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Experimental studies on the influences of temperature, sex and ethynylestradiol on stereological parameters of liver peroxisomes and hepatocyte volume in the platyfish (*Xiphophorus maculatus*)

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Abstract

Peroxisomes are small organelles that are present in almost every eukaryotic cell. They are responsible for important metabolic functions in the cell and are essential to life. The structure and function of those organelles can be influenced by both endogenous and exogenous factors, including abiotic ones (like temperature). Peroxisomes were only viewed as organelles in 1967, acquiring spotlight relevance when it was found that some chemicals evoked the abnormal proliferation of peroxisomes in rodent livers, which in time ultimately developed hepatocellular carcinomas. Such chemicals were called peroxisome proliferators. Some of these have also the ability to exert other effects, such as estrogenic actions. The peroxisome proliferation, and also the beta oxidation of long-chain fatty acids occurring in peroxisomes is tightly regulated by the so-called peroxisome proliferator-activated receptors, which are nuclear receptors that also control other key cellular and metabolic processes.

There are many pollutants present in the marine ecosystem, including peroxisome proliferators and compounds that mimic sex-steroid hormones (and more often estradiol). As such, many of these pollutants are endocrine disrupters. They have the ability to cause physiologic changes in the endocrine system, for instance impacting on liver metabolism, liver cells and hepatocellular organelles (including peroxisomes). The endocrine disruptors have been gaining importance as water pollutants, and fishes are sensible to their effects.

Given the highly suggestive findings of liver peroxisome modulating by estrogenic compounds in brown trout (*Salmo trutta*) and in zebrafish (*Danio rerio*), and not apparently in the same way, it is necessary to know whether this modulation occurs in other models. By a number of practical reasons, such as sexual dimorphism, a non-lobed and an easy to dissect liver, one interesting model is the ovoviviparous platyfish (*Xiphophorus maculatus*) This can be used to tackle not only structural kinetics of the peroxisomes in liver, but also changes occurring in the hepatocytes. These are known to be influenced by sex-steroids, namely in fish, including changes in cell number and size, which are nevertheless poorly studied and correlated with what happens with organelles such as peroxisomes.

With the above in mind, the main objective for this study was to see if there were baseline morphologic differences between sexes, at two temperatures, in the peroxisomes of liver cells and at the same time evaluating some ultrastructural aspects of hepatocytes, also their volume; as this has an impact on the relative and total content of peroxisomes. A second objective was to see if an estrogenic exposure could influence the peroxisomes, and eventually change the load of peroxisomes within the hepatocytes and alter these too.

In a first experience, females and males were separately maintained ay a 22 ºC and 28 ºC for fifteen days. At the end of the assay and after taking basic morphometric measurements, liver pieces were processed for transmission electron microscopy, being

subjected to catalase cytochemistry to tag the peroxisomes. A general qualitative analysis was made in the hepatocytes. Additionally, stereological techniques were used in semithin and ultrathin sections to estimate the volume of hepatocytes, and diverse volume, surface, and number-related parameters, both relative (in relation to the hepatocyte) and absolute. In a second assay, males were exposed to a model estrogen, 17α-ethynylestradiol (EE2), at 75 μL^{-1}, 22 ºC, for twenty one days, and then compared to a solvent control group.

We concluded that males and females of platyfish did not vary as to the qualitative and quantitative structure of the hepatocytic peroxisomes, namely as to the mean volume, surface and spherical-equivalent diameter of the organelle, and as to its relative volume, surface and number in relation to the hepatocyte. However, we concluded that liver cells of males had more peroxisomes and greater total volume and surface of such organelles than those of females. The latter facts were due to a lower mean volume of the female hepatocytes. Females had greater liver weight and also liver somatic index; indicating that they contain a greater number of hepatocytes per liver than males. Such differences could be revealed under a two-way ANOVA that detected a significant effect of sex, irrespective of two tested temperatures (22ºC and 28ºC). Exposure to EE2 at 75 μL^{-1} for fifteen days did not induce significant changes both in the liver weight, hepatocytes volume and also in relative and absolute numbers, volumes and surfaces of peroxisomes. The ultrastructural study of the hepatocytes revealed baseline differences between males and females, at both temperatures, with the latter sex having cells richer in rough ER. Also, males under EE2 exposure had hepatocytes ultrastructuraly alike to those of normal females.

We suggested that platyfish male and female hepatocytic peroxisomes do not differ as to their qualitative aspects and quantitative structural characteristics, like individual size and relative quantity per hepatocyte. Also, we reasoned that temperature does not seem to have a major influence as to the structure of peroxisomes in platyfish. Also, and at least under a subacute exposure regime, a significant estrogenic stimulus was unable to induce structural changes in the liver cell peroxisomes, not supporting the notion that peroxisome regulation by estrogens as seen in brown trout exists in all fishes.

Finally, our hypothesis that hepatocytes of male and female platyfish could have baseline quantitative differences was viewed as likely true, as males had, overall, bigger hepatocytes than females. Also, the latter were richer in rough ER, which indirectly (but strongly) supported that estrogenic hormones may have a functional role in governing the hepatocyte differences between sexes. Because of such regulation, the total number of peroxisomes per cell differed between sexes, with females having smaller peroxisomal amounts per hepatocyte. Consequently, considering that these cells can be subjected to hormonal influences, namely by estrogens, the total peroxisome amount in hepatocytes (and therefore in all liver) may vary between sexes. All this can be explored in the future.

Resumo

Os peroxissomas são pequenos organelos que estão presentes em quase todas as células eucaróticas e são responsáveis por importantes funções metabólicas celulares sendo, essenciais à vida. A estrutura e função desses organelos podem ser influenciadas por vários fatores endógenos e exógenos, incluindo abióticos (tais como a temperatura). Apenas em 1967 os peroxissomas foram reconhecidos como organelos, adquirindo uma importante relevância quando se descobriu que alguns produtos químicos provocam uma proliferação anormal dos peroxissomas em fígados de alguns roedores modelo, podendo levar ao desenvolvendo de carcinoma hepatocelular. Estes produtos químicos foram designados por proliferadores peroxissomais. Alguns destes produtos têm também a capacidade de exercer outros efeitos, tais como ações estrogénicas. A proliferação de peroxissomas, e também a beta-oxidação de ácidos gordos de cadeia longa realizada em peroxissomas, são reguladas por recetores ativados pelos proliferadores peroxissomais (PPARs, do inglês *Peroxisome Proliferator-Activated Receptors*); sendo eles recetores nucleares que também controlam outros importantes processos celulares e metabólicos.

Existem muitos poluentes no ecossistema marinho, incluindo proliferadores de peroxissomas e compostos que mimetizam hormonas esteróides sexuais (mais frequentemente o estradiol). Como tal, muitos destes poluentes são desreguladores endócrinos que possuem a capacidade de causar alterações fisiológicas no sistema endócrino, por exemplo com, impactos sobre o metabolismo e células hepáticas e até sobre organelos hepatocelulares (incluindo peroxissomas). Tais compostos, têm vindo a ganhar grande importância como poluentes aquáticos, já que os peixes são sensíveis aos seus efeitos.

Face aos dados muito sugestivos quanto a uma modelação de peroxissomas por parte de compostos estrogénicos, pelo menos em truta (*Salmo trutta*) e peixe-zebra (*Danio rerio*), ainda que não necessariamente da mesma forma, justifica-se saber se esta modelação ocorre noutros modelos biológicos. Por uma série de razões práticas, tais como um marcado dimorfismo sexual, um fígado não lobulado e fácil de dissecar, o plati (ou plátio) (*Xiphophorus macultus*) surge-nos como um modelo interessante que pode ser utilizado não só para estudar a cinética estrutural dos peroxissomas no fígado, mas também as mudanças que ocorrem nos hepatócitos. Estes já são conhecidos por serem influenciados por esteróides sexuais, nomeadamente em peixes, por exemplo quanto número e tamanho das celulas, ainda que as modificações estejam pouco estudadas e correlacionadas com o que sucede com os organelos, tais como os peroxissomas.

Considerando o acima exposto, o objetivo principal deste estudo foi verificar se havia diferenças morfológicas de base entre sexos, em duas temperaturas diferentes, no que respeita a peroxissomas dos hepatócitos e, ao mesmo tempo, avaliar alguns aspetos ultra-estruturais dos hepatócitos e também o seu volume celular; dado que este tem impacto sobre o conteúdo relativo e total de peroxissomas. O segundo objetivo foi verificar se uma exposição estrogénica poderia influenciar os peroxissomas, e, eventualmente, alterar o seu conteúdo total dentro dos hepatócitos, bem como modificar os próprios hepatócitos.

Num primeiro ensaio, fêmeas e machos foram mantidos separadamente a 22 ºC e 28 ºC por 15 dias. No final da experiência e depois de tiradas as medidas morfométricas dos peixes, os fígados foram processados para microscopia eletrónica de transmissão, sendo feita a técnica citoquímica da catalase para induzir a marcação de peroxissomas. Posteriormente foi feita uma análise qualitativa dos hepatócitos, aplicando-se técnicas estereológicas em cortes semifinos e ultrafinos, para se estimar o volume celular e outros parâmetros, como sejam o volume, superfície e número de peroxissomas, tanto relativos (em relação ao hepatócito) como absolutos. Numa segunda experiência, machos foram expostos a um composto estrogénico, o 17α-etinilestradiol (EE2), numa concentração de 75 μL^{-1}, a 22 ºC, por vinte e um dias sendo depois comparados com um grupo controlo.

Concluímos que machos e fêmeas de plati não variam quanto à estrutura qualitativa e quantitativa dos peroxissomas hepáticos, nomeadamente quanto ao volume médio, superfície e diâmetro-esférico equivalente do organelo, bem como o seu volume, superfície e número relativo em relação ao hepatócito. No entanto, observamos que, globalmente os hepatócitos dos machos possuiam mais peroxissomas, assim como maiores volumes e superfícies totais de peroxissomas que as fêmeas. Tais fatos foram devidos ao menor volume médio dos hepatócitos estimado das fêmeas. Estas tinham um maior peso de fígado, bem como um maior índice hepatossomático; indicando que as fêmeas continham um maior número de hepatócitos por fígado que os machos. Estas diferenças foram reveladas por uma *two-way* ANOVA, que detetou um efeito significativo do sexo, independentemente das temperaturas testadas (22 ºC e 28 ºC). A exposição a 75 μL^{-1} EE2 em durante 15 dias não induziu diferenças significativas, tanto no peso do fígado, como no volume dos hepatócitos e nem no volume, número e superfície relativos e absoluto dos peroxissomas. O estudo ultra-estrutural dos hepatócitos revelou diferenças entre machos e fêmeas em ambas as temperaturas, sendo os hepatócitos das fêmeas mais ricos em reticulo endoplasmático rugoso. Além disso, os machos expostos a EE2 mostraram hepatócitos ultra-estruturalmente mais similares aos de fêmeas normais.

Por último, a nossa hipótese quanto a existirem diferenças quantitativas de base entre os hepatócitos de machos e fêmeas de plati, parece ser verdadeira, dado que os machos tinham, em geral, hepatócitos maiores. Além disso, os hepatócitos das fêmeas eram mais ricos em reticulo endoplasmático rugoso, o que indiretamente (mas muito sugestivamente) reforça a ideia de que as hormonas estrogénicas podem ter um papel funcional na regulação das diferenças encontradas em hepatócitos de sexos diferentes. Devido a tal regulação, o número total de peroxissomas por célula diferiu entre os sexos, com as fêmeas a terem menos massa peroxissomal por hepatócito. Consequentemente, considerando que estas células podem estar sujeitas a grandes influências hormonais, designadamente por estrogénios, as quantidades de peroxissomas nos hepatócitos (e, portanto, no fígado) poderá variar entre sexos. Tudo isto pode ser explorado no futuro.

Abbreviation List

ADH - Alcohol dehydrogenase

DAB – 3,3'-Diaminobenzidine

E2 - 17β-oestradiol

EE2 – 17α-Ethynylestradiol

ER – Endoplasmic reticulum

GSI – Gonado-somatic index

LSI – Liver somatic index

PAH – Polycyclic aromatic hydrocarbon

PCB – Polychlorinated biphenyl

PPAR – Peroxisome proliferator-activated receptor

PPRE – Peroxisome proliferator response element

ROS – Reactive Oxygen Species

RXR – Retinoid X receptor

TEM – Transmission electron microscopy

Tris – Tris(hydroxymethyl)aminomethane

Introduction

Peroxisomes – Features and Functions

Peroxisomes are single membrane cytoplasmic organelles that exist in almost every eukaryotic cell, usually roundish and not exceeding the 1.5 μm in diameter. They have a proteinaceous matrix (Gould *et al*., 2000) and can exhibit variability in their morphology and function depending on the species but also on the cell type (Rocha 2006). The number of peroxisomes per cell can range from a few, to hundreds or even thousands (in mammals). They are involved in various vital functions, among them, they are responsible for the β-oxidation of fatty acids, etherlipids biosynthesis, purine and estradiol catabolism, metabolism of cholesterol, drugs and xenobiotics, detoxification of hydrogen peroxide, which is a toxic and very reactive compound formed in oxide reactions (Islinger *et al*., 2010; Dzhekova-Stojkova *et al*., 2001). Therefore, according to their functions peroxisomes are very rich in enzymes, being catalase the most important one. Catalase is an essential redox enzyme; it decomposes the H_2O_2 into H_2O. There is a technique that uses 3,3'-diaminobenzidine (DAB) to form a stained product that allows seeing peroxisomes in the microscope (Novikoff and Goldfischer, 1969). This helped studying the genesis and kinetics of the organelle. Indeed the genesis of peroxisomes is a topic of great interest. There are three theories for the formation of peroxisomes. The first one says that new peroxisomes are divided from older peroxisomes (Fahimi *et al.,* 1993). The second one is apologist that some peroxisomes are generated from a ghost peroxisome that incorporates proteins from the cell matrix. Only after this incorporation, it is called a mature peroxisome and is ready to form other peroxisomes (Lodish *et al*., 2004). Still a third later theory proposes the contribution of the endoplasmic reticulum (ER) in the formation of peroxisomes. Some peroxisomal proteins enter the ER, concentrate in one area that later separates from the ER, forming a vesicle that matures into a peroxisome (Tabak *et al.,* 2006).

Peroxisome Proliferator-Activated Receptor and Peroxisome Proliferators

The protein content in peroxisomes can be modeled by Peroxisome Proliferator Activated Receptors (PPARs) (Ilsinger *et al.,* 2010). They are nuclear receptors that govern diverse cellular and metabolic processes. There are three subtypes of PPARs (α, β/δ and γ) with different distribution among the tissues. However, they are expressed mainly in tissues with a high fatty acid oxidation, such as the liver, heart and skeletal muscle (Jalouli *et al*., 2003). The PPARs are strongly involved in the regulation of lipid metabolism. PPARs form heterodimers with Retinoid X Receptor (RXR) which can

connect to a specific DNA sequence called Peroxisome Proliferator Response Element (PPRE). The PPRE region is upstream of the target gene (Ziouzenkova and Plutzky, 2008). This is how they are involved in the regulation of genes related to lipid utilization and storage, lipoprotein metabolism and other functions of lipid metabolism and regulation (Kim *et al*., 2009). Also, proliferation of peroxisomes can be evoked by PPARα ligands and it is well established that different species have different responses to peroxisome proliferators. For example, rodents show a high peroxisomal enzyme induction, whereas humans do not (Ammerschlaeger *et al*., 2004). In fish, peroxisome proliferation can be affected by natural factors like, water temperature, salinity, season or even eating habits (Fahimi and Cajaraville, 1995). But they can also be affected by xenobiotics such as, industrial plasticizers, hypolipidemic drugs, herbicides, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), solvents and some leukotriene antagonists (Bosgra *et al.,* 2005; Cajaraville *et al.,* 2003; Woodyatt *et al*., 1999). Peroxisome proliferators typically have the ability to increase the size and abundance the peroxisomes in hepatocytes, with posterior induction of liver tumors when administrated chronically to mice (Lai, 2004). Long term exposure causes liver hypertrophy with cell hyperplasia in rodents, which eventually leads to hepatocellular carcinoma (Bosgra *et al.,* 2005). Indeed, it was established a link between peroxisomal proliferation and carcinogenesis (Reddy *et al.,* 1980). The administration of peroxisome proliferators to rodents induce many liver alterations that are summarized in the Table 1.

Table 1 - Effects of peroxisome proliferation in rodents. Adapted from Dzhekova-Stojkova *et al.* (2001).

Liver weight	• Liver enlargement due to both hyperplasia and hypertrophy; • Increased DNA and protein synthesis;
Morphological changes	• Increased number and size of peroxisomes; • Increased smooth endoplasmic reticulum; • Increased number of mitochondria; • Lysosomal changes and lipofuscin deposition; • Liver nodules and hepatocellular carcinoma;
Biochemical changes	• Selective induction of peroxisomal enzymes: marked induction of peroxisomal fatty acid β-oxidation enzymes, but only a small increase in catalase activity; • Induction of the other microsomal and cytosolic enzymes; • Inhibition of glutathione peroxidase, glutathione transferase and superoxide dismutase activities.

The hepatocyte growth and peroxisome proliferation seen in rodents was not observed in guinea-pig, dog, rhesus monkey and non-human primates (Bosgra *et al.,* 2005). Also, humans are considered to be less sensitive or not responsive to the dose that reacts in rodents. Fish respond more like primates (Fahimi and Cajaraville, 1995). Since peroxisomes are involved in cholesterol homeostasis (Bosgra *et al.,* 2005), hypopolipidemic drugs are used in the control of plasma cholesterol and triglyceride levels. So, it is now clear that humans react to peroxisome proliferators' exposure, but the response is very different from the rodents (Bosgra *et al*., 2005; Vu-Dac *et al.,* 1995; Woodyatt *et al.,* 1999).

In addition to the above considerations, there is also a great biomedical interest in studying peroxisomes due the fact that they are associated to some fatal human diseases. These diseases can be divided into two groups (Wanders, 2004): those related to deficiencies in the biogenesis of peroxisomes and those related from deficiencies in their enzymes. There are also defects in the PPARs, and for example humans with mutations in the PPARγ are associated with hypertension, insulin resistance and diabetes mellitus (Schinner *et al.,* 2002).

Peroxisome Regulation and Estrogenic Influences

As previously mentioned, through interaction and activation of PPARα, peroxisome proliferators have the ability to increase the size and number of peroxisomes in certain murine models, but also to influence the activity of some peroxisomal enzymes. Some studies focused on the intake of vitamin E for protection against peroxisome proliferators (Calfee-Mason *et al.,* 2004). Among other authors, Hertz *et al.* (1993) defended that the biological effects triggered by some peroxisome proliferators are very similar to those evoked by the thyroid hormones. They can induce liver activities that were considered to be thyroid-hormone-dependent. In summary, the peroxisome regulation is a very complex process and it is related to the endocrine system. With all this in mind this dissertation focused on the potential of modeling by estrogenic exposure, while searching for baseline hints about a more broad sex-steroids control by looking at differences between sexes.

Typically estrogens are considered the female sex hormones and androgens are viewed as the male sex hormones; despite androgens are not exclusive to males and androgens are not synonym of females. Both groups of hormones have multiple sites of activity and innumerous biological actions. They are synthesized from cholesterol and the biological pathway of sexual hormones is very complicated. Their release is regulated by feedback mechanisms from the hypothalamus and hypophysis (Geyer *et al.,* 2001). Xenoestrogens and xenoandrogens are synthetic substances that mimic the effects of estrogens and androgens. Some industrial compounds and pesticides have little or none

estrogenic activity, but, after absorption, they can be metabolized to other compounds that have bigger estrogenic potency than the parent chemicals (Geyer *et al*., 2001). Estrogens have a biological effect in adipose tissue. Female rodents that suffer ovariectomy become obese and when they are treated with 17β-oestradiol (E2) the condition can be reversed (Kim *et al*., 2009). Lipid metabolism is affected by many hormonal signals, like insulin, thyroid hormone, retinoic acid, glucocorticoids and estrogen (Kim *et al.,* 2009). In brown trout (*Salmo trutta*), Rocha *et al.* (1999) described for the first time that there is a negative correlation between the ovary maturation and the size of hepatocytic peroxisomes. There were morphologic alterations in peroxisomes but with no changes in their number; indeed, only the volume of peroxisomes varied (both that of the individual organelle and the one concerning the total amount per liver cell). The structural modifications occurred naturally during the reproductive cycle and in both sexes, with no known exposure to exogenous compounds. In particular, there was a decrease in the organelle volume and in the activity and expression of peroxisomal enzymes as females underwent vitellogenesis and ovary maturation; at these periods they were under largely exposed to endogenous estradiol. Afterwards, these data was correlated to and agreed with the findings of Castro *et al*., (2009), when it was measured the gene expression of 17β-hydroxysteroid dehydrogenase type 4 during the reproductive cycle. The corresponding enzyme plays an important role in the β-oxidation of fatty acids and, it seems, also in estradiol catabolism. A link between E2 and peroxisomes was first established in fish by Veranic and Pipan (1992). These authors showed that zebrafish males exposed to E2, for 20 to 30 days, reduced the diameter of their liver peroxisomes, while increasing both their numerical and surface density, but not the relative volume of the organelles in the cell.

The Influence of Steroids and Their Mimics in Liver

The liver is a vital organ responsible for a huge number of metabolic processes, and where the exogenous compounds are metabolized and eventually excreted. The liver is a key responsible for storage and metabolism of nutrients. Since it is responsible for maintaining part of the metabolic homeostasis of the body, hepatotoxicity leads to serious consequences. All major functions of the liver can be altered by acute or chronic exposure to xenobiotics. One famous example in hepatoxicology was derived from the use of a pharmaceutical that was a peroxisome proliferator compound: the troglitazone (Rezulin®). This was an antidiabetic drug that – acting like a PPAR agonist – was used in the treatment of type 2 diabetes. This compound was ultimately removed from the market after hundreds of patients that used this drug suffered from liver failure (Chojkier, 2005).

It has been recognized that steroid hormones influence both healthy and diseased liver (Colantoni *et al.,* 2000; Eagon, 2010; Huggins and Yao, 1959). The latter responds to estrogens by increasing the synthesis and secretion of many serum glycoproteins, like, ceruloplasmin, corticosteroid-binding globulin, thyroid-binding globulin and testosterone-estradiol-binding globulin (Colantoni *et al*., 2000; Eagon *et al.,* 1985). There are many studies that use steroid hormones, and particularly sex-steroids and their mimics, to induce changes in the liver. For example, liver vitellogenin can be used as a biomarker for assessing endocrine disruption by estrogens for aquatic organisms. Van der Ven *et al.* (2003a) applied four histological methods to better measure the induction of vitellogenin after exposure of zebrafish (*Danio rerio*) to E2, and concluded that male vitellogenin production could be an indicator of exposure to compounds with (estrogenic) endocrine disruption activity. Islinger *et al.* (2003) studied the effects of 17α-ethinylestradiol (EE2) in liver zebrafish and demonstrated that the hepatocytes developed stacks of ER, accompanied with large Golgi fields with electron dense material. There were changes that indicated an enhanced hepatocellular activity. Arnold *et al*., (1996) exposed rainbow trout (*Oncorhynchus mykiss*) to endosulfan (a pesticide with endocrine disruption properties, by mimicking female steroids), showing that the compound induced pathological changes in mitochondria and peroxisomes of hepatocytes, and morphological alterations in their ER and Golgi complex. Estrogens were also implicated in different liver diseases, such as alcoholic injury (Eagon, 2010). In acute fatty liver of pregnancy, estrogen causes mitochondrial injury by decreasing β-oxidation of fatty acids, altering the function of mitochondrial proteins and enzymes, and causing ultrastructural damage; for instance mitochondrial enlargement (Grimbert *et al*., 1995). In another study, Enomoto *et al*., (1999) documented the increased sensitivity of Kupffer cells to endotoxin by very high doses of estriol (a weak estrogen that even so appears in high levels during pregnancy). Other interesting examples exist relating steroids and liver, such as the case of alcohol dehydrogenase (ADH) (the principal enzyme responsible for alcohol metabolism), that has been shown to be under the control of sex hormones (Colantoni *et al.*, 2000). Sex steroid hormones have been also implied in the modulation of reactive oxygen species (ROS) and to act as antioxidant or pro-oxidant agents according to their concentration and the system studied (Chao *et al.*, 1994; Colantoni *et al.*, 2000; Nathan and Chaudhuri, 1998).

Finally, within the context of this work it is important to stress that even the number and volume of hepatocytes can be modeled by sex-steroid hormones, particularly by estrogenic (and xenoestrogenic) action, as experimentally shown from fish (Korsgaard and Mommsen, 1993; Peute *et al*., 1985) to rat (Hornstein *et al*., 1992; Mayol *et al.*, 1992).

The Platyfish

The platyfish (*Xiphophorus maculatus*) is a tropical species, native from North and Central America, member of the Poecilidae family, order Cyprinodontiformes. We choose this fish herein because it has several characteristics advantageous for our aims. Platyfish is much less used than the currently prime small fish model, the zebrafish, despite it is bigger than the latter species, having a larger non-lobed liver that is very easy to dissect; with practical advantages when compared with other small fish models. It is thus possible to do conduct biochemical tests that are more difficulty when using zebrafish. Also, more parallel studies can be made, for instance morphological, molecular and biochemical evaluations, taking advantage of the generous liver size and ease of manipulation. The platyfish males and females are phenotipically distinct, so it is possible to divide groups of fish per sex with 100% guarantee, which is not so granted with zebrafish. Finally, platyfish is easy to maintain and breed. It is ovoviviparous, thus being an additional model in fish biology. The use of platyfish in hepatology and peroxisome biology is largely unexplored.

Objectives

Considering all the above, this work started addressing the following questions:

1. Does male and female platyfish differ as to qualitative and quantitative structural characteristics of their liver peroxisomes and hepatocytes? Do abiotic factors known to influence the gonad maturation, namely temperature, also impact on the size/number of liver peroxisomes and hepatocytes volume in this species?
2. Can estrogenic stimuli promote modifications in the quantitative morphology of the hepatocytic peroxisomes and in the volume of hepatocytes in the platyfish?

The two above baseline questions are sustained in two hypotheses:

1. At least in certain fishes there is a hormonal modeling, namely by sex steroids, of the morphology and function of both the hepatocytes and their peroxisomes;
2. Moreover, and again at least in certain fishes, estrogenic stimulation should be able to promote decreases in the volume of liver cells and their peroxisomes, while not necessarily reducing the number of such organelles per hepatocyte.

To respond to the above questions we made a first experiment tackling differences regarding peroxisomes and hepatocytes, which could suggest regulation by sex steroids, by comparing both sexes at a higher (28 ºC) *versus* lower (22 ºC) temperature. Later on we made a subacute assay exposing males to a model xenoestrogen (ethinylestradiol), and then studying the same kind of structural targets as in the first assay. The analyses were based on qualitative and stereological methods, at light and electron microscopy.

Materials and methods

Animals and Experiences

The platyfish used in both experiences were provided from our usual supplier, Aquários Virgínia (Porto, Portugal). Before the experience began, they were maintained in quarantine for two weeks. All fish were considered healthy.

Experiment 1 – Influences of Water, Temperature in Males and Females

For the first assay, forty fish were selected from a batch of eighty previously acclimated fishes. Twenty males and twenty females were put in four tanks, ten per tank. Each tank only had one sex, either male or female, totaling two male tanks and two female tanks. They were kept in the same conditions, animal density, photoperiod of 12h/12h and temperature of 26 ºC. After a period of fifteen days for acclimatization, the temperature of one tank with males and one with females was reduced (1ºC/day) and then maintained at 22 ºC, while in the other two tanks temperature was raised (1ºC/day) and then maintained at 28 ºC. Fish stayed in these stable conditions for fifteen days, with water quality being monitored each other day, for pH, ammonium and nitrites. Temperatures were measured and registered every day.

Experiment 2 – Exposure of Males to Ethynylestradiol

In the sub-acute exposure to a xenoestrogenic compound, it was used 30 male fish in two tanks. They were initially kept under equal conditions as the first experience, as to animal density, photoperiod of 12h/12h, and temperature of 26 ºC. After a fifteen day period of acclimatization, the temperature was reduced (1ºC/day) and then maintained at 22ºC. The first (randomly assigned) tank was exposed to EE2 at a nominal concentration of 75 μgL^{-1}. Fish were exposed in these conditions for twenty-one days. Water and EE2 concentrations were renewed daily. At same time, water quality was evaluated by pH, ammonium and nitrites levels monitoring. Temperatures were measured and registered every day.

Tissue Processing for Microscopy

Animals were first anaesthetized and killed by immersion in an overdose of phenoxyethanol, then weighed and finally measured for length. They were opened and the liver and gonads were removed and weighed. The liver was then cut in half, with one half being randomly picked and chopped again in half. One of the resulting pieces was randomly chosen and then sliced into very tiny pieces with the adequate size for catalase

cytochemistry detection at electron microscopy level. The liver fragments were fixed for 1 hour in a solution of 2.5% glutaraldehyde in 0.2M cacodylate buffer (pH 7.2). After fixation, the liver fragments were rinsed twice for 30 minutes in 0.1M cacodylate buffer (pH 7.2) and then rinsed again for 20 minutes in a 0.1M Tris-HCl (pH 8.5) solution. For catalase detection, pieces were incubated for 2 hours (at 37 ºC) in a medium containing 0.12% of H_2O_2 and 2mg mL^{-1} of DAB in 0.1M Tris-HCl buffer (pH 8.5) (Veenhuis and Bonga, 1979). After incubation, the pieces were washed twice in 0.1M Tris-HCl (pH 8.5) for 20 minutes and then in 0.1M cacodylate buffer (pH 7.2). A post-fixation in 0.1M cacodylate buffered 1% OsO_4 with 1.5% $K_3Fe(CN)_6$ was carried out for 2 hours. Then, the material was washed in 0.1M cacodylate buffer (pH 7.2) for 10 minutes. After dehydration in ethanol, the pieces were embedded in epoxy resin at 60 ºC for 2 days. The blocks were trimmed and cut with an ultramicrotome (Supermova, Leica Reichert). Various 1 µm thick semithin sections were stained with a mixture of 1:1 of 1% methylene blue and 1% azur II, for light microscopy observation. Unstained ultrathin sections were put into 300 mesh square copper grids and observed with a JEOL 100CXII transmission electron microscope, operated at 60 kV.

Stereological Methodology

In order to obtain the relative and absolute parameters, it was used the procedure applied by Rocha *et al*., 1999. For each fish, five randomly selected tissue blocks were analyzed. From each block, one grid was observed and photographed four times, using a simple random scheme; sequentially moving the microscope stage to select the four fields of view (disregarding those areas that were not hepatocytes). A total of approximately 20 images per animal were taken at a magnification of x 5.300. The photographs were further printed at a final magnification of x15,900. A total of 600 TEM fields were analyzed

The volume, surface and numerical densities of peroxisomes, considering the hepatocyte as the reference space, were the primary parameters to be estimated, being designated, respectively, as V_v, S_v and N_v (peroxisome, hepatocyte).

The volume densities were determined by using a classical manual stereological technique based on point counting (Weibel, 1979):

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

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 (hepatocytes) and *k* is the ration 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 nucleus.

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

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

In which (p ÷ l) is the ratio of test points to test lines length (according to magnification) for a particular grid; Σ I(s) is the sum of all intersection counts of test lines across boundaries of the structures (peroxisomes); Σ P(r) is the sum of all points falling over the reference space (hepatocytes). The same formula was used for a direct estimation of the surface-to-volume ration (S/V) of peroxisomes, but in which Σ P(r) referred instead to points falling over the peroxisomes themselves.

The numerical densities of peroxisomes were estimated by analyzing the same images used for point and intersection, according to the technique of Weibel and Gomez (Weibel, 1979):

$$N_v(\text{structure, reference space}) = \left(N_A^{3/2} + V_V^{1/2}\right) \times (K+\beta)$$

In which N_A is the number of structures (peroxisomes) per unit of reference space (hepatocytes), counted using a unbiased rectangular counting frame bearing forbidden lines (Gundersen, 1977); V_v is the volume density of peroxisomes within the cell; β is a shape coefficient (the value of 1.382 for spherical particles was adopted, this because the majority of peroxisomes was fairly roundish); K is a constant which depends on the size distribution of the objects. It was shown that K may be safely 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, hepatocyte) ÷ N_V (peroxisome, hepatocyte)

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

The spherical equivalent mean diameter $\bar{d}_{sphere}$ of a peroxisome was calculated using the classical formula: $\bar{v}_N$ (peroxisome) = $^1/_6 \times \pi \times \bar{d}_{sphere}$.

The nuclear volume ($V_{nucleus}$) was estimated using the nucleator method (Gundersen *et al.,* 1988), applied to semithin sections, where the nuclei of hepatocytes were sampled by the so-called “single-section” dissector (for which each nucleolus was considered a unique sampling particle). This sampling scheme is not strictly unbiased, but it can suffice for the sake of comparisons (Howard and Reed, 2004); for these, the method was consistently applied to the different groups being compared. It consisted in generating two isotropic orthogonally oriented lines from the nucleolus centre, and the distances from the center to the nuclear envelope were recorded along the lines. From a series of these measurements, the mean nuclear volume (μm^3) was estimated from the following formula:

$$V_{nucleus} = \frac{4\pi}{3} l_n^{-3}$$

In the formula, l_n refers to the distances from the sampling point (nucleolus centre) to the edge of the particle of interest (nucleus). Two stained semithin tissue sections where sampled per slide. All cells with an evident nucleolus were sampled and all estimating procedures were carried out under oil immersion (x100 objective lens) with a microscope (Olympus, BX50), equipped with a motorized stage (Prior) for stepwise displacements in *x-y* directions and with a CCD camera (Sony) connected to a PC monitor. The whole system was controlled by CAST-Grid software version 1.5 (Olympus Denmark A/S, Denmark).

To estimate the cell (hepatocyte) volume, the formula used was:

$$V_{cell} = V_{nucleus} \div V_v(\text{nucleus, cell})$$

After obtaining the cell volume it was then possible to estimate the mean total peroxisome volume, total peroxisome number and total peroxisome surface within the hepatocyte. The formulas used to these calculations were:

Total peroxisome volume: $V(\text{peroxisome}) = V_v(\text{per, cell}) \times V_{cell}$

Total peroxisome number: $N(\text{peroxisome}) = N_v(\text{per, cell}) \times V_{cell}$

Total peroxisome surface: $S(\text{peroxisome}) = S_v(\text{per, cell}) \times V_{cell}$

Statistical Analysis

The Statistica 10.0 for Windows was used to analyze the data. A two-way ANOVA was applied to test the effects of gender, temperature and gender *vs* temperature interaction, for each parameter, after checking the assumptions of normality and homogeneity of variances. Tuckey and Newman-Keuls post-hoc tests for multiple

comparisons between means were further applied. Since there was not a significant difference, the data was grouped in gender and then applied a one-way ANOVA. By default, differences were considered significant when $p \leq 0.05$. Data transformation was made in some cases to warrant normality and homogeneity. Non-parametric tests (Kruskal-Wallis ANOVA, followed by Mann-Whitney U tests) were also applied when data transformation failed to grant homogeneity of the data sets; it was verified that the results corroborated the statistical significances obtained with the parametric approach.

Results

Experiment 1 – Morphometric Data of the Animals

In Table 2 we give the length, weights and the liver somatic and gonado-somatic indexes (LSI and GSI, respectively) of animals from the temperature assays. The high temperature males and females are those that were held at 28 ºC. The low temperature males and females are the fishes that were tested at 22 ºC.

Table 2 - Morphometric data from the platyfish males and females from the first experiment. Results are shown as mean ± standard deviation.

	Lenght (cm)		Weight (g)		Index (%)	
	Total	**Fish**	**Liver**	**Gonad**	**LSI**	**GSI**
High Temp. Males	4.2 ± 0.2	1.2 ± 0.3	0.025 ± 0.009	0.016 ± 0.003	2.0 ± 0.4	1.4 ± 0.4
High Temp. Females	4.1 ± 0.1	1.4 ± 0.2	0.037 ± 0.008	0.080 ± 0.082	2.7 ± 0.5	5.8 ± 5.6
Low Temp. Males	4.2 ± 0.2	1.2 ± 0.2	0.024 ± 0.006	0.016 ± 0.007	1.9 ± 0.4	1.2 ± 0.5
Low Temp. Females	4.1 ± 0.3	1.4 ± 0.3	0.033 ± 0.006	0.055 ± 0.082	2.5 ± 0.8	3.7 ± 5.5

When comparing all morphometric data separately, *i.e*., considering all four groups in the Table 2, there were no significant differences between groups. However, by grouping the data into males and females, and then comparing both sexes, significant differences appeared in two parameters: total liver weight and LSI. There was thus a sex effect, as shown in Figures 1 and 2, with the females displaying a bigger liver weight and a greater LSI than males. Finally, despite two of the females from the 28 ºC group were found to be pregnant, this did not significantly increased the mean gonad weight and GSI.

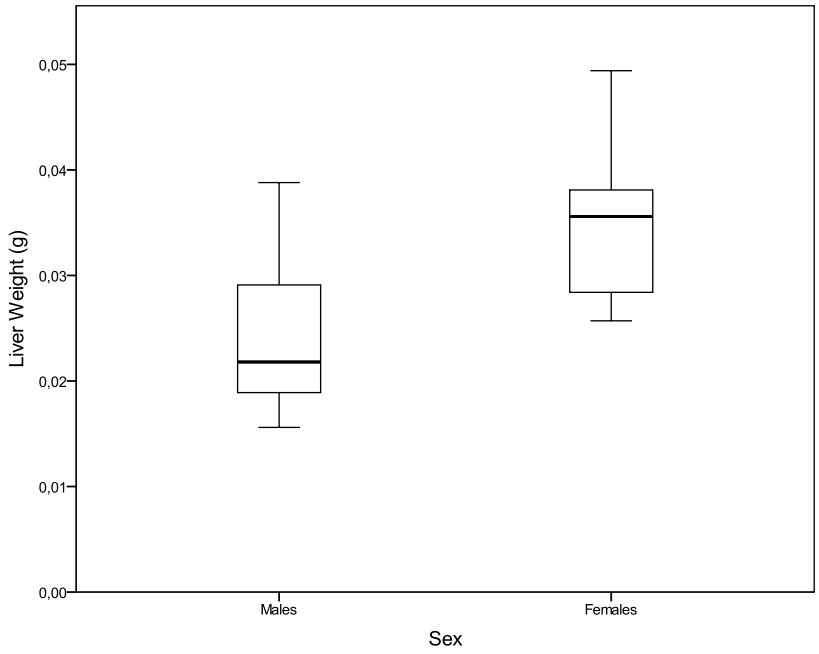

Figure 1- Liver weights (g) of platyfish males and females (high and low temperature grouped). Box-plot with minimum, first quartile, median, third quartile and maximum. Groups differed statistically (*p*<0.05).

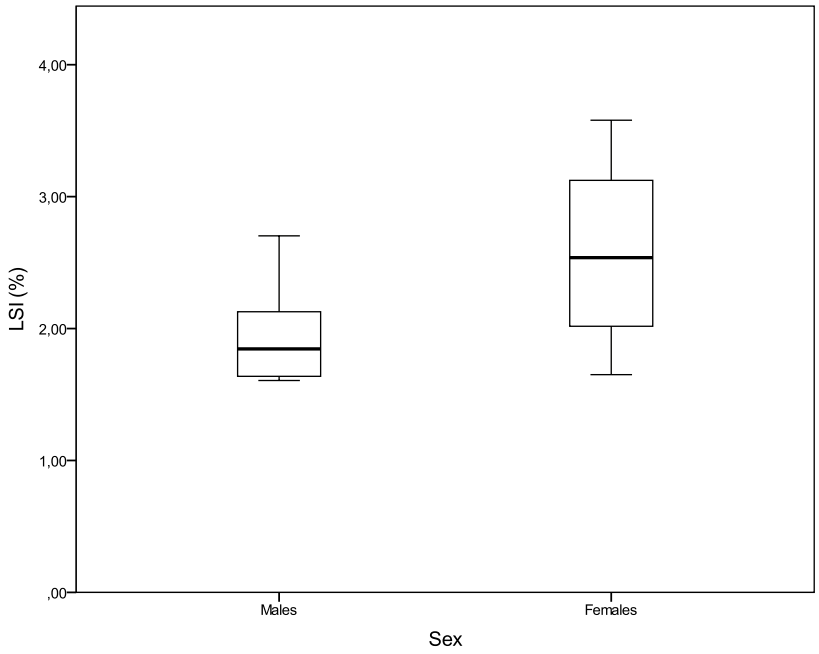

Figure 2 - LSI (%) of platyfish males and females (high and low temperature grouped). Box-plot graphic with minimum, first quartile, median, third quartile and maximum. Groups differed statistically (*p*<0.05).

Experiment 1 – Ultrastructural Aspects of Peroxisomes and Hepatocytes

With catalase cytochemistry, DAB interacts with peroxidase reaction and form a dense product that allow us to more easily identify peroxisomes as they are stained. Due to their proteinaceous matrix, they appear with very dense granular content (Figure 3). As expected for fish hepatocytes, peroxisomes did not have a crystalloid core. In general they were round (Figure 3B), but sometimes they presented a more oval form (Figure 3A).

There were clear differences between male and female hepatocytes, at the two temperatures. As shown in Figures 4 to 7, male livers have huge lipid drops and females do not. Also, female hepatocytes tend to have a more developed rough ER than males.

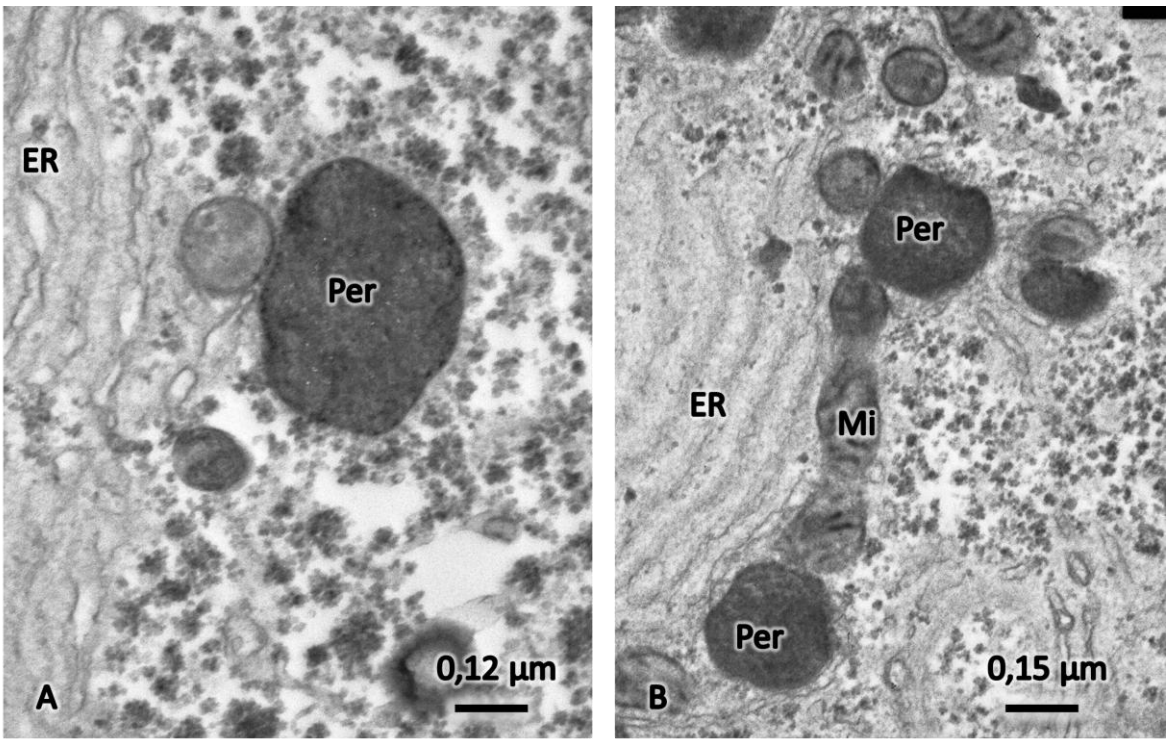

Figure 3 - Peroxisome in detail, from a low temperature female hepatocyte. A) Note the dark stained peroxisome (Per) B) More roundish Per then in A). Mitochondria (Mi), Endoplasmic Reticulum (ER).

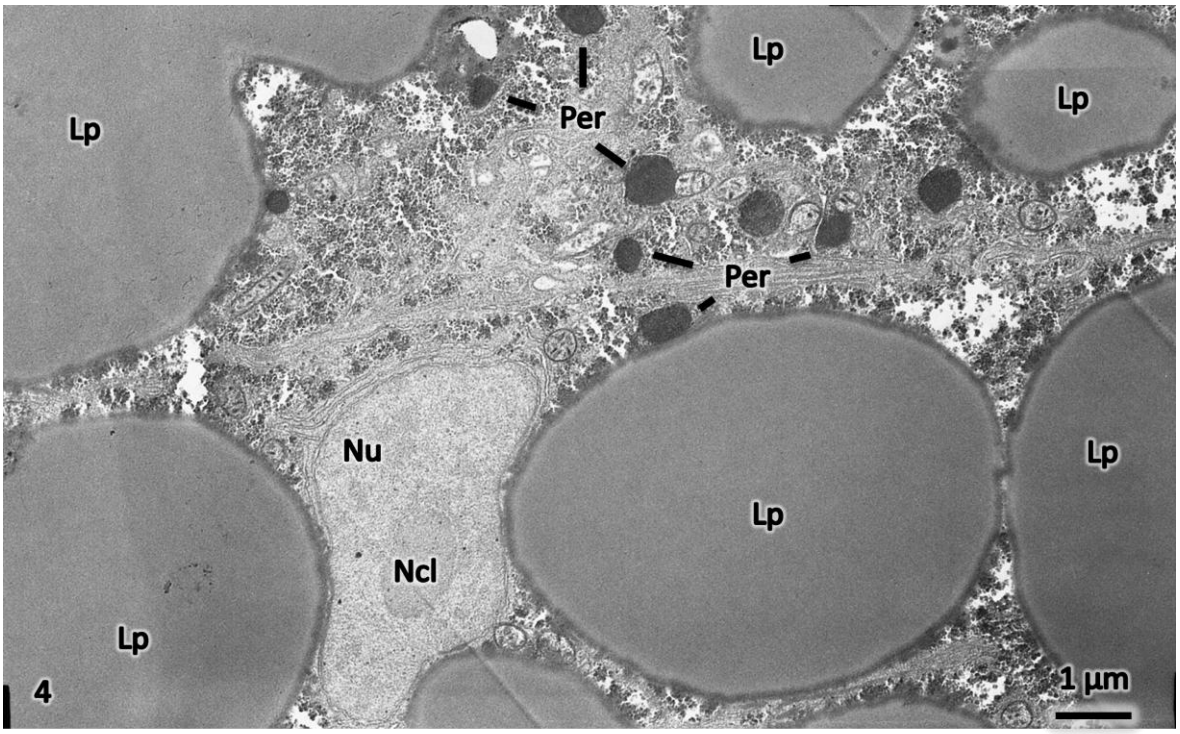

Figure 4 - High temperature male hepatocyte. Nucleolus (Ncl), Nucleus (Nu), Peroxisome (Per), Lipid droplets (Lp).

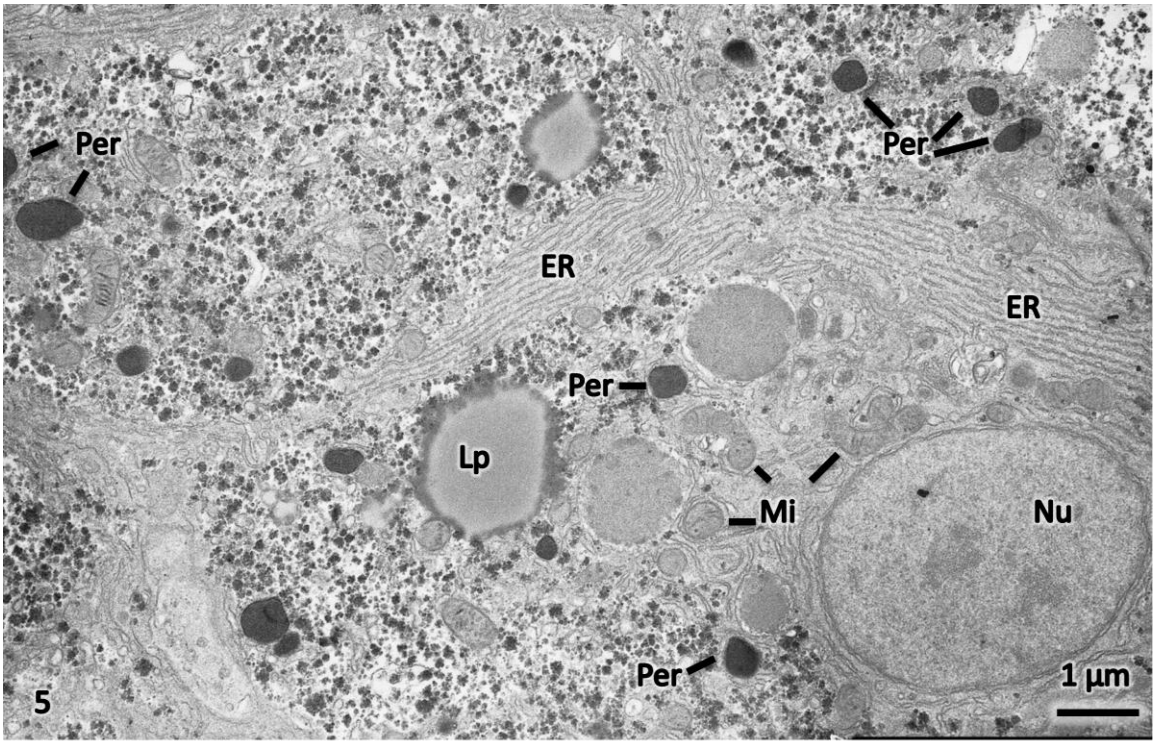

Figure 5 - High temperature female hepatocyte. Nucleus (Nu), Mitochondria (Mi), Endoplasmic Reticulum (ER), Peroxisome (Per), Lipid droplets (Lp).

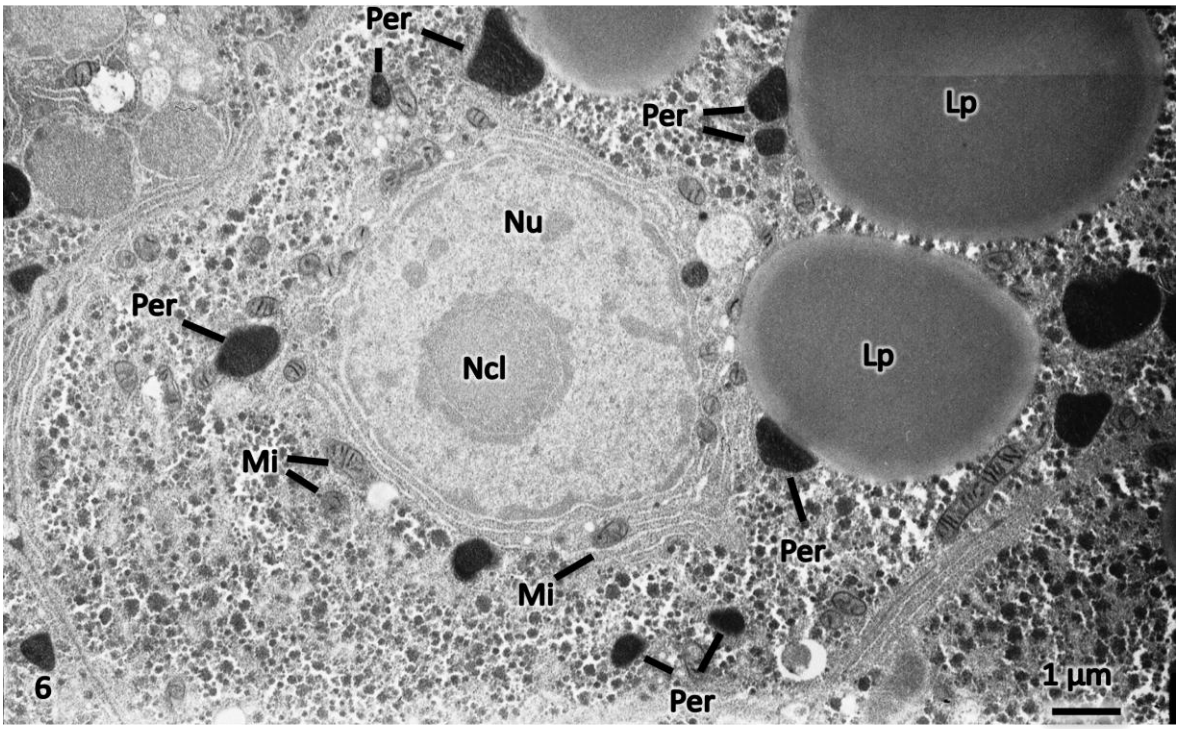

Figure 6 - Low temperature male hepatocyte. Nucleolus (Ncl), Nucleus (Nu), Mitochondria (Mi), Peroxisome (Per), Lipid droplets (Lp).

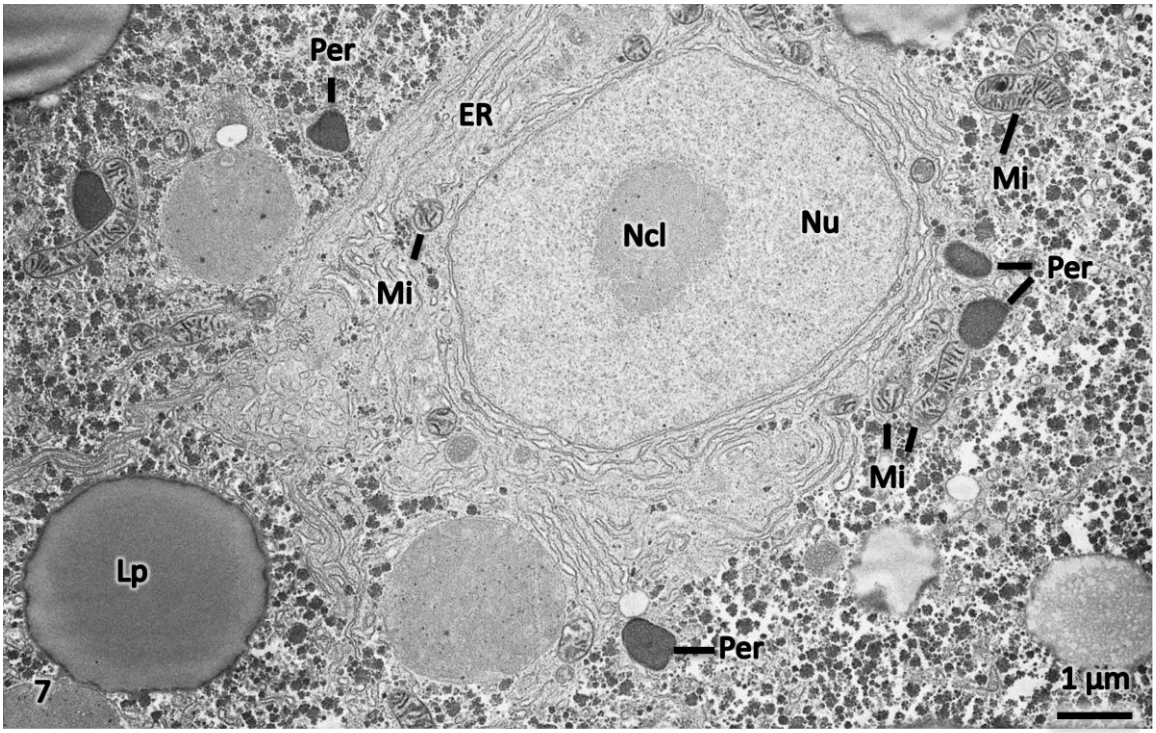

Figure 7 - Low temperature female hepatocyte. Nucleolus (Ncl), Nucleus (Nu), Mitochondria (Mi), Endoplasmic Reticulum (ER), Peroxisome (Per), Lipid droplets (Lp).

Experiment 1 – Stereological Parameters of Hepatocytes

The nuclear and cell volume of the hepatocytes are given in the Table 3. After comparing the four groups from Table 3 separately, there were no significant differences between groups. However, the two-way ANOVA detected a significant sex effect. So, after grouping the males and females and comparing both sexes it appeared a significant difference in the hepatocyte volume (V_{cell}) as shown in Figure 3.

Table 3 - Nuclear and cell volumes of hepatocytes, from platyfish males and females held at low (22 ºC) and high (28 ºC) temperatures. Results are show as means± standard deviation.

	$V_{nucleus}$ (μm^3)	V_{cell} (μm^3)	V_V (Nu,Cell) (μm^3)
High Temp. Males	38.2 ± 2.6	1380 ± 523	0.031 ± 0.013
High Temp. Females	37.2 ± 3.1	798 ± 417	0.056 ± 0.024
Low Temp. Males	36.1 ± 6.3	2296 ± 1645	0.024 ± 0.015
Low Temp. Females	46.7 ± 6.3	1047 ± 417	0.053 ± 0.029

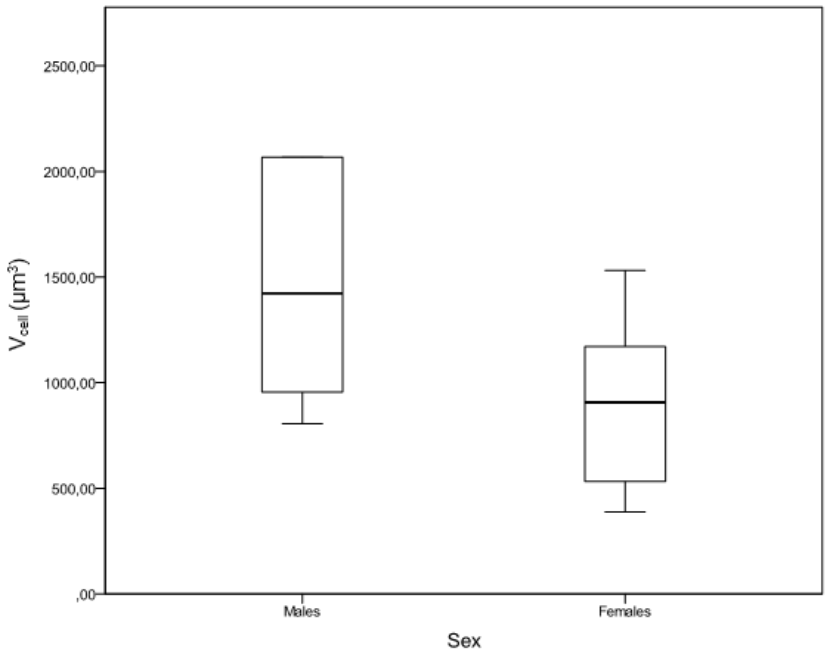

Figure 8 – Hepatocyte volume (μm^3) of platyfish males and females (high and low temperature animals grouped). Box-plot graphic with minimum, first quartile, median, third quartile and maximum. Groups differed statistically ($p<0.05$).

Experiment 1 – Stereological Parameters of Peroxisomes

The Table 4 shows data for three relative stereological parameters of peroxisomes. There were no statistically significant differences between the experimental groups. Also,

and contrary to cell volume (see above), there was no significant effect of sex under ANOVA, and so data were not further considered for statistics applied to fish grouped only per sex.

Table 4 - Peroxisomes volumes and surface, from platyfish males and females held at low (22 ºC) and high (28 ºC) temperatures. Results are show as means ± standard deviation.

	N_V (Per,Cel) (No./µm^3)	V_V (Per,Cel) (µm^3)	S_V (Per,Cel) (µm^{-1})
High Temp. Males	0.054 ± 0.008	0.013 ± 0.002	0.088 ± 0.015
High Temp. Females	0.052 ± 0.012	0.014 ± 0.004	0.092 ± 0.028
Low Temp. Males	0.064 ± 0.010	0.018 ± 0.005	0.127 ± 0.003
Low Temp. Females	0.049 ± 0.021	0.012 ± 0.003	0.084 ± 0.019

Table 5 displays data for three absolute stereological parameters of peroxisomes. Considering groups separated by both sex and temperature, the two-way ANOVA was not significant as to the interaction of the two tested factors, but highlighted a significant effect of sex. As such, further post-hoc analysis was done by grouping the fish only per sex, and thus joining the two temperatures. Globally, males and females were shown to differ significantly (p<0.05) as to the total peroxisome number, volume and surface per cell, with males consistently showing greater values that females (Figures 9 to 12).

Table 5 - Total peroxisome volume, number and surface, from platyfish males and females held at low (22 ºC) and high (28 ºC) temperatures. Results are show as means ± standard deviation.

	V (peroxisome) (µm^3)	N (peroxisome) (No.)	S (peroxisome) (µm^2)
High Temp. Males	18.3 ± 9.1	73.1 ± 23.6	124.8 ± 59.9
High Temp. Females	11.2 ± 6.3	44.4 ± 32.5	74.5 ± 40.7
Low Temp. Males	41.0 ± 30.2	136.6 ± 82.9	274.7 ± 195.7
Low Temp. Females	12.9 ± 5.6	53.0 ± 31.4	91.2 ± 40.3

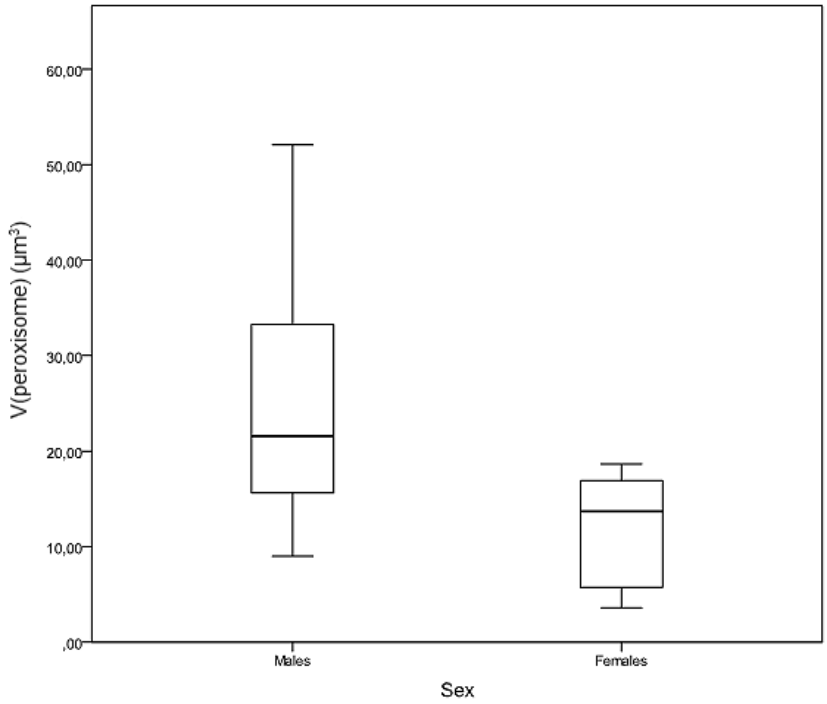

Figure 9 - Total peroxisome volume in the cell (µm^3), in males and females platyfish. Box-plot with minimum, first quartile, median, third quartile and maximum. Groups differed statistically (*p*<0.05).

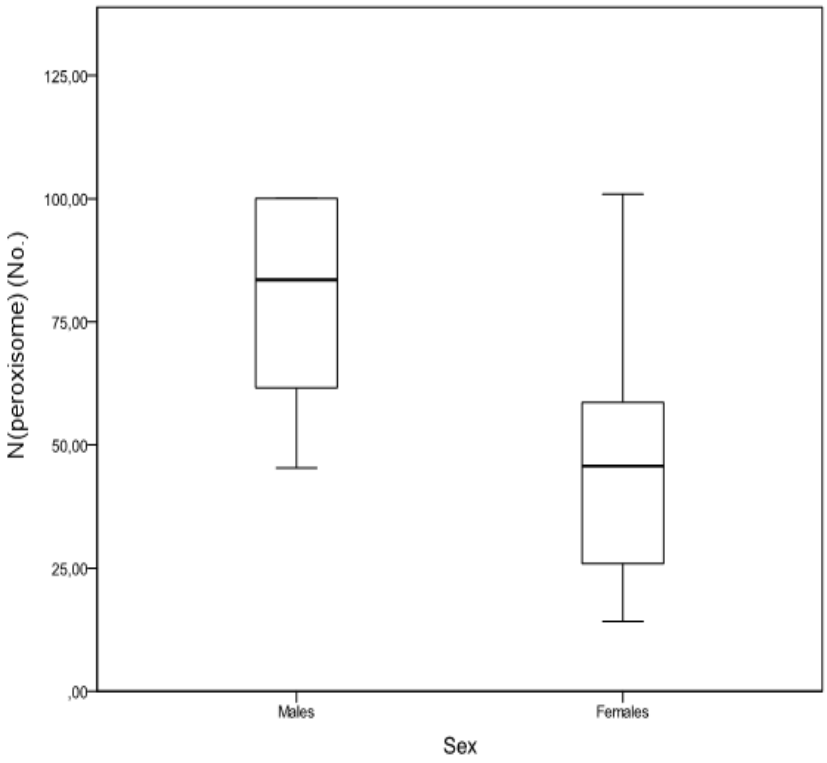

Figure 10 - Total peroxisome number in the cell (No.) in males and females platyfish. Box plot with minimum, first quartile, median, third quartile and maximum. Groups differed statistically (*p*<0.05).

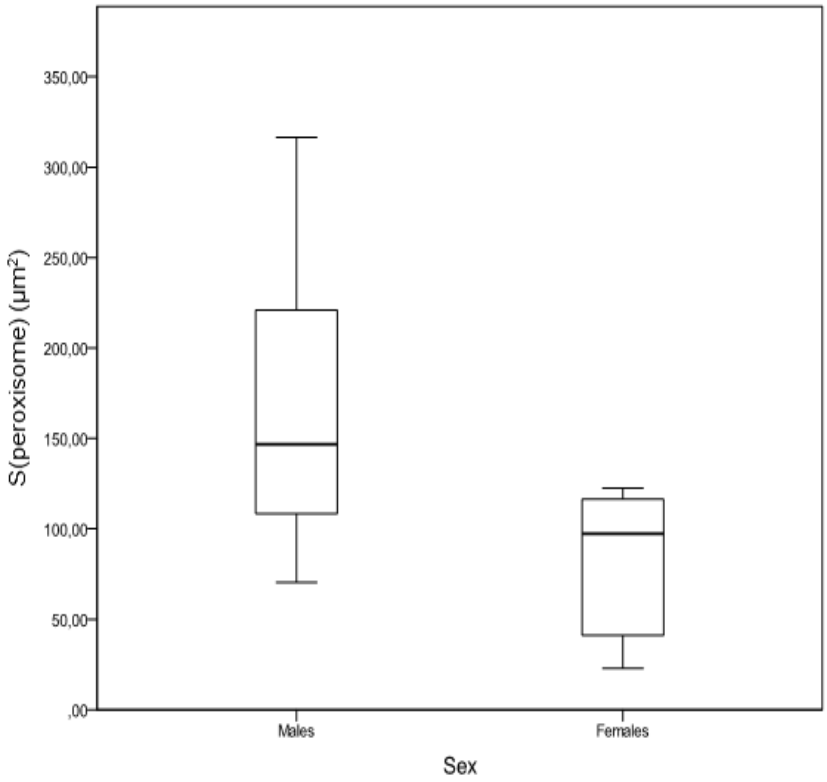

Figure 11 - Total peroxisome surface in the cell (µm^2) in males and females platyfish. Box plot with minimum, first quartile, median, third quartile and maximum. Groups differed statistically (p<0.05).

Experiment 2 – Morphometric Data of the Animals

Table 6 displays the length, weights and the liver- and gonad-somatic indexes for control *vs* fish under estrogen stimulation. Statistics did not reveal significant differences.

Table 6 - Morphometric data from the platyfish control male group and exposed males to EE2 from the second experiment. Results are shown as mean ± standard deviation.

	Lenght (cm)		Weight (g)		Index (%)	
	Total	Fish	Liver	Gonad	LSI	GSI
Control	3.9 ± 0.4	1.2 ± 0.1	0.025 ± 0.008	0.012 ± 0.003	2.2 ± 0.6	1.1 ± 0.3
Exposed	4.5 ± 0.3	1.6 ± 0.4	0.030 ± 0.006	0.015 ± 0.005	2.0 ± 0.2	1.1 ± 0.2

Experiment 2 – Ultrastructural Aspects of Peroxisomes and Hepatocytes

The findings in the control group (Figure 12) were consistent with the ones from the males in Experiment 1. However, when observed the images taken from the males that were exposed to EE2, there were two visible differences (Figure 13): 1) the absence of huge lipid droplets; and 2) the presence of a highly developed ER. These properties were more alike those displayed by female hepatocytes.

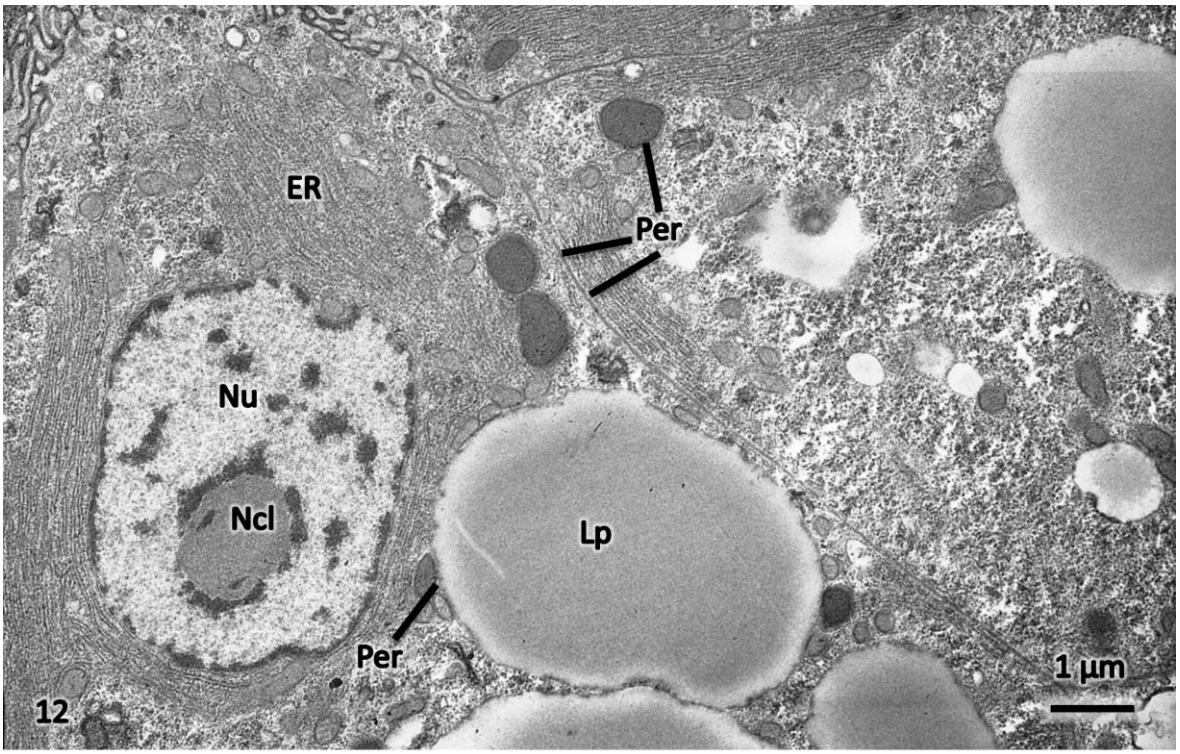

Figure 12 - Control male hepatocyte. Nucleolus (Ncl), Nucleus (Nu), Peroxisome (Per), Lipid (Lp).

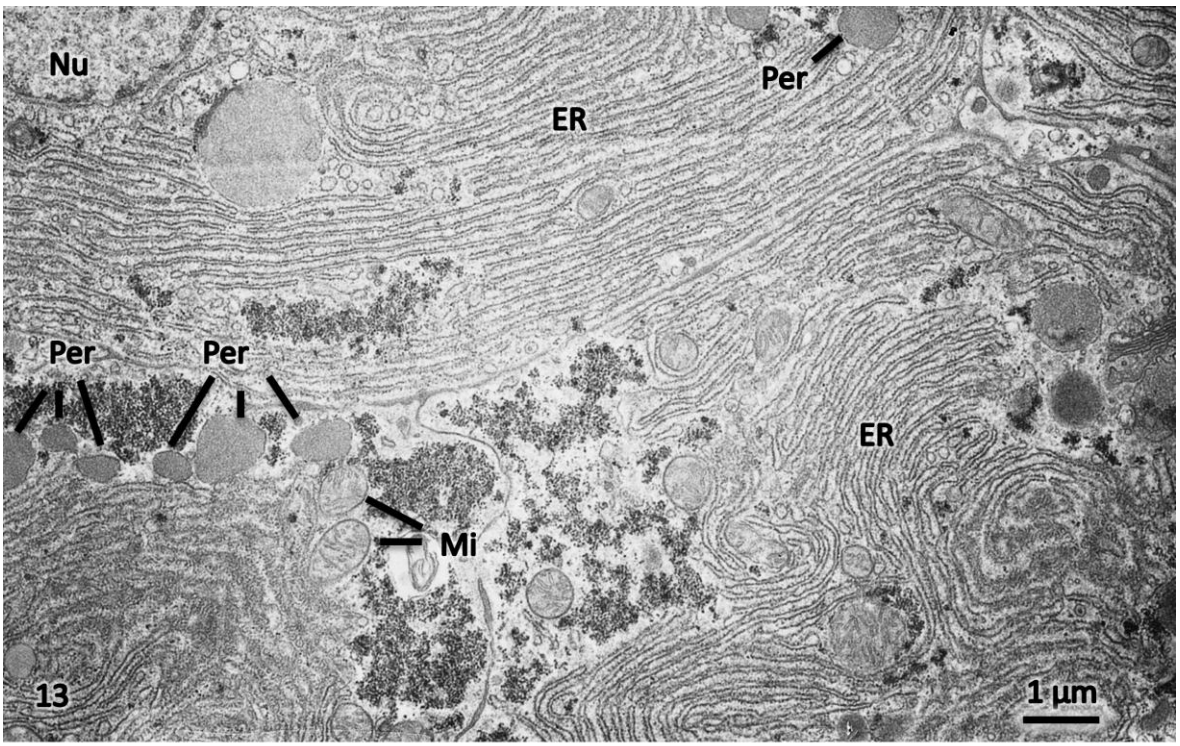

Figure 13 - Exposed male hepatocyte. Nucleus (Nu), Mitochondria (Mi), Endoplasmic Reticulum (ER), Peroxisome (Per).

Experiment 2 – Stereological Parameters of Hepatocytes

Although there were some differences in the ultrastructure of hepatocytes, in the stereological parameters depicted in Table 7 there were not — no significant differences were found.

Table 7 - Nuclear (relative and absolute) and cell volumes of hepatocytes, from platyfish control male group and males exposed to EE2. Results are show as mean ± standard deviation.

	$V_{nucleus}$ (μm^3)	V_{cell} (μm^3)	V_V (Nu,Cell) (μm^3)
Control	43.1 ± 5.1	1189 ± 619	0.042 ± 0.015
Exposed	37.3 ± 3.9	566 ± 120	0.070 ± 0.025

Experiment 2 – Stereological Parameters of Peroxisomes

There were also no significant differences in the stereological parameters of peroxisomes from Tables 8 and 9.

Table 8 - Relative number, volume and surface of peroxisomes, from platyfish control male group and males exposed to EE2. Results are show as mean ± standard deviation.

	N_V (Per,Cel) (No./μm^3)	V_V (Per,Cel) (μm^3)	S_V (Per,Cel) (μm^{-1})
Control	0.028 ± 0.010	0.005 ± 0.003	0.054 ± 0.023
Exposed	0.029 ± 0.009	0.006 ± 0.001	0.050 ± 0.014

Table 9 - Total peroxisome volume, number and surface, from platyfish control male group and males exposed to EE2. Results are show as mean ± standard deviation.

	V (peroxisome) (μm^3)	N (peroxisome) (No.)	S (peroxisome) (μm^2)
Control	6.6 ± 4.7	37.3 ± 33.0	67.2 ± 54.2
Exposed	3.6 ± 1.3	16.9 ± 7.6	28.9 ± 12.8

Discussion

Rocha *et al.* (1999) described for the first time morphologic quantitative alterations in brown trout liver peroxisomes during the annual breeding cycle. In both genders, the dimensions of peroxisomes and their total volume per hepatocyte changed. In February and September female peroxisomes were smaller, but in May, both sexes were virtually identical. The number of peroxisomes per cell never varied. Significant correlations were made between peroxisome dimensions and the maturation status of the gonads. These facts launched the hypothesis that peroxisomes could be influenced by sex steroids, like it was the case of other structural and functional compartments of the teleost hepatocyte. It was also known at the time that that natural factors such as, water salinity, temperature, season and feeding habits could exert changes in both peroxisomes and hepatocytes in fishes (Rocha *et al*., 1999). In this context, our first approach was to study if there were any baseline morphologic differences between sexes, evaluated at two temperatures, as to peroxisomes, while looking also at aspects of their containing cells, *i.e.*, hepatocytes.

When analyzing our data sets divided by sex and temperature we could not prove significant differences. However, in several occasions the ANOVA highlighted a significant effect of the sex of the fish. So, within scope of the tested temperatures, this single factor did not seem crucial per se or when interacting with sex for influencing the diverse targets we looked at. However, facing the relatively high interanimal variability in the generality of the parameters, and the fact that for this work we analyzed so far only 5 fish per group, we cannot rule out the possibility that a greater sample could provide statistical significance for certain parameters. This idea is reinforced by the fact that many statistically significant differences could be revealed under the scope of two-way ANOVA only when grouping the animals per sex, and consequently raising the “n”. Anyway, facing the amount of work involved and the time to conduct this dissertation, it would be impossible to conduct more assays or add and study more individuals per group in the scope of the present analysis. With this in mind we will discuss to the point below the aspects deserving interpretation.

Experiment 1

Despite the focus of this work was the cell and organelle level it was very important to have in mind eventual differences in the gross morphometric parameters. In this vein, it was important to register that our males and females did not differ in either length or weight. However, and overall, females had heavier livers and showed a higher LSI than males. This was not really an unexpected finding, as such kind of difference have been noted before, both in seasonal breeding species, like the brown trout (Rocha *et al.,* 1999), or in regular spawners, like the zebrafish (Madureira *et al*., 2012). Anyway, our results on

the LSI pointed to the presence of baseline structural and functional differences between sexes in the tested conditions. Typically, under normal conditions a greater liver mass in fish females is associated with a need to produce proteins, such as eggshell elements and particularly the vitellogenins, which are hepatic precursors of egg-yolk proteins, and so to be integrated in the growing oocytes (Arukwe and Goksøyr, 2003; Matozzo *et al*., 2008).

The hepatocellular mechanisms underlying liver weight differences in fish are still poorly studied. In theory, the inter sex differences we saw could be explained by females having either a higher number of hepatocytes and/or bigger ones. According to the current theories derived from quantitative studies conducted in salmonids, the females have increased liver masses at the cost of having more hepatocytes, smaller than usual at the peak of the endogenous estrogenic stimuli (Hampton *et al*., 1989; Rocha *et al*., 2009). Despite platyfish is not an annual seasonal spawner like trouts, our results are consistent with what is described in the cited literature, as our females had smaller hepatocytes than those of males. So, and despite we did not measured the total hepatocyte number, it is legitimate to propose that the higher liver weight and LSI in females of platyfish was due to a greater number of hepatocytes; this likely connected with the continuous ovary needs.

In accordance with the above, herein female hepatocytes typically displayed a more developed rough ER than males. This backs the notion that platyfish females should have a higher baseline rate of hepatic protein production for export to the ovary, at least within a range of temperatures compatible with breeding, as the two we tested. Again, it seems that despite this platyfish has an asynchronous ovarian maturation, it seems that under normal circumstances the platyfish females ultrastructure of hepatocytes resembles that of vitellogenic females of seasonal breeders, with synchronous or group-synchronous ovary maturation patterns. This was well characterized in brown trout (Rocha *et al*., 2010).

Despite the above discussed global differences between males and females, and contrary to our expectations, there were no significant differences as to the quantitative morphology of the peroxisomes; this when looking at their individual size and also relative contents (number, volume, surface) within the hepatocyte. Indeed, we had hypothesized that it would be possible that female peroxisomes would be smaller than those of males. This idea is connected with a broader hypothesis under testing nowadays, according to which smaller peroxisomes (with a decreased activity) in females would favor not fatty acid oxidation in liver, but the use of long chain fatty acids for producing the lipoproteins the ovary needs to mature oocytes (Batista-Pinto *et al*., 2009; Castro *et al*., 2009; Rocha *et al.,* 1999). At this point, we can infer that in platyfish, male and female hepatocytes

have similarly sized peroxisomes, which have equal relative volume, surface and number within the hepatocyte. However, the scenario changes when we analyzed total amounts.

In fact, a significant sex effect in ANOVA revealed that the male sample population had bigger hepatocytes. So, and as the relative quantities of peroxisomes per cell were similar between sexes, in the end hepatocytes in males had significantly greater quantities of peroxisomes, in relation to their total volume, surface and also number. This latter situation is similar to that found in brown trout, in advanced gonadal maturation (Rocha *et al.,* 1999). The findings from the platyfish and brown trout models suggest that fishes with quite different reproduction strategies and underlying ovary maturation events do not have exactly similar structural dynamics as to intersex specificity of the liver peroxisomes. The intersex differences in the platyfish seem to be governed primarily by the hepatocyte size, rather than by also the size of organelles, as saw in the brown trout. Under the hypothesis of an endocrine (mainly estrogenic) control of the peroxisome size, maybe this different scenario among dissimilar species is due to the fact that whereas female salmonids (and those of other annual breeders) have huge seasonal changes in the levels of plasma estradiol (Pankhurst, 2008; Jordanova *et al*., 2011), platyfish and other asynchronous fish do not, additionally having much lower plasmatic levels of sex-steroids Pankhurst, (2008). Also, if we think that only part of the oocytes of an asynchronous ovary are in vitellogenesis, maybe there is no physiological need in platyfish females for an individual reduction of the peroxisome size as part of a shift in the use of long chain fatty acids (as we cited above, proposed for trout). Definitive conclusions can only be derived from comparative studies, including experimental ones, like testing estrogenic influences in diverse settings.

Experiment 2

This assay was based on the exposure of male platyfish to EE2 at 75 μgL^{-1}. The concentration used in this first trial is close to the 96 μgL^{-1} dose of E2 previously tested in platyfish (Kinnberg *et al*., 2000), higher than the dosage of E2 (50 μgL^{-1}) that was able to decrease the size of liver peroxisomes in the brown trout model (Rocha *et al*., 2003), and much higher than levels proved to induce estrogenic effects in small fish models, like for instance the 10 μgL^{-1} of E2 and of EE2 that evoked impacts in zebrafish liver, including changes in peroxisome markers enzymes (Ortiz-Zarragoitia and Cajaraville, 2005).

In this experiment we did not found significant changes regarding the quantitative parameters measured. However, we noted ultrastructural changes in the fish exposed to the estrogenic stimulus, namely a greater development of the rough ER. This contrasts in certain extent with our expectation, at least regarding some parameters. For example, Kinnberg *et al*., (2000) demonstrated that a 96 μgL^{-1} dosage of E2 for 28 days significantly

reduced the GSI of male platyfish while inducting vitellogenin production. Facing this result we could think that under our test conditions we could get a comparable result, in view of that EE2 is more or less similarly potent compared with E2; and even can be more potent in experimentally inducing vitellogenin production (Thorpe *et al*., 2003) . Anyway, effects of estrogenic stimuli in male fish GSI are not as linear as it would seem. For instance, in another experiment, Kristensen *et al*., (2005) exposed males of guppies (*Poecilia reticulata* – a viviparous species much like the platyfish) to 10, 50, and 200 ngL^{-1} of EE2, for 108 days, which was enough to induce a number of effects, but only altering (increasing) the GSI at the lowest concentration; there was a decreasing trend disclosed for the other doses. As to data from other fish models, for example the GSI of males of zebrafish exposed for 3 weeks to 10 or 25 ngL^{-1} EE2 was significantly reduced (Belt *et al*., 2001). Considering these and other examples in the literature, it seems that depending on the model, amount and type of estrogenic compound, and also exposure conditions, impacts on the male GSI are not always visible or change in the same way.

The above discussion on variability of responses can be applied to the fact that the platyfish LSI did not change with the exposure to EE2. It was shown in male rainbow trout (*Oncorhynchus mykiss*) that 62 days of exposure to 100 ngL^{-1} (but not when exposed to 10 ngL^{-1}) induced an increase in the LSI, compared with those of the controls (Schultz *et al*., 2003). In the same vein, male medaka (*Oryzias latipes*) exposed for 7 days to 5, 50, or 500 ngL^{-1} of EE2 resulted in statistically greater LSI. In opposite, the LSI was lower in EE2-exposed males of sand gobies (*Pomatoschistus minutes*), using a dose of only 11 ng L^{-1}, for 1 to 4 weeks (Saaristo *et al*., 2010). Other examples could be cited, most pointing towards an increase of LSI with estrogenic stimulation, but other revealing no changes or decreases. In our case, the EE2 as experimented did not evoke any liver hypertrophy or the contrary,

We could hypothesize that the negative results in our experiment with EE2 could be derived from an experimental flaw that would impair (or not favor) a physiological action of the chemical. Eventually, the real concentration of EE2 in water and/or time of exposure could have been not enough to really trigger estrogenic effects. However, the fact is that the hepatocytes of males exposed to EE2 showed signs of “feminization”, *ie*, they doubtless revealed more richness in rough ER than the control fish, and much looked like the hepatocytes of females from the Experiment 1. This type of liver “feminization” under estrogenic exposure is often described in literature, and denotes that male hepatocytes started vitellogenesis (van der Ven *et al*., 2003b). We cannot prove at this time that this was the case in our study, but this ultrastructural fact is strong evidence that our experiment evoked at least some expected change in the liver of exposed males.

Further evidence that the EE2 stimulus exerted some effect in platyfish males was the relative paucity of hepatocytes in lipid droplets when compared to control fish. For example, Pawlowski *et al*., (2004) exposed fathead minnow (*Pimephales promelas*) males for 3 weeks to various concentrations of EE2 (from 0.1 to 100 ngL^{-1}), seeing a decrease in the GSI at 10 and 100 ngL^{-1}. Also, exposed fathead minnow males reduced the amount of lipids and glycogen in the liver, especially at higher exposure concentrations.

Final evidence suggesting that there was some level of estrogenic stimulation and liver cell impacts is the fact that, despite no statistical significance could be proved, when comparing the volume of hepatocytes between experimental groups, the mean liver cell volume in exposed platyfish was merely 48% that of control animals, and the number of peroxisomes was only 45%. These reductions did not have statistical significance but they may have some biological meaning, and are worth further exploring in experimental terms. We wonder if the assay did not last time enough to model the targets of our concern.

Despite the above, we concluded that under our assay settings EE2 did not evoke statistical significant changes in the mean volume of liver cells or in targeted stereological parameters of peroxisomes. So, at least in this fish model, the dynamics of the liver cell and of its peroxisomes do not seem to be so sensitive to modeling by estrogenic stimuli. Anyway, for a definitive conclusion it would be useful to measure the real concentration in the water that the EE2 attained in our study (samples were taken for posterior analysis). Also, considering that in the work of Kinnberg *et al*., (2000) estrogenic stimulation for about a month triggered significant changes in the GSI of male platyfish (necessarily involving a more profound hepatic remodeling), we think it would be worth a longer assay (eventually testing more dosages) than the one conducted in the first place. In terms of environmental relevancy, dosages in the range that we tested may be not so interesting, but that is not the case with the aim of understanding mechanisms and establishing new modeling ways.

Conclusion

From this study we concluded that males and females of platyfish did not vary as to the qualitative and quantitative structure of their hepatocytic peroxisomes, namely as to the mean volume, surface and spherical-equivalent diameter of the organelle, and as to its relative volume, surface and number in relation to the hepatocyte. However, we concluded that liver cells of males had more peroxisomes and greater total volume and total surface of such organelles than those of females. The latter facts were due to a lower mean volume of the female hepatocytes. Females had greater liver weight and also liver somatic index; indicating that they contained a greater number of hepatocytes per liver than males. Such differences could be revealed under a two-way ANOVA that detected a significant effect of sex, irrespective of two tested temperatures (22ºC and 28ºC). Exposure to 75 μL^{-1} of EE2 for twenty one days did not induce significant changes both in the liver weight, hepatocytes volume and also in relative and absolute numbers, volumes and surfaces of peroxisomes. The ultrastructural study on hepatocytes revealed natural differences between males and females, at both temperatures, with the latter sex being richer in rough ER. Also, males under EE2 exposure had hepatocytes ultrastructuraly alike to those of normal females.

The collected facts make us suggest that platyfish male and female hepatocytic peroxisomes did not seem to differ as to their qualitative aspects and quantitative structural characteristics, like individual size and relative quantity per hepatocyte. Temperature did not seem to have a major influence as to the structure of peroxisomes in platyfish. Also, and at least under our subacute exposure regime, a significant estrogenic stimulus did not seem to be able to induce structural changes in liver cell peroxisomes, not supporting the notion that peroxisome regulation by estrogens as seen in brown trout exists in all fishes.

Finally, our hypothesis that hepatocytes of platyfish males and females could have baseline quantitative differences is most likely true, as males had, on the whole, bigger liver cells (and with more voluminous nuclei). Also, the latter were richer in rough ER, which indirectly (but nevertheless strongly) supports that estrogenic hormones may have a functional role in governing the hepatocyte differences between sexes. Because of such regulation, the total number of peroxisomes per cell differed between sexes, with females having a smaller total peroxisomal load per hepatocyte. Consequently, taking into account that these cells can be subjected to hormonal influences, including by estrogenic actions, the total peroxisome amount in hepatocytes (and hence in the all liver) may vary between sexes. This issue can be much further explored in the future, including in functional terms.

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