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Expression profiles of LHβ, FSHβ and their gonadal receptor mRNAs during sexual differentiation of *Xenopus laevis* tadpoles

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ABSTRACT

The gonadotropins, luteinising hormone (LH) and follicle stimulating hormone (FSH), are important hormones regulating reproductive biology in vertebrates, especially the processes of steroidogenesis and gamete maturation. Despite the role of gonadotropins during the reproductive cycle in amphibians is well established, much less is known about the functional maturation of the hypothalamus–pituitary–gonad axis during larval development. Therefore, the present study aimed to analyze the expression profiles of hypophyseal LHβ and FSHβ mRNA and of their corresponding gonadal receptors (LH-R, FSH-R) in *Xenopus laevis* tadpoles during their ontogeny and sexual differentiation. The first significant elevation of LHβ and FSHβ mRNA was observed at late premetamorphosis. A clear raise of LHβ mRNA was present during prometamorphic stages especially in males, while the LH-R only slowly increased during ontogeny with highest levels during metamorphic climax. In contrast, FSHβ mRNA expression only slightly increased during ontogeny, however in both sexes the FSH-R mRNA was considerably elevated at prometamorphosis and further at metamorphic climax. Our results suggest that LHβ and LH-R mRNA expression might be involved in initial maturation events of gametes, at least in males, while the gradually increase of FSH-R mRNA coincided with the advancing process of gamete maturation in both sexes. The present study provides for the first time evidence based on expression of gonadotropins and their corresponding gonadal receptors that the hypothalamus–pituitary–gonad axis evolves already at early stages of ontogeny and sexual differentiation in amphibians.

1. Introduction

The hypothalamus–pituitary–gonadal (HPG) axis is the most important endocrine axis regulating reproductive biology in vertebrates and the major players are the same in amphibian, fish, mammals and humans (Norris, 2007). The main functions of gonadotropins were assigned to gonadal maturation, proliferation of germ and their somatic cells, or the stimulation of steroidogenesis (Swanson et al., 2003; Weltzien et al., 2004). In amphibians, research on the HPG axis focused mostly on the developmental appearance or localization of GnRH (D'Aniello et al., 1995; Li and Lin, 2000; Troskie et al., 2000) and gonadotropins (Pinelli et al., 1996; Miranda et al., 1998; Fiorentino et al., 1999), while a few studies explored the potential of steroidal feedback mechanisms on the pituitary gonadotropins to interfere with environmental compounds (Mosconi et al., 2002; Urbatzka et al., 2006). However, despite of the sound knowledge regarding the role of the HPG axis for the regulation of reproduction in adult animals, surprisingly little is known about the functional maturation of the HPG axis in anuran species.

The amphibian species *Xenopus laevis*, the South African clawed toad, has been often used as model species concerning endocrinology and demonstrated to be a useful animal model to study alterations of the reproductive hormone system in amphibians (Kloas et al., 1999; Levy et al., 2004; Urbatzka et al., 2009). In *X. laevis*, LH appeared in the pituitary between developmental stage 42 (Moriceau-Hay et al., 1982) or stage 50 (Ogawa et al., 1995), and the amounts did not change until reaching stage 66 (Ogawa et al., 1995). So far, the available data on gonadotropins during development are limited by the low sensitivity of immunohistochemistry, the sole analysis of LH and the lack of discrimination between male and female animals. The aim of this study was to analyze the expression profiles of LHβ and FSHβ mRNA in the pituitary and of LH-R and FSH-R in the gonads of male and female *X. laevis* tadpoles during ontogeny and sexual differentiation (See Fig. 1). The first data were analyzed for gonadotropin and for gonadotropin receptors mRNA expression in amphibians using sensitive real-time PCR technique. The information on the expression pattern of gonadotropin and gonadotropin receptor mRNA should

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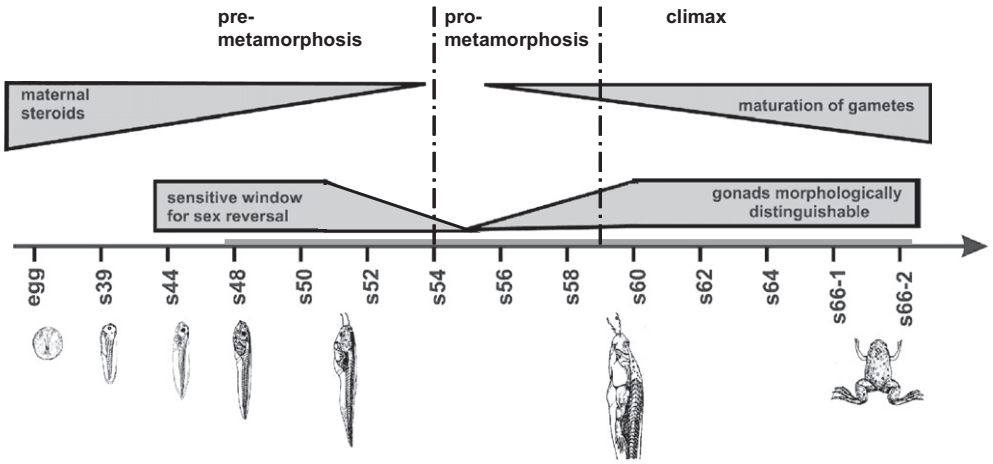

Fig. 1. Model of sexual differentiation in *Xenopus laevis*. The gray line indicates the developmental stages analyzed in this study.

provide insights into the functional maturation of the HPG axis during larval development of *X. laevis* tadpoles.

2. Material and methods

2.1. Animals

X. laevis were taken from the breeding stock of the Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany and experiments were performed in accordance with the institutional committee and national guidelines for animal protection. Adult *X. laevis* were injected with human chorionic gonadotropin (SIGMA, Deisenhofen, Germany) into the dorsal lymph sac to induce spawning as described previously (Kloas et al., 1999). Fertilized eggs and tadpoles were reared in 50 L tanks containing reconstituted tap water using deionised water supplemented with 2.5 g marine salt (Tropic Marin Meersalz, Tagis, Dreieich, Germany) per 10 L. Tanks were aerated and temperature was adjusted to 22 ± 1 °C. The light:dark cycle was 12:12 h. Ten days after fertilization tadpoles were transferred to 10 L glass aquaria at a density of 25 tadpoles per tank. Water was renewed every Monday, Wednesday and Friday and tadpoles were fed daily with Sera Micron (Sera GmbH, Heinsberg, Germany).

2.2. Sampling

The developmental stages of the tadpoles were determined according to the Normal Table of *X. laevis* (Nieuwkoop and Faber, 1994). Tadpoles were collected at developmental stages 48, 50, 52, 54, 56, 58, 60, 62, 64, 66-1, and 66-2. Stage 66-1 referred to tadpoles that finished metamorphosis and were directly collected while stage 66-2 frogs were sampled 2 weeks after reaching stage 66. Samples of brain including the pituitary and gonads with the attached kidney tissue were dissected and directly snap-frozen in liquid nitrogen. Males and females can be distinguished by gross morphology starting from stage 56 and therefore samples were taken separately for both sexes from stages 56 to 66-2. At least six tadpoles were collected per developmental stage and per sex.

2.3. RNA-Isolation and reverse transcription

Extraction of total RNA was performed by using RNA extraction kits of Quiagen (RNeasy Micro Kit, RNeasy Mini Kit, Hilden, Germany) according to the instructions of the manufacturer and included on-column DNAse ingestion (Quiagen). Transcription of RNA into cDNA was accomplished by reverse transcriptase reaction (RT) and was performed with 1 μg total RNA for all samples. For RT 7.5 pM poly (dT) primer (Biometra, Göttingen, Germany), 10 mM dNTP (Qbiogene, Heidelberg, Germany) and 10 U AMV reverse transcriptase (Finnzymes, Finland) were applied in a 30 μl reaction.

2.4. Real-time RT-PCR

Amplifications of cDNA specific for luteinising hormone (LHβ), follicle stimulating hormone (FSHβ), luteinising hormone receptor (LH-R), follicle stimulating hormone receptor (FSH-R), β-2-microglobulin (B2M), histone 4 (H4), ornithine decarboxylase (ODC), ribosomal protein L8 (rpL8) and elongation factor 1 – α (EF) of *X. laevis* were carried out by using real-time RT-PCR with SYBR green in a thermal cycler (Stratagene, Mx3000P). The following thermal cycling conditions were applied: 7 min 40 s at 95 °C followed by 40 cycles of 17 s at 95 °C, 25 s at 62 °C, 25 s at 72 °C. Melting curve analyzes were performed with the following setting, 40 s at 95 °C, 30 s at 55 °C and 30 s at 95 °C. For all PCR reactions, cDNA was diluted 1:5 (brain–pituitary samples) or 1:10 (gonad–kidney samples) and 2 μl of diluted cDNA were mixed in a 20 μl reaction with 10 mM dNTPs (Qbiogene), 1:40.000 SYBR (Invitrogen, Karlsruhe, Germany), 1:200 ROX (Invitrogen), 2 mM $MgCl_2$, 100–400 pM forward and reverse primer and 1 U of Platinum Taq-Polymerase (Invitrogen). In order to improve the efficiencies of the PCR reactions, the conditions for FSH-R and LH-R were adapted to the following: FSH-R, 3 mM $MgCl_2$, 59 °C; LH-R, 4 mM $MgCl_2$, 65 °C, cycles with 25 s at 95 °C, 40 s at 62 °C and 40 s at 72 °C. EF, B2 M, L8, H4 and ODC were tested regarding their suitability as housekeeping genes in this study and were analyzed with real-time PCR under the described conditions.

Primers were designed according to sequences published in the National Center for Biotechnology Information (NCBI) and are listed in the Supplementary Table 1. To test specificity of the PCR products the bands were run on an agarose gel, extracted (QIAquick, Gel extraction kit, Qiagen, Hilden, Germany) and forwarded to automated sequencing (SeqLab, Sequence Laboratories, Göttingen, Germany). Sequence comparisons by BLAST and sequence alignments (BioEdit) confirmed the specificity of amplified PCR products.

2.5. cDNA quantification

The cDNA quantity of brain–pituitary and gonad–kidney samples were analyzed by alkaline hydrolysis of RNA and a