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Research paper

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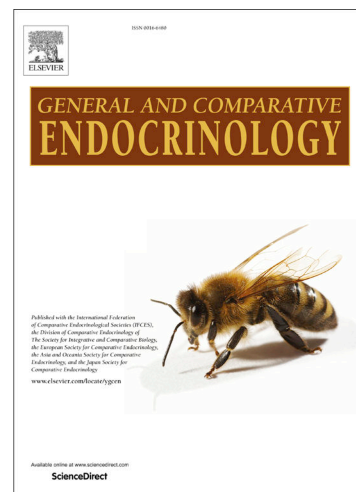

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Characterization of StAR protein of *Rhinella arenarum* (Amphibia, Anura)

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Abbreviations:

StAR: Steroidogenic Acute Regulatory

UTR: untranslated region

ORF: open reading frame

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ABSTRACT

The steroidogenic acute regulatory (StAR) protein performs the delivery of cholesterol from the outer to inner mitochondrial membrane. This is considered the rate-limiting step of acute steroid production, widely studied in mammals. However, there are only few reports regarding the characterization and expression of StAR protein in non-mammalian vertebrates. In this study, StAR protein sequence of *Rhinella arenarum* has been characterized and deduced from interrenal and testis cDNA sequences. StAR encodes a 285 amino acid protein with a conserved domain containing putative lipid binding sites. *In vitro* incubations showed that expression of StAR mRNA in testis, determined by qPCR, and testosterone synthesis determined by radioimmunoassay were stimulated after treatment with hCG and 8Br-cAMP. However, StAR mRNA expression results obtained with hCG show a higher stimulation than those obtained with 8Br-cAMP, even though steroidogenic production is the same with both treatments.

KEYWORDS: StAR, AMPHIBIANS, TESTIS, STEROIDOGENESIS.

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1. INTRODUCTION

The steroid synthesis occurs in specific structures such us testes, adrenal/interrenal glands and brain. Final products depend on the presence and quantity of enzymes that constitute the steroidogenic pathway in specific cells. However, the first step of steroidogenesis pathway is shared by all those tissues (Miller, 1988). This step corresponds to the transformation of cholesterol into pregnenolone, catalyzed by the enzyme cytochrome P450 cholesterol side chain cleavage (Cyp11A1), which is located in the mitochondrial inner membrane (Farkash et al., 1986). Cholesterol must be delivered from the outer to the inner mitochondrial membrane, a process considered to be a rate-limiting step of steroidogenesis requiring de *novo* protein synthesis. Moreover, this step is hormonally regulated (for a complete revision see Miller 2017).

In mammals, this step is widely studied and it has been suggested that StAR (Steroidogenic Acute Regulatory) protein possesses the necessary characteristics to fulfill the role of the acute regulator (Stocco and Sodeman, 1991; Clark et al., 1994). In addition, the idea that StAR protein plays an indispensable role in steroidogenesis comes primarily from observations that mutations in the human StAR gene result in the almost complete inability of these individuals to synthesize steroids. Moreover, StAR knockout mice have essentially the same phenotype that is seen in human pathology (Lin et al., 1995; Caron et al., 1997). The revision of Miller (2016) provides a summary of the mutations in StAR that had been discovered so far.

StAR was originally characterized as a cAMP-induced 37 kDa mitochondrial protein in MA10 testicular cells and it is expressed in nearly all steroid-producing cells (Stocco and Sodeman, 1991). In the murine and human StAR sequence, two separate phosphorylation sites by PKA were identified in serine 56/57 and serine 194/195 (Arakane et al., 1997). Despite the importance of StAR as the major regulator of

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steroidogenesis in mammals, studies in non-mammalian models are scarce, but those studies assessing a characterization of this protein suggest that StAR seems to be strongly conserved among vertebrates. Among non-mammalian models, StAR has been studied in fishes (Kusakabe et al., 2002; 2009; Evans and Nunez, 2010), in birds (Bauer et al., 2000; Johnson et al., 2002) and in amphibians. In this group several anuran species have been studied, such as *Xenopus laevis*, *Glandirana rugose* and *Lithobates catesbeiaus* (Bauer et al., 2000; Maruo et al., 2008; Kim et al., 2009; Paden et al., 2010) and, only one urodelean species, the giant salamander *Andrias davidianus* (Wang et al., 2018).

Moreover, similary to most vertebrates, in amphibians, gonadotrophins regulate gonadal function. In *R. arenarum*, an anuran amphibian specie, a study on 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 specie (Volonteri et al., 2013). Furthermore, testicular steroids synthesis can be *in vitro* evoked by both gonadotropins, human recombinant LH and human recombinan FSH (hrLH and hrFSH), as well as by hCG (Pozzi and Ceballos, 2000, Canosa and Ceballos, 2002, Canosa et al., 2003; Pozzi et al., 2006; Volonteri and Ceballos, 2010).

Considering the importance of StAR as one of the key step of the steroidogenic pathway, the main objective of this paper is to characterize the cDNA sequence of StAR from *R. arenarum* in interrenal glands and testes, and also to study the hCG or cAMP regulation of the mRNA expression in testicular tissue by using quantitative real time PCR method.

2. MATERIALS AND METHODS

2.1 Materials

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All the primers used and the GeneRacer Kit were from Invitrogen (Carlsbad, CA). TRIzol Reagent was from Life Technologies (MA, USA). Gen Elute^{TM} Direct mRNA Miniprep Kit, tricaine methanesulfonate (MS222) and 8Br-cAMP were obtained from Sigma–Aldrich Inc. (St. Louis, MA). Oligo (dTs) were obtained in Biodynamics (Buenos Aires, Argentina). RNase-free DNase RQI, 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). FastSart Universal SYBR Green Master (Rox) and RNAse inhibitor were obtained from Roche (Mannheim, Germany). Human chorionic gonadotropin (hCG) was from Elea Laboratory (Buenos Aires, Argentina). [1,2,6,7 (n)-^{3}H]testosterone (75.5 Ci/mmol) were from PerkinElmer (MA, USA). Testosterone antibody was obtained from The Colorado State University (CO, USA). The scintillation cocktail for androgen determination was OptiPhase-Hi safe 3 (Wallac Co, Turku, Finland).

2.2 Animals

Reproductive male toads of *R. arenarum* were collected in a nonagricultural area near Buenos Aires City (Argentina) during 2016-2018 period (n = 11) all year long. Animals were maintained in the animal facility of the Facultad de Ciencias Exactas y Naturales, Buenos Aires, Argentina, with free access to water and fed with live crickets and zophobas under natural conditions of light and temperature. For tissue preparation, animals were over-anaesthetized by immersion in 1% neutralized aqueous solution of MS222 (Gentz, 2007). To avoid the variability due to diurnal changes in hormones production, organs 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

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Laboratorio (Protocol nº 21), Facultad de Ciencias Exactas y Naturales, Buenos Aires, Argentina.

2.3 Tissue collection and RNA isolation

Three animals were used for the initial characterization of StAR. Interrenal gland was chosen due to fact of the high proportion of steroidogenic cells in comparison to testes (Pozzi and Ceballos, 2000; Regueira et al., 2013). For tissue expression studies, brain, skin, liver, interrenal gland, testes and Bidder organs were rapidly excised and stored at -80 ºC until RNA isolation. Total RNA was extracted using the TRIzol reagent. Only for testes, total mRNA was extracted using Gen Elute^{TM} Direct mRNA Miniprep Kit from another three animals. The amount of RNA was quantified by using a Qubit Fluorometer (Invitrogen, Carlsbad, CA) and RNA integrity was checked by 1% agarose gel electrophoresis.

2.4 Reverse transcription (RT)

Prior to reverse transcription, all RNA samples were pretreated with RNase-free DNase RQI to remove genomic DNA contamination. Transcription of RNA into cDNA was carried out by reverse transcriptase reaction as follows: 2 µg of RNA was incubated with 0.5 µg oligo (dT) primer (sequence: 5'-TTT TTT TTT TTT TTT-3') in a final volume of 10 µl, at 70ºC for 5 min. After incubation, samples were placed rapidly on ice. Reverse transcription was performed by adding to each sample a mixture of DEPC treated water, 5 µl AMV RT-buffer, 0.25 µl dNTPs mix (25 mM each), 30 U AMV Reverse Transcriptase and 40 U RNAse inhibitor in a final volume of 25 µl. The incubation was carried out at 48ºC for 45 min and ended by heating at 95ºC for 5 min.

2.5 Oligonucleotides

Oligonucleotides used as PCR primers for PCR, for 3' and 5' rapid amplification of cDNA ends (RACE) are listed in Table 1 and for real time PCR (qPCR) are listed in

Table 2. Degenerate primers sequences were chosen according to Seung-Chang et al. (2009). All specific primers used for PCR were synthesized based on StAR partial sequence obtained from the degenerate PCR by using DNAMAN Program (Huang and Zhang, 2003). After sequencing of StAR mRNA isolated from interrenal gland the same program was used to design new specific primers for characterizing the messenger isolated from testis.

For qPCR, specific primers for StAR were synthesized based on StAR sequence obtained from 3´ and 5´ RACE by using Beacon Designer 6.0 (PREMIER, Biosoft). Primers for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, reference gene) were synthesized according to Volonteri et al. (2013).

2.6 PCR of StAR

The reaction mixture for degenerate PCR of interrenal gland consisted in 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 1 μM each primer and 1.25U Go*Taq* DNA polymerase, in a final volume of 50 μl. The conditions for PCR were 5 min at 94 ºC followed by 35 cycles of 94 °C (30 s) denaturing, 58 °C (30 s) annealing, and 72 °C (1 min) extension, ended by a final extension for 72 °C (5 min). A 600 bp fragment was amplified, purified with Accuprep purification kit and sequenced.

For testicular StAR mRNA isolation, the conditions used were the same but with annealing temperature describes in Table 1. Products of PCRs were purified and sequenced as previously described.

2.7 3' and 5' RACE

Both 3' and 5' RACE for *R. arenarum* StAR were carried out using the GeneRacer Kit. Briefly, for 3' RACE, first-strand cDNA was synthesized from 1 μg of interrenal 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 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.33 µM of each RACE primer, 1 µM of each GeneRacer primer, and 1.25 U *GoTaq* DNA polymerase in a total volume of 25 µl. PCR conditions were 94 ºC (5 min) followed by 25 cycles of 94 ºC (30 s) denaturing, 68 °C (30 s) annealing, 70 °C (20 s) extension, ended by a final extension for 70 °C (5 min).

2.8 qPCR

Quantitative real-time (qPCR) was performed on cDNA samples of testes in duplicates using a QuantStudio™ 3 and 5 Real-Time PCR system (Life Technologies, USA). To determine optimal primer concentrations PCRs were conducted for each primer set as described in section 2.6, under the following conditions: 95 °C for 30 s followed by 30 cycles of 95 °C (30 s), 59 °C (30 s) and 72 °C (20 s). The lowest primer concentration that resulted in single band after gel electrophoresis was chosen for qPCR. Amplicon was sequenced and compared to toad StAR sequence and to other sequences of the GenBank using the NCBI-BLAST tool to confirm primer specificity.

All qPCR reactions were performed in a 30 µl volume containing 7.5 µl cDNA dilution, 15 µl FastSart Universal SYBR Green Master (Rox) and 3.75 µl of 2.5 µM each primer (Table 2). Conditions were as follows: 95 °C for 10 min followed by 40 cycles of 95 °C (20 s) denaturing, 59 °C (30 s) annealing and 72 ºC (20 s) extension, ended by a final incubation at 95 °C (15 s).

GAPDH was used as a reference gene *(see 2.5 Oligonucleotides)*. Melting curves were obtained by using a dissociation protocol to verify the presence of a single peak for the target gene. Relative quantification describes the change in the expression of the StAR gene of treated group relative to an untreated control. The fold of change in the StAR gene, normalized to GAPDH, was calculated using the formula $2^{-\Delta(\Delta CtA - \Delta CtB)}$, in which

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$\Delta CtA = (Ct_{StAR\ treated} - Ct_{GAPDH\ treated})$ and $\Delta CtB = (Ct_{StAR\ control} - Ct_{GAPDH\ control})$, being Ct the threshold cycle (Livak and Schmittgen, 2001).

2.9 Sequence analyses

Partials sequences obtained by 3' and 5' RACE or PCR using mRNA from testes were compared to other sequences of the GenBank using the NCBI-BLAST tool. In addition, these sequences were assembled using CAP3 Sequence Assembly Program (Huang and Madan, 1999). The putative open reading frame (ORF) and the amino acid sequence of *R. arenarum* StAR protein were predicted using the ORF Finder from the NCBI followed by a BLAST alignment to choose the ORF corresponding StAR. The signal peptide and conserved domains were predicted with ExPASy Bioinformatics Resource Portal (Artimo et al., 2012) and tools of BLAST program.

2.10 Sequence alignments and molecular phylogenetic 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 Clustal Omega program. To determine the relationship of toad StAR protein with StAR from other species, the deduced amino acid sequence of the putative *R. arenarum* StAR protein was analyzed. All the sequences were aligned using the Clustal Omega algorithm and a molecular phylogenetic analysis performed using maximum likelihood method in MEGA program version 6 (Tamura et al., 2013). One thousand iterations were used to generate a bootstrap consensus tree, which was rooted using chondrichthyes fish's sequences.

2.11 *In vitro* treatments with hCG and 8Br-cAMP

After characterizing interrenal and testicular StAR and assessing its tissue-specific distribution the effect of hCG and 8Br-cAMP on testicular StAR mRNA expression was analyzed.

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Testes from five animals were quickly dissected under sterile conditions, placed on Leibovitz's (L15) medium plus 10mM Hepes, 10% fetal bovine serum plus antibiotic (penicillin, 100 IU/ml and streptomycin, 0.1 mg/ml), and antimycotic (amphotericin B, 0.25 mg/ml) (Canosa and Ceballos, 2002) and fat bodies, mesorchia, and Bidder's organ were removed. Fetal bovine serum was inactivated at 55°C and depleted of steroids by charcoal:dextran treatment before use. Both testes from each animal, weighing between 190 and 220 mg, were cut into approximately 2-mm-thick slices (20 ± 3 mg), with scalpels.

For StAR mRNA quantification, both testes from each toad were cut into six slices. One slices from each testis were randomly transferred to one well of culture plates and incubated for 4 hours at 28°C with shaking in 4 ml of L-15 culture medium with (treated) or without (basal) steroidogenesis regulators (40 UI/ml hCG; 1mM 8Br-cAMP) (Supplementary Figure 1). Experimental design allows having a replica of each treatment per animal. After the incubations, 1 ml of the medium was stored at -20ºC for the subsequent androgens determination by RIA. Testicular fragments were processed using the Miniprep Gene Elute Direct mRNA kit. Obtained mRNA was quantified and processed to cDNA as described in section 2.4.

2.12 Determination of androgen by radioimmunoassay

Basal and stimulated testosterone production was analyzed in incubation media by radioimmunoassay. Testosterone antibody was employed in a dilution of 1:125,000. The buffer used was 10 mM phosphate buffered saline, 1% gelatin, and 20 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4. Because the cross reactivity of testosterone antibody with dihydrotestosterone was 35%, results were expressed as androgen production (Fernández Solari et al., 2002). The sensitivity of the assay was 10 pg. Steroids were assayed in triplicate. Intra and interassay coefficients of variation

were 7.6% and 9.4%, respectively. Androgen production was expressed as pg per ml of the medium. Charcoal–Dextran method was used to separate bound and free hormones. Radioactivity in the supernatant (bound) was determined by liquid scintillation counting with Wallac 1409 DSA equipment (Wallac Co, Turku, Finland).

2.13 Statistics

Results were expressed as mean ± standard error of mean, and analyzed and compared by using analysis of variance (ANOVA) test followed by Tukey's multiple comparisons tests to detect significant differences among treatments (Steel and Torrie, 1980). Androgen production and qPCR quantification results were compared by means of a randomized block ANOVA design in which there is more than one observation per treatment within each block. In all the cases each toad was considered as a block. Statistical analyses were performed with STATISTICA 6.0 (StatSoft, Inc., OK). Differences were considered significant with $p < 0.05$. Before statistical analysis, data were tested for normality and homoscedasticity by mean of Lilliefors and Bartlett's test, respectively. Data were not transformed because they satisfied the ANOVA assumptions.

3. RESULTS

3.1 Sequence analyses, alignments and molecular phylogenetic analysis

The sequence of StAR was characterized by rapid amplification of cDNA ends from total interrenal RNA and by RT-PCR from testis mRNAs. Comparison between testis and interrenal glands showed that sequences in both tissues are identical. The full length cDNA from *R. arenarum* is a 1350 bp sequence containing 176 bp of 5'–untranslated region (UTR) and 316 bp of 3'-UTR (Accession KT719384). The cDNA sequence was confirmed by BLAST analyses. The open reading frame encodes for a peptide of 285 amino acids (Accession AMD33476), with a mitochondrial signal peptide of 26 amino

acids. The mature protein has two consensus sequences for PKA phosphorylation (R-R/K-X-S/T) at positions 53-57 and 192-195 (indicated in a gray box in Figure 1), characteristics of the sequence of StAR protein as mentioned in the introduction. There is a START_START1-like domain at the C-terminal end of the deduced amino acid sequence, corresponding to a START domain of cholesterol binding (cd 08905). This analysis was performed using the NCBI database of conserved domains (Marchler-Bauer et al., 2015). In total, 44 amino acids are indicated in the database as possible lipid binding sites. Among the 32 species sequences used in the alignment, 25 amino acids are conserved (56.8%) and 11 replaced with conservative substitutions (25%). The putative single-point mutation sites of human lipoid CAH (indicated in bold in Figure 1) are conserved or conservatively substituted (L275V) in *R. arenarum*.

After the analysis, the StAR precursor protein sequence was compared with the sequences of representative species from different vertebrate groups existing in the database: 5 anurans, 1 urodelean, 2 chondrichthyes, 1 sarcotpterygii, 10 actinopterygii, 5 species of the sauropsida class and 8 mammals. Alignments with Clustal Omega showed that STAR of *R. arenarum* is almost identical to other members of Dicroglossidae and Ranidae families (91.19-90.14 %) and exhibits more differences with *Silurana tropicalis* and *X laevis* (87.07-87.36 %), two members of the Pipidae family, and with the salamander *A. davidianus* (80.70 %), one urodelean amphibian.

In order to determine phylogenetics relationships, the deduced amino acid sequence of the putative *R. arenarum* StAR protein was included in a molecular phylogenetic analysis with 33 StAR sequences of other vertebrates (Figure 2). This analysis indicated that StAR from *R. arenarum* is more closely related to proteins of species of Dicroglossidae and Ranidae families than from other species.

3.2 Tissue distribution of *R. arenarum* StAR mRNA

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StAR expression in the toad was detected by RT-PCR in the interrenal gland and in testes. However, the expression in testicular tissue seems to be lower than in interrenal gland. Under the experimental conditions of this paper no signal was detected in brain, Bidder organ, skin, and liver. Figure 3 shows a representative agarose gel performed with cDNA obtained from different tissues. Primers used for all tissues were the same employed to obtained a fragment from testis mRNA (StAR-F-2Test and StAR-R-2Test), detailed in Table 1. Bands corresponding to StAR were detected only in testes and interrenals whereas GAPDH is expressed in all the tissues studied, confirming the tissue-specific expression of StAR.

3.3 Effect of hCG and 8Br-cAMP on StAR mRNA levels and androgen production in *R. arenarum* testes

The effect of both gonadotropins and 8Br-cAMP on StAR mRNA expression was quantified by qPCR. Our results suggest that, even if hCG stimulates the expression of StAR, the activation of PKA with 8Br-cAMP seems to have a minor effect (Figure 4). Despite this difference of hCG and 8Br-cAMP on StAR expression, the production of androgens is equally stimulated by both treatments (Figure 5).

4. DISCUSSION

The available information on StAR protein in amphibians corresponds to species of Ranidae, Dicroglossidae and Pipidae families (Bauer et al., 2000, Maruo et al., 2008; Kim et al., 2009, GenBank). In this sense, this study constitutes the first description of StAR protein sequence, tissue distribution and protein regulation in a member of the Bufonidae family.

In the phylogenetic analysis, *Potamotrygon hystrix* and *Rhincodon typus*, two cartilaginous fish, were used as an external group for the rooting of the trees and its consequent polarization of characters. As a consequence, the StAR protein sequence

obtained for *R. arenarum* is located within the group of amphibians, which has a robust branch since it was formed in 99% or more of the cases during the testing of the tree. Even though there are few families represented, the tree topology arrangement used in this study is the same current phylogenetic tree suggested for amphibians (AmphibiaWeb, 2018). Our results also suggest that *R. arenarum* (Bufonidae) is close to *Nanorana Parkeri*, a species of the Dicroglossidae family, and both are, in turn, close to species from the Ranidae family, *Lithobates catesbeianus* and *Glandirana rugosa*. Moreover, species from the Pipidae family are grouped into a separate branch within the amphibian node. Phylogenetic analysis based on the alignment of multiple sequences of StAR protein from different species attempts to evaluate the magnitude of the phylogenetic component in the variability of the sequence.

Analysis of the amino acid sequence deduced from *R. arenarum* StAR mRNA reflects that this protein is conserved among all vertebrate species analyzed in this work. This is particularly observed within anurans, since it has between 87 and 91% identity with StAR sequences of other members of the group. However, this percentage of identity decreases when the toad is compared with the urodelean *A. davidianus*, a giant salamander of Cryptobranchidae family (Wang et al., 2018).

Furthermore, the comparison of the sequence of *R. arenarum* with amniotes suggests that the accumulation of amino acid changes is greater in mammals, whose percentages of identity are between 70% and 65%, while sauropsids share a percentage of identity of 75%. Within the anamniotas, higher percentages of identity were obtained between *R. arenarum* and fish species of earlier origin, such as *A. transmontanus*, (76%) *Anguilla japonica* (73.68%) and *Anguilla australis* (74%) (see Supplementary Table 2). The alignments and analysis of identity and similarity of amino acid sequences offer valuable information about the conservation of protein domains, structures and the

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functions associated with them. Studies assessing the conservation of these sites in different species allow evaluating their importance in the maintenance of their function or even in the emergence of a new one. Also, the characterization of these domains allows expanding our knowing about other proteins with similar functions. For example, the MLN64 protein shares a domain homologous to StAR and also stimulates steroidogenesis (Watari et al., 1997). Taking into account the fundamental role of StAR protein in mammalian steroidogenesis, this study characterized the amino acid sequence deduced from cDNA of *R. arenarum* StAR mRNA to contribute to the knowledge of steroid synthesis in amphibians. The comparison of the primary structure of StAR protein between *R. arenarum* and other vertebrate species reflects a high degree of conservation, particularly in the domains previously described for human StAR, N-terminal mitochondrial import sequence and C-terminal domains (Arakane et al., 1996; Wang et al., 1998; Clark, 2012), linked to the functionality of the protein. Within C-terminal domains there are the two possible consensus sequences for PKA phosphorylation (Arakane et al., 1997), which show no changes in most of the species analyzed in this work. In addition, the residues that contribute to the formation of the hydrophobic tunnel structure for the transport of lipids (Tsujishita and Hurley, 2000), and the amino acids in which mutations produce Congenital Lipoid Adrenal Hyperplasia (CLAH) disease in humans (Bose et al., 2000; Stocco, 2002; Chen et al., 2005; Flück et al., 2005), were also strongly conserved, suggesting that the function of StAR is also preserved. The role of StAR in the synthesis of steroids has been extensively studied in humans, suggesting that mutations in its gene cause the potentially lethal disease CLAH, characterized by an almost complete inability of the newborn to synthesize steroids, and by the accumulation of lipid droplets in the cytoplasm of steroidogenic cells. This defect in the synthesis of steroids is caused by the

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failure in the transport of cholesterol to the inner mitochondrial membrane, demonstrating that StAR has an essential role in this step. In addition, StAR null mice show a phenotype that is essentially identical to the human pathology (Stocco, 2002). Results in this study suggest that in *R. arenarum*, those amino acids involved in CLAH in humans are identical to the normal form of human StAR or substituted conservative.

In this study the expression of StAR mRNA was evaluated in different steroidogenic and non-steroidogenic organs in *R. arenarum*, such as brain, testes, interrenal, Bidder organ, skin and liver. During first experiments, total RNA was extracted from mentioned tissues and only one band was obtained, corresponding to expression of StAR with the cDNA from interrenal gland. After unsuccessful tests with cDNA obtained from testis, total mRNA extraction for this organ was performed. From this it was possible to obtain results of StAR expression in testes. This difficulty could be presented taking into account distribution and quantity of steroidogenic cells present in testes (Pozzi y Ceballos, 2000). Unfortunately, it was not possible to obtain sufficient amount of tissue from Bidder's organ in order to get quantifiable results. This organ has been described by Scaia as an organ with the possibility of de novo synthesis of steroids in this species (Scaia et al, 2011) and, considering that it is the main source of plasma estradiol in adult males (Scaia et al, 2013, 2019), it is an interesting tissue to analyze the expression of StAR. Despite the fact that the presence of GAPDH is evident in all organs analyzed, a band corresponding to a fragment of StAR was only detected in the interrenal gland and in testis. Moreover, the presence of StAR mRNA in testis has already been described in other anuran amphibians, such as *L. catesbeianus* (Paden et al., 2010), *Pelophylax nigromaculata* (Jia et al., 2018), *Rana chensinensis* (Bai et al., 2017) and *Pelophylax esculentus* (Burrone et al., 2012). In *L. catesbeianus* and *P. esculentus* the expression has also been detected in the brain (Paden et al., 2010;

Santillo et al., 2017). Only in *L. catesbeianus* it has been also detected in the skin and interrenal gland (Paden et al., 2010). Interrenal expression in the bullfrog should be inferred since authors used the steroidogenic gland attached to the kidney (Paden et al., 2010). The fact that in this study we were not able to identify the presence of StAR in brain, skin and Bidder´s organ is not enough to affirm that StAR is not present in these organs. It is worth mentioning that even if total mRNA was extracted in testes, in other organs total RNA was extracted with TRIzol, so future studies could assess mRNA extraction in these organs. However, other scenarios also deserve further discussion in this regard. For example, in the case of the Bidder´s organ, the lack of signal could be related to the fact that this structure contains previtellogenic, early and late vitellogenic oocytes, surrounded by active and follicular cells in proliferation (Scaia et al., 2011; Scaia et al., 2016). The lack of signal of StAR in this case could be explained because bidderian oocytes could provide a large amount of mRNA, and it is possible that not all the bidderian follicles have follicular cells expressing the Cyp450scc, so not all of them would require cholesterol transport to the mitochondrial inner membrane. In the case of brain, Santillo et al (2017) reports the expression of StAR gene in different brain regions of *P. esculentus*. Their results show that StAR expression in telencephalon and diencephalon-mesencephalon in males increases during reproductive period. Similarly to *P. esculentus*, *R. arenarum* is also an anuran with seasonal breeding, so it would be interesting to study the seasonality of expression of StAR assessing specific brain areas instead of whole brains and using mRNA instead of total RNA, since there could be differences in total RNA quantities among different brain areas and mRNA representation could be impaired. Moreover, even if there are no reports of StAR expression in liver, expression of StAR has been detected in skin of anurans (Paden et al., 2010). However, this study refers to skin in *L. catesbeianus*, which is

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phylogenetically distant from *R. arenarum* and presents a skin with important differences in their morphology and histology. As a consequence, considering that these structural differences between skin of both species could explain a lower sensitivity of RNA extraction in *R. arenarum*, future studies could further explore experimental conditions to improve the quality and sensitivity of this method in skin in order to confirm the presence or the absence of StAR in skin of *R. arenarum*. Regarding the presence of StAR in amphibian testis, there is only one *in vivo* study in particular in *L. catesbeianus*, showing that StAR mRNA levels increase at 2 and 4 hours post injection of hCG (Paden et al., 2010). In *R. arenarum* it has been also demonstrated that *in vitro* treatment of testis with doses of hCG that stimulate steroid synthesis also produces an increase in StAR mRNA, but this was not detected when the protein was measured under the same experimental conditions (Czuchlej et al., 2018). Differences in both evidences could be explained by the fact that in this case an heterologous antibody was used for the Western blot technique, raising the possibility that this method was not sensitive enough for detecting these differences.

In the perciform *Micropogonia undulatus*, *in vitro* studies show that testicular treatments with hCG for 6 and 24 hours did not affect the level of the StAR mRNA, although it is increased in ovarian follicles after 24 hours of incubation (Nunez and Evans, 2007). The presence of StAR mRNA has been also detected in testes of teleost *Clarias gariepinus* and evidence suggests that it increases significantly during the preparatory period of its reproductive cycle. Moreover, *in vitro* and *in vitro* studies in females of the same species, suggest that hCG treatment increases StAR mRNA expression in mature oocytes (Sreenivasulu et al., 2009).

Finally, this study constitutes the first evidence showing what happens with StAR mRNA levels when PKA activity is directly stimulated by 8Br-cAMP treatment in amphibian testes. Our results suggest that, even though PKA activation can increase the synthesis of androgens in a similar way as hCG does, the increase in StAR expression is lower when

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compared to hCG treatment. Similar results were obtained in interrenal gland of *Oncorhynchus mykiss.* In that case, *in vitro* stimulation of interrenal glands with dibutyryl-cAMP during 6 or 18 hours caused a significant increase in the synthesis of cortisol when compared to the control, although no changes were observed in the expression of StAR mRNA (Hagen et al., 2006). Our results showing that StAR mRNA expression increases more with gonadotropin than with AMPc analog suggest that the gonadotropin could induce StAR expression by at least two mechanisms, one which involves cAMP and other which does not. In mouse Leydig cells, several cAMP-independent steroidogenesis-regulating pathways have already been described (Stocco et al., 2005; Manna et al., 2006).

CONCLUSION

In conclusion, this study analyzes the StAR sequence in both testis and interrenal glands of *R. arenarum*, and assesses the effect of gonadotropins and an AMPc analog on testicular StAR expression and androgen production. Results here suggest a high degree of conservation of the protein and its domains associated with its functionality. Moreover, the fact that StAR expression increases more with gonadotropins than with an AMPC analog, a different pattern when compared to androgen production, suggests that testicular StAR expression involves both a cAMP-dependent and a cAMP-independent steroidogenesis-regulating pathways. This study highlights the importance of molecular phylogenetic analysis and of exploring non-traditional species to increase our knowledge on mechanisms involved in steroidogenesis regulation. Future studies should explore further methodological conditions to increase the sensitivity and explore StAR expression and distribution in other organs of *R. arenarum.*

ACKNOWLEDGMENTS

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REFERENCES

Amphibiaweb 2018. https://amphibiaweb.org/taxonomy/index.html. University of California, Berkeley, CA, USA.

Arakane, F., King, S.R., Du, Y., Kallen, C.B., Walsh, L.P., Watari, H., Stocco, D.M., Strauss, J.F., 1997. Phosphorylation of steroidogenic acute regulatory protein (StAR) modulates its steroidogenic activity. J. Biol. Chem. 272, 32656-32662.

Arakane, F., Sugawara, T., Nishino, H., Liu, Z., Holt, J.A., Pain, D., Stocco, D.M., Miller, W.L., Strauss, J.F., 1996. Steroidogenic acute regulatory protein (StAR) retains activity in the absence of its mitochondrial import sequence: Implications for the mechanism of StAR action. Proc. Natl. Acad. Sci. USA. 93, 13731-13736.

Artimo, P., Jonnalagedda, M., Arnold, K., Baratin, D., Csardi, G., de Castro, E., Duvaud, S., Flegel, V., Fortier, A., Gasteiger, E., Grosdidier, A., Hernandez, C., Loannidis, V., Kuznetsov, D., Liechti, R., Moretti, S., Mostaguir, K., Redaschi, N., Rossier, G., Xenarios, I., Stonkinger, H., 2012. ExPASy: SIB Bioinformatics Resource Portal. Nucleic Acids Res. 40, W597-W603.

Bai, Y., Lia, X-Y., Liu, Z-J., Zhanga, Y-H., 2017. Effects of octylphenol on the expression of StAR, CYP17 and CYP19 in testis of *Rana chensinensis*. Environ. Toxicol. Pharm. 51, 9-15.

Bauer, M.P., Bridgham, J.T., Langenau, D.M., Johnson, A.L., Goetz, F.W., 2000. Conservation of steroidogenic acute regulatory (StAR) protein structure and expression in vertebrates. Mol. Cell. Endocrinol. 168, 119-125.

Journal Pre-proofs

Bose H.S., Sato S., Aisenberg J., Shalev S.A., Matsuo N., Miller W.L., 2000. Mutations in the steroidogenic acute regulatory protein (StAR) in six patients with congenital lipoid adrenal hyperplasia. J. Clin. Endocrinol. Metab. 85, 3636-3639.

Burrone, L., Raucci, F., Di Fiore, M.M., 2012. Steroidogenic gene expression following D-aspartate treatment in frog testis. Gen. Comp. Endocrinol. 175, 109-117.

Canosa, L.F., Ceballos, N.R., 2002. 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., Rosemblit C., Ceballos N.R., 2003. Steroid production in toads. J. Steroid Biochem. Mol. Biol. 85, 227-233.

Caron K.M., Soo S.C., Wetsel W.C., Stocco D.M., Clark B.J., Parker K.L., 1997. Targeted disruption of the mouse gene encoding steroidogenic acute regulatory protein provides insights into congenital lipoid adrenal hyperplasia. Proc. Natl. Acad. Sci. USA. 94, 11540-11545.

Chen X., Baker B.Y., Abduljabbar M.A., Miller W.L., 2005. A genetic isolate of congenital lipoid adrenal hyperplasia with atypical clinical findings. J. Clin. Endocrinol. Metab. 90, 835-840.

Clark, B.J., Wells J., King, S.R., Stocco D.M., 1994. The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. J.Biol. Chem. 269, 28314-28322.

Clark, B.J., 2012. The mammalian START domain protein family in lipid transport in health and disease. J. Endocrinol. 212, 257-275.

Czuchlej, S.C., Volonteri, M.C., Regueira, E., Ceballos, N.R., 2018. Effect of glucocorticoids on androgen biosynthesis in the testes of the toad *Rhinella arenarum* (Amphibia, Anura). J. Exp. Zool.1, 10. DOI 10.1002/jez.2232.

Evans, A.N., Nunez, B.S., 2010. Regulation of mRNAs encoding the steroidogenic acute regulatory protein and cholesterol side-chain cleavage enzyme in the elasmobranch interrenal gland. Gen. Comp. Endocrinol.168, 121-132.

Farkash, Y., Timberg, R., Orly, J., 1986. Preparation of antiserum to rat cytochrome P-450 cholesterol side chain cleavage, and its use for ultrastructural localization of the immunoreactive enzyme by protein A-gold technique. Endocrinology. 118, 1353-1365.

Fernández 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.

Flück C.E., Maret A., Mallet D., Portrat-Doyen S., Achermann J.C., Leheup B., Theintz G.E., Mullis P.E., Morel Y., 2005. A novel mutation L260P of the steroidogenic acute regulatory protein gene in three unrelated patients of Swiss ancestry with congenital lipoid adrenal hyperplasia. J. Clin. Endocrinol. Metab. 90, 5304-5308.

GeneBank https://www.ncbi.nlm.nih.gov/genbank

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

Hagen, I.J., Kusakabe, M., Young, G., 2006. Effects of ACTH and cAMP on steroidogenic acute regulatory protein and P450 11β-hydroxylase messenger RNAs in rainbow trout interrenal cells: Relationship with in vitro cortisol production. Gen. Comp. Endocrinol. 145, 254-262.

Huang, X.Y., Madan, A., 1999. CAP3: A DNA Sequence Assembly Program, Genome Res. 9, 868-877.

Huang Y.Y., Zhang L., 2003. Rapid and sensitive dot-matrix methods for genome analysis. Bioinformatics. 20, 460-466.

Journal Pre-proofs

Journal Pre-proofs

Jia, X., Liu, Z., Lu, X., Tang, J., Wu, Y., Du, Q, He, J., Zhang, X., Jiang, J., Liu, W., Zheng, Y., Ding, Y., Zhu, W., Zhang, H., 2018. Effects of MCLR exposure on sex hormone synthesis and reproduction related genes expression of testis in male *Rana nigromaculata*. Environ. Pollut. 236, 12-20.

Johnson, A.L., Solovieva, E.V., Bridgham, J.T., 2002. Relationship between steroidogenic acute regulatory protein expression and progesterone production in hen granulosa cells during follicle development. Biol. Reprod. 67, 1313-1320.

Kim, S-C., Oh, S-D, Ahn, R-S., Soh, J., Kwon, H-B., 2009. Molecular cloning and expression of steroidogenic acute regulatory protein from bullfrog (*Rana catesbeiana*). Gen. Comp. Endocrinol. 162, 146-152.

Kusakabe, M., Todo, M., McQuillan, H.J., Goetz, F.W., Young, G., 2002. Characterization and expression of steroidogenic acute regulatory protein and MLN64 cDNA in trout. Endocrinology. 143, 2062-2070.

Kusakabe, M., Zuccarelli, M.D., Nakamura, I., Young, G., 2009. Steroidogenic acute regulatory protein in white sturgeon (*Acipenser transmontanus*): cDNA cloning, sites of expression and transcript abundance in corticosteroidogenic tissue after an acute stressor. Gen. Comp. Endocrinol. 162, 233-240.

Lin D, Sugawara T, Strauss JFIII, Clark BJ, Stocco DM, Saenger P, Rogol A, Miller WL. 1995. Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis. Science. 267, 1828-1831.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta C}$T method. Methods. 25, 402-408.

Manna PR, Chandrala SP, Jo Y, Stocco DM. 2006. cAMP-independent signaling regulates steroidogenesis in mouse Leydig cells in the absence of StAR phosphorylation. J. Mol. Endocrinol. 37, 81-95.

Journal Pre-proofs

Journal Pre-proofs

Marchler-Bauer, A., Derbyshire, M.K., Gonzales, N.R., Lu, S., Chitsaz, F., Geer, L.Y., Geer, R.C., He, J., Gwadz, M., Hurwitz, D.I., Lanczycki, C.J., Lu, F., Marchler, G.H., Song, J.S., Thanki, N., Wang, Z., Yamashita, R.A., Zhang, D., Zheng, C., Bryant, S.H., 2015. CDD: NCBI´s conserved domain database. Nucleic Acids Res. 43, 222-226.

Maruo, K., Suda, M., Yokoyama, S., Oshima, Y., Nakamura, M., 2008. Steroidogenic gene expression during sex determination in the frog *Rana rugosa*. Chemosphere. 80, 41-45

Miller, W.L., 1988. Molecular biology of steroid hormone. Endocr. Rev. 9, 295-318.

Miller W.L., 2016. Disorders in the initial steps of steroid hormone synthesis. J. Steroid Biochem. Mol. Biol. 165, 18-37.

Miller, W.L., 2017. Steroidogenesis: unanswered questions. Trends Endocrinol. Metab. 28, 771-793.

Nunez, B.S., Evans, A.N., 2007. Hormonal regulation of the steroidogenic acute regulatory protein (StAR) in gonadal tissues of the Atlantic croaker (*Micropogonias undulatus*). Gen. Comp. Endocrinol. 50, 495-504.

Paden, N.E., Carr, J.A., Kendall, R.J., Wages, M., Smith, E.E., 2010. Expression of steroidogenic acute regulatory protein (StAR) in male American bullfrog (*Rana catesbeiana*) and preliminary evaluation of the response to TNT. Chemosphere.80, 41-45.

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.

Pozzi, A.G., Rosemblit, C., Ceballos, N.R., 2006. Effect of human gonadotropins on spermiation and androgen biosynthesis in the testis of the toad *Bufo arenarum* (Amphibia, Anura). J. Exp. Zool. A Comp. Exp. Biol. 305, 96-102.

Regueira, E., Scaia, M.F., Volonteri, M.C., Ceballos, N.R., 2013. Anteroposterior variation of the cell types in the interrenal gland of the male toad of *Rhinella arenarum* (Amphibia, Anura). J. Morphol. 274, 331-343.

Santillo A., Falvo S., Di Fiore M., Chieffi Baccari G., 2017. Seasonal changes and sexual dimorphism in gene expression of StAR protein, steroidogenic enzymes and sex hormone receptors in the frog brain, Gen. Comp. Endocrinol. 246, 226-232.

Scaia, M.F., Regueira, E., Sassone, A.G., Volonteri, M.C., Ceballos, N.R., 2011. The Bidder´s organ of the toad *Rhinella aremarum* (Amphibia, Anura). Presence of steroidogenic enzymes. J. Exp. Zool A. Ecol. Genet. Physiol. 15, 439-446.

Scaia M.F., Regueira E., Volonteri M.C., Ceballos N.R., 2013. Estradiol production by the Bidder's organ of the toad *Rhinella arenarum* (Amphibia, Anura). Seasonal variations in plasma estradiol. J. Exp. Zool. 319A, 355-364

Scaia M.F., Czuchlej S.C., Cervino N.G., Ceballos N.R., 2016. Apoptosis, proliferation and presence of estradiol receptors in the testes and Bidder´s organ of the toad *Rhinella arenarum* (Amphibia, Anura). J. Morphol. 77, 412-423.

Scaia M.F., Volonteri, M.C., Czuchlej S.C., Ceballos N.R., 2019. Estradiol and reproduction in the South American toad *Rhinella arenarum* (Amphibian, Anura). Gen. Comp. Endocrinol. 273, 20-31.

Seung-Chang, K., Sung-Dug, O., Ryun-Sup, A., Jaemog, S., Hyuk-Bang, K., 2009. Molecular cloning and expression of steroidogenic acute regulatory protein from bullfrog (*Rana catesbeiana*). Gen. Comp. Endocrinol 162, 146-152.

Sreenivasulu, G., Sridevi, P., Sahoo, P.K., Swapna, I., Ge, W., Kirubagaran, R., Dutta-Gupta, A., Senthilkumaran, B., 2009. Cloning and expression of StAR during gonadal cycle and hCG-induced oocyte maturation of air-breathing catfish, *Clarias gariepinus*. Comp. Biochem. Physiol. B. 154, 6-11.

Steel, R.G.D., Torrie, J.H., 1980. Principles and procedures of statistics. A biomedical approach. Chapter 9. New York. McGraw Hill.

Stocco, D.M., 2002. Clinical disorders associated with abnormal cholesterol transport: mutations in the steroidogenic acute regulatory protein. Mol. Cell. Endocrinol. 191, 19-25.

Stocco, D.M., Sodeman, T.C., 1991. The 30-kDa mitochondrial proteins induced by hormone stimulation in MA-10 mouse Leydig tumor cells are processed from larger precursors. J. Biol. Chem. 226, 19731-19738.

Stocco, D.M., Wang, X.J., Jo, Y., Manna, P.R., 2005. Multiple signaling pathways regulating steroidogenesis and steroidogenic acute regulatory protein expression: more complicated than we thought. Mol. Endocrinol. 19, 2647-2659.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30, 2725-2729.

Tsujishita, Y., Hurley, J.H., 2000. Structure and lipid transport mechanism of a StAR-related domain. Nat. Struct. Biol. 7, 408-414.

Volonteri, M.C., Ceballos, N.R., 2010. Mechanism of hCG-induced spermiation in the toad *Rhinella arenarum* (Amphibia, Anura). Gen. Comp. Endocrinol. 169, 197-202.

Volonteri M.C., Regueira E., Scaia M.F., Ceballos N.R., 2013. Characterization and seasonal changes in LHβ and FSHβ mRNA of *Rhinella arenarum* (Amphibia, Anura). Gen Comp Endocrinol. 187, 95-103.

Journal Pre-proofs

Wang, Q., Tian, H., Meng, Y., Xiao, H., Li, W., Hub, Q., 2018. Characterization and evolution analysis of Wt1 and StAR genes in *Andrias davidianus*. Gene Reports. 13, 158-165.

Wang, X., Liu, Z., Eimerl, S., Timberg, R., Weiss, A.M., Orly, J., Stocco, D.M., 1998. Effect of truncated forms of the steroidogenic acute regulatory protein on intramitochondrial cholesterol transfer. Endocrinology. 139, 3903-3912.

Watari, H., Arakane, F., Moog-Lutz, C., Kallen, C.B., Tomasetto, C., Gerton, G.L., Rio, M.C., Baker, M.E., Strauss, J.F. III., 1997. MLN64 contains a domain with homology to the steroidogenic acute regulatory protein (StAR) that stimulates steroidogenesis. Proc. Natl. Acad. Sci. USA. 94, 8462–8467.

FIGURE CAPTIONS

Figure 1: Multiple alignment of StAR protein of different vertebrates. by Clustal Omega. The number of amino acids is shown on the right. Asterisk (*): single, fully conserved residue; colon (:): residues with strongly similar properties; dot (.): residues with weakly similar properties. Two potential sites of protein kinase A-mediated serine phosphorylation are marked in grey. Mutations resulting in lipoid CAH (M144R, L157P, E169K, E169G, R182L, R182H, R193X, Q212X, R217T, A218V, M225T, W250X, Q258X, L260P and L275P) are indicated in bold. The first 26 amino acids of the C-terminal end correspond to the mitochondrial orientation sequence Gaps in the amino acid sequences are indicated with a dash (-). The single letter code is used for the amino acids.

Figure 2: Phylogenetic analysis of StAR of different vertebrates. Molecular phylogenetic analysis was performed using the maximum likelihood method using the MEGA program version 6. Values on the tree represent bootstrap scores of 1000 trials,

indicating the reliability of each branch. Branch lengths are proportional to the number of amino acid changes. Refer to Supplementary Table 1 material for GenBank accession numbers.

Figure 3: mRNA expression of StAR in different tissues of *R. arenarum*. GAPDH was used as expression control. NC: negative control, T: testis, IG: interrenal gland, BO: Bidder's organ. Primers used for all tissues were StAR-F-2Test and StAR-R-2Test, detailed in Table 1. Only for testes, cDNA comes from mRNA extraction.

Figure 4: Effects of hCG and 8Br-cAMP on the expression of StAR mRNA. Fragments of testis were cultured with hCG (40 IU/ml) or 8Br-cAMP (1mM) (treated) and without (basal). After incubation, fragments of testis were processed as indicated in Materials and methods. mRNA StAR and GAPDH level were determinates by qPCR. Analysis of relative mRNA expression were calculated using $2^{-\Delta(\Delta CtA-\Delta CtB)}$ method, in which $\Delta CtA = (Ct_{StAR\ treated} - Ct_{GAPDH\ treated})$ and $\Delta CtB = (Ct_{StAR\ control} - Ct_{GAPDH\ control})$, being Ct the threshold cycle. GAPDH was used as a reference gene. Results are expressed as means ± SE. Different letters means significant differences among treatments, n = 5 ($p<0.05$).

Figure 5: Effects of hCG and 8-Br-cAMP on androgen production. Fragments of testis were cultured with hCG (40 IU/ml) or 8-Br-cAMP (1mM) (treated) and without (basal). After incubation androgen production was determined by RIA. Results are expressed as means ± SE. Different letters mean significant differences among treatments, n = 5 ($p<0.05$).

Supplementary Table 1: Species name used in alignment and phylogenetic analysis and access number to GenBank databases protein.

Supplementary Table 2: Percent identity and similarity between aligned sequences.

Supplementary Figure 1: Experimental design for StAR mRNA expression and androgen production.

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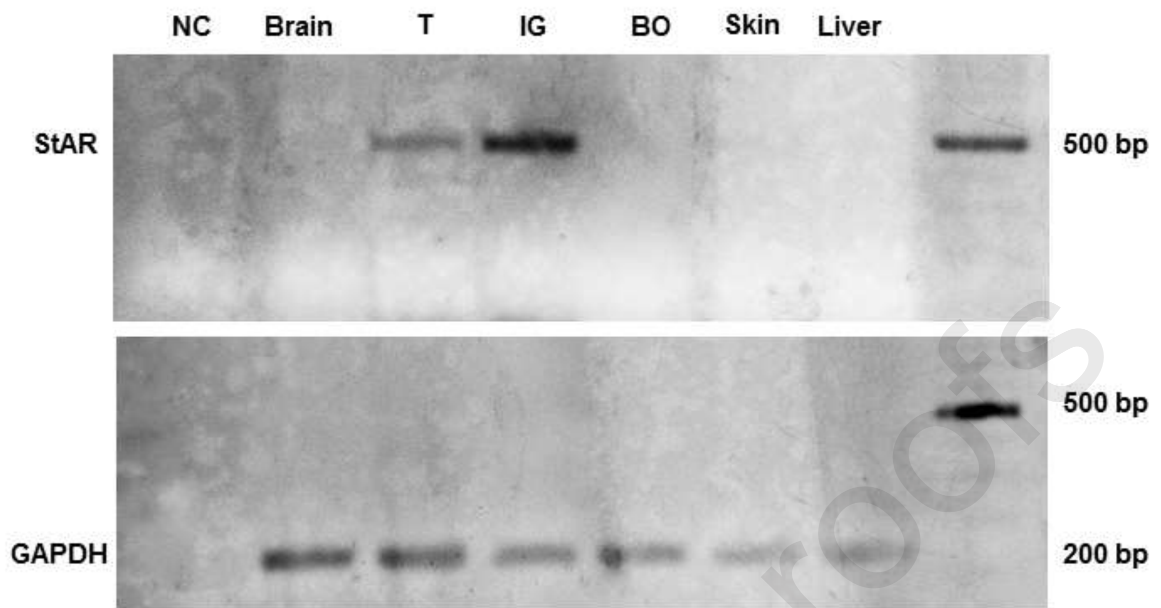

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Table 1

Primer	Utilization	Tm (ºC)	Primer sequences (5´- 3´)
StAR-F	Degenerate PCR	58	CCT GCM ACH TTC AAR YTS TG
StAR-R	Degenerate PCR	58	ACA AMT CCC YTC TGY TCV GGC AT
StAR-3` RACE	RACE 3´	74	GAAGCCGTGGTGGAGAAGCCATTGGATA
StAR-3` nested RACE	RACE 3´	70	GCCATTGGATAGTGTCTATGGAGAGCTG
StAR-5` RACE	RACE 5´	72	CCACCACGGCTTCAAGCTTAAAGACCTT
StAR-5` nested RACE	RACE 5´	74	CCGATGTCCGGGAGGACTTTACTGAGAA
GeneRacer™ 5´	RACE 5´	74	CGACTGGAGCACGAGGACACTGA
GeneRacer™ 5´ Nested	RACE 5´	78	GGACACTGACATGGACTGAAGGAGTA
GeneRacer™ 3´	RACE 3´	76	GCTGTCAACGATACGCTACGTAACG
GeneRacer™ 3´ Nested	RACE 3´	72	CGCTACGTAACGGCATGACAGTG
StAR-F-1Test-SP	PCR from Test	56	TGCTACAGACTCCAGAGAATAGA
StAR-R-1Test-SP	PCR from Test	58	TCTCCCTGCTTGAGGTAAGA
StAR-F-2Test-SP	PCR from Test	60	GGGAGAAGAAGCCCTAAAGAAG
StAR-R-2Test-SP	PCR from Test	61	GATGATGAGGGTGAACGGTTAG
StAR-F-3Test-SP	PCR from Test	61	CAGGACCCAGTCAATGGATAAA
StAR-R-3Test-SP	PCR from Test	60	CCGCCTTCTCGTGTGTTATTA

IUB code: **R** = AG, **Y** = CT, **M** = AC, **S** = GC, **B** = CGT, **H** = ACT, **V** = ACG.

SP= specific primer. F = Forward. R = Reverse.

Table 2

Primer real time	**Primer sequences (5´- 3´)**	**Amplicon Size (pb)**
StAR-F-SP	GCATTGACTTAAAGGGCTGG	
StAR-R-SP	GAACGGTTAGCAGAGTGACAG	180
GAPDH-F-SP	CCCATCACCGTCTCTCAGG	
GAPDH-R-SP	CGCTTGGCACCTCCTTTC	200

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Research Highlights

StAR of *Rhinella arenarum* is a protein with a conserved domain containing putative lipid binding sites.
StAR mRNA expression was detected in the interrenal gland and testis.
In testis, StAR mRNA expression was more stimulated with hCG treatments than with 8Br-cAMP ones.
Both hCG and 8Br-cAMP treatment stimulated androgen synthesis with the same intensity.

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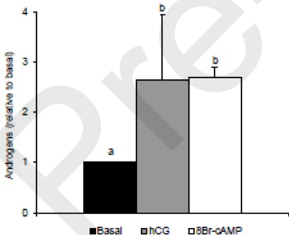
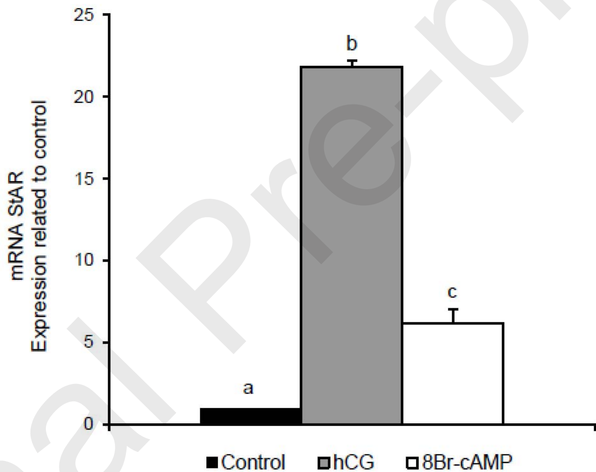


Androgens (relative to basal)
4
3
2
1
0
a
b
b
Basal
hCG
8Br-cAMP



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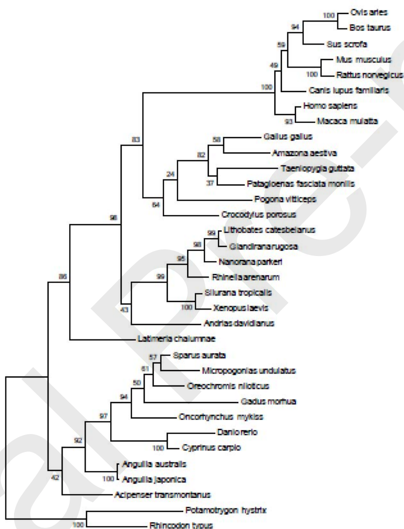

Ovis aries
Bos taurus
Sus scrofa
Mus musculus
Rattus norvegicus
Canis lupus familiaris
Homo sapiens
Macaca mulatta
Gallus gallus
Amazona aestiva
Taeniopygia guttata
Patagioenas fasciata monilis
Pogona vitticeps
Crocodylus porosus
Lithobates catesbeianus
Glandirana rugosa
Nanorana parkeri
Rhinella arenarum
Silurana tropicalis
Xenopus laevis
Andrias davidianus
Latimeria chalumnae
Sparus aurata
Micropogonias undulatus
Oreochromis niloticus
Gadus morhua
Oncorhynchus mykiss
Danio rerio
Cyprinus carpio
Anguilla australis
Anguilla japonica
Acipenser transmontanus
Potamotrygon hystrix
Rhincodon typus
0,05

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H.sapiens          MLLATFKLCAGSSYRHMRNMKGLRQQAVMAISQELNRRALGGPTPSTWINQVRRRSLLG 60
M.musculus         MPLATFKLCAGSSYRHMRNMKGLRHQAVLAIGQELNRRALGDSSP-GWMGQVRRRSLLG 59
D.rerio            MLPATFKLCAGISYRHMRNMKGLRKNAMIAIHHELNKL--SGPGASTWINHIRRRSLLS 58
O.mykiss           MLPATFKLCAGISYRHMRNMKGLRKNAMVAIHHELNML--AGPNPSSWISHVRRRSLLS 58
S.aurata           MLPATFKLCAGISYRHMRNMKGLRKNAMVAIHHELNML--AGPSPSNWTSQVRRRSLLS 58
L.chalumnae        MIPATFKLCAGISYRHLRNMTGLRRTATVAIAQELKKI--AGPGPSNWTSQVCRRSLLS 58
A.transmontanus    MLPATFKLCAGISYRHLRNMTGLRQTATVVAIHHELKRF--AGPSPSNWTSQVRRRSLLS 58
G.gallus           MLPATFKLCAAISYQHLRNVTGLRRQAAVAISQELSKLSCRSTGPSPSTWISQVRRRSLLS 60
A.davidianus       MLPATFKLCAGISYRRVRNMTGLRRTATVAIAQHLERLAAAGPSPSNWIHHVRRRSLLS 60
X.laevis           MLPATFKLCAGISYRHLRNMTGLRRTAAVALTHLEKLALVGPGPGKWINQIRRRSILS 60
S.tropicalis       MLPATFKLCAGISYRHLRNMTGLRRTAAVALTHLEKLALVGPGPSRWTNQVRRRSVLS 60
R.arenarum         MLPATFKLCAGISYRHLRNMTGLRRTAAVALSHLEKLALVGPGPSQWTNQVRRRSLLS 60
N.parkeri          MLPATFKLCAGISYRHLRNMTGLRRTAAVALSHLEKLALVGPGPSQWTNQVRRRSLLS 60
L.catesbeianus     MLPATFKLCAGISYRHLRNMTGLRRTAAVALSHLEKLALVGPGPSQWTNQVRRRSLLS 60
G.rugosa           MLPATFKLCAGISYRHLRNMTGLRRTAAVALSHLEKLALVGPGPSQWTNQVRRRSLLS 60
                   *: *******. **:*:**:.**:: * :*: :.*.          *:.:: :* **

H.sapiens          SRLEE-TLYSDQELAYLQQGEEAMQKALGILSNQEGWKKESQQDNGDKVMSKVVPDVGKV 119
M.musculus         SQLEA-TLYSDQELSYIQQGEVAMQKALGILNNQEGWKKESQQENGDEVLSKMVPDVGKV 118
D.rerio            SPIAE-ETYSEADQCTVQQGQEALQKSISILGDQDGWQTETESTNGEKVMSKVLPGIGKF 117
O.mykiss           SRIREEQGYNEAEVSYVKQGEEALQKSISILGDQDGWTTETIIAANGDKVLSKVLPDVGKV 118
S.aurata           SRIREVEGYSEAEMSYVKQGEDALQKAISILGDQDGWTTETRTVAANGDKVLSKTLPDIGKV 118
L.chalumnae        SRLEE-KPYSEAELSYVKQGEEALQKSISILSDQEGWKTEIVAKNGDKVLSKVLPDIGKI 117
A.transmontanus    SRIEE-KPYSEVEMSYTKQGEEALQKSISILSDQDNWKTEIVAANGDKVLSKVLPDIGKV 117
G.gallus           SRLEE-KPYSEMEMSYTKQGEEALQKSISILSEQDGWKTEIVADNGDKVLSKVLPDIGKV 119
A.davidianus       SRLEE-NPYSEVEMSYTKQGEEALKKSKNILEGQDGWKTEIVAQNGDKVLSKVLPDIGKV 119
X.laevis           SRLEE-KTLNDVEMSYTKQGEEALKKSMNILSDQDGWKTETVAENGDKVLSKVLPDIGKV 119
S.tropicalis       SRLEE-KTLNEGEMAYTKQGEEALKKSMNILGDQDGWKTETVAENGDKVLSKVLPDIGKV 119
R.arenarum         SRLDE-KPYNEVEMSYTKQGEEALKKSMSILGDQDGWKTEIVAENGDKVLSKVLPDIGKV 119
N.parkeri          SRLDE-KVYSEIETSYTKQGEEALKKSMSILGRQDGWKTEIVAENGDKVLSKVLPDIGKV 119
L.catesbeianus     SRLEE-KVYNEAEISYTKQGQEALKKSMSILGRQDGWKTEIVAENGDKVLSKHLPDIGKV 119
G.rugosa           SRLEE-KVFNEAEISYTKQGQEALKKSMSILGRQDGWKTEIVAENGDKVLSKVLPDIGKV 119
                   *  :      .: : : .*:;:**: *::*: ;.**  *:.*  *     **::*;** :*.:**:

H.sapiens          FRLEVVVDQPMERLYEELVERMEAMGEWNPNVKEIKVLQKIGKDTFITHELAAEAAGNLV 179
M.musculus         FRLEVVVDQPMDRLYEELVDRMEAMGEWNPNVKEIKVLQKIGKDTVITHELAAEAAGNLV 178
D.rerio            FKLEVTLEQQTGDLYDELVDNMEQMGEWNPNVKQVKILQKIGQETMITHEISAETPGNVV 177
O.mykiss           FKLEVLLDQRSDNLYEELVGNMEQMGEWNPNVKQVKILQKIGQETMVTHEVSGPTPGNVV 178
S.aurata           FKLEVMLEQHPDNLYEELVGNMEQMGDWNPNVKQVKILQKIGQDTMVTHEVSAETPGNVV 178
L.chalumnae        FKLEVVLDQQTDNVYEELVGNMEQMGEWNPSVKQVKILQKIGKDTMITHEVAAETPGNIV 177
A.transmontanus    FKLEVVLDQYPDRLYEELVDNMEQMGEWNPNVKQVKILQKIGKDTMITHEMAAETAGNIV 177
G.gallus           FRLEVVVDQPLDAVYSELVDNMEQMGDWNPSVKEVKILQRVGKDTLITHETAAAPPGNIV 179
A.davidianus       FKLEVVLDQPLSSLYGELVDRMEQMGDWNPNVKEIKILQKIGKDTVITHEAAETVGKTV 179
X.laevis           FKLEAVVEKPLDNVYGELVDNMEKMGEWNPNVKEVKILQKIGKDTVITHEKAAETPGNTV 179
S.tropicalis       FKLEAVVEKPLDNVYGELVDNMEKMGEWNPNVKEVKILQKIGKDTVITHEKAAETPGNTV 179
R.arenarum         FKLEAVVEKPLDSVYGELVDNMEKMGEWNPTVKEIKILQRIGKDTVITHEKAAETPGNII 179
N.parkeri          FKLEAVVEKPLERVYGELVDNMEKMGEWNPNVKEIKILQRIGKDTVITHEKAAETAGNIV 179
L.catesbeianus     FKLEAVVEKPLDRVYGELVDNMESMGEWNPNVKEIKILQRIGNDTVITHEKAAETAGNIV 179
G.rugosa           FKLEAVVERPLDRVYGELVDKMESMGEWNPNVKEIKILQRIGNDTVITHEKAAETAGNIV 179
                   *:**. :::    :* ***   ** **:***.**::*:**::*:*:.:*** :.     *:::

H.sapiens          GPRDFVSVRCAKRRGSTCVLAGMDTDFGEMPEQKGVIRAENGPTCMVLHPLAGSPSKTKL 239
M.musculus         GPRDFVSVRCTKRRGSTCVLAGMATHFGEMPEQGGVIRAENGPTCMVLHPLAGSPSKTKL 238
D.rerio            GPRDFVNVRHAKRRGSTCFLAGMSTQHPGMPEQKGFVRAENGPTCSVMRPSADDPNKTKF 237
O.mykiss           GPRDFVSVRCAKRRGSTCFLAGMSTQHPTMPEQRGVVRAENGPTCSVMRPSADDPNKTKF 238
S.aurata           GPRDFVSVRCAKRRGSTCFLAGMSTQHPKMPEQRGVVRAENGPTCSVMKPCAEDPNKTKF 238
L.chalumnae        GPRDFVSVRCSKRRGSGCFLAGIATRFAEMPEQKGYVRAENGPTCSVLHPSAEDPTKTKF 237
A.transmontanus    GPRDFVSVRCAKRRGSTCFLAGMSTRYTGMPEQKGFIRAENGPTCSVLRPNAEDPTKTKF 237
G.gallus           GPRDFVSVRCSRRRGSTCVLAGMSTTHGAMPEQQGFIRAENGPTCMVLRPLAGSPSQTKL 239
A.davidianus       GPRDFVSVRCSKRRGSTCVLAGMSTRFGAMPEQKGVIRAENGPTCMVLRPFAEFPTRTKL 239
X.laevis           GARDFVSVRCSKRRGSTCILAGMSTRFGGMPEQKGFVRGENGPTCMVLRPLAEDISKTKL 239
S.tropicalis       GARDFVSVRCSKRRGSTCILAGMSTRFGGMPEQKGYIRGENGPTCMVLRPLAEDISKTKL 239
R.arenarum         GPRDFVSVRCSKRRGSTCILAGMSTRFSGMPEQKGYVRAENGPTCMVLHPVLEDPSKTKL 239
N.parkeri          GPRDFVSIRCNKRRGSTCILAGMSTRFGGMPEQKGYIRAENGPTCMVLHPVLEDPSKTKL 239
L.catesbeianus     GPRDFVSVRCNKRRGSTCILAGMSTRFGGMPEQKGYIRAENGPTCMVLHPVLEDPSKTKL 239
G.rugosa           GPRDFVSVRCNKRRGSTCILAGMSTRFGGMPEQKGYVRAENGPTCMVLHPVLEDPSKTKL 239
                   * ****.:*  :****:*.***: * .   **** * :*.*:****:*::*      .:**:

H.sapiens          TWLLSIDLKGWLPKSIINQVLSQTQVDFANHLRKRLESHPASEAR---C 285
M.musculus         TWLLSIDLKGWLPKTIINQVLSQTQIEFANHLRKRLEASPASEAQ---C 284
D.rerio            TWLLSLDLKGWIPKTVINRVLSQTQVDFVNHLRDRMASGG-SIDAAIAC 285
O.mykiss           TWLLSIDLKGWIPKTIINKVLSQTQVDFANHLRQRMADNSVSMEMAAAC 287
S.aurata           TWLLNIDLKGWIPKTIINKVLSQTQVDFANHLRQRMAN-NVSMEMAHAC 286
L.chalumnae        TWLLSIDLKGWLPKSIINQVLSQTQVDFAQHLRQRMAVSPFAMATNV-C 285
A.transmontanus    TWLLSIDLKGWLPKTIINQVLSQTQMDFAQHLRDRMETIALPC------ 280
G.gallus           TWLLSIDLKGWLPKTIINQVLSQTQVDFAKHLRQRMARAAGC------- 281
A.davidianus       TWLLSIDLKGWLPKSIINQVLSQTQMDFANHLRKRMTTSNALTALNI-C 287
X.laevis           TWLLSIDLKGWLPKSIINQVLSQTQVDFAKHLRSRMTSSSNA--LSL-C 285
S.tropicalis       TWLLSIDLKGWLPKSIINQVLSQTQVDFAKHLRSRMVSSSNA--LSL-C 285
R.arenarum         TWLLSIDLKGWLPKSIINQVLSQTQVDFAKHLRNRVAMSTAS--LSL-C 285
N.parkeri          TWLLSIDLKGWLPKSIINQVLSQTQVDFAKHLRSRVAMSS-N--LSL-C 284
L.catesbeianus     TWLLSIDLKGWLPKSIINQVLSQTQVDFAKHLRSRVAMSS-N--VSL-C 284
G.rugosa           TWLLSIDLKGWLPKSIINQVLSQTQVDFAKHLRSRVAMYS-N--VSL-C 284
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