Seasonal and Morphological Variations of Brown Trout (Salmo trutta f. fario) Kidney Peroxisomes: A Stereological Study

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Abstract: Literature about fish kidney peroxisomes is scarce. To tackle this caveat, a stereological approach on renal peroxisome morphological parameters was performed for the first time in a fish, establishing correlations with maturation stages as it was previously done in brown trout liver. Three-year-old brown trout males and females were collected at the major seasons of their reproductive cycle. Trunk kidney was fixed and processed for catalase cytochemistry. Classical stereological methods were applied to electromicrographs to quantitate morphological parameters. Different seasonal variation patterns were observed between genders, and between renal proximal tubule segments I and II. In males, peroxisomes from proximal tubule segment II had a relatively higher volume and number in May, being individually bigger in February. Females presented similar trends, though with less marked variations. Overall, males and females did not show exactly the same seasonal patterns for most peroxisomal parameters, and no correlations were found between the latter and the gonado-somatic index (GSI). Hence, and despite the variations, the morphology of renal peroxisomes is not strictly correlated with gonad maturation kinetics, therefore suggesting that kidney peroxisome morphology is not seasonally modulated by sex steroids, like estradiol, as it seems to happen in liver peroxisomes.

Key words: teleost, renal microbodies, reproductive cycle, morphometry, ultrastructure

INTRODUCTION

The study of peroxisomes became a significant research area especially because of their association with fatal hereditary human diseases (Wanders, 2014; Waterham et al., 2015). The discovery of many chemicals which induce peroxisome proliferation is another aspect of interest in peroxisome research (Cajaraville & Ortiz-Zarragoitia, 2006; Schrader et al., 2016), especially because a great number of proliferators also induce hepatocellular tumors (Michalk et al., 2004).

Quantitative studies have been done in mammals in order to investigate peroxisomal associated diseases as well as experimental induction of peroxisome proliferation and ageing (Beier & Fahimi, 1991; Beier et al., 1993; Stefanini et al., 1995; Ozaki et al., 2001). The application of stereological methods proved to be very useful in the detection of peroxisome alterations in cancer, steatosis, alcohol consumption, and nephrectomy (Decraemer et al., 1993, 1995, 1996, 1997). However, and despite the importance of comparative perspectives, in non-mammalian vertebrates quantitative studies are still very scarce (Orbea et al., 1999; Rocha et al., 1999; Madureira et al., 2015). In addition, the fact that many pollutants which are released into water systems are peroxisome proliferators warns for the danger of xenobiotic-induced peroxisomal impacts in aquatic organisms (Cajaraville & Ortiz-Zarragoitia, 2006).

In addition, it was also documented that fish peroxisomes undergo alterations in their enzymatic activities, as well as morphological changes apparently modulated by sex steroids, and particularly by estradiol (Veranic & Pipan, 1992; Rocha et al., 1999; Ibabe et al., 2002; Madureira et al., 2015). Nevertheless, such studies focused on liver peroxisomes and so the influences of those factors are still unknown in renal peroxisomes. We hypothesize that these may change too, namely because sex and seasonal effects were already detected in other structural components of the brown trout kidney (Resende et al., 2010).

Fish kidneys are dorsal retroperitoneal organs located along the body cavity, being typically fused (partially or totally); in salmonids the fusion is almost complete (Hibiya, 1982). Also, two differentiated parts of fish kidney are commonly identified (but not always distinct macroscopically): the head and the trunk kidney (Ogawa, 1961; Hibiya, 1982).
The head kidney is composed essentially of hematopoietic tissue. Renal tubules are very scarce in this portion, but become more frequent towards the caudal part. The trunk kidney has numerous nephrons, much more frequent and developed in freshwater than in marine species, and ducts that are nevertheless surrounded with hematopoietic tissue, rich in pigmented macrophages. In salmonids, nephron segmentation has been described at light and electron microscope level (Anderson & Loewen, 1975; Hentschel & Elger, 1987; Elger et al., 2000; Resende et al., 2010). According to such studies, the “proximal nephron” is composed of the classical Malpighi glomerulus and Bowman’s capsule, a short neck segment, the proximal tubule (with a first and a second portion) and the intermediate segment. The existence of a distinct neck segment as well as an intermediate segment is not very clear. In addition, the “distal nephron” is made of the distal tubule, the collecting tubule and the collecting duct. The latter links the nephron and the collecting ducts that in turn coalesce forming the mesonephric duct or ureter.

As in other vertebrates, it is well established that fish kidney peroxisomes are mainly located in epithelial cells of tubules (Elger et al., 2000). However, the distribution profile of these organelles along the renal tubules could vary among different fish species (Oulmi et al., 1995b; Elger et al., 2000). The finding of peroxisomes in renal proximal tubule epithelial cells is unanimous, as well as the absence of references to peroxisomes in the distal tubule. Nevertheless, different descriptions in a qualitative or semi-quantitative view do exist when the two segments of proximal tubules are compared (Elger et al., 2000). In addition, and to our best knowledge, a quantitative morphological study of peroxisomes in renal tubules has not been made. As such, this work was made with three purposes: (1) to investigate structural changes of kidney tubule peroxisomes occurring in adult brown trout during the year; (2) to disclose if those alterations are tubule segment specific and gender dependent; (3) to find possible correlations with the reproductive status. Several stereological parameters of peroxisomes were estimated, namely the relative number and the absolute and individual size values of these organelles. These findings, adding to the knowledge about hepatic peroxisomes, will widen the baseline phenotypic characteristics for understanding peroxisome “behavior” in brown trout under the influence of sex steroids. The study might also provide new clues about other factors that may influence the kinetics of these organelles.

**Material and Methods**

**Animals**

Three-year-old adult male and female brown trout (*Salmo trutta f. fario*) were randomly collected by net fishing from a pool at a state aquaculture station (Posto Aquícola do Torno, Amarante, Portugal). Collection took place at major seasons of the brown trout natural reproductive cycle: February (post-spawning), May (early vitellogenesis), September (advanced vitellogenesis), and December (pre-spawning). Per season, five males and five females were sacrificed, thus summing to 40 fish used in this study. Animals from each period were in the same developmental stage. Before being sacrificed, the fish were held in observation tanks for 12–24 h. All fish were considered healthy. The mean body standard length and weight, as well as the gonado-somatic index (GSI) and the reno-somatic index (RSI) were measured.

**Tissue Processing**

Animals were anesthetized by immersion in a 1 mL/L aqueous solution of ethylene glycol monophenyl ether, and then weighed and measured in length. Before kidney collection, perfusion was made with a 5 IU/mL heparinized isosmotic buffer for salmonids (Cascales et al., 1997), composed of NaCl 0.74%, KCl 0.04%, CaCl2 0.02%, MgSO4 0.15%, NaHCO3 0.03%, NaH2PO4·H2O 0.05%, Na2HPO4·2H2O 0.16%. For improving the perfusion the most posterior part of the fish was cut allowing a cannulation of either the vein or the artery (each at a time) with simultaneous escape of both perfusate and blood by either one or the other vessel. During perfusion, the fish was maintained on top of an ice bed. The perfusion was carried out at 4°C, with a physiological flow rate of about 5 mL/min/kg of body weight (Hampton et al., 1985) during the time necessary to eliminate the organ residual blood. Before removing the kidney, we split its head (cranial) and trunk portions by cutting the organ at the virtual line where the enlarged anterior portion is continued with the much more elongated posterior part. The trunk kidney was then removed, weighed and sliced into 4 mm thick sections (with the first cut made at random between 1–4 mm from the anterior edge of the trunk kidney). Systematic samplings were then performed for obtaining final samples made of very tiny pieces possessing adequate size for the catalase cytochemistry procedure at electron microscopy level. This practical operational collection system assures equal sampling probabilities for all pieces of tissue (Gundersen, 1986). The tissue sections were fixed for 2 h in a solution of 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 at 4°C. Gonads were also removed and weighed. After fixation, kidney pieces were rinsed for 2 h at 4°C in 0.1 M phosphate buffer pH 7.4. For catalase detection, pieces were incubated for 2 h at 37°C in a medium containing 0.12% of H2O2 and 2 mg/mL 3,3′-Diaminobenzidine (DAB) in 0.1 M Tris-HCl buffer pH 8.5 (Veenhuis & Bonga, 1979). After incubation, the samples were washed twice at 4°C, first in 0.1 M Tris-HCl buffer pH 8.5 for 1 h, and subsequently in 0.1 M phosphate buffer pH 7.4 for 30 min. Post-fixation in 0.1 M phosphate buffered 1% OsO4 with 1.5% K3Fe(CN)6 was carried out for 2 h at 4°C. After dehydration in ethanol, the pieces were embedded in an epoxy resin. Unstained ultrathin sections of segments I and II of the proximal tubules were observed with a JEOL 100CXII transmission electron microscope (TEM), operated at 60 kV.
Stereological Methodology

In order to estimate relative and absolute parameters of kidney peroxisomes, we followed the general procedures applied to liver peroxisomes by Rocha et al. (1999). Five tissue blocks from each gender and from every season were used for analysis at TEM level. From each block, one grid was observed and photographed based on a systematic design carried out over areas with proximal tubules (disregarding those areas presenting mainly lumens, extra-tubular components, and distal tubules). A total of ~50 fields of proximal tubule segment I and 20 fields of proximal tubule segment II were taken at a magnification of 5,300×, and further printed at a final magnification of 15,900×. A total of 2000 TEM fields were analyzed in this study.

The volume, surface, and numerical densities of peroxisomes, considering the tubule cell as the reference space, were the primary parameters to be estimated, being designated, respectively, as $V_V$, $S_V$ and $N_V$ (peroxisomes, tubule cell).

The volume densities were estimated using a classical and unbiased manual stereological technique based on point counting as detailed by Weibel (1979):

$$V_V (\text{structure, reference space}) = \frac{\sum P(s) \times 100}{k \times \sum P(r)},$$

in which $P(s)$ is the total number of points of all structural component, $P(r)$ the total number of test points lying over the reference space (tubule cells), and $k$ the ratio between the number of points of the grid used for the structure analysis and for the reference space. A multipurpose test-grid containing two sets of points was used, in which $k = 16$ for the $V_V$ of peroxisomes and $k = 1$ for the $V_V$ of tubule cell nucleus.

Surface densities were also estimated by the use of an unbiased rectangular counting frame bearing forbidden lines according to magnification (Weibel, 1979). The value of 1.1 was adopted in this study for $V_V$ based on the previous work of Rocha et al. (1999). In the latter work, the implementation of the Weibel and Gomez method (model-based stereology) for brown trout peroxisomes was validated against the “gold-standard” design-based method of the physical dissector (Sterio, 1984); exceedingly time consuming and difficult to apply at the electron microscopy level. It is important to stress here that since its publication the Weibel & Gomez (1962) method has been often confirmed to provide accurate estimates for several biological targets—namely, in liver (Rocha et al., 1999) and kidney (Buzello, 2000; White & Bilous, 2004)—even when the structures under study are only roughly approximate to the standard geometrical shapes.

The mean peroxisomal volume ($\pi _N$) and surface ($\alpha _N$) estimations in the number-weighted distribution required the combination of some relative parameters, as follows:

$$\pi _N (\text{peroxisome}) = \frac{V_V (\text{peroxisome, tubulecell})}{N_V (\text{peroxisome, tubulecell})},$$

$$\alpha _N (\text{peroxisome}) = \frac{S_V (\text{peroxisome, tubulecell})}{N_V (\text{peroxisome, tubulecell})}.$$

The spherical equivalent mean diameter $\bar{d}_{\text{sphere}}$ of a peroxisome was calculated using the classical formula:

$$\bar{d}_{\text{sphere}} = \frac{1}{6} \times \pi \times \bar{d}_{\text{sphere}}.$$

Statistical Analysis

Statistica 6.0 for Windows was used to analyze the data sets. A two-way ANOVA was applied to test the effects of season, gender, and season by gender interaction, for each parameter, after checking the assumptions of normality and homogeneity of variances. The Tukey post-hoc tests for multiple comparisons between means was further applied. By default, differences were considered significant when $p \leq 0.05$. Data transformation was made in some cases to warrant normality and homogeneity. Non-parametric tests (Kruskal–Wallis ANOVA and Mann–Whitney U-tests) were also applied when data transformation failed to normalize the data sets; it was verified that the results always corroborated the parametric significances. Correlation tests were used to find significant linear associations between selected variables.

RESULTS

Brown Trout Biological Parameters

Biological parameters of the animals from each season sample are presented in Table 1, namely the standard body weight, body length, GSI, and RSI.
**Table 1.** Fish Biological Parameters.

<table>
<thead>
<tr>
<th>Months</th>
<th>Gender</th>
<th>Weight (g)</th>
<th>Length (cm)</th>
<th>GSI (%)</th>
<th>RSI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>February</td>
<td>♀</td>
<td>617.9 (0.19)</td>
<td>35.8 (0.06)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.6 (0.23)&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>0.7 (0.29)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>673.0 (0.08)</td>
<td>35.4 (0.05)</td>
<td>1.6 (0.50)&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>0.7 (0.12)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>May</td>
<td>♀</td>
<td>809.0 (0.16)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40.0 (0.06)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.8 (0.04)&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>0.5 (0.08)&lt;sup&gt;Ab&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>796.5 (0.16)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.4 (0.05)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.2 (0.33)&lt;sup&gt;Bb&lt;/sup&gt;</td>
<td>0.6 (0.05)&lt;sup&gt;Bb&lt;/sup&gt;</td>
</tr>
<tr>
<td>September</td>
<td>♀</td>
<td>526.3 (0.20)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32.8 (0.06)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.1 (0.22)&lt;sup&gt;Ac&lt;/sup&gt;</td>
<td>0.4 (0.06)&lt;sup&gt;Ab,c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>558.8 (0.22)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.5 (0.08)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.3 (0.16)&lt;sup&gt;Bc&lt;/sup&gt;</td>
<td>0.5 (0.08)&lt;sup&gt;Bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>December</td>
<td>♀</td>
<td>579.6 (0.18)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.5 (0.06)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.00 (0.10)&lt;sup&gt;Ad&lt;/sup&gt;</td>
<td>0.4 (0.15)&lt;sup&gt;Ac&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>697.7 (0.17)</td>
<td>35.8 (0.05)</td>
<td>3.9 (0.18)&lt;sup&gt;Bd&lt;/sup&gt;</td>
<td>0.7 (0.09)&lt;sup&gt;Ba&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>Different uppercase superscript letters represent differences between genders within each month.

<sup>a,b</sup>Different lowercase superscript letters represent differences among months within a gender.

For the lower cases, the presence of at least one similar letter means differences absence. Only values with different letters are significantly different (p ≤ 0.05).

GSI, gonado-somatic index; RSI, reno-somatic index.

**Table 2.** Relative Stereological Parameters of Proximal Tubules Segments I and II From Brown Trout Along its Reproductive Cycle.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Proximal Tubule I</th>
<th>Proximal Tubule II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S&lt;sub&gt;V&lt;/sub&gt; (P, Cell I) (μm&lt;sup&gt;2&lt;/sup&gt;/μm&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>V&lt;sub&gt;V&lt;/sub&gt; (P, Cell I) (%)</td>
</tr>
<tr>
<td>Sex</td>
<td>♀</td>
<td>♂</td>
</tr>
<tr>
<td>February</td>
<td>0.05 (0.15)</td>
<td>0.05 (0.19)</td>
</tr>
<tr>
<td>May</td>
<td>0.07 (0.40)</td>
<td>0.06 (0.15)</td>
</tr>
<tr>
<td>September</td>
<td>0.06 (0.26)</td>
<td>0.05 (0.21)</td>
</tr>
<tr>
<td>December</td>
<td>0.06 (0.17)</td>
<td>0.04 (0.41)</td>
</tr>
</tbody>
</table>

Results are shown as mean (CV). CV is the coefficient of variation (CV = standard deviation/mean).

<sup>A,B</sup>Different uppercase superscript letters represent differences between genders within each month.

<sup>a,b</sup>Different lowercase superscript letters represent differences among months within a gender; the presence of the same letter means absence of differences.

S<sub>V</sub>, V<sub>V</sub>, N<sub>V</sub>—surface, volume and numerical densities of peroxisomes (P) in relation to the tubule cell.

**Peroxisomal Stereology**

The results of the two-way ANOVAs of all stereological parameters from proximal tubule segment I are summarized in Supplementary Table 1 and from proximal tubule segments II in Supplementary Tables 2 and 3. The detailed stereological data of peroxisomes are shown in Tables 2–4.

**Supplementary Tables 1–3**

Supplementary Tables 1–3 can be found online. Please visit https://doi.org/10.1017/S1431927616011995

In the proximal tubule segment I, significant ANOVAs were detected for the relative stereological parameters of peroxisomes, such as the V<sub>V</sub>, S<sub>V</sub>, and N<sub>V</sub>; independent effect of sex in all parameters, independent effect of season in N<sub>V</sub>, and no significant ANOVAs for the sex and season interaction effect (Supplementary Table 1). Further analysis showed no seasonal or gender variations (at the same breeding period) occurring in this kidney segment, which was further confirmed by the absence of significances in the post-hoc tests (Table 2).

Contrary to the relative peroxisome parameters, the individual absolute dimensions of peroxisomes (d<sub>Sphere</sub>, d<sub>3V</sub>, and V<sub>P</sub>) from proximal tubule segment I were deeply influenced by the season, whereas the sex and the sex and season interaction did not exert effects (Supplementary Table 1). In males, none of the parameters underwent variation either among seasons or between genders (Table 3). However, in
Table 3. Absolute Stereological Parameters of Individual Peroxisomes of Segments I and II Proximal Tubules from Brown Trout Kidney Along its Reproductive Cycle.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>( \bar{d}_{sphere} (\mu m) )</th>
<th>( \tau_N (\mu m^3) )</th>
<th>( \nu_N (\mu m^3) )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td>( \varphi )</td>
<td>( \delta )</td>
<td>( \varphi )</td>
</tr>
<tr>
<td>Proximal tubule I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>February</td>
<td>0.31 (0.16)( ^a )</td>
<td>0.24 (0.33)</td>
<td>0.90 (0.09)</td>
</tr>
<tr>
<td>May</td>
<td>0.19 (0.24)( ^b )</td>
<td>0.20 (0.18)</td>
<td>0.67 (0.22)</td>
</tr>
<tr>
<td>September</td>
<td>0.22 (0.20)</td>
<td>0.26 (0.21)</td>
<td>0.77 (0.21)</td>
</tr>
<tr>
<td>December</td>
<td>0.19 (0.37)( ^b )</td>
<td>0.22 (0.22)</td>
<td>0.67 (0.26)</td>
</tr>
<tr>
<td>Proximal tubule II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>February</td>
<td>0.17 (0.05)( ^{AA} )</td>
<td>0.38 (0.33)( ^{Ba} )</td>
<td>0.58 (0.03)( ^a )</td>
</tr>
<tr>
<td>May</td>
<td>0.12 (0.22)( ^b )</td>
<td>0.16 (0.22)( ^b )</td>
<td>0.50 (0.23)</td>
</tr>
<tr>
<td>September</td>
<td>0.17 (0.10)( ^a )</td>
<td>0.20 (0.41)( ^b )</td>
<td>0.63 (0.08)( ^a )</td>
</tr>
<tr>
<td>December</td>
<td>0.11 (0.29)( ^b )</td>
<td>0.19 (0.68)( ^b )</td>
<td>0.41 (0.25)( ^b )</td>
</tr>
</tbody>
</table>

Results are shown as mean (CV). CV is the coefficient of variation (CV = standard deviation/mean).

\( ^a \), \( ^b \), Different uppercase superscript letters represent differences between genders within each month.

\( ^{AA} \), \( ^{Ba} \), Different lowercase superscript letters represent differences among months within a gender.

Table 4. Relative Stereological Parameters of Segments I and II Proximal Tubules from Brown Trout Kidney Along Its Reproductive Cycle.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>( S_V ) (P, Cyt) (( \mu m^2/\mu m^3 ))</th>
<th>( V_V ) (P, Cyt) (%)</th>
<th>( N_V ) (P, Cyt) (no./( \mu m^3 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td>( \varphi )</td>
<td>( \delta )</td>
<td>( \varphi )</td>
</tr>
<tr>
<td>Proximal tubule I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>February</td>
<td>0.07 (0.22)</td>
<td>0.06 (0.19)</td>
<td>0.90 (0.22)</td>
</tr>
<tr>
<td>May</td>
<td>0.09 (0.39)</td>
<td>0.08 (0.17)</td>
<td>0.90 (0.36)</td>
</tr>
<tr>
<td>September</td>
<td>0.08 (0.29)</td>
<td>0.07 (0.21)</td>
<td>0.90 (0.28)</td>
</tr>
<tr>
<td>December</td>
<td>0.07 (0.15)</td>
<td>0.06 (0.37)</td>
<td>0.70 (0.23)</td>
</tr>
<tr>
<td>Proximal tubule II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>February</td>
<td>0.09 (0.23)( ^{AA} )</td>
<td>0.03 (0.91)( ^{Ba} )</td>
<td>1.00 (0.23)( ^a )</td>
</tr>
<tr>
<td>May</td>
<td>0.14 (0.09)( ^{Ab} )</td>
<td>0.10 (0.32)( ^{Bb} )</td>
<td>1.30 (0.10)</td>
</tr>
<tr>
<td>September</td>
<td>0.11 (0.24)</td>
<td>0.07 (0.33)( ^{Bb} )</td>
<td>1.20 (0.26)</td>
</tr>
<tr>
<td>December</td>
<td>0.11 (0.24)( ^{AA} )</td>
<td>0.02 (0.75)( ^{Ba} )</td>
<td>1.10 (0.31)( ^a )</td>
</tr>
</tbody>
</table>

Results are shown as mean (CV). CV is the coefficient of variation (CV = standard deviation/mean).

\( ^{AA} \), \( ^{Ba} \), Different uppercase superscript letters represent differences between genders within each month.

\( ^{Ab} \), \( ^{Bb} \), Different lowercase superscript letters represent differences among months within a gender.

The presence of the same letter means differences absence.

\( S_V \), \( V_V \), \( N_V \) – surface, volume and numerical densities of peroxisomes (P) in relation to the cytoplasm of the tubule cell.

females, the \( \bar{d}_{sphere} \) and \( \tau_N \) changed along the year and with similar kinetics, with highest values in February, significantly decreasing to the lowest values in May and December, though only marginally significant between February and May (Fig. 1). Between these months (in September) a slight increasing trend (not statistically significant) in the peroxisome size was observed. The \( \tau_N \) tended to the same variation pattern, although none of the differences was statistically significant.

The individual absolute dimensions of peroxisomes in proximal tubule segment II depended greatly from sex and seasons effects, not only by an independent action but also by their interaction, excepting to the \( \tau_N \)'s, in which only a sex effect was observed by a significant ANOVA (Supplementary Table 3). Further analysis revealed that, in males, \( \tau_N \) did not vary, whereas \( \tau_N \) and \( \bar{d}_{sphere} \) presented changes along the year and with a similar variation pattern for both parameters (Table 3). Thus, male peroxisomes were significantly bigger in February, decreasing in May and maintaining smaller dimensions onwards to September and December (Fig. 2). Despite the absence of statistically significant differences for the \( \tau_N \)'s, values pointed to the same variation pattern. In females, the seasonal pattern was similar for \( \tau_N \), \( \bar{d}_{sphere} \), and \( \bar{d}_{sphere} \), though different from the males; the highest dimensions of peroxisomes were found in February and September (Fig. 3). Differences between genders were only detected in February, where males had bigger peroxisomes than females (Figs. 2, 3).

No effects of sex, season, or sex and season interaction were detected in the size of peroxisomes from proximal tubule segment I relative to the cytoplasm volume of the tubule cell (Supplementary Table 1). However, a sex and season independent effect in the number of peroxisomes per \( \mu m^3 \) of cytoplasm was detected, but not a sex and season interaction.
interaction effect. Further analysis of the results did not confirm those significant ANOVAs and only a marginal significance was observed in females between February and May, being the lowest $N_V$ found in the former (Table 4).

In peroxisomes from proximal tubule segment II strong independent effects of sex and season in the size and volume of these organelles in relation to the cytoplasm of the tubule cell (Supplementary Table 2) were detected. The equivalent $N_V$ of peroxisomes was also sharply influenced by sex and season effects acting independently, but also the sex and season interaction effect was detected (Supplementary Table 2). Further analysis (Table 4) revealed a different variation pattern for males and females in relation to all the mentioned parameters. In males, the $S_V$ and $V_V$ were significantly higher in May, decreasing onwards to the lowest values observed in December. The same pattern was shown for the $N_V$ of peroxisomes; with a significantly high number of peroxisomes per cytoplasm of cell tubule observed in May, which gradually decreased to the lowest numbers found in December and February. In females, the $V_V$ did not show any variation during the year, whereas $S_V$ presented a significant variation between February and May, where peroxisomes were smaller in February and bigger in May in relation to the tubule cell cytoplasm. The variation pattern of the relative number of peroxisomes in females was also different from that found in males, having higher peroxisome numbers in May and December, and lowest numbers in February and September. Significant differences between genders were detected in February, May, and December.

No statistically significant correlations were found between the GSI and any of the stereological parameters measured in peroxisomes. However, some correlations between the latter and the RSI were found, particularly in proximal tubule segment II (Supplementary Table 4). In proximal tubule segment I of females, the individual peroxisome dimensions were positively correlated with the RSI, whereas the relative number of those organelles was negatively correlated with that index. In proximal tubule segment II, positive correlations between the RSI and the individual dimensions of peroxisomes were found just for females, as registered in tubule segment I. On the contrary, the relative dimensions and number of peroxisomes in proximal tubule II cells exhibited negative correlations with the RSI only in males.

Supplementary Table 4
Supplementary Table 4 can be found online. Please visit https://doi.org/10.1017/S1431927616011995
The scarcity of data concerning fish kidney peroxisomes from proximal tubule segments I and II epithelial cells. We anticipated differences in the peroxisomal number between those two segments of proximal tubules as described for other fish species, in the few studies concerning this issue (Veenhuis and Wendelaarborga, 1977; Oulmi et al., 1995a, 1995b; Johkura et al., 2000), in which peroxisomes were said to be more frequent in segment II epithelial cells of the proximal tubule. However, those descriptions were always made based on qualitative or at most semi-quantitative perspectives. We quantitatively demonstrated for the first time, not only that in female brown trout renal peroxisomes were more abundant—with more numerous peroxisomes per unit cell volume—in the epithelial cells of proximal tubule II than in proximal tubule I, but also the extent to which they differed. Further, we showed that differences are not only gender dependent, but also that they may fade at points of the breeding cycle, a fact that was not known to this date.

The relative peroxisome volume and number in proximal tubules experienced seasonal alterations, though more pronounced in segment II than in segment I. In proximal tubule segment II, variations in the relative number of peroxisomes did occur during the year for both genders, females having more peroxisomes than males in all seasons, and with higher relative numbers in May and December (males had more peroxisomes in May and September). For the relative peroxisomal dimensions, it was shown that both genders had greater volumes in the seasons displaying higher numbers of peroxisomes (except for the females in December). The individual peroxisome dimensions showed an opposite variation pattern when compared with the relative ones. In males, bigger peroxisomes were found in February, when proximal tubule segment II epithelial cells had less peroxisome number and volume. In females, the differences were not so striking, but again February and September were the seasons in which peroxisomes were bigger, but less in number and occupying less cell volume. Data on morphological and biochemical peroxisomal variations associated to seasonality does exist for the hepatic peroxisomes of fish, for the gray mullet (Mugil cephalus), although the seasonality was related to the environmental pollution status and not with the fish reproductive cycle (Orbea et al., 1999). Also, the seasonal variations observed herein in the renal peroxisomes do not seem correlated with breeding cycle events, as characterized herein by the GSI. Thus, the results obtained in the present study showed that the regulation of kidney peroxisome morphology is quite different from that described in the hypothesis of a sex steroidal regulation (particularly via estradiol), suggested by the quantitative study in brown trout liver peroxisomes (Rocha et al., 1999). In that work, these organelles showed along the annual reproductive cycle changes in their morphological features, such as the size and the number. Particularly in females, the peroxisome dimensions were quite negatively correlated with the GSI, being smaller at vitellogenesis, when the GSI was highly increased (Rocha et al., 1999).

**Discussion**

The scarcity of data concerning fish kidney peroxisomes was an encouraging motivation to develop this study. In addition, the fact that hepatic peroxisomes from brown trout seem to be modulated by events connected with the breeding cycle, namely by estradiol, which influences morphological and functional variations of these organelles, led to new questions about peroxisomal physiology and need of more fundamental knowledge (Rocha et al., 1999). Thus, a stereological study was performed for quantifying size- and number-related parameters of brown trout renal peroxisomes, taking into account the effects of gender, season and their interaction. Quantification considered the different portions of the kidney proximal tubule. This zonal differentiation perspective was not only due to the fact that epithelial cells from different segments of the proximal tubule differ in morphology and function (Elger et al., 2000; Resende et al., 2010), but also because it is known that there are morphological segment-specific responses when fish kidney is exposed to several toxins (Pritchard and Bend, 1984; Oulmi et al., 1995a, 1995b). Thus, it is likely that natural factors, such as sex and seasonality, might also modulate or induce segment-specific responses.

In this study, seasonal variation patterns were detected for some stereological parameters of renal peroxisomes as well as the existence of differences between the peroxisomes from proximal tubule segments I and II epithelial cells. We anticipated differences in the peroxisomal number between those two segments of proximal tubules as described for other fish species, in the few studies concerning this issue (Veenhuis and Wendelaarborga, 1977; Oulmi et al., 1995a, 1995b; Johkura et al., 2000), in which peroxisomes were said to be more frequent in segment II epithelial cells of the proximal tubule. However, those descriptions were always made based on qualitative or at most semi-quantitative perspectives. We quantitatively demonstrated for the first time, not only that in female brown trout renal peroxisomes were more abundant—with more numerous peroxisomes per unit cell volume—in the epithelial cells of proximal tubule II than in proximal tubule I, but also the extent to which they differed. Further, we showed that differences are not only gender dependent, but also that they may fade at points of the breeding cycle, a fact that was not known to this date.

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The fact that the segment II proximal tubules had more peroxisomes per cell volume unit and that these organelles displayed more changes in their morphology along the year, indicates a potentially specific involvement of those organelles in kidney function, namely as segment II of the proximal tubule was described as the preferential compartment for nitrogen compound catabolism in teleost fishes (Hickman & Trump, 1969). Moreover, and taking into account the freshwater nature of brown trout, variations in the proximal tubule ultrastructure may also be related with the needs of appropriate urine dilution, in which proximal tubules are extremely important. In fact, as to the ionic and osmotic homeostasis, whereas segment I is responsible in higher extent for reabsorption, segment II is more devoted to ion secretion (Larsen & Perkins, 2001).

The observed annual variations in peroxisome morphology were different in both genders and some correlations between the peroxisome structural parameters and the RSI were found, particularly in males. However, the absence of significant correlations between the variations of the stereological parameters of brown trout renal peroxisomes and the GSI suggested, again, that in kidney and at least at a morphological level, sex steroids do not seem to exert an influence on peroxisomes. Thus, other factors and hormones might govern the observed gender differences. For example, stress and particular fish behavior induce specific hormone secretion, which can influence kidney physiology and morphology (Larsen & Perkins, 2001). However, the regulation of fish kidney morphophysiology by hormones is still quite understudied and unveiling the cellular mechanisms by which they act is still a challenge to tackle.

In conclusion, renal peroxisomes from adult brown trout underwent annual changes in their morphology with different variation patterns in females and in males. These variations were observed especially in the peroxisomes from proximal tubule segment II from both genders, and seemed not to be correlated with the animal’s reproductive status. A correlation between those variations and kidney physiology in order to accomplish a hydrostatic homeostasis seems to be a plausible hypothesis to explain the modifications. By establishing the normal seasonal variation pattern of brown trout kidney peroxisomes, this study further shows that season and gender must be taken into account when considering changes in those organelles.

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