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SEASONAL AND TOXICOLOGICAL STUDY OF BROWN TROUT (*Salmo trutta*) KIDNEY AND LIVER PEROXISOMES

Dissertação de Candidatura ao grau de Doutor em Ciências Biomédicas, submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

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Ao Jorge, que dá cor à minha vida e inspira o meu coração,

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“Equipped with his five senses, man explores the universe around him and calls the adventure Science.”

Edwin Powell Hubble

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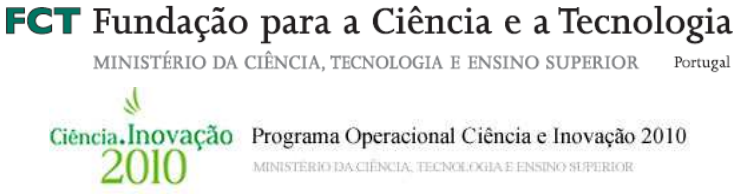
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No cumprimento do disposto no Decreto-Lei nº 216/92 de 13 de Outubro, declara-se que a autora desta Dissertação participou na concepção e na execução do trabalho experimental que estiveram na origem dos resultados apresentados, bem como na sua interpretação e na redacção dos respectivos manuscritos.

Nesta tese inclui-se um artigo científico publicado numa revista internacional, resultante de uma parte dos resultados obtidos no trabalho experimental, referenciado como:

Resende, A. D., E. Rocha, and A. Lobo-da-Cunha (2005). Activity of purine catabolism enzymes during the reproductive cycle of male and female brown trout (*Salmo trutta*). In *Trends in Comparative Endocrinology and Neurobiology*, vol. **1040**. *Annals of the New York Academy of Sciences*, pp. 444-447.

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ABSTRACT

The discovery of severe inherited human diseases caused by disorders in peroxisomes was a great impetus for studying these vital organelles with a widespread distribution in eukaryotic cells. Peroxisomes were also a target for many toxicological studies, since many chemicals induce their proliferation (particularly in hepatocytes from sensitive species) and some of these chemicals were associated to the induction of hepatic tumours. These connections were a starting point for the investigations concerning this organelle in aquatic organisms, particularly in liver and kidney since these are the peroxisome richest organs. Studies in brown trout revealed that female hepatic peroxisomes presented morphological and biochemical variation patterns related to the breeding cycle, suggesting a sex steroid modulation on these organelles. Further, it was questioned if that regulation could exist in peroxisomes from other organs, particularly in kidney. Finally, and because very little is known about seasonal changes of fish kidney, we sought it would be useful to make a detailed stereological study of the excretory kidney in our trout model. Hence, the first purpose of this thesis was to quantitatively characterize the brown trout trunk kidney, providing baseline knowledge for better understanding the potential variations and morphofunctional correlations. Secondly, we wanted to investigate possible morphological and biochemical alterations of the kidney peroxisomes and their eventual correlation with the gonad maturation status. Thirdly, for increasing the knowledge about the effects of chemicals which induce oxidative stress in fish, and that may interfere with peroxisomes, we started an approach to verify whether or not structural and physiological alterations occur in trout hepatic and renal peroxisomes under exposure to a reference pesticide. Finally, and taking into account that hepatic urate oxidase, a peroxisomal enzyme involved in purine catabolism, exhibited in brown trout a variation pattern of activity highly correlated with the ovary maturation degree, we examined other purine catabolism enzymes for seasonal changes, in the liver and also in the kidney.

In this study, the brown trout trunk kidney was for the first time qualitatively described and quantitatively characterized using a stereological approach, offering a differential analysis among four annual periods, namely February, May, September and December, and also a male versus female comparison. Qualitatively, the stroma and parenchyma tissues were structurally similar to those described in other freshwater teleost species. Quantitatively, some differences in several renal components volume, such as renal corpuscles, proximal tubule segments I and II, collecting tubules, new growing tubules and vacuolized tubules, were detected between genders and among seasons. Although only few linear

correlations between these volumes and the gonado-somatic index (GSI) were found, more correlations were confirmed between the volume parameters and the reno-somatic index (RSI). Thus, these gender dependent seasonal variations along the year suggested a morphological adaptation of renal components in order to accomplish physiological needs. These results constitute a baseline for future studies which design and interpretation could be influenced by morphological variations and their physiological consequences, as well as a reference in the analysis of abnormal changes in toxicological contexts.

In brown trout males and females collected in the same four annual periods mentioned above, stereological and biochemical analyses of the renal peroxisomes were performed. The quantitative structural parameters covered the relative volume, surface and number of peroxisomes, and spectrophotometric measurements targeted activities of peroxisomal enzymes, namely catalase, D-alanine oxidase and palmitoyl-CoA oxidase. At transmission electron microscopy level, quantitative morphological variations were detected in the renal tubule peroxisomes not only among seasons but also between genders, particularly in those from the proximal tubule segment II, in which they appeared more numerous than in other segments and with more size variations along the year. Despite the changes, there were no correlations between these stereological parameters and the GSI. In relation to the peroxisomal enzymes, urate oxidase and L-α hydroxy acid oxidases A and B activities were not detected in the renal homogenates. Palmitoyl-CoA oxidase activity was detected, but without significant variations along the year. On the contrary, catalase presented lower activities in September and December, in both males and females. D-alanine oxidase also showed similar variations for both genders, with lower activities not only in September and December but also in February. Negative correlations between the GSI and both catalase and D-alanine oxidase activities were found, being stronger in the former. Although, in kidney this correlation was found for both genders, whereas in liver was detected only in females. These findings suggest that renal peroxisomes seem not be morphologically modulated by events related or derived from the gonad maturation, and in consequence by sex steroids contrarily to aspects of their physiology. However, that modulation does not seem similar as noted in liver, since different seasonal patterns were found for each organ. Whereas female steroids, and particular estradiol, seem to highly influence hepatic peroxisomes, the renal peroxisomes are probably influenced by other hormone signalling, and without ultrastructural implications. Moreover, significant linear correlations between the D-alanine oxidase activities and the total peroxisome number and volume per gram of kidney, in both genders, were found after analysis of data sets derived from parameters

presented in Chapters 2, 3 and 4. Contrarily to the D-alanine oxidase, the catalase activity did not show any correlation with the cited peroxisome morphological parameters.

The subcellular location of some purine catabolism enzymes was investigated herein in brown trout. This approach seemed justifiable by the large reported variability among species for the localization of the enzymes involved in that metabolic pathway. Thus, crude cell fractions were firstly obtained after centrifuge fractionation of the total liver homogenate, and further submitted to spectrophotometric measurements of several enzymatic activities. It was well known that urate oxidase is a peroxisomal enzyme in vertebrates, including fishes, and that the studied salmonids lack allantoicase, which imply that allantoic acid has to be the excretion product of purine catabolism; facts that were confirmed also in brown trout. In addition, the xanthine oxidoreductase was detected only in the dehydrogenase form, as it happens in some organisms. Moreover, in brown trout liver, xanthine dehydrogenase and allantoinase were considered herein as cytosolic enzymes, since their activities were largely detected in the supernatant fraction.

The variation pattern of some purine catabolism enzymes during the annual reproductive cycle of brown trout was investigated, measuring spectrophotometrically their activities in renal and hepatic total homogenates. Urate oxidase, allantoinase and allantoicase activities were not detectable in renal homogenates, as also allantoicase activity in hepatic homogenates. In female liver, xanthine dehydrogenase, urate oxidase and allantoinase presented similar variation patterns with higher activities in May and decreasing in September and December, being hepatic urate oxidase undetectable in the latter period. On the other hand, male liver showed similar variation patterns as described for females. In the case of kidney xanthine dehydrogenase from females, a similar variation pattern as described for liver was shown, but in males higher activities were found not only in May but also in December. Negative correlations between the enzymatic activities and the GSI were found, particularly in females. Thus, our data indirectly suggested that sex hormones influence brown trout liver purine catabolism, being such modulation stronger in females than males and being the peroxisomal enzyme urate oxidase the most affected enzyme.

Finally, for examining to which extent peroxisomal enzymes might be altered by fish being exposed to oxidative stressors, a first 15 days waterborne exposure assay to the pesticide paraquat was made. Its hypothesized influence in the biochemistry of brown trout renal and hepatic peroxisomes was evaluated spectrophotometrically measuring the activity of three peroxisomal enzymes - catalase, D-aminoacid oxidase and urate oxidase - at days 0, 7 and 15 of the assay. Either in liver or in kidney homogenates, no differences were

found between control and paraquat groups for all the enzymes, both at 7 and 15 days. Curiously, despite the absence of control *vs.* treated significant differences in the enzymatic activity of kidney samples, it was detected a tendency towards an increase of catalase activity and a decrease of D-alanine oxidase activity at 15 days, in both control and paraquat groups. Thus, the study warned that normal changes may occur over time, at least in our test model, which is relevant information for future planning. Herein, the absence of urate oxidase in fish kidney was again confirmed. We concluded that at least for the used nominal dose of 0.3 mg L^{-1} of paraquat, the renal and hepatic peroxisomes do not seem to be functionally affected. However, to investigate the full toxicological potential of paraquat, and other reference pollutants, on peroxisomes, further works with other concentrations and exposure conditions (time, mixtures, etc.) will be performed.

RESUMO

A descoberta de algumas doenças humanas congénitas causadas por deficiências peroxissomais deu um grande impulso para o aumento do interesse no estudo destes organelos vitais, presentes na generalidade das células eucarióticas. Os peroxissomas tornaram-se também alvo de estudos toxicológicos, uma vez que vários compostos químicos induzem proliferação peroxisomal (sobretudo em hepatócitos de espécies sensíveis) e alguns desses proliferadores estão também associados ao desenvolvimento de tumores hepáticos. Estes factos foram um ponto de partida para uma abordagem deste tema em organismos aquáticos, dando especial atenção ao fígado e ao rim, já que estes são os órgãos mais ricos nestes organelos. Estudos em truta fário mostraram que os peroxissomas hepáticos das fêmeas apresentam padrões de variação, a nível morfológico e bioquímico, relacionados com o ciclo reprodutivo, sugerindo uma modulação destes organelos por parte de hormonas sexuais. Posteriormente, questionou-se se este mecanismo de regulação actuaria também nos peroxissomas de outros órgãos, nomeadamente no rim. Finalmente, e uma vez que pouco se sabia sobre variações sazonais no rim de peixes, pensámos ser útil um estudo estereológico do rim excretor no nosso modelo, a truta fário. Assim, um dos objectivos desta tese foi caracterizar quantitativamente o rim posterior da truta fário, de forma a obter conhecimentos de base que ajudem a uma melhor compreensão de potenciais variações e correlações morfofuncionais. Pretendeu-se também investigar a existência de possíveis alterações morfológicas e bioquímicas nos peroxissomas renais, eventualmente correlacionáveis com o grau de maturação da gónada. Com o propósito de aumentar o conhecimento no que respeita ao efeito de químicos de stress oxidativo em peixes, e respectiva interferência com os peroxissomas, foi desenvolvido um trabalho experimental com peroxissomas renais e hepáticos da truta fário quando exposta a um pesticida de referência, tendo em vista estudar as possíveis alterações estruturais e funcionais destes organelos. Finalmente, e sabendo que a urato oxidase hepática, uma enzima peroxissomal envolvida no catabolismo das purinas, apresenta um padrão anual de variação fortemente correlacionado com o grau de maturação do ovário de truta fário, foi nosso intuito verificar a variação anual de outras enzimas envolvidas na mesma via metabólica, não apenas no fígado, mas também no rim da mesma espécie.

Neste estudo, e pela primeira vez, o rim posterior da truta fário foi descrito qualitativamente e caracterizado quantitativamente através de métodos estereológicos, analisando-se quatro períodos do ciclo reprodutivo, nomeadamente, em Fevereiro, Maio,

Setembro e Dezembro, e comparando também machos com fêmeas. Qualitativamente, o estroma e o parênquima apresentaram características estruturais similares às descritas para outras espécies de teleósteos de água doce. Quantitativamente, foram detectadas algumas diferenças, quer entre sexos quer entre estações, quanto ao volume relativo de alguns componentes renais, tais como os corpúsculos renais, segmentos I e II dos túbulos proximais, túbulos colectores, túbulos em formação e túbulos vacuolizados. Encontrou-se um reduzido número de correlações lineares entre os parâmetros estereológicos e o índice gonado-somático, mas mais correlações foram confirmadas entre os referidos parâmetros estereológicos e o índice reno-somático. Assim, estas variações sazonais e as dependentes do sexo sugerem uma adaptação morfológica dos componentes renais de forma a satisfazer necessidades fisiológicas. Estes dados constituem uma base a ter em conta em estudos futuros, cujo desenho experimental e interpretação dos resultados possa estar comprometida pela influência de variações morfológicas e respectivas consequências fisiológicas, bem como uma referência na análise de resultados de ensaios toxicológicos.

Em seguida, efectuou-se uma análise estereológica e bioquímica dos peroxissomas renais de machos e fêmeas de truta fário colhidos nos quatro períodos referidos anteriormente. Para tal, os parâmetros estruturais quantitativos estimados foram o volume, a superfície e o número relativo de peroxissomas, e as medições espectrofotométricas de actividades de enzimas peroxissomais. Quantitativamente, e a nível ultraestrutural, foram encontradas variações morfológicas nos peroxissomas renais dos túbulos, não só entre os quatro períodos de colheita, mas também entre sexos. Tais variações incidiram especialmente no segmento II dos túbulos proximais, segmento onde os peroxissomas foram observados em maior número e com maiores variações de tamanho ao longo do ano. No entanto, e apesar destas diferenças, não foram encontradas correlações entre os parâmetros estereológicos e o índice gonado-somático. Relativamente às enzimas peroxissomais, nos homogeneizados renais de truta fário não foram detectadas actividades da urato oxidase e das L-α hidroxiácido oxidases A e B. A actividade da palmitoil-CoA oxidase foi detectada e manteve-se estável ao longo do ano. Pelo contrário, a catalase apresentou as mais baixas actividades em Setembro e Dezembro, tanto nos machos como nas fêmeas. A actividade da D-alanina oxidase também apresentou variações similares em ambos os sexos, com as mais baixas actividades não só em Setembro e Dezembro, como também em Fevereiro. Foram encontradas correlações negativas entre o índice gonado-somático e o padrão de variação das actividades enzimáticas da catalase e da D-alanina oxidase, tendo sido

mais forte no caso da catalase. Contudo, no rim esta correlação existe em ambos os sexos, enquanto que no fígado foi detectada apenas nas fêmeas. Estes resultados sugerem que os peroxissomas renais não são morfologicamente influenciados por processos relacionados ou derivados da maturação das gónadas e, consequentemente, por esteróides sexuais, contrariamente a aspectos do seu metabolismo. Além disso, este sistema de modulação não parece ser igual ao sugerido para o fígado, já que os padrões de variação encontrados para cada um dos órgãos são diferentes entre si. Os esteróides femininos, particularmente o estradiol, parecem influenciar fortemente os peroxissomas hepáticos, enquanto que os renais são provavelmente influenciados por outros sinais hormonais sem aparente implicação ultraestrutural. Com o propósito de integrar os resultados referentes aos capítulos 2, 3 e 4, os respectivos dados foram analisados em actividades enzimáticas de catalase e D-alanina por grama de rim e em números e volumes totais de peroxissomas por grama de rim. Desta análise, foram encontradas correlações lineares significativas entre o padrão de variação da actividade da D-alanina oxidase e o número e volume totais de peroxissomas, mas não com o padrão de variação da catalase.

A localização subcelular de algumas enzimas envolvidas no catabolismo das purinas foi investigada na truta fário. Este estudo foi efectuado devido à grande variabilidade referida entre espécies no que respeita à localização subcelular das enzimas envolvidas nesta via metabólica. Assim, obtiveram-se várias fracções celulares após fraccionamento diferencial dos homogeneizados totais de fígado, as quais foram depois sujeitas a medições de várias actividades enzimáticas por métodos espectrofotométricos. É já conhecido que em vertebrados, incluindo peixes, a urato oxidase é uma enzima peroxissomal, bem como o facto de que todos os salmonídeos estudados não possuem alantoicase, implicando que o produto de excreção nestes organismos seja o ácido alantóico, factos estes confirmados também na truta fário. Além disso, a xantina oxidorredutase foi apenas detectada na forma de desidrogenase, tal como acontece em alguns organismos. Após este estudo, as enzimas xantina desidrogenase e alantoinase do fígado da truta fário foram consideradas citosólicas, uma vez que as suas actividades foram essencialmente detectadas nos sobrenadantes.

Foi também investigado o padrão de variação anual de enzimas envolvidas no catabolismo das purinas na truta fário, medindo espectrofotometricamente as suas actividades nos homogeneizados totais de rim e fígado. No rim, não foram detectadas actividades das enzimas urato oxidase, alantoinase e alantoicase. No fígado das fêmeas, os padrões de variação encontrados para as actividades da xantina desidrogenase, da

urato oxidase e da alantoinase foram similares, com actividades mais altas em Maio, diminuindo em Setembro e Dezembro, sendo mesmo indetectável a actividade da urato oxidase nestes meses. Além disso, no fígado dos machos foram encontrados padrões de variação semelhantes aos das fêmeas. No rim, a xantina desidrogenase das fêmeas apresentou também um padrão de variação similar ao já descrito, mas nos machos detectaram-se picos de actividade, não só em Maio, mas também em Dezembro. Adicionalmente, e particularmente nas fêmeas, foram encontradas correlações negativas entre os padrões de variação das actividades enzimáticas e o índice gonado-somático. Indirectamente, estes resultados sugerem uma influência por parte de hormonas sexuais no catabolismo das purinas do fígado da truta fário, sendo no entanto esta modulação mais forte nas fêmeas do que nos machos e afectando particularmente a actividade da enzima peroxissomal urato oxidase.

Finalmente, e com o objectivo de verificar de que forma as enzimas peroxissomais podem ou não ser alteradas quando os peixes são expostos a compostos que originam stress oxidativo, foi levado a cabo um ensaio experimental durante 15 dias de exposição ao pesticida paraquat. A possibilidade de uma influência na bioquímica dos peroxissomas renais e hepáticos da truta fário foi avaliada espectrofotometricamente pela medição das actividades de três enzimas peroxissomais - catalase, D-alanina oxidase e urato oxidase - aos 0, 7 e 15 dias. Quer nos homogeneizados hepáticos, quer nos renais, não foram encontradas diferenças, para nenhuma das enzimas, entre os grupos controlo e tratado, aos 7 e aos 15 dias. Apesar da ausência de diferenças significativas nas actividades enzimáticas das amostras renais dos grupos controlo *versus* tratado, foram encontradas tendências para um aumento da actividade da catalase e uma diminuição na da D-alanina oxidase durante os 15 dias de experiência, em ambos os grupos. Assim, o estudo alerta para a ocorrência de variações normais ao longo do tempo, pelo menos no nosso modelo animal, constituindo estes dados informação útil para futuros planeamentos experimentais. Além disso, a ausência de actividade de urato oxidase no rim foi mais uma vez confirmada neste estudo. Assim, considerando o uso de paraquat numa dose de 0,3 mg L^{-1}, podemos concluir que os peroxissomas renais e hepáticos não parecem ficar funcionalmente afectados. Contudo, de forma a investigar o verdadeiro potencial de toxicidade do paraquat, e outros poluentes de referência, nos peroxissomas, será necessário desenvolver novos trabalhos com outras concentrações e condições de exposição.

RÉSUMÉ

La découverte de quelques maladies humaines congénitales dues à des déficiences des peroxysomes a énormément augmenté l'intérêt par l'étude de ces organelles, présentes chez la plupart des cellules eucaryotes. Les peroxysomes sont aussi objet d'études toxicologiques, une fois que plusieurs composés chimiques induisent une prolifération peroximal (surtout dans les hépatocytes des espèces sensibles) et quelques de ces proliférateurs sont associés au développement de tumeurs hépatiques.

Basés sur ces faits nous avons essayé une abordage de ce thème chez les animaux aquatiques, notamment dans le foie et le rein, une fois que ces organes sont les plus riches dans ces organelles. Des études chez la truite fario ont mis en évidence que les peroxysomes hépatiques des femelles présentaient des patrons de variation, au niveau morphologique et biochimique, en rapport avec le cycle reproductif, suggérant une modulation de ces organelles par les hormones sexuelles. Après ça, nous nous avons demandé si ce mécanisme de régulation agirait sur les peroxysomes d'autres organes, notamment sur les reins. Finalement il nous a paru utile l'étude stéréologique du rein excréteur chez notre modèle d'étude la truite fario, une fois que les variations saisonnières dans le rein des poissons étaient mal connues. Ainsi, un des objectifs de ce travail était la caractérisation quantitative du rein postérieur de la truite fario pour une meilleure connaissance des variations et corrélations saisonnières.

Nous avons aussi décidé d'étudier l'existence de variations morphologiques et biochimiques dans les peroxysomes rénaux, éventuellement corrélés avec le grade de maturation de la gonade. Dans le but d'étudier l'effet des composés chimiques du stress oxydatif chez les poissons et leur rapport avec les peroxysomes nous avons développé un essai avec des peroxysomes rénaux et hépatiques de la truite fario soumise à une exposition à un pesticide de référence, étudiant les variations structurelles et fonctionnelles de ces organelles. En plus, une fois qu'il est connu que l'urate oxydase hépatique, enzyme peroxymal associée au le catabolisme des purines, présente un patron annuel de variation fortement corrélé avec le grade de maturation de l'ovaire chez la truie fario, nous avons étudié la variation d'autres enzymes impliquées dans cette voie métabolique soit dans le foie soit dans le rein.

Dans ce travail nous décrivons qualitative et quantitativement le rein postérieur de la truite fario pour la première fois, en utilisant des méthodes stéréologiques, au cours de quatre périodes du cycle reproductif notamment dans les mois de Février, Mai, septembre et

Décembre et en comparant males et femelles. Du point de vue qualitatif, le strome et le parénquima présentent des caractéristiques structurelles similaires à celles décrites pour d'autres espèces de teleostes d'eau douce. Du point de vue quantitatif, quelques différences sont observées, soit entre genres soit entre saisons, en ce qui concerne le volume relatif de quelques composants rénaux : corpuscules rénaux, segments I et II des tubules proximales, tubules collecteurs, tubules en formation et tubules vascularisés. Nous avons observé un nombre réduit de corrélations linéaires entre les paramètres stereologiques et l'indice reno-somatique. Ainsi, ces variations saisonnières et les dépendantes du sexe suggèrent une adaptation morphologique des components rénaux de façon à satisfaire des besoins physiologiques. Ces résultats doivent être présentes lorsque quelqu'un veut dans le futur faire des études dont le dessin expérimental et l'interprétation puisse être sous l'influence des variations morphologiques et physiologiques aussi bien que dans l'analyse des résultats d'essais toxicologiques.

Nous avons fait aussi une analyse stéréologique et biochimique des peroxysomes rénaux de males te femelles de la truite fario collectés dans les quatre périodes décrites auparavant. Pour cela les paramètres quantitatifs étudies furent le volume, la surface et le nombre relatif de peroxysomes et aussi la mesure en spectrophotométrie de l'activité des enzymes des peroxysomes. Du point de vue quantitatif, et au niveau structural, plusieurs variations morphologiques étaient observées dans les peroxysomes rénaux des tubules soit entre les quatre périodes de récolte soit entre les sexes. Ces variations concernaient principalement le segment II des tubules proximaux, segment où les peroxysomes furent observés en plus grand nombre et des variations toute au long de l'année. Cependant, et malgré ces différences, aucune corrélation entre les paramètres stéréologiques et l'indice gonado-somatique ne puisse être établie. En ce qui concerne les enzymes des peroxysomes, aucune activité de l'urate oxydase ou de la L - α hydroxiacide oxydase n'était observée. L'activité de la palmitoil.CoA oxydase fût observée stable tout au long de l'année. Par contre, la catalase présentait les plus faibles activités en septembre et en Décembre, soit chez les mâles soit chez les femelles. L'activité de la D-alanine oxydase présentait aussi des variations similaires chez les deux sexes, les plus faibles activités étant observées en Septembre, Décembre et aussi Février. Des corrélations négatives furent établies entre l'indice gonado-somatique et le patron des variations des activités enzymatiques de la catalase et de la D-alanine oxydase, plus forte chez la catalase. Cependant, dans le rein cette corrélation existe entre les sexes, tandis que dans le foie elle n'existe que chez les femelles. Ces résultats suggèrent que les peroxysomes rénaux ne sont pas morphologiquement sous l'influence des processus en rapport avec la

maturation des gonades et en conséquence par les stéroïdes sexuels, au contraire de quelques aspects de leur métabolisme. En plus, ce système de modulation ne semble pas être le même de celui du foie, une fois que les patrons de variation trouvés dans chaque organe sont différents entre eux.

Les stéroïdes féminins, en particulier l'oestradiol, semblent influencer fortement les peroxysomes hépatiques, tandis les rénaux sont probablement influences par d'autres signaux hormonaux sans une implication ultrastructurale évidente. Dans le but d'intégrer les résultats concernant les chapitres 2, 3 et 4 les donnés respectifs furent analysés pour l'activité enzymatique de catalase et de la D-alanine par gramme de rein et aussi en nombre et volume totaux de peroxysomes par gramme de rein. Des corrélations linéaires significatives furent établies entre le patron de variation de l'activité de la D-alanine et le nombre et volume totaux de peroxysomes, mais non avec le patron de variation de la catalase.

La localisation subcellulaire de quelques enzymes impliquées dans le catabolisme des purines fut étudiée chez la truite fario, en raison de la grande variabilité entre espèces reportées. Ainsi, plusieurs fractions cellulaires ont été obtenues après fractionnement différentiel des homogénats totaux de foie, et l'activité des différentes enzymes mesurée par spectrophotométrie. Il est bien connu que chez les vertébrés, poissons inclus, l'urate oxydase est une enzyme peroxysomal et aussi que touts les salmonidés étudiés ne présentent pas l'allantoicase, impliquant que le produit d'excrétion de ces animaux soit l'acide allantoïque ; ces résultas étaient confirmés chez la truite fario. En plus, la xanthine oxydoréductase n'était observée que sous la forme de desydrogenase, comme observé chez d'autres organismes. Après, cette étude, les enzymes xanthine desydrogenase et allantoinase du foie de la truite fario etaient classées comme cytosoliques, une fois que leurs activités furent essentiellement observés dans les surnageants.

Nous avons étudie aussi le patron de variation annuelle des enzymes impliquées dans le catabolisme des purines chez la truite fario, mesurant par spectrophotométrie leur activité dans le foie et le rein. Dans le rein, aucune activité des enzymes urate oxydase et, allantoinase et allantoicase était observé. Dans le foie des femelles les patrons de variation trouves pour l'activité de la xanthine dehydrogenase, de l'urate oxydase et de la allantoinase étaient similaires, les activités plus fortes observées en Mai, diminuant en Septembre et Décembre, l'activité de l'urate oxydase étant même indectetable à cette époque. En plus, dans la fois des males furent observés des patrons de variation semblables à ceux des femelles. Dans le rein la xanthine dehydrogenase des femelles

présentait aussi un patron similaire, mais chez les males des pics d'activité furent observés non seulement en Mai mais aussi en Décembre. En plus, et particulièrement chez les femelles, des corrélations négatives entre les patrons de variation des activités enzymatiques et l'indice gonado-somatique furent établies. Ces résultats suggèrent, d'une façon indirecte, une influence par des hormones sexuelles dans le catabolisme des purines dans le foie de la truite fario, cette modulation etant cependant plus forte chez les femelles que chez les males et concernant, dans le peroxysome, surtout l'activité de l'enzyme urate oxydase.

Pour savoir si les enzymes des peroxysomes peuvent être modifiées et de que façon, quand les poissons sont exposés à des composés provocant le stress oxydative, nous avons établi un essai expérimental pendant 15 jours d'exposition au pesticide paraquat. La possibilité d'une influence sur la biochimique des peroxysomes rénaux et hépatiques de la truite fario fut évaluée par la mesure par spectrophotométrie des activités de trois enzymes des peroxysomes – la catalase, la D-allanine oxydase et l'urate oxydase – aux jours 7 et 15. Malgré l'absence de différences significatives dans les activités enzymatiques dans les échantillons rénaux des groupes contrôle versus traites, des tendances pour une augmentation de l'activité de la catalase et une diminution de la D-allanine oxydase au cours des 15 jours de l'essai, dans les deux groupes. Ainsi, cette étude alerte pour l'occurrence des variations normales au cours du temps, au moins dans notre modèle, l'ensemble des résultats fournissant information utile pour des planning expérimentaux futures. En plus, l'absence d'activité de l'urate oxydase dans le rein fut confirmée dans cette étude. Ainsi, si nous considérons l'utilisation du paraquat dans un dosage de 0,3 mg L^{-1}, nous pouvons conclure que les peroxysomes rénaux et hépatiques ne semblent pas être fonctionnellement affectes. Cependant, de façon a établir le vrai potentiel de toxicité, dans les peroxysomes, du paraquat et d'autres polluants de référence nous devrons développer d'autres essais à des différentes concentrations et conditions d'exposition.

ABBREVIATIONS

AADHAPR	Acyl/Alkyl - DHAP
ADHAPS	Alkyl-DHAP Synthase
ALD	Adrenoleukodistrophy
cAMP	Cyclic Adenosine Monophosphate
ANOVA	Analysis of Variance
AOX	Acyl-CoA Oxidase
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium Chloride
Co	Coenzyme
CO_2	Carbon Dioxide
CV	Coefficient of Variation
DAB	3,3'-Diaminobenzidine
DHAP	Dihydroxyacetone Phosphate
DNA	Deoxyribonucleic Acid
DPIP	2,6 – Dichlorophenolindophenol
EDTA	Ethylenediaminetetraacetic Acid
EM	Electron Microscopy
EROD	Ethoxyresorufin-O-deethylase
FAD	Flavin Adenine Dinucleotide
FMN	Flavin Mononucleotide
GSI	Gonado-somatic Index
HCl	Hydrogen Chloride
H_2O_2	Hydrogen Peroxide
HSI	Hepato-somatic Index
IRD	Refsum Child Disease
$K_3Fe(CN)_6$	Potassium Ferricyanide
KCl	Potassium Chloride
LC_{50}	Lethal Concentration 50
$MgSO_4$	Magnesium Sulphate
$MnSO_4$	Manganese Sulphate
MOPS	3-(N-Morpholino)propanesulfonic Acid
NaCl	Sodium Chloride
NAD^+	Nicotinamide Adenine Dinucleotide

$NADP^+$	Nicotinamide Adenine Dinucleotide Phosphate
$Na_2HPO_4•2H_2O$	Disodium Phosphate Dihidrogenate
$NaH_2PO_4•H_2O$	Sodium Dihydrogen Phosphate 1-hydrate
$NaHCO_3$	Sodium Bicarbonate
NALD	Neonatal Adrenoleukodistrophy
NaOH	Sodium Hydroxide
NH_3	Ammonium Hydroxide
O_2^-	Superoxide Radical
$OH^•$	Hydroxyl Radical
OsO_4	Osmium Tetroxide
Pex or *PEX*	Peroxin
PH	Peroxisomal Hydratase-dehydrogenase-isomerase (Multifunctional Enzyme)
PMP	Peroxisomal Membrane Protein
PMSF	Phenylmethylsulfonyl Fluoride
PP	Peroxisomal Proliferator
PPAR	Peroxisome Proliferator Activated Receptor
PTI	Proximal Tubule Segment I
PTII	Proximal Tubule Segment II
PTS	Peroxisomal Targeting Signal
ROS	Reactive Oxygen Species
RSI	Reno-somatic Index
RXR	Retinoic X Receptor
SKL	Serine – Lysine – Leucyne
SOD	Superoxide Dismutase
TEM	Transmission Electron Microscopy
Tris-HCl	Tris(Hydroxymethyl)aminomethane hydrochloride
Triton X-100	t-Octylphenoxypolyethoxyethanol
X-ALD	Adrenoleukodistrophy linked to X chromosome
ZS	Zellweger Syndrome

CHAPTER 1

GENERAL INTRODUCTION

1.1. The Peroxisome

1.1.1. Discovery

The name “peroxisome” was used for the first time in 1965 by Christian de Duve and coworkers (de Duve, 1965; de Duve and Baudhuin, 1966) but it was J. Rhodin who discovered them approximately ten years earlier, when he observed mouse kidney proximal tubule cells with a transmission electron microscope. To an electron-dense organelle surrounded with a single membrane he called “microbody”. Since its discovery, microbodies have been widely investigated and their presence was revealed in almost all eukaryotic cells (Lazarow and Fujiki, 1985). Peroxisome definition is based in its main characteristic: the presence of H_2O_2 (hydrogen peroxide) producing oxidases and catalase that degrades it (de Duve, 1965; de Duve and Baudhuin, 1966). Indeed, the term microbodies is applied nowadays to all organelles similar to peroxisomes as the glyoxysomes from plants and some fungi (Breidenbach and Beevers, 1967; Maxwell *et al.*, 1977; Veenhuis *et al.*, 1987) and the glycosomes of the parasite trypanosomatids (Opperdoes and Borst, 1977). Glyoxysomes and glycosomes are also single-membrane organelles but functionally different from peroxisomes. Thus, in addition to the enzymes responsible for the β-oxidation, plant glyoxysomes located in the germinating seeds also possess key enzymes of glyoxylate cycle, such as isocitrate lyase and malate synthase (Michels *et al.*, 2005). On the other hand, glycosomes contain the enzymes involved in the major part of the glycolytic pathway (Michels *et al.*, 2005).

1.1.2. Morphology and Composition

Peroxisomes can be described as cytoplasmic organelles with a finely granular matrix bounded by a single membrane (Figure 1), normally spherical or oval in shape with a diameter ranging from 0.1 to 1.5 µm, or eventually bigger (3 µm) as in rat kidney peroxisomes (Barrett and Heidger, 1975) and in mullet *Mugil cephalus* (Orbea *et al.*, 1999). However, their morphological and biochemical features (number, size, shape and enzymatic composition) can vary depending on the species, cell types and physiological state. In relation to the peroxisomal enzymatic content, catalase is the most abundant enzyme, but they also possess oxidases, acyl-transferases, desidrogenases, amino-transferases, glyoxilic acid cycle enzymes and enzymes for the plasmalogens and biliary acids synthesis. Peroxisomes can be found in several organs such as liver, kidney (Masters and Crane, 1995b), testis (Nemali *et al.*, 1988), intestine (Roels *et al.*, 1991) and

brain (Lazo *et al.*, 1991) among others, in which they can present specific enzymatic composition. Despite the existence of peroxisomes in several organs, literature about these organelles in fishes focuses essentially liver peroxisomes as a target for many of those studies.

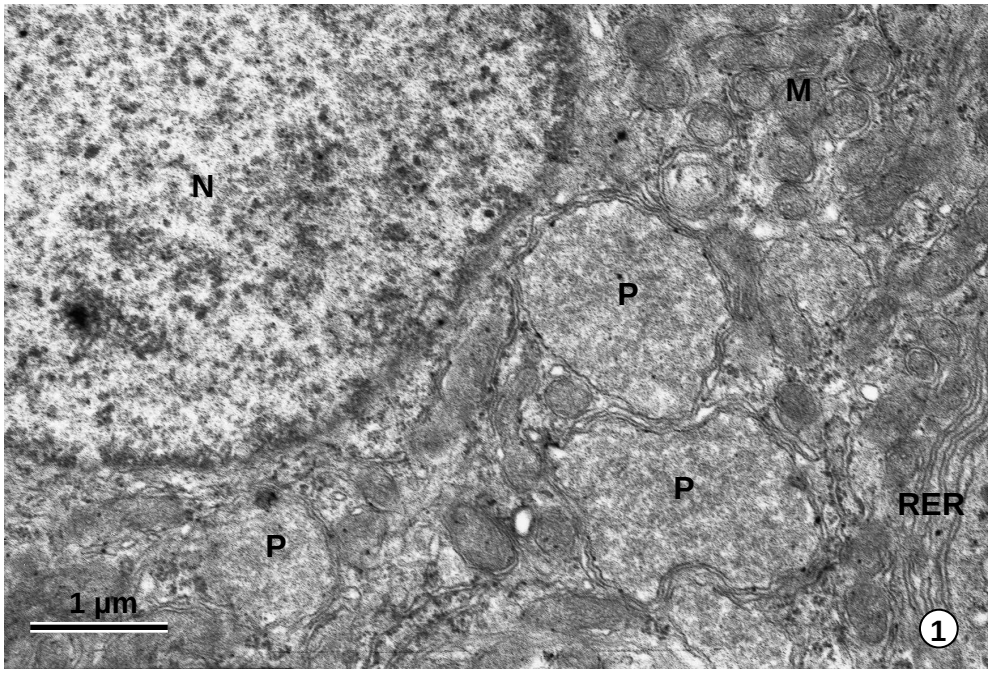


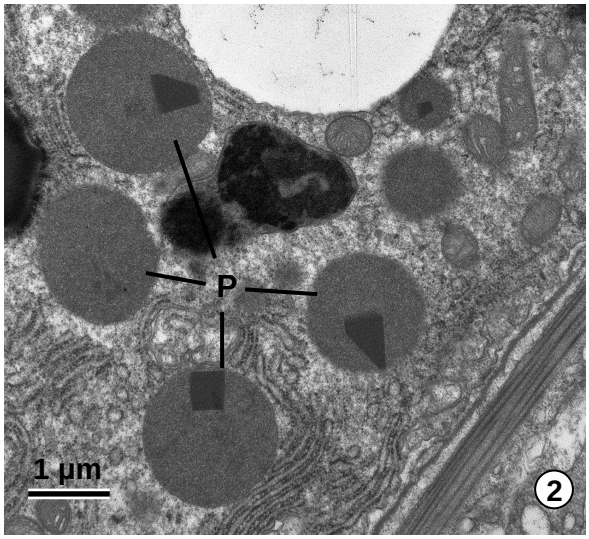
FIGURE 1. Transmission electron microscope (TEM) image of peroxisomes (P) from proximal tubules of brown trout kidney. Peroxisomes are located close to the nucleus (N), surrounded with mitochondria (M) and cisternae of rough endoplasmic reticulum (RER).

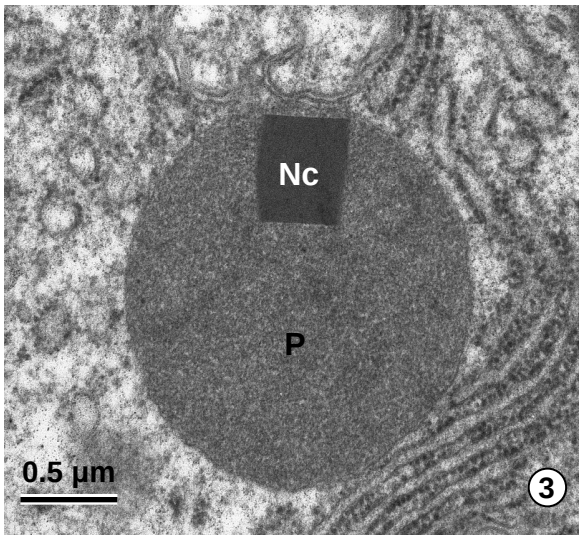
Crystalline cores, also named nucleoid are frequent in the peroxisomal matrix (Figure 2 and 3). Other crystalline structures could also be observed close to the peroxisomal membrane - the marginal plate. These inclusions are formed due to the existence of some insoluble proteins in high concentrations, which crystallize (Veenhuis *et al.*, 1981; Zaar and Fahimi, 1991; Fahimi *et al.*, 1993b). Normally, the nucleoid is associated with the existence of urate oxidase (Volkl *et al.*, 1988) and the inexistence of this enzyme is associated with peroxisomes devoid of nucleoids, as happens in liver peroxisomes of some primates, which does not possess neither urate oxidase nor nucleoid (Usuda *et al.*, 1988a; Yeldandi *et al.*, 1990). Nevertheless, exceptions occur like in rat and human kidney peroxisomes which exhibit nucleoid but no urate oxidase activity, and some non-mammalian vertebrates possess high levels of urate oxidase in hepatic peroxisomes but

lack crystalline structures (Masters and Holmes, 1977). The simultaneous presence of urate oxidase in the crystalline core and in the peroxisomal matrix was also described (Vandenmunckhof *et al.*, 1994). Other enzymes such as xanthine oxidase were also reported in peroxisomal cores of rodent hepatocytes (Angermuller *et al.*, 1987). Although common in organisms from different taxonomic groups, nucleoids are rarely found in glioxysomes and glycosomes. The existence of marginal plates gives an angular shape to peroxisomes and it consists in cristalized L-α-hydroxyacid oxidase B (Zaar, 1992). Mammalian renal peroxisomes seem to have more frequently these inclusions, but they can also be found in other organs (Sima, 1980; Gorgas and Zaar, 1984; Zaar *et al.*, 1991).

Peroxisomes are dynamic organelles able to modify their morphology and their enzymatic content influenced by different environmental factors, resulting in alterations of their metabolic capabilities. For this reason, many studies have been performed in order to verify peroxisomal changes in response to hypolipidaemic drugs and other chemicals (Reddy *et al.*, 1982).

The correct identification of peroxisomes in tissues at electron microscopy level is based not only on their morphological features, but also on the cytochemical demonstration of some of their enzymatic activities. The detection of catalase is widely used for this purpose (Figure 4), in which 3,3'-diaminobenzidine (DAB) behaves as electron donor on the peroxidatic reaction of catalase, in which this enzyme oxidize DAB in the presence of H_2O_2, producing polymeric complexes with osmium (Essner, 1974). Catalase activity was also detected in many fishes like in other aquatic organisms, and used to identify these organelles through DAB labelling (Kramar *et al.*, 1974; Veenhuis and Wendelaarbonga, 1977; Goldenberg *et al.*, 1978; Braunbeck *et al.*, 1987; Braunbeck and Volkl, 1991; Lallier and Walsh, 1991; Cajaraville *et al.*, 1992; Lobo-da-cunha, 1995; 1997). The use of monoclonal antibodies for immunocytochemistry in conjugation with protein A-gold is sometimes applied to identify peroxisomal enzymes and to verify its localization in the peroxisome at electron microscopy level (Yokota *et al.*, 1987; Usuda *et al.*, 1988b). In addition, centrifugal fractionation is another methodology which has been used in peroxisomal studies. This technique is performed to achieve pure peroxisomal fraction and nowadays much literature exists with different procedures regarding this methodology (Leighton *et al.*, 1968; Wattiaux *et al.*, 1978; Appelkvist *et al.*, 1981; Opperdoes *et al.*, 1984; Crane *et al.*, 1985; Hajra and Wu, 1985; Hartl *et al.*, 1985; Volkl and Fahimi, 1985).





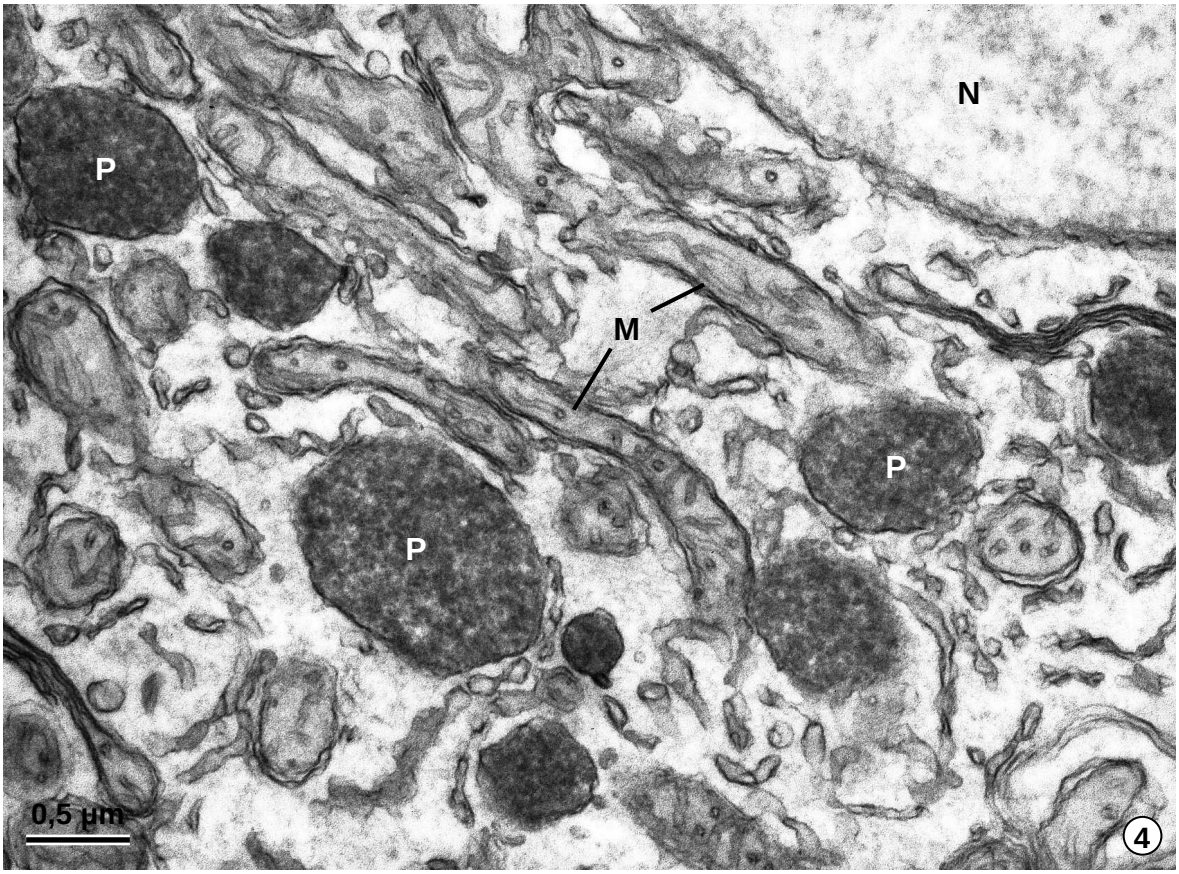


FIGURE 2. and **FIGURE 3.** Electron micrograph of peroxisomes (P) from the mollusc *Bulla striata*, showing the nucleoid (Nc) located in their matrix. **FIGURE 4.** TEM micrograph of renal proximal tubules of brown trout showing DAB stained peroxisomes (P) surrounded with mitochondria (M) and located close to the nucleus (N). Images 3 and 4 were kindly provided by Prof. Alexandre Lobo da Cunha.

If separation of peroxisomes is set with disruption of the peroxisomal membrane and the release of their content, a differential centrifugal fractionation by compartments will give us the knowledge of the location of each enzyme (de Duve and Baudhuin, 1966). This procedure is useful to determine which enzymes are located in the peroxisomal core, matrix or membrane, and also contributes to a better understanding of the peroxisomal metabolism.

Fish peroxisomes vary their morphological and physiological features with species, and with other factors, such as gender or environmental conditions. In brown trout, liver peroxisomes present a spheroidal (or at most ovoidal) shape without nucleoids or marginal plates and its size ranges in average from 0.4 – 0.6 µm of spherical equivalent diameter (Rocha *et al.*, 1999).

Rocha *et al.* (1999) described some peroxisomal alterations in the liver of brown trout. They observed that hepatic peroxisomes from vitellogenic females are smaller and with decreased relative and total volume and surface per cell, when compared with other breeding cycle periods. These variations were also negatively correlated with the gonado-somatic index (GSI). However, the relative and total peroxisome number per cell did not significantly change along the year. Moreover, males also presented seasonal alterations in hepatic peroxisomes, but with a different pattern, with size being positively correlated with the GSI. In addition to these morphological variations, functional changes also occurred. Peroxisomal enzymatic activities showed along the year patterns of variation that were also correlated with the GSI (Rocha *et al.*, 2001; Resende *et al.*, 2005). These data strongly suggested an influence of 17β-estradiol in female trout hepatic peroxisomes.

1.1.3. Function

Peroxisomes were considered organelles of little importance and their true functions were unknown for long time. Currently, their fundamental role in cell physiology is recognized. Peroxisomes are important organelles because they actively participate in many vital metabolic pathways as the synthesis of bile acids, plasmalogens and cholesterol, the β-oxidation of long chain fatty acids and the catabolism of purines, prostaglandins, leucotrienes, aminoacids and xenobiotics, and the elimination of some reactive oxygen species. The relevance of each peroxisomal function is dependent from the cellular type where the peroxisomes are located and the environmental conditions. Nonetheless, in fishes, the knowledge about peroxisome function is still very limited and mainly regarding β-oxidation and purine catabolism pathways.

In humans, alterations or absence of those peroxisomal functions are directly related with severe clinical symptoms. Nowadays almost twenty diseases associated to peroxisomal disorders are known, which could be classified in two types depending on its origin: the peroxisome biogenesis defects and the lack or deficiency of peroxisomal enzymes (Wanders, 2004; Wanders and Waterham, 2006a). The most common diseases associated to peroxisomal biogenesis are the Zellweger (cerebrohepatorenal) syndrome (ZS) (Danks *et al.*, 1975; Kelley, 1983), the neonatal adrenoleukodystrophy (NALD) (Stewart *et al.*, 1994) and the Refsum child disease (IRD), where genes involved in the codification of some peroxisomal membrane proteins involved in biogenesis (peroxins) suffers several types of mutations, resulting in a morphological abnormality of peroxisomes as well as their reduced numbers or even their absence (Schutgens *et al.*, 1986; Moser, 1993; Subramani, 1998; Wanders, 2004; Wanders and Waterham, 2006a). From the second group of diseases, the well-known lipid metabolism disease called adrenoleukodystrophy is a disease linked to the X chromosome (X-ALD), which is caused by a mutation in the ALD gene resulting a defective enzyme involved in peroxisomal membrane transport (Moser *et al.*, 1992; Moser, 1993; Wanders, 2004; Wanders and Waterham, 2006a). Another example from the last class of disorders, the rhizomelic form of chondrodysplasia punctata is an example of a deficiency of several peroxisomal enzymes (Schutgens *et al.*, 1986; Moser, 1993; Wanders, 2004; Wanders and Waterham, 2006a). However, in this last group, peroxisomes appear morphologically normal and at usual levels in terms of number.

1.1.3.1. Fatty acids β-oxidation

The β-oxidation is a major function of peroxisomes, consisting in the breakdown of fatty acid molecules in units of two carbons in the form of acetyl-CoA (Figure 5). This function is shared with mitochondria in animal cells, but not in yeast and plant cells where peroxisomes are exclusively responsible for β-oxidation (Mannaerts and Vanveldhoven, 1993; Reddy and Mannaerts, 1994; Wanders and Waterham, 2006b).

In peroxisomes, and before oxidation of saturated or unsaturated fatty acids, an activation of those molecules is needed, *i.e.* a transformation in CoA esters. This first step is regulated by several acyl-CoA synthetases generally located in the peroxisomal membrane (Mannaerts and Debeer, 1982). Inside the peroxisome, the Acyl-CoA esters are metabolized through a four step reaction: first, a dehydrogenation by the acyl-CoA oxidase into 2-trans-enoyl-CoA; second, the enoyl-CoA hydratase hydrate the last product

into L-3-hydroxiacyl-CoA; third, the 3-hydroxyacyl-CoA dehydrogenase performs another dehydrogenation into 3-keto-acyl-CoA, and finally a thyolitic cleavage by the 3-ketoacyl-CoA thiolase occur resulting in acetyl-CoA molecule and an acyl-CoA derivative shorter two carbon units, which could restart the peroxisomal β-oxidation cycle till be in the appropriate size to be transported and oxidized in mitochondria (Lazarow, 1978; Hashimoto and Hayashi, 1987; Reddy and Mannaerts, 1994; Singh, 1997). Further, the final products of peroxisomal β-oxidation are fully oxidized in mitochondria or could be integrated in other metabolic pathways (Masters and Crane, 1992; Reddy and Mannaerts, 1994; Fan *et al.*, 1996).

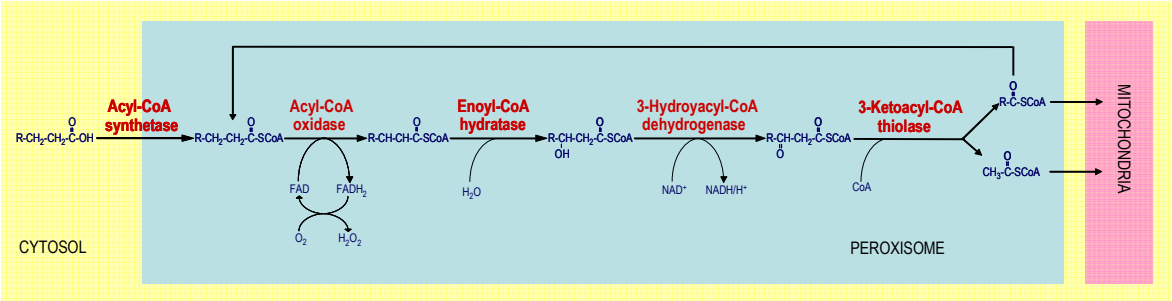


FIGURE 5. β-oxidation metabolic pathway occurring in mammalian hepatic peroxisomes. Enzymes appear in red. Shortened fatty acids and acetyl-CoA can be further oxidized in mitochondria or used as substrates for other metabolic pathways. Adapted from Fahimi *et al.* (1993a).

Two β-oxidation enzymes, namely acyl-CoA oxidase (AOX) and the bifunctional enzyme (PH) enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase, were identified in several fish species, such as rainbow trout *Onchorynchus mykiss* (Baldwin *et al.*, 1990; Yang *et al.*, 1990; Donohue *et al.*, 1993; Scarano *et al.*, 1994), in medaka *Oryzias latipes* (Scarano *et al.*, 1994), channel catfish *Ictalurus punctatus* (Gallagher and Digiulio, 1991) and gilthead seabream *Sparus aurata* (Pedrajas *et al.*, 1996). Carnitine acetyltransferase, a required enzyme to the transport of the long-chain acyl groups from fatty acids into the mitochondria or the peroxisome was studied in ray *Raja erinacea* (Kramar *et al.*, 1974) and in goldfish *Carassius auratus* (Stewart *et al.*, 1994).

There are significant differences between peroxisomal and mithocondrial β-oxidation processes. First, the enzymes involved in this metabolic pathway have particular molecular properties and three enzymes are necessary in peroxisomes compared to the four required in mitochondria counterpart (Hashimoto, 1982; Mannaerts and Vanveldhoven, 1992; 1993; Masters and Crane, 1995b). Second, mitochondria usually

metabolizes the common fatty acids or straight chain fatty acids (molecules with 16 to 20 carbons) till complete oxidation through Krebs cycle and concomitant ATP production, whereas peroxisomal β-oxidation is not directly coupled to ATP synthesis, instead, mainly long and very long chain fatty acids are partially degraded to shorter chain molecules. Another difference is the formation of H_2O_2 in peroxisomes resulting from the acyl-CoA oxidase step (Masters and Crane, 1992). Moreover, peroxisomes accept other substrates in this metabolic pathway, as dicarboxylic acids (Kolvraa and Gregersen, 1986) and bile acid precursors (Kase *et al.*, 1986; Bjorkhem, 1992; Russell and Setchell, 1992), as well as prostaglandins (Schepers *et al.*, 1988) and xenobiotics (Yamada *et al.*, 1987; Yoshida *et al.*, 1990). In addition, peroxisomes also seem to be more active in the oxidation of unsaturated fatty acids as palmitoleic, oleic or polyunsaturated acids (Masters and Crane, 1995b; Wanders and Waterham, 2006b).

1.1.3.2. Cholesterol synthesis

Cholesterol and dolichol, as others isoprenoids products, are synthesized via mevalonate pathway, sharing the same initial steps till farnesyl pyrophosphate production (Goldstein and Brown, 1990). Cholesterol is required to assure membrane integrity, which is extremely important for cellular equilibrium and it is also a precursor of steroid hormones and bile acids. In turn, dolichol is a membrane component involved in protein glycosilation (Burda and Aebi, 1999) and might also function as a cell membrane free radical scavenger (Bizzarri *et al.*, 2003). Some controversial exists around the localization of cholesterol synthesis enzymes, being particularly the participation of peroxisomes still well-understanded, because conflicting data have been published from last decades (Mannaerts and Vanveldhoven, 1993; Aboushadi *et al.*, 1999; Olivier *et al.*, 2000; Wanders and Waterham, 2006b; Weinhofer *et al.*, 2006). However, more recently this issue was re-investigated and authors reported that some enzymes involved in the cholesterol biosynthesis, such as phosphomevalonate kinase, isopentenyl diphosphate isomerase, mevalonate kinase and farnesyl diphosphate synthase, were clearly peroxisomal enzymes (Kovacs *et al.*, 2007).

Cholesterol degradation results in several bile acids intermediates, namely dihydroxicoprostanoic and trihydroxicoprostanoic acids (Pedersen, 1993), which are excreted in bile participating in the digestion and lipid absorption. These compounds are metabolized to CoA esters and further shortened in peroxisomes by β-oxidation (Kase *et al.*, 1983; 1986; Prydz *et al.*, 1986) via trihydroxycoprostanoyl-CoA oxidase (Pedersen, 1993).

1.1.3.3. Ether-phospholipid biosynthesis

Ether-phospholipids are important components in the structural and functional mantainence of biological membranes. This class of compounds may occur in one of two forms: the plasmanyl-phospholipids or the plasmenyl-phospholipids (plasmalogens) (Nagan and Zoeller, 2001; Gorgas *et al.*, 2006; Wanders and Waterham, 2006b). In mammals, aproximately 20% of the phospholipids from the biological membranes are constituted by plasmalogens, which could contain ethanolamine or choline as the head group, being the former the predominant in human tissues, particularly abundant in organs such as brain, heart and skeletal muscle, whereas the latter occur with higher levels in heart and skeletal muscle (Wanders and Waterham, 2006b). Peroxisomes are involved in the ether-phospholipids synthesis, since the first steps of this metabolic pathway occurred in these organelles, namely the esterification of dihydroxyacetone phosphate (DHAP) by the DHAP acyltransferase and the exchange of the long-chain fatty acid from the DHAP to a long-chain fatty alcohol via alkyl-DHAP synthase (ADHAPS) activity (Nagan and Zoeller, 2001). These two enzymes are exclusively peroxisomal enzymes, whereas the acyl/alkyl-DHAP reductase (AADHAPR) could be found bounding either to the membrane of peroxisomes or to the endoplasmatic reticulum, and which is involved in the third step of ether-phospholipids synthesis (Nagan and Zoeller, 2001; Wanders and Waterham, 2006b). Further steps of plasmalogens synthesis occur in the endoplasmic reticulum. The physiological role of plasmalogens is still being studied, although indications for their participation in processes such as membrane dynamics, intracellular signalling, cholesterol transport and metabolism, oxidative stress and polyunsaturated fatty acid metabolism have been proposed (Nagan and Zoeller, 2001; Gorgas *et al.*, 2006; Wanders and Waterham, 2006b). In addition, several human diseases are also related with deficiencies of some of the enzymes intervenient in this metabolism (Nagan and Zoeller, 2001; Gorgas *et al.*, 2006; Wanders and Waterham, 2006b).

1.1.3.4. Purine catabolism

Purine degradation is a strongly divergent pathway from species to species. All vertebrates present the same pathway reactions from the nucleotides until uric acid formation (Figure 6). First, purine nucleotides such adenine, guanine and their derivatives are converted to xanthine, followed by the transformation into uric acid under xanthine oxidoreductase action (Hayashi *et al.*, 2000).

As birds, terrestrial reptiles and some insect species, higher primates excrete uric acid as

the final product of purine catabolism in consequence of mutations in the urate oxidase gene which result in a stop codon, leading to the absence of that enzyme activity (Yeldandi *et al.*, 1990; Wu *et al.*, 1992). In humans, this lost of urate oxidase activity is the origin of symptoms as the gout pain. Other mammals and some reptiles degrade uric acid into allantoin by urate oxidase activity (Friedman *et al.*, 1985; Usuda *et al.*, 1988a). Amphibians and fishes possess a longer metabolic pathway, in which allantoinase transform allantoin into allantoic acid, and allantoicase and ureidoglycollate lyase degrade it further into urea and glyoxylate (Scott *et al.*, 1969; Hayashi *et al.*, 2000). For some of those species, urea is the excretion product, whereas for others which posses urease, the final product is ammonia in result of urease activity.

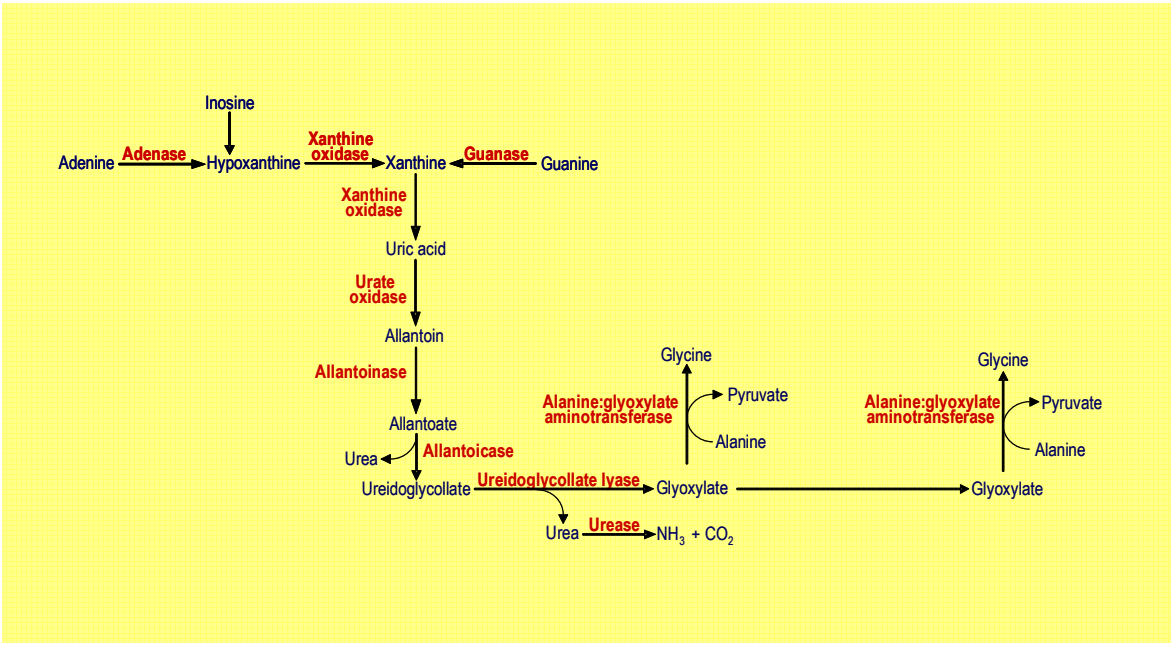


FIGURE 6. Metabolic pathway of purine degradation in the liver of some freshwater fish species. Adapted from Sakuraba *et al.* (1996).

The location of each enzyme involved in this metabolic pathway varies from species to species, but, in general, uric acid formation occurs in the cytosol and its degradation in the peroxisomes. However, Angermuller *et al.* (1987) described the presence of xanthine oxidase in rat hepatic peroxisomes as also Scott (1969) in liver and kidney from certain amphibian and bird species. On the other hand, urate oxidase is always located in peroxisomes, in the nucleoid (Baudhuin *et al.*, 1965; Volkl *et al.*, 1988) or in the matrix (Noguchi *et al.*, 1979). The location of allantoinase and allantoicase is more controversial,

but, generally, allantoinase was reported in the peroxisomal matrix of some organisms whereas allantoicase occurs in the peroxisomal membrane (Hayashi *et al.*, 1989). However, Yeldandi *et al.* (1995) performed immunocytochemical studies which revealed a mitochondrial localization for allantoinase and a cytosolic one for allantoicase, in frog liver and kidney. Some authors classified two groups of fishes depending on the presence and localization of those two last enzymes - in freshwater species allantoinase was located in the cytosol whereas in marine fishes this enzyme was cytosolic and peroxisomal (Fujiwara *et al.*, 1989; Hayashi *et al.*, 1989; Sakuraba *et al.*, 1996). Allantoicase was described as a peroxisomal matrix enzyme for some species and a peroxisomal membrane for others (Hayashi *et al.*, 1989). This enzyme is particularly interesting in an evolutionary point of view because in amphibians it was observed in association with cytosolic allantoinase, which was further lost in higher organisms (Hayashi *et al.*, 1989; Usuda *et al.*, 1994; 2000). Urate oxidase was identified as a peroxisomal matrix enzyme in two mackerel species, the *Pneumatophorus japonicus* and *Trachurus trachurus* (Noguchi *et al.*, 1979) and as a peroxisomal membrane linked enzyme in the carp *Cyprinus carpio* (Goldenberg, 1977; Goldenberg *et al.*, 1978). On the contrary, in most of the reported cases, xanthine oxidase was always identified as a cytosolic enzyme (Moriwaki *et al.*, 1999).

1.1.3.5. Polyamines, amino acids and glyoxylate catabolism

Polyamines are compounds involved in regulation of cellular growth and differentiation, although their actual function is not entirely clear. However, it is known that polyamines are able to bind nucleic acids, especially DNA (Feuerstein *et al.*, 1990; 1991; Tippin and Sundaralingam, 1997; Deng *et al.*, 2000). Polyamine oxidase is a peroxisomal enzyme present in several mammalian organs, such as kidney, liver and intestine (Beard *et al.*, 1985; Vandenmunckhof *et al.*, 1995), and which appear with very high levels during peroxisomal and cellular proliferation (Hayashi *et al.*, 1989). This enzyme is responsible for the final polyamine catabolic pathway, namely the spermine and N-acetylspermidine conversion into putrescine (Wanders and Waterham, 2006b).

Amino acid metabolism involve several enzymes such D-amino acid oxidase, D-aspartate oxidase, L-pipecolate oxidase, alanine:glyoxylate aminotransferase I and L-α-hydroxyacid oxidase (types A and B) (Yokota *et al.*, 1985; Noguchi, 1987; Angermuller and Fahimi, 1988; Wanders *et al.*, 1989; Zaar *et al.*, 1989; Zaar and Fahimi, 1991; Zaar, 1992; Danpure, 1993; Mannaerts and Vanveldhoven, 1993; 1996) (Figure 7).

D-amino acid oxidase and L-α-hydroxyacid oxidase were already described in the carp

Cyprinus carpio liver peroxisomes (Kramar *et al.*, 1974; Goldenberg *et al.*, 1978). In the kidney peroxisomes of the three-spined stickleback *Gasterosteus aculeatus trachurus* and in the intestinal mucosa of the goldfish *Carassius auratus* it was also identified D-amino acid oxidase (Connock, 1973; Veenhuis and Wendelaarbonga, 1977). In turn, glycolate oxidase was characterized in hepatic peroxisomes of carp *C. carpio*, prawn *Penaeus japonicus* and Atlantic horse mackerel *Trachurus trachurus* (Goldenberg *et al.*, 1978; Noguchi *et al.*, 1979). Sea bream *Sparus aurata* liver peroxisomes also presented the antioxidant enzyme Cu,Zn-superoxide dismutase (Pedrajas *et al.*, 1996).

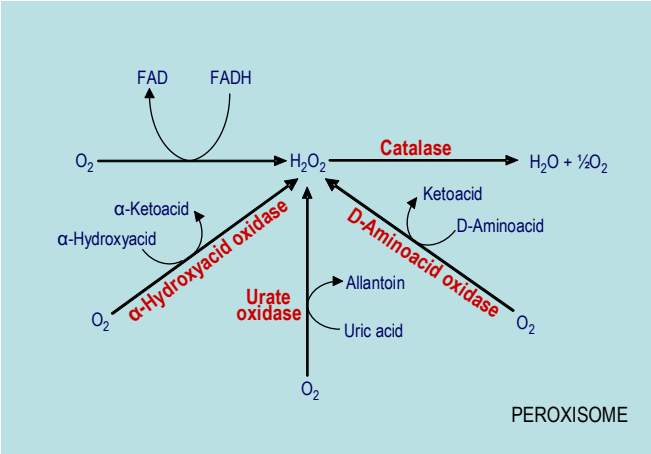


FIGURE 7. Illustration of the metabolic pathways of some peroxisomal enzymes.

D-aminoacid oxidase was one of the first enzymes found in peroxisomes (de Duve and Baudhuin, 1966) which, as D-aspartate oxidase, has the ability to oxidase the D-isomeres. Although the first enzyme acts in neutral and basic aminoacids, instead of the acidic aminoacids as D-aspartate oxidase (Zaar *et al.*, 1989; Wanders and Waterham, 2006b). Ketoacids, ammoniac and hydrogen peroxide are the end products of these reactions (Mannaerts and Vanveldhoven, 1993). Although the true function of these enzymes is still unclear, it is known that D-amino acids are present in the peptidoglycans of the bacterial cell wall and their participation in the D-aminoacid catabolism of intestinal microbial flora was pointed as a possibility (Hoeprich, 1965; Konno *et al.*, 1989). Hamilton *et al.* (1987) hypotised that these enzymes catalyzes reactions with different substrates than D-aminoacids, such as cysteamine, L-cysteine or L-cysteinylglycine. From those reactions result final products which possibly participate in hormones formation, *e.g.* insulin. Moreover, some D-amino acids were identified, in free form or as part of neuropeptides, in

the nervous tissue of some molluscs, amphibians and vertebrates, where they seem to play essential neuromodulatory functions (Fujisawa *et al.*, 1992; Daniello *et al.*, 1993a; 1993b; Hashimoto *et al.*, 1993; Yasudakamatani *et al.*, 1995; Zaar *et al.*, 2002).

Alanine:glyoxylate aminotransferase I is an enzyme which was identified in rabbit, guinea pig, rat and human peroxisomes where it plays a crucial role in the glyoxylate pathway (Figure 7). In this metabolism, amino acids work as amino donors and glyoxylate and pyruvate as amino receptors (Danpure, 1993; Mannaerts and Vanveldhoven, 1993; Wanders and Waterham, 2006b). The peroxisomal enzyme L-α-hydroxyacid oxidase A mediates the transformation of glycolate in glyoxylate, and this last product in oxalate (Mannaerts and Vanveldhoven, 1993). Glyoxylate could also be transformed in glycine by alanine:glyoxylate aminotransferase I. In cases of absence of this enzyme, oxalate is accumulated in high levels causing calcium oxalate deposition and renal failure – Prymary Hyperoxaluria Type I (Danpure, 1993). Type A of L-α-hydroxyacid oxidase is mainly a hepatic peroxisomal enzyme, also found in kidney peroxisomes of some species (Yokota *et al.*, 1985), which preferably metabolize short-chain aliphatic L-α-hydroxyacids into specific ketoacids and hydrogen peroxide. While type B is part of kidney peroxisomes marginal plates (Zaar *et al.*, 1991), oxidizing a diversity of other hydroxyacids as the long chain and aromatic L-α-hydroxyacids, as well as its catalytic function of the α-amino acids oxidative deamination (Zaar and Fahimi, 1991; Mannaerts and Vanveldhoven, 1993; Masters and Crane, 1995b). The resulting carbon units from all those reactions are then used in gluconeogenesis which occurs in liver and kidney (Mannaerts and Vanveldhoven, 1993).

L-lysine degradation could occur through two processes: the saccharopine and the L-pipecolate pathways (Mannaerts and Vanveldhoven, 1993; Wanders and Waterham, 2006b). The L-pipecolate metabolism occurs actively in brain while in other tissues the saccharopine pathway predominate (Hutzler and Dancis, 1968; Chang, 1978; 1982). The peroxisomal enzyme L-pipecolate oxidase acts in this metabolic pathway, and its substrate – L-pipecolate - could be accumulated in tissues and body fluids from peroxisomal deficiency patients (Lazarow and Moser, 1989).

All of these peroxisomal oxidases and transaminations of amino acids are interconnected with citric acid cycle intermediates suggesting a very important role of peroxisomes in cellular amino acid degradation (Masters and Crane, 1995a).

1.1.3.6. Metabolism of xenobiotics

Xenobiotics like drugs, herbicides and phthalate plasticizers, are metabolized mostly in liver, resulting in their deactivation by enzyme activities and excretion, especially in urine and bile. The xenobiotics excretion is easier when these molecules acquire a carboxyl group via ω-oxidation and consequently are shortened via β-oxidation into more polar molecules (Mannaerts and Vanveldhoven, 1993). Peroxisomal β-oxidation is more effective in xenobiotic acyl compounds degradation than the mitochondrial counterpart (Yamada *et al.*, 1987; Yoshida *et al.*, 1990).

1.1.3.7. Metabolism of reactive oxygen species (ROS)

In peroxisomal respiration, H_2O_2 is formed (Deduve and Baudhuin, 1966), with concomitant oxygen consumption, after oxidation of specific substrates by peroxisomal enzymes such as urate oxidase, D-aminoacid oxidase, D-aspartate oxidase, α-hydroxycacid oxidase, oxalate oxidase, pipecolic acid oxidase, polyamine oxidase, palmitoyl-CoA oxidase, pristanoyl-CoA oxidase, glutaryl-CoA oxidase, trihydroxycoprostanoyl-CoA oxidase (Schrader and Fahimi, 2004). Even in less extent, other toxic molecules could also be produced as consequence of the conversion of xanthine dehydrogenase in xanthine oxidase under specific conditions (Engerson *et al.*, 1987) or several cytochrome membrane proteins activities (Lopez-Huertas *et al.*, 1997; 1999), like the superoxide radical (O_2^-) which in the presence of transition metals originate the extremely reactive hydroxyl radical ($OH^\bullet$) (Lazarow, 1980b). Further, H_2O_2 and their intermediates are detoxified by catalase, glutathione peroxidase and some superoxide dismutases (Dhaunsi *et al.*, 1993; Singh, 1997), in water and oxygen. The H_2O_2 decomposition could be performed by two reactions – the catalytic and the peroxidatic pathways, being the second process the most incident, in which ethanol, methanol, quinones or nitrites, among others, are used as substrates (Chance and Oshino, 1971). Additionally to the presence of those enzymes involved in the ROS metabolism in the cytoplasm or organelles, such as mitochondria or lysosomes, they are also found in the peroxisomal membrane or matrix (Orbea *et al.*, 2000; Schrader and Fahimi, 2004). This fact confers to peroxisomes an extremely important role in cell protection due to the maintenance of their normal physiology even when they are under action of toxic oxygen metabolites.

In addition to the described functions, peroxisomes also contain enzymes which are

involved in other metabolic pathways, such as pipecolic acid metabolism, phytanic acid α-oxidation, hexose monophosphate production and fatty acid elongation (Vandenbosch *et al.*, 1992; Mannaerts and Vanveldhoven, 1993; Singh, 1997; Wanders and Waterham, 2006b).

1.1.4. Biogenesis

Initially, peroxisomes were believed to derive directly from the endoplasmatic reticulum because membranes of these two organelles were persistently observed in close proximity in rat liver electron microscopy studies (Novikoff and Goldfisc.S, 1969; Novikoff and Novikoff, 1972). Nonetheless, in the 1980's many studies concerning this subject concluded that the membrane and matrix peroxisomal proteins were codified by nuclear genes, then syntethized in free cytosolic rybosomes and post-translationally imported to peroxisomes (Goldman and Blobel, 1978; Fujiki *et al.*, 1984; Lazarow and Fujiki, 1985; Imanaka *et al.*, 1996). In addition, studies in yeast also contributed to the indication that peroxisomes may multiply by division (Veenhuis *et al.*, 1979). With these findings, in the middle 1980's a new theory was developed – both fusion and fission of existing peroxisomes could originate new peroxisomes (Lazarow and Fujiki, 1985). Under this theory, new peroxisomes can result from the division of pre-existing ones, which import membrane and matrix proteins afterwards, was generally accepted (Lazarow, 1980c; 1980a; Gorgas, 1985; Fahimi *et al.*, 1993b). This hypothesis implies that every cell must posseses since its origin at least one peroxisome and the assurance of transmiting one of these organelles during mitosis.

More recently, the above last hypothesis was questioned since peroxisomes in yeast and mammal species could appear even in the absence of pre-existing ones (Matsuzono *et al.*, 1999; South and Gould, 1999; Sacksteder and Gould, 2000; South *et al.*, 2000; Tabak *et al.*, 2006). However, the fact that in the yeast species *Yarrowia lipolytica* and *Hansenula polymorpha* some peroxisomal membrane proteins (or their derivatives) are only found in peroxisomes and endoplasmic reticulum, renewed attention was attained to the participation of this last organelle in the peroxisomal biogenesis (Titorenko *et al.*, 1997; Titorenko and Rachubinski, 2001; Faber *et al.*, 2002). Some authors believe in the autonomous origin of peroxisomes but with endoplasmatic reticulum contribution (Geuze *et al.*, 2003). Indeed, Tabak *et al.* (2006) proposed just recently a new concept for the biogenesis of peroxisomes (Figure 8). According to the theory, some peroxisomal proteins go into the endoplasmic reticulum capturing its membrane and subsequently exclude the

endoplasmic reticulum inherent proteins from the captured area. Thus, this newly formed specialized area in the endoplasmic reticulum is released and matures into metabolic active peroxisomes. Although, this model raises the question of how the peroxisomal membrane is released from the endoplasmic reticulum (Tabak *et al.*, 2006).

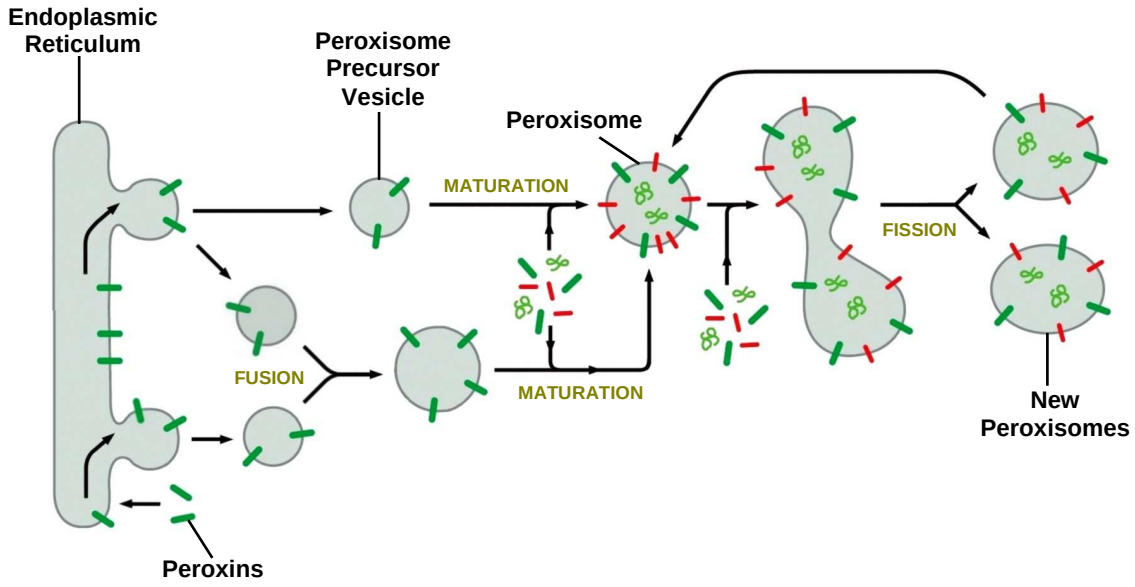


FIGURE 8. Peroxisome biogenesis. Adapted from Alberts *et al.* (2008) and Tabak *et al.* (2006).

Nowadays this subject is still controversial and not totally explained despite the many studies concerning this issue. Peroxisomal biogenesis can be regarded as an integration of several processes, the peroxisomal membrane biosynthesis, the matrix proteins import and the proper peroxisome growth and division (Sacksteder and Gould, 2000).

It is already known that most peroxisomal proteins contain specific aminoacids sequences in the C-terminal as SKL (Serine-Lysine-Leucyne) – the peroxisomal targeting signal 1 (PTS1) (Gould *et al.*, 1987), which function as a signal for protein import into peroxisomes or even in glioxysomes (Distel *et al.*, 1992) and glycosomes (Blattner *et al.*, 1992). Another signal sequence can also be found in or near the protein N-terminus, but showing less conservative sequences – the PTS2 (Zhang *et al.*, 1993; McCammon *et al.*, 1994). PTS1 is found approximately in 50% of the mammalian peroxisomal proteins, whereas PTS2 are being part of a smaller proteins group (Subramani, 1998). However, there are some peroxisomal proteins that do not exibhit any of these PTS (Subramani, 1998). In

addition to the PTSs, a peroxisome-specific protein import machinery is needed to the peroxisome protein traffic, which is responsible for matrix protein targeting and import, and membrane proteins targeting and import (Subramani, 1998).

Advances of all those theories and research in peroxisomal biogenesis were mainly based in two models, yeast species and mammalian cell cultures, mainly because peroxisomal development imply different strategies among species (Veenhuis *et al.*, 2003).

Peroxisomal membrane proteins (PMP) can be originated from the endoplasmic reticulum. In addition, the recruitment of components to the peroxisomal membrane and matrix are mediated by other peroxisomal proteins. Actually, the name “peroxins” is given to all the proteins involved in peroxisome biogenesis and the genes which coded to those proteins are identified as *PEX* (Distel *et al.*, 1996). So far, more than 30 different peroxins were already cloned (Waterham and Cregg, 1997) and it is well known that deficiencies in that genes are associated with several peroxisomal disorders (Subramani, 1998). Peroxins work as receptors or chaperone of PMP, which can possess PTS1 and/or PTS2 sequences and mediate the protein traffic through the peroxisome (Snyder *et al.*, 2000; Fransen *et al.*, 2001). Examples of some peroxins features are followed: Pex5p is a PTS1 receptor, Pex7p is a PTS2 receptor, Pex13p is a peroxisome-associated docking protein which binds to Pex5p, Pex14p is also a docking protein which attachs to Pex5p and Pex7p, Pex2p, Pex10p and Pex12p are zinc containing proteins which are peroxisomal integral membrane proteins, Pex1p and Pex6p are ATPases of the AAAfamily, Pex4p is a ubiquitin-conjugating enzyme, Pex11p is involved in peroxisome proliferation, Pex8p is a matrix protein containing both PTS1 and PTS2 signals which participate in biogenesis, Pex16p and Pex17p are peripherical peroxisomal membrane proteins which seems to be involved in the import of some matrix proteins (Subramani, 1998). In addition to the important role of peroxins in peroxisomal protein traffic, peroxins also regulate the number and dimension of peroxisomes during their proliferation (Yang *et al.*, 1990).

Despite that, notable progress has been made to increase knowledge about peroxisomal protein import and biogenesis, questions such as the endoplasmic reticulum participation and the detailed protein import mechanisms constitute a matter of great interest and promising new findings, namely because peroxisomes are unique organelles regarding their biogenesis characteristics (Subramani, 1998).

1.1.5. Proliferation

Peroxisomal proliferation can be enhanced by the presence of some chemicals, including xenobiotics (Lazarow and Deduve, 1976; Veenhuis *et al.*, 1987; Gorgas and Krisans, 1989; Veenhuis and Harder, 1989; Sakai and Subramani, 2000). Development of modern life imputs into environment a great quantity of new compounds with toxicological potential, including herbicides, insecticides, plastifiers and hipolipidermic drugs, capable of inducing peroxisomal proliferation, especially in the liver of sensitive species (Beier and Fahimi, 1991; Braunbeck and Volkl, 1991; Braunbeck, 1993). Those compounds are denominated as peroxisomal proliferators (PPs), being structurally different from each other except for the existence of an acidic function, mainly constituted by a carboxyl group (Bentley *et al.*, 1993; Lake, 1995).

In the middle 1960's it was noted by Hess *et al.* (1965) that the continued use of clofibrate (a hypolipidermic drug) causes a marked increase in peroxisome numbers of rat livers. In sequence to these first findings, numerous studies involved other hypolipidermic drugs have been performed in order to understand the peroxisome proliferation phenomenum.

It is well established that rats under chronically exposition of approximately 30% of all known PPs develop tumours, especially hepatics (Lai, 2004), establishing an association between peroxisomal proliferation and carcinogenesis (Reddy *et al.*, 1980). The available literature about this issue gives special attention to mammals, but in fishes and other aquatic animals, works are still reduced (Fahimi and Cajaraville, 1995).

Toxicological studies in aquatic organisms demonstrated an induction of peroxisomal proliferation (Yang *et al.*, 1990; Scarano *et al.*, 1994; Cajaraville *et al.*, 2003; Cajaraville and Ortiz-Zarragoitia, 2006), revealing the potential of those organisms to behave as water pollution indicators for PPs pollution. Some peroxisomal enzymes, namely those involved in lipid metabolism, are increased in their activities simultaneously to peroxisomal proliferation (Nemali *et al.*, 1989; Reddy *et al.*, 1992). Moreover, not only the peroxisomal enzymes are influenced when there is proliferation, as mitochondrial and endoplasmic reticulum enzymes are induced with this process too (Masters and Crane, 1995b).

Estrogens are in several ways related with peroxisomes. First, these organelles seem to be involved in cholesterol synthesis, which is a precursor of steroid hormones as estradiol. Moreover, estrogens can in certain conditions behave as peroxisomal proliferators (Ortiz-Zarragoitia and Cajaraville, 2005), influencing the morphology and physiology of these organelles. In another way, and at least in some mammals and apparently also in fish, steroid hormone levels are influenced by peroxisomes, since these organelles possess

the (multifunctional) enzyme 17β-hydroxyesteroid dehydrogenase, that despite its important role in fatty acids β-oxidation is also capable of inactivating estradiol by oxidasing it into estrone (Markus *et al.*, 1995; Corton *et al.*, 1997; Huyghe *et al.*, 2006).

In 1990, it was discovered that the peroxisomal proliferation phenomenon is mediated by some members of the nuclear hormone receptors superfamily, the PPARs (peroxisome proliferator activated receptors), which are activated in the presence of PPs (Issemann and Green, 1990; Kliewer *et al.*, 2001) as well as fatty acids (Sorensen *et al.*, 1998). PPARs present three isotypes, PPARα, PPARβ and PPARγ, coded by three different genes which have been identified in several organisms, such as rodents, humans, amphibians and fishes among others (Issemann and Green, 1990; Dreyer *et al.*, 1992; Sher *et al.*, 1993; Kliewer *et al.*, 1994). The PPARs subfamily is related to the retinoid and thyroid hormone receptor subfamilies, since those first proteins need to be coupled with the retinoid X receptor (RXR) to form heterodimers and then bind to DNA (Kliewer *et al.*, 1992; Keller *et al.*, 1993). The PPARs isotypes possess different target genes, thus different physiological functions are attributed to each isotype (Escher and Wahli, 2000). However, all the enzymes produced in response of that activation are associated with lipid metabolism and homeostasis (Lemberger *et al.*, 1996; Sorensen *et al.*, 1998). In mammals, PPARα is known as a regulator of lipid oxidation, while the comparatively under studied PPARβ seems to be involved in lipid synthesis and turnover, and in cell differentiation, and finally, PPARγ plays an essential role in adipocyte differentiation and it is also implicated in the inflammatory response (Tontonoz *et al.*, 1994; Lee *et al.*, 1995; Braissant and Wahli, 1998; Jiang *et al.*, 1998; Ricote *et al.*, 1998). Specific tissue expression is demonstrated by changed levels of different PPARs isotypes, which also vary from species to species (Sorensen *et al.*, 1998). Nevertheless, in brown trout, PPARα is found mainly in white muscle, heart and liver, whereas PPARβ is highly expressed in testis, heart, liver, white muscle and trunk kidney, and PPARγ limits its expression to the trunk kidney and liver (Batista-Pinto *et al.*, 2005). Nowadays, PPARs and their action mechanism is a matter for many studies, not only in mammals but also in other species, including fishes (Motojima, 1993; Escriva *et al.*, 1997; Kliewer *et al.*, 1999; Andersen *et al.*, 2000; 2001; Robinson-Rechavi *et al.*, 2001; Ibabe *et al.*, 2002; Zhang and Young, 2002; Batista-Pinto *et al.*, 2005; Leaver *et al.*, 2007).

1.2. The Brown Trout

Brown trout and sea trout are members of the **Salmonidae** family, having distinct habitats

despite they belong to the same species (*Salmo trutta*). The former is mainly a freshwater fish, whereas the later behaves as an anadromous fish, migrating to the ocean after birth, where they spend most of their life cycle, and returning to the freshwater only during their breeding period to spawn. Moreover, sea trout corresponds to the trutta morpha, whereas brown trout includes two morphs: the *lacustris* morpha with a potamodromous behaviour, in which the fish migrate from lakes into rivers and streams to reproduce, and the morpha *fario*, the stream resident population, migrating only within the freshwater (Elliott, 1994; Watson, 1999). In this study, *Salmo trutta* f. *fario* was chosen mainly because it is the native trout of Portugal rivers with a particular commercial interest to the reacreational fisheries industry and due to its good manageable size, its relatively short generation time and the facility in obtaining healthy fishes. Also, as rainbow trout, this species can be a good experimental model, being inclusively used as a sentinel organism in environmental studies. In addition, this species has been the target of stock maintenance programs in several European countries (Laikre *et al.*, 1999). In Portugal, these programs were also implemented, though currently brown trout is not considered in extinguishing danger (Laikre *et al.*, 1999).

Indeed, brown trout is native to Europe and western Asia, but was introduced in North America in 1883 and now is also found in New Zeeland, Australia, Asia and South America. It is a carnivorous species which lives in cold and well oxygenated rivers and lakes. Its lifespan is variable, depending on the habitat, but specimens with up to 20 years of age were already observed (Klemetsen *et al.*, 2003). Breeding time depends on the location latitude and commonly, in the North Hemisphere, the natural spwaning season occurr between September and March after the second year (Klemetsen *et al.*, 2003). Although in Portugal, brown trout from captivity usually spawns in late December or January. Females lay about 2,000 eggs per kilogram of body weight. After external fertilization, the young fishes hatch in the following spring. For an adult brown trout the reproductive cycle basically includes a spawning season occurring in December/January and a gonad maturation period, named vitellogenic season in females (Figure 9). In males, the same months corresponds to the testis maturation period, but the classification of the trout reproductive cycle periods is tipically based on ovarian histological criteria (Selman *et al.*, 1987; Washburn *et al.*, 1990). In each of those seasons, the gonads present typical cytological characteristics and variations of the GSI (Washburn *et al.*, 1990).

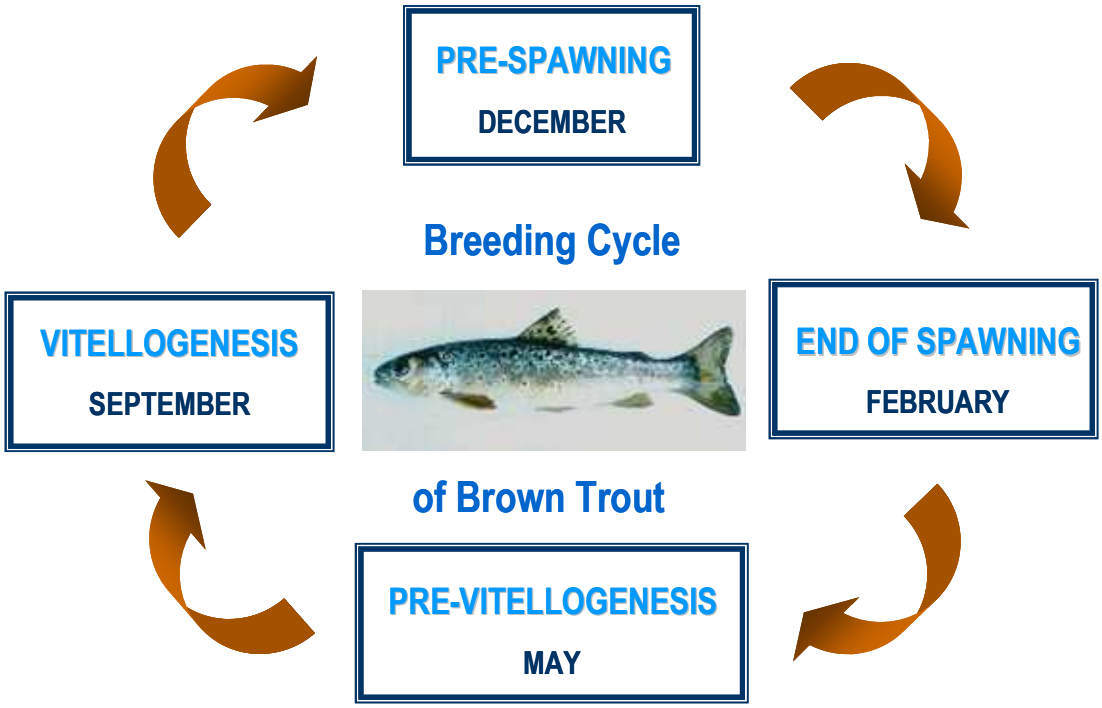


FIGURE 9. Squematic representation of brown trout reproductive cycle, named considering the vitellogenesis process occurring in females. The months presented in the squeme were only to refer the collecting periods used in the experimental work of this thesis. Photo from Klaus Bussel.

As a teleost fish, brown trout belongs to the major group of vertebrates with more than 20,000 species, and logically shares anatomic features with other species, namely the organ localization illustrated in Figure 10. The liver generally presents an anteroventral location in the abdominal cavity while the elongated kidney has a retroperitoneal position.

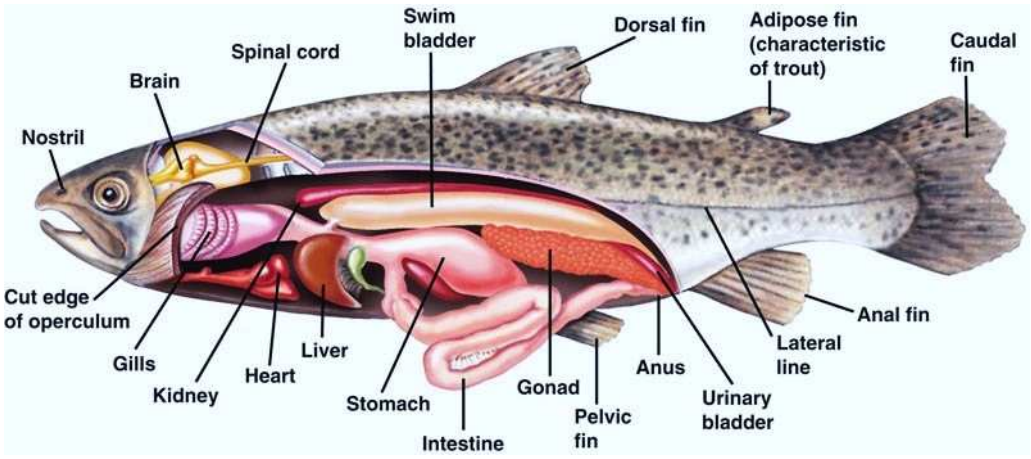


FIGURE 10. Illustration of brown trout anatomy.

1.3. Fish Kidney

Most fishes excrete their nitrogenous wastes as ammonia. Some waste components are diffused through the gills and others are removed by the kidney. In addition to the excretory function, kidney behaves as an ionic equilibrium organ. Indeed, the kidney (or opisthonephros for lower vertebrates) is the excellence organ of osmoregulation, where the nephron is the functional unit for urine formation.

Fish renal morphology has been studied since the 1960's, but still remains incomplete for most species, even when considering the most studied ones. The specialization and the structural variations of species due to environmental adaptations turn them into a huge subject that is largely understudied (Anderson and Loewen, 1975). However, some literature examples are shown in Table 1. In addition to those presented in Table 1, authors as Hentschel and Elger have been contributed to the knowledge of fish renal morphology in a comparative perspective (Hentschel and Elger, 1987, 1989; Elger *et al.*, 2000).

As usual in fish, trout kidney is a dorsal retroperitoneal organ located along the body cavity. It is delimited dorsally by the vertebrae, dorsolaterally by the ribs and ventrally by the swim bladder (Figure 10). Teleosts renal circulation is established by the existence of a venous portal system, in which venous blood from fish caudal and trunk regions supplies the blood sinuses between the tubuli and the interstitial tissue by the caudal vein and intercostals veins, respectively (Hentschel and Elger, 1987). Two differentiated parts of fish kidney are clearly identified: the head and the trunk kidney. The head kidney is composed essentially by hematopoietic tissue, in which it can be found undifferentiated stem cells, blast cells, immature and mature stages of red and white blood cells, as well as a great amount of pigment granules (melanin) essentially in association with macrophages – the melanomacrophages.

In some teleosts, melanomacrophages are organized in the melanomacrophage centers, whereas in salmonids, those cells appeared with a spread distribution (Amin *et al.*, 1992). Renal tubules are very scarce in this portion but become more frequent towards the caudal part. The trunk kidney possesses numerous nephrons and ducts that are nevertheless surrounded with hematopoietic tissue (Anderson and Loewen, 1975). Actually, this organ appears as a fusion of two kidneys in salmonids, whereas in other fish species, such as carp *C. carpio* and goldfish *C. auratus*, the two portions are macroscopically distincts (Hibiya, 1982).

TABLE 1. Summary of published studies about fish kidney

Species	Habitat	References
English sole (*Parophrys vetulus*)	Marine	(Bulger and Trump, 1968)
Bluegill (*Lepomis macrochirus*)	Freshwater	(Hickman and Trump, 1969)
Southern flounder (*Paralichthys lethostigma*)	Marine	(Hickman and Trump, 1969)
Pink salmon (*Oncorhynchus gorbuscha*)	Marine	(Newstead and Ford, 1960)
Sea lamprey (*Petromyzon marinus*)	Marine	(Youson and McMillan, 1970b, 1970a, , 1971a, 1971b, 1971c)
Atlantic hagfish (*Myxine glutinosa*)	Marine	(Heatheve.Mj and McMillan, 1974)
Trouts (*Salmo aguabonita, S. gairdneri, S. trutta, Salvelinus fontinalis*)	Freshwater	(Anderson and Loewen, 1975)
Elasmobranch dogfish (*Scyliorhinus canaliculus*)	Marine	(Hentschel, 1991)
Rainbow trout (*Oncorhynchus mykiss*)	Freshwater	(Kendall, 1978; Oulmi *et al.*, 1995a, 1995b)
Pacific salmons (*Oncorhynchus spp*)	Marine	(Maksimovich *et al.*, 2000)
Sturgeon (*Acipenser nacarii*)	Freshwater	(Ojeda *et al.*, 2003)

Fish kidney is also composed of endocrine elements, namely the interrenal (or “adrenocortical”) cells, the corpuscules of Stannius and the juxtaglomerular cells. The first cell type is found typically in small groups around the blood vessels of the head kidney, being the counterpart of mammals suprarrenal, which produces cortisone (important for osmorregulation). Chromaffin cells can also be present between the interrenal cells, which produce “stress hormones”, as adrenalin and noradrenalin. Corpuscules of Stannius are located in the anterior half of kidney and possibly they are involved in calcium metabolism and regulation of blood pressure (Amin *et al.*, 1992). The juxtaglomerular cells are found

in the glomerular blood vessel walls, wherein the proteolytic enzyme renin is produced. This enzyme is responsible for the production of the hormone angiotensin II, which in turn regulates blood pressure and urine formation (Amin *et al.*, 1992).

1.3.1. Structure of the Nephron

By definition, the nephron (from greek νεφρός (nephros), meaning "kidney") is composed of a renal corpuscule (glomeruli) and the renal tubule which is typically differentiated in two portions – proximal and distal (Anderson and Loewen, 1975). These portions are homologous to those of higher vertebrates, though the distal tubule is less well defined in that homology (Hentschel and Elger, 1987). For some authors, the collecting tubule could be considered as part of the renal tubule, although in general, it is not a section from the proper nephron, representing the terminal part of the unbranched renal tubule. The segment (loop) of Henle present in most of vertebrates is absent in fish nephron. Blood is ultrafiltrated in the glomeruli and the filtrate passes through the tubule where primary urine is altered by tubular epithelia due to their reabsorptive and secretory properties.

The characterization of nephron segments varies among fishes, because some species lack some segments, and also the cellular structure could be different within the same portion for different species, especially because the major function of this organ is directly correlated with fish habitat under adaptation to water of different salinities. One example is the fact that there are fishes that lack renal corpuscules (Elger *et al.*, 2000).

In salmonids, nephron segmentation has been studied for more than 40 years at light and electron microscope level (Figure 11) (Newstead and Ford, 1960; Anderson and Loewen, 1975; Elger and Hentschel, 1986; Hentschel and Elger, 1987; Elger *et al.*, 2000). The “proximal nephron” is composed of the glomerular epithelium, the epithelium of 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 and still produces some controversy among authors (Elger and Hentschel, 1986). The “distal nephron” is constituted by the distal tubule, the collecting tubule and the collecting duct. The collecting tubule established the link between the nephron and the collecting ducts that in turn coalesce forming the mesonephric duct or ureter (Elger *et al.*, 2000). In some fish species, the posterior part of ureter can possess an enlarged region, which corresponds to the urinary bladder (Hibiya, 1982).

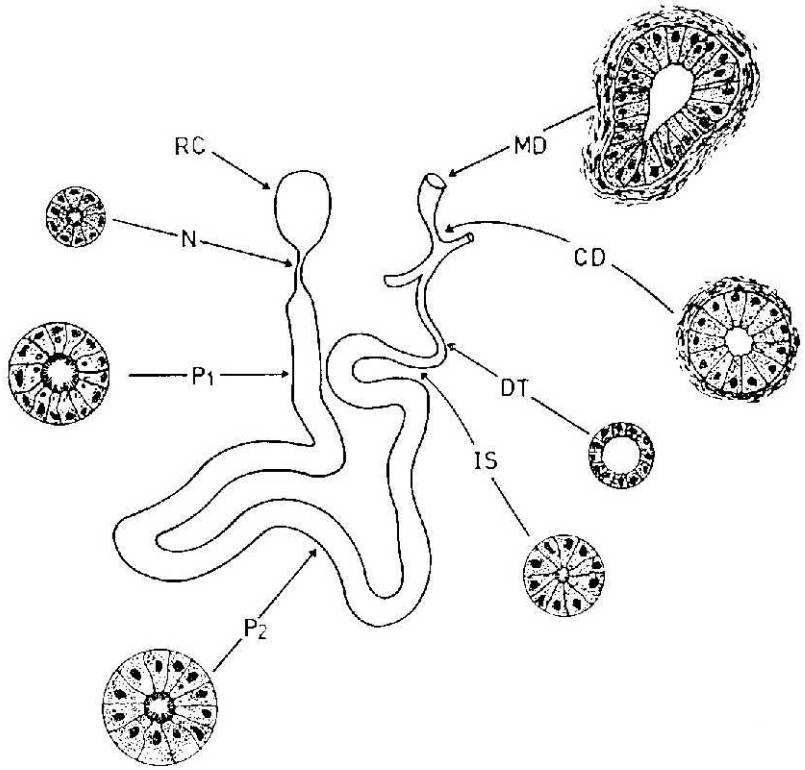


FIGURE 11. Trout nephron and duct system squeme. RC - renal corpuscule, N - neck segment, P_1 - proximal tubule segment I, P_2 - proximal tubule segment II, IS – intermediate segment, DT – distal tubule, CD – collecting duct, MD – mesonephric duct. Taken from Anderson and Loewen (1975).

The mentioned glomerular and multisegmental nephron is considered the central nephron type, which can be subdivided in other types (Elger *et al.*, 2000). As above stated, some fishes do not possess glomeruli, which are refered as aglomerular kidneys. This type is predominantly found in marine species and euryhaline fishes in sea water, such as toadfish (family Batracoididae), anglerfish (order Lophiiformes), sea horses (genus *Hippocampus*) and needlefish (family Belonidae). Contrary to the multisegmental nephron, other type denominated as “atubular” nephron may be found in Myxinoidea species, though not present in other agnathan subgroup such as Petromyzontia (lampreys). Chondrostei, Holostei and Teleosts, such as eels, salmonids and ostariophysids (*e.g.* carps and catfishes) possess a long multisegmental nephron, whereas in marine teleosts of different orders, such as percids (family Percidae), killifish (order Cyprinodontiformes), flounder (order Pleuronectiformes) and sticklebacks (family Gasterosteidae) the nephron is shorter because distal segment is absent (Elger *et al.*, 2000).

In general and when comparing with freshwater species, marine fishes exhibit smaller

glomeruli and a shorter segment I of proximal tubule. These features are in accordance to their different physiology, as marine species have to accomplish low filtration and low reabsorption rates in order to excrete more concentrated urine, contrary to the highly diluted urine produced from freshwater species (Elger *et al.*, 2000).

The **renal corpuscule** encloses the glomerulus and the Bowman's capsule. The glomerulus is characterized by a cluster of anostomosed capillaries surrounded with the parietal and visceral layers of the Bowman's capsule, both in contact with the urinary space laying between them. Glomerular renal circulation is established with afferent arteriole supplying the blood and an efferent arteriole exiting this structure with the filtrated blood. The parietal layer of Bowman's capsule consists of a basement membrane where squamous cells with flattened nuclei are layered. The visceral layer is constituted by an epithelium of a particular cell type – the podocytes (Anderson and Loewen, 1975; Elger *et al.*, 2000).

In the transition between the Bowman's capsule parietal layer and the **neck segment** 4 to 6 rows of cuboidal cells appear, becoming abruptly columnar with basal nuclei and the opposite surface bearing a myriad of cilia, characteristic features from neck portion (Anderson and Loewen, 1975; Hentschel and Elger, 1987). These cells essentially display elongated mitochondria, Golgi complex, multivesicular bodies and lipid droplets, well developed rough and smooth endoplasmic reticulum, lysosomes, glycogen granules and a great density of free ribosomes. The junctional complexes existing between those cells are also prominent. The neck segment is present only in the renal tubules of some fishes, such as lampreys, elasmobranchs, polypterids, holosteans, some teleosts and lungfish (Hentschel and Elger, 1989). Elger *et al.* (2000) suggested that the characteristic presence of multiciliary cells in this segment may be in order to propulsion the fluids into the tubule.

The passage from the neck segment to the first portion of the proximal tubule is characterized by an increase in cell height, as well as in lumen diameter and in outside diameter. The **proximal tubule segment I** (PTI) is characterized by columnar epithelial cells possessing a high brush border, basally or subcentrally located spherical nuclei, presence in the apical cytoplasm of an "endocytic apparatus" formed by vesicles and vacuoles, and also of lisosomal bodies in high density (Anderson and Loewen, 1975; Hentschel and Elger, 1987). These two last morphological characteristics are specific from PTI and suggest that proximal tubule participate in protein reabsorption. Mitochondria are large but scarce in the cytoplasm of these cells, and are absent in their apical zone. Multiple peroxisomes are found in this first portion of proximal tubule, as rough and

smooth endoplasmic reticulum cisternae and free ribosomes. Adjacent cells are connected basolaterally by interdigitations and apically by junctional complexes as tight junctions and desmosomes. This segment is present in all vertebrates except in aglomerular teleosts (Elger *et al.*, 2000).

In several teleosts as in trout, the transition of PTI and PTII epithelium is abrupt contrary to the gradual transition seen in other cases, such as the plaice *Pleuronectes platessa* (Elger *et al.*, 2000). The cells of the **proximal tubule segment II** (PTII) are columnar and taller than in PTI, with centrally located oval nuclei, with small round or elongated vesicles in the apical cytoplasm, and presenting a brush border lower than in PTI as well as lower vesicular density in the apical zone. Mitochondria are dispersed troughout the cytoplasm, with larger ones preferentially located in perinuclear region and lacking in the apical zone. Filamentous mitochondria basally located are lined up parallel to the cell axis with invaginations of the cell membrane. Golgi stacks are also present in this segment as well as small cisternae of rough and smooth endoplasmic reticulum and lysosomes. Adjacent cells are attached to each other by junctional complexes, mainly tight and gap junctions, but some desmosomes could also be found (Anderson and Loewen, 1975; Elger *et al.*, 2000). As in PTI segment, numerous invaginations could be observed in the lateral and basal cell membranes. The presence of taller cells results in a decrease of the luminal diameter towards the intermediate segment (Anderson and Loewen, 1975). A major function from the PTII is the active fluid and ion secretion (Larsen and Perkins Jr., 2001).

Among the common cells of the PTI and PTII, other cell types can be additionally found, namely multiciliary cells and small spindle-shaped cells lacking brush border which may be amoeboid (Elger *et al.*, 2000).

A very short ciliated terminal portion of PTII can be distinguished and defined as **intermediate segment**, which is characterized by multiciliary cells abundance (Elger *et al.*, 2000). The ciliated cells are cuboidal in shape with centrally located nuclei and a cytoplasm occupied by round mitochondria, smooth endoplasmic reticulum, small vacuoles and multiple free ribosomes (Anderson and Loewen, 1975). This segment is not present in all fish species, but it was identified in freshwater fish, such as the Senegal bichir, *Polypterus senegalus*, the popefish/redfish, *Erpetoichthys calabaricus*, and several lungfish, as well as elasmobranchs. In other species, as lampreys, this segment is characterized by the presence of flattened cells between the proximal tubule columnar cells and the cuboidal or low columnar cells from distal tubule, and do not possess multiciliary cells. On the contrary, the intermediate segment of teleosts is not a distinct segment, presenting in turn a mixture of the PTII and distal segment cell types (Elger *et*

al., 2000). Lacking of a distinct intermediate segment is also a renal morphological feature of the sturgeon (family Acipenseridae) and bowfin *Amia calva* (Elger *et al.*, 2000).

The existence of multiciliary cells in this portion suggests a propulsion function to the fluid through the renal tubule, in similarity with the neck segment (Elger *et al.*, 2000). Specialy in marine species, this segment may have a very important role in the impediment of stone formation, since a combination of a very low urine flow with a secretion of ions in the proximal tubule II happened in the intermediate segment (Elger *et al.*, 2000).

The **distal segment** is typical in freshwater salmonids and it is identified by the presence of low cuboidal epithelial cells, elaborate membrane amplifications, high density of mitochondria arranged perpendicular to the basal lamina and parallel to the cell membrane infoldings, and few short microvilli in the cell apex (Anderson and Loewen, 1975; Elger *et al.*, 2000). This segment presents an epithelium which permits high ion permeability and low hydraulic conductance, features that are in agreement with the main function of a freshwater kidney which is the production of diluted urine (Hentschel and Elger, 1987). In addition, the active ion transport through these cells should require high energy level which is in accordance with the existence of a great density of mitochondria in this segment.

Urine is ultimately discharged in the **collecting tubule**, which is characterized by the presence of an epithelia which progressively change from cuboidal to columnar with basally located nuclei and some smooth muscle cells and connective tissue surrounding the basement membrane. Usually, the lumen of this portion is irregular in shape (Anderson and Loewen, 1975). The collecting system establishes the connection between the nephron and the archinephric ducts of fish mature kidneys (Elger *et al.*, 2000).

The **mesonephric duct** is the last branch of the collecting duct system, in which small ducts united to larger ones, and in turn these joined to eachother till reach the mesonephric duct, which became larger towards the caudal part. Ultrastructurally, it presents a tall columnar epithelium which converts into a pseudostratified, surrounded with many layers of smooth muscle cells. Normally, the lumen of the mesonephric duct appears irregular in shape (Anderson and Loewen, 1975).

1.4. A Brief Note on Fish Liver

Liver is an essential organ in metabolism, present in all vertebrates; it comprises many important body functions such as glycogen storage, lipid handling, protein synthesis and

detoxification. In fishes, this organ has been for long time a target for morphological studies with a phylogenetic perspective (Elias, 1949b; 1949a; Elias and Bengelsdorf, 1952; Yamamoto, 1962; Yamamoto *et al.*, 1965; Hampton *et al.*, 1988; Robertson and Bradley, 1992; Rocha *et al.*, 1994). However, the relevance of fish liver studies for scientists increased since this organ began to be used in environmental and carcinogenic studies, where it revealed to be a good model for biomarker and comparative toxicological assays (Hampton *et al.*, 1985b; Hinton, 1989).

The hepatic structure show many different aspects among species, sometimes even within species of the same family (Hampton *et al.*, 1985b; 1987; Robertson and Bradley, 1992; Rocha and Monteiro, 1999). Also, contrarily to what happens in the mammalian liver, the traditional lobulation of this organ is not present in fishes. It is also extremely difficult to distinguish all afferent (portal veins) from efferent (centrolobular veins) areas (Rocha and Monteiro, 1999). For all this, it is reasonable not use for fishes exactly the same nomenclature usually applied to the mammalian liver, *e.g.* centrolobular, Kiernan spaces, Glisson triangules, periportal or zonal (Hampton *et al.*, 1985b; 1988). Nevertheless, altertative terminology for fish liver is being advanced over time (Hampton *et al.*, 1989; Rocha *et al.*, 1995; Figueiredo-Fernandes *et al.*, 2007).

In relation to the cellular constitution, aproximately 80% of liver volume is occupied by hepatocytes (Hampton *et al.*, 1989; Rocha *et al.*, 1997). The other cellular types are biliary epithelial cells, endothelial cells, Ito cells and macrophages (Hampton *et al.*, 1985a; 1988; 1989). Kupfer cells are generally absent, but other types of macrophages are found in this organ, as well as rodlet cells and eosinophilic granular cells (Figueiredo-Fernandes *et al.*, 2006).

Tubular arrangement of fish hepatocytes was recognized in rainbow trout, *Onchorynchus mykiss* (Hampton *et al.*, 1985b; Schar *et al.*, 1985; 1988; 1989), in Atlantic salmon, *Salmo salar* (Robertson and Bradley, 1991; 1992) and in brown trout *Salmo trutta* (Rocha *et al.*, 1994). Hinton (1993) proposed the term “tubular muralium” to denominate the three-dimensional (3D) organization of hepatocytes. Inside the “tubules”, components of biliary tree are disposed in canaliculus, preductules or ductules, in which sinusoids surrounded them through the parenchyma. However, the spatial arranjment of hepatocytes in fishes is a matter of debate and several three-dimensional arrangements may actually exist though probably dependent on the species (Eduardo Rocha, personnal communication).

The qualitative ultrastructure of fish hepatocytes is similar to that observed in the liver of other vertebrates. Generally, one spherical nucleus with a prominent nucleolus is present, although is frequent the presence of two nucleoli. The cytoplasm is rich in organelles,

typically of very active cells, as shown by the abundancy of rough reticulum endoplasmic cisternae, the great number of mitochondria, peroxisomes and Golgi stacks. Lysosomes and residual bodies are also part of the organeolar content of hepatocytes. The abundancy of lipid droplets, lipofuscin deposits as well as glycogen granules is variable, existing hepatocytes rich in glycogen or in lipid, or even in both (Rocha and Monteiro, 1999).

It is known that fish liver presents natural variations, which are related with age, sex, season and nutritional condition (Hinton and Lauren, 1990; Segner and Braunbeck, 1990). Those changes imply not only liver weight, but also hepatocyte morphology namely in its organeolar content, according to its metabolic activity (Rocha *et al.*, 2003). Xenobiotic compounds, such as xenoestrogens, could also induce variations in liver ultrastructure (Arukwe and Goksoyr, 1998).

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1.5. Aims

Since 1970's peroxisomes of aquatic organisms have been a target for some studies, including their morphology and physiology in fish hepatocytes. The influence by natural environmental factors and toxicological conditions, which could induce peroxisomal proliferation, was a great motive for research in this field.

It is already known, from previous studies of our research group, that in brown trout, hepatic peroxisomes are highly correlated with gonadal development, thus deeply influenced by sexual hormones, namely 17-β estradiol. Especially in females, those organelles change their morphology and enzymatic content during the breeding cycle. Rocha *et al.* (1999; 2001) observed for the first time that during vitellogenesis period, peroxisomes appear smaller, lower in catalase and almost without urate oxidase activities. Xenobiotic compounds could also cause similar alterations, even in less extent, suggesting that hepatic peroxisomes are regulated by estrogens.

However, very limited studies concerning renal peroxisomes are available in literature. Since kidney is one of the peroxisome richest organs, this work has the intention to contribute to a better knowledge about estrogenic regulation in kidney peroxisomal structure and function. For that purpose, we proposed some objectives which are described in detail as follows:

- To enhance the information about trunk kidney morphology of brown trout, in qualitative and quantitative perspectives.
- To investigate seasonal variations of kidney peroxisomes morphology and their eventual correlations with the gonadal maturation status, that could point to an influence of sexual hormones on peroxisome morphology. To achieve this objective, stereological methods will be applied during the reproductive cycle of brown trout in both genders.
- In the same vein as above, to verify whether the gonadal maturation correlates with biochemical changes of kidney peroxisomes, accessing the latter using peroxisomal enzymatic measurements in both genders of brown trout and along the year.
- To study kidney and liver purine catabolism considering the enzymatic variation pattern exhibited by some of the involved enzymes in this pathway along the breeding cycle.
- To examine the subcellular location in brown trout of some purine catabolism enzymes.

- To search if the toxic paraquat has any influence in brown trout renal and hepatic peroxisomes, using the methodology mentioned in last item.

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CHAPTER 2

HISTOLOGICAL AND STEREOLOGICAL CHARACTERIZATION OF BROWN TROUT (*SALMO TRUTTA*) TRUNK KIDNEY

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Histological and Stereological Characterization of Brown Trout (*Salmo trutta*) Trunk Kidney

Abstract

When compared to mammals, the large macro- and microscopic variability in kidney morphology among different fish species impairs a more comprehensive understanding on its function and structure. Thus, a quantitative study of brown trout trunk kidney was performed, considering the hypothesis of seasonal and gender effects. Therefore, three-year-old specimens of both genders were collected in four seasons of their reproductive cycle. Kidney was processed for light and electron microscopy. With stereological methods, the relative volumes of renal components were estimated. Qualitatively, the general nephron structure of brown trout is similar to that described for other glomerular teleost species, including other trouts. Quantitatively, however, differences in the relative volume of renal corpuscules, proximal tubule segments I and II, collecting tubules, new growing tubules and vacuolized tubules were detected. In males from February, lower volumes of proximal tubules segments II, collecting tubules and renal corpuscules were found accompanied by the highest values of vacuolized tubules, whereas the greatest volumes were found in September for collecting tubules and renal corpuscules and in December for proximal tubules segment II. Females from September presented higher volumes of proximal tubule segments I, collecting tubules and renal corpuscules comparing to the lowest values of February, showing this last period the highest values of new growing and vacuolized tubules. Differences between genders were detected in February, for the volume of the proximal tubule segments I, in May and December for the volume of proximal tubule segments II, and in December, for the volume of renal corpuscules. Despite seasonal changes, only two linear correlations were found in

females between the GSI and some of the renal portions volume, whereas more linear correlations between the latter and the RSI were found in both genders. Thus, in this study, it was verified that some brown trout renal components undergo gender dependent seasonal variations along the year suggesting a morphological adaptation of the components in order to accomplish physiological needs. These findings are relevant for interpreting abnormal changes in toxicological contexts, and constitute a baseline for launching new studies to know which factors govern the morphological variations and their functional consequences.

Introduction

The fish urinary system is composed of the kidney, extra-renal urinary ducts and the urinary bladder, playing a vital role not only in excretion but also in osmoregulation (Varsamos *et al.*, 2005). These facts contribute to the relevancy of understanding this organ, both in a structural and functional (including toxicological) perspective and for building comparative approaches. Despite the significant number of articles focusing on the fish kidney, the existence of large variations in kidney anatomy, histology and cytology related to the habitat turns the existing knowledge insufficient when the interest is directed to an unstudied fish species (Elger *et al.*, 2000).

In salmonids, head and trunk kidney do not present an anatomical division, but structurally and functionally they are clearly distinct (Hibiya, 1982). The anterior part, or head kidney, works mainly as an haematopoietic organ, whereas nephrons and ducts responsible for the excretion and osmoregulation functions are found in the trunk kidney, though intermingled with some spread haematopoietic tissue. The latter and the remaining connective tissue constitute the renal stroma (Anderson and Loewen, 1975). Several authors described the nephron of salmonids as a glomerular and multisegmental nephron, composed of a renal corpuscle and a renal tubule (Anderson and Loewen, 1975). The renal tubule is generally subdivided into several segments, distinct in morphology and function, such as the proximal tubule segment I, proximal tubule segment II, distal tubule and collecting tubule (Elger *et al.*, 2000). In trout, that nephron type is associated with its freshwater habitat, which implies the production of very diluted (hyposmotic) urine. For this, the glomeruli presence is essential to increase the filtration capacity as well as the existence of a long renal tubule in order to accomplish high ion reabsorption rates (Elger *et al.*, 2000). In addition, the size of each renal tubule portion is also dependent on the species habitat and on the ability of each organism to adapt to different salinities. For this

reason, characteristics such as small proximal tubules segment I and small or absent distal tubules are usually in association with small glomeruli or even their absence, typical features normally found in stenohaline species (Elger *et al.*, 2000).

Althought salmonids share renal macroscopical and microscopical features, tubule portions present some ultrastructural differences among species from the same family (Anderson and Loewen, 1975; Oulmi *et al.*, 1995a; Elger *et al.*, 2000). Moreover, factors such as age and environmental conditions are known as contributors of nephron heterogeneity (Hentschel and Elger, 1987). However, the study of fish kidney is still an undervalued matter, particularly with the application of quantitative methods. Currently, only few semi-quantitative studies were performed in this organ and those, to our knowledge, were always associated with toxicological settings (Rojik *et al.*, 1983; Oulmi *et al.*, 1995a, 1995b; Oulmi and Braunbeck, 1996; Kamunde *et al.*, 1997).

Therefore, the goal of this study was to characterize qualitatively and quantitatively the trunk kidney of brown trout, providing baseline data for further descriptive or experimental studies. The study also addressed the question of whether or not the gender and season exhibit their effects in key renal excretory components of adults of this species. In order to do that, an evaluation of the percentage of distribution of each nephron component and stroma in the kidney was carried out using stereological methods at light microscopy.

Material and Methods

Animals

Five males and five females of three-year-old brown trout (*Salmo trutta* f. *fario*) were collected randomly by net fishing within a state aquaculture station (Posto Aquícola do Torno, Amarante, Portugal) at major seasons of their natural reproductive cycle: after spawning (February), early vitellogenesis (May), advanced vitellogenesis (September), and pre-spawning (December). Before being sacrificed, the sampled fish were held in observation tanks for 12 to 24 h.

Tissue Processing

Animals were deeply anaesthetized by immersion in 1 ml L^{-1} aqueous solution of ethylene glycol monophenyl ether, and then weighed and measured in length. Before kidney fixation, a perfusion was made with a 5 IU ml^{-1} heparinised isosmotic buffer for salmonids

(Cascales *et al.*, 1997), composed of: NaCl 0.74%, KCl 0.04%, $CaCl_2$ 0.02%, $MgSO_4$ 0.15%, $NaHCO_3$ 0.03%, $NaH_2PO_4 \bullet H_2O$ 0.05%, $Na_2HPO_4 \bullet 2H_2O$ 0.16%. For improving the perfusion, the most posterior part of the fish was cut off, allowing a direct cannulation of either the vein or the artery with simultaneous escape of both perfusate and blood. During the perfusion, fish was maintained on top of ice. The perfusion was carried at 4 ºC and with a physiological flow rate of about 5 ml min^{-1} kg^{-1} of body weight (Hampton *et al.*, 1985) during the time necessary to eliminate the organ residual blood. The trunk kidney was removed, weighed and cut into ≈ 4 mm thick slices (with the first cut made at random between 1-4 mm from the anterior edge of the kidney). A systematic selection was carried out and sampled pieces were processed for light and electron microscopy. Gonads were also removed and weighted.

For light microscopy, kidney pieces were fixed in a solution of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 ºC during 48h. After fixation, the pieces were dehydrated in ethanol, cleared in xylene and embedded in paraffin. From each block, sections 3 µm thick were cut and stained with hematoxylin-eosin. Thus, a mean of four systematically sampled sections were analyzed per animal. For transmission electron microscopy, pieces were fixed for 2 h in a solution of 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 ºC. After fixation, kidney pieces were rinsed for 2 h (at 4 ºC) in 0.1 M phosphate buffer (pH 7.4). A post-fixation in 0.1 M phosphate buffered 1% OsO_4 with 1.5% $K_3Fe(CN)_6$ was carried out for 2 h at 4 ºC. After dehydration in ethanol, the pieces were embedded in an epoxy resin. Semithin sections of trunk kidney were stained with methylene blue/azure II, observed with an Olympus BX-50 light microscope and photographed with an Olympus CAMEDIA C-5050 digital camera. Ultrathin sections of segments I and II of the kidney proximal tubules were observed in a JEOL 100CXII transmission electron microscope, operated at 60 kV.

Stereological Analysis

According to previous descriptions of fish kidney (Anderson and Loewen, 1975; Elger *et al.*, 2000), in this study we operationally divided the organ into the following components: 1) renal corpuscules; 2) proximal tubule segment I epithelium and its lumen (including the neck segment portion); 3) proximal tubule segment II epithelium and its lumen; 4) distal tubule epithelium and its lumen; 5) collecting tubule epithelium and its lumen; 6) haematopoietic tissue with the melanomacrophage aggregates; 7) new growing tubules, and 8) vacuolized tubules.

At light microscopy, a stereological approach was designed to estimate the volume densities (V_V) of the different structural elements within the kidney, being all the organ the reference space. The V_V was obtained by a long established and unbiased stereological technique based on point counting (Weibel, 1979):

$$V_V \text{ (structure, reference space)} = \sum P(s) \div [\, k \times \sum P(r) \,]$$

In which $\sum P(s)$ is the total number of points within each structural component; $\sum P(r)$ is the total number of test points lying over the reference space; and *k* is the ratio between the number of points of the grid used for the structure of interest and for the reference space. A two lattice pointing grid with a ratio 1:6 was used, being k either 1 for most frequent structures, namely the proximal tubules, or 6 for all the other described components. The estimations of V_V were made with the help of a stereological workstation composed of a microscope (Olympus, BX-50), a motorised stage (Prior) for stepwise displacement in the x-y directions (1 µm accuracy), and a CCD video camera (Sony) connected to a 17'' PC monitor (Sony). The whole system was controlled by the software Olympus CAST-Grid System (version 1.50). A final magnification of x 1608 in the monitor image allowed a clear recognition of all kidney components. The first field was randomly selected, but thereafter, a systematic field sampling was carried out by stepwise movements of the stage in the x and y directions, with a $step_{x,y}$=500 or 600 µm according the kidney size (smaller step for smaller kidneys). Under the x 40 objective lens, a mean of 292 sampled fields per animal were analysed using live images, over which the virtual stereological grids were superimposed.

The volume estimations of the proximal tubule segment I and II lumina were performed in TEM images (for granting adequate resolution). The stereological method was implemented according to the detailed description given in the chapter 3.

Statistical Analysis

All data are presented by a mean of each animal group (5 fishes per group) from every season and gender, followed by the respective coefficient of variations (CV = standard deviation ÷ mean). The Statistica 6.0 for Windows software was used to analyse the data. After testing the normality and the homogeneity of variances, a two-way ANOVA was performed to detect the effects of season, gender and season by gender interaction, on each parameter. The Tukey and Newman-Keuls post-hoc tests for multiple comparisons between means were further applied. Differences were considered significant for $p \leq 0.05$. When a difference was significant according Newman-Keuls but not Tukey post-hoc tests,

we considered it marginally significant. Data transformation was made in some cases to warrant normality and homogeneity. Non-parametric tests (both Kruskal-Wallis ANOVA and Mann-Whitney U) were also applied when data transformation was not well succeeded - the results always corroborated the significances derived from parametric methods. Correlation tests were used to find linear associations.

Results

Brown Trout Biological Parameters

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

TABLE 1.

Fish biological parameters

Month	Gender	Weight (g)	Length (cm)	GSI (%)	RSI (%)
February	♀	617.9 (0.19)	35.8 (0.06)^{b}	0.6 (0.23)^{Aa}	0.7 (0.29)^{a}
	♂	673.0 (0.08)	35.4 (0.05)	1.6 (0.50)^{Ba}	0.7 (0.12)^{a}
May	♀	809.0 (0.16)^{a}	40.0 (0.06)^{a}	0.8 (0.04)^{Ab}	0.5 (0.08)^{Ab}
	♂	796.5 (0.16)^{a}	38.4 (0.05)^{a}	0.2 (0.33)^{Bb}	0.6 (0.05)^{Bb}
September	♀	526.3 (0.20)^{b}	32.8 (0.06)^{b}	11.1 (0.22)^{Ac}	0.4 (0.06)^{Ab,c}
	♂	558.8 (0.22)^{b}	33.5 (0.08)^{b}	5.3 (0.16)^{Bc}	0.5 (0.08)^{Bc}
December	♀	579.6 (0.18)^{b}	33.5 (0.06)^{b}	20.00 (0.1)^{Ad}	0.4 (0.15)^{Ac}
	♂	697.7 (0.17)	35.8 (0.05)	3.9 (0.18)^{Bd}	0.7 (0.09)^{Ba}

Different upper case superscript letters represent differences between genders within each month; different lower case superscript letters represent differences among months within a gender; the presence of the same letter means differences absence. Only values with different letters are significantly different ($p \leq 0.05$). The GSI and the RSI were estimated by the formula: (gonad or kidney weight $\times$ 100) $\div$ body weight.

Qualitative morphology

All renal components were clearly identified by light microscopy as illustrated in images taken from the thin paraffin sections that were stereologically analysed (Figure 1) and from the semithin epoxy sections used for additional qualitative characterization (Figure 2).

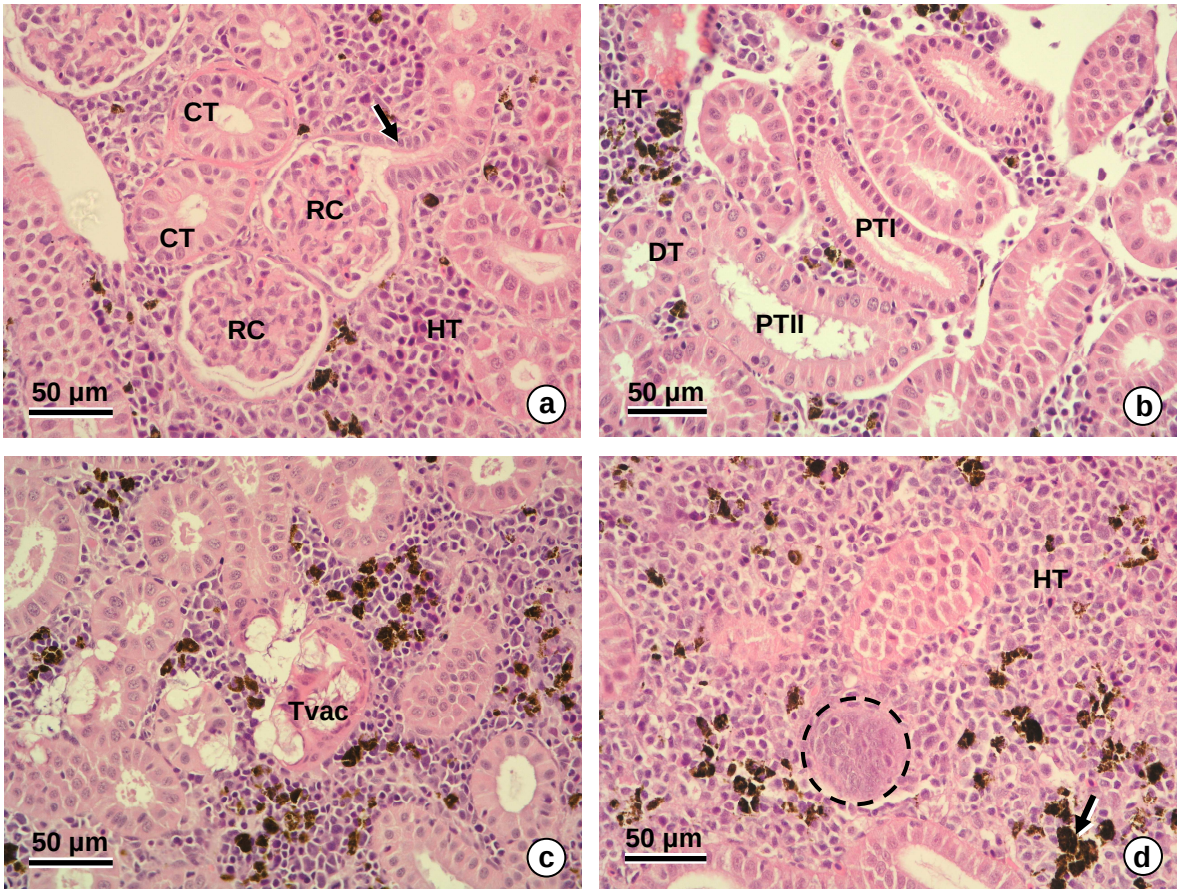

FIGURE 1. Light micrographs of paraffin sections from brown trout trunk kidney, showing the different stereologically analysed components. **a)** Among the several structures present in this figure, two renal corpuscle (RC) and two collecting tubules (CT) stand out. Note the connection between one of the renal corpuscle and the initial portion of a proximal tubule, the neck segment (arrow). HT- haematopoietic tissue. **b)** Image showing several segments of renal tubules, first (PTI) and second (PTII) segments of the proximal tubule and distal tubule (DT). HT- haematopoietic tissue. **c)** Image with extremely rare vacuolized tubules (Tvac), with apparent peritubular vacuolization in some areas, surrounded with other renal tubule types and haematopoietic tissue. The vacuolized cells seem to be degenerating. Note that parasites were not seen associated with this peculiar condition. **d)** Photograph showing a tubule interpreted as in formation (circle) among other renal components, mainly haematopoietic tissue (HT) with some melanomacrophage aggregates (arrow).

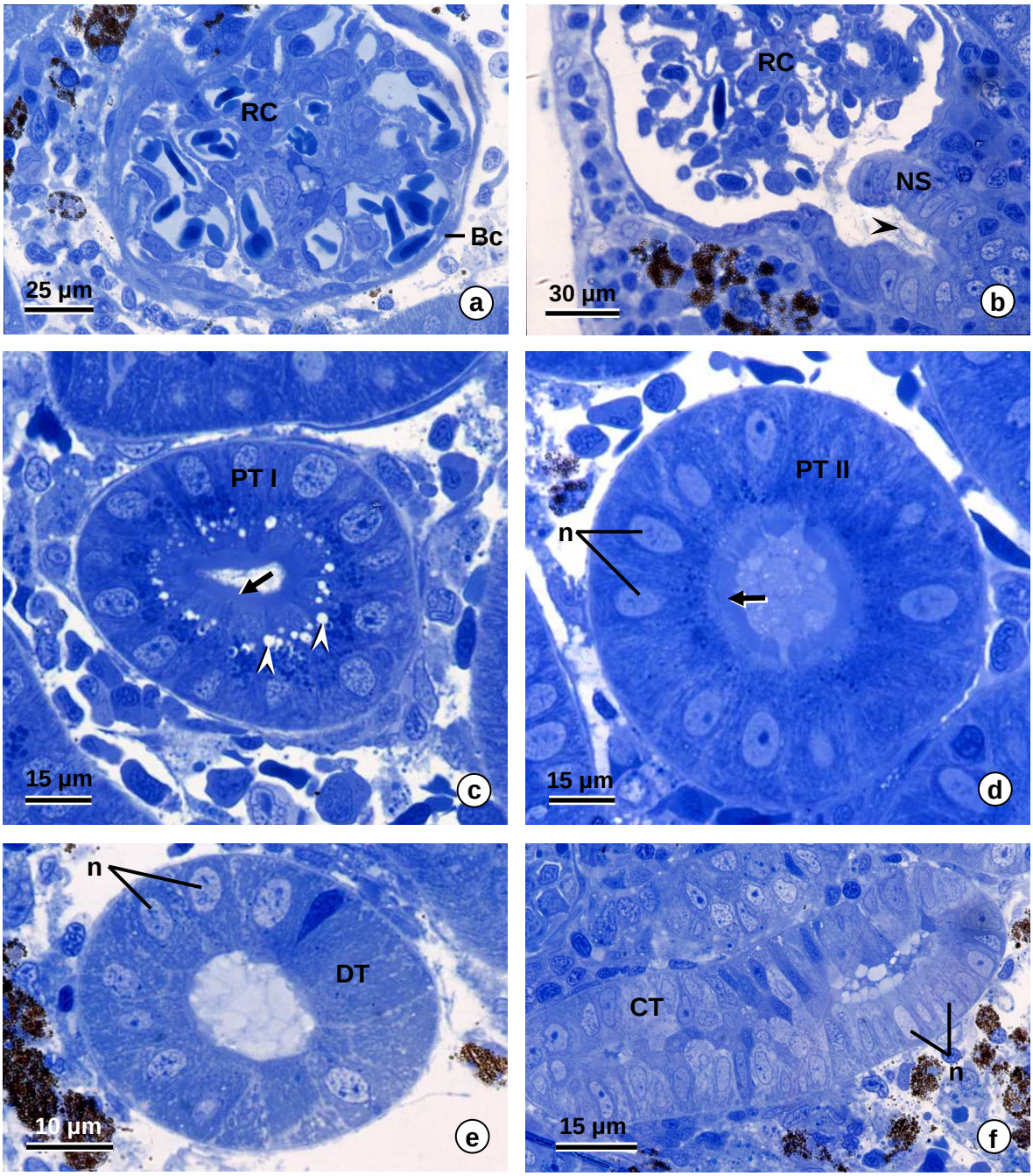

FIGURE 2. Light micrographs of semithin sections from brown trout trunk kidney showing different nephron portions surrounded with haematopoietic tissue. Note the melanin pigment in black. **a)** Renal corpuscle (RC), with the Bowman's capsule (Bc) surrounded capillaries wherein erythrocytes can be depicted. **b)** A renal corpuscle and the initial portion of renal tubule, the neck segment (NS). Verify the presence of cilia (arrow head) from epithelial cells in the lumen. **c)** Proximal tubule segment I (PT I). Observe the basally located nuclei (n) and the characteristic apical cell portion, plenty of endocytic vesicles (arrow heads) and an evident brush border (arrow). **d)** Proximal tubule segment II (PT II) showing the epithelial cells with central nuclei (n) and apical brush border (arrow). **e)** Distal tubule (DT), with the characteristic epithelium possessing basal nuclei (n) and devoid of brush border. **f)** Collecting tubule (CT) longitudinal section, showing the columnar epithelium with basal nuclei (n).

Each glomerulus displayed anastomosed renal capillaries surrounded with the Bowman's capsule, which together constitute the renal corpuscle (Figures 1a, 2a and 2b). This is connected with the renal tubule by a very short neck segment, being characterized by an abrupt epithelium transition, from cuboidal to columnar, in which the columnar cells showed basal nuclei and cilia towards the lumen (Figures 1a and 2b). The first segments of proximal tubules were identified in paraffin and semithin sections by their columnar epithelium with basal nuclei, prominent brush border and a very developed endocytic system of vesicles, vacuoles and lysosomes located in the cell apex (Figures 1b and 2c). Sometimes, the proximal tubule segment I appeared with an eosinophilic staining.

Proximal tubules segments II were distinct from the other renal tubule segments because they have a high columnar epithelium with central nuclei and a brush border (Figures 1b and 2d). Distal tubules were characterized by the absence of brush border, and display a low cuboidal epithelium with basal nuclei and great density of mitochondria (Figures 1b and 2e). Collecting tubules were distinguished by the columnar epithelia with basal nuclei and the presence of some smooth muscle cells and connective tissue surrounding the basement membrane (Figures 1a and 2f). Unusual tubules were also found, namely extremely rare vacuolized tubules, which had an epithelium partly or mainly occupied by tubular epithelial cells and/or phagocytes, engorged with small to huge vacuoles or spaces, which due to their disrupted architecture, with degenerating cells, could not be classified as any of the typical tubules (Figure 1c). Vacuolated cells (presumed macrophages) appear peritubularly. Another rare aspect was the observation of tubules (highly cellular) still in formation, which also could be assigned neither in proximal, nor in distal nor in collecting tubules (Figure 1d). Surrounding all the described components there was haematopoietic tissue with some melanomacrophage aggregates (Figures 1 and 2).

Quantitative morphology

The two-way ANOVA results for the relative volume of each analysed renal component are presented in Table 1, and the stereological data are showed in Table 2.

As to the relative volume of the renal stroma, it was detected a significant ANOVA with an independent sex effect, although by further post-hoc analysis a significant difference was not detected between genders in any season of the reproductive cycle.

The relative volume of the proximal tubule segment I epithelium showed a significant ANOVA with a sex and season interaction effect, plus a marginally significant ANOVA for

an independent season effect (Table 1). By post-hoc analysis, significant differences were found between genders, in February, where males presented almost twice the relative volume of the female proximal tubule segment I. Differences among seasons were also found, in which females from February presented half of the proximal tubule segment I relative volume presented by females from September (Table 2 and Figure 3a). The relative volume of the proximal tubule segment I lumen did not vary between genders or among seasons; thus, a significant ANOVA was not detected (Tables 1 and 2).

TABLE 1.

Summary of two-way ANOVAs showing the effects of age, season and their interaction in the volume density (V_V) of all brown trout kidney components. Kid – kidney, Str – renal stroma, PTI – proximal tubule segment I, Ln – lumen, PTII – proximal tubule segment II, DT – distal tubule, CT – collecting tubule, Corp – renal corpuscule, Tnew – new tubules, Tvac – vacuolized tubules.

Parameters	Sex		Season		Interaction (Sex × Season)	
	Fisher's F ratio	*p* value	Fisher's F ratio	*p* value	Fisher's F ratio	*p* value
V_V (Str, Kid)	9.13	0.005	2.04	0.128	1.57	0.217
V_V (PTI, Kid)	0.57	0.456	2.79	0.056	4.12	0.014
V_V (Ln PTI, PTI)	1.51	0.228	1.04	0.390	0.48	0.696
V_V (PTII, Kid)	39.95	<0.001	4.40	0.011	1.19	0.330
V_V (Ln PTII, PTII)	4.99	0.033	0.89	0.457	0.54	0.656
V_V (DT, Kid)	0.01	0.905	5.49	0.004	0.19	0.902
V_V (Ln DT, DT)	0.07	0.792	2.39	0.087	0.29	0.834
V_V (CT, Kid)	7.77	0.009	8.55	<0.001	2.62	0.068
V_V (Ln CT, CT)	3.43	0.073	0.83	0.489	0.97	0.418
V_V (Corp, Kid)	8.87	0.005	12.52	<0.001	2.51	0.076
V_V (Tnew, Kid)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
V_V (Tvac, Kid)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Degrees of freedom (df): df(sex) = 1; df (season) = 3; df (interaction) = 3; df (residual) = 32. n.a. – not applicable, a non-parametric analysis of the data was used.

TABLE 2.

Volume density (V_V) of each renal component in relation to the kidney of brown trout during the reproductive cycle. Kid – kidney, Str – renal stroma, TI – proximal tubule segment I, Ln – lumen, TII – proximal tubule segment II, DT – distal tubule, CT – collecting tubule, Corp – renal corpuscule, Tnew – new tubules, Tvac – vacuolized tubules.

Parameters	February		May		September		December	
	♀	♂	♀	♂	♀	♂	♀	♂
VV (Str, Kid)	74.34 (0.05)	66.98 (0.09)	70.83 (0.09)	61.97 (0.03)	66.31 (0.10)	66.86 (0.08)	67.43 (0.09)	63.03 (0.07)
V_V (TI, Kid)	5.66^{Aa} (0.27)	9.29^{B} (0.28)	8.37 (0.25)	8.34 (0.13)	10.05^{b} (0.32)	8.32 (0.18)	7.37 (0.17)	6.42 (0.15)
V_V (Ln TI, TI)	11.52 (0.49)	15.26 (0.45)	15.13 (0.38)	16.82 (0.18)	17.14 (0.23)	16.12 (0.08)	15.16 (0.36)	18.58 (0.29)
V_V (TII, Kid)	14.63 (0.10)	20.14^{a} (0.17)	14.67^{A} (0.21)	22.27^{B} (0.08)	14.25 (0.21)	17.84^{a} (0.22)	16.77^{A} (0.21)	25.14^{Bb} (0.15)
V_V (Ln TII, TII)	5.34 (1.46)	11.06 (0.60)	11.05 (0.56)	13.48 (0.27)	8.42 (0.60)	9.78 (0.51)	5.26 (0.57)	12.46 (0.61)
V_V (DT, Kid)	1.08 (0.54)	0.91 (0.21)	1.03 (0.20)	1.22 (0.36)	1.60 (0.24)	1.53 (0.18)	1.86 (0.41)	1.80 (0.48)
V_V (Ln DT, DT)	0.42 (0.81)	0.38 (0.41)	0.30 (0.31)	0.39 (0.40)	0.27 (0.09)	0.27 (0.08)	0.23 (0.40)	0.23 (0.39)
V_V (CT, Kid)	2.60^{a} (0.39)	1.64^{a} (0.36)	3.51 (0.46)	4.23^{b} (0.30)	5.73^{b} (0.11)	3.79^{b} (0.61)	5.15 (0.40)	2.81 (0.33)
V_V (Ln CT, CT)	0.15 (0.16)	0.25 (0.59)	0.17 (0.23)	0.27 (0.62)	0.22 (0.32)	0.20 (0.51)	0.14 (0.30)	0.18 (0.17)
V_V (Corp, Kid)	1.11^{a} (0.37)	0.67^{a} (0.24)	1.49 (0.31)	1.64^{b} (0.18)	1.90^{b} (0.30)	1.55^{b} (0.08)	1.36^{A} (0.37)	0.60^{Ba} (0.27)
V_V (Tnew, Kid)	0.23^{a} (0.66)	0.10 (0.80)	0.10 (0.94)	0.32 (0.80)	0.16^{a} (0.37)	0.11 (0.95)	0.07^{b} (0.68)	0.06 (0.31)
V_V (Tvac, Kid)	0.34^{a} (1.02)	0.37^{a} (1.12)	0.00^{b} (2.24)	0.02^{b} (2.24)	0.00^{b}	0.00^{b}	0.00^{b}	0.13^{a} (0.80)

Results are shown as mean and coefficient of variation (standard deviation/mean) in brackets. Different upper case superscript letters represent differences between genders within each month; different lower case superscript letters represent differences among months within a gender; the presence of the same letter means absence of differences.

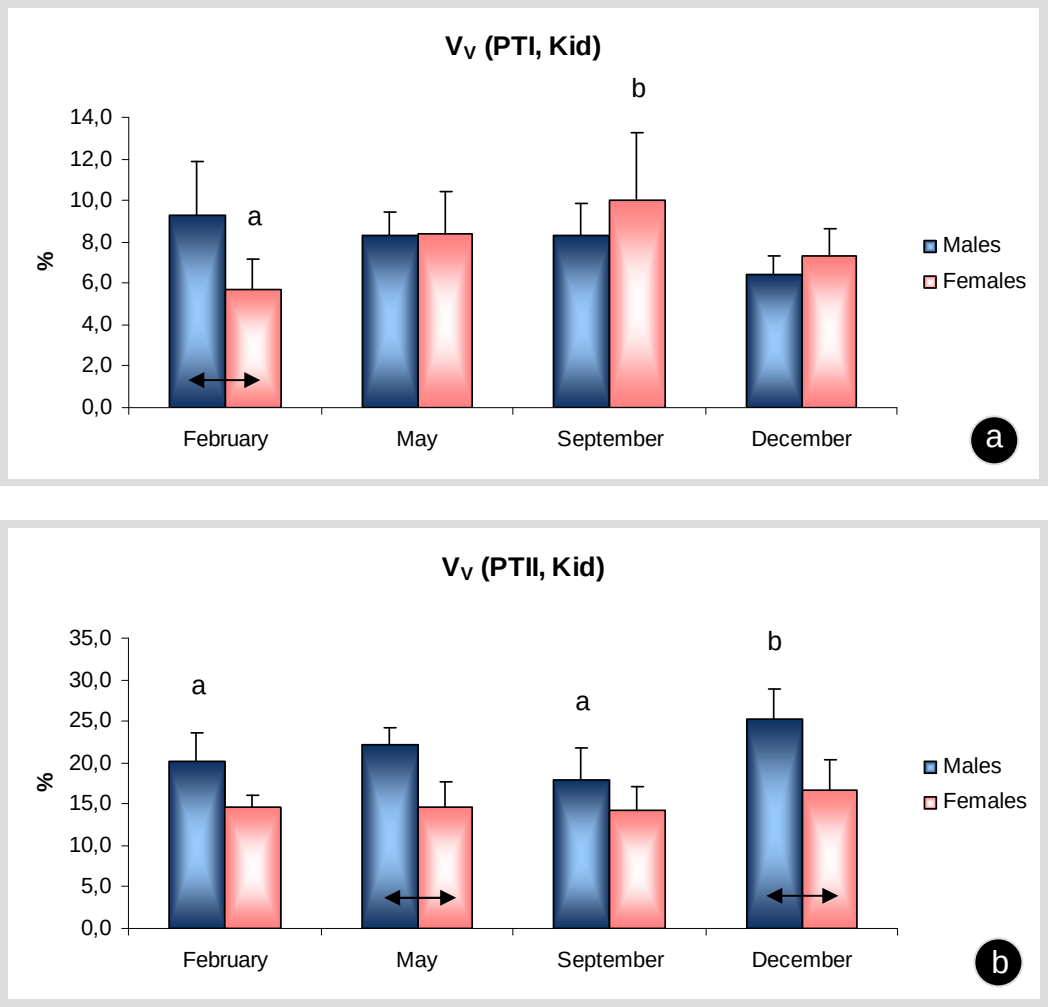

FIGURE 3. Relative volume (%) of the proximal tubules along the brown trout breeding cycle. **a.** Proximal tubule segment I (PTI). **b.** Proximal tubule segment II (PTII). Significant differences ($p \leq 0.05$) among months within a gender are represented by different letters; differences between genders within each month are illustrated by arrows. Vertical bar = standard deviation.

Respecting to the relative volume of the proximal tubule segment II epithelium, significant ANOVAs were detected with gender and season effects acting independently, but not for the effect of their interaction (Table 1). Further analysis showed that males differed from females in May and December, in which males showed almost 60% more relative volume of the segment II. Differences among seasons were only found in males, between the minimum relative volumes of September and the maximum of December, and marginally between February and December (Table 2 and Figure 3b). Additionally, the lumen relative volume also showed a significant ANOVA with an independent sex effect, although by further analysis significant differences were found neither between genders in the same

season nor among seasons for the same gender (Tables 1 and 2).

The relative volume of the distal tubule epithelium showed a significant ANOVA with an independent season effect, whereas the relative volume of its lumen did not (Table 1). However, and for both structural components, further post-hoc analyses did not show significant differences among seasons for each gender (Table 2).

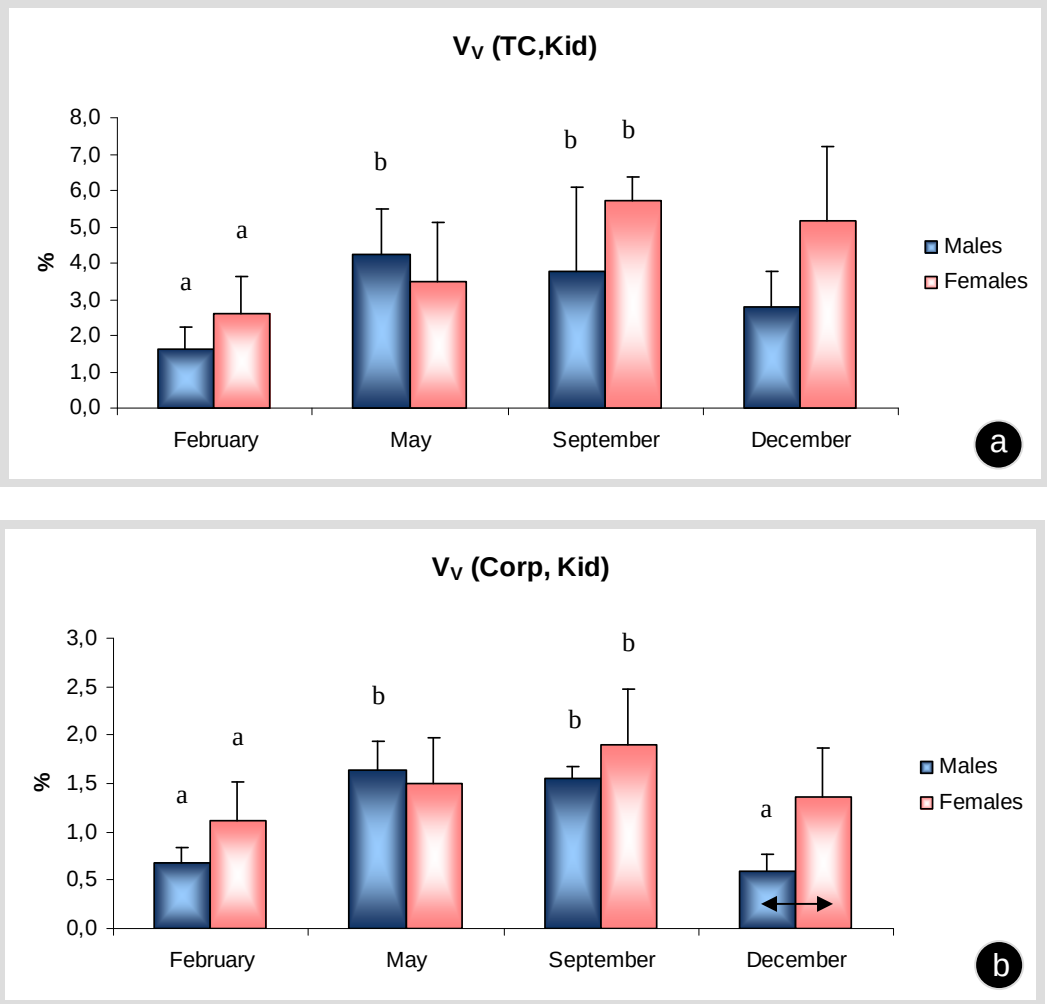

FIGURE 4. Relative volume (%) of some renal structures along the brown trout breeding cycle. **a.** Collecting tubules (TC). **b.** Renal corpuscules (Corp). Significant differences ($p \leq 0.05$) among months within a gender are represented by different letters; differences between genders within each month are illustrated by arrows. Vertical bar = standard deviation.

As to the relative volume of collecting tubule epithelium, significant ANOVAs were detected for the sex and season effects acting independently (Table 1). By the contrary,

there were no differences for the respective lumen. By post-hoc analysis, significant differences were not observed between genders in a season, although the collecting tubules of males from February were significantly lower in relative volume when compared to the highest values of May, and marginally significant with the values of September (Table 2 and Figure 4a).

A significant ANOVA with independently gender and season effects was detected for the relative volume of renal corpuscles (Table 1). Further analysis showed only a significant value between genders in December, but in which females had twice the volume of corpuscules in comparison to males (Table 2 and Figure 4b). Additionally, significant differences in the relative volume of renal corpuscules were found among seasons. In females, between the lower values of February and the highest of September, whereas in males from December and February presented the lowest corpuscules volume, which were significantly different from the highest values of May and September (Table 2 and Figure 4b).

After a non-parametric analysis, only the females presented significant differences for the relative volume of the new growing tubules, being February the season where the highest volumes were found compared with the values of September and December (Table 2 and Figure 5a).

The relative volume of the vacuolized tubules was also non-parametrically analysed and significant differences existed in both genders (Table 2 and Figure 5b). Indeed, February was almost the only season where this type of tubules was seen in females; thus, they significantly differed from the other seasons. In males, February and December were the months in which vacuolized tubules were observed and quantified with the values being significantly different from the ones (practically zero) estimated in May and September.

Additionally, linear correlations were found between the seasonal pattern of the relative volume of some renal structures and the GSI and/or the RSI. In females, correlations with the GSI were observed: 1) a positive correlation with the relative volume of collecting tubules ($r = 0.53$; $p = 0.015$), and 2) a negative one with the relative volume of vacuolized tubules ($r = -0.62$; $p = 0.003$). On the contrary, males did not present correlations with the GSI. In relation to the RSI, females presented negative (nevertheless weak) correlations between that index and the relative volume of collecting tubules ($r = -0.45$; $p = 0.048$), as also with the relative volume of renal corpuscules ($r = -0.49$; $p = 0.029$), plus a positive (stronger) correlation between the RSI and the relative volume of vacuolized tubules ($r = 0.72$; $p < 0.001$). In males, negative correlations were found between the RSI and the: 1)

relative volume of collecting tubules (r = -0.57; p = 0.008); 2) relative volume of renal corpuscules (r = -0.84; p < 0.001). Also, there was a positive correlation between the male RSI and the relative volume of vacuolized tubules (r = 0.84; p < 0.001).

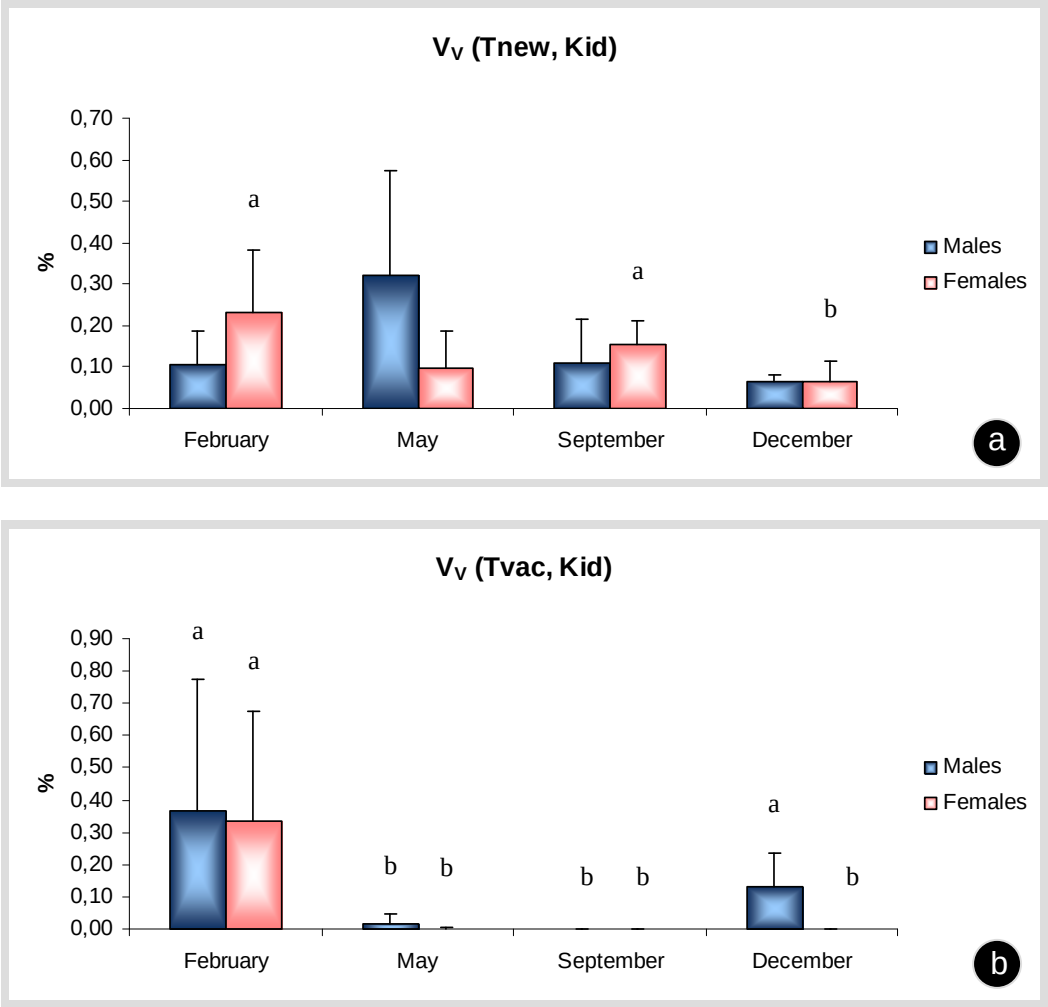

FIGURE 5. Relative volume (%) of some renal tubules along the brown trout breeding cycle. **a.** New growing tubules (Tnew). **b.** Vacuolized tubules (Tvac). Significant differences (p≤0.05) among months within a gender are represented by different letters; differences between genders within each month are illustrated by arrows. Vertical bar = standard deviation.

Discussion

Fish renal morphology has been studied for a long time, but the vast heterogeneity of this organ among fish species makes the available literature still quite insufficient for a detailed picture. Studies quantitatively characterizing the renal structure are even more rare

(Kamunde *et al.*, 1997) and mostly regarding toxicological studies (Oulmi *et al.*, 1995a, 1995b; Oulmi and Braunbeck, 1996; Weber *et al.*, 2003; Triebskorn *et al.*, 2004). Thus, this work aimed not only a qualitative characterization of the several components from brown trout kidney but also a quantitative analysis, with stereological methods to provide baseline data to support morphofunctional correlations, and also to determine whether changes existed in the renal components during the male and female reproductive cycle.

After visualization of thin and semithin sections of brown trout kidney, we encountered the usual renal morphological structures, such as corpuscules, proximal, distal and collecting tubules, as described in the literature for freshwater fishes and particularly for several trout species (Anderson and Loewen, 1975; Elger *et al.*, 2000). Thus, identification of the basic renal structures was straightforward, permitting their quantification with a high degree of accuracy. Nonetheless, unusual structures were also observed and quantified, such as the growing and the vacuolized tubules. The identification of these tubules as being one of the typical tubule portions was not possible. Vacuolized tubules were mostly observed in February, being almost absent in the other seasons. By now, descriptions of vacuolized tubules in fish were found in literature associated to pesticides and heavy metals exposure (Rangsayatorn *et al.*, 2004; Zhang *et al.*, 2005; Velmurugan *et al.*, 2007a; 2007b), which could suggest that in February our fishes were exposed to some kind of unknown water contaminant. However, this hypothesis seems highly unlikely facing, for instance, the location of the aquaculture station (Torno, Amarante, Portugal), high in the mountains and feed with plenty pristine water, with no records of pollution influence. Moreover, the vacuolated tubules depicted in literature, so far, clearly differ in aspect from those found herein, and, additionally, pesticide exposures elicited other tubular and non-tubular lesions, which could not be found in our fish (Rangsayatorn *et al.*, 2004; Zhang *et al.*, 2005; Velmurugan *et al.*, 2007a; 2007b). Another aspect was the very low percentage of affected tubules, which was not really consistent with pollution exposure, and seemed to point more to focal tubular remodelling. In agreement, the linear negative correlation of vacuolated tubules with the GSI in females, and a similar trend being seen in males, might point to some kind of connection with breeding as pointed for other organisms, such as the reptile water snake *Nerodia sipedon* (Krohmer *et al.*, 2004). Another interesting aspect was the consistent and positive correlation between the RSI and the relative volume of the vacuolated tubules, thus suggesting some connection between the presence of the vacuoles and the seasonal kidney turnover, poorly studied so far. Despite a parasitic nature could not be sought, no signs of infection existed in those weird tubules, including absence of peritubular inflammation.

Despite attaining only some of the structural components, the quantitative results demonstrated that brown trout kidney had morphological changes during the reproductive cycle. Renal corpuscules, proximal, collecting and vacuolized tubules were the renal structures which showed seasonal variations in their relative volume as well as differences between genders. Moreover, the alterations happened in the tubule epithelium only, since no variations in lumina existed. Data revealed that renal corpuscules and collecting tubules had the same variation pattern along the year suggesting that - despite the fact that all renal components structure is modulated in accordance to the urine production specific needs (Larsen and Perkins Jr., 2001) - a common factor seemed to influenced those two renal components. Furthermore, both structures presented negative correlations with RSI in either gender indicating that those structures occupied less 3D space when the kidney had an increased relative weight. There was only one positive correlation between GSI and the relative volume of female collecting tubules. Probably, at the period around the breeding season (starting earlier for males than females), hormones which regulate urine formation and which could be influenced in some way by reproductive factors, might be responsible for those variations (Larsen and Perkins Jr., 2001).

Another point of consideration is the fact that new growing tubules were observed in all sampled periods for both genders, and that their relative volume changed along the year in females. Analysing that variation pattern, in which maximum values were found in February whereas the minimum values were observed in December, and the opposite variation pattern of the collecting tubules relative volume, it could be speculated that the majority of new growing tubules from February could originate collecting tubules in order to establish a recovering from their minimum values observed in the same period. Anyway, before accepting such hypothesis, there are key questions to be solved regarding new tubules, not only herein but in general, for fish. Which cells originate the new tubules? Are they capable or merging with the existing network or do they form solely new nephrons? The patterns found justify plainly further investigation, namely looking at the 3D connections that the growing darker tubules establish or not with the existing kidney components.

Among the several structures which compose brown trout kidney, renal stroma occupied an annual global average of 67% of the total organ volume, which can be further divided in 1% of renal corpuscules, 8% of proximal tubules segment I, 18% of proximal tubules segment II, 1.5% of distal tubules, 4% of collecting tubules, 0.2% of tubules in formation and 0.1% of vacuolized tubules. Although, if we consider the nephronic tissue volume as

the reference space, the mean volumes proportions can be overally divided in 3% of renal corpuscules, 24% of proximal tubules segment I, 55% of proximal tubules segment II, 4.5% of distal tubules, 12% of collecting tubules, 0.6% of new tubules and 0.3% of vacuolized tubules. These results are moderately distant from the published data for some teleosts species belonging to Perciformes order and Ostariophysi superorder, and even with major differences in some aspects, like in the volume of distal tubules (Kamunde *et al.*, 1997). For those fish species the results found were, on average 3.84-6.54% of renal corpuscules, 0.50-1.11% of neck segments, 67.17-69.72% of proximal segments, 18.16-20.55% of distal segments and 5.66-7.41% of collecting tubule-collecting duct system. Thus, despite the different values of our results and the published ones in relation to the proximal tubules, in which 79% was the relative volume of these tubules found in brown trout kidney nephronic tissue (proximal tubules segment I including neck segments, plus proximal tubules segment II) instead of the approximately 70% revealed by Kamunde *et al.* (1997) for the same tubules type, and also in relation to the collecting tubules, 12% in brown trout against the approximately 6% of published data, they are in the same order of magnitude. The comparison between the relative volumes of distal tubules of those fish species displayed a bigger difference, 4.5% in brown trout against the 19% of the studied teleosts species. The similarity of some of the results likely lies on the fact that all the referred species are freshwater fishes; thus, they have to produce much diluted urine, which imply renal features such as the existence of large glomeruli for high filtration rates and long proximal tubules for the consequent high rates of filtrate reabsorption, as well as the presence of distal tubules (Hentschel and Elger, 1987; Elger *et al.*, 2000). Nevertheless, the discrepancy of the results found for distal tubules can be explained by the existence of great variation in the length of renal tubules according not only to the species and its habitat, but also because of an age-related degree of nephron differentiation, described as an individual heterogeneity (Hentschel and Elger, 1987).

In conclusion, we described the qualitative histology of brown trout kidney, showing that all usual components described for freshwater species exist and can be discriminated both using paraffin sections and semithin epoxy sections. Also, we described for the first time a rare event, mainly appearing at post-spawning, consisting in tubules with a peculiar vacuolation (from mild to a severe degree). The cause and significance of such vacuolized tubules is now at stake. Also, we showed that gender and seasonal changes exist in some common kidney components, which necessarily imply that there is a seasonal kinetics of events that must be based on some sort of remodelling according to the physiological needs of the animals. The most striking changes attained the corpuscules and collecting

tubules, suggesting seasonal fluctuations in the filtration rate and urine production, unstudied yet. This study supports future approaches for understanding the significance of the changes and also how they are governed, likely by hormones but also by other factors.

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CHAPTER 3

SEASONAL AND MORPHOLOGICAL VARIATIONS OF BROWN TROUT (*SALMO TRUTTA*) KIDNEY PEROXISOMES – A STEREOLOGICAL STUDY

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Seasonal and Morphological Variations of Brown Trout (*Salmo trutta*) Kidney Peroxisomes – A Stereological Study

Abstract

Literature about fish kidney peroxisomes is still scarce. In this work a stereological approach on renal peroxisome morphological parameters was performed for the first time in a fish, establishing baseline fundamental data for peroxisomes in kidney tubules and also aiming the detection of possible correlations with the maturation stage as it was observed in brown trout liver. Thus, three-year-old brown trout males and females were collected in February (after spawning), May (early vitellogenesis), September (advanced vitellogenesis) and December (pre-spawning). For peroxisomal detection, trunk kidney was fixed and processed for catalase cytochemistry. Classical stereological methods were applied on electron microscopic photographs to estimate relative volumes, surfaces and numbers of peroxisomes. Besides, their mean surface, volume and spherical equivalent diameter were thereafter derived. Different seasonal variation patterns were observed not only between genders, but also between renal proximal tubule segments I and II. Most of the variations in the size and number of peroxisomes were in the proximal tubule segment II, where it was detected a higher abundance of these organelles. In males, peroxisomes from proximal tubule segment II had a relatively higher volume and number in May, being individually bigger in February. In the same parameters, females presented similar trends as males, though with weaker 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 GSI. Hence, and despite the variations, the morphology of renal peroxisomes seems not strictly correlated with the gonad kinetics, so suggesting that kidney peroxisomes morphology is not seasonally modulated by sex steroids, like estradiol, as it seems to happen in liver peroxisomes. Any way, our study demonstrates that gender and season must be taken into account when interpreting changes in fish kidney peroxisomes, namely in toxicological scenarios.

Introduction

The study of peroxisomes became a significant research area especially because of their association with some hereditary human diseases (Roels *et al.*, 1991; 1993; Masters and Crane, 1995). The discovery of many chemicals which induce peroxisome proliferation is other aspect of interest attributed to peroxisome research (Reddy *et al.*, 1982; Reddy and Rao, 1987; Fahimi and Cajaraville, 1995), especially because a great number of those proliferators also induce hepatocellular tumours (Reddy and Lalwai, 1983).

Quantitative studies have been done in mammals in order to investigate several peroxisomal associated diseases as well as experimental conditions inducing peroxisome proliferation and peroxisomal ageing (Beier and Fahimi, 1991; 1992; Beier *et al.*, 1993; Stefanini *et al.*, 1995). In these works, the application of stereological methods revealed to be very useful in situations connecting peroxisomes with cancer, steatosis, alcohol consumption or nephrectomy studies (Decraemer *et al.*, 1991; 1993; 1994; 1995; 1996; 1997). However, in other vertebrates and invertebrates the literature focusing quantitative studies is reduced, although the emphasis given by some authors to the importance of comparative perspectives (Fahimi and Cajaraville, 1995; Lobo-da-cunha, 1995; 1997; Rocha *et al.*, 1999). Additionally, the fact that many pollutants which are released into water systems are peroxisome proliferators warns for the danger of xenobiotic induced peroxisomal disorders in aquatic organisms. As a consequence, some studies concerning fish liver and kidney alterations were already made, in which toxic compounds caused cellular damage and organeolar alterations in both organs, with physiological implications (Rojik *et al.*, 1983; Thophon *et al.*, 2004; Fishelson, 2006). Particularly related to peroxisomal alterations, literature focused toxicological exposure effects, covering not only the induction of morphological changes, such as the increase in number and volume of these organelles, but also modifications on their biochemical properties, such as the increase of catalase and some oxidases activities (Braunbeck and Volkl, 1991; Braunbeck *et al.*, 1992; Braunbeck, 1993; Arnold *et al.*, 1995; Oulmi *et al.*, 1995b; Zahn and Braunbeck, 1995; 1996; Oulmi and Braunbeck, 1996; Zahn *et al.*, 1996; Schramm *et al.*, 1998; Braunbeck and Strmac, 2001; Cajaraville and Ortiz-Zarragoitia, 2006). Semi-quantitative and morphometric peroxisomal evaluations from fishes exposed to chemicals, such as linuron and atrazine, indicated a general increase of peroxisome number, especially in liver (Braunbeck *et al.*, 1992; Oulmi *et al.*, 1995b; Oulmi and Braunbeck, 1996; Braunbeck and Strmac, 2001; Strmac and Braunbeck, 2002). Published data is mainly reported in liver, being the kidney studies limited to a few works (Oulmi *et al.*, 1995a, 1995b; Oulmi and Braunbeck, 1996; Ozaki *et al.*, 2001).

In addition, it was also documented that fish peroxisomes undergo alterations in their enzymatic activities under certain conditions of salinity, temperature, season and feeding habits (Braunbeck *et al.*, 1987; Fahimi and Cajaraville, 1995), as well as morphological changes apparently modulated by sex steroids, and particularly by estradiol (Veranic and Pipan, 1992; Rocha *et al.*, 1999; Ibabe *et al.*, 2002). Nevertheless, such studies focused on liver peroxisomes and so the influences of those factors are still unknown in renal peroxisomes.

It is well established that in fish kidney, as seen in other vertebrates, 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 the 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. Additionally, and to our best knowledge, a quantitative morphological study of peroxisomes in renal tubules was not made.

This work was made with three purposes: 1) to investigate structural changes of kidney tubules peroxisomes occurring in adult brown trout along 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 relative and absolute individual size values of these organelles. These findings, adding to the knowledge about hepatic peroxisomes, will give a better overview of peroxisome “behaviour” in brown trout under sex steroids influence, eventually providing other clues for factors that influence the kinetics of those organelles.

Material and Methods

Animals

Five adult males and five adult females of three-year-old 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 trout natural reproductive cycle: February (post-spawning), May (early vitellogenesis), September (advanced vitellogenesis) and December (pre-spawning). Before being

sacrificed, the fish were held in observation tanks for 12 to 24 h. All fish were considered healthy. The mean body standard length and weight, as well as the gonado-somatic (GSI) and the reno-somatic (RSI) indexes are presented in Table 1 of Chapter 2.

Tissue Processing

Animals were anaesthetized by immersion in a 1 ml L^{-1} aqueous solution of ethylene glycol monophenyl ether, and then weighed and measured in length. Before kidney collection, a perfusion was made with a 5 IU/ml heparinised isosmotic buffer for salmonids (Cascales *et al.*, 1997), composed of: NaCl 0.74%, KCl 0.04%, $CaCl_2$ 0.02%, $MgSO_4$ 0.15%, $NaHCO_3$ 0.03%, $NaH_2PO_4 \bullet H_2O$ 0.05%, $Na_2HPO_4 \bullet 2H_2O$ 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 at 4 ºC and with a physiological flow rate of about 5 ml min^{-1} kg^{-1} of body weight (Hampton *et al.*, 1985) during the time necessary to eliminate the organ residual blood. The trunk kidney was 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 kidney). Systematic samplings were then performed for obtaining final samples made of very tiny pieces possessing the adequate size for catalase cytochemistry procedure at electron microscopy level. This 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 ºC. Gonads were also removed and weighted. After fixation kidney pieces were rinsed for 2 h (at 4 ºC) in 0.1 M phosphate buffer (pH 7.4). For catalase detection, pieces were incubated for 2 h (at 37 ºC) in a medium containing 0.12% of H_2O_2 and 2 mg ml^{-1} of DAB in 0.1 M Tris-HCl buffer (pH 8.5) (Veenhuis and Bonga, 1979). After incubation, the pieces were washed twice at 4 ºC, firstly 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. A post-fixation in 0.1 M phosphate buffered 1% OsO_4 with 1.5% $K_3Fe(CN)_6$ was carried out for 2 h at 4 ºC. 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, 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, extratubular components and distal tubules). A total of approximately 50 fields per animal (30 fields of proximal tubules segment I and 20 fields of proximal tubules segment II) were taken at a magnification of × 5,300, and further printed at a final magnification of × 15,900. A total of 2,000 TEM fields were analysed 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 (Weibel, 1979):

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

in which P(s) is the total number of points within each structural component, P(r) is the total number of test points lying over the reference space (tubule cells) and *k* is the ratio between the number of points of the grid used for the structure of 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.

The surface densities were estimated also by the use of an unbiased manual stereological technique based on point and intersection counting (Baddeley *et al.*, 1986):

$$S_V \text{ (structure, reference space)} = 2 \times (p \div l) \times [\sum I(s) \div \sum P(r)]$$

in which $(p \div l)$ is the ratio of test points to test lines length (according to magnification) for a particular grid, $\sum I(s)$ is the sum of all intersection counts of test lines across boundaries of the structures (peroxisomes) and $\sum P(r)$ is the sum of all points falling over the reference space (tubule cells).

The numerical densities of peroxisomes were estimated on the fields used for point and intersection counting, and according the technique of Weibel and Gomez (Weibel, 1979):

$$N_V \text{ (structure, reference space)} = (N_A^{3/2} \div V_V^{1/2}) \times (K \div \beta)$$

in which N_A is the number of structures (peroxisomes) per unit area of reference space

(tubule cells), counted using an unbiased rectangular counting frame bearing forbidden lines (Gundersen, 1977), V_V is the volume density of peroxisomes within the tubule cell, β is a shape coefficient (the value of 1.382 for spherical particles was adopted, because the majority of peroxisomes in our material were fairly roundish) and K is a constant which relates with the size of the objects. It was shown that K may be disregarded or replaced by an arbitrary number between 1.02 and 1.1 (Weibel, 1979). The value of 1.1 was adopted in this study for K based in the previous work of Rocha *et al.* (1999).

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

$\bar{v}_N$ (peroxisome) = V_V (peroxisome, tubule cell) ÷ N_V (peroxisome, tubule cell)

$\bar{s}_N$ (peroxisome) = S_V (peroxisome, tubule cell) ÷ N_V (peroxisome, tubule cell)

The spherical equivalent mean diameter $\bar{d}_{Sphere}$ of a peroxisome was calculated using the classical formula: $\bar{v}_N$ = 1/6 x π x $\bar{d}_{Sphere}$.

Statistical Analysis

The Statistica 6.0 for Windows was used to analyse the data. 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. Tukey and Newman-Keuls post-hoc tests for multiple comparisons between means were further applied. By default, differences were considered significant when $p \leq 0.05$. When a difference was significant according to Newman-Keuls but not Tukey post-hoc tests, we considered it marginally significant. 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 linear associations.

Results

Peroxisomal Stereology

The results of the two-way ANOVAs of all stereological parameters from proximal tubule

segments I are summarized in Table 1 and from proximal tubule segments II in Tables 2 and 3. The stereological data of peroxisomes are shown in Tables 4, 5 and 6.

TABLE 1.

Summary of two-way ANOVAs showing the effects of age, season and their interaction on all peroxisomal parameters from proximal tubule segment I: surface density (S_v), volume density (V_v), numerical density (N_v), spherical-equivalent diameter ($\overline{d}_{Sphere}$), surface ($\overline{s}_N$), volume ($\overline{v}_N$). P – peroxisome, Cell – proximal tubule cell, Cyt – cytoplasm.

Parameters	Sex		Season		Interaction (Sex × Season)	
	Fisher's F ratio	*p* value	Fisher's F ratio	*p* value	Fisher's F ratio	*p* value
S_v (P, Cell)	4.40	0.044	1.81	0.167	0.10	0.962
V_v (P, Cell)	4.46	0.043	0.55	0.651	0.70	0.976
N_v (P, Cell)	5.90	0.021	2.99	0.046	1.13	0.353
$\overline{d}_{Sphere}$	0.12	0.732	4.58	0.009	1.76	0.175
$\overline{s}_N$	0.65	0.427	2.61	0.069	1.17	0.336
$\overline{v}_N$	0.08	0.777	4.84	0.007	1.79	0.169
S_v (P, Cyt)	3.88	0.058	2.30	0.097	0.07	0.973
V_v (P, Cyt)	3.45	0.073	1.07	0.374	0.02	0.996
N_v (P, Cyt)	7.29	0.011	4.21	0.013	0.89	0.459

Degrees of freedom (df): df(sex) = 1; df (season) = 3; df (interaction) = 3; df (residual) = 31.

In the proximal tubule segment I, significant ANOVAs were detected for the relative stereological parameters of peroxisomes, such as the V_V, S_V and N_V: independent effect of sex in all parameters, independent effect of season in N_V and no significant ANOVAs for the sex and season interaction effect (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 4).

TABLE 2.

Summary of two-way ANOVAs showing the effects of age, season and their interaction of all measured peroxisomal parameters from proximal tubule segment II: surface density (S_v), volume density (V_v), numerical density (N_v). P – peroxisome, Cell –proximal tubule cell, Cyt – cytoplasm.

Parameters	Sex		Season		Interaction (Sex × Season)	
	Fisher's F ratio	*p* value	Fisher's F ratio	*p* value	Fisher's F ratio	*p* value
S_v (P, Cell)	54.13	<0.001	13.33	<0.001	2.15	0.114
V_v (P, Cell)	40.98	<0.001	8.11	<0.001	1.86	0.158
N_v (P, Cell)	62.80	<0.001	12.82	<0.001	4.36	0.011
S_v (P, Cyt)	53.81	<0.001	12.78	<0.001	2.51	0.077
V_v (P, Cyt)	38.21	<0.001	7.33	<0.001	2.03	0.131
N_v (P, Cyt)	64.15	<0.001	12.14	<0.001	4.92	0.007

Degrees of freedom (df): df(sex) = 1; df (season) = 3; df (interaction) = 3; df (residual) = 31.

TABLE 3.

Summary of non-parametric ANOVAs showing the effects of season in both genders of all measured peroxisomal parameters from proximal tubule segment II: spherical-equivalent diameter ($\overline{d}_{Sphere}$), surface ($\overline{s}_N$), volume ($\overline{v}_N$).

Parameters	Season (*p* value)	
	♀	♂
$\overline{d}_{Sphere}$	0.009	0.029
$\overline{s}_N$	0.014	0.510
$\overline{v}_N$	0.009	0.029

Contrary to the relative peroxisome parameters, the individual absolute dimensions of peroxisomes ($\bar{d}_{Sphere}$, $\bar{s}_N$ and $\bar{v}_N$) from proximal tubule segment I were deeply influenced by the season, whereas the sex and the sex and season interaction did not exert effects (Table 1). In males, none of the parameters underwent variation either among seasons or between genders (Table 5). However, in females, the $\bar{d}_{Sphere}$ and $\bar{v}_N$ changed along the year and with a 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 (Figure 1). Between these months (in September) a slight increasing trend (not statistically significant) in the peroxisome size was observed. The $\bar{s}_N$ 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 $\bar{s}_N$, in which only a sex effect was observed by a significant ANOVA (Table 3). Further analysis revealed that, in males, $\bar{s}_N$ did not vary, whereas $\bar{v}_N$ and $\bar{d}_{Sphere}$ presented changes along the year and with a similar variation pattern for both parameters (Table 5). Thus, male peroxisomes were significantly bigger in February, decreasing in May and maintaining smaller dimensions onwards to September and December (Figure 2). Despite the absence of statistical significant differences for the $\bar{s}_N$, values pointed to the same variation pattern. In females, the seasonal pattern was similar for $\bar{s}_N$, $\bar{v}_N$ and $\bar{d}_{Sphere}$, though different from the males; the highest dimensions of peroxisomes were found in February and September (Figure 3). Differences between genders were only detected in February, where males had bigger peroxisomes than females (Figures 2 and 3).

TABLE 4.

Relative stereological parameters of proximal tubules segments I and II from brown trout along its reproductive cycle. S_v, V_v, N_v – surface, volume and numerical densities of peroxisomes (P) in relation to the tubule cell.

	Proximal Tubule I					
Parameters	S_v (P, Cell I) ($\mu m^2 / \mu m^3$)		V_v (P, Cell I) (%)		N_v (P, Cell I) (no. / μm^3)	
Sex	♀	♂	♀	♂	♀	♂
February	0.05 (0.15)	0.05 (0.19)	0.68 (0.19)	0.54 (0.17)	0.06 (0.13)	0.06 (0.39)
May	0.07 (0.40)	0.06 (0.15)	0.72 (0.37)	0.59 (0.15)	0.10 (0.23)	0.08 (0.17)
September	0.06 (0.26)	0.05 (0.21)	0.68 (0.25)	0.58 (0.27)	0.08 (0.13)	0.06 (0.24)
December	0.06 (0.17)	0.04 (0.41)	0.60 (0.20)	0.52 (0.48)	0.09 (0.34)	0.06 (0.50)
	Proximal Tubule II					
Parameters	S_v (P, Cell II) ($\mu m^2 / \mu m^3$)		V_v (P, Cell II) (%)		N_v (P, Cell II) (no. / μm^3)	
Sex	♀	♂	♀	♂	♀	♂
February	0.07 (0.18) ^{Aa}	0.02 (0.89) ^{Ba}	0.80 (0.18) ^{A}	0.34 (0.74) ^{Ba,c}	0.12 (0.17) ^{Aa}	0.02 (0.71) ^{Ba}
May	0.12 (0.10) ^{Ab}	0.08 (0.33) ^{Bb}	1.11 (0.12)	0.83 (0.37) $^{b, c}$	0.24 (0.27) ^{Ab}	0.14 (0.41) ^{Bb}
September	0.09 (0.27) ^{a}	0.06 (0.36) ^{b}	0.91 (0.29)	0.57 (0.33) ^{c}	0.13 (0.19) ^{a}	0.09 (0.50)
December	0.09 (0.21) ^{Aa}	0.02 (0.71) ^{Ba}	0.88 (0.25) ^{A}	0.18 (0.72) ^{Ba}	0.22 (0.25) ^{Ab}	0.04 (0.96) ^{Ba}

Results are shown as mean (CV). CV is the coefficient of variation (CV = standard deviation/mean). Different upper case superscript letters represent differences between genders within each month; different lower case superscript letters represent differences among months within a gender; the presence of the same letter means absence of differences.

TABLE 5.

Absolute stereological parameters of individual peroxisomes of segments I and II proximal tubules from brown trout kidney along its reproductive cycle. S_v, V_v, N_v – surface, volume and numerical densities of peroxisomes (P) in relation to the tubule cell.

Proximal Tubule I

Parameters	$\overline{d}_{Sphere}$ (µm)		$\overline{s}_N$ (µm^2)		$\overline{v}_N$ (µm^3)	
Sex	♀	♂	♀	♂	♀	♂
February	0.31 (0.16) [a]	0.24 (0.33)	0.90 (0.09)	0.79 (0.23)	0.12 (0.16) [a]	0.09 (0.33)
May	0.19 (0.24) [b]	0.20 (0.18)	0.67 (0.22)	0.75 (0.13)	0.07 (0.24) [b]	0.08 (0.14)
September	0.22 (0.20)	0.26 (0.21)	0.77 (0.21)	0.91 (0.15)	0.09 (0.20)	0.10 (0.21)
December	0.19 (0.37) [b]	0.22 (0.22)	0.67 (0.26)	0.72 (0.29)	0.07 (0.37) [b]	0.08 (0.22)

Proximal Tubule II

Parameters	$\overline{d}_{Sphere}$ (µm)		$\overline{s}_N$ (µm^2)		$\overline{v}_N$ (µm^3)	
Sex	♀	♂	♀	♂	♀	♂
February	0.17 (0.05) [Aa]	0.38 (0.33) [Ba]	0.58 (0.03) [a]	1.06 (0.44)	0.07 (0.05) [Aa]	0.15 (0.33) [Ba]
May	0.12 (0.22) [b]	0.16 (0.22) [b]	0.50 (0.23)	0.60 (0.20)	0.05 (0.22) [b]	0.06 (0.22) [b]
September	0.17 (0.10) [a]	0.20 (0.41) [b]	0.63 (0.08) [a]	0.72 (0.33)	0.07 (0.10) [a]	0.08 (0.41) [b]
December	0.11 (0.29) [b]	0.19 (0.68) [b]	0.41 (0.25) [b]	0.74 (0.61)	0.04 (0.29) [b]	0.07 (0.68) [b]

Results are shown as mean (CV). CV is the coefficient of variation (CV = standard deviation/mean). Different upper case superscript letters represent differences between genders within each month; different lower case superscript letters represent differences among months within a gender.

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 (Table 1). However, it was detected a sex and season independent effect in the number of peroxisomes per µm^3 of cytoplasm, but not a sex and season 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 6).

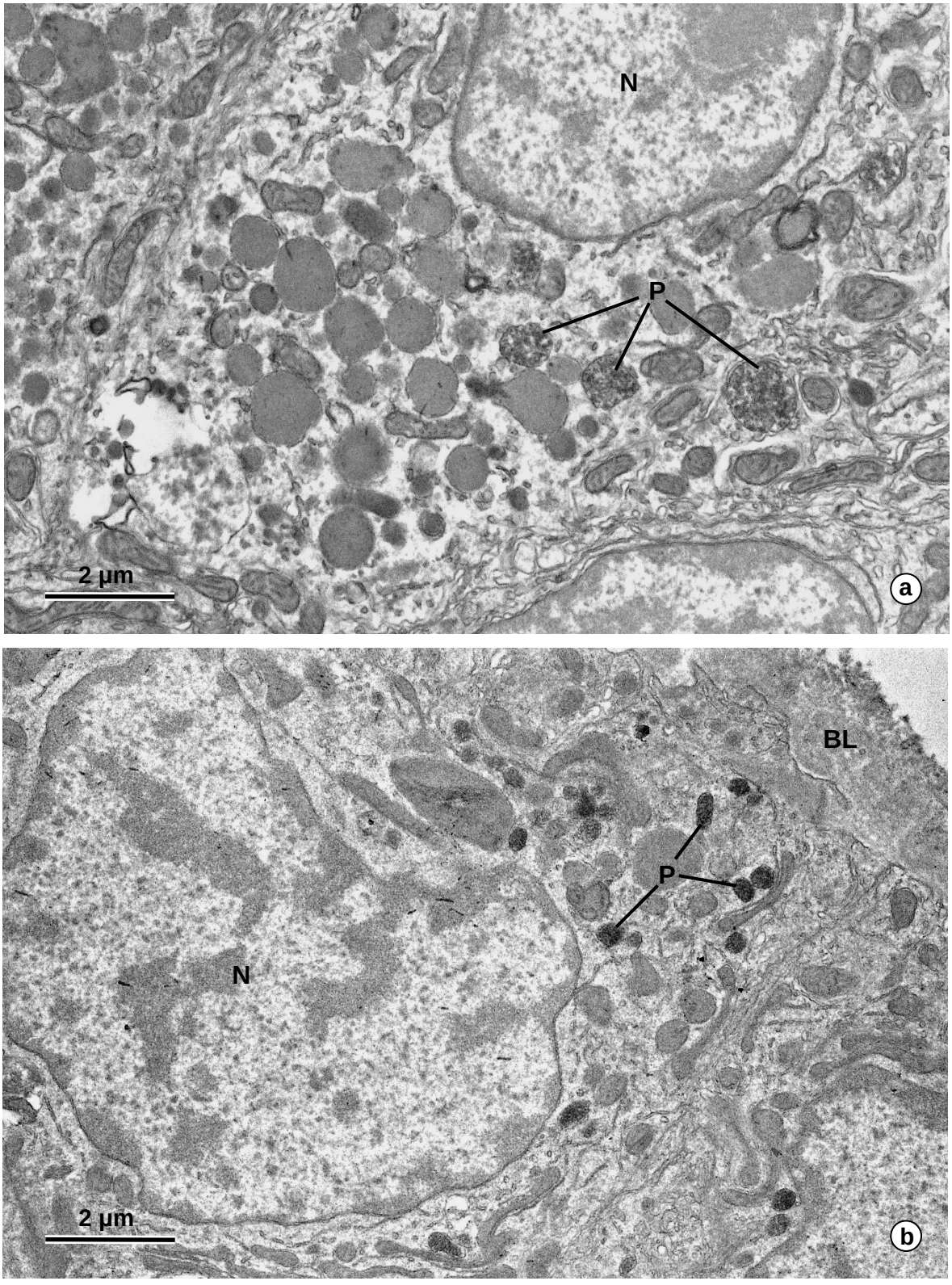

FIGURE 1. Ultrathin sections of proximal tubule segment I from female brown trout kidney taken in February (a) and in December (b), with peroxisomes (P) stained after DAB reaction. N - nucleus, BL – basal lamina.

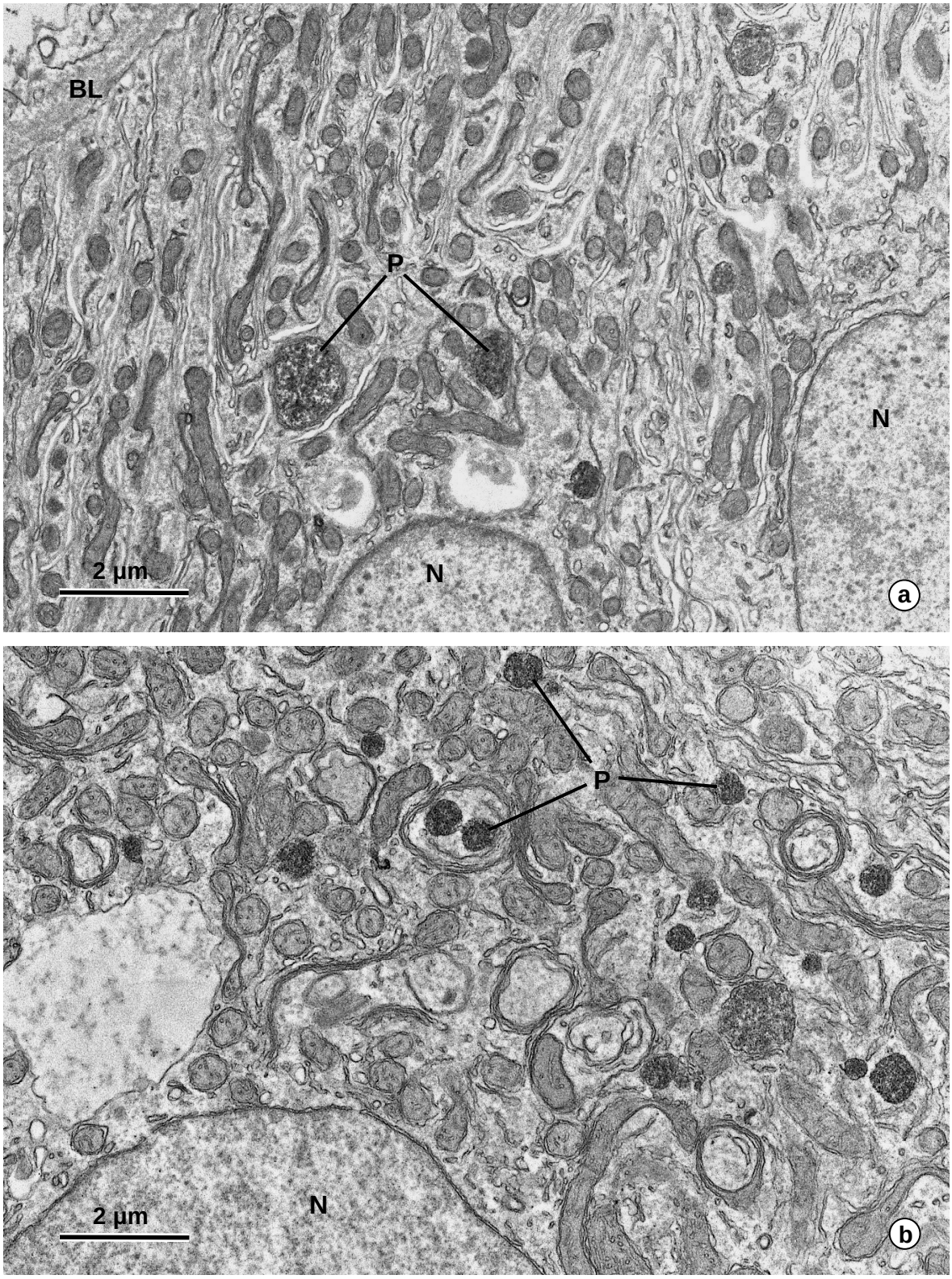

FIGURE 2. TEM images of proximal tubule segment II from male brown trout kidney taken in February (a) and in May (b), with peroxisomes (P) evidenced by DAB staining for catalase detection. N - nucleus, BL – basal lamina.

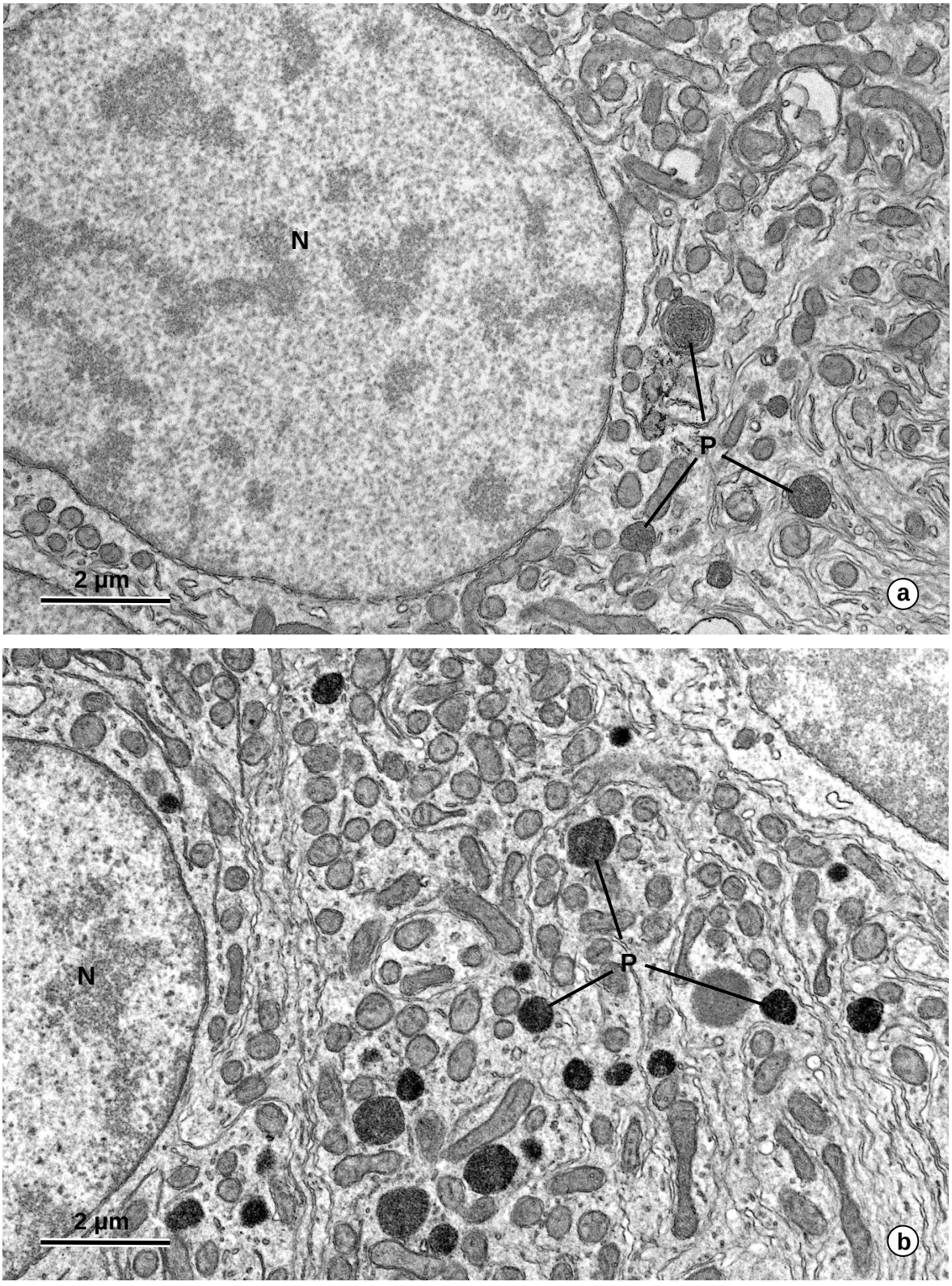

FIGURE 3. Micrographs of proximal tubule segment II from female brown trout kidney taken in February (a) and in May (b), with peroxisomes (P) cytochemically stained for catalase. N - nucleus.

TABLE 6.

Relative stereological parameters of segments I and II proximal tubules from brown trout kidney along its reproductive cycle. S_v, V_v, N_v – surface, volume and numerical densities of peroxisomes (P) in relation to the cytoplasm of the tubule cell.

	Proximal Tubule I					
Parameters	S_v (P, Cyt) ($\mu m^2 / \mu m^3$)		V_v (P, Cyt) (%)		N_v (P, Cyt) (no. / μm^3)	
Sex	♀	♂	♀	♂	♀	♂
February	0.07 (0.22)	0.06 (0.19)	0.90 (0.22)	0.70 (0.18)	0.07 (0.20) ^{a}	0.07 (0.37)
May	0.09 (0.39)	0.08 (0.17)	0.90 (0.36)	0.80 (0.15)	0.12 (0.23) ^{b}	0.10 (0.21)
September	0.08 (0.29)	0.07 (0.21)	0.90 (0.28)	0.80 (0.26)	0.10 (0.13)	0.08 (0.24)
December	0.07 (0.15)	0.06 (0.37)	0.70 (0.23)	0.60 (0.50)	0.12 (0.31)	0.08 (0.44)
	Proximal Tubule II					
Parameters	S_v (P, Cyt) ($\mu m^2 / \mu m^3$)		V_v (P, Cyt) (%)		N_v (P, Cyt) (no. / μm^3)	
Sex	♀	♂	♀	♂	♀	♂
February	0.09 (0.23) ^{Aa}	0.03 (0.91) ^{Ba}	1.00 (0.23) ^{A}	0.40 (0.85) $^{Ba, c}$	0.15 (0.21) ^{Aa}	0.03 (0.72) ^{Ba}
May	0.14 (0.09) ^{Ab}	0.10 (0.32) ^{Bb}	1.30 (0.10)	1.00 (0.35) $^{b, c}$	0.29 (0.26) ^{Ab}	0.17 (0.40) ^{Bb}
September	0.11 (0.24)	0.07 (0.37) ^{b}	1.20 (0.26)	0.70 (0.33) ^{c}	0.17 (0.15) ^{a}	0.11 (0.50) ^{b}
December	0.11 (0.24) ^{Aa}	0.02 (0.75) ^{Ba}	1.10 (0.31) ^{A}	0.20 (0.79) ^{Ba}	0.28 (0.26) ^{Ab}	0.04 (0.97) ^{Ba}

Results are shown as mean (CV). CV is the coefficient of variation (CV = standard deviation/mean). Different upper case superscript letters represent differences between genders within each month; different lower case superscript letters represent differences among months within a gender; the presence of the same letter means differences absence.

In peroxisomes from proximal tubule segment II it was noted strong independent effects of sex and season in the size and volume of these organelles in relation to the cytoplasm of the tubule cell (Table 2). 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 (Table 2). Further analysis (Table 6) 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 significant 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 along 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 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.

TABLE 7.

Results of statistically significant correlations (Pearson or Spearman correlation coefficients) between the RSI and the peroxisome stereological parameters from brown trout kidney (n = 20, for each correlation)*

Proximal Tubule I				**Proximal Tubule II**			
	♀			♂		♀	
Parameters	r	*p* value	Parameters	r	*p* value	r	*p* value
RSI *vs.* N_v(P, Cell I)	- 0.49	0.035	RSI *vs.* N_v(P, Cell II)	- 0.59	0.006	n.s.	n.s.
RSI *vs.* N_v (P, Cyt)	- 0.53	0.021	RSI *vs.* N_v (P, Cyt)	- 0.62	0.003	n.s.	n.s.
RSI *vs.* $\overline{v}_N$	+ 0.60	0.006	RSI *vs*. S_v(P, Cell II)	- 0.53	0.016	n.s.	n.s.
RSI *vs.* $\overline{d}_{Sphere}$	+ 0.60	0.006	RSI *vs.* S_v (P, Cyt)	- 0.56	0.011	n.s.	n.s.
			RSI *vs.* V_v (P, Cyt)	- 0.46	0.043	n.s.	n.s.
			RSI *vs.* $\overline{v}_N$ **	n.s.	n.s.	+ 0.52	0.026
			RSI *vs.* $\overline{d}_{Sphere}$ **	n.s.	n.s.	+ 0.52	0.026

* The RSI was computed as: (kidney weight × 100) ÷ body weight. n.s. – not significant.

** Non-parametric correlations.

No correlations were found between the GSI and any of the stereological parameters measured in the peroxisomes. However, some correlations between the latter and the RSI were found, particularly in the proximal tubule segment II (Table 7). 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 the tubule segment I. On the contrary, the relative dimensions and number of peroxisomes in the proximal tubule cell II exhibited negative correlations with the RSI only in males.

Discussion

The scarcity of data concerning fish kidney peroxisomes was an encouraging motive to develop this study. Additionally, 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 the peroxisomal physiology and a need of more fundamental knowledge. Thus, a stereological work 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. The 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, but also because it is known that there are morphological segment-specific responses when fish kidney is exposed to several toxics (Anderson and Loewen, 1975; Pritchard and Bend, 1984; Hentschel and Elger, 1987; Oulmi *et al.*, 1995b, 1995a; Elger *et al.*, 2000). In those studies, the peroxisomes from proximal tubule segment II cells had morphological alterations with lower toxic doses than those of the proximal tubule segment I (Oulmi *et al.*, 1995b, 1995a). Thus, it is possible 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 Wendelaarbonga, 1977; Oulmi *et al.*, 1995b, 1995a; Johkura *et al.*, 2000), in which

peroxisomes were said to be more frequent in the segment II epithelial cells of the proximal tubule. However, those descriptions were always made based on qualitative or at most semi-quantitative perspectives. From our approach, we quantitatively demonstrated for the first time, and in accordance with the mentioned published data for other fish species, not only that brown trout renal peroxisomes were more abundant (in females) 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 the proximal tubule segment II, variations in the relative number of peroxisomes did occur along 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 to the relative ones. In males, bigger peroxisomes were found in February, when proximal tubule segment II epithelial cells had less peroxisomes 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 grey 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 peroxisomes morphology are 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). Actually, 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).

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 since segment II of proximal tubule was described as the preferential compartment for the nitrogen compounds catabolism in teleost fishes (Hickman and Trump, 1969). Moreover, and taking into account the freshwater nature of brown trout, the variations in the proximal tubules ultrastructure may be also 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 the segment I is responsible in higher extent for reabsorption, segment II is more devoted to ion secretion (Larsen and Perkins Jr., 2001). Kidney function is modulated by several hormones, such as vasoactive hormones and prolactin (Larsen and Perkins Jr., 2001). Besides the antidiuretic effect of arginine vasotocin by lowering the glomerular filtration rate, this hormone seems also to exhibit an effect in kidney tubules of trout stimulating the cAMP production and increasing water reabsorption (Warne *et al.*, 2002). Additionally, other vasoactive hormone, angiotensin II, is mentioned to cause glomerular morphological alterations during acclimation (Brown *et al.*, 1990). By other hand, prolactin is described as a hormone which induces the urine production in freshwater fish by increasing the glomerular filtration rate and decreasing the epithelium permeability with induction of nephron ultrastructural changes (Larsen and Perkins Jr., 2001; Manzon, 2002). It is known that in eurythermal fish, some of the mentioned hormones, namely arginine vasotocin and prolactin, are influenced by seasonal environmental factors as temperature and photoperiod (Manzon, 2002; Martin *et al.*, 2004; Balment *et al.*, 2006). Hence, the kidney peroxisomal variations we observed in brown trout might be connected in some way to those hormones effects, since at least some of the latter changes surely culminate in more or less energy demand, partially connected to peroxisome β-oxidation.

The observed annual variations on 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 behaviour induce specific hormone secretion, which can influence kidney physiology and morphology (Larsen and Perkins Jr., 2001). However, the regulation of fish kidney

structure and function by hormones is still quite understudied and the cellular mechanisms by which they act are a challenge to tackle many questions.

In conclusion, renal peroxisomes from adult brown trout suffered 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 be correlated with the animal reproductive status. A correlation between those variations and kidney physiology in order to accomplish a hydrostatic homeostasis seems to be a plausible hypothesis. This study, by establishing the normal seasonal variation pattern of kidney peroxisomes, further warned that season and gender must be taken into account when considering changes in those organelles.

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CHAPTER 4

SEASONAL VARIATIONS OF ENZYMATIC ACTIVITIES OF BROWN TROUT (*SALMO TRUTTA*) KIDNEY PEROXISOMES

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Seasonal Variations of Enzymatic Activities of Brown Trout (*Salmo trutta*) Kidney Peroxisomes

Abstract

Recently it was discovered that estrogens can influence the morphology and physiology of brown trout liver peroxisomes. In a seasonal study, a negative correlation between gonad maturation and the size of the hepatic peroxisomes was found, as well as between the later and the activity of some peroxisomal enzymes. However, in kidney, another peroxisome rich organ, nothing is known about an eventual peroxisomal regulation by estrogens. In this study, some peroxisomal enzyme activities, such as catalase, D-alanine oxidase and palmitoyl-CoA oxidase, were measured under spectrophotometric methods during four different phases of the brown trout breeding cycle, in order to verify if those activities show seasonal and gender related variations, and how they compare with those in liver. Catalase activity presented in males and females a similar variation pattern, being negatively related with gonad maturation. D-alanine oxidase activity presented alterations in the same course for males and females, and again a negative correlation with the GSI was found, though weakly than for catalase and only in males. Palmitoyl-CoA oxidase activity did not show variations during the year. Urate oxidase and L-α hydroxy acid oxidases A and B activities were not detected in renal homogenates of brown trout. Thus, it seems that, at least in some physiological aspects, kidney peroxisomes are also influenced by sex hormones, although not exactly as it happen in liver. As such, a common modulation mechanism for both organs may not exist since they do not show the same seasonal pattern, and estradiol seems to exert a key and major influence in liver. Eventually, kidney peroxisomes changes may be predominantly influenced by other sex hormones, such as testosterone, or other, and under a less strict control as seen in liver.

Introduction

There is evidence that morphological changes in brown trout liver peroxisomes occur along the year showing a variation pattern correlated with the breeding cycle and gonad maturation kinetics, especially in females (Rocha *et al.*, 1999). Hence, it was suggested that estrogens (key to ovary maturation) possibly modulate in some way the peroxisomal physiology. Corroboration of this hypothesis started in that animal model when enzymatic activities from hepatic peroxisomes were measured by spectrophotometric methods. Those studies indicated that peroxisomal enzymes, such as catalase, urate oxidase, palmitoyl-CoA oxidase and glycolate oxidase showed variations during the year, so that a reduction in size of female peroxisomes along September/December is accompanied with declining activity of these peroxisomal enzymes (Rocha *et al.*, 2001; Rocha *et al.*, 2004b). Indeed, catalase, urate oxidase and palmitoyl-CoA activities fall in females in that period.

Peroxisomes are able to decompose and generate hydrogen peroxide by the action of their enzymatic content, namely catalase and several oxidases, which are responsible for the crucial role of these organelles in many metabolic processes, such as the metabolism of reactive oxygen species, fatty acids β-oxidation, purine and amino acids catabolism, and other (Tolbert, 1981; Vandenbosch *et al.*, 1992). Inclusively, abnormalities in one or more of those enzymes are associated to congenital human diseases, that despite rare can be clinically severe or fatal (Masters and Crane, 1995).

Therefore, those dynamic organelles have been a target for numerous studies in several fields, including medicine, biology, toxicology and comparative physiology. Despite the fact that most research was carried out in mammals and yeasts, it is well established that pollutants, including peroxisome proliferators, feeding habits, water salinity, temperature, as well as seasonality, exert morphological and biochemical alterations in peroxisomes from fishes and other aquatic organisms (Braunbeck *et al.*, 1987; Braunbeck and Volkl, 1991; Fahimi and Cajaraville, 1995; Oulmi *et al.*, 1995; Oulmi and Braunbeck, 1996).

In animals, the liver has been the elected organ for peroxisome studies, being kidney and other peroxisome rich organs comparatively understudied in that respect. Hence, and taking into account what happened in brown trout liver peroxisomes, as well as the variations described in the previous Chapter, which focused on kidney peroxisomes morphology, this work was performed to increase our information about renal peroxisomes physiology. Thus, two main questions were raised: 1) do renal peroxisome enzymatic activities show alterations along the year similarly to the variations observed in hepatic peroxisomes?; 2) if there are variations, are they correlated with the seasonal

reproductive cycle? In order to clarify this matter, the enzymatic activities of catalase and some peroxisomal oxidases were measured using spectrophotometric methods along one year in both genders.

Material and Methods

Animals

Three-year-old brown trout (*Salmo trutta* f. *fario*), ten males and ten females, were collected by random net fishing within a state aquaculture station (Posto Aquícola do Torno, Amarante, Portugal) at the major seasons of their natural reproductive cycle: after spawning (February), early vitellogenesis (May), advanced vitellogenesis (September), and pre-spawning (December). Gonado-somatic index (GSI) was used to assess gonad maturation, and the reno-somatic index (RSI) was also estimated. Before being sacrificed, always in the morning period, the fish were kept under rest in quarantine tanks for 12 to 24 h.

Chemicals

Cofactors and substrates for enzyme assays and BSA were obtained from Sigma-Aldrich (Dorset, U.K.). All other chemicals were of reagent grade and were purchased from Merck (Darmstadt, Germany).

Preparation of Tissue Homogenates

Fishes were deeply anaesthetised by immersion in a 1 ml L^{-1} aqueous solution of ethylene glycol monophenyl ether and then weighed and measured in length. Before tissue collection for homogenization, the kidney was perfused with heparinized (5 IU/ml) isosmotic buffer for salmonids (Cascales *et al.*, 1997), (NaCl 0.74%, KCl 0.04%, $CaCl_2$ 0.02%, $MgSO_4$ 0.15%, $NaHCO_3$ 0.03%, $NaH_2PO_4 \bullet H_2O$ 0.05%, $Na_2HPO_4 \bullet 2H_2O$ 0.16%) at 4 ºC and with a physiological flow rate of about 5 ml min^{-1} Kg^{-1} of body weight (Hampton *et al.*, 1985). For improving the perfusion, the most posterior part of the fish was cut, allowing a direct cannulation of either the vein or the artery with simultaneous escape of both perfusate and blood. After removal, the trunk kidney was immediately weighted. Subsequently, these organs were minced in chilled homogenization buffer with a pH of 7.4 (250 mM sucrose, 5 mM MOPS, 1 mM EDTA, 0.1% ethanol saturated with PMSF and Triton X-100 0.5%) (Goldenberg, 1977) and then homogenized in the same buffer using a Potter-Elvejhem homogenizer at 1,000 rpm, held at 4 ºC. The homogenized volume was

adjusted to 5 ml g^{-1} of kidney and filtered through a net of about 95 µm mesh. Subsequently a centrifugation was carried out at 10,000 g during 10 min. The supernatants were collected, aliquoted, and stored at -80 ºC in eppendorfs until enzymatic measurements were performed.

Biochemical Measurements

All assays were run in a spectrophotometer connected to a circulating water system for temperature regulation in the cuvette compartment. Enzymatic assays were performed in duplicate, and with two different dilutions per sample, to calculate an enzymatic activity mean value, using appropriate dilutions to obtain linear activity in time and proportional to the amount of protein. The activities were assayed at 25 ºC, except for catalase which was performed at 20 ºC.

The evaluation of catalase activity was based on the methodology previously described by Aebi (1984) and applied to brown trout by Rocha *et al.* (2003). In detail, a diluted sample was added to the incubation medium contained 50 mM sodium phosphate buffer (pH 7) and 10 mM H_2O_2. The consumption of the H_2O_2 was assessed by a decrease of the absorbance at 240 nm of wavelength for 30 seconds and the activity was expressed by the first-order rate constant (k) for degradation of H_2O_2:

$$k = (1 \div \Delta t) \times \ln (c_1 \div c_2)$$

in which c_1 and c_2 are the concentrations at t = 0 and t = 30 seconds, respectively. Catalase activity was expressed by s^{-1} mg^{-1} of protein.

The measurement of peroxisomal oxidase activities was based on H_2O_2 production, according the procedure of Cablé *et al.* (1993) and Rocha *et al.* (2003). For these assays the incubation mixture contained 50 mM potassium phosphate buffer (pH 8.3), 0.082 mM 4-amino-antipyrine, 1 mM phenol, 2 IU/ml of horseradish peroxidase, 0.01 mM FAD and 10 mM azide, this last compound was added in order to minimize the interference of catalase (Leupold *et al.*, 1985). For L-α hydroxy acid oxidases A and B assays, 0.01 mM FMN was added to the incubation mixture instead of FAD (Vamecq, 1990). To this medium were added specific substrates for each oxidase: 20 mM D-aminoacid oxidase, 1 mM uric acid, 0.1 mM palmitoyl-CoA and 20 mM sodium glycolate. The enzymatic reaction was started by the addition of a diluted sample of kidney homogenate to the medium; a cuvette without substrate was used as reference. The production of H_2O_2 by peroxisomal oxidases was measured by an increase of the absorbance at 500 nm during 10 min. A non-specific reaction was only detected for urate oxidase activity. For this

enzyme, a baseline was subtracted for each assay (medium without sample). The oxidase activities were calculated taking into account the calibration line (Rocha *et al.*, 2003) equation:

$$[H_2O_2] = 185.07 \times \text{Absorbance}$$

Peroxisomal oxidases activities were expressed in nmol min^{-1} mg^{-1} of protein.

Total protein content was determined according to Lowry *et al.* (1951) using BSA as standard and results are expressed in BSA equivalents.

Statistical Analysis

Data are presented by mean and its coefficient of variation (CV = standard deviation ÷ mean). All the remaining data are presented graphically by a mean of each animal group (10 fishes per group) from every season and gender, with a bar corresponding to the respective standard deviation. Statistica 6.0 for Windows software was used to stastically analyse the data. After testing the normality and the homogeneity of variances, a two-way ANOVA was performed to detect the effects of season, gender and season by gender interaction, on each enzyme. Tukey and Newman-Keuls post-hoc tests for multiple comparisons between means were further applied. Differences were considered significant for $p \leq 0.05$. When a difference was significant according Newman-Keuls but not Tukey post-hoc tests, we considered it marginally significant. Data transformation was made in some cases to warranty normality and homogeneity. Non-parametrics tests were also applied in parallel, and the results always corroborated the parametric approach. Kruskal-Wallis ANOVA and Mann-Whitney U non-parametric tests were used. Correlation tests were employed to find significant linear associations.

Results

Animals Biological Parameters

Biological parameters of the animals from each season sample, such as the standard body weight and the body length are presented in Table 1, whereas the GSI and the RSI are shown in Figure 1.

TABLE 1.

Morphometric data of brown trout from each seasonal sample group.

Month	Gender	Weight (g)	Length (cm)
February	♀	626.6 (0.19)	36.1 (0.06)[a]
	♂	682.0 (0.09)	35.7 (0.04)
May	♀	770.7 (0.19)[a]	38.6 (0.08)[a]
	♂	796.0 (0.13)[a]	38.2 (0.05)[a]
September	♀	526.2 (0.16)[b]	33.2 (0.05)[b]
	♂	564.7 (0.16)[b]	33.6 (0.06)[b]
December	♀	614.9 (0.22)	34.4 (0.07)[b]
	♂	758.6 (0.29)[a]	36.4 (0.09)

Different letters represent differences among months within a gender. Only values with different letters are significantly different ($p \leq 0.05$).

The GSI presented a similar seasonal pattern for males and females, though being the variations amplitude wider in females. These had highest GSIs at the vitellogenic period and spawning season, and the lowest in February and May. Significant differences between genders occurred in every sampling season, as also significant changes happened among all months within each gender. RSI also presented variations along the year. Though, these changes in the RSI were much more subtle than those found in the GSI. Females presented the lowest values of RSI in September and December and the highest in February, whereas males also showed the highest RSI in February, but the lowest indexes were in May and September.

Enzymatic Measurements

For catalase activity, significant ANOVA was found in season ($p \leq 0.001$), gender ($p \leq 0.01$) and season and gender interaction ($p \leq 0.01$). In female kidney, this enzyme activity was higher in February and May declining in September and December, when the lowest activity levels were detected (Figure 2). In males, significant higher catalase activities

were measured in February and May comparing with September and in a less extent with December, and between September and December the males catalase activity variation was also significant. Between genders, significant differences in catalase activities were only detected in February and September.

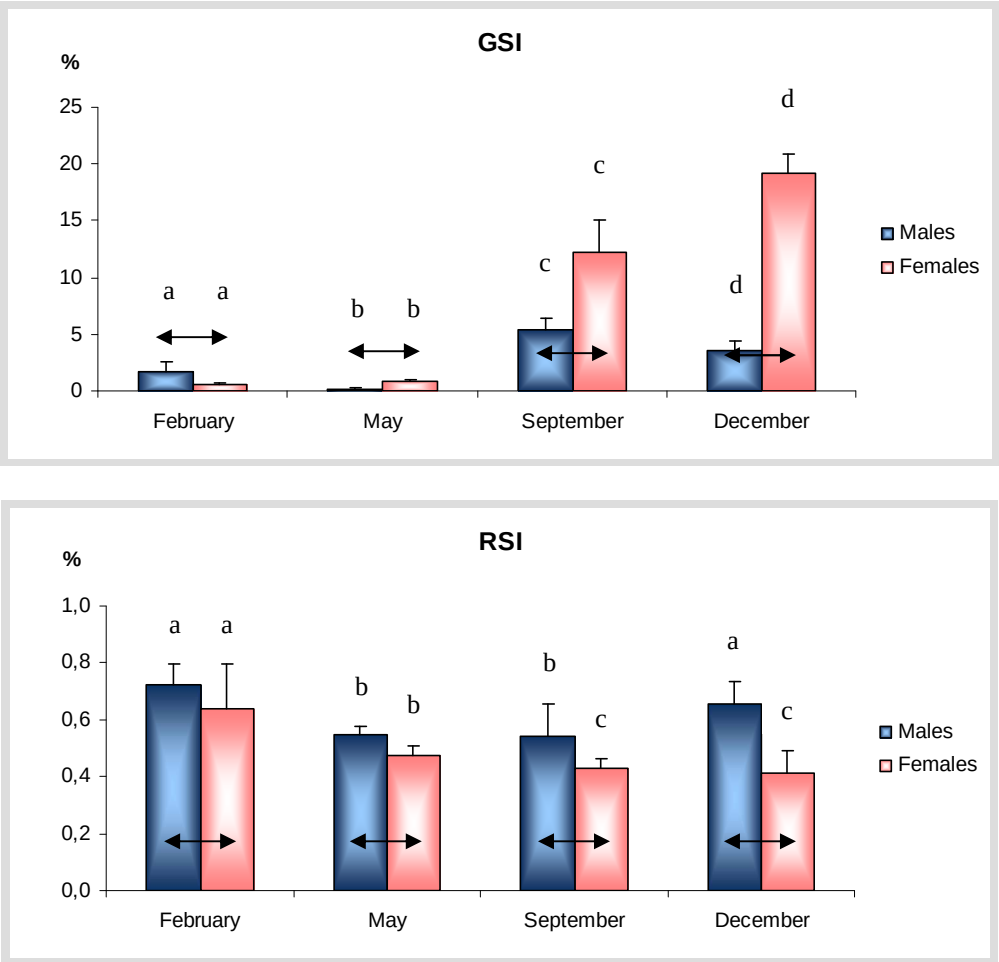

FIGURE 1. Gonado-somatic (GSI) and reno-somatic (RSI) indexes in four periods of brown trout reproductive cycle. Differences among months within a gender are represented by different letters; differences between genders within each month are represented by arrows. To simplify, just $p \leq 0.05$ was used to considered significant differences. Vertical bar = standard deviation. The GSI and the RSI were estimated by the formula: (gonad or kidney weight × 100) ÷ body weight.

The palmitoyl-CoA oxidase activity of kidney did not show significant difference either among seasons, or between genders (Figure 2). In addition, the activity of urate oxidase and L-α hydroxy acid oxidases A and B was not detectable.

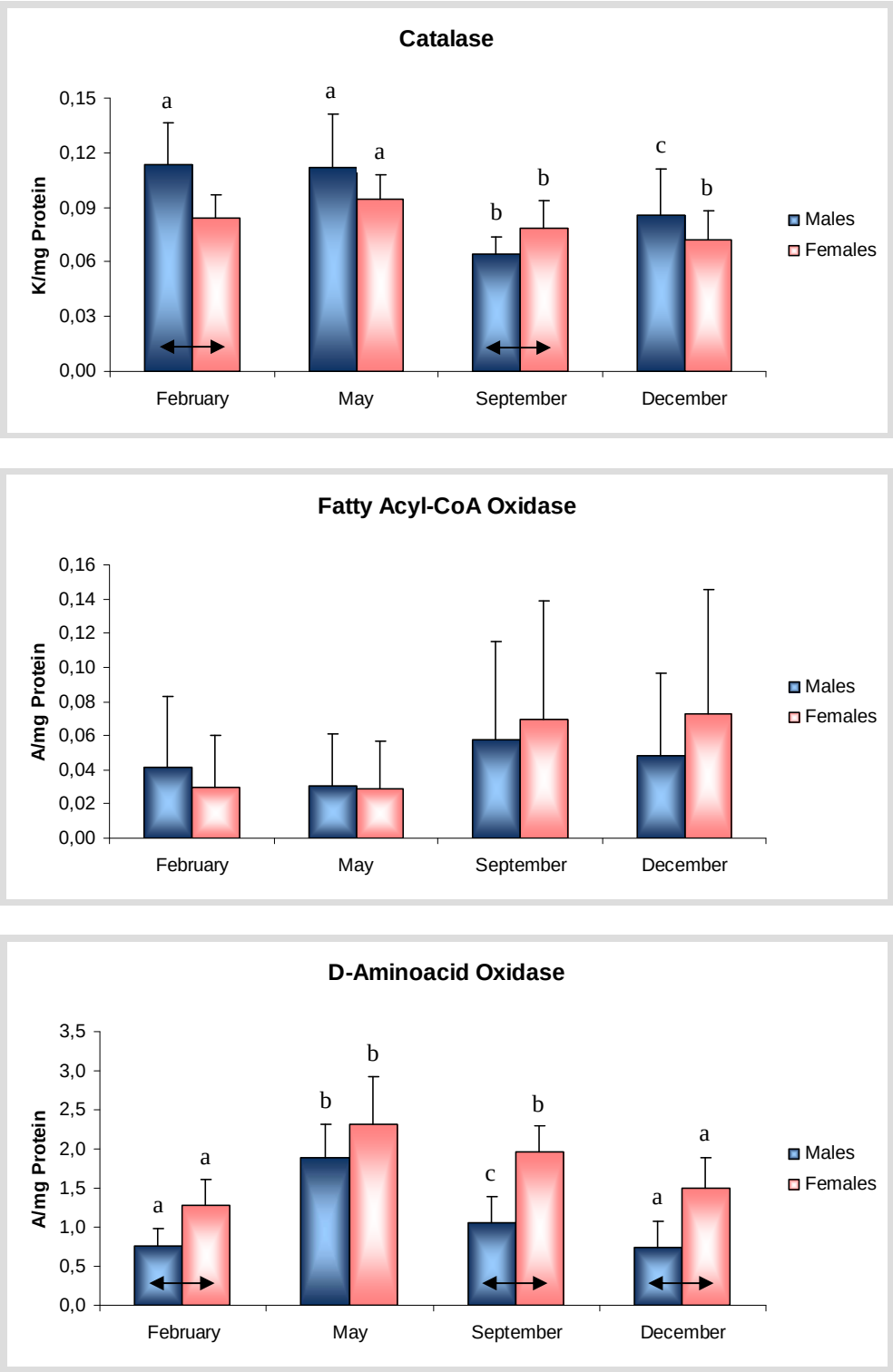

FIGURE 2. Peroxisomal enzymatic activities in four seasons of brown trout breeding cycle. Enzyme activities (A) expressed as nmol min^{-1}. Differences among months within a gender are represented by different letters; differences between genders within each month are represented by arrows. Vertical bar = standard deviation. To simplify, just $p \leq 0.05$ was used to considered significant differences.

The D-amino acid oxidase presented a similar variation pattern in male and female kidney, with the highest activity being seen in May, decreasing to the lowest values in December and February. The male enzymatic activities declined sharply than in females, as shown in Figure 2. Differences between genders within each month were detected in February, September and December (Figure 2). For this enzyme significant ANOVA were found for season and gender effects ($p \leq 0.001$), but not for their interaction.

Several correlations between some peroxisomal enzymatic activities and the GSI of brown trout kidney were revealed by statistical analysis. In males, negative correlations between enzymatic activities and the GSI were observed, being stronger for catalase ($r = -0.72$; $p < 0.001$) than for D-amino acid oxidase ($r = -0.39$; $p = 0.015$). In females, just for catalase a negative correlation was found ($r = -0.42$; $p = 0.006$) with the GSI. Negative correlations between the enzymatic activities and the RSI were found only for the D-alanine oxidase, both in males ($r = -0.49$; $p = 0.002$) and in females ($r = -0.37$; $p = 0.02$).

Discussion

There are many studies including measurements of peroxisomal enzyme activities, but the majority were performed to verify toxicological effects of pollutants, including peroxisome proliferators compounds (Cancio *et al.*, 1998; Braunbeck and Strmac, 2001; Cajaraville and Ortiz-Zarragoitia, 2006). On the other hand, there are fewer investigations regarding the seasonality of those enzymes, and they were mainly related to feeding habits and temperature changes (Moyes *et al.*, 1991; de Brito-Gitirana and Storch, 2002). Seasonal effects influenced by the animal reproductive cycle were only described in some molluscs (Cancio *et al.*, 1999; Orbea *et al.*, 1999), being brown trout the first fish species to be documented on these correlations (Rocha *et al.*, 2001; Rocha *et al.*, 2003; Rocha *et al.*, 2004a; Rocha *et al.*, 2004b). Our study was made to investigate the normal morphofunctional variations of the brown trout kidney peroxisomes, contributing to the needed fundamental knowledge.

Liver was the most studied organ in those investigations, since hepatocytes have revealed excellent properties of environmental-induced cell plasticity. Other peroxisome rich organs, such as kidney, are understudied concerning this matter; consequently, little is known about the possible morphofunctional variations of their peroxisomes. In fact, there is no published data related to season and gender effects in renal peroxisomes.

For that reason, this study was designed essentially with two purposes: to verify if kidney peroxisomal enzymes suffer seasonal alterations in their activities during the brown trout reproductive cycle and to observe possible correlations with those changes and sex steroid levels.

The absence of urate oxidase and L-α hydroxy acid oxidases A and B activities in brown trout kidney homogenates is in accordance with data from other teleost species, in which neither urate oxidase nor L-α hydroxy acid oxidase activities in renal peroxisomes were detected (Veenhuis and Wendelaarbonga, 1977). In addition, the L-α hydroxy acid oxidase A is described in mammals as a typical hepatic peroxisomal enzyme (Zaar and Fahimi, 1991), whereas the type B isoenzyme is known as being an enzyme located in the marginal plates of kidney peroxisomes (Zaar *et al.*, 1991). Thus, despite other location could not be ruled out a prior, the fact that brown trout do not possess marginal plates in their peroxisomes is in agreement with the absence of that enzymatic activity.

Significant variations in the activity of kidney peroxisomal enzymes among four seasons of brown trout breeding cycle were observed, as well as differences between males and females. Moreover, most of the detected variations possessed correlations with the GSI, which reflects the body content in sex steroid hormones, which appear in higher levels during advanced gonadal maturation and breeding. Thus, in these periods, the GSI presented also upper values in both genders, being particularly high in vitellogenic females. The biological parameters as well as the enzyme activity profiles did not seem to be influenced by the differences in body weight seen among groups, which is compatible with the notion that despite natural differences in size, all animals were fully adult with breeding potential.

In relation to the seasonality of brown trout kidney peroxisomes, catalase presented a similar variation pattern in males and females, with the enzyme activity diminishing when the GSI increased. Besides, negative correlations between these two parameters for both genders existed. These results are in agreement with findings in liver, in which a similar variation pattern of catalase activity was found (Rocha *et al.*, 2001; Rocha *et al.*, 2004b). Nevertheless, in liver the differences were only significant in females, contrarily to what occurs in kidney, where significant differences were observed in both genders, being even more relevant in males. This fact draws attention to the possible participation of different steroid hormones in the peroxisomal regulation in different organs, such as testosterone. This sexual hormone has a variation pattern similar for both brown trout males and females, in which high levels of testosterone are found in September and December, then

after falling to very low levels in February and May (Rocha *et al.*, 2004b). In fact, this variation pattern is also similar to the described for 17-β estradiol in the same fish species, although this hormone is only found in relevant levels in females (Rocha *et al.*, 2004b). Contrarily to what happened in kidney, estradiol seems the key player hormone for liver peroxisomes morphofunctional regulation, with males and females differing in seasonal pattern during the breeding cycle and with diverse effects seen in animals experimentally exposed to that hormone (Rocha *et al.*, 1999; Sole *et al.*, 2000; 2001; 2004a; Rocha *et al.*, 2004b; Ortiz-Zarragoitia and Cajaraville, 2005).

It is thought that the peroxisomal D-alanine oxidase is a FAD-containing flavoenzyme responsible to catalyze the dehydrogenation of D-amino acids into their correspondent imino acids, which are further hydrolyzed to α-keto acids and ammonia (Pilone, 2000; Tishkov and Khoronenkova, 2005). The origin of D-amino acids, which are metabolized by that enzyme, could be from endogenous or exogenous sources. Actually, it is already documented in carp, that D-alanine concentration is dependent from the animal nutritional habits, *e.g.* diets containing exogenous free amino acids (Daniello *et al.*, 1993; Sarower *et al.*, 2003a; 2003b; Abe *et al.*, 2005). In addition, differences of salinity seem to influence the D-amino acid oxidase synthesis in aquatic invertebrates, such as certain crustaceans and molluscs (Abe *et al.*, 2005). Data concerning this enzyme and peroxisome variations is mentioned in yeasts by Pollegioni *et al.* (2007), in which induction of this enzyme activity is accompanied by an increase of the peroxisomal size as well as peroxisomal biogenesis. Taking into account the results from previous and actual Chapters, the high values of relative volume of brown trout kidney peroxisomes from proximal tubule segment II coincided with the high D-amino acid oxidase activity levels. Although, the size of peroxisomes from both proximal tubule segments I and II decreased when the enzymatic activity levels are increased. Particularly in males, not only the relative size of peroxisomes had the same annual variation pattern than the observed for D-amino acid oxidase activity, but also the relative number of peroxisomes pointed to a similar variation. In addition, our results showed an annual pattern in the D-alanine oxidase activity of renal peroxisomes weakly correlated with the brown trout GSI and RSI. Indeed, despite the fact that males and females had the same variation pattern, only males presented a significant negative correlation with the reproductive cycle. From the seasonal pattern, it seems that this enzyme is slower in response to the gonad maturation (and so corresponding to hormonal influences) when comparing with catalase, since D-alanine oxidase activity was lower in February when the GSI had already fall (and with it, the sex hormones). In accordance, at advanced vitellogenesis and pre-spawning, when males and females

presented the major differences in GSI, the enzymatic activity was also different between males and females, as in post-spawning season, probably due to the slow response to the lower steroid hormone levels. In fact, in early vitellogenesis, the period with fewer differences in gonad maturation between males and females, it was also the period when renal D-alanine oxidase activity does not differ between genders.

The palmitoyl-CoA oxidase is a peroxisomal enzyme involved in fatty acids β-oxidation. This metabolic pathway occurs in a great extent in fish liver, and hepatic peroxisomes have a large participation in that process (Crockett and Sidell, 1993). In brown trout females the activity of this enzyme in hepatic peroxisomes is diminished during latter vitellogenesis and spawning season (Rocha *et al.*, 2001; Rocha *et al.*, 2004b), periods where lipids are mainly mobilised to vitellogenin synthesis and further incorporated in the gonads, while in the post-spawning and early vitellogenesis lipids are essentially used to energy purposes (Bon *et al.*, 1997). In addition, it is known that hepatic palmitoyl-CoA oxidase decrease in consequence of high estradiol levels (Rocha *et al.*, 2004a). Thus, the absence of significant sex dependence and seasonality in the variation pattern of this enzyme in renal peroxisomes might be explained by the fact that fish kidney is not a metabolizing organ, whereas the ionic balance and the excretion are their major functions (Elger *et al.*, 2000), apparently not needing great shifts in fatty acids β-oxidation.

In conclusion, our hypothesis that peroxisomes are modulated by sex steroid hormones was reinforced. However, in renal peroxisomes that modulation seems to be weaker than in liver. So, the same hormonal stimulus may produce different effects on peroxisomes depending on the organ. By the similarities we saw between males and females herein, we hypothesize that in kidney other sex hormones, namely testosterone, rather than estradiol, can have a major involvement in influencing peroxisomes.

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CHAPTER 5

SUBCELLULAR DISTRIBUTION OF SOME PURINE CATABOLISM ENZYMES IN BROWN TROUT (*SALMO TRUTTA*) LIVER

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Subcellular Distribution of Some Purine Catabolism Enzymes in Brown Trout (*Salmo trutta*) Liver

Abstract

Subcellular location of purine catabolism enzymes is of great interest in comparative studies due to the large variability among species. This metabolic pathway reflects in large extent a phylogenetic evolution with the loss of several enzymes in certain species, with consequences in the end products that are excreted. Particularly in fishes, some of those enzymes are described as cytosolic for some species and as peroxisomal for others. To identify the intracellular location of some purine catabolism enzymes in brown trout liver a centrifugal fractionation technique was applied to obtain crude cell fractions, in which enzyme activities were measured using spectrophotometric methods. Xanthine oxidase/xanthine dehydrogenase, allantoinase and allantoicase activities were assayed plus some cell organell marker enzymes. Related to the purine catabolism enzymes in brown trout liver, published data points that urate oxidase is a peroxisomal enzyme, as in other vertebrates. Herein, xanthine oxidase and allantoicase activities were not detected in brown trout liver fractions, which is in accordance with the fact that in some organisms the xanthine oxidoreductase enzyme is only found or predominantly found in the form of xanthine dehydrogenase and that in salmonids the purine catabolism pathway ends with allantoic acid as final product. Finally, xanthine dehydrogenase and allantoinase were considered to be located in the cytosol, since more than 85% of their activities were recovered in the supernatant fraction.

Introduction

Purine catabolism vary significantly from species to species resulting in different end products, due to a successive enzyme loss during phylogenetic evolution, such as urate oxidase, ureidoglycollate lyase, allantoicase, allantoinase and urate oxidase (Noguchi *et al.*, 1979; Takada and Noguchi, 1983; Usuda *et al.*, 1988; Hayashi *et al.*, 1989; 1994). Hence, purine-degrading enzymes have been a matter of study for several organisms, specially related to their subcellular distribution, molecular structures and other relevant properties. Purine catabolism is one of the pathways in which peroxisomes are implicated, because some of the enzymes involved are reported to be in these organelles, such as urate oxidase and others, in which xanthine oxidase, allantoinase and allantoicase can be included depending the organism species (Yeldandi *et al.*, 1996; Moriwaki *et al.*, 1999; Hayashi *et al.*, 2000).

The first steps of purine degradation, the transformation of adenine and guanine into xanthine and this into urate is common to all organisms, although urate degradation presents a shorter process in mammals, wherein fishes and amphibians have a more extended pathway (Hayashi *et al.*, 2000). The enzymes involved in these first purine degradation reactions are adenase and guanase, which produce hypoxanthine and xanthine. The transformation of hypoxanthine into xanthine and further to uric acid is mediated by xanthine oxidoreductase. Uric acid is the end product of purine catabolism in certain primates including humans, which excrete this product in result of the absence of urate oxidase activity (Yeldandi *et al.*, 1995; Hayashi *et al.*, 2000). In fact, the urate oxidase gene do exist in those species but mutations suffered during evolution culminated in its silencing (Yeldandi *et al.*, 1991; Wu *et al.*, 1992). In other animal groups such as fish and amphibian, the uricolytic pathway occurs, in which urate is degraded to urea through the action of several enzymes, such as urate oxidase which hydrolyse uric acid to allantoin, being catabolised by allantoinase to allantoic acid, which is further degraded to ureidoglycollate and urea by allantoicase. In marine invertebrates, ureidoglycollate is then degraded to glyoxylate and urea by ureidoglycollate lyase, and urease further hydrolyses urea to NH_3 and CO_2 (Hayashi *et al.*, 2000).

It is important to notice that xanthine oxidoreductase can be found in two distinct and convertible enzymatic forms, the dehydrogenase form which uses NAD+ as cofactor, and the oxidase form, which uses oxygen as electron acceptor (Frederiks and Vreeling-Sindelarova, 2002). In healthy mammals, xanthine dehydrogenase is the predominant form and representing about 80% or more of total xanthine oxidoreductase activity (Kooij

et al., 1992; Kooij *et al.*, 1994; Frederiks and Bosch, 1996). Under pathological circumstances, such as tissue injury due to ischemia, the dehydrogenase form can be reversibly transformed into the oxidase (Engerson *et al.*, 1987; McKelvey *et al.*, 1988). However, in chicken and bivalves the enzyme was only found in the form of xanthine dehydrogenase (Dykens and Shick, 1988; Hattori, 1989; Cancio and Cajaraville, 1997).

Despite variations among distinct phylogenetic animal lineages related to purine catabolic pathway, the excretion products also varies between organisms from phylogenetically closer groups, such as salmonids which excrete allantoic acid whereas other freshwater teleosts excrete urea and glyoxylic acid (Urich, 1994).

This work was performed in order to recognize the subcellular location of some purine catabolism enzymes, particularly xanthine oxidoreductase (dehydrogenase and oxidase) and allantoinase, in crude cell fractions of brown trout liver, and to confirm the absence of allantoicase. In addition, marker enzyme activities were also measured under standard spectrophotometric procedures to characterize each fraction, such as succinate dehydrogenase for mitochondria, arylsulphatase for lysosomes, catalase and D-aminoacid oxidase for peroxisomes and NADPH cytochrome c reductase for microsomes.

Material and Methods

Animals

Four 2 year-old brown trout (*Salmo trutta* f. *fario*) were selected randomly by net fishing within aquaculture station (Posto Aquícola do Torno, Amarante, Portugal). Fish collection occurred in June. Before being sacrificed, the randomly sampled fish were kept in well-aerated and dechlorinated water for approximately 12 h. Fishes presented a mean length of 27.5 cm (coefficient of variation, CV = 0.09) and a mean liver weight of 3.3 g (0.3).

Chemicals

Cofactors, substrates for enzyme assays and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (Dorset, U.K.). All other chemicals were of reagent grade and were purchased from Merck (Darmstadt, Germany).

Preparation of Tissue Homogenates

Fishes were anaesthetised by immersion in a 1 ml L^{-1} aqueous solution of ethylene glycol

monophenyl ether and then measured in length. Before tissue homogenization, liver was perfused with a heparinized (5 IU/ml) isosmotic buffer for salmonids (Cascales *et al.*, 1997), composed of NaCl 0.74%, KCl 0.04%, $CaCl_2$ 0.02%, $MgSO_4$ 0.15%, $NaHCO_3$ 0.03%, $NaH_2PO_4 \bullet H_2O$ 0.05%, $Na_2HPO_4 \bullet 2H_2O$ 0.16%, via caudal vein and caudal artery (alternately), at 4 ºC and with a physiological flow rate of about 5 ml min^{-1} Kg^{-1} of body weight (Hampton *et al.*, 1985). After removal, liver was immediately weighted. Afterwards, the organ was minced in chilled homogenization buffer with a pH of 7.4 (250 mM sucrose, 5 mM MOPS, 1 mM EDTA, 0.1% ethanol saturated with PMSF and Triton X-100 0.5%) (Goldenberg, 1977) and then homogenized in the same buffer using a Potter-Elvejhem homogenizer at 1000 rpm, held at 4 ºC. The homogenized volume was filtered through a net of about 95 µm mesh and adjusted to 5 ml g^{-1} of liver - A fraction (Cajaraville *et al.*, 1992). Subsequently a differential centrifugation of A fraction was carried out according to Völkl and Fahimi (1985). This methodology generated several fractions, where each fraction was presumed enriched in different organelles. The fraction B (enriched in mitochondria and lysosomes), was obtained after centrifugation at 2,000g during 10 min. To acquire fraction D (enriched in peroxisomes), was applied a centrifugation at 20,000g during 30 min. For fraction E (enriched in microsomes), a centrifugation at 100,000g during 1h was carried out, in which the supernatant constituted the fraction F, the cytosolic fraction. All centrifugations were performed at 4 ºC. The pellets (B, D and E) were resuspended in an appropriate volume of buffer. The total homogenate A and fractions B, D and E were treated with Triton X-100 at a final concentration of 0.5% (v/v). All fractions were stored at -80 ºC in eppendorfs until enzymatic measurements were performed.

Biochemical Measurements

All assays were run in a spectrophotometer connected to a circulating water system for temperature regulation in the cuvette compartment. Total protein content was determined according to Lowry *et al.* (1951) using BSA as standard and results are expressed in BSA equivalents. Enzymatic assays were performed in duplicate, with two different dilutions of each sample to calculate a mean value and to assure results reproducibility. Appropriate sample dilutions were used in order to obtain enzymatic activities linearity in time and proportional to the amount of protein. The activities of peroxisomal enzymes were assayed at 25 ºC of temperature, except for catalase which was performed at 20ºC.

The evaluation of catalase enzymatic activity was based on the methodology previously described by Aebi (1984). In detail, the incubation mixture contained 50 mM sodium phosphate buffer (pH 7), 10 mM hydrogen peroxide (H_2O_2) and a diluted sample. The

consumption of the H_2O_2 was evaluated by a decrease of the absorbance at 240 nm for 30 seconds and the activity was expressed in s^{-1} by the first-order rate constant (k) for degradation of H_2O_2:

$$k = (1 \div \Delta t) \times \ln (c_1 \div c_2)$$

in which c_1 and c_2 are the concentrations at t = 0 and t = 30 seconds, respectively.

The measurement of the peroxisomal D-aminoacid oxidase activity was based on H_2O_2 production and according the procedures of Cablé *et al.* (1993) and Rocha *et al.* (2003). For this assay the incubation mixture contained 20 mM of substrate (D-alanine), 50 mM potassium phosphate buffer (pH 8.3), 0.082 mM 4-amino-antipyrine, 1 mM phenol, 2 IU ml^{-1} of horseradish peroxidase, 0.01 mM FAD and 10 mM azide, this last compound was added in order to minimize the interference of catalase (Leupold *et al.*, 1985). The enzymatic reaction was started by the addition of a diluted sample to the medium. Medium without substrate was used as reference. The production of H_2O_2 by this peroxisomal oxidase was measured by the increase of the absorbance during 10 min at 500 nm. The calculation of the oxidase activities was expressed in μM taking into account the calibration line (Rocha *et al.*, 2003) expression:

$$[H_2O_2] = 185.07 \times \text{Absorbance}$$

D-aminoacid oxidases activity was expressed by nmol. min^{-1}. mg^{-1} of protein.

Measurements of succinate dehydrogenase activity followed the methodology described by Schoner *et al.* (1967). In detail, the incubation mixture contained 50 mM potassium phosphate buffer (pH 7.5), 5 mM sodium succinate, 1 mM potassium cyanide, 0.1 mM 2,6-dichlorophenol indophenol (DPIP) and a diluted sample. Calculation of enzymatic activity was based in the DPIP molar extinction coefficient at 600 nm ($\varepsilon = 17.3\ mM^{-1}\ cm^{-1}$).

To the estimation of arylsulphatase enzymatic activity was applied the method of Worwood *et al.* (1973). The incubation mixture contained 0.5 mM acetate buffer (pH 5.6), 20 mM nitrocatechol sulphate and a diluted sample. The reaction was stopped after 5 and 10 min of incubation by 1 ml of 1 M sodium hydroxide addition. The nitrocatechol molar extinction coefficient at 515 nm was used to calculate the activity ($\varepsilon = 12.4\ mM^{-1}\ cm^{-1}$).

Measurements of NADPH cytochrome c reductase activity were based in the method described by Johannesen *et al.* (1977). Incubation mixture contained 0.1 M Tris-HCl buffer (pH 7.6), 10 mM potassium cyanide, 0.4 mM oxidized cytochrome c, 3 mM NADPH and a diluted sample. The reaction started with the addition of NADPH. Calculation of enzymatic activity was based in the reduced cytochrome c molar extinction coefficient at 550 nm (ε =

19.6 mM^{-1} cm^{-1}).

Xanthine oxidase and xanthine dehydrogenase assays were performed according to Lallier and Walsh (1991). For xanthine oxidase the incubation mixture consisted of 50 mM potassium phosphate buffer (pH 8.0) and 0.2 mM hypoxanthine solution (1.5 g L^{-1} in 0.125 N NaOH) and a diluted sample. Some assays also contained in the incubation mixture oxonic acid 2 mM, a urate oxidase inhibitor. The formation of urate was measured at 293 nm during 10 min. Calculation of enzymatic activity was based in the molar extinction coefficient of urate at 293 nm of absorbance (ε = 12.61 mM^{-1} cm^{-1}). For xanthine dehydrogenase the incubation mixture contained 50 mM potassium phosphate buffer (pH 8.0), 1.5 mM NAD^+, 0.2 mM hypoxanthine solution (1.5 g L^{-1} in 0.125 N NaOH) and a diluted sample. The production of NADH was measured for 10 min at 340 nm. Calculation of enzymatic activity was based in the molar extinction coefficient of NAD^+/NADH at 340 nm of absorbance (ε = 6.221 mM^{-1} cm^{-1}).

Measurements of allantoinase activity followed the method of Piedras *et al.* (1998). Incubation mixture contained 20 mM Tris-HCl (pH 7.5), 10 mM allantoin, 1 mM $MnSO_4$ and a diluted sample. The reaction was stopped after 5, 10 and 15 min of incubation by adding 100 µl HCl 0.15 N and boiled during 10 min. At the end of this step, allantoate is transformed in glyoxylate. The reaction second step started with the addition of 100 µl potassium phosphate buffer 0.4 M (pH 7.0) and 100 µl phenylhydrazine 25 mM. After 10 min of reaction is added 0.5 ml HCl 37% and 100 µl potassium ferrycianide 50 mM and maintained more 15 min. These two last incubations were made at room temperature. Calculation of allantoinase activity was based in a titration curve performed with several known glyoxylate concentrations determined by the absorbance at 535 nm.

Allantoicase activity was measured according methodology described by Piedras *et al.* (1998). Incubation mixture contained 20 mM Tris-HCl (pH 7.5), 50 mM allantoate and a diluted sample. The reaction first step was stopped after 5, 10 and 15 min of incubation by adding 100 µl NaOH 0.5 N. At the end of this step, ureidoglycollate is transformed in glyoxylate. The reaction second step started with the addition of 400 µl potassium phosphate buffer 0.4 M (pH 7.0) and 100 µl phenylhydrazine 25 mM. After 10 min of reaction is added 0.5 ml HCl 37% and 100 µl potassium ferrycianide 50 mM and maintained more 15 min. These two last incubations were made at room temperature. Calculation of allantoicase activity was based also in the titration curve performed with several known glyoxylate concentrations determined at 535 nm, as already mentioned in allantoinase assay.

Data Presentation

Percentage distribution [(protein or enzyme activity in one fraction ÷ ∑ of protein or enzyme activity in fractions B to F) × 100] and relative specific activity [percentage distribution of the enzyme in one fraction ÷ percentage distribution of protein in that fraction] were calculated according to published literature (Goldenberg *et al.*, 1978; Rocha *et al.*, 2003).

Catalase activity is expressed as s^{-1} g^{-1} of liver and s^{-1} mg^{-1} of protein. For all other enzymes, results are given in nmol min^{-1} g^{-1} of liver and nmol min^{-1} mg^{-1} of protein.

In tables, data is presented as the mean of 4 fishes and its coefficient of variation (CV = standard deviation ÷ mean).

Results

Enzymatic activities

The enzymatic activities of the total homogenate A and fractions B, D, E and F revealed by the relative specific activity and the percentage of distribution of marker enzymes is presented in Table 1 and 2. The organeolar enrichment distribution of each fraction is schematically shown in Figure 1.

Catalase and D-aminoacid oxidase, as peroxisomal marker enzymes, showed highest activities in crude peroxisomal fraction (D fraction). As expected for peroxisomal enzymes, specific activities of these two enzymes were about four times as higher in D fraction than in total homogenate A (Table 1). The pattern exhibited for these enzymes was similar for both, but clearly different from the marker enzymes of other organelles, excepting succinate dehydrogenase (Figure 1). Furthermore, the distribution of these enzymes in other fractions than D presented very low percentages, when compared with the around 70% presented in fraction D. Actually, for both enzymes, the exhibited pattern demonstrated that fraction D is enriched about 4-fold in peroxisomes and mitochondria and twice in lysosomes.

TABLE 1.

Specific and per g enzyme activities in brown trout liver

Enzymes	Activity per g of liver	Specific activity				
	s^{-1} g^{-1} (*) or nmol min^{-1} g^{-1}	s^{-1} mg^{-1} (*) or nmol min^{-1} mg^{-1}				
		A	**B**	**D**	**E**	**F**
Peroxisomal marker						
Catalase	52.1 (0.29)	0.9 (0.29)	0.9 (0.42)	3.6 (0.22)	0.1 (0.61)	0.01 (0.24)
D- aminoacid oxidase	345.7 (0.04)	5.5 (0.11)	4.4 (0.35)	21.2 (0.29)	1.1 (0.32)	1.8 (0.12)
Mitochondrial marker						
Succinate Dehydrogenase	230.5 (0.22)	4.0 (0.16)	2.72 (0.59)	15.73 (0.38)	0.39 (1.15)	0.61 (0.72)
Lysosomal marker						
Aryl Sulphatase	1.7 (0.16)	0.03 (0.15)	0.060 (0.25)	0.056 (0.20)	0.008 (0.18)	0.011 (0.33)
Microsomal marker						
NADPH Cytochrome C Reductase	1.6 (0.10)	0.03 (0.20)	0.01 (0.96)	0.04 (0.26)	0.10 (0.19)	0.02 (0.28)
Supernatant enzymes						
Xanthine Dehydrogenase	251.5 (0.02)	4.4 (0.15)	0.4 (0.27)	1.2 (0.80)	4.4 (0.35)	9.2 (0.19)
Allantoinase	2453.8 (0.33)	43.1 (0.37)	4.2 (0.32)	11.3 (0.56)	20.8 (0.44)	78.3 (0.40)
	Protein per g of liver	**Total protein per fraction**				
	(mg g^{-1})	(mg)				
	58.2 (0.14)	194.9 (0.23)	57.4 (0.33)	46.9 (0.16)	25.6 (0.29)	133.1 (0.23)

A- liver homogenates; B- crude mitochondrial/lysosomal fraction; D- crude peroxisomal fraction; E- crude microsomal fraction; F- cytosolic fraction. (*) Catalase activity is expressed by the first-order rate constant (s^{-1}) and all other enzymes activities are expressed in nmol min^{-1}, per mg of protein or per g of liver. Values are presented as mean and coefficient of variation between brackets.

Fraction B was enriched in mitochondria and lysosomes with higher activities in their marker enzymes, succinate dehydrogenase and arylsulphatase (Figure 1). However, the first enzyme showed higher specific activities in fraction D as well as a similar distribution pattern to the peroxisomal marker enzymes, indicating a high presence of mitochondria in the crude peroxisomal fraction. On the contrary, arylsulphatase presented identical specific activities in fractions B and D, added to the percentage distribution pattern of

Figure 1, where it is possible to see an enrichment of fraction B and D in lysosomes of about 2-fold.

Fraction E is mostly constituted of microsomes like is shown by the highest specific activity of its enzyme marker, the NADPH cytochrome c reductase, measured in that fraction. In addition, the percentage distribution pattern showed that fraction E is enriched in those organelles in about 3 times (Figure 1).

TABLE 2.

Percentage distribution of the marker enzymes

Enzymes	**Percentage Distribution (%)**			
	B	**D**	**E**	**F**
Peroxisomal marker				
Catalase	22.4 (0.29)	75.8 (0.08)	1.5 (0.44)	0.4 (0.31)
D-aminoacid oxidase	16.5 (0.44)	65.4 (0.16)	1.8 (0.32)	16.3 (0.26)
Mitochondrial marker				
Succinate Dehydrogenase	16.8 (0.83)	73.5 (0.26)	1.1 (1.16)	8.6 (0.75)
Lysosomal marker				
Aryl Sulphatase	43.1 (0.17)	35.4 (0.21)	2.6 (0.28)	18.9 (0.24)
Microsomal marker				
NADPH Cytochrome C Reductase	7.0 (0.60)	22.4 (0.16)	32.4 (0.07)	38.2 (0.14)
Cytosolic enzymes				
Xanthine Dehydrogenase	1.5 (0.30)	3.9 (0.82)	8.0 (0.31)	86.6 (0.01)
Allantoinase	2.1 (0.18)	4.4 (0.21)	4.4 (0.10)	89.1 (0.01)
Protein	21.4 (0.15)	18.3 (0.16)	9.8 (0.18)	50.6 (0.02)

Percentage distribution of enzymes and protein in brown trout liver fractions. B- crude mitochondrial/lysosomal fraction; D- crude peroxisomal fraction; E- crude microsomal fraction; F- cytosolic fraction. Values are presented as mean and coefficient of variation between brackets.

Xanthine oxidase and allantoicase activities were not detectable in any fraction of brown trout liver homogenates, even when oxonic acid was used to inhibit urate oxidase activity. Xanthine dehydrogenase and allantoinase showed highest specific activities in the supernatant fraction (F fraction) which encloses the cytosolic components (Table 1), being almost twice the specific activities found in the total homogenate A. Those two enzymes

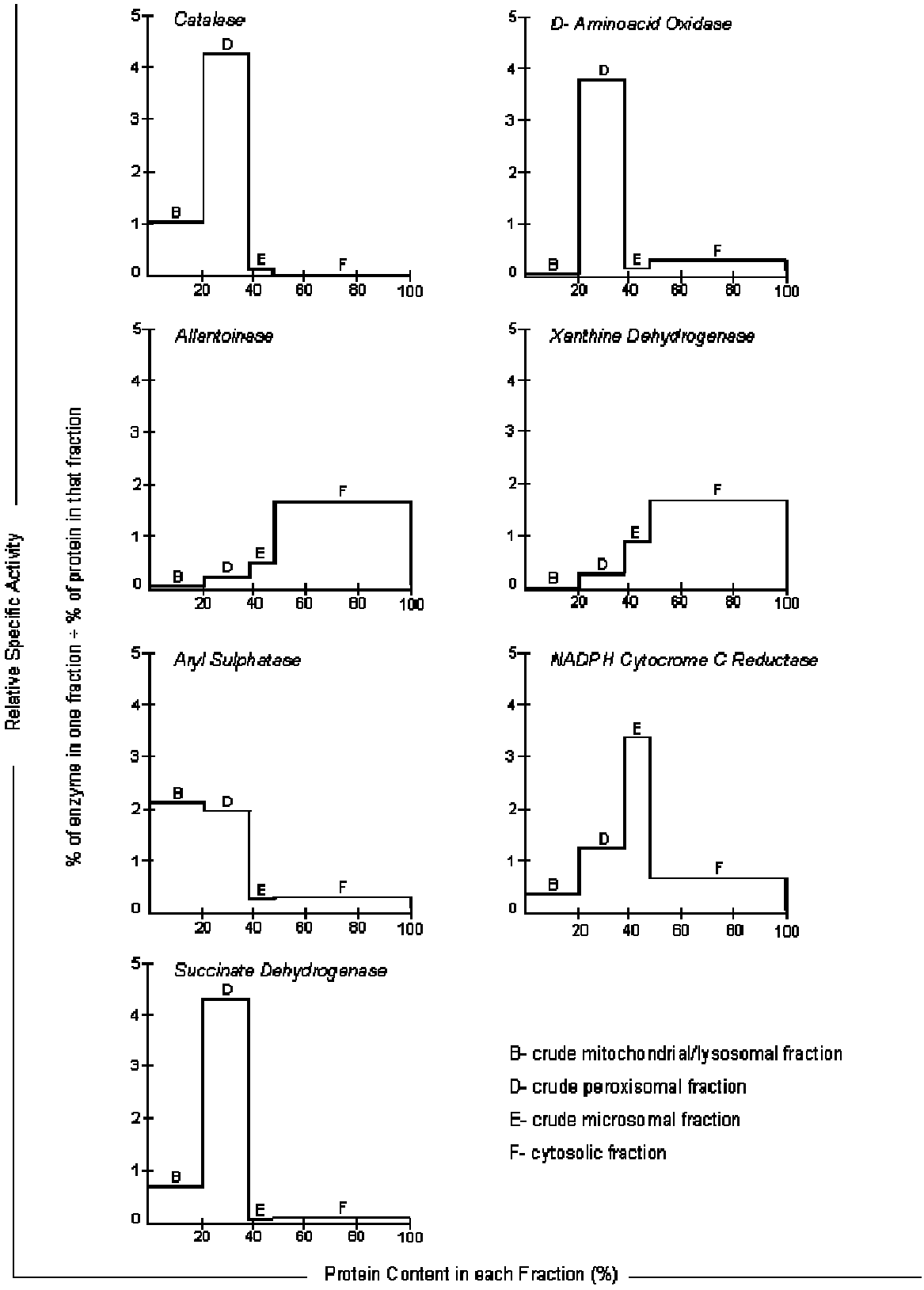

FIGURE 1. Distribution of relative specific activities of several marker enzymes in B, D, E and F fractions from brown trout liver.

presented a similar pattern of distribution in liver fractions, as shown in the respective graphs of Figure 1, where it is evident the major percentage of xanthine dehydrogenase and allantoinase activities in fraction F, more than 85% according Table 2, when compared with the less than 10% found in each of the remain fractions. For these two enzymes the distribution pattern was clearly different from the other enzymes.

Discussion

Several studies on peroxisomes applied differential and density gradient centrifugation techniques in order to achieve peroxisomal fractions. These methods have been optimized along the years by several researchers, since peroxisomes present different densities depending on the studied organism and tissue, and thus different gradient mediums are applied to optimize each situation, such as sucrose, Metrizamide, Nycodenz and Percoll gradients (Leighton *et al.*, 1968; Wattiaux *et al.*, 1978; Appelkvist *et al.*, 1981; Fujiki *et al.*, 1982; Hajra and Wu, 1985; Volkl and Fahimi, 1985; Lazo *et al.*, 1991; Gouveia *et al.*, 1999). However, peroxisomal studies, as also the application of the isolation techniques, were performed essentially in mammals, being other vertebrates and invertebrates species much less studied (Lobo-da-cunha, 1995, 1997; Cancio and Cajaraville, 2000; Rocha *et al.*, 2003). Nonetheless, it is well known that fish and mammal peroxisomes are functionally similar, being part of the same metabolic pathways and possessing identical enzymatic content. Peroxisomal enzymes such as catalase, D-aminoacid oxidase, urate oxidase, allantoinase, fatty acyl-CoA oxidase are among the enzymes that could be found in fish peroxisomes (Kramar *et al.*, 1974; Noguchi *et al.*, 1979; Sakuraba *et al.*, 1996; Cancio and Cajaraville, 2000).

In brown trout liver, purines are catabolized to xanthine or hypoxanthine and via uric acid and allantoin to the excretion product allantoate, contrarily to what happens with other teleost species, which have a longer catabolism pathway (Urich, 1994). Due to the great variability among organisms, special attention has been paid to this metabolism, not only in relation to the extent of the pathway, but also to the enzymes which are involved, for example, the presence *vs.* absence of activity as consequence of gene silence, as well as their tissue distribution and their intracellular compartmentalization.

Hence, this work had the purpose of clarify the localization of xanthine oxidase / xanthine dehydrogenase and allantoinase, two purine catabolism enzymes, in brown trout hepatocytes. Thus, a subcellular fractionation methodology was used to isolate organelles in separate fractions and then the enzyme activities were measured in each fraction. Our

results pointed to a scarcity of contamination by other organelles in the microsomal (E) and cytosolic (F) fractions. For this fractions our results suggested that we ended up with less contamination when comparing with other authors, either in fish or rat (Volkl and Fahimi, 1985; Rocha *et al.*, 2003). On the contrary, fractions B and D seem to present considerable contamination, particularly by mitochondria in fraction D. Perhaps, this fact was the reason for the unexpected high levels of succinate dehydrogenase activity in the peroxisomal fraction added to the very low activity values in the mitochondrial/lysosomal fraction. Nevertheless, catalase and D-amino acid oxidase activities were higher in the peroxisomal fraction with very low values in B fraction. Actually, the results of those enzymes in B fraction were similar with the findings by Völk and Fahimi (1985) and in a less extent with the results of Rocha et al. (2003).

In respect to tissue distribution and subcellular location of purine catabolism enzymes, it was depicted that adenase, guanase and xanthine oxidase were generally recognized as cytosolic enzymes in the hepatocytes of a great number of fish species (Moriwaki *et al.*, 1999), such as mackerel (Noguchi *et al.*, 1979), carp (Goldenberg, 1977) as well as in the digestive gland cells from a mussel species (Cancio and Cajaraville, 1999). Our results pointed also to a cytosolic localization of xanthine dehydrogenase in brown trout, since its enzymatic activity was detected mostly in fraction F, which is in line with the described by some authors for many fish species (Goldenberg, 1977; Noguchi *et al.*, 1979). The absence of xanthine oxidase activity in brown trout liver could be due to the fact that, in some species as chicken and marine bivalves, xanthine oxidoreductase is only found in the xanthine dehydrogenase form (Dykens and Shick, 1988; Hattori, 1989; Cancio and Cajaraville, 1997). On the contrary, in rat liver parenchymal cells xanthine oxidoreductase was reported in the peroxisomal core as also urate oxidase (Angermuller *et al.*, 1987), or even in the peroxisomal matrix (Frederiks and Vreeling-Sindelarova, 2002). In addition, it is well established that urate oxidase is present in peroxisomes from vertebrate liver and also in the digestive gland of certain crustacean species, associated with the nucleoid when it exists, or in the peroxisomal matrix when the enzyme is in the soluble form (Hruban and Swift, 1964; Baudhuin *et al.*, 1965; Noguchi *et al.*, 1979; Hayashi *et al.*, 2000). However, allantoinase and allantoicase subcellular location is a matter of major variability and still great uncertainty for a vast number of organisms (Usuda *et al.*, 1994; Yeldandi *et al.*, 1995).

Several ultrastructural locations for allantoinase were ascertained by different authors: in the mitochondria of frog liver and kidney (Usuda *et al.*, 1994; Yeldandi *et al.*, 1995), in the peroxisomal matrix of mackerel fish liver and of prawn digestive gland (Noguchi *et al.*,

1979), simultaneously in the peroxisomes and in the cytosol of some marine fish species (Fujiwara *et al.*, 1989; Hayashi *et al.*, 2000), and only in the cytosol of carp hepatocytes and other freshwater fishes such as sunfish, largemouth bass, gray mullet and pale chub (Goldenberg, 1977; Fujiwara *et al.*, 1989; Hayashi *et al.*, 2000). It was obvious by data analysis that allantoinase is a cytosolic enzyme in brown trout hepatocytes, because there was a great discrepancy of this enzyme activity values between cytosolic fraction (F) and any of other fractions. Our finding is in agreement with the last cited authors, corroborating the idea that allantoinase is a cytosolic enzyme in all freshwater fishes.

Allantoicase has been described in the cytosol of frog liver and kidney (Usuda *et al.*, 1994; Yeldandi *et al.*, 1995) and also in freshwater species such as carp, crussian carp, sunfish, largemouth bass, gray mullet and pale chub (Goldenberg, 1977; Fujiwara *et al.*, 1989), as well as in the peroxisomal membrane of mackerel fish liver and prawn digestive gland (Noguchi *et al.*, 1979; Hayashi *et al.*, 1989), and also in the peroxisomal matrix and in the cytosol of some marine fish species (Fujiwara *et al.*, 1989; Hayashi *et al.*, 1989). Nevertheless, in brown trout liver this enzyme is absent because any activity of it was detected. Despite the fact that in most fish liver urate could be degraded as a maximum into urea, because urease is lacking (Fujiwara *et al.*, 1989), the inexistence of allantoicase in brown trout hepatocytes is in accordance with the fact that salmonids (at least those studied so far) excrete allantoic acid (Urich, 1994).

The controversy around allantoinase and allantoicase goes further with descriptions in several amphibian species liver, in which those two enzymes are associated to the same protein, appearing as an allantoinase-allantoicase complex, located firstly in the cytosol (Takada and Noguchi, 1983) and further reported to be located in the mitochondria (Usuda *et al.*, 1994). Exceptions to the existence of this complex seem to occur in other amphibian species (Masuda *et al.*, 2001).

In summary, the paucity in organelles marker enzymes in the cytosolic fraction (F) allow us to conclude that in brown trout liver both xanthine dehydrogenase and allantoinase are cytosolic enzymes. As a consequence, in this fish some of the products of the respective purine catabolism reactions have the ability of crossing the peroxisomal membrane, in both ways, in order to complete this metabolic pathway. Xanthine oxidase is lacking and the absence of allantoicase supports allantoic acid as the end product of the purine catabolism in brown trout.

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CHAPTER 6

ACTIVITY OF PURINE CATABOLISM ENZYMES DURING THE REPRODUCTIVE CYCLE OF MALE AND FEMALE BROWN TROUT (*SALMO TRUTTA*)

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Activity of Purine Catabolism Enzymes during the Reproductive Cycle of Male and Female Brown Trout (*Salmo trutta*)

Abstract

In order to detect predicted changes in purine catabolism during the annual reproductive cycle of brown trout, we measured the activity of xanthine dehydrogenase, urate oxidase and allantoinase. In kidney, only xanthine dehydrogenase was detected. In female liver and kidney, the activity of these enzymes was higher in May and decreased during vitellogenesis, urate oxidase being undetectable in this period. In male liver, a similar variation pattern was found, but in kidney high activities were found both in May and December. These results suggest an influence of sex hormones in trout purine catabolism, especially in females.

Introduction

Purine catabolism is more complete in fish and amphibians than in higher vertebrates (Hayashi *et al.*, 2000). After degradation of purine nucleotides into xanthine, this product is metabolised by xanthine oxidoredutase to produce uric acid. Animals containing urate oxidase produce allantoin, which is the end product of this pathway in most mammals. In lower vertebrates, allantoin is hydrolysed by allantoinase into allantoic acid, which can be further metabolised. But even among fish the final product of purine catabolism is not always the same. While salmonids excrete allantoic acid (Urich, 1994), other teleosts possessing allantoicase and ureidoglycollate lyase degrade purines into urea and glyoxylic acid (Hayashi *et al.*, 2000).

Urate oxidase is a peroxisomal enzyme, and in some fish allantoinase and allantoicase were also detected in peroxisomes. Taking into account that in brown trout the volume of liver peroxisomes and urate oxidase activity are reduced during vitellogenesis (Rocha *et al.*, 1999; 2001), this study aims to verify which variations occur in other purine catabolism enzymes during the reproductive cycle.

Material and Methods

Ten three-years-old farmed trout (*Salmo trutta* f. *fario*) of each gender were collected at the major seasons of their natural reproductive cycle: after spawning (February), early vitellogenesis (May), advanced vitellogenesis (September), and pre-spawning (December). Gonado-somatic index was used to assess gonad maturation. Preparation of liver and kidney homogenates was based on Rocha *et al.* (2003). Samples were stored at -80ºC until measurement. Enzyme activities were assayed at 25 ºC: xanthine dehydrogenase according Lallier and Walsh (1991), urate oxidase as described by Rocha *et al.* (2003), and allantoinase following Piedras *et al.* (1998) (at pH 7.5). Protein was determined according to Lowry *et al.* (1951). Two-way ANOVA and Newman-Keuls post-hoc test were applied.

Results and Discussion

Higher values of gonado-somatic indexes found in September and December (Fig. 1a) indicate an advanced stage of gonadal maturation, corresponding to a period of high sex hormones levels. Activity variations of purine catabolism enzymes in liver and kidney are

presented in Figs.1b to 1d.

For all enzymes, a significant annual and gender effect was seen as assessed by ANOVA and subsequent testing (p<0.05). Significant differences are shown in Fig. 2. In kidney, only xanthine dehydrogenase was present and in female liver urate oxidase activity was undetectable during vitellogenesis (from September to December). Purine catabolism enzyme activities presented a similar annual variation pattern in both genders, with the exception of xanthine dehydrogenase in male kidney, which activity increased from September to December. In general, these enzyme activities decline during vitellogenesis, when sex hormones levels are higher. Data suggest an influence of sex hormones in trout purine catabolism, mainly in females, being urate oxidase much more affected than the other enzymes.

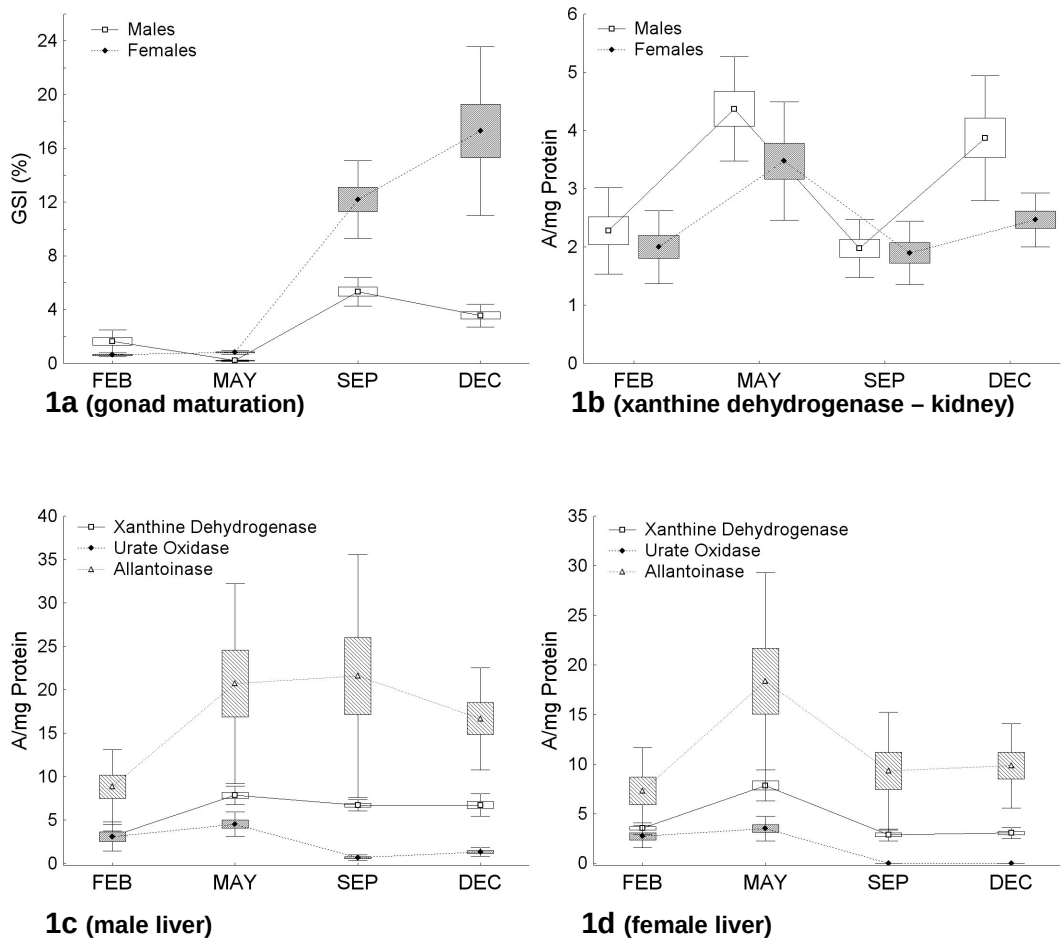

FIGURE 1. a) Gonado-somatic indexes (GSI) in four seasons of brown trout reproductive cycle. **b)** Activity of xanthine dehydrogenase in male and female kidney. **c)** and **d)** Activities of purine catabolism enzymes in male liver (**c**) and female liver (**d**). Enzyme activities (A) expressed as nmol.min^{-1}. Central mark: Mean; Box: Mean ± SE; Whisker: Mean ± SD.

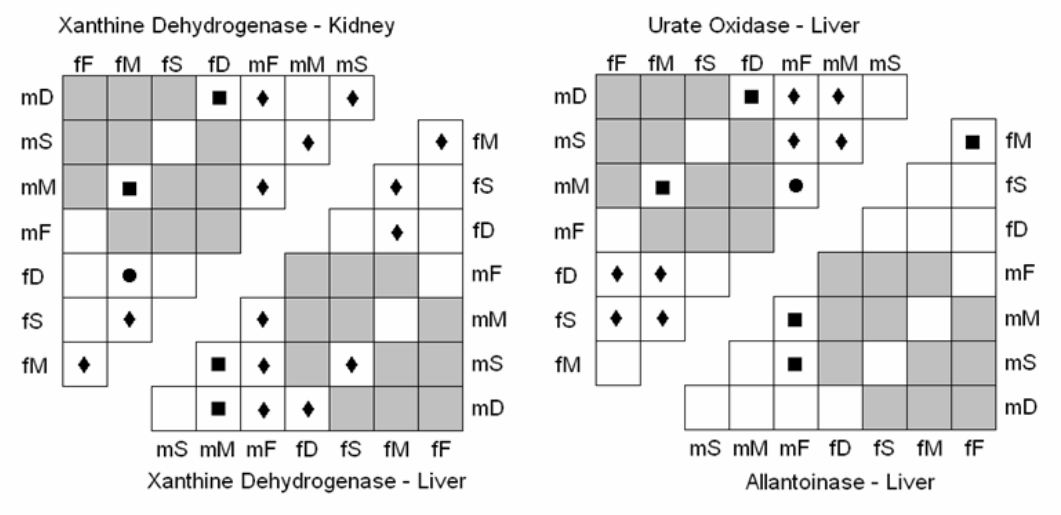

FIGURE 2. Significant variations: ♦(p<0.001), ● (p<0.01), ■ (p<0.05). Grey areas correspond to not relevant comparisons. f: females; m: males; F: February; M: May; S: September; D: December.

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CHAPTER 7

ENZYME ACTIVITIES OF BROWN TROUT PEROXISOMES UNDER PARAQUAT EXPOSURE

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Enzyme Activities of Brown Trout (*Salmo trutta*) Peroxisomes under Paraquat Exposure

Abstract

Environmental pollutants exert deleterious effects particularly in aquatic organisms, inducing the intracellular formation of reactive oxygen species, which consequently conduce to cellular damage. Paraquat, as a potent herbicide, can be highly toxic for fish, causing morphological and biochemical alterations in several organs. Since peroxisomal enzymes are implicated in the reactive oxygen species and xenobiotic metabolisms, a subacute exposition of brown trout to a waterborne relevant environmental concentration of that toxic was performed in order to verify if some peroxisomal enzymes show significant alterations, particularly from liver and kidney peroxisomes. Thus, spectrophotometric measurements of catalase and D-aminoacid oxidase activities were assayed in liver and renal homogenates, plus urate oxidase in liver. In liver, all those enzymes did not show differences between control and paraquat groups, both after 7 and 15 days of exposure. In kidney, any difference in the enzymatic activities between control and paraquat groups was also detected, but during the experiment it was observed an increase of catalase and a simultaneous decrease of D-amino acid oxidase in both groups. Hence, at least for the used concentration of paraquat, hepatic and renal peroxisomes are not affected by that toxic. Nevertheless, further research would be conducted with other concentrations and toxic mixtures in order to acquire a truly assessment of paraquat toxicological potential for peroxisomes.

Introduction

It is currently known that peroxisomes are deeply implicated in detoxification mechanisms such as xenobiotic and reactive oxygen species metabolism. Specific peroxisomal enzymes involved in those pathways are responsible for the deactivation and neutralization of toxic compounds, because they are able to transform toxic oxygen metabolites as superoxide and hydroxyl radicals into non or less toxic products (Pedrajas *et al.*, 1996; Masters and Crane, 1998; Masters, 1998; Schrader and Fahimi, 2004; Parvez and Raisuddin, 2006b; 2006a). Environmental pollution has been increasing in the last decades as a result of industrial and agricultural technological developments and from the vast array of daily urban activities. The aquatic environment is especially affected since it is the end-point of many contaminants such as pesticides and herbicides. These toxic compounds imply health hazards for humans and other animals (Cancio *et al.*, 1998; Cajaraville *et al.*, 2000; Strmac and Braunbeck, 2002).

Paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride) (Fig. 1) is a non-selective herbicide with a broad spectrum of activity widely used for crop desiccation and weed control (Tortorelli *et al.*, 1990). This toxic reaches natural waters via runoff from agricultural fields and is accumulated in different aquatic organisms causing several deleterious effects (Gabryelak and Klekot, 1985), since it easily reduced into radical ions generating superoxide anions, which seems to react with unsaturated membrane lipids (Ken *et al.*, 2003).

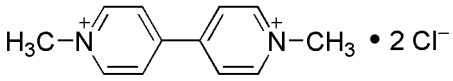

FIGURE 1. Chemical structure of paraquat.

Particularly in fish, it is known that paraquat induces biochemical alterations in gill epithelium, kidney and liver (Dimarzio and Tortorelli, 1994). For instance, it is also documented that some enzymes suffer alterations in their activities, such as the increase of transaminases in two carp species (Rojik *et al.*, 1983), higher activities of glucose-6-phosphate dehydrogenase, glutathione reductase, γ-glutamylcysteine synthetase and glutathione S-transferase in rainbow trout liver (Stephensen *et al.*, 2002; Akerman *et al.*, 2003) and in Nile tilapia (Figueiredo-Fernandes *et al.*, 2006a) as well as the enhanced

SOD and EROD activities in the latter species (Figueiredo-Fernandes *et al.*, 2006a; 2006b). Increasing of protein carbonyls was also detected in the freshwater fish *Channa punctata* (Parvez and Raisuddin, 2005), contrarily to the reduced levels of glutathione in the same species (Parvez and Raisuddin, 2006b). Tortorelli *et al.* (1990) also reported changes in a catfish enzymatic activities caused by paraquat, which subsequently affected the contraction of the heart.

Catalase, D-amino acid oxidase and urate oxidase are typical examples of peroxisomal enzymes. Catalase, as an antioxidant enzyme, was investigated in several fish toxicological studies, generally showing higher activities in the toxic exposed groups (e.g. Braunbeck and Strmac, 2001; Strmac and Braunbeck, 2002; Peixoto *et al.*, 2006). Literature about the effects of toxics in the activities of peroxisomal oxidases is very scarce, although Cajaraville and Ortiz-Zarragoitia (2006) and Cancio *et al.* (1998) reported an increase in the activities of acyl-CoA and D-aminoacid oxidases in mussels exposed to pollutants as well as Arnold *et al.* (1995) mentioned an increase of urate oxidase activity among other enzymes in response to the exposition of rainbow trout to toxic compounds.

In a preliminary approach and in order to increase the knowledge concerning the effect of chemicals which induce oxidative stress on fish peroxisomes, brown trout animals were subacutely exposed to a certain dose of the herbicide paraquat. In peroxisome richest organs (liver and kidney) were measured some peroxisomal enzyme activities, namely catalase and two oxidases (D-aminoacid and urate oxidases), to detect eventual alterations in their activities caused by paraquat exposure.

Material and Methods

Animals and Experimental Design

Two groups of immature (1 year-old) brown trout (*Salmo trutta* f. *fario*) were maintained in two different tanks at an aquaculture station (Estação Aquícola do Rio Ave, Portugal) during 15 days. Body parameters of those fish included weight and length. Fish were acclimatized along one week in the testing tanks, with dechlorinated water running in a recirculating water system, being fed with commercial trout food. The experiment was started by the addition of paraquat diluted in a saline solution to one tank (0.3 mg L^{-1} in nominal concentration). In the control tank only the saline solution was added. During the 15 days of exposure to paraquat, water circulated in the closed system with cleaning maintenance, total water renewal and reposition of paraquat every 2-3 days. Before being

sacrificed, the fish were kept in well-aerated and dechlorinated water for approximately 12 h. Ten fishes were collected in the first day of the experience as control at day 0. After the beginning of the paraquat exposure, ten fishes from each group were collected at day 7 and 15.

Chemicals

Paraquat, cofactors, substrates for enzyme assays and BSA were obtained from Sigma-Aldrich (Dorset, U.K.). All other chemicals were of reagent grade and were purchased from Merck (Darmstadt, Germany).

Preparation of Tissue Homogenates

Fishes were anaesthetised by immersion in a 1 ml L^{-1} aqueous solution of ethylene glycol monophenyl ether and then weighed and measured in length. Before tissue collection for homogenization, liver and kidney were perfused with a heparinized (5 IU/ml) isosmotic buffer for salmonids (Cascales *et al.*, 1997) composed of NaCl 0.74%, KCl 0.04%, $CaCl_2$ 0.02%, $MgSO_4$ 0.15%, $NaHCO_3$ 0.03%, $NaH_2PO_4 \bullet H_2O$ 0.05%, $Na_2HPO_4 \bullet 2H_2O$ 0.16%, at 4 ºC and with a physiological flow rate of about 5 ml min^{-1} Kg^{-1} of body weight (Hampton *et al.*, 1985). For improving the perfusion, the most posterior part of the fish was cut, allowing a direct cannulation of either the vein or the artery with simultaneous escape of both perfusate and blood. After removal, liver and trunk kidney were immediately weighted. Subsequently, the organs were minced in chilled homogenization buffer with a pH of 7.4 (250 mM sucrose, 5 mM MOPS, 1 mM EDTA, 0.1% PMSF and Triton X-100 0.5%) (Goldenberg, 1977) and then homogenized in the same buffer using a Potter-Elvejhem homogenizer at 1,000 rpm, held at 4 ºC. The homogenized volumes were adjusted to 10 ml g^{-1} of liver and to 5 ml g^{-1} of kidney and filtered through a net of about 95 µm mesh. Subsequently a centrifugation was carried out at 10,000 g during 10 min. The supernatants were collected and stored at -80 ºC in eppendorfs until enzymatic measurements were performed.

Biochemical Measurements

All assays were run in a spectrophotometer connected to a circulating water system for temperature regulation in the cuvette compartment. Total protein content was determined according to Lowry *et al.* (1951) using BSA as standard and results are expressed in BSA equivalents. Enzymatic assays were performed in duplicate, with two different dilutions of

each sample to calculate a mean value and to assure results reproducibility. Appropriate sample dilutions were used in order to obtain linearity in time of enzymatic activities and proportional to the amount of protein. The activities of peroxisomal enzymes were assayed at 25 ºC, except for catalase which was performed at 20 ºC.

The evaluation of catalase enzymatic activities was based on the methodology previously described by Aebi (1984) and applied to brown trout by Rocha *et al.* (2003). In detail, the incubation mixture contained 50 mM sodium phosphate buffer (pH 7), 10 mM H_2O_2 and a diluted sample. The consumption of the H_2O_2 was seen by a decrease of the absorbance at 240 nm of wavelength for 30 seconds and the activity was expressed by the first-order rate constant (k) for degradation of H_2O_2:

$$k = (1 \div \Delta t) \times \ln (c_1 \div c_2)$$

in which c_1 and c_2 are the concentrations at $t = 0$ and $t = 30$ seconds, respectively.

The measurement of peroxisomal oxidase activities was based on H_2O_2 production and according the procedure of Cablé *et al.* (1993) and Rocha *et al.* (2003). For these assays the incubation mixture contained 50 mM potassium phosphate buffer (pH 8.3), 0.082 mM 4-amino-antipyrine, 1 mM phenol, 2 IU/ml of horseradish peroxidase, 0.01 mM FAD, 10 mM azide (Leupold et al., 1985), and a diluted sample of either liver or kidney total homogenates. The enzymatic reaction was started by the addition of the correct substrate in the concentrations: 20 mM of D-alanine and 1 mM of uric acid. The production of H_2O_2 by peroxisomal oxidases during 10 min was calculated taking into account a calibration line (Rocha *et al.*, 2003) with the mathematical expression $[H_2O_2] = 185.07 \times$ Absorbance at 500 nm.

Statistical Analysis

The Statistica 6.0 for Windows software was used. After testing the normality and the homogeneity of variances, a two-way ANOVA was performed to detect any effect of paraquat along the exposure, and a one-way ANOVA was used to observe the kinetics along the 15 days in both control and paraquat exposed groups, both analyses for each enzyme. In some cases, transformation of data was needed to warranty normality and homogeneity. Data displaying heterogeneity of variances were analysed using Tukey and Newman-Keuls post-hoc tests in the two-way ANOVA and Dunnett's C test was applied in one-way ANOVA analysis. The significance level was set at $p \leq 0.05$. Data is presented as mean (CV) from 10 fish per group. CV is the coefficient of variation (CV = standard deviation ÷ mean).

Results

Animals from the control and the exposed to paraquat groups showed during experiments very similar mean values in their body and organ parameters, namely the fish weight, the standart fish length, the gonado-somatic index (GSI), the hepato-somatic index (HSI) and the reno-somatic index (RSI) (Table 1).

TABLE 1.

Morphometric data of fishes

	Weight	Length	GSI	HSI	RSI
Control	51.54 (0.27)	15.8 (0.09)	0.001 (0.52)	0.011 (0.16)	0.009 (0.10)
PQ	51.60 (0.21)	15.9 (0.07)	0.002 (0.87)	0.011 (0.19)	0.009 (0.06)

Values of weight and length are expressed as g and cm, respectively, whereas the GSI, HSI and RSI are expressed as % of total body weight. PQ – paraquat exposed group.

Peroxisomal Enzymatic Activities in the Liver

Data of some peroxisomal enzymatic activities (catalase, D-aminoacid oxidase and urate oxidase) are presented in Table 2. In liver, none of those enzymes showed significant differences in their activities between the control and the paraquat exposed group, in each sampling period. Additionally, along the 15 days, the activities of those hepatic enzymes also did not show significant differences in control or in paraquat groups.

Peroxisomal Enzymatic Activities in the Kidney

Regarding the enzymatic activities from brown trout kidney, results showed that also in this organ no significant differences existed between the control and the paraquat groups (Table 3). But when data were analysed in a kinetics perspective in both control and paraquat exposed groups, results revealed a significant increase in catalase activity and, simultaneously, a significant decrease in the D-aminoacid oxidase activity, during the 15 days of experience. No urate oxidase activity was found in kidney samples.

TABLE 2.

Activity of some peroxisomal enzymes from liver of brown trout in the control group and paraquat (PQ) exposed group, at day 0, 7 and 15.

Liver	Day 0	Day 7		Day 15	
		Control	PQ	Control	PQ
Catalase	1.080 (0.09)	1.090 (0.10)	1.048 (0.13)	1.108 (0.22)	1.124 (0.15)
D-Aminoacid Oxidase	3.119 (0.23)	3.559 (0.21)	2.803 (0.21)	3.712 (0.23)	3.373 (0.21)
Urate Oxidase	2.386 (0.33)	2.115 (0.64)	1.948 (0.40)	1.764 (0.78)	1.924 (0.33)

Mean values followed by their coefficients of variation between brackets are presented as enzyme activities (A) mg^{-1} of Protein, in which units are expressed in nmol min^{-1} for oxidases and in s^{-1} for catalase.

TABLE 3.

Activity of some peroxisomal enzymes from kidney of brown trout control group and paraquat (PQ) exposed group, at day 0, 7 and 15.

Kidney	Day 0	Day 7		Day 15	
		Control	PQ	Control	PQ
Catalase	0.069 (0.18)^{a}	0.063 (0.33)^{a}	0.083 (0.20)	0.095 (0.25)^{b}	0.089 (0.17)^{b}
D-Aminoacid Oxidase	1.891 (0.19)^{a}	1.329 (0.38)^{b}	1.830 (0.21)^{a}	1.333 (0.27)^{b}	1.355 (0.33)^{b}
Urate Oxidase	n.d.	n.d.	n.d.	n.d.	n.d.

Mean values followed by their coefficients of variation between brackets are presented as enzyme activities (A) mg^{-1} of Protein, in which units are expressed in nmol min^{-1} for oxidases and in s^{-1} for catalase. n.d. - not detectable. Means with lower case superscript letters represent significant differences among sampling periods within the same group, and for each enzyme. To simplify, the level of significance was always assumed as $p \leq 0.05$.

Discussion

Nowadays, fishes are affected by complex mixtures of contaminants in their habitats. For this reason, it became extremely important the determination of biomarkers which could

indicate a general response to chemical toxicity (Cajaraville *et al.*, 2000; Strmac and Braunbeck, 2002). Moreover, it is equally important to know which metabolic pathways are disrupted by the action of xenobiotics. Catalase expression is commonly altered under toxicant exposure and increases of its activity have been used as biomarkers of cell stress (Arnold *et al.*, 1995; Strmac and Braunbeck, 2002)

Toxics, such as 4-chloroaniline, atrazine, linuron and 4-nitrophenol among others were used in several fish studies in which the authors identified many ultrastructural modifications in liver and kidney cells caused by those toxicants (Braunbeck *et al.*, 1989; Braunbeck, 1993; Oulmi *et al.*, 1995a, 1995b; Oulmi and Braunbeck, 1996; Braunbeck and Strmac, 2001; Strmac and Braunbeck, 2002). Liver and kidney peroxisome proliferation was a toxicological feature reported in almost all of the referred studies. Paraquat is known to cause several morphological alterations and tissue damages. Babatunde *et al.* (2001) observed a linear increase of histological damages in *Oreochromis niloticus* gill architecture with paraquat doses from 9.6 to 16 mg L^{-1}. Rojik *et al.* (1983) described for a paraquat concentration of 10 mg L^{-1} structural damages also in fish gills (chloride cell necrosis and disappearance of cytoplasmic organelles in the epithelial cells) and several stages of morphological changes in liver, namely the absence of heterochromatin, appearance of autophagic vacuoles with glycogen granules, dilatation and vacuolation of the rough endoplasmic reticulum, swollen mitochondria and presence of myelin-like structures indicative of membrane decomposition. However, few studies on fishes concerning kidney changes under paraquat effect are documented (Rojik *et al.*, 1983).

Considering the peroxisome role in detoxification and in accordance to the morphological alterations of these organelles described in literature for aquatic organisms, including salmonids, the potent herbicide paraquat, under environmentally relevant concentrations, could exert some influence on peroxisomal enzyme activities of brown trout liver and kidney (Rojik *et al.*, 1983; Fahimi and Cajaraville, 1995; Cajaraville *et al.*, 1997; Cajaraville and Ortiz-Zarragoitia, 2006).

However, and at least for the studied enzymes, results indicate that paraquat does not induce enzymatic alterations on liver and kidney peroxisomes when administered at 2.5% of the LC_{50} for brown trout (13 mg L^{-1}) (Tomlin, 2000). The used concentration of paraquat was chosen based in several published data, for which no documented signals of cellular necrosis or cellular death were found, and additionally, because that pesticide dosage corresponds to a more realistic situation found in nature (America, 1994). Thus, for an

exposure to 0.3 mg L^{-1} of paraquat, hepatic and renal peroxisomes does not seem affected in their enzymatic activity, contrarily to what happened in other studies which reveals an increase of catalase activity in both organs under that herbicide exposure. However, those investigations were performed with much higher paraquat concentrations, which do not translate a frequent environmental condition. Therefore, we can assume that probably for this paraquat dosage, hepatic peroxisomes are not really needed to maintain the content of reactive oxygen species (ROS) in homeostasis, avoiding an oxidative stress situation. The same assumption can be applied to kidney, despite the fact that this organ is more active in the excretion of physiological compounds and metabolites derived from itself and other organs such as liver. However, the absence of paraquat effect in renal tubules are in accordance with morphological data mentioned by Rojik *et al.* (1983).

We saw that the peroxisomal enzymatic activities in kidney varied with time, not only in paraquat treated group but also in control group. This fact calls attention for the use of juvenile trout kidney samples in toxicological studies with enzymatic measurements, since the time of sample collection could indicate differences in those activities, not due to the experimental toxic effect but otherwise to temporal variations reflecting the normal physiology and adaptations of animals. On the contrary, the liver is an organ which revealed higher stability in relation to its peroxisomal enzymatic content, not influenced by time.

The absence of urate oxidase activity in kidney peroxisomes was once more in conformity with results found in the last chapters of this thesis and in published data from other authors (Urich, 1994). By the other hand, the urate oxidase activities in liver agree with the former detections of this enzyme in juvenile and adult brown trout liver (Rocha *et al.*, 2003; Rocha *et al.*, 2004a; Rocha *et al.*, 2004b; Resende *et al.*, 2005) as well as in other species (Kinsella *et al.*, 1985; Yeldandi *et al.*, 1995; Hayashi *et al.*, 2000; Terjesen *et al.*, 2001).

In conclusion, the subacute toxicity test showed that under a relevant concentration of the herbicide paraquat there were no changes in some peroxisomal enzyme activities, which could signal (biomark) that, in brown trout juvenile, an exposure to a toxicant was occurring or a disrupting effect was being exerted. The study warns for the fact that common biomarker approaches (such as studying catalase activities) may not detect exposure to low concentration of persistent toxicants. We further showed that for subacute (and likely subchronic) testing and with the selected peroxisome marker enzymes, studies in kidney must account with the potential adaptative changes occurring in time, both in control and exposed animals; a phenomenon not occurring in liver.

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CHAPTER 8

FINAL ASPECTS AND CONCLUSIONS

Final Aspects

Before summarizing the overall Conclusions of this Thesis, and for better integrating some results from Chapters 2, 3 and 4, we found it opportune to analyse herein a few selected peroxisome morphological and biochemical data, in terms of total number and volume of peroxisomes per g of kidney and enzymatic activity per g of kidney, respectively (Table 1).

TABLE 1.

Morphometric and biochemical data of brown trout peroxisomes per g of brown trout kidney.

Parameters	Renal Tubule Peroxisomes (mm^3 / g Kidney)		Renal Tubule Peroxisomes (no. / g Kidney)		Catalase (A / g Kidney)		D-alanine oxidase (A / g Kidney)	
Sex	♀	♂	♀	♂	♀	♂	♀	♂
February	1.48 (0.20)	1.16 (0.60)	$2.02E^{10}$ (0.19)	$1,00E^{10}$ (0.48)	7.21 (0.16)	10.36 (0.20)	103.10 (0.08)	62.88 (0.20)
May	2.31 (0.16)	2.27 (0.35)	$4.52E^{10}$ (0.22)	$3,66E^{10}$ (0.40)	6.02 (0.23)	6.14 (0.26)	152.60 (0.23)	98.97 (0.14)
September	1.89 (0.29)	1.42 (0.31)	$2.59E^{10}$ (0.25)	$1,85E^{10}$ (0.44)	5.19 (0.23)	4.19 (0.14)	130.08 (0.19)	70.29 (0.21)
December	1.81 (0.25)	0.75 (0.45)	$4.23E^{10}$ (0.39)	$1.31E^{10}$ (0.72)	5.07 (0.19)	7.03 (0.34)	107.44 (0.35)	54.22 (0.31)

A – s^{-1} for catalase and nmol min^{-1} for D-alanine oxidase.

With this approach we found some linear correlations between seasonal data sets (Table 2). Thus, contrarily to catalase, which did not correlate with either the number or volume of peroxisomes, the activity of D-alanine oxidase was significantly and linearly correlated with both the peroxisome number and volume, mainly in males. As shown in Table 1, the periods in which peroxisomes were more numerous and occupied more volume per g of kidney, also corresponded to those with the highest content in D-alanine oxidase. Hence,

in trout renal peroxisomes, D-alanine oxidase seems to translate in a certain way what happens with the structure of these organelles, globally marking in some extent the morphological changes. This kind of correlations between structure and enzyme activities have been rarely performed, but they can give new perspectives about the dynamic of cell organelles.

TABLE 2.

Statistically significant correlations (Pearson coefficients) between the D-alanine oxidase and catalase activities and the peroxisome volume and number, all per g of brown trout kidney (n = 5, per correlation).

	♂		♀	
Parameters	r	*p* value	r	*p* value
D-alanine *vs.* V Total / g kidney	0.76	<0.001	0.62	0.005
D-alanine *vs.* N Total / g kidney	0.75	<0.001	0.49	0.035
Catalase *vs.* V Total / g kidney	-0,12	0.625	0.20	0.408
Catalase *vs.* N Total / g kidney	-0.26	0.270	0.02	0.922

Conclusions

This Thesis addressed some relations between the gonad maturation status and the kidney and liver of brown trout. Thus, morphological parameters of kidney components, structural and biochemical peroxisome variations, as well as activities of some hepatic and renal purine catabolism enzymes were analysed during the annual reproductive cycle. In addition, it was studied the subcellular localization of two purine catabolism enzymes and, finally, we studied possible implications in the peroxisome physiology by exposure to a toxicant known to act on oxidative stress-induced mechanisms. In the following the main findings are presented:

- After a qualitative description in paraffin and semithin epoxy sections of brown trout kidney, we firstly confirmed that its histology is similar to other freshwater species. Subsequently, new quantitative data about the brown trout kidney morphology was

obtained; in particular, the relative volumes of renal components were estimated for both genders and in four seasons of the annual breeding cycle. It was shown that kidney components vary with gender and also along the year. In addition, an unusual occurrence of vacuolized tubules was found at the post-spawning season, suggesting a sort of remodelling of this organ in response to physiological needs, under influence of yet unknown seasonal events. Despite proximal tubule segments I and II as well as new growing tubules presented relative volume variations between genders and along the year, it was the renal corpuscules and collecting tubules that were the most “affected” renal components; the data suggested that seasonal variations in the filtration rate and urine production may occur.

- According to our data, peroxisome morphology, especially in the proximal tubule segment II, varied in a gender dependent manner and also during the annual reproductive cycle, despite these changes were not linearly correlated with the animal reproductive status (as evaluated by the GSI). Moreover, catalase and D-alanine oxidase activities also varied along the year and most of these values were linearly correlated with the gonadal status. In spite of this, in kidney, the possible influence of sex hormones on peroxisomes is not so strongly supported as in liver and the variation pattern is different in each of these organs; this, because in kidney, both genders showed similar variations. Thus, probably, kidney peroxisomes are influenced by other factors and / or other hormones, but not necessarily estradiol, which exert a major influence in liver. The fact that higher values of testosterone were reported in both males and females during the gonadal maturation, when the referred enzymes presented the lowest activities, suggests that this hormone may play a role in renal peroxisome metabolic regulation.
- We advanced the thesis that brown trout liver purine catabolism may be influenced by sex hormones, because some enzymes of that metabolic pathway presented gender dependent fluctuations with the breeding cycle. Especially in females, and in hepatic homogenates, xanthine dehydrogenase, urate oxidase and allantoinase presented lower activities when the gonad maturation status was more advanced, being urate oxidase undetectable in this period. The same variation pattern was also found for renal xanthine dehydrogenase activity in females, but not in males.
- We showed that, in brown trout, xanthine dehydrogenase and allantoinase, two purine catabolism enzymes are located in the cytosol. Taking into account that urate oxidase is also another enzyme from this metabolic pathway, which acts “between the other two”, and that it is located in the peroxisome, the evidence pointed that

some products from purine catabolism reactions have the ability to cross the peroxisomal membrane in both ways, in order to complete this metabolic pathway. In addition, it was found that brown trout lacks xanthine oxidase and also allantoicase, confirming the excretion of allantoic acid as the end product of purine catabolism in salmonids.

- In the context of testing other toxicological effects on fish peroxisomes, other than those exerted by the much studied peroxisome proliferators, we revealed that the herbicide paraquat does not affect the activities of catalase, D-alanine and urate oxidase from juvenile brown trout liver and kidney peroxisomes, at least under a subacute assay and with environmental relevant concentrations of the chemical. Being catalase often used as a toxicity biomarker in highly polluted scenarios, it was shown herein that this enzyme could not indicate the presence of that herbicide in low amount.

The findings given in this Thesis increase the knowledge about brown trout kidney and liver, in morphological and metabolic perspectives, offering a new view of the kidney as a very dynamic organ, which has both organellar and cellular morphofunctional adaptations along the year, surely to cope with functional needs. Since this organ is yet very poorly studied in fish, this Thesis offers more baseline data for further studies targeting the fish kidney, both in physiological and toxicological contexts.