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Development and optimization of a Q-RT PCR method to quantify *CYP19* mRNA expression in testis of male adult *Xenopus laevis*: Comparisons with aromatase enzyme activity

June-Woo Park [a,*], Markus Hecker [a], Margaret B. Murphy [a], Paul D. Jones [a], Keith R. Solomon [b], Glen Van Der Kraak [g], James A. Carr [c], Ernest E. Smith [e], Louis du Preez [f], Ronald J. Kendall [e], John P. Giesy [a,d]

[a] *Department of Zoology, National Food Safety and Toxicology Center, Center for Integrative Toxicology, Michigan State University, East Lansing, MI 48824, USA*
[b] *Centre for Toxicology and Department of Environmental Biology, University of Guelph, Guelph, Ontario, Canada N1G 2W1*
[c] *Department of Biological Sciences, Texas Tech University, Lubbock, TX 79409, USA*
[d] *Department of Biology and Chemistry, City University of Hong Kong, Kowloon, Hong Kong, SAR, China*
[e] *Department of Environmental Toxicology, The Institute of Environmental and Human Health, Texas Tech University, Lubbock, TX 79416, USA*
[f] *School of Environmental Sciences and Development, North-West University, Potchefstroom 2520, South Africa*
[g] *Department of Integrative Biology, University of Guelph, Ontario, Canada NIG 2W1*

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Abstract

Due to limitations of the currently used enzymatic assays, it is difficult to determine aromatase activity in testicular tissue of amphibians. Quantitative reverse transcription polymerase chain reaction (Q-RT PCR) is a sensitive and reliable technique to detect low amounts of mRNA for specific genes. This study was designed to develop and optimize a SYBR Green I-based Q-RT PCR method to quantify *CYP19* mRNA in testicular tissue from male *Xenopus laevis*. Four quantification methods for measuring *CYP19* mRNA expression were compared. The established test system proved to be highly sensitive (detectable mRNA copies <10), reproducible (interassay CV<5.4%, intraassay CV<0.9%), precise and specific for the *CYP19* gene. To confirm the validity of the applied test system, an ex vivo testicular and ovarian explant study with a known inducer of aromatase, forskolin, was conducted. Forskolin induced *CYP19* gene expression in both ovarian (3.7-fold) and testicular (2.6-fold) explants. Of the four quantification methods, the absolute standard curve and the comparative C_T method appear to be optimal as indicated by their highly significant correlation ($r^2=0.998$, $p<0.001$). In conclusion, we recommend the comparative C_T method over the standard curve method because it is more economical in terms of both cost and labor. Although both aromatase activity and *CYP19* mRNA were clearly detectable in testes of *X. laevis*, both aromatase enzyme activity and *CYP19* gene expression were very low. Also, no significant relationships were found between aromatase enzyme activity and gene expression. This is likely due the fact that the aromatase enzyme may have been dormant at the developmental stage the frogs were in during the experiment.

Keywords: Amphibians; Estrogen synthesis; Frog; Gene quantification; Molecular methods; Optimization

1. Introduction

The cytochrome P450 enzyme aromatase is the key enzyme that catalyzes the conversion of androgens to estrogens and represents the rate-limiting step in estrogen biosynthesis. The protein that catalyzes the aromatization of steroid hormones is encoded by the *CYP19* gene (Thompson and Siiteri, 1974; Simpson et al., 1994). Estrogens, especially estradiol-17β

* Corresponding author. 218C Aquatic Toxicology Laboratory, National Food Safety and Toxicology Center, Michigan State University, East Lansing, MI 48824, USA. Tel.: +1 5174323100x171; fax: +1 5174322310.
E-mail address: parkju11@msu.edu (J.-W. Park).

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(E2), have been shown to play a key role in ovarian development, reproductive function and sexual differentiation in various amphibian species (Miyashita et al., 2000; Miyata and Kubo, 2000; Kuntz et al., 2003a; Kato et al., 2004). Thus, disruption of either activity or production of this enzyme is likely to result in altered developmental or reproductive biology of organisms. Due to its key function in estrogen biosynthesis and associated reproductive processes, aromatase has been considered as an important endpoint to assess the exposure to compounds that may interact with reproductive endocrinology in vivo and in vitro (Sanderson et al., 2002; Hayes et al., 2002; Rotchell and Ostrander, 2003).

Recently, concern was raised about the potential of triazine herbicides to interact with the endocrine system of male frogs by inducing aromatase resulting in an increase of endogenous estrogen production and subsequently causing feminization or demasculinization of males (Hayes et al., 2002). Although studies by Sanderson et al. (2002) and Roberge et al. (2004) have found that high concentrations of triazine herbicides can induce aromatase in mammalian cells in culture, to date there have been no reports of this mechanism of action being observed in vivo in amphibians. This may be due to the fact that testicular aromatase enzyme activities are often low and are thus difficult to detect because they are near the detection limits of the commonly used enzymatic assays (Hecker et al., 2004). Therefore, to increase our ability to determine possible changes in aromatase activity in the testis, a more sensitive test system is needed that allows for detecting even subtle changes. One way to examine the potential for such subtle effects on the expression of aromatase activity is by measuring the changes in the expression of *CYP19* mRNA. Quantitative (real-time) reverse transcriptase polymerase chain reaction (Q-RT PCR) is a sensitive and flexible technique that can detect small quantities of mRNA in small amounts of tissue (Bustin, 2000, 2002). This technique, which amplifies the number of copies of mRNA many times, can theoretically measure as little as a single molecule of the target mRNA (Linz et al., 1990; Bej et al., 1991).

There have been few studies analyzing *CYP19* gene profiles in the African clawed frog (*Xenopus laevis*) or in amphibians in general (Miyashita et al., 2000; Akatsuka et al., 2004; Kuntz et al., 2004). None of above studies, however, have focused on adult males and, to our knowledge, Q-RT PCR methods using reliable quantification methods have not yet been applied to quantify the gene expression levels of *CYP19* in testes of *X. laevis*. It is known that *CYP19* is differentially expressed based on the sex or life-stage in most vertebrate species (Miyashita et al., 2000; Liu et al., 2004; Sakata et al., 2005; Forlano and Bass, 2004) and that one cannot simply extrapolate between sexes, especially with regard to effects of chemical exposure. Therefore, the objective of this study was to develop and optimize a Q-RT PCR procedure to measure the expression level of *CYP19* in testicular tissue of male *X. laevis.* To facilitate accurate quantification, a cDNA standard was produced that could be used for the determination of absolute copy numbers of *CYP19* mRNA in addition to the relative quantification determined by comparison to the expression of housekeeping genes. Furthermore, we compared *CYP19* gene expression in males with aromatase enzyme activities to establish a link between expression and function of gonadal aromatase in male *X. laevis*.

2. Materials and methods

2.1. Animals

Adult male *X. laevis*, 30–50 g, were purchased from Xenopus Express (Plant City, FL, USA). Each frog was treated with 0.06% NaCl upon their arrival at the laboratory to reduce the risk of possible infections. Frogs were acclimated for several weeks at the Michigan State University's Aquatic Toxicology Laboratory before the experiment was initiated. During acclimation, animals were held in 600-L fiberglass tanks under flow-through conditions. The photoperiod was 12:12-h light/dark. Frogs were fed Nasco frog brittle (Nasco, Fort Atkinson, WI, USA) three times per week ad libitum.

2.2. Isolation of total RNA and first-strand cDNA synthesis

Total RNA was isolated from gonad tissues of 14 male *X. laevis* using the SV Total RNA Isolation System (Promega, Madison, WI, USA) following the manufacturer's specifications with minor modifications to maximize the efficiency of total RNA isolation. Briefly, tissues were homogenized using a Kontes pestle and lysed in microcentrifuge tubes with guanidine thiocyanate and β-mercaptoethanol mixture. After centrifugation to remove precipitated proteins and cellular debris, nucleic acids were precipitated with ethanol and bound to a glass fiber membrane. All samples were treated with RNase-free DNase I at room temperature for 15 min to remove the chromosomal DNA. RNA integrity was checked by denaturing agarose gel electrophoresis (not shown) and 260:280 nm absorbance ratio (2.33 ± 1.03) using a DU530 UV/VIS spectrophotometer (Beckman Coulter, Inc., CA, USA). Concentrations of total RNA were determined using the RiboGreen™ RNA quantitation reagent (Molecular Probes, Inc., OR, USA) in a TD700 laboratory fluorometer (Turner BioSystems, Sunnyvale, CA, USA). Purified RNA was stored at −80 °C until further analysis.

A sample containing 500 ng of total RNA was used to synthesize single-strand cDNA in accordance with the manufacturer's directions (SuperScript™ First-Strand Synthesis System for RT PCR, Invitrogen, CA, USA). Briefly, prior to reverse transcription, total RNA was treated with DNAse I to remove potential chromosomal DNA. Then, 1.25 μL of $_{12-18}$Oligo(dT) (0.5 μg/μL) and 10 mM dNTP mix were added to the total RNA, and incubated at 65 °C for 5 min. The reaction was stopped by chilling the test solution on ice. Reaction mixture (10× RT buffer, 25 mM $MgCl_2$, 0.1 M DTT and recombinant ribonuclease inhibitor) was added to the RNA/primer mixture and incubated at 42 °C for 2 min. SuperScript II reverse transcriptase (1.25 μL of 50 U M-MLV) was added and the reaction mixture was incubated at 42 °C for 50 min, followed by a second incubation at 70 °C for 15 min. To confirm complete removal of possible genomic contamination, a negative control (sample without reverse transcriptase) was run in parallel in the Q-RT PCR system, which resulted in no

amplification of the PCR product (data not shown). To improve sensitivity of the PCR to amplify the *CYP19* mRNA from cDNA, the RNA template from the cDNA:RNA hybrid molecule was removed by digestion with *Escherichia coli* RNase H (2 U/μL) after first-strand cDNA synthesis took place.

2.3. Real-time PCR using SYBR Green I

To determine the accumulation of the PCR product, SYBR Green I dye was used as a real-time reporter of the presence of double-stranded DNA. The expression level of *CYP19* mRNA was normalized to an internal control gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Both cDNA sequences were obtained from the public GenBank database of NCBI. The *X. laevis CYP19* gene primer [forward primer: 5′CGGTTCCA-TATCGTTACTTCC3′, reverse primer: 5′GCATCTTCCTCT-CAATGTCTG3′, amplicon length (bp): 140] was designed in our laboratory based on consideration of GC content, length, secondary structure and melting temperature of the primer using the program Beacon Designer 2 (PREMIER Biosoft Intl., Palo Alto, CA, USA). Sequences for the *GAPDH* gene primer [forward primer: 5′GCT CCT CTC GCAAAG GTC AT3′, reverse primer: 5′GGG CCA TCC ACT GTC TTC TG3′, amplicon length (bp): 101] was obtained from the published literature (Wiechmann and Smith, 2001). Primer specificity was verified by a single distinct peak obtained during the melting curve analysis of the SYBR Green-based RT PCR system and by DNA sequencing of the PCR amplicons separated by gel electrophoresis. Best results were obtained at a dilution of the reverse-transcribed samples of 1/4 and 1/20 for *CYP19* and *GAPDH*, respectively. All PCR reactions were performed in a SmartCycler® II (Cephid, Sunnyvale, CA, USA). PCR master mix was prepared on ice with 10× SYBR Green I buffer containing 3 μL of $MgCl_2$ (25 mM/1.5 mL), 0.5 μL of dNTP mix with dUTP (12.5 mM/1 mL), proper primers (sense primer/antisense primer, 9.8 pM/μL:7.3 pM/μL for *CYP19* and sense primer/antisense primer, 9.3 pM/μL:11.3 pM/μL for *GAPDH*), 0.65 units of AmpliTaq Gold™ DNA polymerase (5 U/μL) and 0.25 units of AmpErase (1 U/μL). 5 μL of diluted reverse-transcribed samples were added to 20 μL of the PCR master mix. The PCR reaction mix was denatured at 95 °C for 10 min before the first PCR cycle. The thermal cycle profile was: (1) denaturation for 15 s at 95 °C, (2) annealing for 30 s at 60 °C and (3) extension for 30 s at 72 °C. A total of 50 PCR cycles was used for amplification due to the low *CYP19* copy numbers in many of the samples.

2.4. Synthesis of plasmid DNA standards

PCR products of *CYP19* and *GAPDH* were separately ligated into the pGEM T vector (Promega, Madison, WI, USA) following manufacturer's specifications. Sequence validity of the cloned amplicons was confirmed by automatic DNA sequencing and followed by a BLAST2 analysis (National Center for Biotechnology Information [NCBI (www.ncbi.nlm.nih.gov), Bethesda, MD, USA] with their corresponding sequences in GenBank. The concentrations of purified plasmids (*CYP19* plasmid DNA and *GAPDH* plasmid DNA) that spanned the target regions for forward and reverse primers were measured by using TD700 laboratory fluorometer (Turner design, CA, USA) with molecular probes' RiboGreen™ DNA quantitation reagent (Molecular Probes, Inc., OR, USA). These measured plasmids were converted to copy numbers/μL according to below formula (Eq. (1)):

$$\text{Number of DNA molecules per } \mu\text{L} = (\text{ng}/\mu\text{L} \times 1.515 \div N_{\text{bp}}) \times 6.023 \times 10^{11} \quad (1)$$

where N_{bp} = size of dsDNA (plasmid size plus DNA insert size) expressed as bp.

To evaluate PCR efficiency, uniformity and linear dynamic range of each Q-RT PCR assay, standard curves for *CYP19* and *GAPDH* were constructed using serial dilution of PCR product-inserted plasmid DNA standards (1×10^1–1×10^6 copies/μL).

2.5. Quantification of CYP19 mRNA expression

There are two methods that are commonly used for the analysis of data obtained from the RT PCR system. These include relative measurements, where the change in expression of the mRNA of interest is compared to that of an internal housekeeping gene that is assumed to be unaffected by the study treatment(s) (comparative C_T method). This method does not require any standards and is generally sufficient to demonstrate changes in gene expression. The more accurate method is to develop standards of either mRNA or the appropriate cDNA so that a standard curve can be developed to which the results of the PCR from a sample can be compared (absolute standard curve method). To assure the accuracy of measurements, both methods were applied and the results compared.

2.5.1. Comparative C_T method

This method in which the expression of the *CYP19* target gene (cDNA made from mRNA) was normalized to that of *GAPDH* in each RT PCR reaction (referred to as C_T) is the most commonly used method. Differences between median ΔC_T of test group and ΔC_T of each sample were expressed as $\Delta\Delta C_T$. The fold difference ($2^{\Delta\Delta CT}$) of gene expression in a *CYP19* was calculated for each sample. While this method is accurate and generally gives reliable results, the absolute quantification method, which relies on a standard curve for each gene, is more accurate.

2.5.2. Absolute standard curve method

In addition to the comparative method, an absolute method, based on standard curves developed for each transcript was generated from a dilution series of synthesized plasmid cDNA standards and a linear regression model was applied to quantify the data (Eq. (2)).

$$Y = aX + b \quad (2)$$

where $Y = C_T$ value, a = the slope of the standard curve, X = logarithm of the total copy numbers and $b = y$-intercept.

The amount of mRNA present in the original RNA extract was determined in the Q-RT-PC method. Data was expressed as

the C_T value, which is the cycle number when a reaction reaches the threshold (level of detection of increasing fluorescence) (Girault et al., 2002). Determination of transcript abundance (mean of C_T value) of the *CYP19* and the *GAPDH* genes were conducted in triplicate. The copy numbers of *CYP19* and *GAPDH* cDNA were calculated (Eq. (2)). To compensate for variations in RNA amount and RT efficiency, the copy number of *CYP19* was normalized to that of the internal gene (*GAPDH*). *GAPDH* was selected as the internal control (housekeeping gene) because it has been reported to be expressed at lesser levels than other housekeeping genes, such as β actin and 18S rRNA (Wiechmann and Smith, 2001). *GAPDH* is a consistently expressed gene, making it suitable as an internal standard for Q-RT PCR assays (Raaijmakers et al., 2002). Expression ratio (ER) of mRNA copy numbers between *CYP19* and *GAPDH* in the same sample was also calculated (Eq. (3)).

$$\mathrm{ER} = \text{mRNA copy number of } \textit{CYP}19 \;/\text{mRNA copy number of } \textit{GAPDH} \quad (3)$$

Quantitative (real-time) RT PCR efficiencies were calculated as follows (Eq. (4)).

$$\text{Efficiency } (\%) = [[10^{(-1/a)}]-1] \times 100 \quad (4)$$

where a is the slope of the standard curve derived from Eq. (3).

2.6. Confirmation of test system using positive controls

To confirm the validity of the developed methods, testicular and gonadal tissues from adult *X. laevis* were exposed to a model compound, forskolin (Sigma-Aldrich, St. Louis, MO), that is known to induce *CYP19* ovarian gene expression (Watanabe and Nakjin, 2004). Briefly, ovarian and testicular tissues were harvested and plated in Medium 199 (Hepes supplemented with 0.1 mM IBMX and 1 μg/mL 25-hydroxycholesterol) in 24-well plates (Corning, NY) (testis: approx. 0.1 g/well, ovary: approx. 0.5 g/well). Prior to transferring tissue from male frogs to plates each testis was dissected into eight pieces of equal size. Testicular fragments from all animals were then combined and four pieces were randomly assigned to each well to minimize variation of *CYP19* gene expression due inter-individual differences. Exposure concentrations were 0 and 100 μM forskolin using DMSO as solvent carrier. A solvent control was run in the forskolin experiment to test for possible effects of DMSO on *CYP19* gene expression. Experiments were conducted over a time period of 20 h at 25 °C. After exposure, *CYP19* gene expression was measured in tissue using the methods to be described above. Due to limitations in the amount of tissue available, no measurements of aromatase activity could be conducted in parallel.

2.7. CYP19 aromatase activity

Aromatase activity was measured following the protocol of Lephart and Simpson (1991) with minor modifications. Less than 0.5 g of gonadal tissue was homogenized in 600 μL of ice-cold gonad buffer (50 mM KPO_4, 1 mM EDTA, 10 mM glucose-6-phosphate, pH 7.4). The homogenate was incubated with 300 nM ^{3}H-androst-4-ene-3,17-dione (25.9 Ci/nmol; Lot No. 3467-067; Cat. No. NET-926; New England Nuclear, MA, USA), 0.5 IU/mL glucose-6-phosphate (Sigma Cat. # G6378) and 1 mM NADP (Sigma Cat. # N-0505) at 37 °C and 5% CO_2 for 90 min. Tritiated water released from each sample was extracted and activity determined by liquid scintillation counting. Aromatase activity was expressed as pmol androstenedione converted/h/mg protein. The specificity of the reaction for the substrate was determined by use of a competitive test with non-labeled androstenedione and the use of the specific aromatase inhibitor fadrozole (Novartis Pharma AG, Basel, CH). Addition of large amounts of androstenedione reduced tritiated water formation to the concentrations found in the tissue blanks. Furthermore, addition of fadrozole during the tritium-release assay reduced aromatase enzyme activity in a dose-dependent manner with concentrations of 5 μM and greater resulting in complete inhibition of enzyme activity to the levels measured in the blanks. This demonstrated that the activity being measured was specific for aromatase. Protein concentrations were determined using the Bradford assay (Bradford, 1976) with bovine serum albumin as the protein standard (Sigma-Aldrich, St. Louis, MO, USA).

2.8. Statistical analysis

Statistical analyses in this study were conducted using SYSTAT 10 (SPSS Inc., Chicago, IL, USA). Data sets were tested for normality using Kolmogorov–Smirnov's one sample test. The Pearson correlation analysis was used to evaluate the relationship between *CYP19* enzyme activity and *CYP19* mRNA expression, and a linear regression model was used to quantitatively determine relationships among gene quantification methods in the Q-RT PCR system. The Student's *t*-test was used to examine differences in gene expression between *CYP19* and *GAPDH*. The criterion for significance in all statistical tests was $p<0.05$.

3. Results

3.1. RT PCR amplification efficiencies, linearity and reproducibility

Specificity of the PCR reaction, accuracy of mRNA quantification and sensitivity and linearity of SYBR Green-based Q-RT PCR for *CYP19* and *GAPDH* in adult male *X. laevis* were determined. Real-time PCR amplification curves for the two genes obtained with the SmartCycler® were very reproducible and indicated that primers were selective and effective in producing the specific PCR products (Figs. 1A and 2A). The melting curves (Figs. 1C and 2C) generated at the end of the PCR reaction show that all amplicons of the *CYP19*/*GAPDH* plasmid DNA standard had the same melting temperature (81 °C). This result indicates that no primer–dimers were formed during the reactions (Figs. 1C and 2C). To further validate the specificity of the assay, gel electrophoresis (1.5% agarose) was performed on the PCR products obtained from

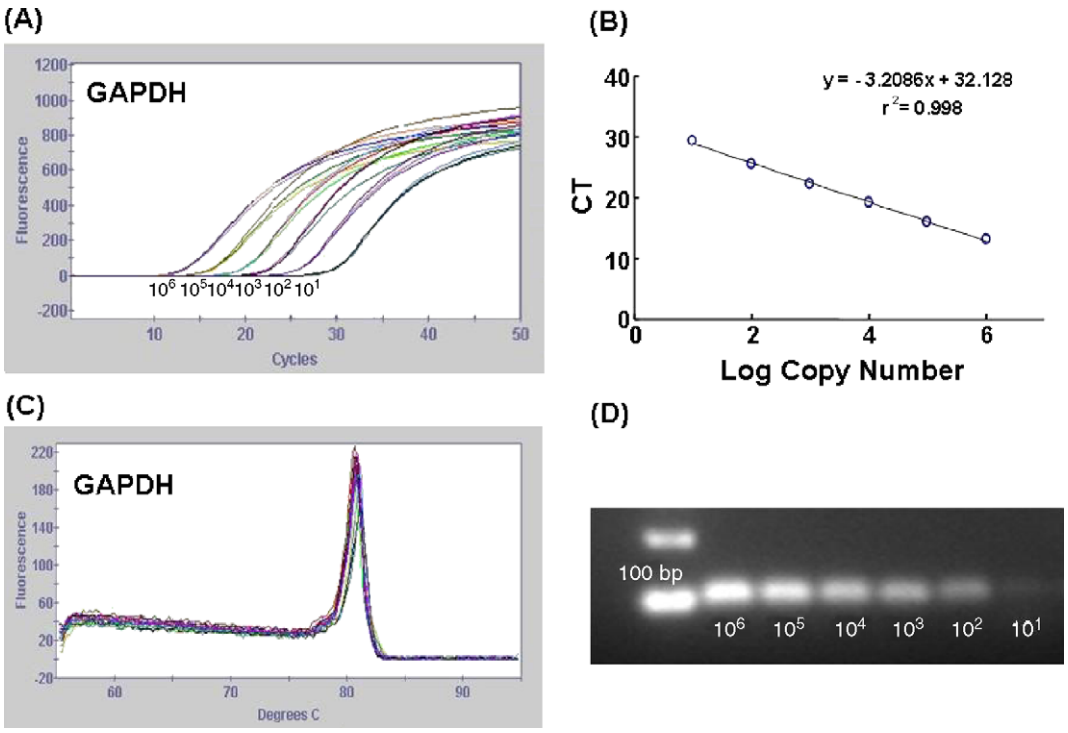

Fig. 1. *GAPDH* plasmid DNA standard curve. (A) Amplification curves of six dilutions of *GAPDH* plasmid DNA standard from 1×10^1 to 1×10^6 copies/μL. (B) *GAPDH* plasmid DNA standard curve plotting the log copies/μL (x) of *GAPDH* plasmid DNA against C_T (y), the equation was calculated by linear regression analysis ($r^2 = 0.998$ and 105.7% of PCR efficiency). (C) Melting curve of PCR products, showing specificity of the reaction. (D) 1.5% agarose gel electrophoresis of the PCR products in the serially diluted samples.

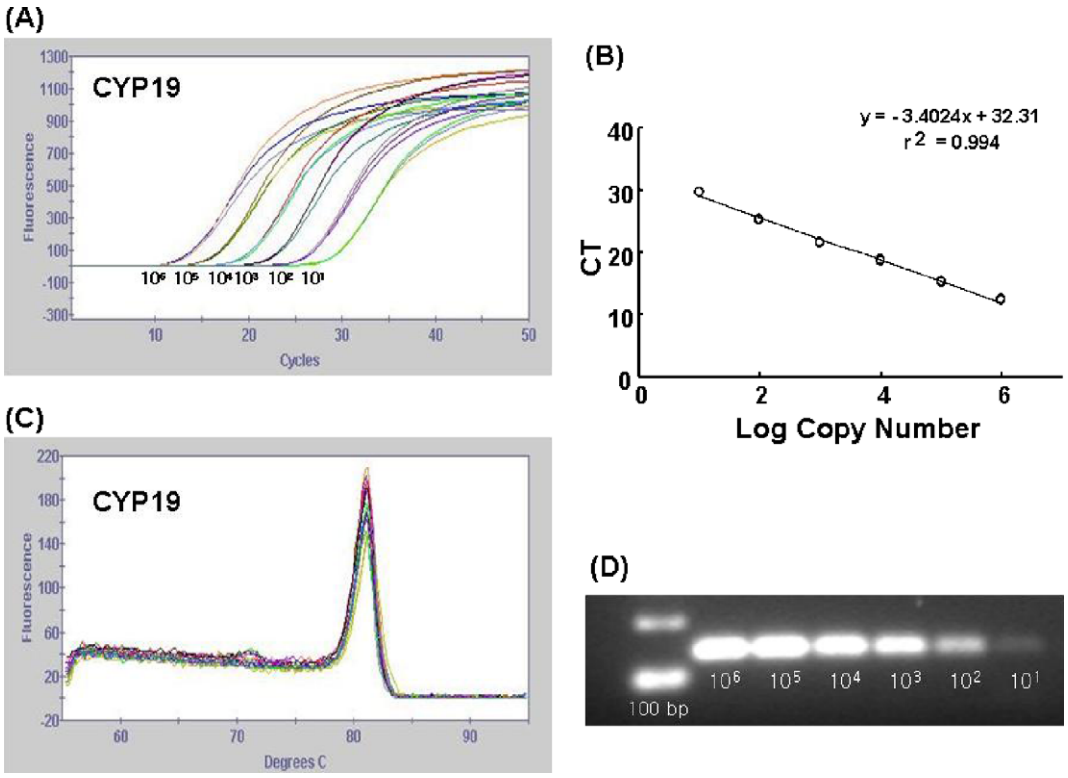

Fig. 2. *CYP19* plasmid DNA standard curve. (A) Amplification curves of six dilutions of *CYP19* plasmid DNA standard from 1×10^1 to 1×10^6 copies/μL. (B) *CYP19* plasmid DNA standard curve plotting the log copies/μL (x) of *CYP19* plasmid DNA against C_T (y), the equation was calculated by linear regression analysis ($r^2 = 0.994$ and 96.7% of PCR efficiency). (C) Melting curve of PCR products, showing specificity of the reaction. (D) 1.5% agarose gel electrophoresis of the PCR products in the serially diluted samples.

serially diluted plasmid DNA standards (Figs. 1D and 2D). The results from the gel electrophoreses demonstrate that the amplification was specific for the ~140 bp and ~101 bp products of *CYP19* and *GAPDH*, respectively.

The accuracy of mRNA quantification, and sensitivity and linearity of SYBR Green-based Q-RT PCR were examined using a 10-fold serial dilution of each plasmid DNA standard. Efficiencies during the exponential phase were 96.7% and 105.7% for *CYP19* and *GAPDH*, respectively. The relationship between threshold cycle (C_T) and the log copy number of plasmid DNA standard was linear with $r^2 > 0.99$ for both genes, indicating that the C_T values changed proportionally with serial dilution of the samples. The reproducibility of the techniques within and between assays was tested, using serial dilutions of *CYP19* and *GAPDH* plasmid cDNA standards. Intraassay variabilities were assessed by evaluating the coefficient of variation (CV) for three replicates in each dilution within one run (Table 1). Interassay variabilities were assessed by conducting three different assays performed in triplicate of each dilution over a period of 3 days (Table 1). Intraassay CVs of C_T for both genes were very small (<1.2%), indicating that the assays were highly reproducible for determining expression of both genes. Although greater than intraassay CVs, interassay C_T values were also small with CVs<5.4% for both genes.

3.2. Comparison of different quantification methods for CYP19 gene expression

Serial dilutions (1×10^1 to 1×10^6 copies/μL) of *CYP19* and *GAPDH* plasmid DNA standards were used to quantify gene amplification rates for the genes of interest. The results demonstrated that the SYBR Green-based Q-RT PCR assay allowed for the quantification of small amounts of *CYP19* mRNA (10 copies/reaction) in all 14 adult male *X. laevis*. Initial copy numbers for both genes in all 14 samples were determined by use of the standard curve method. *GAPDH* exhibited significantly greater abundances of the transcript with a mean C_T value of 22.9±0.62 (mean±S.D.) than *CYP19* with a mean C_T value of 28.1±1.4 ($p<0.001$). Mean copy numbers for all samples were 25.2±23.4 copies/μL and 802.61±350.9 copies/μL for *CYP19* and *GAPDH*, respectively.

Table 1
Reproducibility and precision of standard curve method for *CYP19* and *GAPDH* plasmid DNA

	Intraassay [a]			Interassay [b]		
	C_T mean values [c]	S.D. [d]	CV [e]	C_T mean values	S.D.	CV
CYP19 plasmid DNA (copies/μL)						
1×10^6	12.37	0.04	0.31	12.57	0.23	1.79
1×10^5	15.18	0.06	0.36	15.41	0.32	2.08
1×10^4	18.56	0.16	0.84	18.62	0.15	0.83
1×10^3	21.53	0.11	0.50	21.84	1.16	5.33
1×10^2	25.18	0.11	0.44	25.65	0.72	2.80
1×10^1	29.60	0.03	0.11	28.77	0.83	2.90
GAPDH plasmid DNA (copies/μL)						
1×10^6	13.11	0.12	0.95	13.20	0.08	0.61
1×10^5	16.02	0.18	1.12	16.03	0.10	0.64
1×10^4	19.23	0.10	0.54	19.32	0.10	0.50
1×10^3	22.22	0.11	0.49	22.60	0.47	2.06
1×10^2	25.52	0.04	0.15	25.81	0.35	1.34
1×10^1	29.28	0.05	0.16	29.73	1.02	3.43

[a] Intraassay was assessed by evaluating the coefficient variation (CV) for each dilution of the plasmid using three replicates within run.
[b] Interassay was assessed by evaluating the coefficient variation (CV) for each dilution of the plasmid using three assays with three replicates over 3 different days.
[c] Average of number of cycles when fluorescence crosses threshold.
[d] S.D.=standard deviation from the mean.
[e] CV=coefficient of variation (%).

Table 2
Diverse gene expression quantification methods and aromatase enzyme activities in individual male *X. laevis*

Replicate	C_T ratio [a]	$2^{\Delta\Delta CT}$ [b]	ER [c]	*CYP19* copy [d]	Aromatase activity (fmol/h/mg protein)
1	1.260	0.443	0.014	9.961	3.095
2	1.293	0.267	0.008	6.301	5.063
3	1.259	0.442	0.014	9.436	2.760
4	1.194	1.057	0.034	9.184	4.886
5	1.207	1.045	0.031	24.781	9.454
6	1.211	1.000	0.030	24.669	3.210
7	1.260	0.421	0.013	7.565	1.779
8	1.200	1.214	0.036	34.603	21.144
9	1.195	1.437	0.041	63.769	13.057
10	1.313	0.182	0.006	3.497	11.907
11	1.164	1.950	0.059	30.842	8.621
12	1.168	1.807	0.055	27.991	19.842
13	1.255	0.526	0.016	16.070	15.669
14	1.171	2.042	0.058	84.733	9.362

[a] C_T value of *CYP19*/C_T value of *GAPDH*.
[b] Comparative C_T method.
[c] Expression ratio calculated using standard curve method.
[d] Number of mRNA copies (standard curve method).

Because of a number of factors such as varying amounts of mRNA in the samples, differences in reverse transcription efficiency and potential presence of PCR reaction inhibitors can influence the gene amplification reaction, the use of an internal control is necessary to normalize the measurements. The simplest way to quantify mRNA in RT PCR systems, the use of the C_T value ratio (C_T of target gene/C_T of internal gene), was also applied to quantify *CYP19* gene expression (Table 2). The similar efficiencies observed for the two genes in this PCR assay allow for the use of the comparative C_T method for quantifying *CYP19* gene expression after normalization to gene expression of the internal gene. The fold differences ($2^{\Delta\Delta CT}$) of *CYP19* gene expression of all 14 samples were calculated using the comparative C_T method. The average fold difference was not equal, but very close to 1.0. In addition to the calculations above, *CYP19* gene expression was measured using the standard curve method, where the expression was determined as copy numbers obtained from *CYP19* plasmid standard curve or as ER (Eq. (3)) normalized to the internal control (Table 2).

All four quantification methods were compared to each other using a linear regression (r^2) model to determine the compatibility of different quantification approaches (Fig. 3). The comparative C_T method and the standard curve method were the most highly correlated in all comparisons ($r^2=0.997$,

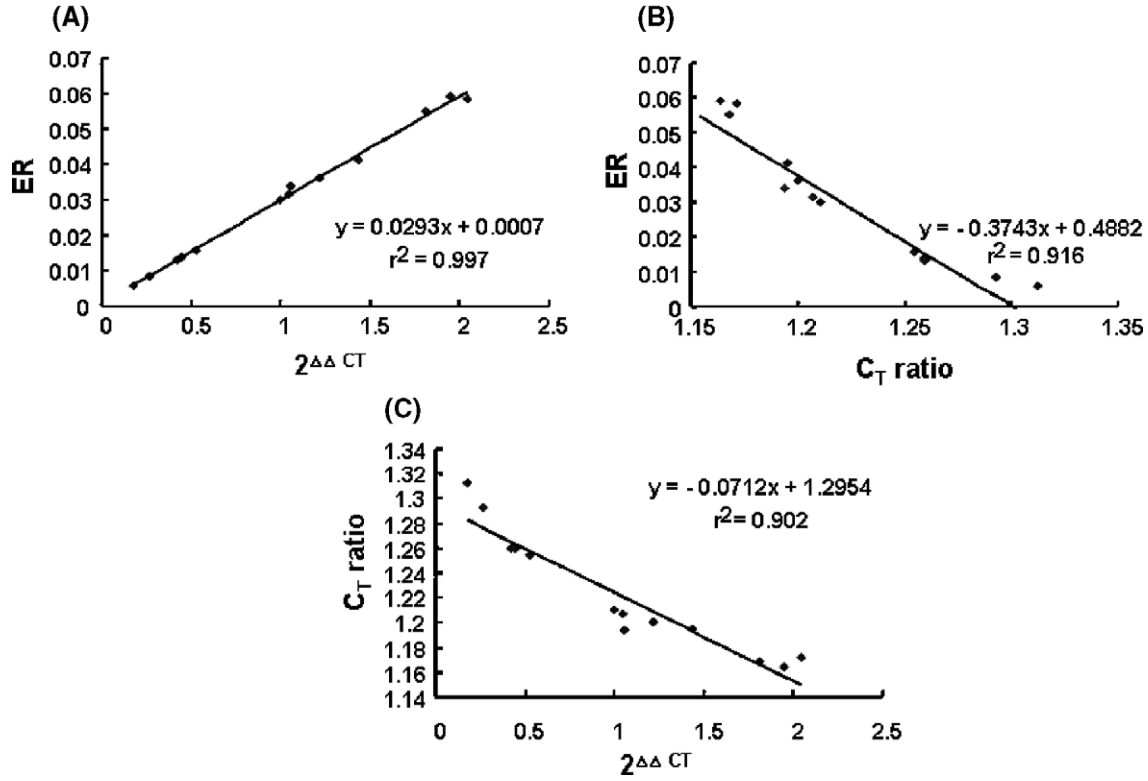

Fig. 3. Comparisons among quantification methods for measuring *CYP19* mRNA expression used in the Q-RT PCR system. ER and $2^{\Delta\Delta CT}$ were calculated from standard curve method and comparative C_T method, respectively. C_T ratio represents the ratio of C_T value of *CYP19* to C_T value of *GAPDH*. (A) Represents comparison of *CYP19* gene expression from standard curve method to that from comparative C_T method. (B) Represents comparison of *CYP19* gene expression from standard curve method to that from C_T ratio of *CYP19*/*GAPDH*. (C) Represents comparison of *CYP19* gene expression from C_T ratio of *CYP19*/*GAPDH* to that from comparative C_T method.

$p<0.001$). The relationship between C_T value ratio and comparative C_T method or C_T value ratio and standard curve method was less strong, with $r^2=0.902$ and $r^2=0.916$, respectively. The coefficient for the correlation between the uncorrected *CYP19* copy number and the results from the comparative C_T method was the lowest overall ($r^2=0.608$, $p=0.001$).

3.3. Comparison of CYP19 gene expression and aromatase activity

Aromatase activity was measurable in all frog testes analyzed with activities ranging from 1.78 to 21.14 fmol/h/mg protein. Variability among individuals was relatively great with a CV of 69%. This variability was similar to those observed for changes in gene expression: 63% for the standard curve method and 64% for the comparative C_T method. However, when comparing aromatase enzyme activities with *CYP19* gene expression determined by either the comparative C_T method or the standard curve method in the same frogs, no significant correlations could be observed ($r=0.404$, $p=0.152$; $r=0.399$, $p=0.158$, respectively) (Table 3).

3.4. Gonadal CYP19 gene expression after exposure to forskolin

Exposure of gonadal tissues to forskolin resulted in an increase of *CYP19* gene expression in both ovarian and testicular explants (Fig. 4). The greatest induction was observed in ovarian tissue with a 3.74-fold induction of *CYP19* gene

Table 3
Pearson correlation coefficients (r) and probabilities (p) between the different parameters measured

	CYP19 copy	ER	$2^{\Delta\Delta CT}$	C_T ratio	Aromatase activity
CYP19 copy	1				
ER	**0.743** (0.002)	1			
$2^{\Delta\Delta CT}$	**0.780** (0.001)	**0.998** (<0.000)	1		
C_T ratio	**−0.669** (0.009)	**−0.957** (<0.000)	**−0.950** (<0.000)	1	
Aromatase activity	0.339 (0.236)	0.399 (0.158)	0.404 (0.152)	−0.334 (0.243)	1

Bold numbers indicate significant correlations. Negative numbers indicated negative relationships.
Refer to Table 2 for explanations.

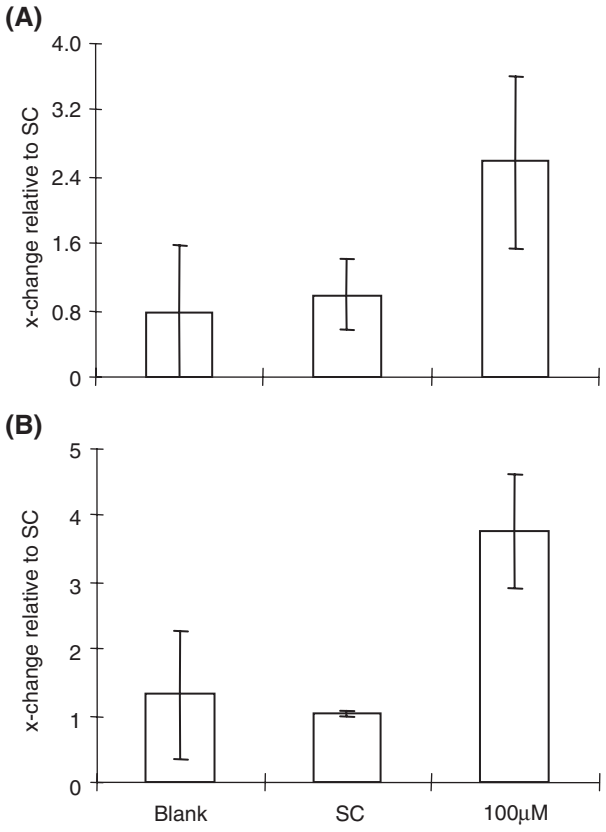

Fig. 4. Fold-change (*x*-change, mean±S.D.) of *CYP19* mRNA in testicular (A) and ovarian (B) explants of *Xenopus laevis* after exposure to 100 μM forskolin (100 μM) for 20 h, using the standard curve method for quantification of mRNA. SC = solvent control (0.1% DMSO).

expression compared to the solvent controls. In testicular tissue *CYP19* mRNA copy numbers were increased 2.62-fold. The above results were achieved using the standard curve method. However, similar patterns were observed when applying other quantification methods such as C_T ratio method (data not shown).

4. Discussion

4.1. Development and optimization of Q-RT PCR system to quantify CYP19 gene expression in male X. laevis

The conditions of SYBR Green-Q-RT PCR analysis for detecting *CYP19* mRNA in testes from male *X. laevis* were established and optimized. The two-step Q-RT PCR method was selected over the one-step method due to its higher sensitivity, lesser risk of primer–dimer formation during PCR reaction and lesser risk of contamination with genomic DNA (Vandesompele et al., 2002). It was possible to detect small quantities of *CYP19* mRNA (as few as 10 copies/reaction) in gonadal tissue (<100 mg) without prior cDNA amplification or a nested PCR approach, which requires a secondary amplification of the target gene using the PCR product from an initial gene amplification to improve sensitivity and specificity.

SYBR Green was chosen for the detection of amplicons during the PCR reaction because it is relatively inexpensive while its sensitivity, reproducibility and dynamic range were comparable to that of the fluorescent probe method (Lekanne Deprez et al., 2002). The melting curve analyses revealed that the obtained signal for both *CYP19* and housekeeping gene were specific, and did not result in the amplification of unwanted gene products. No primer–dimers were formed. The SYBR Green dye detection system proved to be highly sensitive with a method detection limit of as few as 10 copies of the target gene per reaction. The routine treatment of RNA samples with DNase I minimized co-amplification of pseudogenes, which are genetically similar to the original gene but are not expressed, or non-specific DNA the which the primer may have found (Kreuzer et al., 1999).

Quantitative analysis of gene expression is often achieved by normalization to the amplification of housekeeping genes as internal controls. Ideally, the internal control gene should be expressed at a constant level among different cell populations and individuals and should be unaffected by experimental conditions (Thellin et al., 1999). *GAPDH* is a gene that has these characteristics, which make it a useful and effective housekeeping gene to control for these types of variations (Wiechmann and Smith, 2001). The use of the *GAPDH* as an internal control provides more accurate results since it not only compensates for sample-to-sample variations but also circumvents technical problems such as total RNA extraction efficiency and reverse transcription efficiency. However, there are studies that suggested that, in some cases, *GAPDH* might not be appropriate as an internal control for every RT PCR system. Some mammalian species showed unstable gene expression of *GAPDH* during the cell cycle (Mansur et al., 1993) and during different developmental stages (Calvo et al., 1997). A different study with humans found that *GAPDH* mRNA transcription levels can also vary widely among individuals (Bustin et al., 1999). In contrast, in our study, little variation in the expression of *GAPDH* was observed among individuals. This observation indicates that *GAPDH* is a suitable housekeeping gene for determining changes in *CYP19* expression in testes of *X. laevis* that are of similar developmental stage. However, this study was not designed to address effects of different developmental stages on the expression of *GAPDH* and, therefore, when conducting a developmental study the appropriateness of the GAPDH as a housekeeping gene would need to be further validated.

In order to obtain accurate and reproducible results, the PCR reaction should have efficiency as close to 100% as possible. At this efficiency, the template doubles after each cycle. Efficiencies of the PCR reactions were very close to the desired efficiency of 100% for both *CYP19* and *GAPDH*, indicating that the increase in gene expression is directly proportional to the number amplification cycles. Furthermore, the small interassay variabilities among experiments conducted on 3 different days and the low CVs for the calculated C_T values for all experiments demonstrate the reproducibility and precision of the established test system.

In conclusion, the Q-RT PCR method developed to quantify *CYP19* gene expression in male *X. laevis* in this study is sufficiently sensitive to allow the measurement of single digit

copies of total RNA. This sensitive and precise assay is a useful tool that allows for quantifying specific types of mRNA that are expressed at low levels in certain tissues such as *CYP19* in testes of male frogs and that allows for direct comparison of gene expression levels between samples.

4.2. Comparison of different gene quantification methods

In this study, four quantification methods were applied to quantify *CYP19* gene expression and were then compared to identify the optimal method for quantification. In the first method, *CYP19* mRNA copy numbers were calculated from the absolute standard curve obtained by serial 10-fold dilutions of a cloned plasmid standard without referring to the housekeeping gene. This method allowed estimation of the number of copies of *CYP19* mRNA present in the unknown samples. However, estimates of copy numbers of *CYP19* mRNA calculated from the linear equation derived from the absolute standard curve method did not appear to give an accurate estimate of the actual expression of *CYP19* mRNA molecules present in the sample. The inaccuracy of the estimate was indicated by the low correlation of these copy numbers with the housekeeping gene-corrected copy numbers or the calculated ratio from the comparative C_T method. This correlation was improved once the copy numbers were normalized to the internal control (expressed as ER). This demonstrates that the use of an internal control such as *GAPDH* is critical to accurately quantify *CYP19* gene expression profile in male *X. laevis* when using Q-RT PCR. The significant correlations among all three quantification methods using *GAPDH* as the housekeeping gene demonstrate the applicability of all of these methods to quantify *CYP19* gene expression in *X. laevis* testes. However, compared to the very strong relationship between the standard curve and the comparative C_T method ($r^2=0.997$, $p<0.001$), the C_T value ratio was less predictive for the standard curve method ($r^2=0.916$, $p<0.001$) or the comparative C_T method ($r^2=0.902$, $p<0.001$). Thus, we conclude that both the comparative C_T method and the standard curve method are optimal quantification methods to estimate low levels of *CYP19* gene expression in testicular tissue of *X. laevis*. There have been few studies using Q-RT PCR to measure aromatase mRNA expression in male African clawed frogs (Miyashita et al., 2000; Kuntz et al., 2004). These studies reported gene expression of *CYP19* without normalization (Miyashita et al., 2000), or simply by the ratio of *CYP19*/*Sf 1* (Kuntz et al., 2004) and, to date, and to the best of our knowledge, no study has been conducted to measure *CYP19* mRNA level in male *X. laevis* using more accurate RT PCR quantification methods. The results from our study confirm that the simple ratio between housekeeping and *CYP19* gene is not as accurate and sensitive as more sophisticated methods such as the standard curve or comparative C_T method.

The advantage of the comparative C_T method over the absolute standard curve method is that this method eliminates the need to construct a standard curve, which is a time consuming and laborious process, allowing simple quantification of the relative gene expression of paired samples. Therefore, use of the economical and efficient comparative C_T method is recommended as the preferable method to quantify *CYP19* gene expression in testicular tissue of *X. laevis*.

4.3. Comparison of CYP19 gene expression with aromatase enzyme activity

While mRNA quantification of *CYP19* provides important information on the regulation of protein synthesis, it may not directly reflect aromatase enzyme activity due to post-transcriptional control of enzyme activity. An earlier study reported that differences in CYP19 gene expression between males and females were not proportional to aromatase enzyme activity in another amphibian species, the newt, *Pleurodeles waltl* (Kuntz et al., 2004). These authors hypothesized that this lack in correlation might be due to differences in the post-transcriptional regulation of aromatase. Post-transcriptional factors that can influence the net activity of the enzyme aromatase can be either due to modifications of the mRNA that lead to differential translation within a tissue or can be due to post-translational modifications that alter the stability of functionality of the protein (Balthazart et al., 2001; Genissel et al., 2001).

Even though there is evidence that estrogens, which are catalyzed by aromatase, play a stimulatory role in germ cell development including spermatogonial division, germ cell viability and differentiation, acrosome biogenesis and function of the spermatozoa in rodents (O'Donnell et al., 2001), at present little is known of aromatase expression and the role of estrogens in the testis of amphibians. It appears that estrogens are involved in multiple actions of male reproductive system of amphibians during certain developmental stages (Fasano et al., 1989; Cobellis et al., 2002). The fact that aromatase enzyme activities in our study were very low and not correlated with *CYP19* gene expression indicates that this enzyme may have been dormant or at basal levels at the developmental and/or reproductive stage (animals were not in active breeding conditions) the frogs were in during this experiment. However, the confirmation experiments using an inducer of aromatase, forskolin, have demonstrated that *CYP19* gene expression can be modulated (increased) both in ovarian and testicular tissue of *Xenopus*, indicating that stimulation of the enzyme results in a specific response at the gene expression level. This result indicates that the established Q-RT PCR system represents a valid method to determine alterations in the expression of *CYP19* in male testis.

4.4. Implications for toxicological assessment of environmental pollutants

Aromatase regulation and activity play a pivotal role in sexual development and in communicating reproductive processes in vertebrates. While in ovarian tissues the formation of estrogens from androgens via the enzyme aromatase is an essential process for gonadal maturation in males both expression and activity of aromatase are low in the testis during the maturation phase, which mainly depends on androgens.

Accurate transcriptional regulation of the genes encoding steroidogenic enzymes such as aromatase is critical for the regulation of sex steroid homeostasis that is essential for ordinary sexual development processes in animals (Yamada et al., 1995). Thus, improper and untimely changes in *CYP19* gene expression may affect reproductive success in animals (Trant et al., 2001; Kuntz et al., 2003b). Therefore, the quantitative analysis of *CYP19* mRNA expression can be an important marker for detection of developmental and reproductive disruption by EDCs in animals of both sexes. In fact, a series of chemicals have been reported to have the potential to directly or indirectly disturb steroidogenesis simply by interfering with the regulation of *CYP19* gene expression either in vivo or in vitro (Connor et al., 1996; Sanderson et al., 2000; Miyata and Kubo, 2000; Kazeto et al., 2004). In the recent controversy about possible effects of pesticides and/or other environmental contaminants on reproduction and development in amphibian species, it was hypothesized that abnormal sexual development such as compromised reproductive functions and/or characteristics may be due to the induction of aromatase by these chemicals causing a decrease of endogenous androgens in males (Hayes et al., 2002). However, although a series of studies has been conducted to identify effects of the exposure to triazine herbicides on aromatase activity or *CYP19* gene expression in fish or amphibians (Hayes et al., 2002; Kazeto et al., 2004; Lavado et al., 2004; Hecker et al., 2004), it has proved to be difficult to establish a direct link between exposure to these chemicals and changes in gonadal aromatase. As outlined previously, this is likely due to the fact that aromatase enzyme activities are low in adult testicular tissue, often being below or just above the method detection limits of enzymatic assays. The Q-RT PCR technique established in this study represents a method that can help to overcome this difficulty as it is capable of identifying very small amounts of *CYP19* mRNA and has been successfully used to determine gene expression in the testis of *X. laevis*.

In conclusion, the Q-RT PCR system established and optimized in this study represents a highly sensitive, rapid and reliable method to detect and measure very small quantities of *CYP19* mRNA in small amounts of tissue. Although *CYP19* mRNA expression does not seem to directly reflect aromatase enzyme activity in testicular tissue, the developed Q-RT PCR method is a powerful tool due to determine changes in the regulation of protein synthesis of aromatase that will be helpful in researching general regulatory mechanisms in the reproductive endocrinology of *X. laevis*. Furthermore, this method can be used as a highly sensitive marker in toxicological studies to identify effects of environmental contaminants at the pre-translational level of aromatase. Currently, a parallel study is underway that uses this Q-RT PCR method to determine the effects of atrazine on testicular aromatase in *X. laevis*.

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