

# Sexing Frogs by Real-Time PCR: Using Aromatase (*cyp19*) as an Early Ovarian Differentiation Marker

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## Key Words

Aromatase • *cyp17* • *cyp19* • *foxl2* • Gene expression • *Lithobates (Rana) sylvaticus* • Sexual differentiation • *Silurana (Xenopus) tropicalis* • Tadpoles • Wood frog

## Abstract

Most anurans have no identified sex-markers; therefore, alternative methods for identification of early changes in sex ratios are required. In this study, *Lithobates sylvaticus* and *Silurana tropicalis* tadpoles were sampled at different developmental stages covering the entire process of sex differentiation. Three candidate genes known to be involved in sex differentiation in other vertebrate species were selected to develop a method to identify phenotypic sex in frogs: cytochrome p450 aromatase (*cyp19*), forkhead box L2 (*foxl2*) and the cytochrome 17- $\alpha$ -hydroxylase/17,20 lyase (*cyp17*). Cloning of these genes revealed nucleotide identity values ranging between 75–97% when compared to other amphibian species. Gene expression of *cyp17*, *cyp19* and *foxl2* in *L. sylvaticus* adult gonads and gonad-mesonephros complex (GMC) of tadpoles was analyzed by real-time RT-PCR. Results showed clear sexually dimorphic patterns in the expression

of the 3 genes. Our analysis reveals that GMC gene expression levels of *cyp19* alone can be used as a robust predictor of phenotypic sex in *L. sylvaticus* tadpoles. In addition, we validated this method measuring *cyp19* mRNA levels in *S. tropicalis* GMCs. We propose measuring *cyp19* as a tool to study the effects of chemical contaminants (including endocrine disrupting compounds) on amphibian gonadal development and sex ratios in the future.

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In many non-mammalian vertebrates, gonadal development is under the direct influence of steroidogenic enzymes, sex steroids and environmental factors [Bogart, 1987; Scherer, 1999]. Further, sex differentiation in amphibians, fish, reptiles, and birds is a sensitive period that can be altered by exposure to exogenous hormones and endocrine disrupting chemicals [Boggs et al., 2011; Flament et al., 2011; Norris, 2011; Ottinger et al., 2011; Paul-Prasanth et al., 2011; Vajda and Norris, 2011; Warner, 2011]. In contrast, sex steroids are considered as gonadal hormones, which are synthesized as a result of gonadal sex differentiation in mammals. Sex steroids act to estab-

lish the secondary sexual phenotype in most mammalian species [vom Saal, 1989]. In teleost fish, it is the ratio of intragonadal androgens and estrogens during the period of sex differentiation that drives the formation of the ovary versus the testis [Baroiller and D'Cotta, 2001], rather than the action of a single steroid. Generally, gonadal steroidogenesis is active before clear histological differentiation in many non-mammalian vertebrates. For example, as it has been demonstrated in chickens [Scheib, 1983] and turtles [Dorizzi et al., 1991], estrogens are synthesized by morphologically undifferentiated female gonads in frogs [Isomura et al., 2011]. This demonstrates that sex steroids also play an important role in frog sex differentiation.

Studies published in the last decade have reported sexually dimorphic expression of several genes suspected to be involved in frog sex determination and differentiation in tadpoles gonad-mesonephros complex (GMC). The majority of these have focused on a single species, *Glandirana (Rana) rugosa* [Takase et al., 2000a, b; Sugita et al., 2001; Shibata et al., 2002; Aoyama et al., 2003; Ohtani et al., 2003; Oshima et al., 2005, 2006, 2007, 2008, 2009; Yamamura et al., 2005; Iwade et al., 2008; Maruo et al., 2008; Nakamura et al., 2008; Sakurai et al., 2008; Yokoyama et al., 2009; Isomura et al., 2011; Suda et al., 2011a, b]. In this frog species, both genetic and phenotypic sex can be determined [Miura et al., 1998], and gene expression levels in differentiating GMCs can be studied separately in genetic males and females. GMC gene expression patterns during sex differentiation have only been studied in a few other frog species, including *Xenopus laevis* [Semba et al., 1996; Koyano et al., 1997; Takase et al., 1999; Nakajima et al., 2000; Kawano et al., 2001; Lutz et al., 2001; Akatsuka et al., 2005; Osawa et al., 2005; Yoshimoto et al., 2006, 2008; Urbatzka et al., 2007, 2010; Okada et al., 2009], *Silurana (Xenopus) tropicalis* [El Jamil et al., 2008a; Duarte-Guterman and Trudeau, 2011], *Engystomops (Physalaemus) pustulosus* [Duarte-Guterman et al., 2012] or *Lithobates catesbeianus* (formerly *Rana catesbeiana*) [Mayer et al., 2002]. In these reports, 3 genes have consistently exhibited sexually dimorphic expression patterns in gonads of adult frogs and differentiating tadpoles. These are the cytochrome P450 aromatase gene (*cyp19*) that encodes for aromatase, the enzyme responsible for controlling the conversion of androgens into estrogens; the transcription factor forkhead box L2 (*foxl2*) that positively regulates *cyp19* transcription; and the cytochrome P450 17- $\alpha$ -hydroxylase/17,20-lyase (*cyp17*) that encodes for the enzyme responsible for controlling the conversion of progestagens for androgen production.

The wood frog, *L. sylvaticus* (formerly *Rana sylvatica*), is a common and abundant species in northern North America. Sex differentiation in wood frogs is typical for a differentiated gonochoristic species, with undifferentiated gonads developing towards ovaries or testes before metamorphosis is complete [Witschi, 1929]. As shown in other species from the Ranidae family, the course of sex differentiation in wood frogs can be altered by the administration of exogenous hormones [Mackenzie et al., 2003]. However, to our knowledge gene expression profiles of key genes involved in wood frog gonadal differentiation have not been studied previously. For this reason, one of the objectives of the present study was to clone and characterize wood frog *cyp19*, *foxl2* and *cyp17* genes. On the other hand, phenotypic sex identification in frogs has been traditionally achieved by histological approaches, which is time consuming and requires samples from late development stages. In addition, when phenotypic sex is evaluated by histological procedures, no tissue typically remains for molecular studies. For these reasons, we identified the need to develop a molecular tool that allows sex identification and determination of sex ratios in populations and, at the same time, permits the study of the mechanisms involved. Given the sexually dimorphic expression patterns in the tadpole GMC of other frog species, and due to their importance in controlling sex steroid production, we hypothesized that the combined expression pattern of *cyp19*, *foxl2* and *cyp17* could be a useful early marker of sex differentiation in *L. sylvaticus*. We aimed to identify molecular markers for phenotypic sex that could be used more generally in amphibian species, so we have also studied the African frog, *S. tropicalis*. We believe that our approach represents a useful tool to both determine phenotypic sex ratios at early stages of development as well as to analyze the effects of exposure to endocrine disrupting chemical contaminants during frog differentiation in future studies.

## Materials and Methods

### Animals and Sample Collection

Wild adult *L. sylvaticus* frogs (3 males and 3 females) were collected at the Long-term Experimental Wetlands Area at the Canadian Forces Base Gagetown, N.B. Brains, kidneys and gonads were dissected in the laboratory and stored at  $-80^{\circ}\text{C}$  until further analysis. In addition, *L. sylvaticus* tadpoles were collected in the same area from a natural pond at 4 different sampling times during spring 2009. Collections were made when the animals reached a determinate median Gosner developmental stage (Gs) [Gosner, 1960]: Gs26 ( $n = 16$ ), Gs28 ( $n = 16$ ), Gs30 ( $n = 15$ ), and Gs31–42

**Table 1.** Primers and tissues used for gene cloning

| Gene         | Forward primer             | Reverse primer              | Tissue |
|--------------|----------------------------|-----------------------------|--------|
| <i>cyp19</i> | 5'-TGGATTAATGGCGAGGAAAC-3' | 5'-GGYTGGTACCTCATGCTTTCA-3' | Ovary  |
| <i>foxl2</i> | 5'-ATGGCWATYMGGGARAGYBC-3' | 5'-TAKGGRCTGTAGGAGGACGA-3'  | Brain  |
| <i>cyp17</i> | 5'-AGTTTCACCGCAAAATGGTC-3' | 5'-TATGCMACAGCCCATTTCAA-3'  | Brain  |

(n = 52). In the case of Gs26, 28 and 30, the median Gs was targeted during the collections, with all tadpoles collected at the same stage as the median stage of the pond. However, later in development, the variability of stages found in natural ponds increases, and we observed tadpoles from a broad range of stages at any given time. For that reason, in the case of the Gs31–42 sampling, although the animals collected had the same age (were sampled the same day), developmental stages ranged from Gs31–42. Animals were euthanized with MS-222 (Sigma) prior to collections. A ventral incision was made in tadpoles prior to preserved in RNAlater solution (Ambion) at ambient air temperature for <12 h. Tadpoles were then stored at 4°C for 3 days, before the GMC was removed and stored at –80°C until further analysis. This sampling methodology was previously optimized in our laboratory to ensure RNA quality [Navarro-Martín et al., 2012]. The GMC was chosen for ease of dissection and to ensure sufficient amounts of mRNA when studying gonadal differentiation in tadpoles. Some other examples of this approach are available [Urbatzka et al., 2007; Duarte-Guterman and Trudeau, 2011; Duarte-Guterman et al., 2012]. All procedures followed approved animal care protocols of the University of Ottawa and the Canadian Council on Animal Care.

#### *Cloning of L. sylvaticus Genes Involved in Vertebrate Gonadal Differentiation*

Total RNA was isolated from different tissues obtained from adult wood frogs (table 1) using TRIzol, as described by the manufacturer (Invitrogen). The RNA extracted was then resuspended in RNase-free water and stored at –80°C. Five micrograms of total RNA were reverse transcribed to cDNA using Superscript II and 200 ng of random hexamer primers following the manufacturer's instructions. The resulting cDNA was used to amplify the desired genes using native Taq DNA polymerase (Invitrogen) and the respective primers (table 1). In order to obtain the sequence of the desired genes, design of primers based on conserved areas of other frog species sequences was required. Primers were designed using Primer 3 (<http://fokker.wi.mit.edu/primer3/input.htm>). The PCR cycling conditions were: 2 min at 95°C; 40 cycles of 45 s at 95°C, 30 s at 54°C, 45 s at 72°C and a final extension of 10 min at 72°C. PCR products of each sample were resolved on 1% agarose gel stained with ethidium bromide. When the amplified product was not evident or the band was very faint, the expected size was sliced from the gel, and the sample was purified using QIAquick Gel extraction kit (Qiagen). Subsequently, the purified product was re-amplified using the same PCR cycling conditions described above. PCR products were cloned into pCR2.1-TOPO vector and transformed into *E. coli* Topo10 Chemically Competent cells (Invitrogen). Three individual clones per each gene were

sequenced. Obtained sequences were submitted to Genbank. BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>) was used to check for nucleotide identity with other frog and toad species (online supplementary table 1; for all supplement material see [www.karger.com/doi/10.1159/000343783](http://www.karger.com/doi/10.1159/000343783)).

#### *Identification of Molecular Markers to Determine Phenotypic Sex in L. sylvaticus*

**RNA Extractions and cDNA Synthesis.** Total RNA was isolated from adult kidneys and gonads using RNeasy Mini Kit (Qiagen) and from the tadpoles' GMCs using the RNeasy Micro Kit (Qiagen). In all cases, an RNase-free DNase treatment was performed during the extractions. All procedures were carried out following manufacturer's protocols. Total RNA was resuspended in RNase-free water. Concentrations of total RNA, the absorbance ratios at 260 and /280 nm, and at 260 and /230 nm were determined using the NanoDrop 1000 Spectrophotometer (NanoDrop Technologies Inc.). The RNA integrity number determined using the Agilent 2100 Bioanalyzer, was used to assess RNA quality. All quality parameters were optimal (ratios of absorbance around 2 and RNA integrity numbers  $\geq 7$ ). Samples were stored at –80°C until further analysis. Total cDNA tissues were synthesized from 1  $\mu$ g of total RNA, using 200 ng random hexamer primers (Invitrogen) and Superscript II reverse transcriptase (Invitrogen). Due to the small amount of RNA used, the incubation period at 42°C was increased from 50 min to 1.5 h during cDNA synthesis.

**Real-Time RT-PCR Analysis for *L. sylvaticus* *cyp19*, *foxl2*, *cyp17*, and *rpl8*.** Based on the *L. sylvaticus* sequences obtained, gene-specific primers (table 2) were developed using Primer3 (<http://frodo.wi.mit.edu/primer3/input.htm>). Gene location, size, and melting temperatures for each amplicon are summarized in table 2. In addition, a partial sequence of *L. sylvaticus* *rpl8* gene has been previously characterized in our laboratory (Genbank accession number HQ630611) and real-time RT-PCR reactions optimized [Navarro-Martín et al., 2012]. The lack of amplification in control samples not subjected to reverse transcriptase indicated the absence of genomic DNA contamination. SYBR Green I chemistry was used, and a dissociation curve indicating the presence of a single amplification product was performed for each primer pair to verify primer specificity. The identity of the amplicons was further confirmed by sequencing. For each gene, primer concentrations were optimized to ensure maximum amplification and relative standard curves that yielded an efficiency of  $100 \pm 10\%$  (slope between –3.1 and –3.6) and  $r^2 > 0.985$ , according to the manufacturer's instructions (MX3000P real-time polymerase chain reaction system; Stratagene). Standard curves were generated using a serial dilution of cDNA mix from all samples and stages analyzed.

**Table 2.** Real-time RT-PCR primer sets and conditions for *L. sylvaticus*

| Gene         | Primer sequence (5'→3')                              | Amplicon location and size (bp) | Primer concentration* (nM)         | Melting temperature (°C) |
|--------------|------------------------------------------------------|---------------------------------|------------------------------------|--------------------------|
| <i>cyp19</i> | F: TCATTGTGGAAGGTGATTCG<br>R: ATCGTTTCGGTGGACTTCAAA  | 154–250 (97)                    | 225 <sup>a</sup> –600 <sup>b</sup> | 83.5                     |
| <i>foxl2</i> | F: AAGGAGAAGGCGGATGAAAA<br>R: ACTTGGGTGGGGACAGGTAG   | 240–346 (107)                   | 300 <sup>a</sup> –600 <sup>b</sup> | 87.5                     |
| <i>cyp17</i> | F: GCTGTGTATGTTTCGGTGAAGG<br>R: TGTCCAAAGGGATTGTCTGG | 29–135 (107)                    | 450                                | 85.5                     |

\*To obtain optimized conditions for the real-time RT-PCR reactions, different primer concentrations were used in the different tissues. <sup>a</sup> Primer concentrations used in the reactions containing adult gonads and kidneys as well as GMC from tadpoles at developmental Gs36–41. <sup>b</sup> Primer concentrations used in the reactions containing GMC from tadpoles at developmental Gs26, 28 and 30.

Each sample was run in duplicate in optically clear 96-well plates in a final volume of 25 µl. For every gene and PCR run, non-template and non-reverse transcriptase controls were included to confirm the absence of contamination. In all cases, each PCR reaction contained: adult kidney, adult gonad or tadpole GMC cDNA (15.63 or 6.25 ng of RNA transformed to cDNA in adults tissues or in the tadpole GMCs, respectively), 1× Q-PCR buffer (Qiagen), 2.5 mM MgCl<sub>2</sub> (Qiagen), 200 µM dNTPs (Invitrogen), 100 nM passive reference dye (Stratagene), and 1.25 U HotStarTaq (Qiagen). An optimized concentration for each primer set (see table 2) and 0.25× SYBR Green I Dye (Molecular Probes) were added to the reaction. Cycling parameters for *foxl2*, *cyp17* and *rpl8* were as follows: activation step at 95°C for 15 min followed by 40 cycles of 95°C for 15 s, 60°C for 5 s, 72°C for 54 s and 81°C 18 s. In the case of *cyp19*, optimal cycling parameters were as follows: activation step at 95°C for 15 min followed by 40 cycles of 95°C for 30 s, 60°C for 1 min and 72°C for 1 min. Finally, a temperature-determining dissociation step was performed at the end of the amplification phase to ensure the presence of a single amplified product.

#### Validation of the Identified Phenotypic Sex Markers in Frogs Using *S. tropicalis*

*S. tropicalis* was chosen as a second species to validate our phenotypic sex marker method. Gene expression profiles of *cyp19* levels in GMCs of *S. tropicalis* tadpoles from Nieuwkoop and Faber (NF) [Nieuwkoop and Faber, 1994] developmental stages NF52–66, and adult gonads were described by Duarte-Guterman and Trudeau [2011]. Compared to the earlier study, the present study includes earlier developmental stages, including NF48 and 50. In addition, sample size for developmental stages NF52 and 54–55 was increased. In Duarte-Guterman and Trudeau [2011], *cyp19* did not amplify in some of the samples, and we suspect this was due to some detection limitations of the technique used. In the previous study, multiplex real-time RT-PCR reactions were carried out, where different primers compete to amplify different genes in the same reaction. For that reason, we decided also to include and re-analyze some of the samples from the previous study (developmental stages NF52, 55 and 60, and adult testis and ovaries). To avoid detection limitations and to improve amplifica-

tions, the present study was done using single real-time PCR reactions (described below).

**Sample Collection, RNA Extractions and cDNA Synthesis for *S. tropicalis*.** Collection and dissection of GMCs at developmental stages NF48 (n = 16), NF50 (n = 16), NF52 (n = 8), and NF54 (n = 8) were previously done using the same breeding batch described by Duarte-Guterman and Trudeau [2011]. Samples were stored at –80°C until further analysis. Total RNA was obtained using the RNeasy Micro Kit (including the DNase treatment set; Qiagen). All samples were reverse transcribed to cDNA using 1 µg of RNA, 200 ng random hexamers and Superscript II reverse transcriptase (Invitrogen). In addition, cDNA from tadpoles GMCs obtained by Duarte-Guterman and Trudeau [2011] at the developmental stages NF52 (n = 8), NF55 (n = 8) and NF60 (n = 16), and adult gonads (n = 6 ovaries and n = 6 testis) were used. In summary, a total of 12 adult gonads and 80 GMCs were analyzed from *S. tropicalis* tadpoles (NF48 (n = 16), NF50 (n = 16), NF52 (n = 16), NF54–55 (n = 16), and NF60 (n = 16) in the present study.

**Real-Time RT-PCR Analysis for *S. tropicalis cyp19*.** To analyze *cyp19* levels in the *S. tropicalis* samples, the same gene-specific primers designed by Duarte-Guterman and Trudeau [2011] were used in a single PCR reaction. Each sample was run in duplicate in optically clear 96-well plates with a final volume of 25 µl. The lack of amplification in the control samples, including non-template and non-reverse transcriptase controls, were included to confirm the absence of contamination. Each PCR reaction contained: 6.25 ng of RNA transformed to cDNA, 1× Q-PCR buffer (Qiagen), 2.5 mM MgCl<sub>2</sub> (Qiagen), 200 µM dNTPs (Invitrogen), 100 nM passive reference dye (Stratagene), and 1.25 U HotStarTaq (Qiagen). The optimized concentration of 325 nM of the primer set was added to the reaction. Cycling parameters were as follows: activation step at 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 60°C for 1 min, 72°C for 1 min. Finally, a temperature-determining dissociation step was performed at the end of the amplification phase to ensure the presence of a single amplified product. The amplification curve yields an efficiency of 99.2%, a slope of –3.342 and an r<sup>2</sup> of 0.989. On each assay plate, standard curves were generated using a serial dilution of cDNA mix from all samples and stages analyzed.



### Statistical Analysis

To determine the best predictor(s) of phenotypic sex in *L. sylvaticus* tadpoles, 3 steps were followed. First, a 2-step cluster analysis (where the number of clusters was not predetermined) was performed using a combination of stage (as a categorical variable) and/or GMC *cyp19*-, *foxl2*- and *cyp17*-fold change gene expression levels (as continuous variables) from tadpoles of the same age. Developmental stages ranged from Gs31 to 42. The cluster analysis produced 2 clusters, which distinguished individuals with higher and lower gene expression levels, allowing us to classify tadpoles as presumptive males and females (see results section). Second, a discriminant analysis was used to determine which predictor variables were better at discriminating presumptive males and females. Since sex ratios in frog populations are expected to be 1:1 (male:female), equal prior probabilities were assumed at the beginning of the analysis. A cross-validation method was used to calculate the average of correctly classified samples. Third, a 2-step cluster using *cyp19* and *foxl2* gene expression levels was carried out for *L. sylvaticus* GMCs from tadpoles at earlier developmental stages (Gs26, 28 and 30). We found *cyp19* to be the best predictor for *L. sylvaticus* presumptive sexes (see results section). Therefore, to validate our method in *S. tropicalis*, *cyp19* expression was used in a 2-step cluster analysis (where the number of clusters was not predetermined) to classify presumptive males and females.

Significant differences in gene expression levels between sexes in adult frogs were also tested. First, gene expression levels between adult male and female kidneys and gonads were compared using a Student t test. Since no differences were found between males and females in the adult kidneys, data from both sexes were combined. Subsequently, a one-way ANOVA test followed by a multiple comparisons Tukey's post hoc test was carried out to determine differences between adult kidneys, testes and ovaries. In addition, differences in mean gene expression among sexes were compared in Gs30 and 31–42 *L. sylvaticus* GMCs as well as in NF50, 52, 54–55, 60 GMCs and adult gonads from *S. tropicalis*. In all cases and before any analysis, normality and homoscedasticity of variances was verified using Shapiro-Wilk's and Levene's test, respectively. Differences in mean gene expression among sexes were determined by a Student's t-test. Data that violated the assumption of normality was analyzed using the Mann-Whitney test. Furthermore, association between gene expression levels of *cyp19*, *foxl2* and/or *cyp17* at Gs26, 28, 30, and 31–42 was tested in *L. sylvaticus* GMCs. In this case, a Pearson correlation test was performed if the data were normal; otherwise, a Spearman correlation test was performed. Statistical analyses were performed using SPSS 15.0 package. Differences were considered statistically significant when  $p < 0.05$ .

## Results

### *L. sylvaticus* Genes Are Highly Conserved When Compared to Other Frog Species

We sequenced, partially identified and submitted to Genbank *L. sylvaticus* cDNA sequences of the following genes: *cyp19*, JX162018; *foxl2*, JX162019; and *cyp17*, JX162020. Multiple nucleotide alignments of *L. sylvaticus*

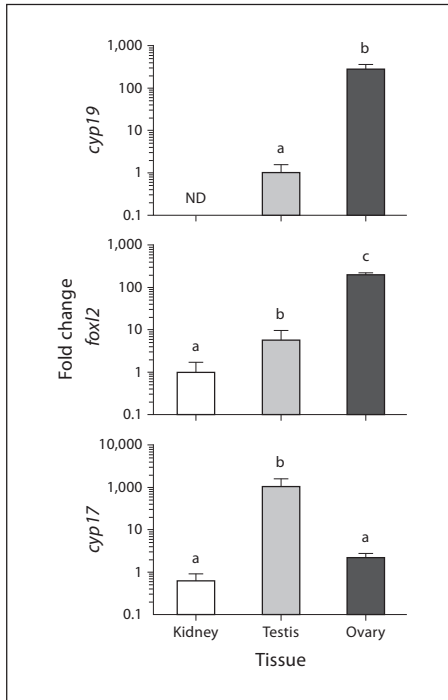
sequences against sequences of other anuran species showed an expected high similarity with *Rana*, *Glandirana* and *Lithobates* spp. (94–97%, online suppl. table 1), while sequence conservation was moderate when compared to *Xenopus* and *Silurana* (75–85%, online suppl. table 1).

### Expression of *cyp19*, *foxl2* and *cyp17* Is Tissue- and Gender-Specific in *L. sylvaticus* Adults

Expression of *cyp19* was not detected in adult male or female *L. sylvaticus* kidneys. Although expression of *foxl2* and *cyp17* was detected in male and female kidneys, no differences between sexes were observed ( $t = -1.927$ ,  $p = 0.126$ ;  $t = -1.412$ ,  $p = 0.231$ ). For this reason, both male and female values were combined (fig. 1). Adult *L. sylvaticus* ovaries were characterized by having significantly higher levels of *cyp19* (260-times,  $t = 10.515$ ,  $p \leq 0.001$ ) and *foxl2* (33-times,  $t = 6.101$ ,  $p = 0.024$ ) and lower levels of *cyp17* (130-times,  $t = -5.597$ ,  $p = 0.005$ ) than adult testes. When compared to testes and ovaries, *foxl2* gene expression was found to be significantly lower (6 and 197-times lower, respectively) in adult *L. sylvaticus* kidneys (ANOVA,  $F = 30.721$ ,  $p \leq 0.001$ ). Kidney *cyp17* levels were lower than those measured in both testes (640-times) and ovaries (5-times), but significant differences were only detected when kidney levels were compared to testes (ANOVA,  $F = 23.617$ ,  $p \leq 0.001$ ).

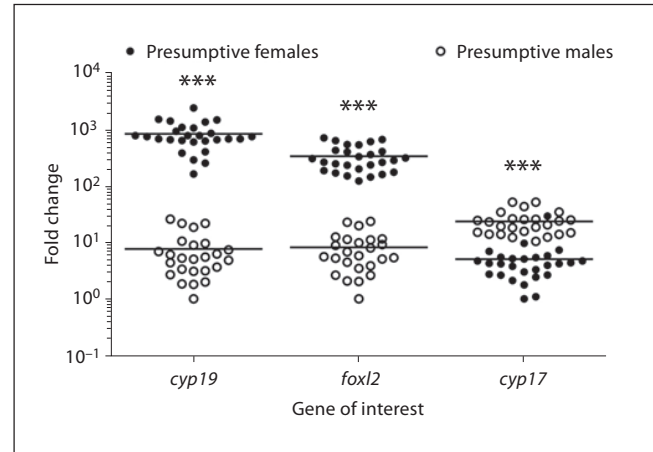
### Gosner Stage Does Not Contribute to the Assignment of Presumptive Phenotypic Sex in *L. sylvaticus* Tadpoles

Gene expression analysis was carried out in *L. sylvaticus* tadpoles of the same age but with different developmental stages, ranging from Gs31 to 42 ( $n = 52$ ). A 2-step cluster analysis using a combination of stage (as a categorical variable) and *cyp19*, *foxl2* and *cyp17*-fold change gene expression levels (fold change as a continuous variable) was carried out. The cluster analysis produced 2 clusters that distinguished individuals with higher and lower gene expression levels. Several studies have demonstrated that sexually differentiated tadpoles and adult frogs present sexually dimorphic gene expression patterns of *cyp19*, *foxl2* and *cyp17* in GMC tissues and adult gonads, respectively (see Introduction and Discussion sections). These studies indicated that *cyp19* and *foxl2* levels are upregulated in adult female gonads and differentiating female tadpole GMC, while *cyp17* levels are highly expressed in adult testes or male differentiating GMCs. For this reason, in the present study individuals



**Fig. 1.** *L. sylvaticus* adult gene expression of *cyp19*, *foxl2* and *cyp17*. Data were normalized to *rpl8* and are presented as fold change in expression relative to the lowest sample value for each gene. Bars represent the mean  $\pm$  SEM for each tissue ( $n = 3$  for ovaries and testes,  $n = 6$  for kidneys). Different letters indicate statistical differences ( $p < 0.05$ ) after ANOVA and a multiple comparison Tukey post hoc test. ND = Non-detected values.

that presented higher *cyp19* and *foxl2* gene expression levels, but lower *cyp17* expression levels, were identified as presumptive phenotypic females, while the ones that presented the opposite pattern were identified as presumptive phenotypic males. Cluster distribution classified 27 tadpoles as presumptive females and 25 as presumptive males. The analysis also revealed that, in animals of the same age, Gosner stage did not contribute to the classification of either cluster: representation of the data by developmental stage clearly shows no difference between different Gosner stage of *cyp19*, *foxl2* or *cyp17* gene expression levels (online suppl. fig. 1). On the other hand, all data for *cyp19*, *foxl2* and *cyp17* gene expression levels contributed to the classification of both clusters. Analysis of *rpl8* gene expression levels revealed no differences between presumptive sexes or stages (online suppl. fig. 1).



**Fig. 2.** Expression of *cyp19*, *foxl2* and *cyp17* in the gonad-meso-nephros complex of *L. sylvaticus* tadpoles during late metamorphosis. Animals at various developmental stages were collected from a natural pond when the median stage in the pond was Gs36 ( $n = 52$ , developmental stages ranged from Gs31 to 42). Data are presented as fold change in expression relative to the lowest sample value for each gene. A 2-step cluster analysis (where the number of clusters was not predetermined) using a combination of developmental stage (as a categorical variable) and/or *cyp19*-, *foxl2*- and *cyp17*-fold change gene expression levels (as a continuous variable) was carried out in all cases to classify tadpoles as presumptive phenotypic males and females (see Materials and Methods and Results sections for more details). Once classified, data were analyzed by Mann-Whitney's test. Bars represent the mean for each presumptive phenotypic sex. Significance is indicated by \*\*\*  $p < 0.001$ .

#### Levels of *cyp19*, *foxl2* and *cyp17* Expression Are Sexually Dimorphic in *L. sylvaticus* Presumptive Phenotypic Females and Males at Late Metamorphic Stages

To be able to determine the relative importance of the variables that classify both presumptive phenotypic females and males, and to calculate significant differences between groups, a discriminant analysis was carried out using expression of *cyp19*, *foxl2* and *cyp17* for Gs31–42 GMCs as the predictor variables. Wilks'  $\lambda$  values range from 0 to 1. The smaller the  $\lambda$  value, the more the predictor contributes to discriminate between the groups [Burns and Burns, 2009]. Results revealed that all predictors significantly contribute to discriminate phenotypic sex (Wilks'  $\lambda = 0.076$ ,  $p = 0.000$ ,  $F = 604.902$  for *cyp19*; Wilks'  $\lambda = 0.102$ ,  $p = 0.000$ ,  $F = 442.594$  for *foxl2* and Wilks'  $\lambda = 0.306$ ,  $p = 0.000$ ,  $F = 113.663$  for *cyp17*). Presumptive phenotypic females expressed significantly higher levels of *cyp19* and *foxl2* (approximately 100-fold and 40-fold

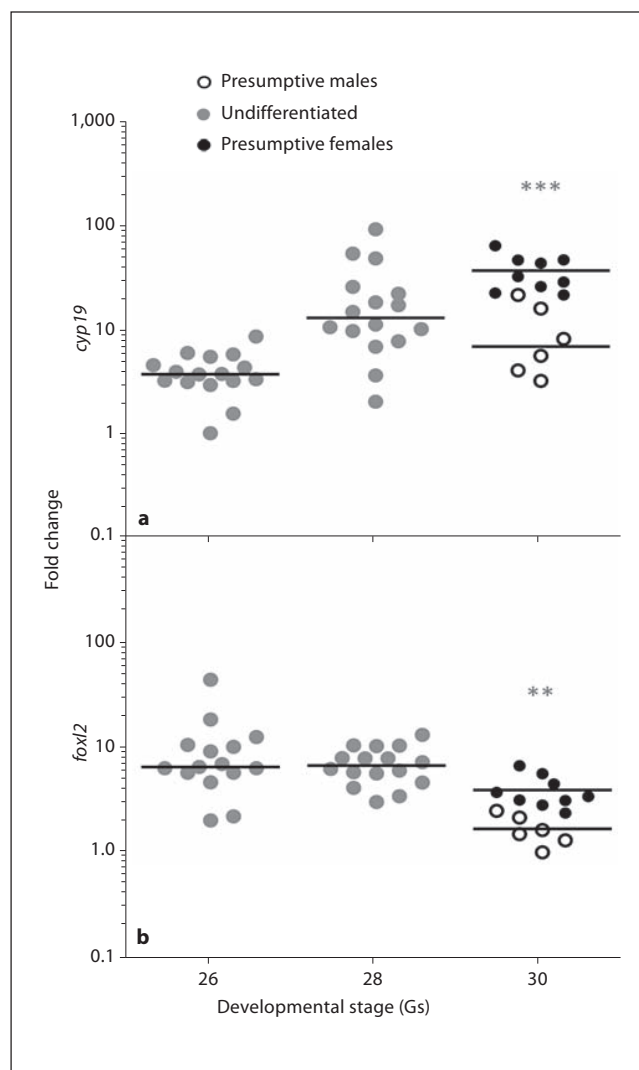
higher, respectively) than presumptive phenotypic males. Presumptive phenotypic females showed 5-fold lower *cyp17* gene expression levels than presumptive phenotypic males (fig. 2). The high canonical correlation (eigenvalue = 30.057;  $r = 0.984$ ) indicates that the function discriminates well between groups. In addition, Wilks'  $\lambda$  indicates that the discriminant function is highly significant ( $p \leq 0.001$ ) with only 3.2% of the variability not explained by the differences among groups. The structure matrix correlations are indicators of the importance of each predictor. An absolute value of 0.3 is considered the cut-off between an important versus a less important variable [Burns and Burns, 2009]. The structure matrix correlation values were 0.634, 0.543 and  $-0.275$  for *cyp19*, *foxl2* and *cyp17*, respectively, revealing that the expression of *cyp17* is a poor predictor. The cross-validated classification showed that overall 100% were correctly classified.

#### *Dimorphic Patterns of cyp19 and foxl2 Gene Expression in L. sylvaticus GMC Can Be First Detected at Gs30*

Only *cyp19* and *foxl2* gene expression levels were analyzed in *L. sylvaticus* samples at early developmental stages (Gs26, 28, and 30) because as indicated in the previous section *cyp17* gene expression levels were found to be a poor indicator of phenotypic sex. Data from animals at Gs26 and 28 were all grouped into a single cluster. In marked contrast, cluster analysis of the Gs30 samples produced 2 distinct groups. For that reason, Gs26 and 28 samples were assigned as undifferentiated tadpoles, while the 2 clusters generated from Gs30 samples were assigned as presumptive phenotypic females and males (fig. 3). At Gs30, presumptive phenotypic females exhibited  $4\times$  and  $2.4\times$  significantly higher levels than presumptive phenotypic males for GMC *cyp19* ( $t = 4.262$ ,  $df = 13$ ,  $p = 0.001$ ) and *foxl2* ( $t = 4.066$ ,  $df = 13$ ,  $p = 0.003$ ), respectively.

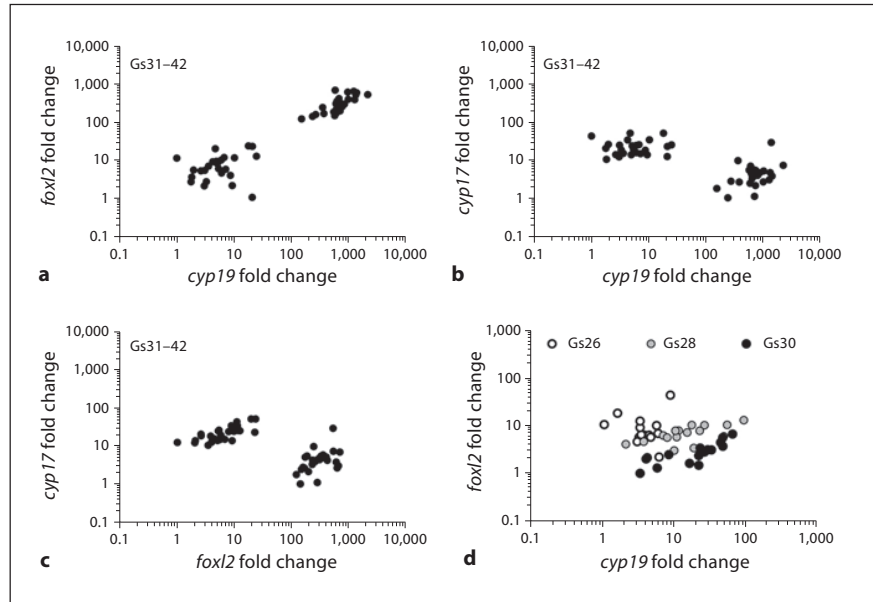
#### *Expression Levels of cyp19, foxl2 and cyp17 Are Highly Correlated in the GMCs of Differentiated L. sylvaticus Tadpoles*

Correlations between *cyp19*, *foxl2* or *cyp17* gene expression levels were determined for samples of *L. sylvaticus* at Gs31–42 (fig. 4a–c). Significant correlations ( $p \leq 0.001$ ) were found between all 3 genes. The highest correlation (Spearman's  $r = 0.890$ ,  $p \leq 0.001$ ; fig. 4a) was between *cyp19* and *foxl2*. Gene expression levels of *cyp17* showed a negative correlation with *cyp19* and *foxl2* (Spearman's  $r = -0.641$ ,  $p \leq 0.001$ ; Spearman's  $r = -0.540$ ,  $p \leq 0.001$ ; respectively; fig. 4b, c). In addition, correlations between *cyp19* and *foxl2* gene expression levels were also



**Fig. 3.** Expression of *cyp19* (a) and *foxl2* (b) in the gonad-mesonephros complex of *L. sylvaticus* tadpoles during early metamorphosis. Animals at different Gs (Gs26, 28 and 30) were collected from a natural pond when the median stage in the pond was Gs26 ( $n = 16$ ), Gs28 ( $n = 16$ ) and Gs30 ( $n = 15$ ), respectively. Data are presented as fold change in expression relative to the lowest sample value for each gene. A 2-step cluster analysis (where the number of clusters was not predetermined) using a combination of *cyp19*- and *foxl2*-fold change gene expression levels was carried out for each stage in order to classify tadpoles as undifferentiated, presumptive phenotypic females or presumptive phenotypic males (see Materials and Methods and Results sections for more details). Once classified, data were analyzed by a Student test. Bars represent the mean for each presumptive phenotypic sex. Significance is indicated by \*\*  $p < 0.005$ , \*\*\*  $p < 0.001$ .

**Fig. 4.** Relationship between *cyp19*, *foxl2* and/or *cyp17* expression in the GMC of *L. sylvaticus* tadpoles. **a** Correlation between *cyp19* and *foxl2* ( $r = 0.890$ ,  $p \leq 0.001$ ). **b** *cyp19* and *cyp17* ( $r = -0.641$ ,  $p \leq 0.001$ ). **c** *foxl2* and *cyp17* ( $r = -0.540$ ,  $p \leq 0.001$ ) in the GMCs at Gs31–42. Due to lack of normality, data were analyzed by Spearman's correlation test. **d** Correlation between *cyp19* and *foxl2* in GMCs of Gs26 (open circles;  $r = 0.451$ ,  $p = 0.092$ ), Gs28 (grey circles;  $r = 0.689$ ,  $p = 0.003$ ) and Gs30 (black circles;  $r = 0.907$ ,  $p \leq 0.001$ ) using Pearson's correlation test. When analyzed together (Gs26, 28 and 30), no significant correlation was found between *cyp19* and *foxl2* ( $r = 0.010$ ,  $p = 0.946$ ). All correlations were performed on an individual tadpole basis. Note that scales of the y-axis vary between genes.



determined for Gs26, 28 and 30 samples (fig. 4d). No significant correlations were found when data from all 3 sampling periods were treated together (Pearson's  $r = 0.100$ ,  $p = 0.946$ ). A different pattern emerged when correlations between *cyp19* and *foxl2* were tested separately at each developmental stage. There appeared to be no correlation between variables at Gs26 (Pearson's  $r = 0.451$ ,  $p = 0.092$ ). In contrast, a significant but weak correlation at Gs28 (Pearson's  $r = 0.689$ ,  $p = 0.003$ ) and a strong significant correlation at Gs30 (Pearson's  $r = 0.907$ ,  $p \leq 0.001$ ) were evident between *cyp19* and *foxl2*.

#### *Clear Dimorphic cyp19 Gene Expression Levels in Developing S. tropicalis GMCs Are Predictive of Phenotypic Sex*

Gene expression levels of *cyp19* were measured in gonads of adult *S. tropicalis* males and females. Cluster analysis clearly confirmed that *cyp19* gene expression levels in the adult females clustered together, while the male values were assigned to a second cluster (fig. 5). In addition, significant differences (Wilcoxon  $W = 21.00$ ,  $p = 0.004$ ) in *cyp19* levels were found between adult testes and ovaries, with ovaries presenting approximately 10,000× more *cyp19* expression than testes. This result supports the idea that *cyp19* gene expression can be used as a female marker because ovaries have higher expression levels than testes. A 2-step cluster analysis was carried out for tadpole GMC at several developmental stages. Only one cluster was evident in the NF48 samples, but clearly

dimorphic patterns were observed at NF50 and subsequent developmental stages (NF52–60), generating 2 well-differentiated clusters. All the NF48 tadpoles were assigned to the undifferentiated group while the 2 clusters generated from NF50 to 60 samples were assigned as presumptive phenotypic females and males. Individuals that clustered with the high *cyp19* levels were designated as females (fig. 5). Significant differences ( $p < 0.005$ ) in *cyp19* gene expression levels were found between presumptive phenotypic females and males at all developmental stages studied (NF50–60).

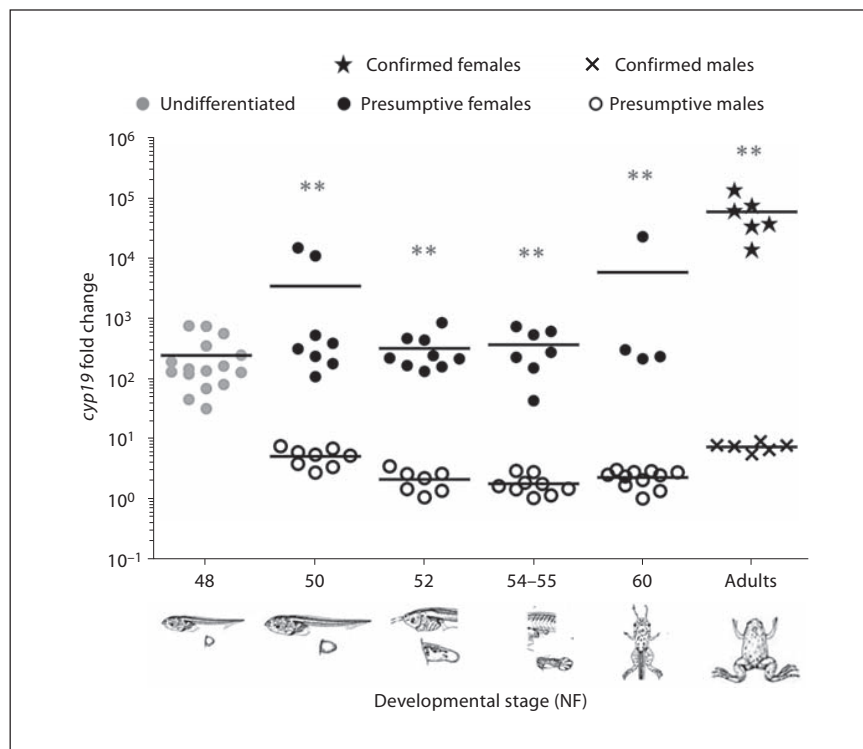
## Discussion

#### *Gender-Specific Patterns of cyp19, foxl2 and cyp17 Can Be Observed in L. sylvaticus Adult Gonads and Developing Tadpole GMCs*

Our results show that expression of *cyp19* was not detected in adult kidneys of either sex, but was clearly differentially expressed in ovaries versus testes. On the other hand, expression of *foxl2* and *cyp17* was detected in adult male and female kidneys, with no difference between sexes. When compared to testes and ovaries, *foxl2* gene expression was found to be significantly lower in adult *L. sylvaticus* kidneys. In addition, kidney *cyp17* levels were significantly lower than levels in the testes, but were not different from ovaries. When studying gonadal differentiation in frogs, dissections of the tadpole GMC



**Fig. 5.** Validation of *cyp19* gonad-mesonephros expression as a marker of phenotypic sex using *S. tropicalis* tadpoles and adults. Gene expression of the cytochrome p450 aromatase (*cyp19*) was analyzed in the adult testes and ovaries as well as in the GMC of *S. tropicalis* tadpoles at different NF developmental stages. A 2-step cluster analysis (where the number of clusters was not predetermined) using *cyp19*-fold change gene expression levels was carried out for each stage in order to classify tadpoles as undifferentiated, presumptive phenotypic females or presumptive phenotypic males (see Materials and Methods and Results sections for more details). Once classified, differences between presumptive sexes within each stage were analyzed by Mann-Whitney's test. Bars represent the mean per each presumptive phenotypic sex. Significance is indicated by \*\*  $p < 0.005$ .



are commonly done to ensure sufficient amounts of mRNA. One possible critique is that the kidney-like tissues of the mesonephros may express levels of the studied genes that may interfere with data interpretation. However, this is not necessarily the case. Our results confirm that *cyp19*, *foxl2* and *cyp17* are expressed in a tissue-specific manner in adults, suggesting that in tadpoles the major contribution to GMC *cyp19*, *foxl2* and *cyp17* levels is from the developing gonadal tissues.

In the present study, dimorphic gene expression levels of *cyp19*, *foxl2* and *cyp17* in the GMC of developing tadpoles (Gs31–42) were observed. Statistical analysis of the expression levels of these genes revealed the existence of 2 obvious clusters, which were assigned to male and female groups. Several studies have shown that dimorphic patterns are also observed in the GMCs of other frog species. For example, genetic female tadpoles undergoing differentiation have significantly higher *cyp19* gonadal expression than males in both *G. rugosa* [Ohtani et al., 2003; Maruo et al., 2008; Oshima et al., 2009] and *X. laevis* [Urbatzka et al., 2007] tadpoles. Similarly, *foxl2* expression was found higher in the GMCs of developing genetic females than in genetic males of *G. rugosa* tadpoles [Oshima et al., 2008]. In contrast, adult males, sex

reversed male tadpoles and differentiating genetic male tadpoles express significantly higher gonadal levels of *cyp17* than adult females or differentiating genetic female of *G. rugosa* tadpoles [Iwade et al., 2008; Maruo et al., 2008; Sakurai et al., 2008; Suda et al., 2011b]. Although genetic sex markers have not been established for many species, including *L. sylvaticus*, we can conclude that these 3 genes are good candidates for studying sex differentiation in frogs.

#### *Molecular Differentiation May Occur Earlier than Morphological Differentiation of the Gonads in L. sylvaticus Tadpoles*

Generally, in fish, the gonadal form of *cyp19* (*cyp19a1a*) can be detected in the developing gonads even before morphological sex differentiation occurs [Nakamura et al., 1998; Baroiller et al., 1999; Blázquez et al., 2009], with aromatization taking place before sex differentiation and directing the fate of the gonad [Kwon et al., 2000]. Although histological analyses were not carried out in the present analysis, our results suggest that dimorphic patterns in the GMC of *L. sylvaticus* tadpoles can be observed previous to morphological differentiation of the tadpole gonads. A study by Witschi [1929] determined that ovar-

ian and testis differentiation can be first detected in 28-day-old *L. sylvaticus* tadpoles. Unfortunately, the age of the wild tadpoles collected for the present study is unknown, since we had no information about when fertilization or hatching occurred in the ponds. However, Witschi [1929] reported that the total length of the tadpoles of that age was around 30 mm, and gonads of smaller tadpoles remained undifferentiated. Interestingly, and although all GMCs at Gs28 were classified as being undifferentiated, a significant but weak correlation between *cyp19* and *foxl2* levels was found. At that stage, total length of our *L. sylvaticus* tadpoles ranged from 17 to 23 mm (data not shown), suggesting that morphological differentiation of the gonads had not yet occurred. This could indicate that sex differentiation at the molecular level was just beginning at this stage of development. Cautious interpretation of these data are warranted, since somatic growth and development are both temperature-dependent and conditions might differ between studies. On the other hand, sex differentiation in tadpoles seems to be more influenced by age than by developmental stage [Ogielska and Kotusz, 2004; and pers. observations]. Our results show that for *L. sylvaticus* tadpoles of the same age, Gosner stage did not contribute to the classification of phenotypic sex. This result suggests that the lack of differences between *L. sylvaticus* gene expression levels at different stages (from Gs31 to 42) most likely indicates that gonadal differentiation in *L. sylvaticus* is also more dependent on age (and/or growth rate) rather than on metamorphic stage. Future studies are needed to determine if molecular differentiation occurs before or during morphological differentiation of the gonads in *L. sylvaticus* tadpoles. Furthermore, the developmental factors that drive the timing of gonadal differentiation in frogs remain unclear.

#### *Development of a Useful Tool to Determine Phenotypic Sex in Frogs Using Gonadal cyp19 Gene Expression Levels*

We carried out a discriminant analysis using gene expression levels in *L. sylvaticus* GMCs (Gs31–42) to determine the importance of the variables used to classify both presumptive males and females. This analysis revealed that all 3 genes clearly contribute to discriminate between groups (i.e. phenotypic sex), but that gene expression levels of *cyp19* is a better predictor of phenotypic sex than *foxl2*, which is a better predictor than *cyp17*. In addition, the structure matrix correlations value for *cyp17* revealed that expression of *cyp17* is a poor predictor. We, therefore, conclude that *cyp19* gene expression levels in the *L. syl-*

*vaticus* GMC is the best discriminating variable to assign phenotypic sex in *L. sylvaticus*. Furthermore, statistical analysis also revealed the existence of significant correlations between the analyzed genes, especially at later stages of development, when gonadal differentiation is taking place. These results reinforce the idea that *cyp19* levels are sufficient to discriminate phenotypic sex during *L. sylvaticus* gonadal differentiation.

One of the aims of the present study was to develop a molecular method to identify phenotypic sex not only in species from the Ranidae family, but that also could be suitable for other frog species. Therefore, we decided to validate our method with one of the model species used in our laboratories, *S. tropicalis*, which belongs to the Pipidae family, estimated to have diverged approximately 135 mya from the Ranidae family [Zhang et al., 2005]. Duarte-Guterman and Trudeau [2011] showed clear dimorphic expression of *cyp19* in the adult males and females gonads of *S. tropicalis*, with females showing significant higher expression levels (around 100× higher) than seen in males. In the same study, *cyp19* levels in the GMC of developing tadpoles (from developmental stages NF52–66) were highly variable within each developmental stage, with some individuals presenting undetectable levels. Multiplex real-time RT-PCR reactions were carried out by Duarte-Guterman and Trudeau [2011], wherein the different primers compete to amplify different genes in the same reaction. These conditions could lead to some detection limitations resulting in undetectable levels of *cyp19* in the GMC samples presenting very low expression levels. The authors suggested that the individuals with higher *cyp19* levels could be presumptive females, whereas the ones with either very low or no *cyp19* expression would most likely be presumptive males. In the present study, we have increased the number of *S. tropicalis* individuals analyzed, included earlier stages, and analyzed samples in single PCR reactions. Our results showed that although only one cluster was found in GMC samples at developmental stage NF48, clear dimorphic patterns were observed from NF50 onwards. A study carried out by El Jamil et al. [2008b] reported that the first signs of morphological differentiation of the testes in *S. tropicalis* appear at NF48, whereas signs of ovarian differentiation were first observed at NF51. They also showed that undifferentiated gonads could be found in tadpoles at developmental stages from NF45 to 52. In our study, we have demonstrated that the first dimorphic differences in *cyp19* gene expression can be found as early as NF50, coinciding with the initiation of morphological differentiation of the gonads. Therefore, in *S. tropicalis*, *cyp19* lev-

els are differentially expressed before the first signs of ovarian differentiation at NF51. In support of this observation, sexually dimorphic levels of GMC *cyp19* in *G. rugosa* have been reported 2 weeks earlier than *cyp17* and *foxl2* [Ohtani et al., 2003; Iwade et al., 2008; Maruo et al., 2008; Oshima et al., 2008, 2009; Sakurai et al., 2008; Suda et al., 2011b]. Together, these results support the use of *cyp19* as an early ovarian differentiation marker in amphibians, as has been proposed for fish [Fernandino et al., 2008; Blázquez et al., 2009].

#### Role of *cyp19* Is Conserved across Vertebrate Species

Several studies have determined a critical role for the enzyme aromatase (Cyp19), during sex differentiation in several non-mammalian vertebrate species. For instance, dimorphic gonadal expression of *cyp19* in favor of females has been identified in chicken embryos [Abinawanto et al., 1996]; ovarian development requires that genetically female embryos achieve a threshold level of *cyp19* gene expression leading to aromatase-dependent estrogen production, whereas the absence of sufficient estrogen leads to the differentiation of the genetic female embryonic gonads into testes [Abinawanto et al., 1996]. Sexually dimorphic levels of *cyp19* mRNA have also been found in the gonads of reptiles [Warner, 2011], fish [Paul-Prasanth et al., 2011] and amphibians [Flament et al., 2011]. Many studies have focused on the involvement of Cyp19 in fish sex differentiation. Ovarian sex differentiation has been related to high expression and activity levels of *cyp19a1a* that produce an increase in estrogen synthesis [Devlin and Nagahama, 2002]. In contrast, testicular differentiation in male teleost fish requires the suppression of *cyp19a1a* in gonads [Piferrer et al., 1994; Guiguen et al., 1999; Navarro-Martín et al., 2009], supporting the idea that *cyp19a1a* expression is critical for ovarian differentiation. Studies in the newt *Pleurodeles waltl*, have also demonstrated that *cyp19* downregulation is necessary for testicular development [Kuntz et al., 2003]. Moreover, *cyp19* levels in female-to-male sex reversed developing gonads of *G. rugosa* were decreased after administration of testosterone [Kato et al., 2004]. In the present study, we have shown that the cloned *L. sylvaticus cyp19* nucleotide sequence is highly conserved when compared to other frog and toads species (with identity values ranging from 77 to 96%). In addition, and as it has been found in other frog species [Ohtani et al., 2003; Urbatzka et al., 2007; Maruo et al., 2008; Oshima et al., 2009; Duarte-Guterman and Trudeau, 2011; Duarte-Guterman et al., 2012], dimorphic patterns of *cyp19* gene expression in developing GMCs were found in tadpoles from 2 different

frog families: the Pipidae (*S. tropicalis*) and the Ranidae (*L. sylvaticus*). This evidence supports the idea that the role of *cyp19* during ovarian differentiation is also conserved in amphibians.

#### Conclusion

Evidence from several studies, including the present one, suggests that *cyp19* plays an important role in amphibian ovarian differentiation. We have successfully cloned *L. sylvaticus cyp19*, *foxl2*, and *cyp17* genes. In all cases, sexual dimorphic expression patterns had been observed in *L. sylvaticus* adult gonads and developing tadpole GMCs. In addition, our data support the development of a new method for determining phenotypic sex in frogs at the time of gonadal differentiation by analyzing *cyp19* gene expression in tadpole GMC. The advantage of using this method is that histological studies are not required to determine phenotypic sex, so gonadal samples can be used for further molecular analysis. We propose this method as a tool to study the effects of endocrine disrupting chemicals on gonadal development and sex ratios.

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