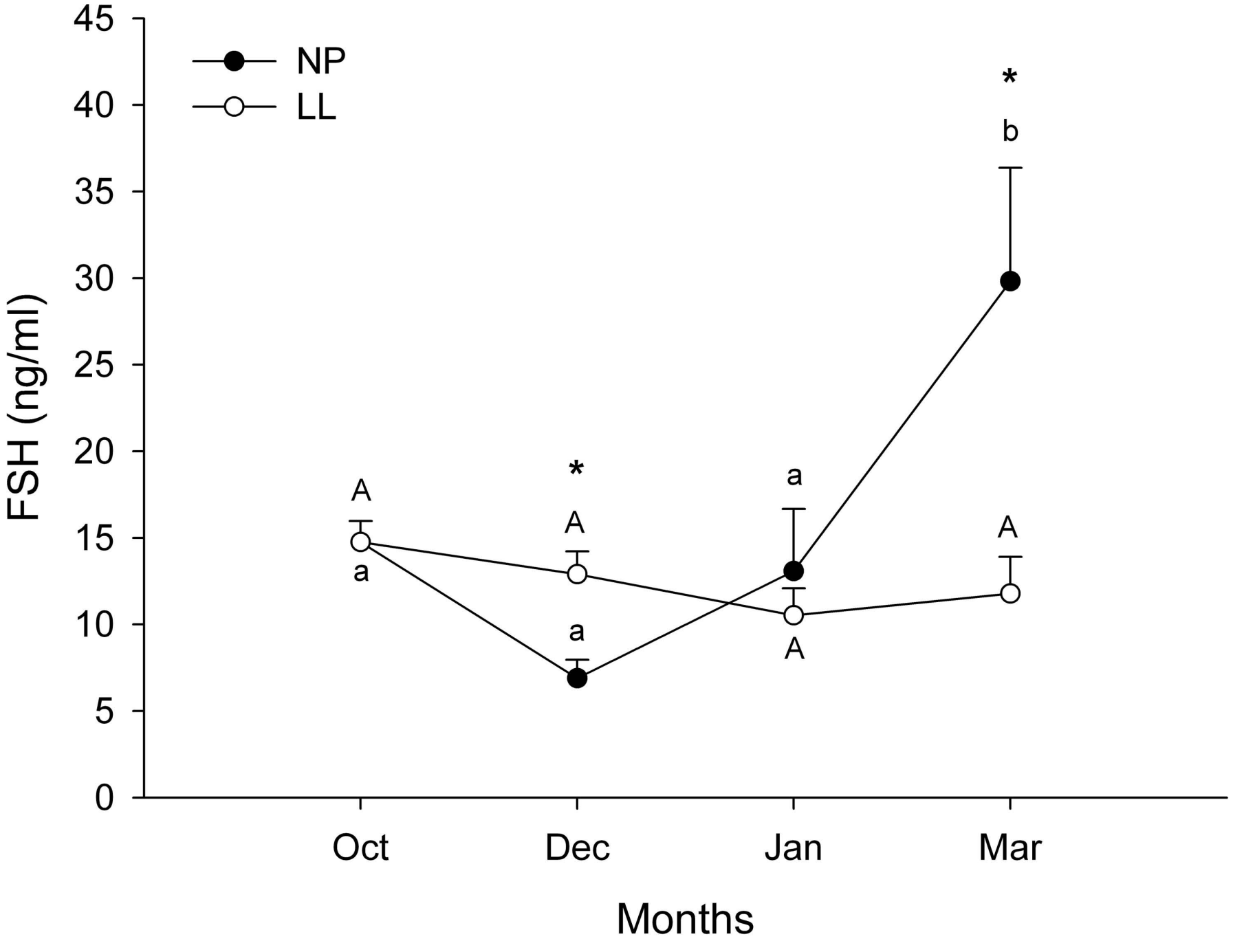
prevtg
evtg
lvtg-pvtg
mat-ovul
atre
Development stage


NP
LL
FSH (ng/ml)
45
40
35
30
25
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15
10
5
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Oct
Dec
Jan
Mar
Months
A
a
*
A
a
a
A
A
*
b
A