General and Comparative Endocrinology 187 (2013) 95–103

Contents lists available at SciVerse ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Characterization and seasonal changes in LHβ and FSHβ mRNA of *Rhinella arenarum* (Amphibia, Anura)

M. Clara Volonteri *, Eleonora Regueira, M. Florencia Scaia, Nora R. Ceballos

Laboratorio de Endocrinología Comparada, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 26 September 2012
Revised 13 March 2013
Accepted 17 March 2013
Available online 22 April 2013

Keywords:
Pituitary
Gonadotropins
Toad
Seasonal variations
Androgens

ABSTRACT

In anurans, two types of gonadotropins were described in several species of Ranidae and Pipidae families but only in one of the Bufonidae family. *Rhinella arenarum* is a bufonid that have the lowest concentration of plasma androgens during the breeding. The objective of this paper was to characterize the cDNA sequence of β subunit of LH and FSH from toad pituitary and study seasonal variation in gonadotropins mRNA using quantitative real-time RT-PCR. The LHβ cDNA is a 636 bp sequence containing an open reading frame (ORF), 45 bp of 5′-untranslated region (UTR) and 174 bp of 3′-UTR. The ORF encodes for a signal peptide of 26 amino acids and a mature protein of 113 amino acids with one N-glycosylation site at the 34th position. The FSHβ cDNA sequence is a 535 bp fragment containing an ORF, 8 bp of 5′-UTR and 152 bp of 3′-UTR. The ORF encodes for a signal peptide of 20 amino acids and a mature protein of 104 amino acids with two N-glycosylation sites at 25th and 42nd positions. Multiple alignments of aminoacid deduced sequences of LHβ and FSHβ (teleosts, amphibians, birds, mammals) showed that all the tetrapods studied conserve 12 cysteins and one (LH) or two (FSH) N-Glycosylation sites. LHβ is closer to teleosts than to mammals and birds while FSHβ is closer to mammals. The analysis of seasonal changes in LHβ and FSHβ mRNA indicates that transcript levels have seasonal variations and that the profile of androgens is opposite to that of the gonadotropins mRNA.

1. Introduction

Pituitary gonadotropins play critical roles in the control of gametogenic and endocrine functions of vertebrate gonads (Kawauchi and Sower, 2006; Norris, 2007). The majority of vertebrate species expresses two gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Both gonadotropins as well as thyroid-stimulating hormone (TSH) and human chorionic gonadotropin (hCG) belong to a family of glycoprotein hormones composed by two non-covalently linked N-glycosylated subunits, the α-subunit, common for all the members of the family, and the β-subunit, specific for each active hormone (Norris, 2007; Pierce and Parsons, 1981).

Several authors have studied the importance of the N-glycosylation of these hormones and how the presence of carbohydrates affects hormonal functions (Baenziger et al., 1992; Manna et al., 2002). Baenziger et al. (1992) proposed that the glycosylation of the β subunits of LH plays a central role in hormone bioactivity by regulating the rate of hormonal clearance from the circulation without affecting the interaction with its receptor. Manna et al. (2002) found that two-point mutations in human LHβ, that provide an extra N-glycosylation consensus site, increase the bioactivity without affecting the affinity for the LH receptor.

In anurans, many papers have reported that bullfrog pituitary has two chemically distinct gonadotropins similar to mammalian LH and FSH (Hanaoka et al., 1984; Hayashi et al., 1992a,b; Licht and Papkoff, 1974; Licht et al., 1977; Papkoff et al., 1976; Takada and Ishii, 1984; Takahashi and Hanaoka, 1981, 1985). In addition, two types of gonadotropins were described in *Rana pipiens* (Zhang et al., 2007), *Xenopus laevis* (Huang et al., 2001), *Bufo japonicus* (Itoh et al., 1990; Itoh and Ishii, 1990; Komoike and Ishii, 2003), *Rana dybowskii* and *Rana nigromaculata* (Kim et al., 1998), and *Rana esculenta* (Pinelli et al., 1996).

In mammals, LH regulates androgen biosynthesis through the interaction with LH-receptor located in Leydig cells (Hall, 1994).

Abbreviations: FSH, follicle-stimulating hormone; LH, luteinizing hormone; LHβ, β subunits of LH; FSHβ, β subunits of FSH; RIA, radioimmunoassay; UTR, untranslated region; ORF, open reading frame.

* Corresponding author. Address: Laboratorio de Endocrinología Comparada, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Pabellón 2, Ciudad Universitaria, C1428EHA Buenos Aires, Argentina. Fax: +54 11 4576 3384.

E-mail addresses: claravolonteri@bg.fcen.uba.ar (M.C. Volonteri), eleonoraregueira@gmail.com (E. Regueira), mflorenciascaia@bg.fcen.uba.ar (M.F. Scaia), nceballo@bg.fcen.uba.ar (N.R. Ceballos).

0016-6480/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ygcen.2013.03.025

In amphibians, gonadotropins are also involved in the regulation of testicular steroidogenesis (Cobellis et al., 2008; Kobayashi et al., 1993; Licht et al., 1983; Pozzi et al., 2006; Pozzi and Ceballos, 2000). However, in several anuran species plasma androgens decline in spring, when reproduction takes place, reaching the lowest values in the summer (Guarino et al., 1993; Itoh et al., 1990; Rastogi et al., 1986), even though LH and FSH rise during the reproductive season (Kim et al., 1998; Licht et al., 1983; Polzonetti-Magni et al., 1998). The fact that plasma gonadotropins increase when androgens decline suggest that some aspects of the regulation of steroidogenesis and gonadotropin synthesis and secretion remain to be explored. Nevertheless, even if the importance of pituitary gonadotropins for steroidogenesis is accepted, little is known about the mechanisms whereby gonadotropins induce this process (Nagahama, 1986).

Rhinella arenarum is a South American toad with a breeding season restricted to springtime i.e., between September and December, and it is characterized for having the lowest concentration of plasma androgens during the breeding (Canosa and Ceballos, 2002a; Canosa et al., 1998; Denari and Ceballos, 2005). Besides, it was suggested that FSH is involved in the regulation of the change in testicular steroidogenesis during the breeding (Canosa and Ceballos, 2002b).

For a better understanding of the reproductive physiology of amphibians, it is important to take into account seasonal changes in the reproductive events, among them the annual variation in gonadotropins synthesis. Taking these considerations into account, the main objective of this paper is to characterize the cDNA sequence of LHβ and FSHβ subunits from *R. arenarum* pituitary as well as to study the seasonal variation in the expression of β subunits mRNA of both gonadotropins by using quantitative real-time RT-PCR method.

2. Materials and methods

2.1. Materials

All the primers used and the GeneRacer Kit were from Invitrogen (Carlsbad, CA). GenElute™ Mammalian Total RNA Miniprep Kit was acquired in Sigma–Aldrich (St. Louis, MA). Oligo(dTs) were obtained in Biodynamics (Buenos Aires, Argentina). RNase-free DNaseI, RT-buffer, dNTPs mix, AMV Reverse Transcriptase, $MgCl_2$ and Go*Taq* DNA Polymerase were purchased in Promega (Madison, WI). Accuprep purification kit was from Bioneer (Daejeon, Korea) and FastSart Universal SYBR Green Master (Rox) was from Roche (Mannheim, Germany). Tricaine methanesulfonate (MS222) was obtained from Sigma–Aldrich (St. Louis, MO).

2.2. Animals

Reproductive male toads of *R. arenarum* were collected near Buenos Aires City during all year long. Animals were maintained with free access to water and fed with crickets and zophobas under natural conditions of light and temperature. For tissue preparation, animals were over-anaesthetized by immersion in 1% aqueous solution of MS222 (Gentz, 2007). To avoid the variability due to diurnal changes in hormone production, both pituitary and blood were always obtained at the same time of the day (between 10 and 12 am). The experiments comply with the Guiding Principles for the Care and Use of Research Animals promulgated by the Society for the Study of Reproduction and with the approval of Comisión Institucional para el Cuidado y Uso de Animales de Laboratorio, Facultad de Ciencias Exactas y Naturales, Buenos Aires, Argentina.

2.3. Tissue collection and total RNA isolation

For gonadotropin characterization and tissue expression studies, brain, spinal cord, heart, liver, kidney, adrenal gland, lung, testis, Bidder organ and pituitary from different animals were rapidly excised and stored at −70 °C until total RNA isolation. Total RNA was extracted using the GenElute™ Mammalian Total RNA Miniprep Kit, RNA samples were quantified with the Qubit Fluorometer (Invitrogen, Carlsbad, CA) and RNA integrity was checked by 1% agarose gel electrophoresis. All RNA samples were pretreated with RNase-free DNaseI to remove genomic DNA contamination prior to reverse transcription.

2.4. Reverse transcription (RT)

Transcription of RNA into cDNA was carried out by reverse transcriptase reaction as follows: 0.5 μg of RNA was incubated with 0.3 μl oligo(dT) primer (sequence: 5′-TTT TTT TTT TTT TTT-3′) in a 20 μl final volume, at 70 °C for 10 min. After incubation, samples were placed rapidly on ice. Reverse transcription was performed by adding to each sample a mixture of DEPC treated water, 4 μl AMV RT-buffer, 2 μl dNTPs mix (25 mM each), and 0.2 μl AMV Reverse Transcriptase. The incubation was carried out at 48 °C for 45 min and ended by heating at 95 °C for 5 min.

2.5. Oligonucleotides

Oligonucleotides used as PCR primers for the 3′ and 5′ rapid amplification of cDNA ends (RACE) and for Real time-PCR are listed in Table 1. Degenerate primers were designed using a Basic Local Alignment Tool (BLAST) based on the known nucleotide sequences of different species (see Table 2 for GenBank Accession numbers).

All specific primers used for real time PCR were synthesized based on LHβ, FSHβ and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, expression control) sequences obtained from the degenerate PCR or 3′ and 5′ RACE.

Table 1
Sequence of oligonucleotide primers used.

Primer	Sequence
LH-F	5′-GAGAARGAVSRCTGYCCA-3′
LH-R	5′-TSARWGCCACRGGGTAGG-3′
FSH-F	5′-TGGTGYKCMGGATACTGC-3′
FSH-R	5′-TCCACARTGACARTCRAC-3′
GAPDH-F	5′-ACATGTTCMARTATGAYTC-3′
GAPDH-R	5′-AAKTTGTCRTKGATGACCT-3′
LH-3′ RACE	5′-CCTGGTTGCTTACTTGTCTGCCGTGC-3′
LH-3′ nested RACE	5′-CCATGTCATCTCGTCAATGCCACCA-3′
LH-5′ RACE	5′-TGGTGGCATTGACGAGATGACATGG-3′
LH-5′ nested RACE	5′-GCACGGCAGACAAGTAAGCAACCAGG-3′
FSH-3′ RACE	5′-GGCTGTCCTGAGAAAGTAAATCCTT-3′
FSH-3′ nested RACE	5′-TCCGGTGGCGGTTGACTGTCACTGT-3′
FSH-5′ RACE	5′-ACAGTGACAGTCAACCGCCACCGGA-3′
FSH-5′ nested RACE	5′-AAGGATTTACTTTCTCAGGACAGCC-3′
LH-F-SP	5′-TCCTTGGTCCTGGTTGCTTACTTG-3′
LH-R-SP	5′-GTGGTCCTTCTCGGCAGATATGG-3′
FSH-F-SP	5′-GAGATTGTGCCTGTATTATGTTC-3′
FSH-R-SP	5′-GATGGTGGAATTGCTCTTTAAC-3′
GAPDH-F-SP	5′-CCCATCACCGTCTCTCAGG-3′
GAPDH-R-SP	5′-CGCTTGGCACCTCCTTTC-3′
GeneRacer™ 5′ Primer	5′-CGACTGGAGCACGAGGACACTGA-3′
GeneRacer™ 5′ Nested Primer	5′-GGACACTGACATGGACTGAAGGAGTA-3′
GeneRacer™ 3′ Primer	5′-GCTGTCAACGATACGCTACGTAACG-3′
GeneRacer™ 3′ Nested Primer	5′-CGCTACGTAACGGCATGACAGTG-3′

IUB code: R = AG, Y = CT, K = GT, M = AC, S = GC, W = AT, B = CGT, D = AGT, H = ACT, V = ACG, N = AGCT. SP = specific primer.

Table 2
GenBank Accession numbers of the mRNA sequences used to synthesize degenerate primers.

Species	LH	FSH	GAPDH
Leopard frog (*Rana pipiens*)	DQ054791	DQ054790	–
Bullfrog (*Rana catesbeiana*)	DQ003309	–	–
Japanese frog (*Rana japonica*)	AB178053	AB178054	–
Marsh frog (*Rana ridibunda*)	AJ311355	–	AB067752
Japanese toad (*Bufo japonicus*)	AB085666	AB085668	–
Pseudolabrus sieboldii	AB300391	–	–
Rainbow trout (*Oncorhynchus mykiss*)	AB050836	–	–
Mouse (*Mus musculus*)	NM008497	NM_008045	-
Human (*Homosapiens*)	NM000894	NM_000510	AB062273
African clawed frog (*Xenopus laevis*)	–	AB175888	NM_001087098
Tropical clawed frog (*Silurana tropicalis*)	–	AB067752	NM_001004949
Newt (*Cynops pyrrhogaster*)	–	–	–
Atlantic salmon (*Salmo salar*)	–	–	BT046468
Rainbow smelt (*Osmerus mordax*)	–	–	BT075447

2.6. Degenerate PCR of LHβ, FSHβ and GAPDH

The PCR using degenerate primers (Table 1) was performed to obtain the partial internal LHβ, FSHβ and GAPDH sequences necessary for RACE primers synthesis. The reaction mixture consisted in 3.5 mM $MgCl_2$, 0.5 mM each dNTP, 1.2 μM each primer and 1.25U Go*Taq* DNA Polymerase, in a final volume of 25 μl. The conditions for all PCR were 5 min at 94 °C followed by 35 cycles of 0.5 min denaturing at 94 °C, 0.5 min annealing at 50 °C (LHβ and FSHβ) or 45 °C (GAPDH), and 0.5 min extension at 72 °C, ended by a final extension for 5 min at 72 °C. A 179 bp fragment for LHβ, 218 bp fragment for FSHβ and 370 bp fragment for GAPDH were amplified, purified with Accuprep purification kit and sequenced.

2.7. 3′ and 5′ RACE

Both 3′ and 5′ RACE for *R. arenarum* LHβ and FSHβ were carried out using the GeneRacer Kit. Briefly, for 3′ RACE, first-strand cDNA was synthesized from 1 μg of intact pituitary total RNA using GeneRacer oligo dT primer. For 5′ RACE, RACE-ready cDNA was generated according to the manufacturer's instructions. All PCR reaction mixtures consisted in 3.5 mM $MgCl_2$, 0.5 mM each dNTP, 1.2 μM of each RACE primer, 1.6 μM of each GeneRacer primer, and 1.25 U *GoTaq* DNA Polymerase in a total volume of 25 μl. PCR conditions were 5 min at 94 °C followed by 35 cycles of 0.5 min denaturing at 94 °C, 0.5 min annealing at 70 °C (LHβ) or 63 °C (FSHβ), and 0.5 min extension at 72 °C, ended by a final extension for 5 min at 72 °C.

2.8. Real time PCR

PCR was performed on cDNA samples in duplicates using an Opticon Monitor system (BioRad, Marnes-la-Coquette, France). Primers were designed using the Beacon Designer 6.0 software (PREMIER Biosoft International) and are described in Table 1. After optimization, PCR reactions were performed in a 10 μl volume containing 2.5 μl cDNA dilution, 5 μl FastSart Universal SYBR Green Master (Rox) and 1.25 μl of each 2.5 μM diluted primer. Conditions were as follows: 5 min at 95 °C followed by 40 cycles of 15 s denaturing at 95 °C, 20 s annealing at 59 °C and 36 s extension at 72 °C, ended by a final incubation for 15 s at 95 °C. Results were analyzed using the standard curve mode according to the manufacturer's recommendations (Opticon Monitor, BioRad, Hercules, CA). GAPDH was used as internal standard (see 2.5 Oligonucleotides).

2.9. Sequence analyses

The nucleotide and amino acid sequences from all species used in this work were downloaded from the GenBank database. Multiple sequences alignments were performed with ClustalW2 program. Both 3′ and 5′ RACE partial sequences were assembled using CAP3 Sequence Assembly Program. The putative open reading frame (ORF) and the amino acid sequence of *R. arenarum* LHβ and FSHβ proteins were predicted using the ORF Finder from the NCBI followed by a BLAST alignment to choose the ORF corresponding to LHβ and FSHβ proteins. The signal peptide and putative cleavage site were predicted using the SignalP 4.0 Server and conserved domains and glycosylation sites were predicted with ExPASy Bioinformatics Resource Portal. Phylogenetic trees were generated based on the aligned amino acid sequences, analyzed with ClustalW2 program by the neighbor-joining method and visualized with TreeView1.6.6.

2.10. Determination of plasma testosterone by radioimmunoassay

Blood samples were obtained with heparinized syringes by cardiac puncture and plasma was separated at 4 °C by centrifugation at 3500 rpm for 10 min. Aliquots of plasma (20 μl) were treated overnight with ten volumes of methanol, evaporated and re-dissolved in radioimmunoassay (RIA) buffer (10 mM phosphate buffer, 1% w/v gelatin, 140 mM NaCl, 20 mM EDTA, pH 7.4). Testosterone was determined as described by Pozzi and Ceballos (2000). Briefly: serial dilutions of the standard curve, non-specific binding, maximum binding, and samples were incubated with 10,000 dpm of [^{3}H] testosterone (80.4 Ci/mmol, Perkin-Elmer, Boston, MA) in a final volume of 0.5 ml at 4 °C for 18–22 h. Testosterone antibody was from The Colorado State University (Co, USA) and was employed in a dilution of 1:125,000 (Pozzi and Ceballos, 2000). Charcoal–dextran method was used to separate bound and free hormones. The sensitivity of the assay was 10 pg/ml. Mean intra-assay variation and inter-assay variation were 7.4% and 12.6%, respectively. Radioactivity was determined by liquid scintillation counting with Wallac 1409 DSA equipment (Wallac Co, Turku, Finland). The scintillation cocktail for all samples was OptiPhase-Hi safe 3 (Wallac Co, Turku, Finland).

2.11. Statistics

Results were expressed as means ± SE, and analyzed and compared by using one way ANOVA test and *a posteriori* contrast by Tukey' test. Since both gonadotropins measured were from the same animal, Bonferroni correction was included in ANOVA. The signification level in all tests was 5%.

3. Results

The sequence of the β subunits of LH and FSH was characterized by rapid amplification of cDNA ends from total pituitary RNA and

the result allowed the identification of the nucleotide sequences of LHβ and FSHβ mRNA. The full length LHβ cDNA from *R. arenarum* is a 636 bp sequence containing an ORF, 45 bp of 5′-untranslated region (UTR) and 174 bp of 3′-UTR. The open reading frame encodes for a signal peptide of 26 amino acids and a mature protein of 113 amino acids with one N-glycosylation site at the 34th position (Accession No. JN031567.1 and Fig. 1). The comparison of the precursor protein with LHβ protein from fish, amphibians, birds and mammals indicated that in all the vertebrate groups analyzed herein there is only one highly conserved N-glycosylation site and 12 conserved cysteins (Fig. 1). Local alignment analysis showed that LHβ of *R. arenarum* is almost identical to another bufonid (92%), and exhibits more differences with several ranids (77 to 81%), *X. laevis* (71%) and *Silurana tropicalis* (71%), while teleosts is the nearest external group, with 50 to 59% identity. The proximity with mammals and teleosts is similar since mammalian identity scores are around 45% (Table 3). Despite these scores, phylogenetic analysis showed that amphibians are closer to mammals than to teleosts (see supplementary material 1)

The cDNA sequence of FSHβ from *R. arenarum* is a 535 bp fragment containing an ORF, 8 bp of 5′-untranslated region (UTR) and 152 bp of 3′-UTR. The open reading frame encodes for a signal peptide of 20 amino acids and a mature protein of 104 amino acids with two N-glycosylation sites at 25th and 42nd positions (Accession No. JX114748 and Fig. 2). The sequence of the precursor protein was compared with FSHβ protein from fish, amphibians, birds and mammals, and results indicate that there is only one fully conserved N-glycosylation site (Fig. 2). The second site observed in *R. arenarum* is not present in the teleosts analyzed but it is fully conserved in tetrapods. In addition, there are nine fully conserved cysteins in the vertebrate studied and three additional cysteins conserved only in tetrapods but not in all the teleosts considered (Fig. 2). Local alignment analysis showed in Table 3 indicated that FSHβ of *R. arenarum* is almost identical to another bufonid, *B. japonicus* (98%), with a lesser similarity to ranids (82–88%), and *X. laevis* and *S. tropicalis* (71% and 67%, respectively). In opposition to what happens with LHβ, for FSHβ teleosts is not the nearest external group since mammals and birds are closer, with 55–60% identity, respectively (Table 3). The same pattern was observed in the phylogenetic analysis of FSHβ (see supplementary material 2).

Real time RT-PCR was used to quantify mRNA expression of pituitary gonadotropins and to characterize the annual pattern of mRNA expression. In order to determine the specificity of the technique, the expression of LHβ and FSHβ mRNA was analyzed in different tissues. Fig. 3 shows a representative agarose gel performed with cDNA obtained from different tissues. Bands corresponding to LHβ and FSHβ were detected only in pituitary, whereas GAPDH is expressed in all the tissues studied, confirming the tissue-specific expression of gonadotropins.

After determining that the real time PCR analysis was specific for gonadotropins, the annual profile of pituitary LHβ and FSHβ mRNA was determined in animals captured all year long. Results shown in Fig. 4 indicate that both gonadotropins have a similar annual profile. LHβ and FSHβ have three peaks along the year, one in spring, another one in the early summer and the last one in the late summer. In addition, both mRNA reach the minimum expression in autumn and winter, and also in November, month corresponding to the late spring. Even if the amount of LHβ and FSHβ mRNAs is similar during the year, there is an exception in February. In this month the amount of LHβ mRNA is almost three times higher than FSHβ mRNA. Another difference is that FSHβ mRNA remains elevated in March when LHβ mRNA decreases.

Data from the different months were grouped according to the reproductive seasons previously described for *R. arenarum*, post reproductive, pre reproductive and reproductive seasons (Denari and Ceballos, 2005). As shown in Fig. 5, the amount of FSHβ mRNA is significantly lower in the pre reproductive season than in the reproductive and post reproductive ones. Regarding LHβ mRNA, there are significant differences between post and pre reproductive season, with intermediate values in the reproductive one.

In order to compare androgen production with the profile of the mRNA of both gonadotropins, plasma concentration of testosterone was determined in animals collected all year long. Results indicate that animals in the reproductive season (September to December) have the lowest levels of androgens while in the pre reproductive season (May to August) they have the highest one (Fig. 4).

4. Discussion

The study of the mechanisms involved in the regulation of the pituitary–gonadal axis makes critical to characterize all the parameters involved and, also, the seasonal changes of all of the actors. Taking into account the limited information regarding amphibian gonadotropins, it is very important to increase the knowledge of the phylogenetic distance between amphibian and other vertebrates as mammals and fish, where there is much more information. Moreover, this knowledge is also an important contribution to amphibian phylogeny since in this aspect it is the first description in one American toad. In addition, the nucleotide sequences of toad gonadotropins are necessary to carry out studies about the regulation of mRNA synthesis. In this study, cDNAs of *R. arenarum* LHβ and FSHβ were characterized in order to contribute to the knowledge of amphibian gonadotropins. Sequence analyses of cDNAs indicate that *R. arenarum* has 92% and 98% homology for LHβ and FSHβ, respectively, with another member of the same family, *B. japonicus*. Moreover, these analyses confirmed that LHβ and FSHβ are highly conserved within anurans since they have between 70% and 98% identities with the other members of the group. The analysis of identity also confirms that in anurans FSHβ seems to be more conserved than LHβ, but has more divergence with teleosts. Identity scores for anuran LHβ indicate that it is more like teleost LHβ than mammalian LHβ while the opposite seems to be true for FSHβ. However, phylogenetic analysis does not indicate the same relationship for LHβ but suggests that amphibians and amniotes exhibit greater phylogenetic proximity than amphibians and teleosts or amniotes and teleosts (see Phylogenetic tree in supplementary material). These conclusions are different to that from Komoike and Ishii (2003) that found after cluster analysis that LHβ from anurans is closer to fish than to birds and mammals while FSHβ has the opposite phylogenetic relationship. In addition, Quérat et al. (2004) suggested that the rate of evolution of the LHβ subunit was particularly high throughout the radiation of mammals while β subunits of FSH and TSH kept quite stable in this lineage.

The comparison of the identities between the soft-shell turtle and different vertebrates indicated that the identity score between reptiles, birds and mammals are larger than between reptiles and fish, suggesting that during the evolution of amniotes FSHβ was more conserved than LHβ (Chien et al., 2005). Therefore, and considering that similar results are described in amphibians, we also suggest that FSHβ has been more conserved during tetrapods evolution than LHβ.

In the vertebrates analyzed herein, all LHβ cysteins are conserved. This fact is expected since cysteins residues are involved in the disulfide bonds formation that is required to produce the correct conformation of the β subunit. This conformation is vital since in turn it allows the heterodimerization with the α chain (Levavi-Sivan et al., 2010). Similar conclusions were published by Zhang et al. (2007) after comparing sequences of other vertebrate species, including Chondrichthyes. On the other hand, FSHβ cysteins, at

```
Rhinella arenarum        --MLPAARMFTPQLI---------------SLVLVAYLSAVQGRRP--CH 31
Bufo japonicus           ---LPAVRMFTPQLI---------------SLVLVTYLSAVQGRLP--CH 30
Rana catesbeiana         --MFSTIRMFSCQLT---------------ILLLFAHLSAVQGRHV--CH 31
Rana pipiens             --MFSTIRMFSCQLT---------------ILLLFAHLSAVQGRHV--CH 31
Rana rugosa              --MFSTIRMFNCQLT---------------ILLLFAHLSAVQGRHV--CH 31
Rana japonica            --------MFSCQLT---------------ILCLFAHLSAVQGRHI--CH 25
Rana ridibundus          --MFSAIRMFNRQLT---------------ILLLFAHLSAVQGRHL--CH 31
Xenopus laevis           --------MFRSQVT---------------LTLLGFYLSAVQGRRL--CH 25
Silurana tropicalis      --------MFRSQVT---------------LTLVAIYLSAVQGRRL--CH 25
Cynops pyrrhogaster      ---------MPPTLA---------------LLLLALSLSAAQGSGL--CH 24
Oncorhynchus mykiss      --------MLGLHVG-----------TLISLLLCIL-LEPVEGSLMQPCQ 30
Danio rerio              --------MLLAGNG-----------VFFLFSLFFL-LAAAQSLVFPRCE 30
Plecoglossus altivelis   --------MLGTSVSCVP--------FLLLLHLLFC-LNPSVAAHLQHCQ 33
Odontesthes bonariensis  MVAQVSITMFPMMMS-----------LFLGVSFFIWPLAPAVAFQLPLCQ 39
Oreochromis mossambicus  ----ISRMLLALMLS-----------LFVGASTFI--LSPAAAFQLPFCQ 33
Mus musculus             -MERLQGLLLWLLLSP----------------SVVWASR---GPLRPLCR 30
Gallus gallus            -MGGAQVLVLMTLLGTPPATTGNPPVAVDPPLAVVGPPMGLGGGGRPPCR 49
Homo sapiens             ----------------------------MKTLQFFFLFCCWKAICCNSCE 22
                                                                   .     *.

Rhinella arenarum        LVNATISAEKDHCPLCVTFTTTICSGHCETKDPVYKTALSAFKQQICTYK 81
Bufo japonicus           LVNATISAEKDHCPVCITFTTTICSGHCWAKDPVYKTALAAVKQKICTYK 80
Rana catesbeiana         LANATISAEKDHCPVCITFTTSICTGYCQTMDPVYKTALSSFKQNICTYK 81
Rana pipiens             LANATISAEKDHCPVCITFTTSICTGYCQTMDPVYKTALSSFKQNICTYK 81
Rana rugosa              LANATISAEKDHCPMCVTFTTTICTGYCETMDPVYKTALSSFKQKICTYK 81
Rana japonica            LANATISAEKDHCPVCITFTTSICTGYCQTMDPVHKTALSSFKQKICTYK 75
Rana ridibundus          LANATISAEKDHCPVCVTFTTTICSGYCQTLDPVHKTALFPFKQKICTYK 81
Xenopus laevis           PTNATISAEKDDCPICVTLTTTVCTGYCLTRDPVFKNALSSVYQNICTYN 75
Silurana tropicalis      PTNATISAEKDDCPICVTLTTTICSGYCLTRDPVFKNALSSVYQNICTYN 75
Cynops pyrrhogaster      LTNATISAEKDDCPICVTFTTSICSGYCHTKELVYKHALSTNSQKVCTYK 74
Oncorhynchus mykiss      PINQTVSVEKEGCPTCLVIQTPICSGHCVTKEPVFKSPFSTVYQHVCTYR 80
Danio rerio              LVNETVSVEKEGCPKCLVFQTTICSGHCVTRDPVYKSPFSTVHQTVCTYR 80
Plecoglossus altivelis   PINQTVSLEKEGCPTCLVFETSICGGHCLTKEPVRRRPYMPVSQHVCTYK 83
Odontesthes bonariensis  PINQTVSLEKDGCARCHPVEATICSGHCVTKDPVMKTWFSNMYQSVCTYQ 89
Oreochromis mossambicus  LINQTVSLEKEGCPSCHPVETTICSGHCITKDPVIKIPFSNVYQHVCTYR 83
Mus musculus             PVNATLAAENEFCPVCITFTTSICAGYCPSMVRVLPAALPPVPQPVCTYR 80
Gallus gallus            PINVTVAVEKDGCPQCMAVTTTACGGYCRTREPVYRSPLGPPPQSACTYG 99
Homo sapiens             LTNITIAIEKEECRFCISINTTWCAGYCYTRDLVYKDPARPKIQKTCTFK 72
                           * *:: *:: *  *  . :. * *:* :   *         *  **:

Rhinella arenarum        EIRYDTIKLPDCLPGTDPFFTYPVALSCDCDLCKMDYSDCTVESSSEPDV 131
Bufo japonicus           DIRYDTIKLPDCLPGTDPFFTYPVALSCDCDLCKMDYSDCTVESSSEPDV 130
Rana catesbeiana         EIRYDTIKLPDCLPGTDPFFTYPVALSCYCDLCKMDYSDCTVESS-EPDV 130
Rana pipiens             EIRYDTIKLPDCLPGTDPFFTYPVALSCYCDLCKMDYSDCTVESS-EPDV 130
Rana rugosa              EIRYDTIKLPDCLPGTDPFFTYPVALSCHCDLCKMDYSDCAVESS-EPDV 130
Rana japonica            EIRYDTIKLPDCLPGTEPILTYPVALSCHCDFCKMDYSDCTVESS-EPDV 124
Rana ridibundus          EIRYDTIKLPDCLPGTDPFFTYPVALSCYCDFCKMDYSDCTVESS-EPDV 130
Xenopus laevis           EIRYDTIKLPDCLPGTDPFFTYPVAVSCKCNQCKMDYSDCTVQSI-GPDF 124
Silurana tropicalis      EIRYDTIKLPDCLPGTDPFFTYPVAVSCKCNQCKMDYSDCTVQSI-GPDF 124
Cynops pyrrhogaster      EIRYDTIKLPNCPPGVDPFITYPVALSCDCNMCKMDYSDCTVQSI-GPEF 123
Oncorhynchus mykiss      DVRYETIRLPDCPPWVDPHVTYPVALSCDCSLCNMDTSDCTIESL-QPDF 129
Danio rerio              DVRYETINLPDCSAGVDPQITYPVALSCDCSLCTINTSDCTIQSL-QPDF 129
Plecoglossus altivelis   DVHYQTVRLPDCPPNVDPFVSFPVALSCECNMCTMDTSDCTIQSL-NPDF 132
Odontesthes bonariensis  EFYYKTFELPDCLPGVDPTVSYPVAVSCHCGGCAMDTSDCTFESL-QPNF 138
Oreochromis mossambicus  DLYYKTFELPDCPPGVDPIVTYPVALSCHCGRCAMDTSDCTFESM-QPDF 132
Mus musculus             ELRFASVRLPGCPPGVDPIVSFPVALSCRCGPCRLSSSDCGGPRT-QPMA 129
Gallus gallus            ALRYERWALWGCPIGSDPRVLLPVALSCRCARCPMATSDCTVQGL-GPAF 148
Homo sapiens             ELVYETVRVPGCAHHADSLYTYPVATQCHCGKCDSDSTDCTVRGL-GPSY 121
                          . :    : .*    :.    *** .* *  *    :**       *

Rhinella arenarum            CVKSR----LDM----- 139
Bufo japonicus               CVKSR----LDM----- 138
Rana catesbeiana             CMKRR----ISI----- 138
Rana pipiens                 CMKSR----ISI----- 138
Rana rugosa                  CMKSR----ISI----- 138
Rana japonica                CMKSR----ISI----- 132
Rana ridibundus              CMKSR----IFI----- 138
Xenopus laevis               CSNSL----VSQFQLIN 137
Silurana tropicalis          CTNSL----VSQFQLIN 137
Cynops pyrrhogaster          CSASR----GFL----- 131
Oncorhynchus mykiss          CITQRVLTDGDMW---- 142
Danio rerio                  CMSQR--EDFPAY---- 140
Plecoglossus altivelis       CMTQT--DFEPAYY--- 144
Odontesthes bonariensis      CMNDI-----DFYYSLQ 150
Oreochromis mossambicus      CMNDI-----PFYY--- 141
Mus musculus                 CDLPH----LPGLLLL- 141
Gallus gallus                CGAPG----GFGGE--- 158
Homo sapiens                 CSFGEMKE--------- 129
            *
```

Fig. 1. Multiple alignment of LHβ amino acid sequence of different vertebrates. Asterisk (∗): single, fully conserved residue; colon (:): residues with strongly similar properties; dot (.): residues with weakly similar properties. N-glycosylation site is marked in grey and conserved cysteins are boxed.

least in the species analyzed in the present paper, are not as fully conserved as in LHβ. While tetrapods have 12 conserved cysteins, all the teleosts analyzed in this paper, with the exception of *Danio rerio*, lack the 3rd cystein described in tetrapods. However, in those teleosts, as the signal peptide cleavage site is closer to the N-terminus, the mature protein has one additional cystein, meaning that in

Table 3
Sequence identities of the deduced LHβ and FSHβ protein from *R. arenarum* with those from other vertebrates and GenBank protein accession numbers.

Species	LH		FSH	
	Accession No.	% Identity	Accession No.	% Identity
Bufo japonicus	BAB93557	92	BAB93558	98
Rana rugosa	BAL15686	81	BAL15684	82
Rana ridibunda	CAC39252	81	CAC39253	85
Rana pipiens	AAY53885	79	AAY53884	85
Rana catesbeiana	AAY21812	78	Q9PS36	88
Rana japonica	BAD16756	77	BAD16757	83
Cynops pyrrhogaster	BAB92959	73	BAB92958	50
Xenopus laevis	AAI70113	71	NP_001084494	71
Silurana tropicalis	XP_002939281	71	XP_002937726	67
Plecoglossus altivelis	AAM92270	51	AAM92269	41
Oreochromis mossambicus	AAS86813	50	AAK83080	32
Odontesthes bonariensis	AAP85607	50	AAP85606	36
Danio rerio	NP_991185	58	NP_991187	36
Oncorhynchus mykiss	NP_001117677	56	NP_001118058	40
Gallus gallus	ADY03193	47	NP_989588	60
Mus musculus	NP_032523	46	NP_032071	56
Homo sapiens	NP_001018090	44	NP_000501	55

those teleosts there are 12 cysteins as in tetrapods but in different order. This difference modifies the formation of disulfide bond and the stability of the heterodimer (Levavi-Sivan et al., 2010). In addition, *D. rerio* lacks the cysteins in positions 10th and 11th. As a consequence, in this species there are only 11 cysteins and cannot form six disulfide bonds. These findings suggest that tetrapods could have a different tertiary structure than teleosts and, consequently, the quaternary structure could be quite different. Nevertheless, other investigations have determined that the position of the 12 cysteins is conserved in LHβ, FSHβ and TSHβ not only in tetrapods but also in fish, suggesting that fish is a variable phylogenetic group very difficult to analyze (Han et al., 2004; Komoike and Ishii, 2003; Quérat et al., 2001). In the LHβ sequence of all the vertebrates analyzed in the present paper there is one N-glycosylation conserved motif (Asn-X-Ser/Thr). However, in FSHβ of *R. arenarum* as well as in the other tetrapods referred there are two N-glycosylation sites while in teleosts there is only one, reinforcing the idea that teleosts constitute a separate line in the evolution of gonadotropins. Chien et al. (2005) and Zhang et al. (2007) observed that in FSHβ the two N-glycosylation motifs are conserved in all the tetrapods examined and also in some primitive fish such as catshark, lungfish, sturgeon and the European eel while one of these motifs is absent in the more derived teleosts. These analyses, together with those of Quérat et al. (2000) suggest that there was a significant divergence in FSHβ during the evolution of teleosts while it was more conserved among tetrapods (Zhang et al., 2007). The importance of the glycosylation pattern in hormonal function was clearly stated several years ago (Manna et al., 2002; Pierce and Parsons, 1981), hence, it could be thought that hormonal activity in teleosts differs from that of tetrapods. As well, the hormonal clearance is affected by the glycosylation pattern (Baenziger et al., 1992).

Seasonal changes in *R. arenarum* pituitary content of LHβ and FSHβ mRNA were studied by quantitative real time PCR. The fact that LHβ and FSHβ mRNA were expressed exclusively in pituitaries confirms the tissue-specificity of the expression as well as the reliability of the results.

In spite of being different parameters, and that the amount of mRNA does not always represent the amount of fully processed gonadotropin, the annual profile of pituitary mRNA in *R. arenarum* is similar to that described for plasma levels of gonadotropins in *R. catesbeiana* (Licht et al., 1983). Both species have minimum levels in autumn and winter seasons while the maximum is detected in warm months like October and February–March for *R. arenarum*, and March to June/July for *R. catesbeiana*. In the frog, both gonadotropins have an identical annual profile with LH being much more elevated than FSH, while in *R. arenarum,* mRNA of LHβ is higher than that of FSHβ only in February. Additionally, Licht et al. (1983) showed that in *R. catesbeiana*, plasma gonadotropins reach the highest concentration in the months corresponding to spring and early summer, when mating occurs. Similarly, in *R. arenarum* both gonadotropins exhibit an increase in mRNA levels in the reproductive season but also in the post reproductive one. In *B. japonicus*, the annual profile of both gonadotropins differs from *R. arenarum* and *R. catesbeiana*. In males of the Japanese toad, plasma LH and FSH levels are clearly dissociated. The concentration of LH increases during March, just prior the breeding, with a second modest increase during October–November. On the other hand, the higher levels of FSH are measured between June and December, months corresponding to summer and autumn (Itoh et al., 1990).

The annual change in gonadotropins mRNA could also be associated with testicular steroidogenesis. During the reproductive season of *R. arenarum* there is a fall in testicular biosynthesis of androgens and an increase in 5α-reduced C21-steroids (Canosa and Ceballos, 2002a) mainly due to a decrease in the activity of the enzyme 17-hydroxylase-C17–20 lyase, (Fernandez Solari et al., 2002). The fact that androgens are low during the breeding when LHβ mRNA increases allow the speculation that androgens could be regulating LHβ mRNA expression by negative feedback and that in the pre reproductive period, when androgens are elevated, LHβ mRNA reaches the minimum expression. However, there are many studies in anurans that show that LH has a similar pattern than androgens, suggesting that LH could be associated to androgen production (Itoh et al., 1990; Licht et al., 1983; Polzonetti-Magni et al., 1998). This speculation is supported by the results described in *B. japonicus* that associates plasma levels of LH with plasma androgens, with both hormones increasing during March, just prior breeding (Itoh et al., 1990) and also in the newt that link LH with androgens regulation (Kano et al., 2005; Tanaka et al., 2004). On the other hand, in *R. arenarum*, the profile of androgens is opposite to that of both gonadotropins mRNA, with the lowest concentration of androgens associated with an increase in LHβ and FSHβ mRNA. Additionally, the increase in LHβ mRNA reported in the present paper seems to be associated with the increase in 5-reduced C21-steroids previously described by Canosa and Ceballos (Canosa and Ceballos, 2002a). Moreover, *R. arenarum* has been defined as having an androgen dissociated reproductive pattern with FSH proposed as the gonadotropin responsible of the inhibition of androgen production (Canosa and Ceballos, 2002b). Then, the increase in FSHβ mRNA detected during the

```
Homo sapiens             -----------MKTLQFFFLFCCWKAICCNSCELTNITIAIEKEECR-FC 38
Mus musculus             ----------MMKLIQLCILFWCWRAICCHSCELTNITISVEKEECR-FC 39
Gallus gallus            -----------MKTLNCYVLLFCWKAICCYSCELTNITIAVEREECE-LC 38
Cynops pyrrhogaster      -----------MTAVYHCALLLGCFVTCCNMCRLSNITIVLEKEQCG-LC 38
Rana catesbeiana         ---------------------------------------------CG-AC 4
Rana pipiens             -----------MEKIFLYGLVLFWNVMLSATCELSNITIVLEKEECG-AC 38
Rana japonica            -----------MEKIFLYGLVLFWNVMLSTTCELSNITIVLEKEECG-AC 38
Rana ridibundus          ---------------FLYGLVLFWNVMLSATCELSNITIVLEKEECG-AC 34
Rana rugosa              -----------MEKIFLYGLVLFWNFMLSATCELSNITIVLEKEECG-SC 38
Rhinella arenarum        -----------MEKFFLYVLVLCWKIIPCATCELSNITIVLEKEECA-AC 38
Bufo japonicus           -----------MEKFFLYMLVLCWKIIPCTTCELSNITIVLEKEECA-AC 38
Xenopus laevis           -----------MERLFVYVLVLCWKMIPCSSCELSNITIVLEKEGCD-TC 38
Silurana tropicalis      -----------MERLFVYVLVLCWKVIPCSACELSNITIVLEKEGCD-TC 38
Odontesthes bonariensis --------MQLVVMAAVL-ALAEMEQGCHLDCYPKNVSIPVES--CG-TT 38
Oreochromis mossambicus --------MQLVVMAAVL-ALAGAEQDCSSGCRPKNISLPVDT--CG--- 36
Plecoglossus altivelis  ------MRMQLVVMAVML-VLVQAESNCEYGCQLANISITLEKTECGSHP 43
Danio rerio             ------MRMRVLVLALLLPVLMSAESECRCSCRLTNISITVESEECG-SC 43
Oncorhynchus mykiss     MYCTHLKMLQLVVMATLWVTPVRAGTDCRYGCRLNNMTITVEREDCH--- 47
                                                                   *

Homo sapiens             ISINTTWCAGYCYTRDLVYKDPARPKIQKTCTFKELVYETVRVPGCAH-H 87
Mus musculus             ISINTTWCAGYCYTRDLVYKDPARPNTQKVCTFKELVYETVRLPGCAR-H 88
Gallus gallus            ITVNATWCSGYCFTRDPVYKYPPVSSVQQICTFKEVVYETVKIPGCGD-H 87
Cynops pyrrhogaster      FEVNTTWCSGYCNTQDPVIKDPLVPFVQSSCTFKDIVYKTVRIPSCGAGI 88
Rana catesbeiana         VSVNATWCSGYCYTKDANLMYPQKSEKQGVCTYTEVIYETVKIPGCAE-N 53
Rana pipiens             VSVNATWCSGYCYTKDPNLMYPQKSEKQGVCTYTEVIYETVKIPGCAE-N 87
Rana japonica            VSVNATWCSGYCYTLDPNLMYPQKSEKQGVCTYTEVLYETVKIPGCAE-N 87
Rana ridibundus          ISVNATWCSGYCYTMDPNLMYPQKSEKQGVCTYTEVIYETVKIPGCAD-N 83
Rana rugosa              ISVNATWCSGYCYTKDPNLMYPQKSEKQGVCTYTEVIYETVKIPGCAE-N 87
Rhinella arenarum        ISVNATWCSGYCDTKDPNLKYPHKSEKQGVCTYTEVTYETVKIPGCPE-K 87
Bufo japonicus           ISVNATWCSGYCDTKDPNLKYPHKSEKQRVCTYTEVTYETVKIPGCPE-K 87
Xenopus laevis           ITVNATWCSGYCLTRDSSSKHPLIKQVQHVCTYTDITYETVKLPGCAE-D 87
Silurana tropicalis      ITVNATWCSGYCLTRDSSSKHPLIRKVQHVCTYTDIIYETVRLPGCAE-N 87
Odontesthes bonariensis EFIHTTICEGLCFNKDSVFISPAGHPEQKICIG-DWYYEVKYTEGCPV-G 86
Oreochromis mossambicus -FVDTTICEGQCFQKDPNFIHTDDWPKQKTCNG-EWSYEVKYTEQCPR-G 83
Plecoglossus altivelis  VIIETTSCTGMCGTKDWNYISSSGQEPQVTCNFKEWSYGTYYMQDCPF-G 92
Danio rerio              VTIDTTACAGLCWTMDRVYPSSMAQHTQKVCNFKNLMYKSYEFKGCPA-G 92
Oncorhynchus mykiss      GSITVTTCAGLCETTDLNYQSTWLPRSQGVCNFKEWSYEKVYLEGCPS-G 96
                           : .* * * *   *     .     * *   :  *       *

Homo sapiens               ADSLYTYPVATQCHCGKCDSDSTDCTVRGLGPSYCSFGEMKE----- 129
Mus musculus               SDSLYTYPVATECHCGKCDSDSTDCTVRGLGPSYCSFSEMKE----- 130
Gallus gallus              PESFYSYPVATECHCETCDTDSTDCTVRGLGPSYCSFSHNGSNQ--- 131
Cynops pyrrhogaster        AESFHSYPVATDCHCGACDTDHTDCTVRGLGPNYCTFSPKQ------ 129
Rana catesbeiana           VNPFYTYPVAVDCHCGRCDSETTDCTVRALGPTYCSLSQD------- 93
Rana pipiens               VNPFYTYPVAVDCHCGRCDSETTDCTVRALGPTYCSLSQD------- 127
Rana japonica              VNPFYTYPVAVDCHCGRCDSETTDCTVRALGPTYCSLSQD------- 127
Rana ridibundus            VNPFYTYPVAVDCHCGRCNSETTDCTVRALGPTYCSLSQD------- 123
Rana rugosa                VNPFYTYPVAVGCHCGRCDAETTDCTVRALGPSYCSLSQD------- 127
Rhinella arenarum          VNPFYTYPVAVDCHCGRCNSETTDCTVRGLGPTHCSL---------- 124
Bufo japonicus             VNPFYTYPVAVDCHCGRCNSETTDCTVRGLGPTHCSLSQD------- 127
Xenopus laevis             VDPFYSYPVAVDCHCDLCNIHTTDCTVRSLGPSYCSLDQEKEDLENN 134
Silurana tropicalis        IDPYYSYPVAIDCHCDLCNIHTTDCTVRSLGPSYCSLGQEKEELGTN 134
Odontesthes bonariensis    ----VTYPVARNCKCGLCNTVYTDCGRLQSDIPSC------------ 117
Oreochromis mossambicus    ----FIYPVARKCECTACN-ANTDCGTLSGYIPSC------------ 113
Plecoglossus altivelis     VDPVLTYPVAKSCACTTCSIASTECDPMHMDMASCLSF--------- 130
Danio rerio                VDSVFVYPVALSCECNQVNSDTTDWGAISPQTTSCSIH--------- 130
Oncorhynchus mykiss        VNP-LFIPVAKSCDCIKCKTDNTDCDRISMATPSCIVNPLEM----- 137
                                  ***  * *   .   *:          *
```

Fig. 2. Multiple alignment of FSHβ amino acid sequence of different vertebrates. Asterisk (∗): single, fully conserved residue; colon (:): residues with strongly similar properties; dot (.): residues with weakly similar properties. N-glycosylation sites are marked in grey and conserved cysteins are boxed. Aminoacids different from cysteins in tetrapods-conserved cysteins sites are shown in bold.

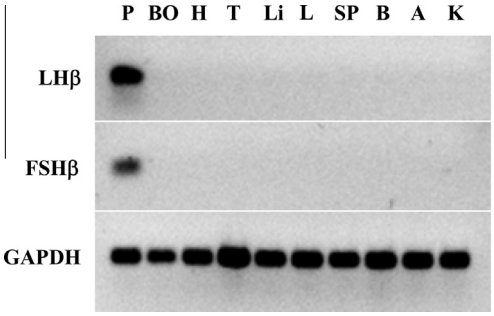

Fig. 3. mRNA expression of LHβ and FSHβ in different tissues of *R. arenarum*. GAPDH was used as expression control. P: pituitary, BO: Bidder's organ, H: heart, T: testis, Li: liver, Lu: lung, SP: spinal cord, B: brain, A: adrenal gland, K: kidney.

breeding could be important for the inhibition of androgens synthesis and the associated-increase in 5α-reduced C21-steroids. Regarding the annual pattern of gonadotropins, the difference with *B. japonicus* could be related to the fact that this species seems to express an androgen-associated reproductive pattern (Itoh et al., 1990; Itoh and Ishii, 1990).

Polzonetti-Magni et al. (1998) also showed that in *R. esculenta* FSH and estradiol reach the highest values at the same time of the year, proposing that FSH could induce estradiol biosynthesis, which in turn could contribute to spermatogonial proliferation (Chieffi et al., 2000). Therefore, it is possible to speculate that in *R. arenarum* FSH is associated with spermatogenesis since FSHβ mRNA is mainly elevated in the post reproductive season, when spermatogonia develop and proliferate (Burgos and Mancini, 1948). In addition, there is an increase of FSHβ mRNA in October, when spermatogonia are also proliferating (Burgos and Mancini,

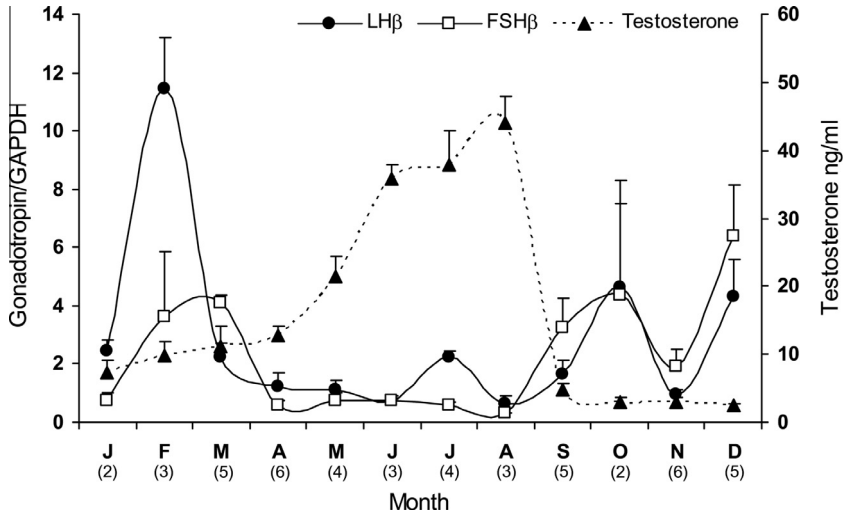

Fig. 4. Annual changes in pituitary content of LHβ and FSHβ mRNA and plasma testosterone in *R. arenarum*. Gonadotropins values are expressed as the mean of gonadotropin/GAPDH ratio within each month ± SE. Testosterone is expressed as the mean of the concentration values (ng/ml) within each month ± SE. Numbers between brackets indicate the number of animals in each month.

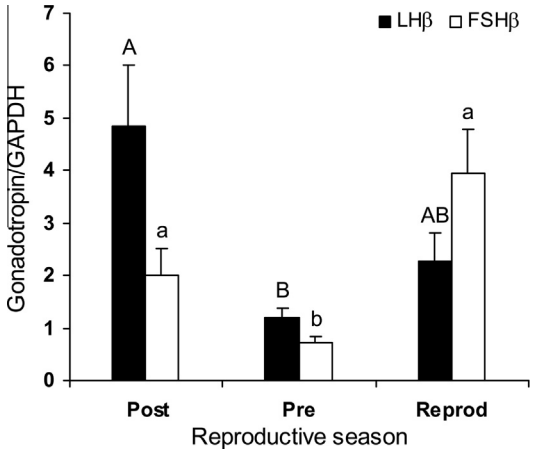

Fig. 5. Seasonal changes in pituitary content of LHβ and FSHβ from *R. arenarum*. Values are expressed as the mean of gonadotropin/GAPDH ratio within each season ± SE. Post: post reproductive season; pre: pre-reproductive season; reprod: reproductive season. Number of animals: post = 16, pre = 14; reprod = 18.

1948). In the red-belied newt, it has been demonstrated that mammalian FSH stimulates the proliferation of secondary spermatogonia and their differentiation into elongated spermatids (Abe and Ji, 1994; Yazawa et al., 2002), suggesting that this seems to be a common mechanism in amphibians. However, in *B. japonicus* the highest concentration of FSH could be measured between the early and late spermatogenesis, indicating that FSH is associated with proliferation and differentiation (Itoh and Ishii, 1990).

In conclusion, this paper reports the characterization of LHβ and FSHβ cDNAs of a bufonid toad, *R. arenarum*. The β subunits of LH and FSH of this species are similar to those of another member of the Bufonidae family, the toad *B. japonicus*, and more distant from the other two families analyzed, Ranidae and Pipidae. In addition, the study of the seasonal changes in the expression of LHβ and FSHβ mRNA indicates that transcript levels have seasonal variations associated with the reproductive cycle of this species.

Acknowledgments

This work was supported by Grants from Universidad de Buenos Aires (UBACYT 20020100100786), Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 2433) and Agencia Nacional de Promoción Científica y Tecnológica de la Argentina (PICT 32219) to NRC.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2013.03.025.

References

Abe, S.-I., Ji, Z.-S., 1994. Initiation and stimulation of spermatogenesis in vitro by mammalian follicle-stimulating hormone in the Japanese newt, *Cynops pyrrhogaster*. Int. J. Dev. Biol. 38, 201–208.

Baenziger, J.U., Kumar, S., Brodbeck, R.M., Smith, P.L., Beranek, M.C., 1992. Circulatory half-life but not interaction with the lutropin/chorionic gonadotropin receptor is modulated by sulfation of bovine lutropin oligosaccharides. Proc. Natl. Acad. Sci. 89, 334–338.

Burgos, M.H., Mancini, R.E., 1948. Ciclo espermatogénico anual del *Bufo arenarum* Hensel. Rev. Soc. Arg. Biol. 24, 328–336.

Canosa, L.F., Ceballos, N.R., 2002a. Seasonal changes in testicular steroidogenesis in the toad *Bufo arenarum* H. Gen. Comp. Endocrinol. 125, 426–434.

Canosa, L.F., Ceballos, N.R., 2002b. In vitro hCG and human recombinant FSH actions on testicular steroidogenesis in the toad *Bufo arenarum*. Gen. Comp. Endocrinol. 126, 318–324.

Canosa, L.F., Pozzi, A.G., Ceballos, N.R., 1998. Pregnenolone and progesterone metabolism by testes of *Bufo arenarum*. J. Comp. Physiol. B 168, 491–496.

Chieffi, P., Colucci-D'Amato, G.L., Staibano, S., Franco, R., Tramontano, D., 2000. Estradiol-induced mitogen-activated protein kinase (extracellular signal-regulated kinase 1 and 2) activity in the frog (*Rana esculenta*) testis. J. Endocrinol. 167, 77–84.

Chien, J.T., Shen, S.T., Lin, Y.S., Yu, J.Y., 2005. Molecular cloning of the cDNA encoding follicle-stimulating hormone beta subunit of the Chinese soft-shell turtle *Pelodiscus sinensis*, and its gene expression. Gen. Comp. Endocrinol. 141, 190–200.

Cobellis, G., Cacciola, G., Chioccarelli, T., Izzo, G., Meccariello, R., Pierantoni, R., et al., 2008. Estrogen regulation of the male reproductive tract in the frog, *Rana esculenta*: a role in Fra-1 activation in peritubular myoid cells and in sperm release. Gen. Comp. Endocrinol. 155, 838–846.

Denari, D., Ceballos, N.R., 2005. 11-hydroxysteroid dehydrogenase in the testis of Bufo arenarum, changes in its seasonal activity. Gen. Comp. Endocrinol. 143, 113–120.

Fernandez Solari, J.J., Pozzi, A.G., Ceballos, N.R., 2002. Seasonal changes in the activity of the cytochrome $P450_{c17}$ from the testis of *Bufo arenarum*. J. Comp. Physiol. B 172, 685–690.

Gentz, E.J., 2007. Medicine and surgery of amphibians. ILAR J. 48, 255–259.

Guarino, F.M., Di Fiore, M.M., Caputo, V., Angelini, F., Iela, L., Rastogi, R.K., 1993. Seasonal analysis of the reproductive cycle in two wild populations of *Rana italica* Dubois. Anim. Biol. 2 (1985), 25–43.

Hall, P.F., 1994. Testicular steroid synthesis: organization and regulation. In: Knobil, E., Neill, J. (Eds.), The Physiology of Reproduction, vol I. Raven Press, New York, pp. 1335–1362.
Han, Y.S., Liao, I.C., Tzeng, W.N., Yu, J.Y.L., 2004. Cloning of the cDNA for thyroid stimulating hormone beta subunit and changes in activity of the pituitary–thyroid axis during silvering of the Japanese eel, *Anguilla japonica*. J. Mol. Endocrinol. 32, 179–194.
Hanaoka, Y., Hayashi, H., Takahashi, H., 1984. Isolation and characterization of bullfrog gonadotropins. Gunma Symp. Endocrinol. 21, 63–77.
Hayashi, H., Hayashi, T., Hanaoka, Y., 1992a. Amphibian lutropin from the bullfrog Rana catesbeiana. Complete amino acid sequence of the subunit. Eur. J. Biochem. 205, 105–110.
Hayashi, T., Hanaoka, Y., Hayashi, H., 1992b. The complete amino acid sequence of the follitropin beta-subunit of the bullfrog, *Rana catesbeiana*. Gen. Comp. Endocrinol. 88, 144.
Huang, H., Cai, L., Remo, B.F., Brown, D.D., 2001. Timing of metamorphosis and the onset of the negative feedback loop between the thyroid gland and the pituitary is controlled by type II iodothyronine deiodinase in *Xenopus laevis*. Proc. Natl. Acad. Sci. USA 98, 7348–7353.
Itoh, M., Inoue, M., Ishii, S., 1990. Annual cycle of pituitary and plasma gonadotropins and sex steroids in a wild population of the toad, *Bufo japonicus*. Gen. Comp. Endocrinol. 78, 242–253.
Itoh, M., Ishii, S., 1990. Changes in plasma levels of gonadotropins and sex steroids in the toad, *Bufo japonicus*, in association with behavior during the breading season. Gen. Comp. Endocrinol. 80, 451–464.
Kano, Y., Nakano, T., Kumakura, M., Wasa, T., Suzuki, M., Yamauchi, K., et al., 2005. Seasonal expression of LHβ and FSHβ in the male newt pituitary gonadotrophs. Gen. Comp. Endocrinol. 141, 248–258.
Kawauchi, H., Sower, S.A., 2006. The dawn and evolution of hormones in the adenohypophysis. Gen. Comp. Endocrinol. 148, 3–14.
Kim, J.W., Im, W.-B., Choi, H.H., Ishii, S., Kwon, H.B., 1998. Seasonal fluctuations in pituitary gland and plasma levels of gonadotropic hormones in Rana. Gen. Comp. Endocrinol. 109, 13–23.
Kobayashi, T., Sakai, N., Adachi, S., Asahina, K., Iwasawa, H., Nagahama, Y., 1993. 17, 20-dihydroxy-4-pregnen-3-one is the naturally occurring spermiation inducing hormone in the testis of a frog, *Rana nigromaculata*. Endocrinology 133, 321–327.
Komoike, Y., Ishii, S., 2003. Cloning of cDNAs encoding the three pituitary glycoprotein hormone subunit precursor molecules in the Japanese toad, *Bufo japonicus*. Gen. Comp. Endocrinol. 132, 333–347.
Levavi-Sivan, B., Bogerd, J., Mañanós, E.L., Gómez, A., Lareyre, J.J., 2010. Perspectives on fish gonadotropins and their receptors. Gen. Comp. Endocrinol. 165, 412–437.
Licht, P., McCreery, B.R., Barnes, R., Pang, R., 1983. Seasonal and stress related changes in plasma gonadotropins, sex steroids, and corticosterone in the bullfrog, *Rana catesbeiana*. Gen. Comp. Endocrinol. 50, 124.
Licht, P., Papkoff, H., 1974. Separation of two distinct gonadotropins from the pituitary gland of the bullfrog *Rana catesbeiana*. Endocrinology 94, 1587–1594.
Licht, P., Papkoff, H., Farmer, S.W., Muller, C.H., Tsui, H.W., Crews, D., 1977. Evolution of gonadotropin structure and function. Recent Prog. Horm. Res. 33, 169–248.
Manna, P.R., Joshi, L., Reinhold, V.N., Aubert, M.L., Suganuma, N., Pettersson, K., et al., 2002. Synthesis, purification and structural and functional characterization of recombinant form of a common genetic variant of human luteinizing hormone. Hum. Mol. Genet. 11, 301–315.
Nagahama, Y., 1986. Testis. In: Pang, P.K.T., Schreibman, M.P., Gorbman, A. (Eds.), Vertebrates Endocrinology: Fundamentals and Biomedical Implications, vol. 1. Academic Press, New York, pp. 399–437.
Norris, D.O., 2007. Vertebrate Endocrinology, fourth ed. Academic Press, San Diego.
Papkoff, H., Farmer, S.W., Licht, P., 1976. Isolation and characterization of luteinizing hormone from amphibian (*Rana catesbeiana*) pituitaries. Life Sci. 18, 245–250.
Pierce, J.G., Parsons, T.F., 1981. Glycoprotein hormones: structure and function. Ann. Rev. Biochem. 50 (1981), 465–495.
Pinelli, C., Fiorentino, M., D'Aniello, B., Tanaka, S., Rastogi, R.K., 1996. Immunohistochemical demonstration of FSH and LH in the pituitary of the developing frog, *Rana esculenta*. Gen. Comp. Endocrinol. 104, 189–196.
Polzonetti-Magni, A.M., Mosconi, G., Carnevali, O., Yamamoto, K., Hanaoka, Y., Kikuyama, S., 1998. Gonadotropins and reproductive function in the anuran amphibian *Rana esculenta*. Biol. Reprod. 58, 88–93.
Pozzi, A.G., Rosemblit, C., Ceballos, N.R., 2006. Effect of human gonadotropins on spermiation and androgen biosynthesis in testes of *Bufo arenarum*. J. Exp. Zool. 305A, 96–102.
Pozzi, A.G., Ceballos, N.R., 2000. Human chorionic gonadotropin-induced spermiation in *Bufo arenarum* is not mediated by steroid biosynthesis. Gen. Comp. Endocrinol. 119, 164–171.
Quérat, B., Sellouk, A., Salmon, C., 2000. Phylogenetic analysis of the vertebrate glycoprotein hormone family including new sequences of sturgeon (*Acipenser baeri*) beta subunits of the two gonadotropins and the thyroid-stimulating hormone. Biol. Reprod. 63, 222–228.
Quérat, B., Tonnerre-Doncarli, C., Genies, F., Salmon, C., 2001. Duality of gonadotropins in gnathostomes. Gen. Comp. Endocrinol. 124, 308–314.
Quérat, B., Arai, Y., Henry, A., Akama, Y., Longhurst, T.J., Jo, J.M.P., 2004. Pituitary glycoprotein hormone b subunits in the australian lungfish and estimation of the relative evolution rate of these subunits within vertebrates. Biol. Reprod. 70, 356–363.
Rastogi, R.K., Iela, L., Delrio, G., Bagnara, J.T., 1986. Reproduction in the mexican leaf frog, *Pachymedusa dacnicolor* II. The male. Gen. Comp. Endocrinol. 62, 23–35.
Takada, K., Ishii, S., 1984. Purification of bullfrog gonadotropin: presence of two subspecies of luteinizing hormone with high isoelectric points. Zool. Sci. 1, 617–629.
Takahashi, H., Hanaoka, Y., 1981. Isolation and characterization of multiple components of basic gonadotropin from bullfrog (*Rana catesbeiana*) pituitary gland. J. Biochem. 90, 1333–1340.
Takahashi, H., Hanaoka, Y., 1985. Characterization of bullfrog gonadotropins molecules in comparison with mammalian hormones. In: Lofts, B., Holmes, W.H. (Eds.), Current Trends in Comparative Endocrinology. Hong Kong University Press, Hong Kong, pp. 187–188.
Tanaka, S., Sakai, M., Hattori, M., Kikuyama, S., Wakabayashi, K., Hanaoka, Y., 2004. Effect of bullfrog LH and FSH on newt testes under different temperatures. Gen. Comp. Endocrinol. 138, 1–7.
Yazawa, T., Yamamoto, T., Jin, Yan, Abe, S.-I., 2002. Follicle stimulating hormone is indispensable for the last spermatogonial mitosis preceding meiosis initiation in newts (*Cynops pyrrhogaster*). Biol. Reprod. 66, 14–20.
Zhang, L., Kessler, A.E., Tsai, P.-S., 2007. Characterization and steroidal regulation of gonadotropin beta subunits in the male leopard frog, *Rana pipiens*. Gen. Comp. Endocrinol. 150, 66–74.