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Quantitative analysis of estradiol-17β-induced changes in the ultrastructure of the liver of the male zebrafish, *Brachydanio rerio*

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Summary. Hepatocytes of male zebrafish, *Brachydanio rerio*, were studied by means of light- and electron-microscopy, following a period of maximally 16 days of in-vivo treatment with estradiol-17β. The responsiveness of the male hepatocytes to this female sex steroid was investigated by use of morphometric methods. The results of this investigation show that the responsiveness was most obvious between 2 and 16 days, as revealed by an increase in cell size, accompanied by a proliferation of the granular endoplasmic reticulum and the Golgi complex. In addition, accumulations of glycogen granules, which are characteristic of hepatocytes in untreated males, had disappeared and lipid droplets had accumulated. These experimentally induced changes in the morphology of the male hepatocyte closely resemble those described for the female hepatocyte during the sexual cycle. It is concluded that the hepatocytes of male zebrafish can be stimulated by estradiol-17β to produce vitellogenin and that in female zebrafish this steroid is a key sex hormone responsible for vitellogenin production by the liver during the natural sexual cycle.

Key words: Liver – Hepatocytes – Ultrastructure – Estradiol – Vitellogenesis – Teleosts

Under laboratory conditions, the female zebrafish is capable of oviposition once every five days. In a previous study it was shown that during this five-day period the ultrastructure of the hepatocyte is subject to several changes (Peute et al. 1978). According to these authors the onset of vitellogenin production is morphologically reflected by an increase in the relative volume of granular endoplasmic reticulum (GER) and Golgi apparatus, as well as by the disappearance of glycogen granules together with the appearance of large lipid droplets. Similar ultrastructural changes, related to vitellogenin production, were observed in hepatocytes of the female rainbow trout, *Salmo gairdneri*, during the annual cycle (Van Bohemen et al. 1981). In several oviparous teleosts vitellogenin production in the liver could be induced with estradiol-17β (Emmerson 1976; Emmerson and Petersen 1976; Campbell and Idler 1976; Van Bohemen 1981; Van Bohemen et al. 1982a; Ng and Idler 1983). Comparable results were obtained in other oviparous vertebrates (Follet and Redshaw 1974; Tata 1976; Wallace 1978).

As the ovaries of *Brachydanio rerio* are able to synthesize estradiol-17β (Lambert and Van Oordt 1974; Lambert 1978), it was assumed that during the reproductive cycle the ultrastructural changes in the hepatocytes were induced by this steroid (Peute et al. 1978). The present study was carried out to test this hypothesis. Male zebrafish, with an expected negligible synthesis of endogenous estrogens, were treated with estradiol-17β. Subsequently, the hepatocytes were studied with the electron microscope.

Materials and methods

Animals. Adult male zebrafish were kept under laboratory conditions in fresh water at 25° C with a 13 h light/11 h dark rhythm. To minimize the variation between the experimental groups, animals were randomly collected and divided amongst the three experimental groups:

Group 0: Ten fish, kept in one aquarium containing 10 l water.

Group I: Three subgroups of 10 animals each, kept in three separate aquaria. Every aquarium contained 10 l water to which 10 µg estradiol-17β was added, dissolved in 0.1 ml dimethyl formamide (DMF; Merck, Darmstadt, FRG). The specimens of groups I.2, I.4 and I.16 were treated for 2, 4 and 16 days, respectively.

Group II: Same as group I, but estradiol-17β was omitted. This group serves as a "vehicle" control group.

Water was renewed at the end of every second day, and the different solutions added. Dry food was given every day until the day before fixation. From each subgroup five animals were chosen at random, killed and small pieces of liver were processed for electron microscopy.

Electron-microscopic techniques. After dissection, three small pieces of the liver of each animal were prefixed at room temperature in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 1 h and postfixed in 2% osmium tetroxide in the same buffer for 1 h at 0° C. Subsequently,

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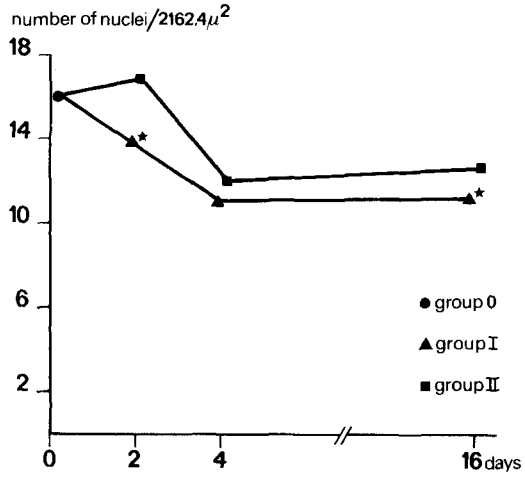

Figs. 1–5. *Group 0:* untreated controls; *group I:* males treated with estradiol-17β in DMF as the solvent; *group II:* males treated with DMF only. Mean values are given (n = 5); *indicates significance between DMF- treated and DMF + estradiol-17β-treated groups ($P < 0.05$)

Fig. 1. Number of nuclei per standard area in semithin sections of the liver of controls (O) and at different times of treatment with estradiol-17β (I) and DMF (II). The number is inversely proportionate to the cell size

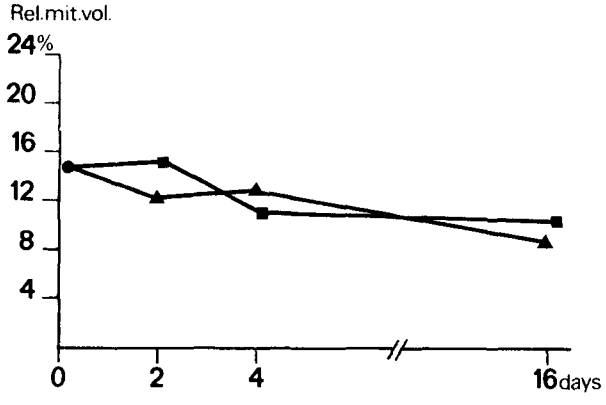

Fig. 2. Relative volume of mitochondria, at different times of treatment

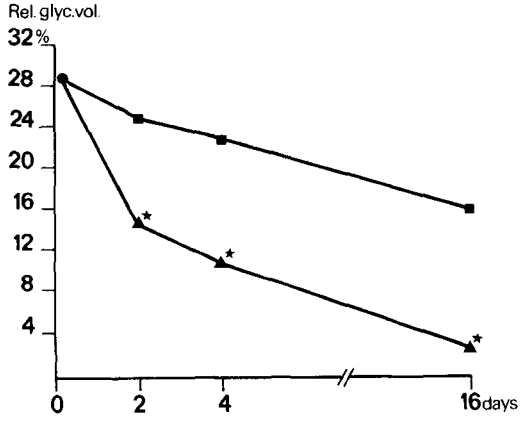

Fig. 3. Relative volume of glycogen concentrations, at different times of treatment

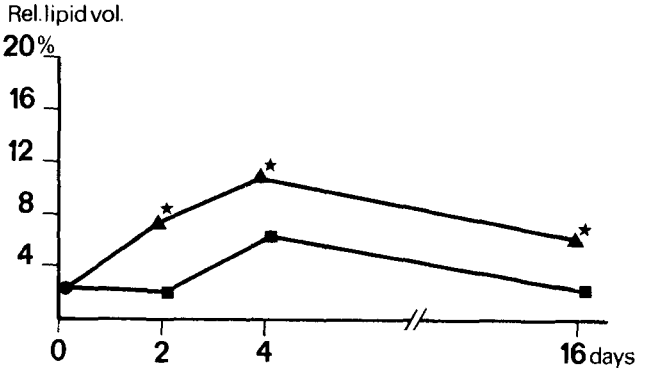

Fig. 4. Relative volume of lipid droplets, at different times of treatment

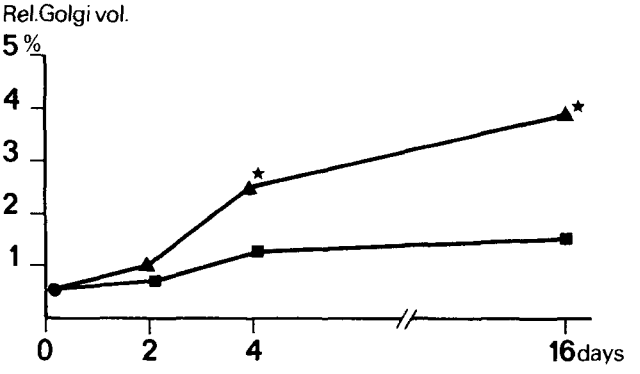

Fig. 5. Relative volume of Golgi area, at different times of treatment

Table 1. Overall mutual differences between the main groups 0 (control), I (estradiol-17β + DMF), and II (DMF)

Parameters	0–I	0–II	I–II
Number of nuclei/surface area	$+^{a}$	–	–
Rel. vol. Golgi area	$+^{a}$	$+^{a}$	$+^{b}$
Rel. vol. mitochondria	–	–	–
Rel. vol. lipid droplets	$+^{c}$	–	$+^{c}$
Rel. vol. glycogen area	$+^{a}$	–	$+^{b}$

$+^{a}$, $P < 0.001$; $+^{b}$, $P < 0.01$; $+^{c}$, $P < 0.05$ and –, not significant

tissue pieces were dehydrated in graded ethanols and embedded in Epon. According to the principle of "stratified random sampling" (Weibel 1969, 1979), thin sections (60 nm) were taken from each liver piece at 50 μm intervals, and stained with uranyl acetate in 70% methanol and lead citrate (Reynolds 1963). Of these sections, micrographs were recorded, with respect to one specified corner of the supporting grid, with a Zeiss EM 9A electron microscope at calibrated magnification. in total, 20 micrographs were recorded and printed at a final magnification of 17400×. The relative volumes of mitochondria, Golgi complex, glycogen concentrations and lipid droplets in the hepatocytes were estimated in each micrograph using the point-counting method with a transparent test-point lattice with 225 systematically spaced points at a distance of 10 mm. Test points falling on the nucleus and on extracellular structures

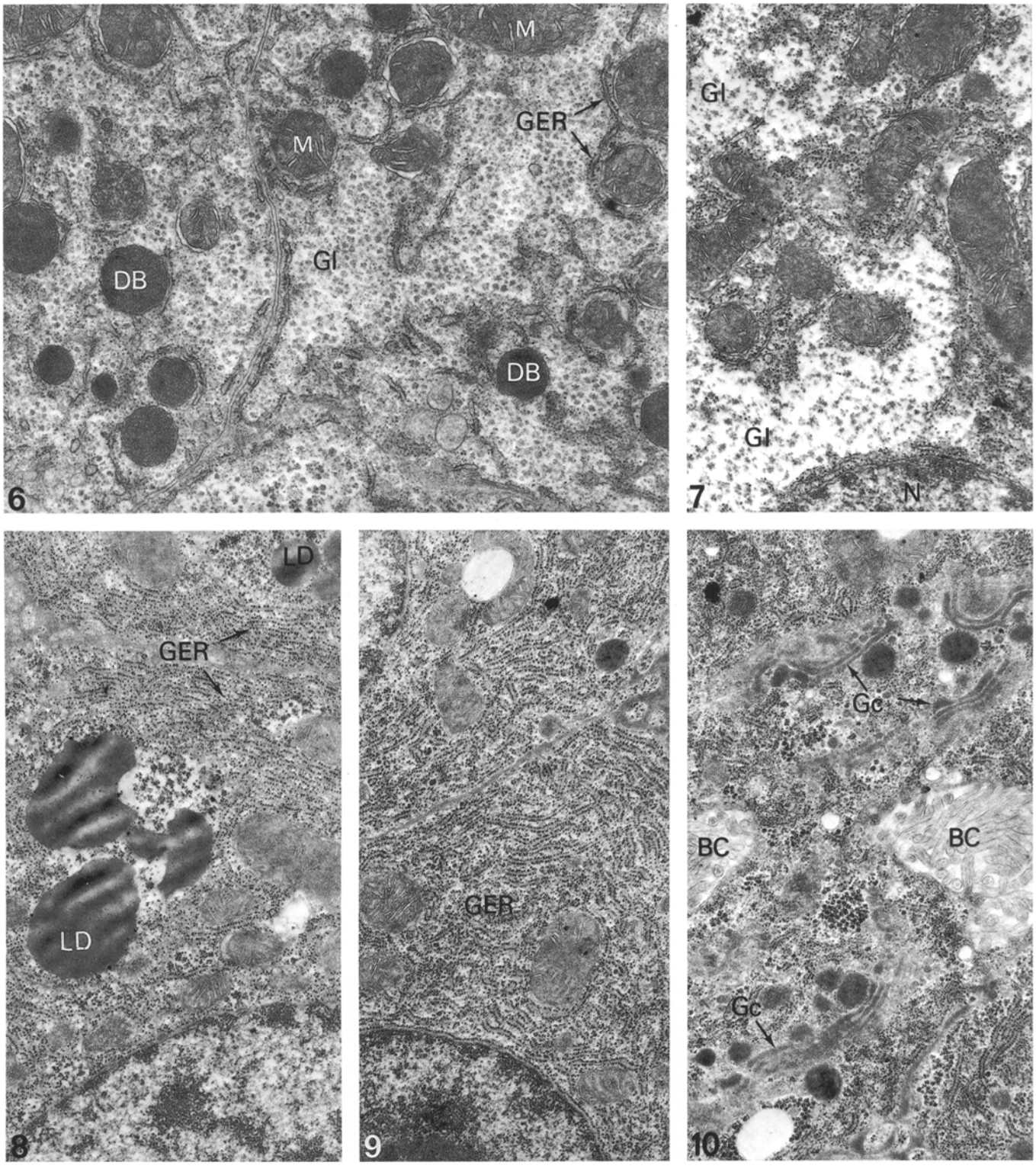

Fig. 6. Hepatocytes of untreated controls (group 0). *DB* dense bodies; *Gl* glycogen area; *M* mitochondrion; *GER* granular endoplasmic reticulum. × 17400

Fig. 7. Hepatocyte after DMF-treatment for two days (group II-2). *Gl* glycogen; *N* nucleus. × 17400

Fig. 8. Hepatocyte after two-day treatment with estradiol-17β in DMF (group I-2). Note the appearance of lipid droplets (*LD*) and an increase in *GER*. × 17400

Fig. 9. Hepatocyte after 16-day treatment with estradiol-17β in DMF (group I-16). Note the strong increase in *GER* (compare with Fig. 6). × 17400

Fig. 10. Hepatocyte after 16-day treatment with estradiol-17β in DMF (group I-16). Note the presence of electron-dense material in all Golgi cisternae (*Gc*). *BC* bile canaliculus occupied by microvilli. × 17400

such as bile canaliculi and blood sinusoids, were subtracted from the total number of test points. Thus, the relative volumes of the various organelles were expressed as percentages of the remaining cytoplasmic volume. In each subgroup of 5 animals, the arithmetic mean of the relative volume of the various cell organelles was calculated from the pooled arithmetic means of each animal, obtained from the individual data of the 20 electron micrographs per animal.

For the statistical evaluation of the results, a Mann-Whitney U-test was used to compare the estradiol-treated group (I) and the vehicle-control group (II) at the various periods of treatment. Significant differences ($P<0.05$) between these two groups are indicated by asterisks in Figs. 1–5. Furthermore, the Mann-Whitney U-test was extended to three pairs of samples, i.e., 0–I.2, 0–I.4 and 0–I.16 to enable an overall comparison between the groups 0 and I. In a similar manner, the groups 0 and II, and the groups I and II were compared (see Table 1).

Light-microscopic techniques. The relative volume of the hepatocytes was estimated in light micrographs from 1-µm thick Epon sections, stained with methylene blue-azur II. At a magnification of 700×, the number of nuclei was counted in an area of 2162.4 µm^2. Since the hepatocytes of *Brachydanio rerio* are uninucleate (Weis 1972; Peute, unpublished results), this number is directly related to the number of hepatocytes in the investigated area (Weibel 1969).

Results

Electron micrographs of hepatocytes of untreated male zebrafish generally show only few and short cisternae of the granular endoplasmic reticulum (GER), which are in close topographical relation with the mitochondria (Fig. 6). The Golgi complex is poorly developed and the contents of its cisternae are not electron dense. Lipid droplets are only incidentally observed. The remainder of the cytoplasm is almost exclusively occupied by glycogen concentrations (Fig. 6). A common feature is the presence of intercellular bile canaliculi, the latter often close to the Golgi area (Fig. 10).

The results of the estimation of the relative volume of hepatocytes, mitochondria, glycogen concentration, lipid droplets and Golgi complex are summarized in Figs. 1–5 and in Table 1. The hepatocytes in the estradiol-treated males have increased in size, as indicated by a decrease in number of cell nuclei per measured area (Fig. 1). This increase is accompanied by a strong development of GER, already noticeable after two days of treatment (Figs. 7, 8). Prolonged treatment results in a further development of GER (Fig. 9). In the DMF-group the hepatocyte volume, as measured by the number of nuclei per unit area, does not differ significantly from the untreated controls, or from the estradiol-treated group (Table 1, 0–II and I–II). The relative mitochondrial volume changes very little after estradiol treatment, or as a result of DMF treatment alone (Fig. 2; Table 1).

The relative volume of the glycogen concentrations, amounting to about 30% in the hepatocytes of untreated controls, decreases to about 3% after 16 days of treatment with estradiol-17β in DMF (Figs. 3, 7–9). With DMF alone no significant decrease was obtained (0–II in Table 1).

Lipid droplets are rarely observed in hepatocytes of the untreated controls, but they are frequently present after two and four days of estradiol treatment and, to a less extent, after four days with DMF alone (Figs. 4, 8). Comparison of Fig. 4 and Table 1 shows that the significant difference between the groups 0 and I, and also between groups I and II is obviously based on the first four days of treatment.

The relative volume of the Golgi complex is less than 0.3% in the untreated controls and increases to almost 4% in hepatocytes of 16 day estradiol-treated males. In DMF alone its relative volume increases to only less than 2% of the cytoplasm after 16 days of treatment (Fig. 5). The increase due to addition of estradiol-17β is significantly stronger than after DMF alone (compare rel. vol. Golgi area, I–II, Table 1). In addition, following estradiol treatment, both the Golgi cisternae and vesicles contain electron-dense material, which is especially obvious in the 16 day-treated group (Fig. 10).

Discussion

The ultrastructure of hepatocytes in untreated male *Brachydanio rerio* resembles that in female fish before spawning, i.e., when most of the liver cells contain high glycogen concentrations, few or no lipid droplets and individual cisternae of GER rather than parallel stacks (Peute et al. 1978). In males, however, the glycogen concentrations are more extended, indicating that the total glycogen content of their liver is higher than that in females. A similar sexual dimorphism concerning liver glycogen content can be observed in other teleosts, such as the white fish, *Coregonus nasus* (Valtonen 1974) and the brook trout, *Salvelinus fontinalis* (Whiting and Wiggs 1978). According to Valtonen (1974) the lower glycogen content of the female liver is caused by the presence of estrogens that inhibit carbohydrate metabolism.

In untreated male *Brachydanio rerio* both the GER and the Golgi complex of the hepatocytes are less developed than those in the female hepatocytes, probably reflecting a lower synthesis and secretion rate for proteins in the male liver, which is not charged with a (cyclic) high production of vitellogenin.

The estradiol-17β-induced changes in hepatocytes of male zebrafish, such as decrease in glycogen concentrations and increase in lipid content, as well as GER and Golgi activity were also observed in hepatocytes of other teleosts treated with this hormone. Olivereau and Olivereau (1979) measured a rapid decrease in glycogen and an increase in the number of lipid droplets in the liver of female freshwater eel treated with estradiol-17β. Similarly, this hormone caused a rapid fall in liver glycogen reserve in female *Barbus conchonius* (Khanna and Singh 1983); in the male Singi fish, *Heteropneustes fossilis,* liver glycogen was depleted and its lipid content increased after injection with estradiol dipropionate (Dasmahapatra and Medda 1982). Recently opposite results were reported for the immature female red grouper, *Epinephelus akaara,* in which estradiol-17β induced a rise in liver glycogen content (Ng et al. 1984). In hepatocytes of *Carassius auratus* the GER was increased and the glycogen depleted after treatment with estrogens

(Ishii and Yamamoto 1970). Similarly, in male *Plecoglossus altivelis* estradiol-17β induced proliferation of GER and Golgi complex in the hepatocytes, additionally accompanied by the appearance of "Female Specific Plasma Protein" in the blood (Aida et al. 1973). Recently, further evidence has been reported for the in-vitro stimulation by estradiol of protein synthesis in salmon hepatocytes, probably involving vitellogenesis (Bhattacharya et al. 1985). It is suggested that vitellogenin is indeed synthesized by the GER and subsequently packed by the Golgi complex (Hahn 1967; Lewis et al. 1976; Carinci et al. 1979; Van Bohemen et al. 1981). Against this background the present results favour the hypothesis that also in *Brachydanio rerio* estradiol-17β triggers vitellogenin production in the liver, since similar changes were observed in hepatocytes of untreated females at the onset of vitellogenesis (Peute et al. 1978).

This does not exclude that under natural circumstances in the female fish other estrogenic hormones are involved in the regulation of vitellogenin synthesis (Van Bohemen 1981; Van Bohemen et al. 1982b). Moreover, recent publications of Khanna and Singh (1983) and Ng et al. (1984) indicate that in the liver estradiol-17β might also stimulate other metabolic processes associated with vitellogenesis and energy requirement of the growing oocytes.

As far as the liver is concerned, it appears that application of DMF as a solvent for steroids may slightly affect the experimental results. In mammals high doses of DMF (100–400 ppm), administered over long periods, caused damage of liver and kidneys (Portman and Wilson 1971). The male zebrafish were exposed to only 10 ppm, which is far below a "no-effect level" (<7300 ppm) observed in *Salmo gairdneri* (Strubat et al. 1982). Therefore it is concluded that, under the present experimental conditions, estradiol-17β is indeed responsible for most of the changes observed in the ultrastructural appearance of the hepatocytes in male *Brachydanio rerio*.

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