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Pex11α in brown trout (*Salmo trutta* f. *fario*): Expression dynamics during the reproductive cycle reveals sex-specific seasonal patterns

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ABSTRACT

A negative correlation between female gonadal maturation kinetics and size variations of hepatic peroxisomes was earlier documented in brown trout, as a probable impact of serum estrogen changes during the reproductive cycle. Herein, we investigated whether the organelle volume/surface dynamics seen in female brown trout liver peroxisomes – without numerical changes within each hepatocyte – is followed by variations in the expression of the membrane peroxisome protein Pex11α gene. For comparison, we also studied males. We find in females a seasonal variation with the highest Pex11α expression in February, which was statistically different from all other tested periods. Overall, the expression of *PEX11α* had over a fivefold decrease from February to September. This period coincides with the reproductive transition between the earlier post-spawning gonadal remodeling and preparatory staging and the pre-spawning period. Males did not show changes. Our approach allowed the first characterization of a peroxin gene in a teleost, the *Pex11α*, while offering a correlation scenario were, as we hypothesized, the peroxisomal size kinetics is paralleled by membrane-related gene alterations (measured herein as proxy of *Pex11α* gene expression). Our data support and expand previous results on the regulation, function and morphology of peroxisome dynamics in brown trout, with a broader interest.

1. Introduction

Peroxisomes are catalase rich organelles present in nearly all eukaryotic cells. They are quite dynamic and versatile, have a complex biogenesis, and play several vital roles, such as the β-oxidation of very long chain fatty acids (Reddy and Hashimoto, 2001; Nuttall et al., 2011). This ample functional task is underscored by their involvement in a number of human serious/fatal diseases related with peroxisomal enzyme defects or peroxisome biogenesis (Schrader and Fahimi, 2008; Ribeiro et al., 2012). In this vein, peroxisome proliferation is a striking example of the cellular response to environmental stimuli. Peroxisome proliferators (PPs) cover a heterogeneous group of compounds (including hypolipidemic drugs) known to cause massive peroxisome proliferation and liver carcinogenesis, namely proved from long ago in rodents (Reddy et al., 1980). Fish have also shown to develop peroxisome proliferation — albeit generally to a much lesser extent than in many rodents — in response to a number of compounds, including some fibrate hypolipidemic drugs, pesticides and even estrogens (Cajaraville et al., 2003). The process is found is invertebrates, such as mollusks (Orbea and Cajaraville, 2006).

In mammals, peroxisome proliferation is mediated by a group of nuclear receptors, the peroxisome proliferator-activated receptors (PPARs). Signal transduction is through receptor activation and binding to peroxisome proliferator receptor elements (PPREs) in the promoter of target genes. Evidence suggests that several factors, including hormonal signals, interact to impact the metabolic pathways taking place in the peroxisome and most importantly its biogenesis (Just et al., 1982; Fagarasanu et al., 2007; Delille et al., 2009). Studies support that comparable mechanisms occur in fishes (Ibabe et al., 2005; Ortiz-Zarragoitia et al., 2006; Bilbao et al., 2009, 2010). Estrogens and the estrogen receptor (ER) pathway apparently influence fish peroxisome dynamics, namely in the liver (Ma et al., 1998; Rocha et al., 2004). In brown trout, *Salmo trutta* f. *fario*, a negative correlation between female ovary maturation and seasonal size changes of the hepatic peroxisomes was shown (Rocha et al., 1999). The more marked aspect of these findings is the parallel decrease in the organelle volume and in the activity of peroxisomal enzymes as females start vitellogenesis and ovary maturation, under estradiol induction (Rocha et al., 2004). Recently, data on the seasonal (annual) expression of PPARs concurred

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with that idea. *PPARα* expression was significantly lower in vitellogenic females, which naturally have higher blood titers of circulating estradiol (Batista-Pinto et al., 2009). Furthermore, a relevant enzyme both in estradiol and very-long-chain fatty acid catabolism, the 17-beta hydroxysteroid dehydrogenase 4 (HSD17B4), changes its expression pattern during the reproductive cycle mimicking the profile of the PPARα (Castro et al., 2009).

The described process should hypothetically impact the chain of events leading to peroxisome biogenesis and/or growth; although no seasonal change in peroxisome number was observed in brown trout liver (Rocha et al., 1999). In mammalian cells, peroxisome growth prior to division comprises a sequence of steps, including elongation, constriction and fission (Koch et al., 2004). The fundamental players in this process are the proteins named peroxins (PEX), in particular PEX11 (Fagarasanu et al., 2007). These were the first proteins to be implicated in peroxisome division (Erdmann and Blobel, 1995). They localize to the peroxisomal membrane and are implicated at least early in the elongation step of the peroxisomal division, as already reported in yeast, in mammals and in plants (Thoms and Erdmann, 2005; Kaur and Hu, 2009). Moreover, a role in transport membrane processes seems also important (van Roermund et al., 2000). In vertebrates, three *Pex11* genes have been described: *Pex11α*, *Pex11β* and *Pex11γ* (Abe and Fujiki, 1998; Abe et al., 1998; Li and Gould, 2002; Thoms and Erdmann, 2005). In yeast, the deletion phenotype implicates larger and fewer peroxisomes (Erdmann and Blobel, 1995); in both mammalian cells and yeast, overexpression of PEX11 proteins may promote peroxisome division whereas their loss inhibits peroxisome metabolism (Li and Gould, 2002). It was shown recently that Pex11 protein family members play subtle distinct roles (Huber et al., 2012). The PEX11α protein is inducible by proliferators and is highly expressed in the liver (Abe et al., 1998; Schrader et al., 1998), whereas PEX11β is constitutively expressed (Abe and Fujiki, 1998). Compensatory loss by both alpha and beta isoforms in knock-out mice has been suggested (Li et al., 2002; Baes and Van Veldhoven, 2006). From a mechanistic point of view, it is interesting that *PEX11α* is apparently regulated by *PPARα* in mice (Shimizu et al., 2004).

In view of the above, and because we observed a marked sex-specific expression pattern of PPARα in the brown trout liver throughout the reproductive cycle, we hypothesize whether Pex11α presented similar seasonal expression changes in this species. In the same vein, we investigated whether the volume and surface area dynamics observed in brown trout liver peroxisomes during the reproductive cycle, especially in females, is followed in parallel by fluctuations in the expression of the membrane protein PEX11α. Such fundamental data have a broad interest.

2. Material and methods

2.1. Pex11α isolation

Total RNA from several tissues of one adult male trout (*Salmo trutta* f. *fario*) was isolated by the guanidine thiocyanate/phenol chloroform extraction method as previously used by us and detailed in Batista-Pinto et al. (2005). cDNA conversion was performed with the oligo (dT)20 primer from the Thermoscript™ kit (Invitrogen). These samples were used to isolate the orthologue of the *PEX11α* in *S. trutta fario*. *PEX11α* and *β* sequences from different vertebrate species were retrieved from Ensembl and GenBank databases. Degenerate oligonucleotide primers were designed in regions of conservation using the CODEHOP software (http://blocks.fhcrc.org/ codehop.html).

Forward and reverse primer sequences were 5′ AGCTGTTCCGGCT GGGNAAYRCNGT 3′ and TCAGCAGGAACAGGAAGGCRTCNARYTG 3′ respectively. The polymerase chain reaction (PCR) was used to isolate the target gene fragment. PCR profile was as follows: 96 °C for 15 min and 40 cycles of 95 °C for 10 s, 58 °C for 30 s and 72 °C for 35 s. An initial fragment of approximately 380 base pairs was extracted from the agarose gel with the illustra GFX PCR DNA & Gel Band Purification Kit (GE Healthcare). Direct sequencing was performed to address the identity of the isolated fragment. To obtain the full cDNA sequence of brown trout *pex*11α, we employed RACE PCR (Clontech) with specific RACE primers (PEXRrace 5′ TAGAAACTGCCTCTCTCTGGTTCTCTGA 3′; PEXFrace 5′ AGAAATGTTGGCCTTGTCCCTGGTA 3′). Single bands were cloned into pGEMT easy vector (Promega). Primers M13F and M13R were used for clone sequencing.

2.2. Phylogenetic analysis

Several *PEX11α* and *PEX11β* vertebrate sequences were collected from the GenBank and Ensembl databases to produce a phylogenetic analysis. The alignment and neighbor-joining tree was generated with ClustalX (Thompson et al., 1997) and visualized with the Treeview program (version 1.6.6) (Page, 1996). The confidence in each node was assessed by 1000 bootstrap replicates. The tree reconstruction was performed using the full-length alignment (without taking into account gaps or ambiguous sites).

2.3. Sampling and determination of the gonadosomatic index

Animal sampling was performed as previously described (Batista-Pinto et al., 2009; Castro et al., 2009). Briefly, three-year-old animals with an average mass of 700 g were randomly sampled by throwing fishing nets into the pools of a governmental aquaculture facility. They were kept with continuous flow of pristine mountain water, under natural temperature and photoperiod, being fed a high quality commercial trout chow (A. Coelho & Castro, Póvoa do Varzim, Portugal), following routine husbandry procedures. Seven males and seven females were sacrificed at each season of the natural reproductive cycle (North of Portugal conditions) (Rocha et al., 1999; Rocha, 2000): February (post-spawning period), May (early gonad maturation onset in both sexes/early vitellogenesis of females), September (advanced gonad maturation/advanced vitellogenesis of females), and December (pre-spawning). After dissection under deep anaesthesia (immersion bath followed by a continuous flow through the gills of a 1 ml/L solution of ethylene-glycol monophenyl ether), livers were immediately snap frozen in liquid nitrogen and then after stored at −80 °C until RNA processing. Gonads were excised so to confirm the sex of the fish and the corresponding reproductive stage of the breeding cycle. For these aims it sufficed the visual inspection of the external anatomy of each gonad together with the calculation of the gonadosomatic index (GSI (%) = 100 × gonad mass/body mass).

2.4. RNA isolation and QPCR

Total RNA extraction was made as above, in every sacrificed fish. All sampled fish were used in the molecular analyses. Afterwards, the samples were cleaned-up with the RNeasy Mini-kit (Quiagen) including an on-column DNAse digesting step. RNA content was measured fluorometrically with the Qubit fluorometer (Quant-it RNA BR assay kit) and 500 ng of total RNA was transcribed into cDNA by using the iScript cDNA synthesis kit (BioRad).

To determine the *PEX11α* expression in brown trout liver samples a real time PCR assay was developed. We designed quantitative PCR primers in the coding region of the *PEX11α* gene using the Beacon Designer 5 (PREMIER Biosoft International). The sequences were as follows: *pex*11ARtF 5′TCAGCACCTCAACGACAACC3′ and *pex11*ARtR 5′CACGAAAGCCCACCACTCC3′ (197 bp of expected size). The comparison of the amplified sequence with other Pex11α teleost orthologues indicates that the real time primers do not outflank an intron. However, to prevent DNA contamination the RNA samples were treated with DnaseI. The final volume of the reaction was 25 μL, and contained 1× iQ SYBR Green Supermix (Bio-Rad), 2 μM of each primer and the