Analysis of Sexually Dimorphic Expression of Genes at Early Gonadogenesis of Pejerrey *Odontesthes bonariensis* Using a Heterologous Microarray

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Key Words

Apoptosis · Gonadogenesis · Microarray · Pejerrey

Abstract

The process of morphological development of a differentiated gonad from an undifferentiated primordium is a very important step of gonadogenesis. Studies on sexually dimorphic gene expression are important to increase our understanding of this process and to investigate how environmental factors such as temperature can regulate gonadal development. The aim of this study was to identify putative genes involved in sex differentiation in pejerrey *(Odontesthes bonariensis)* reared at male- and female-producing temperatures (MPT and FPT, respectively) using a microarray heterologous from the medaka *(Oryzias latipes)*, a closely phylogenetic species. Genes related to numerous processes presented higher expression at MPT, including those involved in muscular contraction, metabolic pathways, developmental processes, and reproduction. Genes induced by FPT were classified under the gene ontology terms of response to stimulus, transport and proteolysis. From genes selected for validation, at MPT *ndrg3* expression was observed in the somatic cells, whereas *pen-2* was detected in germ cells in the caudal portion of the gonads, where no apoptotic signals were observed. Finally, *hsp90* was highly expressed in somatic cells of the gonads at the FPT. The results suggest that the interplay of pro-apoptotic and anti-apoptotic genes is important during the masculinization process and for the prevention of sterility following exposure to warm temperatures.

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The expression of phenotypic sex in vertebrates is the result of 2 closely interrelated processes: sex determination and gonadal differentiation. These processes involve a series of biochemical steps, which can be directed by genetic factors (genotypic sex determination, GSD), and/or by environmental factors (environmental sex determination, ESD) [Barske and Capel, 2008]. In both sex determination systems, the trigger is poorly conserved; however, the molecular mechanisms directing the gonadal differentiation appear to be maintained in the evolutionary process [Yao and Capel, 2005; Ferguson-Smith,

2007; Shoemaker et al., 2007; Piferrer and Guiguen, 2008; Wallis et al., 2008].

Once the final fate of an undifferentiated bipotential gonad is defined during the sex determination period, the next step is the manifestation of sex with the development of the testes or the ovaries. Although the molecular pathways underlying gonadogenesis are well characterized in genetically determined organisms, particularly in mammals [Wilhelm et al., 2007; Cool and Capel, 2009; Hiramatsu et al., 2009], this is less well understood in vertebrates whose sex is under the strong influence of environmental factors.

Our experimental model, the pejerrey *(Odontesthes bonariensis)*, is a teleost species with a remarkable and strong temperature-dependent sex determination (TSD). In this species, all-male or all-female populations can be easily obtained by temperature manipulation [Strüssmann et al., 1997]. Recently, cortisol was proposed as a link between stress and testicular differentiation in pejerrey [Hattori et al., 2009]. Nevertheless, the genes that are differentially expressed during gonadogenesis and potentially participate in the cascade of gonadal differentiation process in pejerrey are largely unknown. Some genes have been identified as 'male-specific' such as *dmrt1*, *amh*, *sox9*, *nr5a1*, *cyp11a1*, and *cyp11b* [Fernandino et al., 2008a; Blasco et al., 2010], whereas others, such as *cyp19a1a*, show 'female-specific' expression [Karube et al., 2007; Fernandino et al., 2008a]. In addition, previous studies suggest that gonadal apoptosis is associated with masculinization in pejerrey and therefore is absent in gonads at feminizing conditions [Strüssmann et al., 2008; Hattori et al., 2009]. However, a large-scale analysis of genes potentially involved in gonadogenesis has not been performed in this or any other TSD species.

The development of expressed sequence tagged (EST) analysis in conjunction with microarrays have produced powerful methods for studying large-scale gene expression. In fish, the use of microarrays for transcriptional profiling has grown considerably during the last years [Douglas, 2006]. There is, however, a general lack of these tools for most non-model fish species as the analyses have typically centered on the study of well-characterized model organisms [Garcia-Reyero et al., 2008]. We [Martyniuk et al., 2006; Popesku et al., 2008; Zhang et al., 2009] and others [Bar-Or et al., 2007; von Schalburg et al., 2008; Williams et al., 2008] have shown that cross-species microarray hybridizations are amenable for the study of diverse biological processes in numerous fishes. In this approach, target RNA and microarray probes are from different, but closely related species.

In this context, we used a heterologous microarray to follow gonadal gene expression during gonadogenesis and increase our understanding on how temperature can regulate gonadal development. The aim of this study was to identify genes using an EST microarray generated from cDNAs of larval gonads of medaka (*Oryzias latipes;* Beloniformes) to probe cDNAs extracted from gonads of pejerrey (Atheriniformes) larvae reared at masculinizing and feminizing temperatures during the gonadal differentiation period. This approach is possible because these 2 species are relatively closely related, e.g. both belong to the series Atherinomorpha, the most successful fish group at the surface layer of the ocean and many freshwater habitats [Setiamarga et al., 2008]. We subsequently validated the cross-species microarray hybridization data by cloning and sequencing 3 pejerrey cDNAs and by using both RT-qPCR and in situ hybridization to analyze their expression. In addition, the pattern of in situ hybridization from one of these genes, which has anti-apoptotic actions, was compared to that of apoptosis occurrence in the gonads.

Materials and Methods

Pejerrey Rearing and Tissue Collection

Fertilized pejerrey *(O. bonariensis)* eggs were obtained by natural insemination using gametes from captive-reared broodstock at the aquatic facilities of the Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico de Chascomús, Argentina. The eggs were incubated at 18 ± 0.5°C in flow-through incubators until hatching at about 14 days post-fertilization. After hatching, approximately 4,000 larvae were divided into two 120-liter tanks and gradually acclimated to each rearing temperature, 17°C or 29°C (±0.5°C). These thermal regimes have been shown to be female-producing temperatures (17°C; FPT) and male-producing temperatures (29°C; MPT) [Strüssmann et al., 1996, 1997]. Fish were reared in flowing brackish water (0.2–0.5% NaCl) under a constant photoperiod (16 h light/8 h dark) and were fed live *Artemia nauplii* and powdered food (Shulet Carassius No. 4, Argentina) to satiation twice daily.

At week 7 post-hatching, fish were anesthetized in benzocaine (50 mg/l) and the gonads of 125 larvae were dissected from each temperature treatment; they were then divided in 2 groups and stored in RNA*later*® solution (Sigma-Aldrich) until use. The remaining fish from the same stock were kept at similar conditions until week 11 when they were sampled for histological determination of sex. Briefly, the larvae were anesthetized as above, immersed in Bouin's solution and processed according to standard protocols for the preparation of hematoxylin-eosin stained histological sections following criteria of Ito et al. [2005]. A second experiment was performed following the same conditions described above. In this case, the gonads from 50 larvae were extracted from each temperature and divided in 2 groups for storage in RNA*later* solution.

The fish were treated in accordance with the UFAW Handbook on the Care and Management of Laboratory Animals (http://www.ufaw.org.uk) and following institutional regulations.

Subtracted Medaka Gonadal cDNA Library

To produce a gonadal enriched cDNA array, RNA was extracted from the gonads of medaka *(O. latipes)* larvae at various developmental stages (5, 12 and 21 days after hatching) using RNeasy (Qiagen). The cDNAs were synthesized using Super SMART™ PCR cDNA Synthesis Kit (Clontech) followed by a subtraction using the Clontech PCR-Select™ cDNA Subtraction Kit according to the manufacturer's protocol (Clontech). Female cDNA was subtracted from male cDNA and vice-versa to obtain sex-specific sets of cDNAs. The subtracted PCR fragments were cloned into pCR4-TOPO vector using TOPO TA Cloning® for Sequencing (Invitrogen). A total of 10,368 clones were sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and further cluster analyses (CAP3) grouped these clones into 2,200 clusters of independent sequences. A total of 3,840 bacterial clones (males: 1,881 clones; females: 1,959 clones) were selected from these clusters by allowing overlap for some of them (more than 1 clone was chosen from some clusters). *Escherichia coli* containing these clones were inoculated into 70 μl LB with 7.5% glycerol and grown at 37°C overnight in 384-well plates. Colony PCR was performed by transferring the overnight-grown cultures into 20 μl PCR mix (AmpliTaq; M13F and M13R primers). The PCR products were purified using FiltrEX™ 384-well glass fiber filter plates (Corning No. 3533). Briefly, 75 μl of KI was added to each well of the 384-well filter plate and the PCR products were transferred to these plates. This step was followed by 1 min incubation at room temperature. The filter plates were drained under vacuum and washed with 200 μl of 80% EtOH. The plates were then centrifuged at 3,300 rpm for 3 min and dried by incubating at 37°C for 10 min. 40 μl of distilled water was added to the filter plate and spun at 3,300 rpm for 3 min for the elution of the DNA into another 384-well plate. The DNA was dried up and 20 μl of 1× Cross Linking Solution (Amersham) was added to the 384-well plates.

A total number of 3,840 clones were spotted by using Gen III microarray spotter (Amersham Bioscience) on Matsunami slides (No. SI53806; Matsunami Glass, Osaka, Japan) in duplicate. Clones from 2 vertical lanes of each 384-well plate (total 10 plates) were arrayed into 12 rows of 12 blocks (each block 10 × 32 format) on the slide. Each block was duplicated and resulted in 7,680 individual spots per slide.

Microarray Hybridization and Scanning

The methods used were similar to those validated previously by some of the authors [Martyniuk et al., 2006; Zhang et al., 2009]. For microarray hybridizations, total RNA was extracted using RNeasy Plus Micro Kit (Qiagen) following the manufacturer's protocol. The total RNA was resuspended in 30 μl of RNase-free water, quantified using Nano-probe (Spectron), and the integrity was analyzed using the Agilent RNA 6000 (Agilent Technologies). The RNA integrity numbers for the samples ranged from 2.05–2.14. The Genisphere Array 900MPX™ cDNA microarray labeling kit (Genisphere), including the recommended buffers for each hybridization step, was used for all microarray hybridizations. The complete hybridization protocol is found at (http://www.genisphere.com/pdf/array900mpx_protocol_v06-22-04.pdf). A

total of 4 microarrays were used: cDNAs from 2 male biological replicates were hybridized against cDNAs from 2 female biological replicates. For each group 1.4 μg of total RNA was used for the first strand cDNA synthesis.

Microarrays were scanned at full speed 10 μm resolution using the ScanArray 5000 XL system (Packard Biosciences/PerkinElmer) using both red and blue lasers. Images were obtained with ScanArray Express software using automatic calibration sensitivity varying PMT gain (PMT starting at 65% for Cy5 and 70% for Cy3) with fixed laser power at 80% and the target intensity set for 90%. Microarray images were opened using QuantArray (Packard Biosciences/PerkinElmer) and raw signal intensity values obtained for duplicate spots of genes.

Data Normalization and Identification of Differentially Expressed Genes

The array quality filter test (AQF) [Sauer et al., 2005] was first applied to check the raw data. The AQF values of all slides were less than the threshold of 0.5. Spots that had been manually flagged due to poor hybridization and spots in which the estimated fluorescence intensity was below or equal to the estimated background signal intensity in either channel were removed prior to further analysis. Normalizations were performed using Lowess [Yang et al., 2002]. Significance analysis of microarray (SAM) method was performed to assess the significance of differential expression of the genes [Tusher et al., 2001]. This technique computes a statistic for each gene and measures the strength of the relationship between gene expression and the response variable. Repeated permutations of the data determine whether the expression of a specific gene was significantly different between test groups. The criterion for significance was q < 5% which is based on the false detection rate (FDR). FDR is the percentage of significant genes identified by chance while the q value is the minimum false discovery rate at which the gene is significant. The q value is an adjusted p value, and it is designed for the FDR analysis using SAM. Genes identified as being differentially regulated were further analyzed using Blast2GO [Conesa et al., 2005].

RT-qPCR

Three genes identified by the microarray analysis as being regulated by temperature were selected for validation using RT-qPCR. Firstly, consensus primers were designed based on sequences from *Oreochromis mossambicus* (GenBank: AY522633) for *pen-2* (presenilin enhancer 2), *Danio rerio* (NM_199797) for *ndrg3* (N-myc downstream regulated 3) and *Paralichthys olivaceus* (DQ662233) for *hsp90* (heat shock protein 90) and their respective medaka ESTs (table 1). Amplification and cloning procedures have been reported previously [Fernandino et al., 2008a, b] with the particular annealing temperatures of 65, 50 and 60°C for *pen-2*, *ndrg3* and *hsp90*, respectively. The sequences cloned from pejerrey were submitted to the GenBank (accession numbers: *pen-2*, GQ381268; *ndrg3*, GQ381269; *hsp90*, GQ381270).

Primer sets for RT-qPCR were then designed using Primer Express Software (Applied Biosystems) with an optimal annealing temperature of 60 ± 2°C (table 1). Primers were tested using cDNA obtained from pejerrey gonads and amplicons were cloned and sequenced to confirm their specificity. The RT-qPCR analyses of gene expression were then carried out using the cDNA previously obtained in both experiments from gonads of pejerrey larvae. RT-qPCRs were assayed on an MX4000® Multiplex Quanti-

Table 1. Oligonucleotide primers used in the study

Oligo name	Forward primer	Reverse primer	Amplicon bp
Cloning procedures			
pen-2	aacctggagcgrvtgcccaatg	agnggtatggtraaggasagg	289
NDRG3	tgttcagctgacygagatc	gtcvatccagcctttagcac	496
HSP90	ccaagctcgactcgggmaarg	gtagaagcccacgccgaactg	215
M13	actggccgtcgttttac	ggaaacagctatgaccatg	
RT-qPCR procedures			
RQpen-2	ttgcctttctgcctttcctgtg	cccagtgctgatcgcttgac	110
RQNDRG3	tccccagcggggtatcgtt	catcacggagggcagcat	61
RQHSP90	gggaaaggaactgaaaatcgaa	cccgtgtcgaccagagtga	69
RQβ-actin	gctgtccctgtacgcctctgg	gctcggctgtggtggtgaagc	200
In situ procedures			
ishpen-2	gtaatacgactcactatagggcaacctggaacgacttcccaatg	gcaattaaccctcactaaaggggacaggtaatctcccacttccc	332
ishNDRG3	gtaatacgactcactatagggctgttcagctgactgagatc	gcaattaaccctcactaaagggtccatccagcctttagcac	539
ishHSP90	gtaatacgactcactatagggcccaagctcgactcgggaaagg	gcaattaaccctcactaaaggggtagaagcccacgccgaactg	258

All primer sequences are shown as 5′ to 3′, left to right.

tative PCR system (Stratagene). The RT-qPCR were performed in 15-µl reaction volumes using the FastStart Universal SYBR Green Master (Roche), 25 ng first strand cDNA, and 5 pmol of each primer. The thermal-cycling conditions consisted of an initial step of 10 s at 95°C followed by 40 cycles of denaturation at 95°C for 10 s and annealing and elongation at 60°C for 30 s, followed by a dissociation curve. Analysis and quantification using the standard curve method were carried out with the MX4000 Software Package (Stratagene). The Ct was automatically calculated and displayed. Expression values for the target genes were normalized using those for *β-actin* as a reference gene.

Student's t test was used to determine if differences in gene expression levels between MPT and FPT were statistically significant. Data are presented as individual points. The analysis was performed using both experiments together. Analyses were done using GraphPad Software Version 4.00 with statistical significance considered at p < 0.05.

In situ Hybridization

Larvae of 7 weeks, all from both temperatures, were fixed in 4% paraformaldehyde (PFA) and subsequently processed as for other histological preparations. In situ hybridizations were performed using probes based on the primer amplicons listed in table 1. The different probes were labeled with a mix of rNTPs including Biotin-16-uridine-5′-triphosphate (Roche Applied Science) and UTPs, ATPs, CTPs, and GTPs (Invitrogen). Briefly, 6-µm thick sections were deparaffinized, dehydrated and pretreated with proteinase K (5 µg/ml) for 7.5 min at room temperature. The reaction was stopped by washing in glycine-PBS buffer (2 mg/ml) for 10 min and the sections were then dipped in 100 mM triethylamine containing 0.25% anhydrous acetic acid for 10 min. For hybridization, sections were covered with 150 µl of Biotin-labeled sense or antisense RNA probe solution (1 mg/ml) and incubated overnight in a moist chamber at 60°C. After hybridiza-

tion, sections were washed in 50% formamide/2× SSC and 0.1× SSC at 60°C, and incubated between 1 and 3 h with Streptavidin-alkaline phosphatase-conjugate (1/2000 dilution; Roche Applied Science). These steps and final detection with NBT/BCIP followed the manufacturer's (Roche Applied Science) protocols.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay for Gonadal Apoptosis

The TUNEL assay was performed according to Hattori et al. [2009]. Briefly, samples were fixed overnight in 4% paraformaldehyde, embedded in Paraplast Plus and sectioned transversally at a thickness of 5 µm. Slides were incubated with 1 µg/ml Proteinase K (Invitrogen) and preincubated with terminal deoxynucleotidyl transferase (TdT) buffer for 10 min at 37°C. Laddered DNA labeling was carried out with 1 mM fluorescein-dUTP (Perkin-Elmer) and 40 units/ml TDT enzymes (Roche) in TdT buffer for 80 min at 37°C. Slides were counterstained with DAPI and observed under a fluorescence microscope (Eclipse E600; Nikon). Images were captured and digitalized with a CCD camera (Penguin 600CL).

Results

Pejerrey Monosex Groups Obtained by Thermal Treatment

Sex ratios in the pejerrey can be easily manipulated with temperature during a critical time frame early in development [Strüssmann et al., 1997]. To confirm if thermal treatments were effective in directing sexual development, gonadal sex was assessed by histology at the end of the experimental period (11 weeks after hatching).
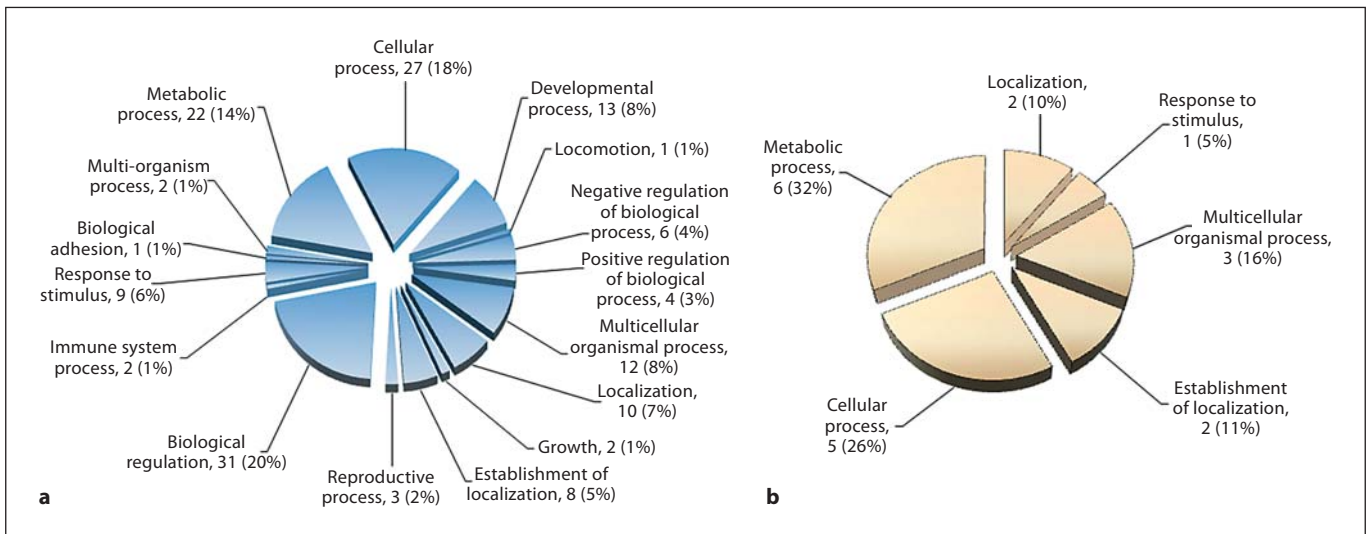
Fig. 1. Gene Ontology (GO) analysis of overexpressed genes in gonad of larvae during the gonadal sex differentiation period. Multilevel GO term categories of biological process of genes differentially expressed in males (**a**) or females (**b**) as determined by Blast2GO analysis of microarray results. The number and percentage of sequences falling into each GO category are indicated.

As expected from previous reports [Strüssmann et al., 1996, 1997], 100% females or 100% males were obtained at the FPT (17°C) and MPT (29°C), respectively.

Microarray Analysis and Annotation of Transcripts Using Gene Ontology (GO)

A medaka microarray platform was used to investigate the gene-expression profile in sexually differentiating gonads of pejerrey. It must be noted that the following results were obtained with larvae reared at different temperatures (e.g. low and high temperatures for production of females and males, respectively).

A higher number of genes were induced in the differentiating testes compared to ovaries, but most of the represented transcripts did not present significant changes in their expression pattern between the MPT and FPT. In males, 6.56% of clones were overexpressed, where as only 0.94% were overexpressed in females from a total of 3,840 clones analyzed. The sequences from 252 spots that were overexpressed in male gonads were linked to a total of 94 known genes through searches (BlastX) in the GenBank database. Of the total spots overexpressed in male gonad, 69 of these spots could not be associated with any known gene and can be considered to code for hypothetical proteins. On the other hand, 36 spots were significantly overexpressed in females and led to the identification of 16 known genes in the GenBank database; however, 16 oth-

er sequences could not be identified. The remaining identified spots were duplicate cDNAs (89 in the MPT and 4 in the FPT).

The identified genes were then grouped according to the GO categorization of Biological Process using Blast-2GO [Conesa et al., 2005] and categorized to multilevel GO terms for either males or females (figure 1a, b, respectively). Genes in numerous processes were induced in males, including those related to muscular contraction, metabolic pathways, developmental processes, and reproduction. In contrast, gene ontology analysis for females indicated that genes in the categories response to stimulus, transport and proteolysis were differentially expressed. A list of the overexpressed genes with an associated GO term is shown in table 2a, b and the complete list of genes is shown as supplementary material (online suppl. table 1, www.karger.com/doi/10.1159/000324423).

Validation of DNA Microarray Data by RT-qPCR

Some of the differentially expressed genes were then selected for validation of the microarray based on their potential interest for future research on pejerrey gonadal development. Two of the selected genes were overexpressed in males: *ndrg3* and *pen-2*. The first has been identified as a gene regulated by androgens and may be related to spermatogenesis in mammals [Zhao et al., 2001; Wang et al., 2009], while *pen-2* has anti-apoptotic

Table 2a. Overexpressed genes in pejerrey larvae gametogenesis reared at male-producing temperature (MPT)

Gene name	Symbol	Source	Fold change	Biological process
Biological adhesion				
Actinin alpha 3b	*Actn3b*	XY	1.4	cell-substrate adhesion
Biological regulation				
Calsequestrin 1 (fast esqueletal muscle)	*Casq1*	XX	1.5	regulation of muscle contraction
Dipeptidyl peptidase IV	*Dpp4*	XY	1.8	regulation of cell-cell adhesion
Ferritin heavy chain	*FTH*	XX	1.3	negative regulation of cell proliferation, cellular iron ion homeostasis
Natriuretic peptide receptor type-C	*npr-c*	XY	1.3	negative regulation of adenylate cyclase activity, phosphoinositide-mediated signaling
Presenilin enhancer 2	*Pen-2*	XY	1.4	negative regulation of apoptosis, positive regulation of catalytic activity
Cellular process				
Alpha actinin 3	*Actn3*	XY	1.3	regulation of apoptosis, cell adhesion, focal adhesion formation
Alpha tubulin-2	*atb2*	XX	1.4	microtubule cytoskeleton organization, establishment and/or maintenance of cell polarity
Alpha-tubulin at 85e	*alphaTub85E*	XY	1.4	mitotic cell cycle spindle assembly checkpoint, microtubule-based movement
Kininogen 1	*kng1*	XY	1.4	negative regulation of cell adhesion, elevation of cytosolic calcium ion concentration, apoptosis
Purinergic receptor P2X, ligand-gated ion channel	*P2rx*	XY	1.3	activation of caspase activity, insemination, regulation of calcium ion transport
RAS oncogen family	*Rab-11b*	XY	2.0	cell motion, small GTPase-mediated signal transduction, regulation of transcription
Synaptonemal complex central 2 element protein 2	*Syce2*	XY	1.5	cell division, meiotic prophase I, synaptonemal complex assembly
Ubiquinol-cytochrome c reductase Core 2	*Uqcrc2*	XX	1.6	mitochondrial electron transport, ubiquinol to cytochrome c
Developmental process				
Desmin	*Desm*	XX	1.3	muscle organ development
P21 (CDKN1A)-activated kinase 2b	*pak2ab*	XY	1.6	blood vessels development
Prosaposin	*Psap*	XX	1.6	epithelial cell differentiation involved in prostate gland development
RAS-related protein 1b	*rap1b*	XY	1.6	cell proliferation
Establishment of localization				
ATPase, Ca++ transporting	*Atp2a1*	XX	1.8	calcium ion transport, cellular homeostasis
Transferrin	*Tsf*	XY	1.5	iron ion transport
Growth				
Opioid growth factor receptor	*OGFR*	XX	1.5	regulation of cell growth
Immune system process				
Complement component C7-2	*C7*	XY	2.4	complement activation, positive regulation of biological process
Complement factor B and C2	*bf/c2*	XY	1.7	activation of plasma proteins in acute inflammatory response
Metabolic process				
B cell RAG-associated protein	*Galnac4s-6st*	XY	1.3	hexose biosynthesis
Carboxypeptidase B1	*Cpb1*	XY	1.5	proteolysis
Creatine kinase, M2-Ck, muscle, muscle isoform 1 and 2	*Ckm*	XX	1.4–1.7	amino acid metabolism, phosphocreatine biosynthesis

Table 2a (continued)

Gene name	Symbol	Source	Fold change	Biological process
Flavoprotein oxidoreductase	Mical2	XY	1.5	cellular aromatic compound metabolic process
Fructose-1,6-bisphosphatase 1	Fbp	XY	1.3	fructose metabolism, gluconeogenesis
Fructose-bisphosphate aldolase A and C	ALDO	XY	1.3–1.5	fructose metabolism, glycolysis
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	XY	1.5	glycolysis, apoptosis
MAP kinase-interacting serine/threonine kinase 1	Mknk1	XX	1.7	protein amino acid phosphorylation
Pro-pol-dUTPase polyprotein	–	XY	2.0	proteolysis, DNA integration, RNA-dependent DNA replication
Short-chain dehydrogenase reductase	DHRS	XY	1.4	oxidation reduction
Urate oxidase	Uox	XY	1.5	purine base catabolic process, oxidation-reduction
Multicellular organismal process				
Actinin alpha 3	Actn3	XY	1.4	muscle contraction
COP9 constitutive photomorphogenic homolog subunit 6	Cops6	XX	1.5	interspecies interaction between organisms
Myosin heavy chain A1, 3, and 4	Mhc	XX	1.5–2.7	muscle contraction, positive regulation of ATPase activity
Myosin light chain 2 and 3	Mlc	XY	1.8–2.5	muscle contraction, positive regulation of ATPase activity
Tropomyosin 1, alpha	Tpm1	XX	1.3	muscle contraction, positive regulation of ATPase activity
Troponin I, fast skeletal	Tnni	XX	1.6	skeletal muscle contraction, regulation of ATPase activity
Smooth muscle alpha-actin	Acta2	XY	1.4	muscle contraction
Reproduction				
Chemokine orphan receptor 7	Cxcr7	XY	1.4	germ cell migration, angiogenesis
N-myc downstream regulated 3	Ndrg3	XY	1.4	spermatogenesis, cell differentiation
Response to stimulus				
Coagulation factor B polypeptide	F13A1	XY	1.5	response to stress, blood coagulation
Complement component C3	C3	XY	1.5	response to chemical stimulus
Fructose-bisphosphate aldolase A	Aldoa	XY	1.6	response to heat
Heparin cofactor II	SERPIND1	XY	1.4	response to external stimulus
Uncoupling protein 4	Ucp4	XY	1.4	response to chemical stimulus, mitochondrial transport
Unknown				
Coiled-coil domain containing 18	CCDC18	XY	1.3	–
Fast white muscle troponin T embryonic/embryonic isoform	–	XX	1.4	–
Fetuin B	Fetub	XY	1.3	biological process
HLA-B-associated transcript 4	Bat-4	XX	1.4	–
Parvalbumin 1	Pvalb1	XY	2.2	–
rRNA intron-encoded homing endonuclease	–	XY	1.3	–
Seminal plasma glycoprotein 120	PP120S	XX	2.5	–
Serine proteinase inhibitor CP9	Serpina1	XY	1.6	–
Transmembrane protein 34	Tmem34	XY	1.6	–
Udp-glucuronosyltransferase	Ugt	XY	1.4	–
Warm temperature acclimatation-related 65-kDa protein	Wap65	XY	2.0	–

List of genes identified by microarray analysis having different statistically significant expression between the gonads of larvae reared at MPT and FPT (q < 5%). The source of the different sequences is indicated by XX for ovary or XY for testis. The genes were grouped according to the GO categorization of Biological Process using Blast2GO.

Table 2b. Overexpressed genes in pejerrey larvae gametogenesis reared at FPT

Gene name	Symbol	Source	Fold change	Biological process
Biological regulation				
Complement factor H precursor	*CFH*	XX	1.6	positive regulation of immune response, regulation of complement activation
Type I collagen alpha 2	*Col1a2*	XY	1.4	transforming growth factor beta receptor signaling pathway, Rho protein signal transduction
Cellular process				
Heat shock protein 90 alpha	*Hsp90a*	XX	1.5	protein folding
S100-A1	*S100A1*	XX	2.0	intracellular signaling cascade, negative regulation of transcription from RNA polymerase II promoter
Thymosin beta	*Tmsb*	XY	1.4	cytoskeleton organization
60S ribosomal protein L7	*RPL7*	XX	1.4	rRNA processing, translational elongation
Localization				
Solute carrier family 38, member 3	*Slc38a3*	XY	2.4	amino acid transport
Metabolic process				
Alpha amylase 2a	*Amy2a*	XX	1.4	carbohydrate metabolic process
Carboxypeptidase A2	*Cbpa2*	XY	2.0	proteolysis
Chymotrypsin b	*Ctrb1*	XY	2.9	proteolysis
Trypsinogen	*Tryp*	XY	1.9	proteolysis
Unknown				
Cytoplasmic actin OlCA1	–	XX	1.5	–
Extracellular matrix protein 1 precursor	–	XY	2.6	–
Gonadal soma-derived growth factor	*Gsdf*	XY	2.8	–
Zinc finger protein 706	*Zn706*	XX	2.1	–

List of genes identified by microarray analysis having different statistically significant expression between the gonads of larvae reared at MPT and FPT (q < 5%). The source of the different sequences is indicated by XX for ovary or XY for testis. The genes were grouped according to the GO categorization of Biological Process using Blast2GO.

actions in fish [Campbell et al., 2006]. *ndrg3* and *pen-2* showed a 3.3- and 1.4- and, 1.7- and 1.4-fold higher expressions in males compared to females by the RT-qPCR and microarray analyses, respectively (fig. 2).

We also confirmed the increased expression of *hsp90* in females. Thus, the expression analyses of this gene by RT-qPCR and microarray showed a 3.4- and 1.5-fold increase, respectively, in gonads of larvae reared at the FPT compared to the MPT (fig. 2).

Localization of pen-2, ndrg3, and hsp90 Expression and Apoptosis in Larval Gonads

The expression of the 3 selected genes was also analyzed in representative histological sections of larvae during the gonadal differentiation period at the MPT and FPT by in situ hybridization. The higher expression of *pen-2* and *ndrg3* in gonads of larvae reared at the MPT was confirmed using in situ hybridization on the gonads of 7-week-old larvae (fig. 3a, 4b). In contrast, no signal was detected in the gonads of larvae reared at the FPT (fig. 3b for *ndrg3*; *pen-2* data not shown). *pen-2* was expressed in germ cells (fig. 4b), whereas *ndrg3* was observed in somatic cells (fig. 3a). The expression of *pen-2* at the MPT showed a clear regionalization along the gonad. Thus, it was found to be expressed only in the caudal/posterior region of the gonad (fig. 4b), whereas no TUNEL signals for apoptosis could be detected (fig. 4f). The third mRNA that we validated, *hsp90*, presented clearly higher expression at the FPT compared to the MPT. The signals were observed in the somatic cells of the gonads, surrounding the germ cells (fig. 3e).

Fig. 2. Relative quantification of ndrg3, hsp90 and pen-2 transcript abundance in the gonads of larvae reared at FPT (17°C) and MPT (29°C) for 7 weeks. Open and filled circles represent the first and the second experiment, respectively. t test analysis showed significant differences between temperatures; ndrg3: t value 8.04, hsp90: t value 3.71 and pen-2: t value 3.61.

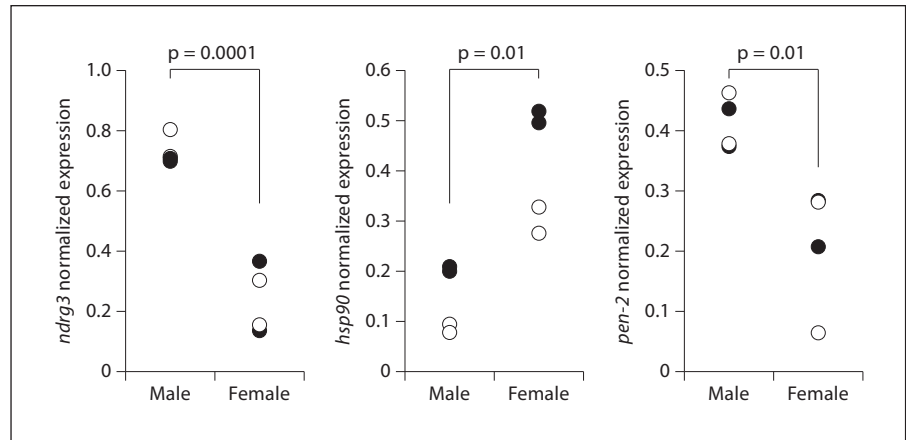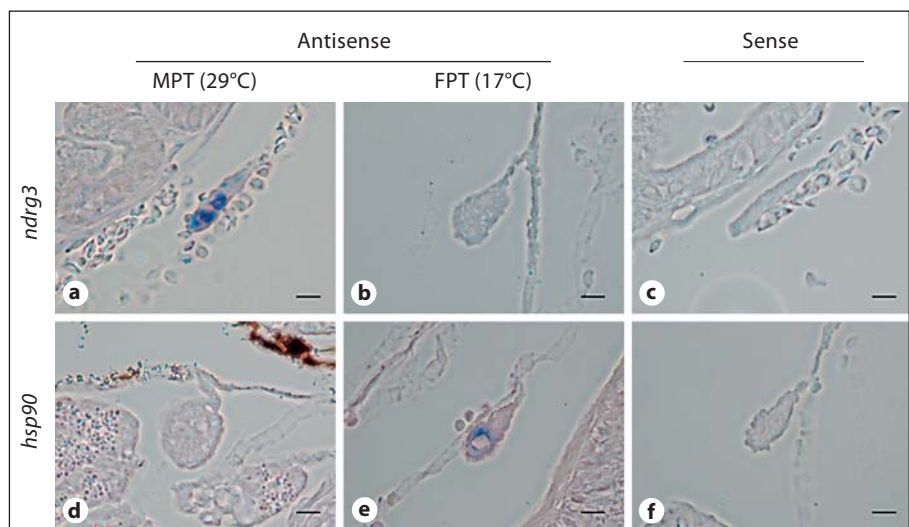**Fig. 3.** Localization of *ndrg3* and *hsp90* expression by in situ hybridization in larvae reared at MPT (29°C; **a**, **c** and **d**) and FPT (17°C; **b**, **e** and **f**) for 7 weeks. Scale bars = 10 μm.

The TUNEL assay showed the occurrence of apoptosis only in the anterior sections of the right lobe of the gonadal primordium in larva reared from the MPT (fig. 4d, f) and little, if any, in the left lobe or the posterior sections of the same gonad. It is important to note that although possibly localized in germ cells, the apoptotic signals were also found in somatic cells (fig. 4d).

Discussion

Although gonadogenesis is a fundamental process, there is little information on the molecular steps required to develop a functional gonad or on the physiological mechanisms following the effects of temperature on gene expression in TSD animals. The pejerrey is an excellent model to study this process because single-sex populations can be obtained through simple temperature manipulation during a critical period of temperature sensitivity early in life [Strüssmann et al., 1997]. Furthermore, the pejerrey is considered by some authors as one of the few fish species with true TSD [Ospina-Álvarez and Piferrer, 2008].

The use of heterologous microarray analysis constitutes a novel tool for nonclassical model species, particularly when the phylogenetic distance between the reference and target species is close. In the present study, both species, medaka (reference) and pejerrey (target), belong to the Atherinomorpha series, and, although the temporal divergence between these species is considered to be relatively high, recent studies using molecular biology tools place Beloniformes and Atheriniformes in a closer
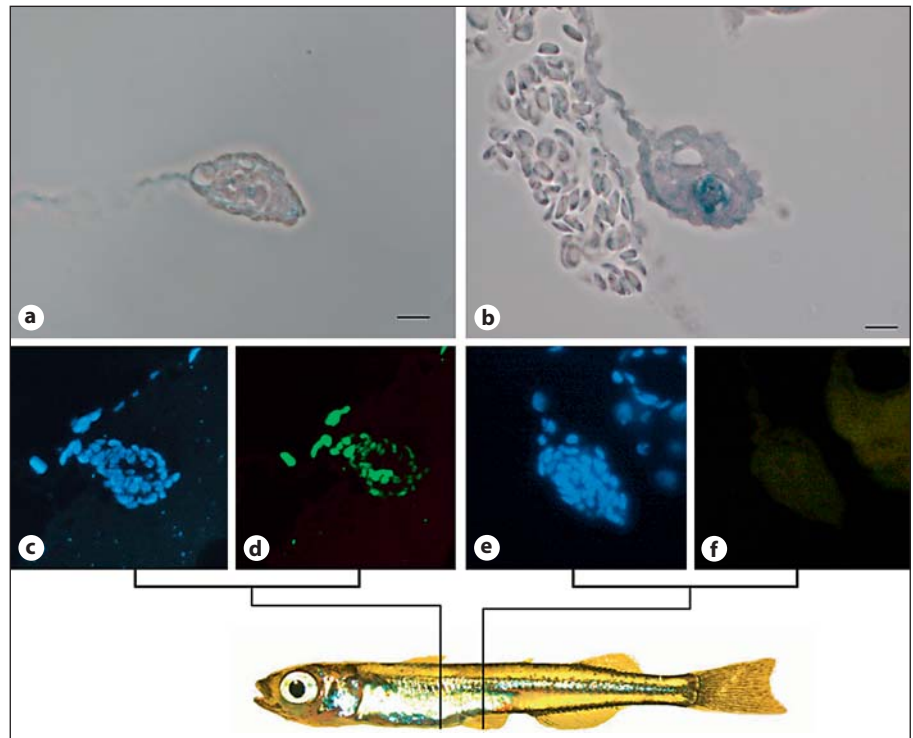
Fig. 4. Gradient of *pen-2* and apoptosis signals in the gonads of larvae reared at MPT (29°C). *pen-2* (in situ hybridization) expression in the anterior (**a**) and posterior (**b**) sections of the gonad. Gonadal apoptosis (TUNEL assay) in the anterior (**d**) and posterior (**f**) sections of the gonad. Panels **c** and **e** show DAPI nuclear staining for visualization of cells. Scale bars = 10 μm.

position than classic phylogeny based on morphological features [Chen et al., 2003; Nelson, 2006; Li et al., 2008].

In the present study, relatively few genes were found to be differentially expressed between sexes during the gonadal differentiation period. The number of overexpressed genes was clearly higher in the gonads from fish obtained from the MPT than from FPT. This difference could be related to the increased metabolism and/or to the homeostatic recovery after high cortisol levels associated with high-rearing water temperatures in this species [Hattori et al., 2009]. That metabolism was accelerated at the MPT can be inferred from the fact that in pejerrey the first morphological signs of gonadal differentiation are usually detected 1–2 weeks earlier in larvae kept at high temperatures than in those reared at low temperatures [Strüssmann et al., 1997; Ito et al., 2005].

The ontological analysis of selected genes in the gonads of males showed that they could be grouped according to different biological processes. Within these processes involved in testicular development and differentiation, it is important to highlight those related to spermatogenesis and efferent duct formation. For example, *ndrg3* was found to be expressed mainly in the testes and prostate of mice and was proposed to play a role in spermatogenesis [Zhao et al., 2001]. In pejerrey, *ndrg3* was

located in somatic cells of presumptive testes, suggesting a similar role in fish. The overexpression of genes involved, either with the muscular structure itself (myosin chain, actin, muscular creatine kinase, and tropomyosin) [Huxley, 1974; Newsholme et al., 1978] or the muscular contraction process (Ca^{2+} transporting sarcoplasmatic reticulum Ca^{2+} ATPase1) [Inesi et al., 2008], most likely relates to the development and differentiation of the efferent ducts. In teleosts, these ducts are lined with contractile cells which are involved in the transport of the seminal fluids [Walter et al., 2005]. Another important gene proposed to be related to the differentiation of the efferent ducts in teleosts is *amh* (anti-müllerian hormone or müllerian-inhibiting substance, *mis*). The expression of *amh* during this period is restricted to the somatic cells in the medullar area of the gonadal primordia, where the efferent ducts develop [Miura et al., 2002; Yoshinaga et al., 2004; Baron et al., 2005; Rodríguez-Marí et al., 2005; Fernandino et al., 2008b].

Two genes identified by the microarray analysis at the MPT are related to temperature adaptation: *wap65* (warm temperature acclimation-related 65 kDa protein) and fructose-bisphosphate aldolase A [Tacchini et al., 1999]. The sequence of wap65 is similar to the mammalian hemopexin gene, and although its functions are unknown,

wap65 was found to be expressed in various tissues of several teleost species when the fish were exposed to high temperatures [Kikuchi et al., 1995; Kinoshita and Ozato, 1995; Choi et al., 2008; Clark and Burns, 2008]. It is important to note that although *wap65* mRNA levels have been shown to increase with temperature in several teleosts, this increase does not occur in a cortisol-dependent manner, for example, when fish are subjected to other stressors such as changes in salinity [Choi et al., 2008]. Thus, despite the high levels of cortisol in pejerrey larvae during sex determination and gonadal differentiation [Hattori et al., 2009], *wap65* transcript levels are probably not affected by cortisol, but by temperature. For these reasons, *wap65* could be a good marker for high-temperature exposure in larvae and adults.

It is known that at least in some teleost larvae, a low number of germ cells is important for the masculinization of the undifferentiated gonad [Kurokawa et al., 2007] and that their depletion is controlled by apoptosis under normal and masculinizing conditions [Uchida et al., 2002, 2004]. In pejerrey, the gonadal primordia undergo apoptosis in presumptive males reared at an intermediate, 'neutral-sex' temperature and in all larvae at the MPT [Strüssmann et al., 2008], but not during the formation of the ovaries suggesting a role for apoptosis in testicular differentiation. The present results are in agreement with previous work whereby apoptotic signals were found to be restricted to the anterior sections of the right gonads, which is most probably related to the antero-posterior and left-to-right gradient of histological sex differentiation in pejerrey [Strüssmann and Ito, 2005; Strüssmann et al., 2008]. However, it is important to note that long exposures to high temperatures can cause degeneration of somatic and germ cells in pejerrey larvae and juveniles, resulting in a clear decrease in the number of germ cells up to complete sterilization [Ito et al., 2008; Strüssmann et al., 2008].

The expression of 2 anti-apoptotic genes *(gapdh* and *pen-2)* was increased in pejerrey raised at MPT relative to those raised at FPT. Particularly, *pen-2* has been shown to play an important and specific role in the survival of cells, protecting them from apoptosis [Silva et al., 2008]. Knock-down of this gene resulted in strong induction of the p53-dependent apoptosis cascade in whole organs and tissues of zebrafish embryos [Campbell et al., 2006]. In mammals, it has also been established that germ cells experience apoptosis via the p53 cascade, and interestingly, this process is inducible by exposure to high temperatures [Ohta et al., 2003]. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) has also been shown to

be necessary in caspase-independent cell death inhibition in HeLa cells [Colell et al., 2007]. Thus, the presence of these anti-apoptotic genes at the MPT could be important for the protection of germ cells in critical portions of the gonad. The pro-apoptotic genes *p2xr1* [North, 2002] and kininogen [Guo et al., 2003] were also found to be overexpressed in male pejerrey gonads. Further studies are needed to determine the localization and mechanism of programmed cell death during teleost gonadal masculinization.

At the FPT, a smaller number of genes showed enhanced expression. Higher expression of carboxypeptidase A2, chymotrypsin B and pretrypsinogen at the FPT indicates effects on gonadal protein metabolism. Another gene with high expression at FPT was *hsp90*. Although traditionally associated with the stress response, it is also involved in normal homoeostatic control mechanisms [Buchner, 1999; Pearl et al., 2008]. HSP90 interacts with numerous proteins, for example, protein kinases, transcription factors, etc., facilitating their stabilization and activation or directing them for proteasomal degradation [Pearl et al., 2008]. HSP90 also interacts with nuclear steroid hormone receptors, thus maintaining the receptors in an inactive state [Beato and Klug, 2000; Smith and Toft, 2008]. For estrogen receptors, HSP90 has the dual role of maintaining receptor inactivity in the absence of estradiol as well as ensuring an efficient hormonal response in mammals [Lee et al., 2002]. In goldfish, hypothalamic *hsp90* was upregulated after aromatase inhibition with fadrozole, implying an association with estrogen signaling [Zhang et al., 2009]. Therefore, the high expression of *hsp90* in pejerrey may be involved in estrogen-dependent processes associated with ovarian differentiation at the FPT.

In summary, the use of cross-species microarray hybridization represents a powerful tool in the identification of potential genes involved in gonadogenesis. The present work is the first to screen thousands of transcripts to identify genes with sexually dimorphic expression in the gonads of the pejerrey, a fish with well-established TSD. Some of the genes found to be overexpressed in the gonads of larvae reared at the high temperature may be directly responding to temperature and are potentially involved in the development of the efferent ducts. Nevertheless, further studies are necessary to determine if this gene-expression pattern is due to sex or a consequence of a general effect of temperature. In addition, some apoptotic genes were identified, supporting the hypothesis that an apoptotic mechanism is necessary for masculinization of the gonadal primordium in this species. The

expression and specific localization of the anti-apoptotic gene *pen-2* suggests that this gene is a good candidate to protect some germ cells from heat-induced degeneration. Many gonadal transcripts regulated by temperature could not be identified and, as yet, have no known functions. This implies that numerous other genes are involved in the process of vertebrate TSD, and that future studies may reveal their novel roles.

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