Histol Histopathol (2001) 16: 423-437

http://www.ehu.es/histol-histopathol

Histology and Histopathology

Cellular and Molecular Biology

The hepatocytes of the brown trout (*Salmo trutta* f. *fario*): A quantitative study using design-based stereology

E. Rocha[1,2], R.A.F. Monteiro[1,2], M.H. Oliveira[1] and M.W. Silva[1]

[1]Laboratory of Histology and Embryology, Institute of Biomedical Sciences Abel Salazar (ICBAS), and [2]Interdisciplinary Center of Marine and Environmental Research (CIIMAR), University of Oporto, Porto, Portugal

Summary. A stereological study was performed on brown trout hepatocytes aiming to disclose whether there are basic gender differences when minimal levels of sex hormones exist, and also to establish a platform for both interspecific comparisons and physiological correlations. We used the so-called "design-based stereology" (with no shape, size or orientation assumptions) and also some new related statistics. Two-year-old brown trout were collected in April, and the livers were fixed by perfusion. From liver slicing to microscopical field selection, systematic sampling was used. Stereology was applied at light and electron microscopy. Target parameters were the relative and total hepatocyte number, the mean individual hepatocyte volume and surface, and also both relative and total volumes, and surfaces, either of organelles or of cell compartments. Observed variability was usually high, but the precision of estimates was proved to be globally adequate facing the true biological variation amongst specimens. Females had more hepatocytes per liver ($1.79x10^9$ *vs.* $1.12x10^9$). Considering the individual hepatocytes, whereas no gender differences were detected in the cell volume, males had higher values of nuclear volume (199 *vs.* 151 μm^3) and surface (170 *vs.* 131 μm^2), endoplasmic reticulum volume (1300 *vs.* 824 μm^3), and microvilli volume (82 *vs.* 54 μm^3) and surface (1445 *vs.* 975 μm^2). However, when dealing with quantities per liver, gender differences were found only in the volumes of dense bodies (56 *vs.* 97 mm^3) and of residual cytoplasm (169 *vs.* 341 mm^3) — both volumes were higher in females. Functional implications of data are discussed, namely that females seem to have basic structural traits for coping with the later demands of breeding. Data also support that structural remodelling of hepatocytes occurs after breeding, urging to pursue seasonal studies (namely on lysosomes). We advanced the hypothesis that genders differ in microvilli surface just to maintain an optimal physiological surface-to-volume ratio. Interspecific similarities and differences were disclosed. For example, the number of hepatocytes/cm^3 of parenchyma of brown trout was much lower than those reported in rainbow trout, but in both trouts females seem to have an higher cell number. In addition, when comparing the size of hepatocytes of brown trout with that from other fish and mammals it was suggested that major interspecific differences exist.

Key words: Hepatocyte, Liver, Teleost, Stereology, Disector

Offprint requests to: Eduardo Rocha, Laboratory of Histology and Embryology, Institute of Biomedical Sciences Abel Salazar (ICBAS), Largo Prof. Abel Salazar no. 2, 4099-003 Porto, Portugal. Fax: 351 22 206 22 32 or 1 801 992 2122. e-mail: erocha@icbas.up.pt

Introduction

For several years, we have been characterizing the normal histology of the brown trout liver, aiming at sound bases for histopathological and physiological approaches. The gathered insight also has a great interest for comparative morphology. We have made both qualitative and quantitative accounts, at light and electron microscopy level (Rocha et al., 1994a,b, 1995, 1996, 1997, 1999). Although highly valuable, qualitative data are nonetheless subjective and not adequate for statistics; so, stereological approaches are also desirable.

From all liver cells, hepatocytes have been the major target for quantification, either in mammals (e.g., Loud, 1968; Weibel et al., 1969; Blouin et al., 1977; Bioulac-Sage et al., 1984; Jack et al., 1990a,b) or in fish. In these, most data came from rainbow trout, *Oncorhynchus mykiss* (e.g., Arnold et al., 1995, 1996a,b; Hampton et al., 1989; Moutou et al., 1997). Despite proved relevant in toxicology (Braunbeck, 1998), stereological data on other fish hepatocytes are rather scarce, but some are available, e.g., for medaka, *Oryzias latipes* (Hinton et al.,1984; Braunbeck et al., 1992), for golden ide, *Leuciscus idus* (Segner and Braunbeck, 1990), for immature brown trout (Schramm et al., 1998), and for an immature demersal fish, *Solea ovata* (Au et al., 1999).

Since the breakthrough of the disector (Sterio, 1984), stereology moved from what is now called "the old assumption-based methods" to the "design-based approaches". Essentially, the old techniques for

estimating particle size and number relied upon strict assumptions of shape, size and orientation of the particle, whereas the design-based methods do not, assuring a priori unbiased estimates not needing validation studies (Gundersen et al., 1988; Howard and Reed, 1998). When correctly used by the experimenter, design-based stereology grants accuracy, i.e., estimates will always vary around the true value. On the contrary, when measuring with model-based estimators – the assumptions being very rarely tested – we might, by chance, stumble across the true answer without knowing it, for bias is invisible and undetectable within any one experiment (Howard and Reed, 1998). Even when comparing control versus experimental groups, bias can be significantly different between groups, despite using exactly the same assumption-based methods (e.g., Mendis-Handagama, 1992). A word of caution is in order to remember that either using the old or the more recent methods, systematic biases can be induced by non-stereological procedures (such as shrinkage), stemming from tissue processing.

In mammals, only two studies seem to exist that have used more recent stereological principles on rat hepatocytes (Jack et al., 1990a,b). As to fish hepatocytes, and except in one of our studies in which modern concepts were introduced (Rocha et al., 1999), all the other studies were solely based on classical stereology and sampling theories (Weibel, 1979). The situation in hepatology contrasts with that in neuroscience, where design-based approaches have been largely used; to a point that editors urge both the authors and the referees to adhere to the design-based unbiased estimates (Coggeshall and Lekan, 1996; Saper, 1996).

This study was focused on hepatocytes, evaluating their number, volume, surface, and dissecting their organeollar compartments in relative and absolute quantities. We used a design-based approach which, in association with recent statistical practices, enabled us to evaluate our procedure, extracting guidelines for further quantification of brown trout hepatocytes in future studies. As in a previous approach (Rocha et al., 1997), young adults were sacrificed at the previtellogenesis period of the female breeding cycle, found to be the best moment for seeking intrinsic sexual differences not too closely related either with vitellogenesis or with spawning.

Materials and methods

Animals

Two-year-old male (n=7) and female (n=7) brown trout (*Salmo trutta fario* Linnaeus, 1758) were randomly fished with a net within the aquaculture pools of the *Posto Aquícola do Torno* (Amarante, Portugal), and fish were sacrificed in four consecutive days. During this time, water was well aerated, daily temperature changed between 13 and 15 °C, pH from 6.5 to 7, and NH_3 <0.005 mg/L. The males weighted 277 g [210-350] and had a total length of 28 cm [26-31], whereas the females weighted 280 g [200-400] and had a total length of 29 cm [26-33]. The animals were being hand fed twice a day with highest quality commercial trout pellets (composition on the label: protein 42%, lipid 18%, cellulose 1.5%, ash 11%, phosphorus 1.8% — from Alpis - A. Coelho & Castro Lda., Póvoa de Varzim, Portugal). Collection took place during late April (middle of previtellogenesis period of female breeding cycle). Ovarian histology was typical of that period, showing mainly growing oocytes mostly in a primordial cortical alveoli stage, and thus with a scarce perinuclear yolk deposition (Selman et al., 1987; Washburn et al., 1990). Testes were atrophic, displaying only spermatogonia.

Fixation and tissue processing

To avoid eventual diurnal variations in the hepatocyte content, all collections were made during the morning. Before fixation, the trout were anaesthetized by immersion in 0.3 ml/L aqueous solution of ethylene glycol monophenyl ether, and then weighed. Liver was fixed by perfusion via hepatic portal vein or one of its major tributaries. Details of the procedure are given elsewhere (Rocha et al., 1994a). Briefly, 2-4 ml of a chilled (15 °C) and heparinized isosmotic Ringer-like solution were perfused at physiological flow rate of about 5.2 ml/min/kg body weight (Schmidt and Weber, 1973; Hampton et al., 1985). Subsequently, 20-40 ml of chilled fixative (15 °C) were introduced as described; fixative was 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.3).

After removal, the liver was quickly weighed and its volume was determined by Scherle's method (1970). The liver was then chopped into 3 mm thick slices. A series of systematic random sampling steps (subsequently applied to groups of smaller tissue fragments) was carried out to obtain a final sample of pieces (<0.5 mm in diameter) to be handled for transmission electron microscopy (TEM). This systematic scheme is known for its high efficiency, assuring a priori equal sampling probabilities for all organ zones (Gundersen, 1986; Gundersen and Jensen, 1987).

The tiny fragments were further immersed in the same fixative as that used in perfusion for 3h (at 4 °C) and then rinsed for 2h in 0.1M phosphate buffer (at 4 °C). Postfixation was done with 1% OsO_4 in 0.1M phosphate buffer, for 2h (at 4 °C). After dehydration in ethanol (50%, 75%, 90%, 95%, at 4 °C, and 100% p.a., at room temperature) and two passages in propylene oxide, the pieces were embedded in epoxy resin at no specific orientation. For achieving this goal, the pieces were rolled inside the resin with the help of dissection needles. Thus, our procedure allowed the generation of randomly oriented sections that may be regarded, for all practical purposes, as isotropic uniform random planes, with no significant chance of bias (Henrique et al., 1997; Mayhew, 1997). Semithin sections were stained with a

mixture of methylene blue-azur II. Ultrathin sections were doubly stained with uranyl acetate (saturated in 50% methanol) and aqueous lead citrate, and then examined in a JEOL 100CXII, at 60 kV.

Stereological analysis

Stereology was implemented at light microscopy (LM) and TEM levels for estimating relative and absolute parameters related to the number, the volume and the surface both of hepatocytes and of several of their components. By applying stereological techniques directly to the sections primary values were derived. These were later used to calculate secondary stereological data.

Stereological methodology and parameters - primary data

At LM level, the unique determined stereological parameter was the numerical density (N_V) of the hepatocytes within the parenchyma. This parameter was estimated by the disector technique (Sterio, 1984). For the purpose, a mean of 6 tissue blocks per animal were used. From each block, a stack of 3 serial 1.0 μm thick sections was made; the first and the third sections were coupled into a physical disector. A rotating stage was used for an easy alignment of the sections. Under a x100 immersion lens, a mean of 4 fields/block were systematically sampled with exactly the same alignment in the two coupled sections, captured by a CCD camera (Sony), and printed with a final magnification of x2,160 (checked with prints of a stage micrometer). Image grabbing and printing was done with an image analysis software (CUE-2, ver 4.5, Olympus). To increase efficiency, both sections of each physical disector were used as the sampling and as the *look-up* sections (Gundersen, 1986), by interchanging their roles, so that a mean total of 8 physical disectors per block (a mean of 48 per animal) were analysed. Because nearly all the brown trout hepatocytes have only one nucleus (Rocha and Monteiro, 1999), the N_V (hepatocytes, parenchyma) can be considered to be virtually equal to the N_V (nucleus, parenchyma). The following formula was used:

$$N_V \text{ (nucleus, parenchyma)} = N_V \text{ (hepatocyte, parenchyma)} = \sum Q^- \div (\sum A \times h)$$

where $\sum Q^-$ is the total number of nuclei that were seen in a reference section, but not seen in the other look-up section; $\sum A$ is the area of an unbiased frame used for sampling the particles to be counted (Gundersen, 1977), summed over all disectors (in practice, this was done by multiplying the frame area - 343 μm^2 - with the total count of the central frame-associated point hitting the reference space); and h is the disector height (2.02 μm). This height was checked by measuring the distances between the block face and a prefixed reference plane (Gundersen, 1986).

At TEM level, the primary parameters to be determined were volume densities (V_V) and surface densities (S_V) of several components; i.e., the relation between the volume or the surface of a structure and the volume of the containing reference space (e.g., cell body). From each animal, a mean of 6 tissue blocks were used. From each block, 1 section was observed, and from that section a mean of 10 fields were quantified, so that a mean total of 60 fields per animal were observed. The fields were systematically sampled by photographing the section areas closely associated with the mesh angles of the squared supporting grid. Photographs were taken at x2800, and morphometry was performed on prints with a final magnification of x8400.

The volume densities (later expressed as %) were estimated by a classical, and unbiased, manual stereological technique based on point counting (e.g., Weibel, 1979):

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

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; 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 multipurpose test-grid containing three sets of points (1:4:16) was superimposed on the prints, and, according to size and frequency of each structure, a particular set of points was used.

Surface densities were also estimated by using a manual stereological technique based on point and intersection counting (e.g., Baddeley et al., 1986) and using the formula:

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

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 intersection counts of test lines across boundaries of the structures of interest (i.e., the nucleus and the microvilli); and $\sum P(r)$ is the sum of points falling over the reference space (i.e., the hepatocyte). The same formula was used for a direct estimation of the surface-to-volume ratio ($\bar{s}_N/\bar{v}_N$) both of the nucleus and of the cell[1], in which P(r) is the sum of points falling over the two referred structures themselves.

Stereological methodology and parameters - secondary data

From the primary data, obtained at LM and TEM levels, secondary parameters were derived by using mathematical relationships. The parameters computed

[1]Two situations were considered in this study: the hepatocyte including microvilli and excluding microvilli. In the latter case, the cell periphery was considered continuous at the base of the microvilli

were:

1. The total number of hepatocytes per liver (N)

$$N\ (\text{hepatocytes}) = N_V\ (\text{hepatocyte, parenchyma}) \times V\ (\text{parenchyma})$$

in which the V (parenchyma) is the mean absolute volume of this hepatic component. Because the N_V was estimated in tissue embedded in epoxy resin, where the low shrinkage (ca 3-5%) can be disregarded (Weibel, 1979), the product $N_V \times V$ in the present study produced an essentially unbiased estimate[2]. The V (parenchyma) was previously determined at LM in the same animals by using the relationship: V_V (parenchyma, liver) V (liver). The technical details and unbiased nature of that estimation was then properly displayed (Rocha et al., 1997); the values were 3255 mm^3 (CV=0.35), for males, and 3497 mm^3 (CV=0.43), for females.

2. The number-weighted mean cellular and nuclear volumes ($\bar{v}_N$)

$$(\bar{v}_N)\ (\text{hepatocyte}) = V_V\ (\text{hepatocyte, parenchyma}) \div N_V\ (\text{hepatocyte, parenchyma})$$

$$(\bar{v}_N)\ (\text{nucleus}) = V_V\ (\text{nucleus, parenchyma}) \div N_V\ (\text{nucleus, parenchyma})$$

The reliability of the $\bar{v}_N$ computed in this way depends on the unbiased determination of both parameters V_V and N_V (Howard and Reed, 1998), granted in our study not only by the stereological techniques adopted, but also by the facts that each V_V was estimated at TEM (where overprojection is negligible for either the cell or the nucleus) and that both V_V (at TEM) and N_V (at LM) were estimated from adjacent sections coming from the same epoxy resin blocks.

3. The number-weighted mean cellular and nuclear surfaces ($\bar{s}_N$)

$$\bar{s}_N\ (\text{hepatocyte}) = \bar{v}_N\ (\text{hepatocyte}) \times \bar{s}_N/\bar{v}_N\ (\text{hepatocyte})$$

$$\bar{s}_N\ (\text{nucleus}) = \bar{v}_N\ (\text{nucleus}) \times \bar{s}_N/\bar{v}_N\ (\text{nucleus})$$

4. The absolute surface per hepatocyte ($\bar{s}_N$) of the microvilli

$$\bar{s}_N(\text{microvilli}) = S_V\ (\text{microvilli, hepatocyte}) \times \bar{v}_N\ (\text{hepatocyte})$$

5. The absolute volumes per cell ($\bar{v}_N$) of both the nucleoli and the cytoplasmic components

$$\bar{v}_N\ (\text{nucleoli}) = V_V\ (\text{nucleoli, nucleus}) \times \bar{v}_N\ (\text{nucleus})$$

$$\bar{v}_N\ (\text{component}) = V_V\ (\text{component, hepatocyte}) \times \bar{v}_N\ (\text{hepatocyte})$$

6. The total volume and surface per liver — i.e., V(component) and S(component) — of the cells and of any of the cell structures:

$$V\ (\text{component}) = \bar{v}_N\ (\text{component}) \times N\ (\text{hepatocytes})$$

$$S\ (\text{component}) = \bar{s}_N\ (\text{component}) \times N\ (\text{hepatocytes})$$

Statistics

All data are presented as group means followed by the respective coefficients of variation (CV = Standard Deviation ÷ Mean). Statistical analysis was made with the software *Statistica* 5.0 for *Windows*. After checking the normality and the homogeneity of variances, genders were compared for each parameter by the unpaired Student's *t* test. Correlation analyses were also made.

The appropriateness of the sampling scheme was evaluated by determining the degree to which the observed relative variance of the group estimate was due to precision of the individual estimates; these ones being estimated by the coefficient of error (conventionally expressed as: CE = SEM÷Mean). Generally, the sampling is thought to be optimal when the observed mean variance of the individual estimates ($\overline{CE}^2$) is about 50% or less than the total observed variance between animals (CV^2). Under these circumstances, the true (inherent) biological variance of a parameter among individuals, ICV^2, should usually make the major contribution to the CV^2 according to the following relationship: $CV^2 = ICV^2 + \overline{CE}^2$ (West et al., 1991, 1996; Howard and Reed, 1998). The $\overline{CE}^2$ were computed as the average of the CE^2 estimated for each animal.

Because a systematic random sampling design was used within individuals and some parameters were derived from ratio estimates based on point counting, sampling variances had to be estimated with formulae that differ from those that are conventionally used on independent random samples (Gundersen and Jensen, 1987; Geinisman et al., 1997). Thus, the individual CEs of the disector estimates (N_V) were calculated with the so-called Matheron's quadratic approximation formula, taking into account the "Nugget effect" (e.g., West et al., 1991, 1996). The CEs of the several V_V and S_V parameters were calculated with the so-called Cochran's approximation formula for ratio estimates (Cochran, 1977; Karlsson and Cruz-Orive, 1991).

The CEs for two-stage estimations of a variable ($\hat{Z}$), resulting from the multiplication or division of two other independent variables (X and Y), assuming null covariance, were estimated for each animal by using the equation: $CE^2(\hat{Z}) \approx CE^2(\hat{X}) + CE^2(\hat{Y})$ (Karlsson and Cruz-Orive, 1992; Howard and Reed, 1998). As the liver volumes were direct determinations by the Scherle's

[2]We found a mean total volumetric shrinkage of 3% both in males and females. Thus, and following the general practice with such small biases, no corrections were performed to our crude results.

method (1970), the respective CEs are literally null for computing any CE^2 (Z).

Results

Animals and liver

The mean liver weights of males and females were, respectively, 3.5g (0.35) and 4.0g (0.26). Genders did not differ either in liver weights, or in body weights and lengths.

Qualitative microscopic observations

Because we have thoroughly described the general histology of the brown trout liver (Rocha et al., 1994a,b,

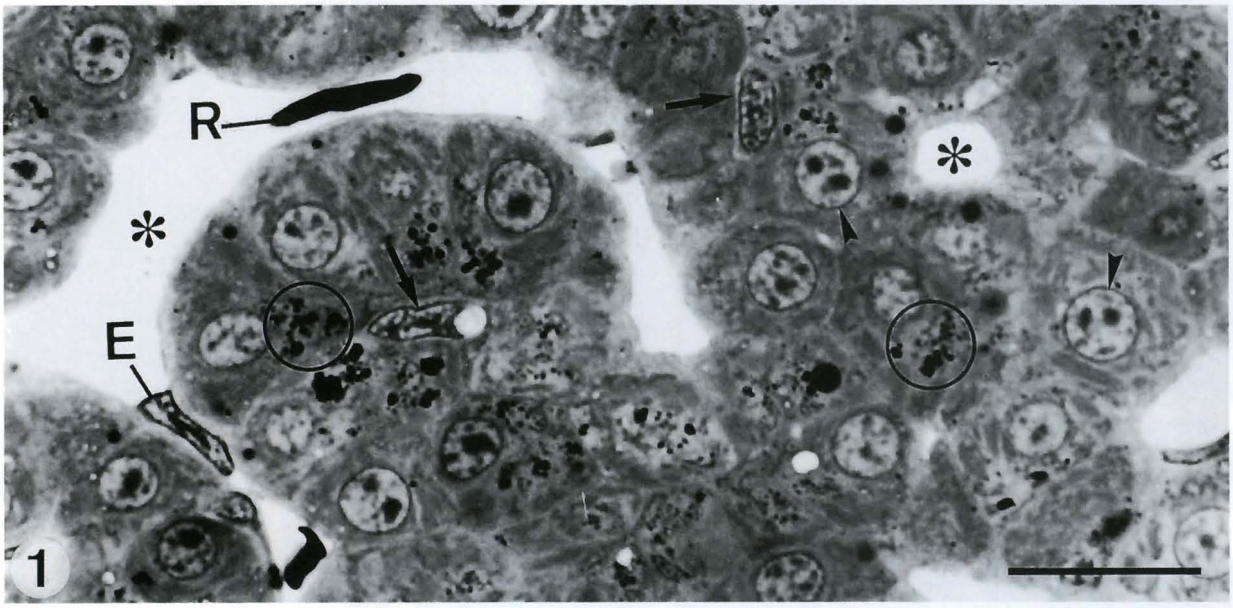

Fig. 1. Light micrograph of a semithin section from a male specimen, illustrating (at the left side of the image) the basic tubular nature of the hepatocytic spatial array, around a biliary axis (arrows). This axis can be often seen when the hepatocytic array assumes a laminar aspect (patent at the right side of the image). Note the scattered roundish nuclei, some bearing two nucleoli (arrowheads), and the presence of dense bodies mostly at the apical pole of hepatocytes. Asterisk - capillaries (*); E- endothelial cell; R - red blood cell. Bar: 20 μm.

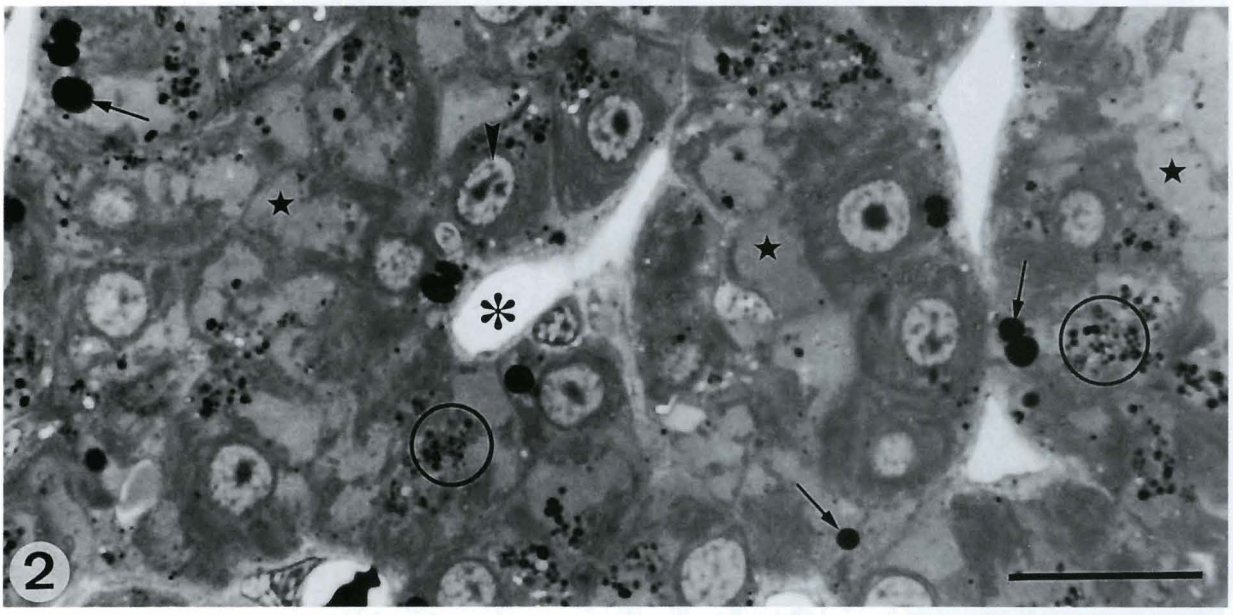

Fig. 2. Light micrograph of a semithin section from a female fish in which the hepatocytes had a high content of glycogen; typically appearing as clear cytoplasmic areas (stars). Note the scattered groups of dense bodies (circles), and the presence of a few lipid droplets (arrows). Arrowhead points to an elongated hepatocytic nucleus. Asterisk: capillaries. Bar: 20 μm.

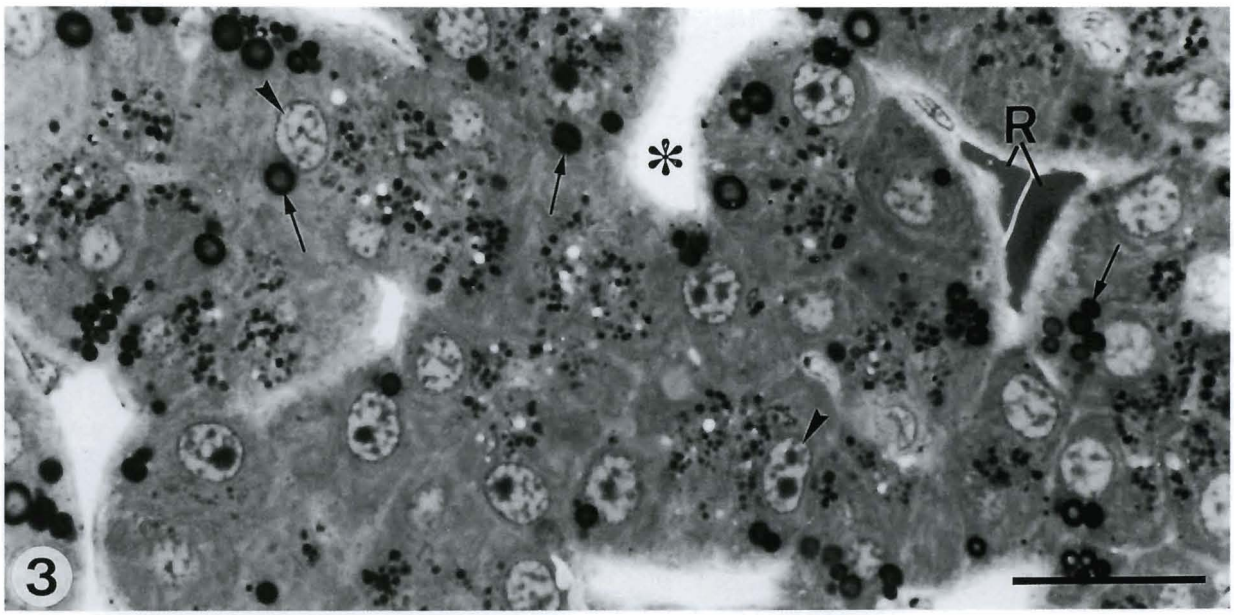

Fig. 3. Light micrograph of a semithin section from a female specimen the hepatocytes of which had virtually no glycogen, but displayed instead many lipid droplets (arrows) and a plethora of dense bodies (compare their abundance with that displayed in hepatocytes of Figs. 1 and 2. Arrowheads indicate elongated hepatocytic nuclei. Asterisk: capillaries. Bar: 20 μm.

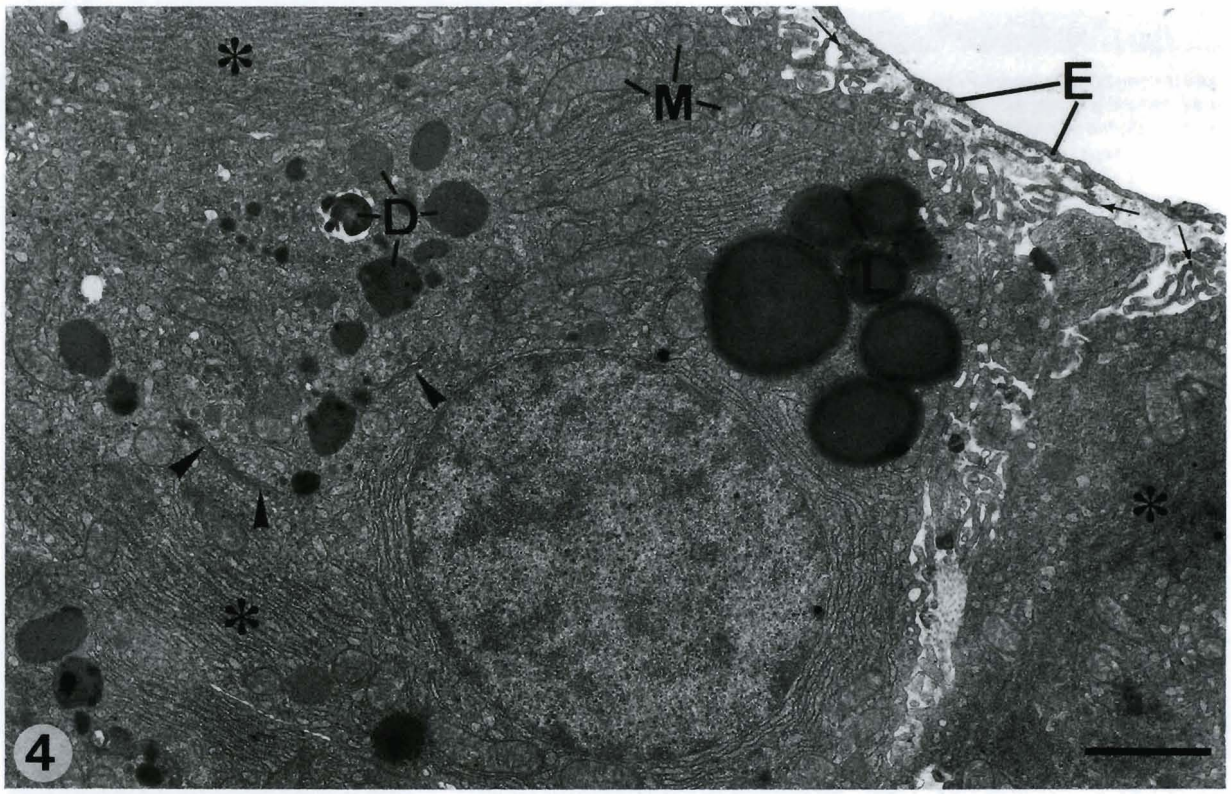

Fig. 4. Electron micrograph of hepatocytes from a male specimen showing a high content in rough endoplasmic reticulum (*), along with several lipid droplets (L), mitochondria (M) and dense bodies (D), but with no glycogen zones. A few Golgi cisternae and vesicles can be seen (arrowheads). Arrows point towards several hepatocytic microvilli within the Disse space. E: endothelial cell. Bar: 2 μm.

1995, 1996, 2000), only a brief account of some morphological qualitative aspects of hepatocytes is given here. This introduces the structures that were quantified in the study and further emphasizes the need for a quantitative approach.

The hepatocytes were radially organized in tubules (with a biliary axis), so tortuous, branched and connected that when seen at light microscopy they usually appeared as plates two or more hepatocytes thick (Figs. 1-3). Though almost all of them had a single nucleus, which was often fairly round or, on the contrary, somewhat irregular or elongated in contour, in some rare occasions there were two nuclei per cell. There were one or two nucleoli per nucleus (Figs. 1-3).

At electron microscope level (Figs. 4, 5), hepatocytes displayed a cytoplasm typically rich in

Table 1. Stereological parameters of the hepatocytes of brown trout, concerned with cell number, volume and surface. The contribution of the estimate precision ($\overline{CE}$) for the total variance ($CV_{among\ animals}$) is also presented.

PARAMETERS	MALES (n=7)		FEMALES (n=7)	
	Mean (CV)	$\overline{CE}^2 \div CV^2$	Mean (CV)	$\overline{CE}^2 \div CV^2$
Numerical density - N_V ($10^6/cm^3$)	358 (0.21)	0.35	475 (0.27)	0.15
Cell number per liver - N (10^9)	1.12 (0.35)**	0.11	1.79 (0.18)**	0.34
Cell volume per liver - V (mm^3)[1]	2787 (0.38)	0.18	3462 (0.27)	0.31
Individual cell volume - $\bar{v}_N$ (μm^3)[1]	2559 (0.26)	0.20	1963 (0.30)	0.13
Cell surface per liver - S (m^2)[1]	2.79 (0.27)	0.44	3.33 (0.23)	0.48
Cell surface per liver - S (m^2)[2]	1.19 (0.31)	0.31	1.56 (0.21)	0.49
Individual cell surface - $\bar{s}_N$ (μm^2)[1]	2585 (0.18)**	0.50	1870 (0.21)**	0.40
Individual cell surface - $\bar{s}_N$ (μm^2)[2]	1092 (0.17)	0.51	885 (0.25)	0.22
Surface-to-volume ratio - $\bar{s}_N/\bar{v}_N$ (μm^{-1})[1]	1.04 (0.19)	0.17	0.97 (0.14)	0.33
Surface-to-volume ratio - $\bar{s}_N/\bar{v}_N$ (μm^{-1})[2]	0.45 (0.10)	0.38	0.47 (0.08)	0.35

[1]: considering microvillli as part of the cell body. (Volumes without microvilli are only about 3% lower in both males and females, moreover, both variability and contribution of estimation precision to total variance are identical to those considering microvilli.); [2]: not including microvillli as part of the cell body. **: significantly different between genders ($p \leq 0.01$).

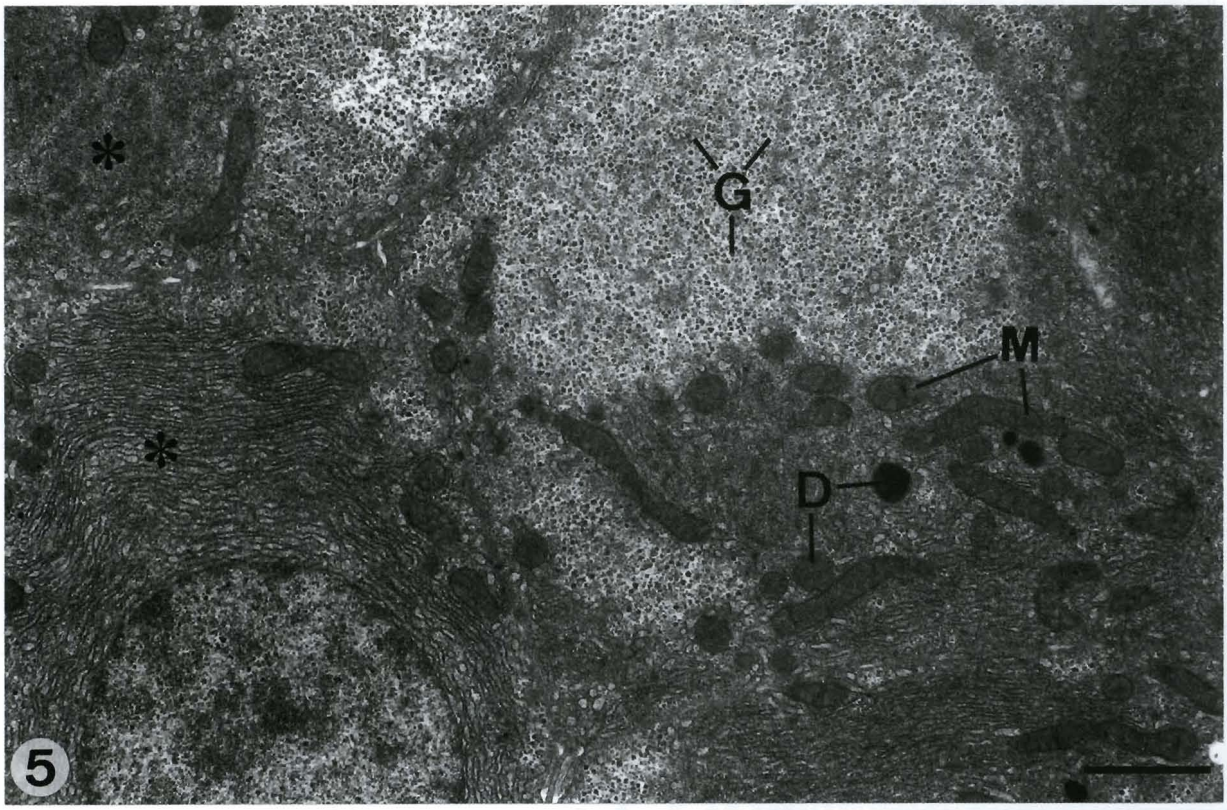

Fig. 5. Electron micrograph of hepatocytes from a female specimen displaying a high content in glycogen (G), along with fields of rough endoplasmic reticulum (*), and scattered mitochondria (M) and dense bodies (D). Bar: 2 μm.

rough endoplasmic reticulum (RER), but which could also possess large amounts of glycogen. Lipid inclusions were also found, either associated to glycogen or to RER fields. These two were defined in this study as the cytoplasmic areas richer either in glycogen or in RER cisternae, respectively, thus including those elements and the closely intermingled cytosol. Furthermore, whenever used in this text, the term reticulum must be understood to include all membrane-bound cytoplasmic cisternae and tubules of the endoplasmic reticulum.

The cytoplasmic plethora of dense bodies included lysosomes (primary, secondary and residual bodies) and peroxisomes. (An extended study on peroxisomes was previously made by us, using catalase as a specific marker - Rocha et al., 1999). From the sinusoidal side of the hepatocytes, numerous slender, tortuous, and often ramified finger-like projections (commonly designated microvilli) extended into the space of Disse. Additionally, from the apices or upper lateral zones of those hepatocytes, true microvilli extend into biliary passages. The hepatocytic cytoplasm near these structures was particularly rich in stacks of Golgi apparatus.

With a qualitative study it was not possible to discern clear differences between genders, either at light or at electron microscopy level. What could be detected was the quite high inter-individual variability in both genders as to the organeollar composition of hepatocytes, specially in the amounts of lipids and glycogen (Figs. 2, 3); e.g., some animals had virtually no glycogen zones in the cells, whereas others displayed areas almost as large as the RER ones.

Quantitative microscopic observations

Original data are organized in four tables. The contribution of the estimate precision to the total variance (i.e., $\overline{CE}^2 \div CV^2$) is presented in all of them. No correlations were found between any of the stereological parameters and either the fish body size or length.

Table 1 displays results concerning hepatocyte number and size parameters. It can be observed that females have a significantly higher total number of hepatocytes (N), but that such difference was only apparent when looking at the relative cell number (N_V); despite the fact that mean N_V for females was about 33% higher than that for males. Another statistically significant gender difference was disclosed in the cell surface ($\bar{s}_N$), but only when considering the contribution of the microvilli, with males presenting a 38% higher mean value. It was noticed that in both genders, microvilli increased the cell surface in more than 200%. It was emphasized that, despite the referred difference in the $\bar{s}_N$, the surface-to-volume ratio ($\bar{s}_N/\bar{v}_N$) was virtually identical in both genders, either considering or not microvilli as part of the cell body. Moreover, no significant differences were detected in the cell surface per liver [S(cell)]. As to the volumes, and despite the mean $\bar{v}_N$ (cell) being about 30% higher in males, the differences were not statistically significant. It was interesting to observe that most of the lower CVs were found in the $\bar{s}_N/\bar{v}_N$, and that for all parameters the ratio $\overline{CE}^2 \div CV^2$ was around 50% or lower.

Table 2 encompasses the splitting of the hepatocyte in its different components, the results being expressed as volume densities. A few points deserve particular attention, such as the fact that the RER and the glycogen zones were the major volumetric components of the cytoplasm, but only the first ones revealed a statistically significant difference between genders. In fact, males presented a higher V_V (reticulum) than females; the 55% higher V_V (glycogen) of females was not enough to be considered statistically relevant when compared to

Table 2. Stereological parameters of the hepatocytes of brown trout: volume densities (Vv - %) of the microvilli, the nucleus and the cytoplasm in relation to cell body, and volume densities (V_V - %) of several specific organelles in relation to their relevant reference spaces (i.e., the nucleus for the nucleolus and the cytoplasm for the remaining organelles). The contribution of the estimate precision ($\overline{CE}$) for the total variance ($CV_{among\ animals}$) is also presented.

STRUCTURES	MALES (n=7)		FEMALES (n=7)	
	Mean (CV)	$\overline{CE}^2 \div CV^2$	Mean (CV)	$\overline{CE}^2 \div CV^2$
Microvilli	3.4 (0.29)	0.13	2.8 (0.19)	0.36
Nucleus[1]	8.1 (0.24)	0.05	8.2 (0.27)	0.07
Cytoplasm[1]	88.5 (0.03)	0.09	89.0 (0.03)	0.14
Nucleolus	3.4 (0.25)	0.26	3.5 (0.09)	0.68
Reticulum	59.8 (0.21)*	0.05	47.3 (0.14)*	0.23
Glycogen	16.5 (0.93)	0.05	25.5 (0.32)	0.19
Mitochondria	9.0 (0.21)	0.10	9.0 (0.15)	0.30
Lipids	3.6 (0.96)	0.10	2.1 (0.85)	0.11
Dense bodies	2.4 (0.24)	0.13	3.3 (0.41)	0.09
Golgi apparatus	1.4 (0.30)	0.13	1.4 (0.28)	0.28
Other	7.3 (0.35)	0.09	11.4 (0.40)	0.14
Nucleus[2]	8.5 (0.24)	0.15	8.5 (0.27)	0.11
Cytoplasm[2]	91.5 (0.02)	0.17	91.4 (0.02)	0.22

[1]: considering microvillli as part of the cell body; [2]: not including microvillli as part of the cell body; *: significantly different between genders ($p \leq 0.05$).

males. One obvious cause for such a result was that, in addition to the high variability found in females, males presented a tremendously high biological variation (CV) in their hepatocytic glycogen content. Another important remark is that lipid droplets account for little cytoplasmic volume, and, what is relevant here, both males and females presented a very high variability (both CVs close to 1). In Table 2, all but one value of the $\overline{CE}^2 \div CV^2$ ratios were far below the 50% "golden rule" (see Methods). In relation to the value of 0.68 reported for the nucleolus in females, and, contrary to what could be thought, the precision of estimating V_V (nucleolus, nucleus) was quite good (CE = 0.07), and so, the 0.68 is actually derived from the fact that the true biological variation ($CV^2 - \overline{CE}^2$) of the female nucleolus was quite low.

Table 3 encloses total volumes per hepatocyte. Some interesting points exist, namely when comparing them with Table 2. Thus, three statistically significant differences were found in the volumes of microvilli, nucleus and reticulum per cell (males having greater values than females). The 58% apparent gender difference regarding the $\bar{v}_N$ (cytoplasm) was not statistically relevant; again, high variability in both genders counteract. Though variability was generally high, except for the nucleus, the $\bar{v}_N$ of glycogen zones and of lipid droplets were by far the most variable structures within hepatocytes (CVs close or over 1). As in Table 2, in Table 3 there are three $\overline{CE}^2 \div CV^2$ ratios that fall well over the desired 50%. However, and again (see above), the results do not mean that the precision of the estimates are inadequate. In fact, what we are facing again is the phenomena of low biological variability combined with relatively good precision estimates; the CE for the $\bar{v}_N$ (nucleus) was estimated in 12-13% in both genders, and the CE for the $\bar{v}_N$ (nucleolus) in females was about 13%.

The analysis of the total volumes per liver [V(component)] of the several hepatocyte constituents, shown in Table 4, revealed a different situation when comparing it with Tables 2 or 3. Statistically meaningful gender differences (greater values now found in females)

Table 3. Stereological parameters of the hepatocytes of brown trout: total volumes (μm^3) per cell of the different components of the hepatocyte [$\bar{v}_N$ (component)]. The contribution of the estimate precision ($\overline{CE}$) for the total variance ($CV_{among\ animals}$) is also presented.

STRUCTURES	MALES (n=7)		FEMALES (n=7)	
	Mean (CV)	$\overline{CE}^2 \div CV^2$	Mean (CV)	$\overline{CE}^2 \div CV^2$
Microvilli	82 (0.23)**	0.44	54 (0.22)**	0.48
Nucleus	199 (0.09)***	1.80	151 (0.10)***	1.72
Cytoplasm	2278 (0.29)	0.16	1758 (0.33)	0.11
Nucleolus	7 (0.31)	0.30	5 (0.11)	1.43
Reticulum	1300 (0.14) **	0.56	824 (0.33) **	0.15
Glycogen	446 (1.10)	0.05	479 (0.65)	0.07
Mitochondria	197 (0.19)	0.45	154 (0.21)	0.36
Lipids	92 (1.15)	0.08	35 (0.82)	0.14
Dense bodies	56 (0.42)	0.12	57 (0.44)	0.13
Golgi apparatus	32 (0.22)	0.50	23 (0.26)	0.49
Other	156 (0.21)	0.55	187 (0.34)	0.19

: significantly different between genders ($p \leq 0.01$); *: significantly different between genders ($p \leq 0.001$).

Table 4. Stereological parameters of the hepatocytes of brown trout: total volumes (μm^3) per liver of the different components of the hepatocyte [V(component)]. The contribution of the estimate precision ($\overline{CE}$) for the total variance ($CV_{among\ animals}$) is also presented.

STRUCTURES	MALES (n=7)		FEMALES (n=7)	
	Mean (CV)	$\overline{CE}^2 \div CV^2$	Mean (CV)	$\overline{CE}^2 \div CV^2$
Microvilli	88 (0.28)	0.49	98 (0.32)	0.28
Nucleus	222 (0.37)	0.26	270 (0.15)	1.19
Cytoplasm	2477 (0.39)	0.17	3094 (0.29)	0.27
Nucleolus	8 (0.45)	0.27	10 (0.21)	0.65
Reticulum	1399 (0.25)	0.46	1451(0.26)	0.40
Glycogen	512 (1.14)	0.05	831 (0.64)	0.10
Mitochondria	221 (0.46)	0.15	272 (0.21)	0.59
Lipids	85 (0.98)	0.12	62 (0.90)	0.13
Dense bodies	56 (0.22)***	0.63	97 (0.32)***	0.36
Golgi apparatus	37 (0.54)	0.19	40 (0.25)	0.71
Other	169 (0.32)**	0.39	341 (0.16)**	1.17

: significantly different between genders ($p \leq 0.01$); *: significantly different between genders ($p \leq 0.001$).

were disclosed in the amount of dense bodies and in the compartment we designated as "other" (encompassing mainly cytosol). No such differences were found in relation to either the reticulum, or nucleus or microvilli. Logically, the observed variability for the total amounts per liver tended to be slightly higher than that reported per cell, with the lipids and glycogen really varying a great deal.

As expected from the results of the previous estimations, despite the fact that almost all of the $\overline{CE}^2 \div CV^2$ ratios were below 50% some were slightly above and others even well above that mark; this is inherent to the cumulative error in multi-leveled estimations. It is to be noted, however, that there is no consistency between genders in that respect, females having four ratios above 50% and males only one. In the case of the V(nucleus) of females the situation was the same as explained above. In the other cases the CEs were 15-17% and for the V(Golgi) of females it was 20%; values we consider only as reasonable estimates facing the CVs.

Table 5 displays data concerning surfaces, both from microvilli and from nucleus. Two statistically significant gender differences were found: in the $\bar{s}_N$ (microvilli) and in the $\bar{s}_N$ (nucleus). In contrast, all the relative surface parameters showed a notable similitude between genders. Moreover, no gender differences existed in the S (microvilli) and S (nucleus). Observed variability among animals was quite low regarding the $\bar{s}_N/\bar{v}_N$ (nucleus) and the $\bar{s}_N$ (nucleus). The precision of estimates seemed adequate in relation to the total observed variability, with the apparent exception of the S (nucleus) in females and of three other results which showed disproportionately high $\overline{CE}^2 \div CV^2$ ratios. As above, the last results were again due to the low observed variability accompanied by a reasonable good precision estimate; in fact, the CE for the $\bar{s}_N/\bar{v}_N$ (nucleus) of males was 5%, and the CE for the (nucleus) was 15% for both genders. As to the S(nucleus), the CE was indeed slightly higher (17%).

Discussion

Using stereological methods not depending on any shape assumption we evaluated the normal structure of young adult brown trout and showed that gender differences do exist. Regarding the individual hepatocytes, genders differed in the nuclear volume and surface, reticulum volume, and microvilli volume and surface. However, when analysing quantities per liver, the differences were exclusively found in the total volumes of both dense bodies and of residual cytoplasm (mainly cytosol with free ribosomes). Finally, females had more hepatocytes per liver.

Our data showed that global implications for the fish were different depending on the analysis of either relative or absolute values (per hepatocyte, per liver, or both). Such parallel approaches have been neglected in most previous works on hepatocytes, despite Weibel et al. (1969) stating that total amounts could be computed either per liver or, eventually, per unit of body weight. Daimon et al. (1984) also emphasized for rat liver that serious interpretation caveats could appear when working only with relative values; which is known as the "reference trap" (Braendgaard and Gundersen, 1986). Because we used design-based stereology to study fish hepatocytes, comparisons with previous data from other fishes and most mammalian studies must be made cautiously regarding many of the parameters. Despite this, comparisons with other data are desirable, not only to know whether or not the estimates from brown trout are of the same magnitude relative to those from other species, but also to look for general trends.

As to hepatocyte number in fishes, Hampton et al. (1989) reported gender differences in 5-year-old rainbow trout; females having 908×10^6 cells/cm^3 of parenchyma and males having only 540×10^6 cells/cm^3. When comparing estimates from brown trout (Table 1), those means are much higher. However, it is relevant that in both trouts females tend to have a higher number of hepatocytes than males. If this is a general phenomenon

Table 5. Stereological parameters of the hepatocytes of brown trout, concerned with the surface of both microvilli and cell nucleus. Contribution of the estimate precision ($\overline{CE}$) for the total variance ($CV_{among\ animals}$) is also presented.

STRUCTURES/PARAMETERS	MALES (n=7)		FEMALES (n=7)	
	Mean (CV)	$\overline{CE}^2 \div CV^2$	Mean (CV)	$\overline{CE}^2 \div CV^2$
Microvilli				
Surface density - S_V ($\mu m^2/\mu m^3$)	0.59 (0.27)	0.16	0.52 (0.24)	0.27
Surface per cell - $\bar{s}_N$ (μm^2)	1445 (0.23)**	0.44	975 (0.23)**	0.49
Surface per liver - S (cm^2)	15451 (0.28)	0.50	17480 (0.29)	0.47
Nucleus				
Surface density - S_V ($\mu m^2/\mu m^3$)1	0.07 (0.24)	0.19	0.07 (0.29)	0.23
Surface-to-volume ratio - $\bar{s}_N/\bar{v}_N$ ($\mu m^2/\mu m^3$)	0.85 (0.03)	3.30	0.87 (0.10)	0.47
Surface per cell - $\bar{s}_N$ (μm^2)	170 (0.07)***	4.80	131 (0.11)***	1.90
Surface per liver - S (cm^2)	1904 (0.39)	0.23	2361 (0.21)	0.67

1: mean values are identical considering or not microvilli as part of the cell body; **: significantly different between genders ($p \leq 0.01$); ***: significantly different between genders ($p \leq 0.001$).

in fishes is not known at this time. In immature golden ide, Segner and Braunbeck (1990) found mean values from 248 to 483x10^6 cells/cm^3, depending on the season. In up to one-year-old medakas of both genders, Hinton et al. (1984) estimated 379x10^6 hepatocytes/cm^3 of liver tissue. From all the data it can be presumed that interspecific differences may exist regarding hepatocyte number. Additionally, more studies are needed to disclose if in adult fishes there are also gender differences across the seasons.

As to the hepatocyte volume and surface, a compilation of data is presented in Table 6. We did not intend to condense here all available papers, but, instead, to group some we found more relevant. Only two of the studies used design-based stereology (Jack et al., 1990a,b). The corresponding estimates are considerably greater than those in brown trout (see Tables 1, 3 and 6), thus suggesting that major interspecific differences in the size of hepatocytes exist. This inference is supported by the fact that most estimates from mammals (Table 6) are much higher than those from fishes. Such a phenomenon is reflected in the smaller values of the mean number of hepatocytic nuclei per 1 ml of liver as estimated in rats: for newborns - 193 to 213x10^6 (Daimon et al., 1984); and for adults - 169x10^6 (Weibel et al., 1969).

From Table 6 it is evident that genders were separately studied only in rainbow trout, females having smaller hepatocytes than males (Hampton et al., 1989); the 63% difference was due to the cytoplasmic volume. We recall that an inverse scenario was found in brown trout, because genders statistically differed only in nuclear size (Table 3). Despite the methodological differences, a global picture may be extracted - in both trouts, male hepatocytes tended to be fewer in number and to have one larger cell compartment: either the nucleus or the cytoplasm.

In mammals, the nuclear volume of the hepatocyte was proved to be correlated with its DNA content, and an increase in volume thus corresponds to an increase in the cell ploidy level (Christie and Le Page, 1961; Romagna and Zbinden, 1981; Watanabe and Tanaka, 1982). If that same relationship also applies to fishes, then brown trout males could have a greater ploidy than the females - this interesting hypothesis must be checked in a future study. A bigger nucleus may, alternatively, represent an increased gene expression with a consequently greater protein synthesis (Christie and Le Page, 1961; Jack et al., 1990a). This hypothesis also seems plausible taking into account the greater RER content we found in male hepatocytes (see Tables 2, 3).

As to the surface area of brown trout hepatocytes: 1) the average male hepatocyte had a significantly higher cell surface; 2) microvilli were uniquely responsible for such a difference (contributing in more than 200% for the total cell surface); and 3) at organ level both genders had the same total hepatocytic surface. These aspects

Table 6. Compilation of some stereological data (mean values) of hepatocytes from fishes and mammals.

REFERENCE	SPECIES	$\bar{v}_N$ (cell)	$\bar{v}_N$ (nucleus)	$\bar{s}_N$ (cell)
Hampton et al. (1989)	Rainbow trout (13 females and 8 males)	Females - 990 Males - 1613	115 136	475[1] 588[1]
Arnold et al. (1995)	Rainbow trout (4 adult males)	1247	156	-
Arnold et al. (1996a)		1190	136	-
Arnold et al. (1996b)		1906	125	-
Moutou et al. (1997)	Rainbow trout (4 immature males)	2471	138	-
Hinton et al. (1984)	Japanese medaka (8 adult fishes)	3480	134	1937
Segner and Braunbeck (1990)[2]	Golden ide (4 immature fishes)	1867 to 3640	158 to 221	-
Weibel et al. (1969)[3]	Rats (Wistar derived) (5 young males)	4940	300	1680
Daimon et al. (1984)	Rats (Wistar strain) (3 fetuses and newborns)	1523 to 3916	341 to 364	-
Jack et al. (1990a)[4]	Rats (Tif:RAlf strain) (3 adult females)	4740[5] 6930[5]	330[5] 233[5]	- -
Jack et al. (1990b)[4]	Rats (Tif:RAlf strain) (3 adult females)	5360	430	3580
Bioulac-Sage et al. (1984)	Humans (a series of biopsies)	11300	800	-

[1]: values that do not include the contribution of microvilli surface; [2]: values estimated for different seasons of the year; [3]: cell volume reports to "mononuclear" hepatocytes and cell surface does not consider the contribution of microvilli; [4]: cell volumes estimated with the unbiased nucleator method (Gundersen, 1988) but nuclear volumes were based on the assumption that particles were perfect spheres; [5]: values for mononuclear (above) and binucleated (below) hepatocytes, sampled with disectors (Sterio, 1984).

seem to reveal that genders differed in microvilli surface just to compensate for small differences in cell volume, thus keeping a steady physiological balance between surface and volume. This idea is supported by the fact that mean values of the cell surface-to-volume ratios - including or excluding microvilli - were nearly equal in both genders (Table 1); the same occurred with the microvilli surface in relation to the cell volume (Table 4). Surface data from rainbow tout (Hampton et al., 1989) is confined to the hepatocyte sinusoidal side (Table 6), but it is relevant that a greater surface area per cell was also found in males, despite the fact that the surface density of microvilli was higher in females.

It was unbiasedly estimated in rats that surface density of microvilli was 0.52 $\mu m^2/\mu m^3$ (Jack et al., 1990b), a value that exactly matches what we found for brown trout (see Table 4). It would be important to have more data from other vertebrates in order to know whether or not throughout phylogeny an optimum relationship between the surface of microvilli necessary to support the same amount of volume of underlying cytoplasm was retained despite the greater cell size and different tri-dimensional positioning of the hepatocytes (Hinton and Couch, 1998).

Before discussing the organeollar composition of hepatocytes, an additional word of caution is relevant here because different criteria were often used for defining unbound cellular compartments (such as RER, cytosol, or glycogen). As to brown trout hepatocytes, let us first analyse the most interesting facts: 1) on average, 47 to 60% of the cytoplasm (40 to 50% of the cell) was reticulum, males having significantly higher relative and total amounts per cell; however, 2) both genders had the same amount of reticulum per liver; and finally, 3) no other gender difference at organ level was found in organelles that primarily govern cell function, such as mitochondria or Golgi apparatus. These data thus suggest that key structural differences give males and females a similar liver metabolic capacity when minimal levels of sexual steroids exist (Fostier et al., 1983; Norberg et al., 1989). Because females showed more hepatocytes with less RER, this may be seen as a basic morphological trait greatly favouring a high expansion potential for coping with the later demands of breeding, namely the vitellogenesis. The bigger liver volume of the "other" portion, mainly cytosol, would also favour such potential. Vitellogenesis was experimentally proved to be related with a marked RER proliferation in zebra fish, *Brachydanio rerio* (Peute et al., 1985). The natural RER expansion was reported for salmonids, e.g., by van Bohemen et al. (1981) who stated that in female rainbow trout during spawning and previtellogenic stages liver had more reduced levels of RER that when in vitellogenesis.

Partially in harmony with our results, in spawning rainbow trout males had a higher total RER volume per cell; but in both genders the RER filled 35% of the cytoplasm (Hampton et al., 1989). Data from both brown and rainbow trouts thus support that in periods other than the vitellogenesis, the hepatocytes of fish females may have in fact less RER than those of males. It is opportune to say that in adult male rainbow trout mean volume densities of RER fields in relation to cell were found to vary from as low as 9% (Arnold et al., 1995) to as high as 31% (Hampton et al., 1989). Considering the apparent plasticity of the RER in hepatocytes of trouts, it is thus understandable that in healthy immature brown trout the RER occupied only 10% of the hepatocyte (Schramm et al., 1998), a rather lower value when compared to those found here, pointing to the fact that besides seasonal influences, age changes can also occur.

As to other cytoplasmic compartments, glycogen deserves a separate comment because it assumed a relatively great spatial relevance (15 to 23% of the cell), despite the great variation (mainly in males). Our results give a morphological support for findings in post-reproductive two-years-old rainbow trout, in which PAS-positive staining intensity for glycogen showed great individual variations (Schär et al., 1985). Moreover, mean values for the relative cytoplasmic volume of glycogen were reported to vary in salmonids from as low as 29% to as high as 55%, in both adults males and juveniles (Arnold et al., 1995, 1996a,b; Moutou et al., 1997; Schramm et al., 1998). In addition to possible true interspecific differences, even within a family, glycogen content can thus greatly vary in a fish group. The poor capability of fishes in handling glucose and other sugars is currently much discussed (Peres et al., 1999), but their individual variability is still an unexplored topic. The relatively low values we found agree with the semiquantitative analysis of van Bohemen et al. (1981) in female rainbow trout, in which the lowest glycogen levels were found in both spawning and previtellogenic stages. Moreover, it was reported that higher temperatures (as in April) induce a decrease in the liver glycogen in several fishes (Dean and Berlin, 1969; Valtonen, 1974; Braunbeck et al., 1987; Segner and Braunbeck, 1990).

A further relevant finding is the higher volume per liver of dense bodies in females (see Table 4). In this study, the dense bodies grouped the peroxisomes and all forms of lysosomes/residual bodies. Based on our results on peroxisomes (Rocha et al., 1999), we can now estimate that at previtellogenesis the lysosomes fill about 1 to 2% of the cell volume respectively for males and females. So it is plausible that lysosomes were in fact the dense bodies available in greater amounts in our previtellogenic females. Considering the lysosome functions, the present results may reflect liver structural changes expected to occur after breeding (van Bohemen et al., 1981; Hampton et al., 1989). Quantitative data on the interhepatocytic macrophages from the same brown trout specimens already support that hypothesis (Rocha et al., 1997).

We believe to have made a significant contribution to improving the knowledge about brown trout liver by solely using design-based stereology, thus not depending on restrictive shape assumptions. In addition, this is also

the first stereological study on hepatocytes in which the precision of all the estimates were evaluated and related with the biological variation, based on recent statistical principles (West et al., 1991, 1996; Karlsson and Cruz-Orive, 1992). That precision should not be regarded as too much entailing because the evaluation of the sampling variances in systematic sampling designs is a topic of continued research in statistics and where only golden rules are available (Geinisman et al., 1997; Schmitz, 1997). Still, such approaches benefit both authors and readers in their ability to properly judge both the data and conclusions.

To design the best combination among sampling, counting and measuring, for achieving reliable estimates with both the minimal effort and cost as possible is not a trivial task when many stereological parameters are targeted, as it happened in this study. Our analysis supports that both sampling and counting efforts at the different levels of analysis were globally adequate facing the true biological variation among animals. Despite the fact that sampling improvements can be equated in the future, it must be taken into account that for reducing the SEM of a group to add more animals often has a greater impact, namely when they largely differ (Gundersen and Østerby, 1980; West, 1993). An important remark on the last aspect is that facing the biological variation we found in the stereological data from cultured fish (not correlated with the body size or length), one would expect an even higher variability in wild brown trout. Nevertheless, it must be also said that irrespective of how much higher the biological variability may be in fishes, or of how much lower precision is aimed for in each particular study, the use of systematic sampling together with stereological unbiased principles has been encouraged in order to get comparable and reliable results (Geinisman et al., 1997).

Acknowledgements. We express our gratitude to Dr. A.R. Santos and Mr. J.M. Gonçalves for printing the negatives. The fishes were generously provided by the Direcção Regional de Agricultura de Entre-Douro e Minho under an official co-operation protocol; in this regard, the commitment of Eng. A. Gonçalves, Eng. A. Maia and Eng. C. Pereira is greatly appreciated. This study was financially supported by the Fundação para a Ciência e Tecnologia (Lisbon, Portugal).

References

Arnold H., Pluta H.-J. and Braunbeck T. (1995). Simultaneous exposure of fish to endosulfan and disulfoton in vivo: ultrastructural, stereological and biochemical reactions in hepatocytes of male rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 33, 17-43.

Arnold H., Pluta H.-J. and Braunbeck T. (1996a). Cytological alterations in the liver of rainbow trout *Oncorhynchus mykiss* after prolonged exposure to low concentrations of waterborne endosulfan. Dis. Aquat. Org. 25, 39-52.

Arnold H., Pluta H.-J. and Braunbeck T. (1996b). Sublethal effects of prolonged exposure to disulfoton in rainbow trout (*Oncorhynchus mykiss*): cytological alterations in the liver by a potent acetylcholine esterase inhibitor. Ecotoxicol. Environ. Saf. 34, 43-55.

Au D.W.T., Wu R.S.S., Zhou B.S. and Lam P.K.S. (1999). Relationship between ultrastructural changes and EROD activities in liver of fish exposed to benzo[a]pyrene. Environ. Pollut. 104, 235-247.

Baddeley A.J., Gundersen H.J.G. and Cruz-Orive L.M. (1986). Estimation of surface area from vertical sections. J. Microsc. 142, 259-276.

Bioulac-Sage P., Dubuisson L., Bonjean P. and Balabaud C. (1984). L'analyse morphométrique du foie humain normal en microscopie électronique. Gastroenterol. Clin. Biol. 8, 856-860.

Blouin A., Bolender R.P. and Weibel E.R. (1977). Distribution of organelles and membranes between hepatocytes and nonhepatocytes in rat liver parenchyma - A stereological study. J. Cell Biol. 72, 441-455.

Braendgaard H. and Gundersen H.J.G. (1986). The impact of recent stereological advances on quantitative studies of the nervous system. J. Neurosci. Methods 18, 39-78.

Braunbeck T. (1998). Cytological alterations in fish hepatocytes following in vivo and in vitro sublethal exposure to xenobiotics - structural biomarkers of environmental contamination. In: Fish ecotoxicology. Braunbeck T., Hinton D.E. and Streit B. (eds). Birkhäuser Verlag Basel. Switzerland. pp 61-140.

Braunbeck T., Gorgas K., Storch V. and Völkl A. (1987). Ultrastructure of hepatocytes in golden ide (*Leuciscus idus melanotus* L.; Cyprinidae: Teleostei) during thermal adaptation. Anat. Embryol. 175, 303-313.

Braunbeck T., The S.J., Lester S.M. and Hinton D.E. (1992). Ultrastructural alterations in liver of medaka (*Oryzias latipes*) exposed to diethylnitrosamine. Toxicol. Pathol. 20, 179-196.

Christie G.S. and Le Page R.N. (1961). Enlargement of liver cell nuclei: effect of dimethylnitrosamine on size and deoxyribonucleic acid content. Lab. Invest. 10, 729-743.

Cochran C. (1977). Sampling techniques. 3rd edn. J Wiley & Sons. New York.

Coggeshall R.E. and Lekan H.A. (1996). Methods for determining numbers of cells and synapses: A case for more uniform standards of review. J. Comp. Neurol. 364, 6-15.

Daimon T., David H., van Zglinicki T. and Marx I. (1984). Morphometric study on the absolute volume of differentiating hepatocytes in rats. Exp. Pathol. 26, 141-149.

Dean J.M. and Berlin J.D. (1969). Alterations in hepatocyte function of thermally acclimated rainbow trout (*Salmo gairdneri*). Comp. Biochem. Physiol. 29, 307-312.

Fostier A., Jalabert B., Billard R., Breton B. and Zohar Y. (1983). The gonadal steroids. In: Fish physiology, reproduction, endocrine tissues and hormones. Vol. IXA. Hoar W.S., Randall D.J. and Donaldson E.M. (eds). Academic Press. pp 277-372.

Geinisman Y., Gundersen H.J.G., van der Zee E. and West M.J. (1997). Towards obtaining unbiased estimates of the total number of synapses in a brain region: problems of primary and secondary importance. J. Neurocytol. 26, 711-713.

Gundersen H.J.G. (1977). Notes on the estimation of the numerical density of arbitrary profiles: the edge effect. J. Microsc. 111, 219-223.

Gundersen H.J.G. (1986). Stereology of arbitrary particles. A review of unbiased number and size estimators and the presentation of some new ones, in memory of William R. Thompson. J. Microsc. 143, 3-45.

Gundersen H.J.G. (1988). The nucleator. J. Microsc. 151, 3-21.

Gundersen H.J.G. and Jensen E.B. (1987). The efficiency of systematic sampling in stereology and its prediction. J. Microsc. 147, 229-263.

Gundersen H.J.G. and Østerby R. (1981). Optimizing sampling efficiency of stereological studies in biology: or "Do more less well!". J. Microsc. 121, 65-73.

Gundersen H.J.G., Bagger P., Bendtsen T.F., Evans S.M., Korbo L., Marcussen N., Møller A., Nielsen K., Nyengaard J.R., Pakkenberg B., Sørensen F.B., Vesterby A. and West M.J. (1988). The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. APMIS 96, 857-881.

Hampton J.A., McCuskey P.A., McCuskey R.S. and Hinton D.E. (1985). Functional units in rainbow trout (*Salmo gairdneri*) liver: I. Arrangement and histochemical properties of hepatocytes. Anat. Rec. 213, 166-175.

Hampton J.A., Lantz R.C. and Hinton D.E. (1989). Functional units in rainbow trout (*Salmo gairdneri*, Richardson) liver: III. Morphometric analysis of parenchyma, stroma, and component cell types. Am. J. Anat. 185, 58-73.

Hinton D.E. and Couch J.A. (1998). Architectural pattern, tissue and cellular morphology in liver of fishes: Relationship to experimentally-induced neoplastic responses. In: Fish ecotoxicology. Braunbeck T., Hinton D.E. and Streit B. (eds). Birkhäuser Verlag Basel. Switzerland. pp 141-164.

Hinton D.E., Lantz R.C. and Hampton J.A. (1984). Effect of age and exposure to a carcinogen on the structure of the medaka liver: a morphometric study. Natl. Cancer Inst. Monogr. 65, 239-249.

Henrique R.M.F., Monteiro R.A.F., Rocha E. and Marini-Abreu M.M. (1997). A stereological study on the nuclear volume of cerebellar granule cells in ageing rats. Neurobiol. Aging 18, 199-203.

Howard C.V. and Reed M.G. (1998). Unbiased Stereology. Three-dimensional measurement in microscopy. Microscopy handbook series 41. Oxford. Bios Scientific Publishers.

Jack E.M., Bentley P., Bieri F., Muakkassah-Kelly S.F., Stäubli W., Suter J., Waechter F. and Cruz-Orive L.M. (1990a). Increase in hepatocyte and nuclear volume and decrease in the population of binucleated cells in preneoplastic foci of rat liver: a stereological study using the nucleator method. Hepatology 11, 286-297.

Jack E.M, Stäubli W., Waechter F., Bentley P., Suter J., Bieri F., Muakkassah-Kelly S.F. and Cruz-Orive L.M. (1990b). Ultrastructural changes in chemically induced preneoplastic focal lesions in the rat liver: a stereological study. Carcinogenesis 11, 1531-1538.

Loud A.V. (1968). A quantitative stereological description of the ultrastructure of normal rat liver parenchymal cells. J. Cell Biol. 37, 27-46.

Karlsson L.M. and Cruz-Orive L.M. (1992). The new stereological tools in metallography: estimation of pore size and number in aluminium. J. Microsc. 165, 391-415.

Mayhew T.M. (1997). Recent applications of the new stereology have thrown fresh light on how the human placenta grows and develops its form. J. Microsc. 186, 153-163.

Mendis-Handagama S.M.L.C. (1992). Estimation error of Leydig cell numbers in atrophied rat testes due to the assumption of spherical nuclei. J. Microsc. 168, 25-32.

Moutou K.A., Braunbeck T. and Houlihan D.F. (1997). Quantitative analysis of alterations in liver ultrastructure of rainbow trout *Oncorhynchus mykiss* after administration of the aquaculture antibacterials oxolinic acid and flumequine. Dis. Aquat. Org. 29, 21-34.

Norberg B., Björnsson B.T., Brown C.L., Wichardt U.-P., Deftos L.J. and Haux C. (1989). Changes in plasma vitellogenin, sex steroids, calcitonin, and thyroid hormones related to sexual maturation in female brown trout (*Salmo trutta*). Gen. Comp. Endocrinol. 75, 316-326.

Peres H., Gonçalves P. and Oliva-Teles A. (1999). Glucose tolerance in gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*). Aquaculture 179, 415-423.

Peute J., Huiskamp R. and van Oordt J.G.D. (1985). Quantitative analysis of estradiol 17ß-induced changes in the ultrastructure of the liver of the male zebra fish, *Brachydanio rerio*. Cell Tissue Res. 242, 377-382.

Rocha E., Lobo-da-Cunha A., Monteiro R.A.F., Silva M.W. and Oliveira M.H. (1999). A stereological study along the year on the hepatocytic peroxisomes of brown trout (*Salmo trutta*). J. Submicrosc. Cytol. Pathol. 31, 91-105.

Rocha E. and Monteiro R.A.F. (1999). Histology and cytology of fish liver: A review. In: Ichthyology: Recent research advances. Saksena D.N. (ed). Science Publishers, Inc. Enfield, New Hampshire, USA, pp 321-344.

Rocha E., Monteiro R.A.F. and Pereira C.A. (1994a). The liver of the brown trout, *Salmo trutta fario*: a light and electron microscope study. J. Anat. 185, 241-249.

Rocha E., Monteiro R.A.F. and Pereira C.A. (1994b). Presence of rodlet cells in the intrahepatic biliary ducts of the brown trout, *Salmo trutta fario* Linnaeus, 1758 (Teleostei, Salmonidae). Can. J. Zool. 72, 1683-1687.

Rocha E., Monteiro R.A.F. and Pereira C.A. (1995). Microanatomical organization of hepatic stroma of the brown trout, *Salmo trutta fario* (Teleostei, Salmonidae): a qualitative and quantitative approach. J. Morphol. 223, 1-11.

Rocha E., Monteiro R.A.F. and Pereira C.A. (1996). The pale-grey interhepatocytic cells of brown trout (*Salmo trutt*a) are a subpopulation of macrophages or do they establish a different cellular type? J. Submicrosc. Cytol. Pathol. 28, 357-368.

Rocha E., Monteiro R.A.F. and Pereira C.A. (1997). Liver of the brown trout, *Salmo trutta* (Teleostei, Salmonidae): a stereological study at light and electron microscopic levels. Anat. Rec. 247, 317-328.

Romagna F. and Zbinden G. (1981). Distribution of nuclear size and DNA content in serial liver biopsies of rats treated with N-nitrosomorpholine, phenobarbital and butylated hydroxytoluene. Exp. Cell Biol. 49, 294-305.

Saper C.B. (1996). Any way you cut it: a new journal policy for the use of unbiased counting methods. J. Comp. Neurol. 364, 5.

Schär T.W., Maly I.P. and Sasse D. (1985). Histochemical studies on metabolic zonation of the liver in the trout (*Salmo gairdneri*). Histochemistry 83, 147-151.

Scherle W. (1970). A simple method for volumetry of organs in quantitative stereology. Mikroskopie 26, 57-60.

Schmidt F.C. and Weber L.J. (1973). Metabolism and biliary excretion of sulfobromophthalein by rainbow trout. J. Fish Res. Bd. Can. 30, 1301-1308.

Schmitz C. (1997). Towards more readily comprehensible procedures in disector stereology. J. Neurocytol. 26, 707-710.

Schramm M., Muller E. and Triebskorn R. (1998). Brown trout (*Salmo trutta f. fario*) liver ultrastructure as a biomarker for assessment of small stream pollution. Biomarkers 3, 93-108.

Segner H. and Braunbeck T. (1990). Adaptative changes of liver composition and structure in golden ide during winter

acclimatization. J. Exp. Zool. 255, 171-185.

Selman K., Wallace R.A. and Barr V. (1987). The relationship of yolk vesicles and cortical alveoli in teleost oocytes and eggs. In: Reproductive physiology of fish. Idler D.R., Crim L.W. and Walsh J.M. (eds). Newfoundland: Memorial University. St. Johns. pp 216.

Sterio D.C. (1984). The unbiased estimation of number and sizes of arbitrary particles using the disector. J. Microsc. 134, 127-136.

Valtonen T. (1974). Seasonal and sex-bound variation in carbohydrate metabolism of the liver of whitefish. Comp. Biochem. Physiol. 47A, 713-727.

van Bohemen Ch.G., Lambert J.G.D. and Peute J. (1981). Annual changes in plasma and liver in relation to vitellogenesis in the female rainbow trout, *Salmo gairdneri*. Gen. Comp. Endocrinol. 44, 94-107.

Washburn B.S., Frye D.J., Hung S.S.O., Doroshov S.I. and Conte F.S. (1990). Dietary effects on tissue composition, oogenesis and the reproductive performance of female rainbow trout (*Oncorhynchus mykiss*). Aquaculture 90, 179-195.

Watanabe T. and Tanaka Y. (1982). Age-related alterations in size of human hepatocytes: a study of mononuclear and binucleate cells. Virchows Arch. (B) 39, 9-20.

Weibel E.R. (1979). Stereological methods. Vol I. Practical methods for biological morphometry. Academic Press. London.

Weibel E.R., Stäubli W., Gnägi H.R. and Hess F.A. (1969). Correlated morphometric and biochemical studies on the liver cell. J. Cell Biol. 42, 68-91.

West M.J. (1993). New stereological methods for counting neurons. Neurobiol. Aging 14, 275-285.

West M.J., Slomianka L. and Gundersen H.J.G. (1991). Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. Anat. Rec. 231, 482-497.

West M.J., Østergaard K., Andreassen O.A. and Finsen B. (1996). Estimation of the number of somatostatin neurons in the striatum: an in situ hybridization study using the optical fractionator method. J. Comp. Neurol. 370, 11-22.

Accepted December 7, 2000