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Weaning experiments with turbot (*Scophthalmus maximus*): electron microscopic study of liver

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Abstract. Weaning, i.e. the change from live to dry food, is a critical stage in the rearing of turbot (*Scophthalmus maximus*). In order to obtain a more detailed understanding of the associated functional processes, an electron microscopical study of liver morphological alterations before, during and after the weaning process was conducted. Three days before the start of weaning, slight malnutrition of fish was suggested by histological features such as nuclear glycogen deposition, mitochondrial swelling, together with a low level of glycogen and lipid stores. Lipid material was repeatedly located within cisternae of endoplasmic reticulum. Intra-cisternal lipid deposition (steatosis) in the liver was dramatically increased 3 d after the start of weaning. At the end of the weaning period, steatosis disappeared while cytoplasmic lipid deposition was enhanced. Although the weaning process dramatically increased hepatocellular steatosis, this phenomenon is obviously not caused by weaning – since it was already present during the pre-weaning period. As a speculation, steatosis in the liver of larval turbot may be the result of ontogenetic changes in intermediary metabolism, with the magnitude of this process being controlled by nutritional factors.

Introduction

Cultivation of turbot, *Scophthalmus maximus*, is of great economical value in European aquaculture (see Iglesias et al. 1987). Problems in turbot culture are encountered mainly during larval rearing and during the subsequent weaning period (e.g. Jones et al. 1981, Bromley and Sykes 1985, Cousin and Baudin-Laurencin 1986). In order to get a more comprehensive understanding of the weaning-associated morphofunctional changes in turbot, we conducted studies on the histological and cytological alterations occurring in the gut (Segner et al. 1988) and in the liver of young turbot during and after the weaning period.

The digestive tract was found to be fully differentiated at the onset of weaning. During weaning, activities of intestinal proteolytic enzymes (trypsin, aminopeptidase) were enhanced and mucosal volume increased significantly (125% 18 d after the start of weaning) (Segner et al. 1988). Volume augmentation of the intestinal mucosa was paralleled by an increase in mucosal surface area. It was concluded that turbot's ability to adapt to the weaning diet is not limited by the functional capacity of the intestine.

In the present paper, we describe changes in liver morphology as they develop during and after the weaning period in young turbot, *Scophthalmus maximus*.

Material and methods

Turbot (*Scophthalmus maximus*) larvae originating from one egg batch were reared by techniques previously described (Witt et al. 1984). Larvae were kept in warm water (mean 16.2 °C range 14.8° to 17.7 °C) until metamorphosis which occurred 24 d after hatch. Water flow was elevated from 1 l min^{-1} a the start of the experiment to 3 l min^{-1} at the end. Water salinity varied between 1.5 and 2‰; oxygen saturation was always greater than 80%; NH_4-N was <0.5 mg N l^{-1} and nitrite <0.1 mg l^{-1}.

Larvae were reared in quadratic tanks (1 m^2, 250 l vol.). As first food, rotifers were used. From Day 16 onwards larvae were fed twice a day with enriched Artemia nauplii (Codes AF, EG; 12 h selco, Artemia Systems), at a prey density of 2 nauplii ml^{-1}. Larvae (24-d-old) were gradually transferred from live to dry food ("weaning"). During a 7-d period, larvae were offered both live Artemia nauplii as well as a dry food. Later, dry food was given exclusively. Automatic feeders were used but additional food was offered manually to control feeding behaviour. The dry food was a commercial formulation (Az 25, TetraWerke, Melle, FRG), which has been used successfully in weaning studies with turbot (Segner et al. 1988). The diet contained 64% crude protein and 13% crude fat. During Days 1 to 9 of weaning dry food was offered as 200 to 500 μm particles; during Days 10 to 18 as 200 to 1000 μm particles; during Days 19 to 28 as 500 to 1000 μm particles; during Days 29 to 42 as >1000 μm; and from Day 42 onwards as sticks (2 mm). Three days before weaning (−3 d) and at various days after the start samples were taken for weight and length analysis as well as for histology. The specific growth rate G was determined according to

$$G=[(\ln W_D-\ln W_d)/(D-d)]\times 100, \quad (1)$$

where W_D is fish weight on Day D, W_d is fish weight on Day d and $(D-d)$ is the time between weighing in days.

For histological studies, 20 specimens were sampled at each sampling date: four were fixed for electron microscopy and 16 for light microscopy (six for paraffin sections, ten for cryo-sections). Light microscopical examinations were mainly used to test to what extent the ultrastructural findings, which were obtained from four individuals only, are representative for a larger sampling group.

From six individuals, whole viscera were fixed in Carnoy's fluid (Pearse 1961) and embedded in paraffin using standard techniques. Sections 7 μm thick were stained for RNA/DNA using methyl-green-thionine (Roque et al. 1965; this method allows a gross assessment, at the light microscopical level, of the performance of the rough endoplasmic reticulum) and for glycogen using Best's carmine (Pearse 1961). In addition, ten individuals were fixed in formalin-calcium and were shock-frozen with liquid nitrogen. Frozen sections 10 μm of digestive tract, including the liver, were cut on a Reichert Frigocut cryostat and stained for lipids with Sudan Black B (Pearse 1961).

For electron microscopy, four specimens were fixed at each sampling date. Livers were dissected under a dissecting microscope and fixed for several days in 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.4 to 7.6. Samples were then rinsed in buffer and postfixed in 2% osmium ferrocyanide (Karnovsky 1971). Tissues were rinsed again in cacodylate buffer, then in 0.05 *M* maleate buffer, pH 5.2, and stained en bloc overnight in 1% uranyl acetate in maleate buffer. Tissues were dehydrated in a graded series of ethanol and embedded in Spurr's resin (Spurr 1969). Semithin sections (0.5 μm) and ultrathin sections were prepared using glass knives on a Reichert Ultramikrotom OM U2. Semithin sections were stained with methylenblue-azur II according to Richardson et al. (1960) and analysed with a Leitz photo-microscope. Ultrathin sections were mounted on uncoated copper grids and the contrast enhanced by staining with alkaline lead citrate. Examination was performed in a ZEISS EM 9 S-2 transmission electron microscope.

Stereologicl analyses were performed using the point counting method of Weibel (1979) as described in a previous paper (Segner and Braunbeck 1990a). For each sampling date, livers of four turbot were analysed, examining 6 to 12 sections from 3 to 6 tissue blocks of each of the four. The volume density V_{Vi} of structures, i.e. the volume fraction or percentage within a given reference volume which is occupied by a given structure, was determined by placing a lattice of test points (P_T) on a micrograph and by determining the number of test points (P_{Pi}) enclosed within profiles of the structure investigated. The volume density V_{Vi} was calculated from

$$V_{Vi} = P_{Pi}/P_T. \quad (2)$$

As a reference volume, hepatocytic tissue was chosen. Thus, test points falling on extrahepatocellular structures (bile canaliculi, sinusoids, stroma tissue, etc) were subtracted from the total number of test points.

Nuclear volume density was measured from light micrographs at a final magnification of 1100× using a test lattice with 391 test points. Volume density of lipid-inclusions as well as volume density of glycogen-fields were measured on electron micrographs at a final magnification of 7200× using a test lattice with 96 test points.

The number of hepatocytes per cm^3 liver tissue was measured indirectly from the number of hepatocyte nuclei. The number of nuclei was calculated according to the formula of Weibel (1979), i.e.

$$N_V = 1/b \times [(N_{An})^{3/2}/V_{Vn}]^{1/2} \quad (3)$$

where N_{An} is the number of nuclear profiles actually counted divided by the test area, V_{Vn} is the nuclear volume density and b is a shape-dependent coefficient (1.38).

Absolute values (μm^3) of hepatocyte volume and cellular lipid volume were calculated assuming a value of 0.82 cm^3 hepatocytic tissue within 1 cm^3 liver tissue.

Statistical differences between mean values from morphometric measurements were compared using the non-parameteric Mann Whitney *U*-test (Sachs 1984). The significance level adopted throughout the study was $p<0.05$.

Table 1. *Scophthalmus maximus*. Specific growth rates and mortality data from weaning experiment with turbot. The results are means from two replicates. The start of weaning was counted as Day 0. For mortality calculations, fish removed for histological examination etc have been considered as alive. Specific growth rate was calculated according to Castell and Tiews (1980)

	Period (d)			
	−3 to 0	0 to +10	+10 to +18	+18 to +50
Mortality %	2.6	0.4	0.4	0.4
Specific growth rate (% d)	8.17		8.06	

Results

Growth

Growth rates and mortality data are summarized in Table 1. Results of growth performance have been discussed previously (Segner et al. 1988) and will be not treated in this communication.

Stereology

Quantitative findings on hepatocyte morphology are given in Table 2. Hepatocellular size was least prior to the start of weaning. Later, a significant increase in cell volume occured, with a maximum at Day 18. Since no equivalent enlargement of nuclear size occured the nucleus-cytoplasmic ratio decreased. Maximum values for both lipid and glycogen, could be measured in liver parenchymal cells at Day 18. Despite the reduction in lipid contents per hepatocyte between Days 18 and 50, the size of single, cytoplasmic, lipid droplets increased, as evidenced by the percentage of hepatocytes containing lipid droplets larger than 4 to 6 μm in diameter.

Table 2. *Scophthalmus maximus*. Stereological data from hepatocytes of turbot, before, during and after weaning

Parameter	Day −3	Day +3	Day +18	Day +50
Hepatocyte volume (μm^3)	2180±268	2810±191	7010±389	5000±275
Nucleus/cytoplasmic ratio	0.13	0.09	0.05	0.06
Lipid vol hepatocyte^{-1} (%)	5.0	30.7	36.5	19.8
Glycogen area hepatocyte^{-1} (%)	7.1	0.5	38.2	26.6
Percent hepatocytes with lipid droplets >4–6 μm diam	0	0	37	82

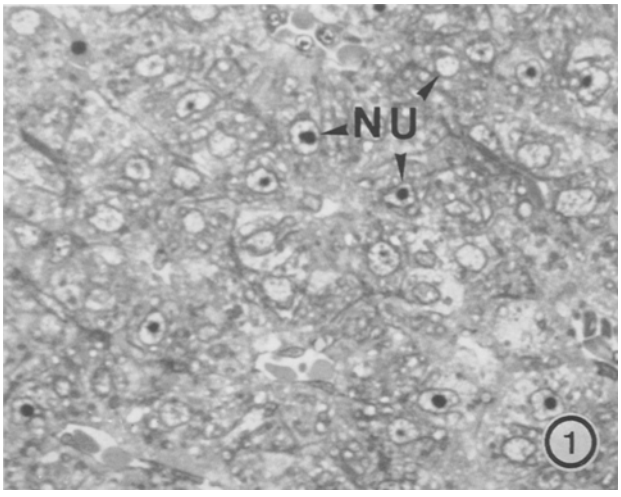
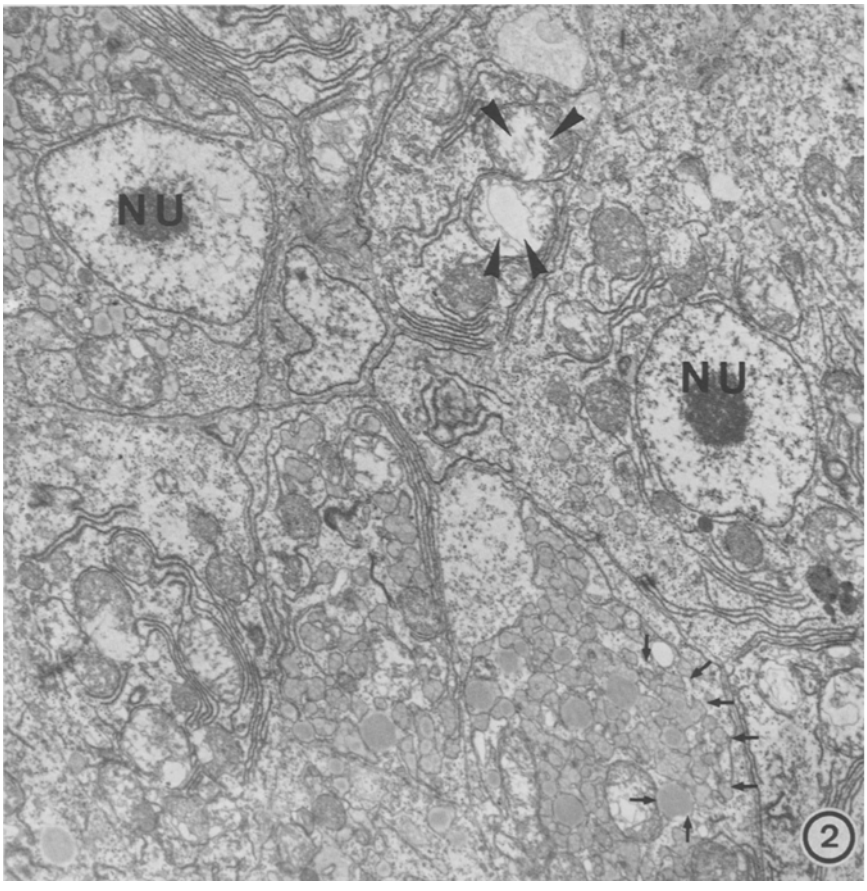
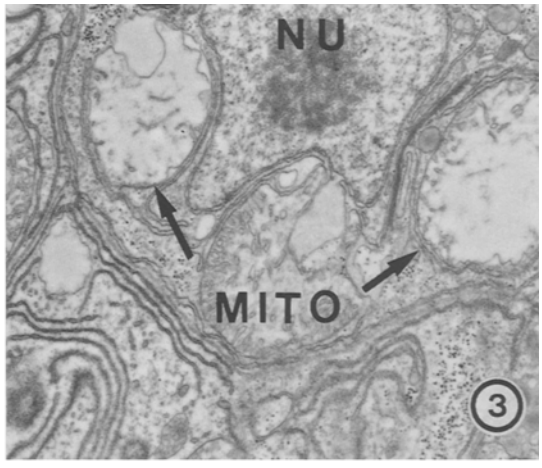
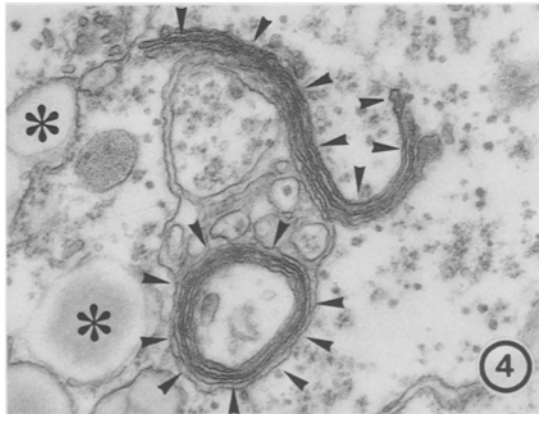


Fig. 1. *Scophthalmus maximus*. Light micrograph of liver parenchyma; 3 d prior to weaning (−3 d); NU: nucleus; Semithin section; Richardson stain; × 500

Figs. 2–4. *Scophthalmus maximus*. Low power electron micrographs of liver parenchyma of turbot 3 d prior to weaning (−3 d). Fig. 2. The cells contain small lipid inclusions (arrows) and oedemic mitochondria (arrowheads). ER cisternae are single-stranded or form small stacks of not more than 3 to 4 cisternae. NU: nucleus; × 3300. Fig. 3. Hepatocyte from turbot. Note the lobulated nucleus (NU) and the swollen mitochondria (MITO), with altered matrical structure; × 7900. Fig. 4. Dictyosomes (arrowheads) from the liver. The circular arrangement of cisternae is typical for pre-weaning fish and was not observed after start of weaning. Isolated, dispersed rosettes of glycogen are present. Asterisk: electron-lucent lipid droplet; × 17 800

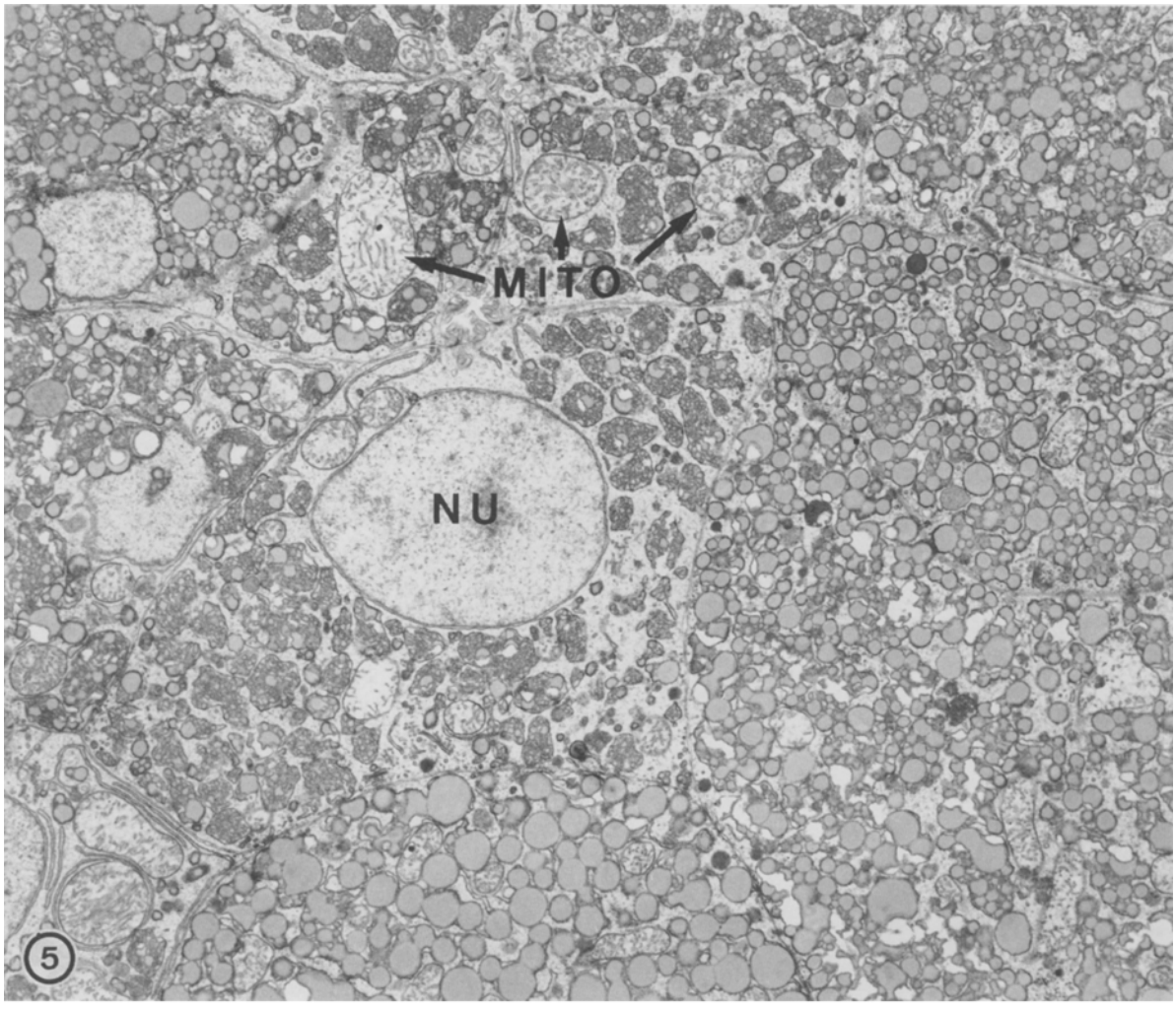






Cytology

Three days before start of weaning (−3 d), at the light microscopical level, the liver parenchyma of turbot larvae displayed predominantly small, moderately basophilic hepatocytes (Fig. 1). In addition, approximately 20% less basophilic hepatocytes occurred which, as revealed by the electron microscope, contained small (1 to 3 μm diam), cytoplasmic droplets not surrounded by a membrane (Fig. 4). However, in some cases small lipid droplets could be also found within the cisternae of the endoplasmic reticulum (ER). A third type of lipid inclusion consisted of osmiophilic droplets with a diameter always less than 0.5 μm, which were enclosed by a smooth membrane. Glycogen deposition was restricted to isolated, dispersed rosettes or small aggregations of rosettes (Fig. 4). The lobulated hepatocellular nuclei displayed an electron-lucent karyoplasm and a prominent nucleolus (Figs. 1 to 3). Mean nuclear volume was 254 μm. Intranuclear glycogen deposits could occasionally be seen. Many mitochondria were swollen (Figs. 2 and 3), exhibiting pale matrices, marginalized cristae and prominent invaginations of the inner membrane. Rupture of mitochondrial membranes, accompanied by infiltration of matrices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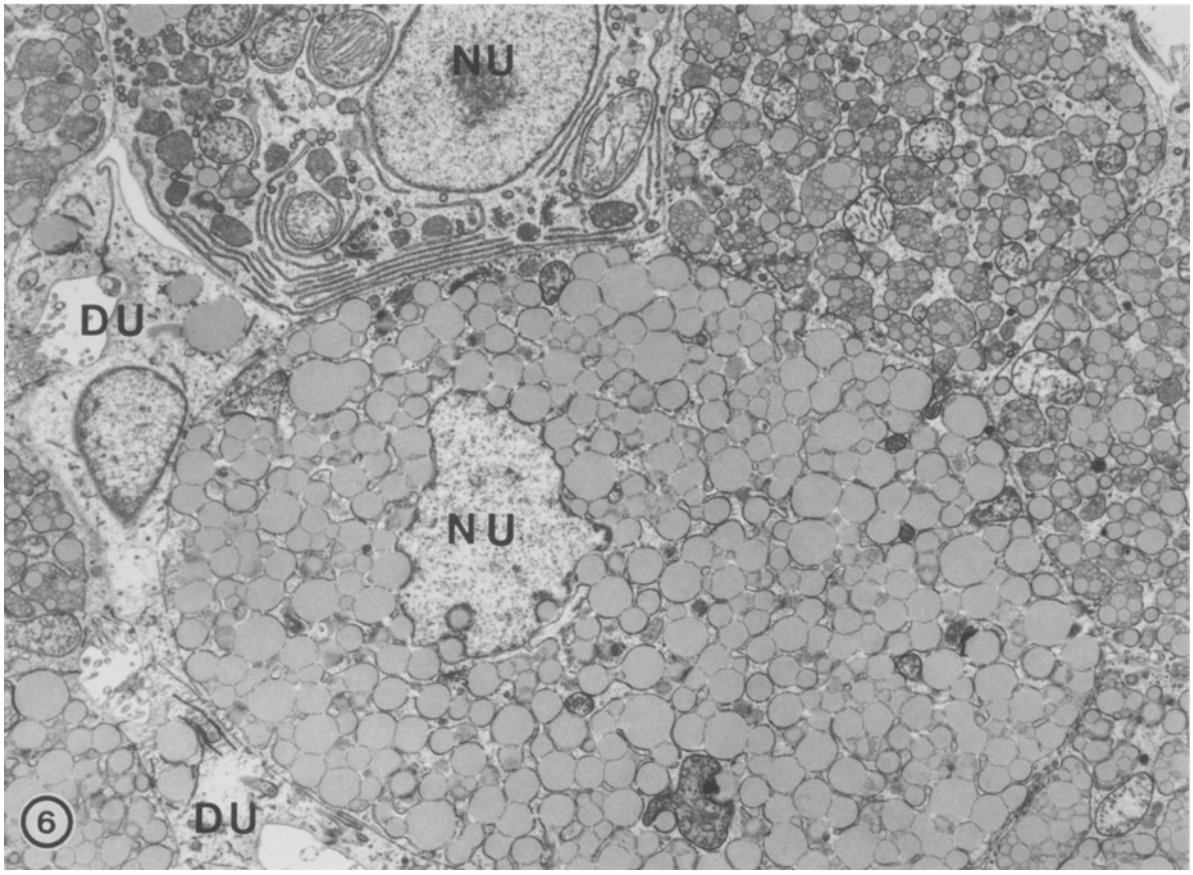
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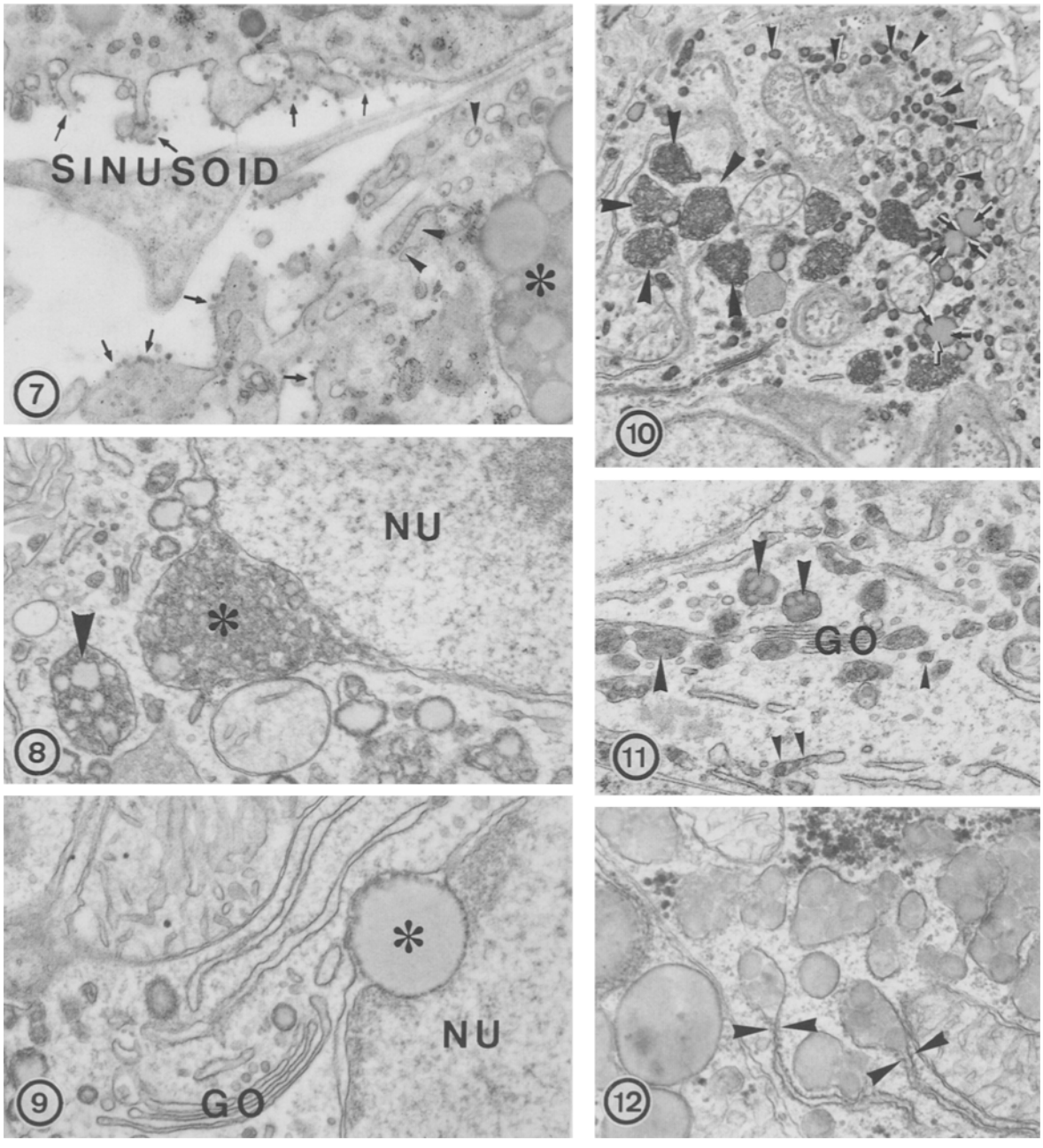


Figs. 7–12. *Scophthalmus maximus*. High power EMS of hepatocytes 3 d after weaning. Fig. 7. Sinusoids and space of Disse are infiltrated with electron-dense lipoprotein particles (arrows) which accumulate along the sinusoidal border of the hepatocytes. Lipoprotein particles are also present within cisternae of the ER close to the sinusoidal border of the hepatocyte (arrowheads). Asterisk: heavy accumulation of lipid material within the ER cisternae; ×17 800. Fig. 8. Lipoprotein clusters are also present within the perinuclear cisterna (asterisk). Note the formation of small lipid droplets within the electron-dense lipoprotein clusters within the ER lumen (arrowhead); NU: nucleus; ×16 500. Fig. 9. Lipid droplet within the perinuclear cisterna (asterisk). GO: Golgi apparatus, not active in lipoprotein secretion; NU: nucleus; ×22 600. Fig. 10. Heterogeneity of membrane-bound lipid materials: small osmiophilic droplets (small arrowheads), electron-dense lipoprotein clusters (large arrowheads) and lipid droplets (arrows) are present; ×10 700. Fig. 11. Lipoproteins, single (small arrowheads) and in clusters (large arrowheads) in the ER. GO: lipoprotein-secreting Golgi apparatus; ×18 000. Fig. 12. Lipid vesicles within the ER lumen. Arrowheads: transition form a "normal" ER cisterna to a lipid-filled cisterna; ×28 000

Figs. 5–6. *Scophthalmus maximus*. Low-power electron micrograph of hepatocytes of turbot 3 d after weaning (+3 d). Fig. 5. Note the drastic increase of lipid material compared to Fig. 2. MITO: mitochondrion; NU: nucleus; ×5500. Fig. 6. Single hepatocytes appear like a "lipid-filled sac" (cell lower center). Note the intercellular variance with respect to lipid contents and to the association of lipid droplets with electron-dense material. DU: bile ductular epithelial cell; NU: nucleus; ×3200

by glycogen particles occurred in some cases. Cisternae of rough ER were single stranded or arranged as stacks of not more than 3 to 4 cisternae (Figs. 2 and 3). The number of lysosomes and peroxisomes was low. The Golgi apparatus was surrounded by a small number of vesicles indicating low activity. Golgi cisternae sometimes formed circles (Fig. 4).

Three days after start of weaning (+3 d), liver parenchymal cells of turbot were characterized by a drastic increase in lipid content (Figs. 5 and 6, Table 2). Glycogen, on the other hand, was almost absent. With respect to organelle structure, a reversion of mitochondrial swelling was obvious in the majority of cells. Golgi secretory activity was enhanced; circular cisternae were absent.

Lipid moieties were present in morphologically different forms. First, a low number of electron-lucent cytoplasmic storage droplets occurred. Their diameters were usually less than 3 μm. Secondly, smooth membrane-bound, osmiophilic, lipid droplets, which were observed before the onset of weaning, were again present. Thirdly, high numbers of electron-dense particles were encountered within cisternae of rough ER (Figs. 7 and 11). According to their diameters (300 to 700 nm) these particles may be interpreted to represent lipoproteins. This assumption is further supported by the observation that these electron-dense particles were also frequently bound in blood and extracellular spaces (Fig. 7). Moreover, within these electron-dense accumulations of these assumed lipoproteins within the ER cisternae (Figs. 10 and 13 to 17) or within outpochings of the perinuclear space (Fig. 8), electron-lucent lipid droplets developed (Figs. 8, 9, 10 and 13) thus indicating again the lipid nature of the particles. The volume of the intracisternal lipid droplets increased by coalescence with other droplets (Figs. 14, 15 and 16). At the same time the amount of the surrounding electron-dense material was reduced correspondingly. Finally, lipid droplets could fill the whole cisternal lumen (Fig. 12). The membrane-bound lipid droplets were released into the cytoplasm by rupture of the ER membrane (Fig. 17). Lipid accumulation within cisternal compartments varied considerably between individual cells (Figs. 5 and 6).

Lipoprotein-bearing cisternae and vesicles were encountered in the vicinity of the cis-face of the Golgi apparatus (Fig. 11). Such vesicles are known to transport lipoproteins from the ER to the Golgi complex. Within the Golgi apparatus, lipoprotein particles were seen in the peripheral extensions of the cisternae and in the secretory vacuoles of the trans-face. However, less than 50% of the dictyosomes contained lipoproteins.

Eighteen days after start of weaning (+18 d), i.e. after 10 d of exclusive feeding on dry diet, a re-organization of hepatocyte structure was evident. A separation of the cytoplasm into areas containing mainly glycogen and/or lipid, and into areas containing mainly organelles ("cytoplasmic compartmentation", Segner and Braunbeck 1988) was seen the majority of cells. Mitochondria exhibited no swelling. The abundance of the RER, which was preferentially organized in stacks, was increased, intracisternal, lipid droplets disappeared, whereas cytoplasmic storage droplets, with diameters of 4 to 10 μm, became dominant. Generally, hepatocellular lipids had experienced a prominent augmentation (see Table 2). Secretion of lipoproteins by the Golgi apparatus appeared to be more active than 3 d after onset of weaning as the majority of dictyosomes contained lipoprotein-filled secretory vacuoles.

Fifty days after start of weaning (+50), organelle structure and cytoplasmic compartmentation were well developed. The lipid reserves of the hepatocytes were stored in 1 to 3 large cytoplasmic, lipid droplets. Glycogen rosettes were arranged in extended cytoplasmic fields. Both hepatocellular glycogen and lipid content were moderately reduced, when compared to Day +18. Intracisternal lipid was completely absent.

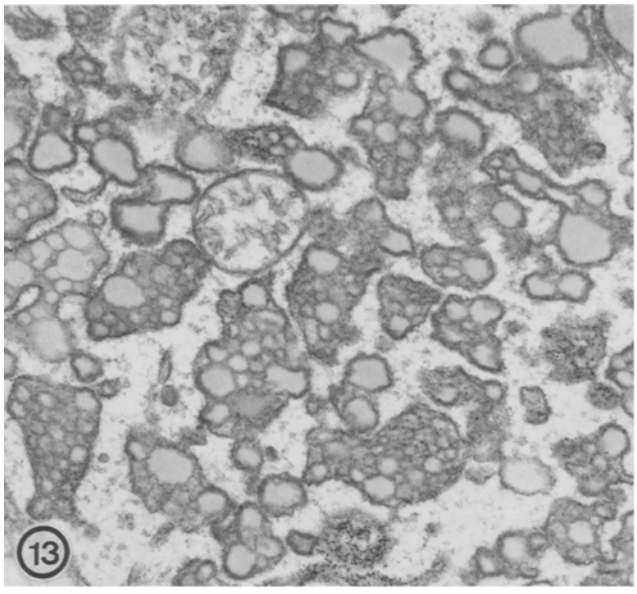
Discussion

According to structural data for liver presented in this study, distinct ultrastructural differences exist between turbot from the pre-weaning, early weaning and post-weaning period, indicating metabolic shifts.

Three days before onset of weaning, liver structural features such as low hepatic glycogen and lipid reserves, swollen mitochondria, nuclear glycogen inclusions and partly altered Golgi morphology indicated metabolic disturbances. Similar morphological symptoms have been reported from livers of other teleost species subjected to malnutrition or food deprivation (e.g. Storch et al. 1983, Verreth et al. 1987, Braunbeck and Segner unpublished data). Starvation as the cause of the observed alterations can probably be excluded since, according to visual examination of feeding behaviour and gut contents, turbot ingested the live food organisms. The metabolic disturbances may be explained either by an inadequate nutritional composition of the live prey, a very critical aspect (see Bromley and Howell 1983), or by the physiological stress of metamorphosis, since pre-weaning turbot had passed metamorphosis just recently. Hepatic disturbances related to metamorphosis have been described for milkfish, *Chanos chanos* (Segner and Braunbeck 1990 b).

The outstanding feature of turbot liver structure during the early phase of weaning was the drastic increase in lipid content together with a pronounced steatosis. The following questions must therefore be discussed: (1) what

Figs. 13–17. *Scophthalmus maximus.* Lipid droplet genesis in and release from the endoplasmic reticulum (ER) in hepatocytes of turbot 3 d after weaning (+3 d). Fig. 13. Formation of lipoprotein clusters within the ER lumen, with a high percentage of dark material; ×14 300. Fig. 14. Enhanced percentage of lipid material, still surrounded by the ER membrane. Coalescence of lipid droplets is obvious (see also Figs. 15 and 16); ×14 900. Fig. 15. Lipid vesicles increase by coalescence with smaller vesicles. Arrowheads: apposition of small droplets to a large droplet; asterisks: aggregation of numerous small droplets within the cisternal lumen ×12 100. Fig. 16. The lipid component dominates the dark material. Arrowheads: Golgi apparatus not active in lipoprotein secretion; arrows: cell border; ×16 600. Fig. 17. Release of lipid vesicles from the ER into the cytoplasm by rupture of the ER membrane (arrowheads). ×34 700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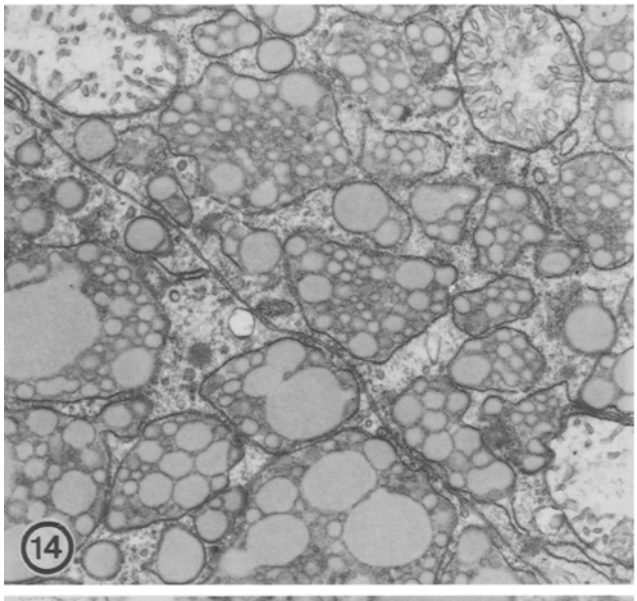
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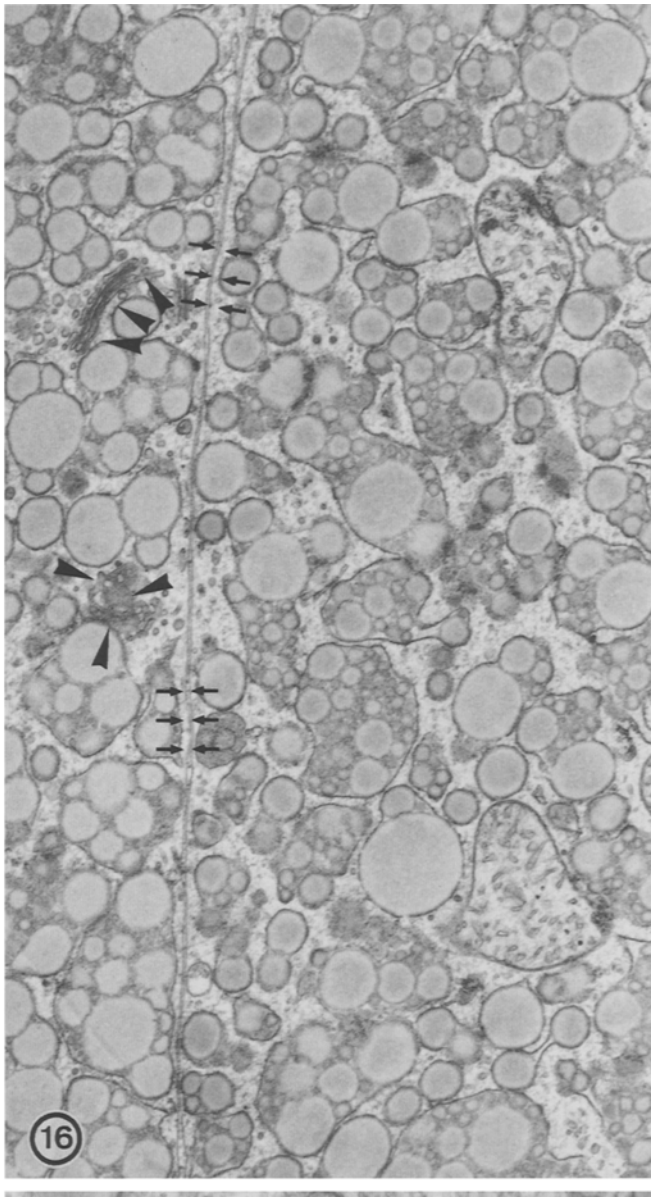
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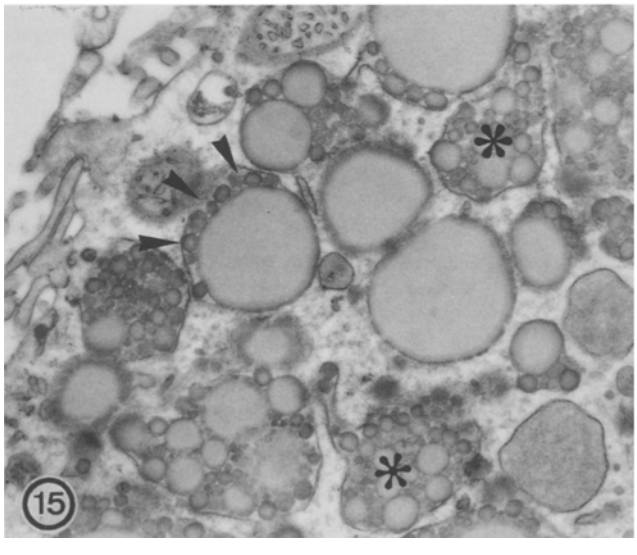
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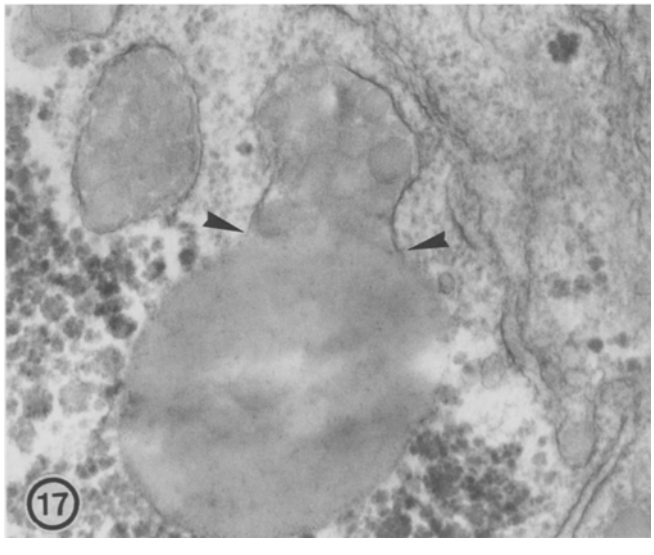
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are the reasons for the enhanced presence of lipids; and (2) why do lipids accumulate within the cisternae of the ER instead of being stored in the cytoplasm or secreted as lipoproteins?

Enhanced presence of lipids in the liver of turbot after the start of weaning can be explained partly by dietary change. Turbot was in a malnourished state prior to the onset of weaning; thus, weaning may have induced a "refeeding" response, which is usually accompanied by enhanced deposition of reserves (see Miglavs and Jobling 1989). On the other hand, since some intracisternal lipid was already found in the liver or turbot prior to weaning, lipid formation in the liver of larval turbot seems to be governed primarily by non-dietary factors. The change in diet obviously only influenced the magnitude of liver lipid formation. We speculate that changes in hepatic lipid metabolism of larval turbot are triggered by ontogenetic factors. For mammals, profound changes in lipid metabolism are known to occur during early life-history (e.g. Coleman et al. 1987). Recently, Munilla-Moran et al. (1989) demonstrated that the cytoplasmic enzymes malic enzyme, isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, which are all involved in NADPH generation for lipid synthesis and modification, increase during ontogenesis of turbot. Interestingly enough, thyroid hormones, which are important regulators of ontogenesis and of lipid metabolism (e.g. Goodridge et al. 1974), peak during metamorphosis of pleuronectid larvae (Inui et al. 1989). Studies on the role of ontogenetic factors upon lipid metabolism of larval turbot from hatching to weaning are currently being undertaken.

The normal intra-hepatocellular transfer of lipids or lipoproteins has been described for mammals e.g. by Stein and Stein (1967), Glaumann et al. (1975) and Bell et al. (1981). For teleosts, only the report of Vernier (1975) exists, which reveals no deviations from the mammalian model. Lipid accumulation within the ER, as it occurred during the early weaning phase, is not a normal constituent of the pathway. Thus, steatosis indicates a disturbance in hepatocellular lipid transfer and metabolism (Lomabrdi 1966, Hey et al. 1988). As causative agents of steatosis in mammalian liver, a large variety of drugs and toxic substances have been identified (Philipps et al. 1987). Also in fish liver, chemicals such as the insecticide lindane were shown to induce hepatic steatosis (Braunbeck et al. 1990). Nutritional factors as cause of steatosis were recently identified by Deplano et al. (1989). The latter authors considered a deficient lipoprotein synthesis to be responsible for lipid accumulation. In the present study, steatosis in turbot liver probably resulted from the combined action of heavy lipid accumulation in the liver after the start of weaning, together with a reduced functional capacity or overcharging of the ER and Golgi apparatus. Low rates of apoprotein synthesis by the ER or low secretory activity of the Golgi apparatus, probably due to the metabolic disturbances of the preweaning period, hinder secretion of lipids as lipoproteins. As a consequence, lipids would accumulate within the cisternae of the ER. Also, an overcharging of the transfer capacity for lipoproteins from the ER to the Golgi apparatus may explain intracisternal lipid accumulation. This step in the metabolic pathway of lipoproteins is known to be rate-limiting in mammals (Chao et al. 1986) and its blockage leads to the observed phenomena. In rat liver, Hamilton et al. (1986) described intracisternal lipid accumulation together with a low frequency of lipoproteins in the Golgi apparatus, to occur after feeding with orotic acid, an agent which restricts translocation of nascent lipoprotein particles from the ER to the *cis* Golgi compartment.

Eighteen days after start of weaning, the dis-equilibrium situation in hepatic lipid transfer was no longer present. Lipoproteins could still be observed within the ER and the dictyosomes, but an intracisternal microvesicular lipid deposition no longer exist. On the contrary, lipoprotein secretion by the Golgi apparatus was clearly enhanced. Numerous large lipid droplets were now deposited within the cytoplasm. In mammals, enhanced hepatic lipid deposition represents a pathologic situation. Also in teleosts, "fatty liver" can indicate degenerative changes of hepatic metabolism (see Roberts and Bullock 1989). However, a number of teleost species use the liver to store lipids (e.g. Lie et al. 1986, Segner and Braunbeck 1988). Pleuronectids belong to this group of fish (Adron et al. 1976, Timoshava 1981, Segner and Möller 1984). Therefore, we consider the lipid deposition in turbot (18 and particularly 50 days after start of weaning) to be the expression of a well-fed status rather than indicating pathological syndrome.

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