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Aquat. Living Resour. 15 (2002) 283–292

Aquatic Living Resources

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Original article

Insulin and hepatic cholestasis during the early post-embryonic development of gilt-head sea bream, *Sparus aurata*

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Received 11 March 2002; accepted 18 July 2002

Abstract

This work is aimed at demonstrating the influence of insulin in the triggering of hepatic cholestasis in young gilt-head sea bream larvae reared under experimental conditions. The results are based on an immunological assay of insulin and an ultrastructural study of the yolk syncytial layer and the liver. Compared to fed larvae, fasting gilt-head sea bream larvae displayed a substantial increase in insulin level correlated with dysfunction of the yolk syncytial layer and hepatic histopathology. This correlation was verified in larvae reared in sea water containing insulin. Ultrastructural observations suggested that insulin affects lipoprotein secretion by the yolk syncytial layer. The failure of plasmatic lipoproteins would then cause dysfunction of the biliary lipid secretion mechanisms and, hence, trigger hepatic cholestasis. These results in gilt-head sea bream show for the first time the influence of insulin in the triggering of hepatic cholestasis during the early development of a vertebrate. © 2002 Ifremer/CNRS/Inra/IRD/Cemagref/Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

Résumé

Insuline et cholestase hépatique au cours du développement postembryonnaire précoce de la daurade, *Sparus aurata*. Notre but est de montrer l'influence de l'insuline dans le déclenchement de la cholestase hépatique chez de jeunes larves de daurade élevées en conditions expérimentales. Nos résultats s'appuient sur le dosage immunologique de l'insuline et sur une étude ultrastructurale du syncytium périvitellin et du foie. Comparées aux larves nourries, les larves de daurade à jeun montrent un accroissement important du taux d'insuline corrélé à un dysfonctionnement du syncytium périvitellin et à une histopathologie hépatique. Cette corrélation est vérifiée chez des larves élevées dans de l'eau de mer contenant de l'insuline. Les observations ultrastructurales suggèrent une influence de l'insuline au niveau de la sécrétion des lipoprotéines par le syncytium périvitellin. La disparition des lipoprotéines plasmatiques aurait alors comme conséquence un dysfonctionnement des mécanismes de la sécrétion des lipides biliaires et donc un déclenchement de la cholestase hépatique. Ces résultats chez la daurade montrent pour la première fois l'influence de l'insuline dans le déclenchement de la cholestase hépatique au cours du développement précoce d'un vertébré. © 2002 Ifremer/CNRS/Inra/IRD/Cemagref/Éditions scientifiques et médicales Elsevier SAS. Tous droits réservés.

Keywords: Teleost; Insulin; Cholestasis; Lipid metabolism; Ultrastructure

1. Introduction

Fish rearing operations experience the problem of diet unsuitability for the nutritional requirements of larvae, which can subsequently develop serious pathologies. Much research has been undertaken on the development of digestive capability during the first weeks of life in order to improve feeding conditions. Thus, in gilt-head sea bream, we have examined the change from endotrophy to exotrophy, and several of our experiments have concerned lipid metabolism (Diaz et al., 2002), especially the first signs of fat intestinal absorption (Diaz et al., 1997a) and biliary lipid secretion mechanisms (Diaz et al., 1997b). During this work, it was found that most of the feeding conditions tested—including those giving the best growth and survival rates—could trigger hepatic cholestasis (Diaz et al., 1998). Parallel research on the hormonal mechanisms regulating carbohydrate and lipid metabolisms (Guyot et al., 1998) led us to focus on insulin, the level of which increases para-

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PII: S0990-7440(02)01183-X

doxically in young fasting larvae while an important histopathology occurs. In order to confirm a causal link between the high insulin level and the triggering of cholestasis, we undertook a complementary study. A batch of larvae, considered as control, was reared in the dark and in sea water containing glucose, conditions required to avoid temporary cholestasis (Diaz et al., 1994). At the same time, a second batch of larvae, reared under the same conditions, was subjected to the addition of insulin to the medium.

2. Materials and methods

2.1. Developmental phases

Two stages of post-embryonic development were considered in gilt-head sea bream, *Sparus aurata* L, prelarva from hatching (day 0) to mouth opening (day 3–4) and larva after mouth opening. With regard to diet, three stages can be distinguished: (1) an endotrophic stage, when the prelarva lives on its yolk reserves; (2) an endo–exotrophic stage starting at mouth opening, characterized by depletion of the yolk reserves and beginning of feeding; (3) an exotrophic stage, when feeding is the only source of energy, starting from day 15 to day 17.

2.2. Experimental design

Gilt-head sea bream prelarvae and larvae from eggs spawned by captive broodstock were supplied by the Palavas IFREMER Centre (Hérault, France). The larvae were reared in cylindrical–conical recycled water tanks containing 500 l sea water at a density of 100 individuals per litre. The sea water was maintained at 38‰ salinity and at 21 °C during experimentation.

In all our experiments, larvae were maintained in the dark until mouth opening. Larvae used for immunoassays, reared under continuous light, were separated into two batches: one fasted, while the second batch was fed rotifers (*Brachionus plicatilis*). The complementary experiment larvae, maintained in the dark, were also separated into two batches. The first one was reared in sea water containing 5 g l^{-1} glucose. The second batch was reared in sea water containing 5 g l^{-1} glucose and 240 μg l^{-1} porcine insulin (Velosuline, Nordisk).

2.3. Morphological studies

For electron microscopy study, five larvae of each batch were sampled every day from hatching to day 8, then on day 10 for fasting larvae, from hatching to day 8, then at day 10, 13, 16, 19, 21 and 24 for fed larvae and day 4, 6, 8 and 9 for both batches reared with glucose.

Larvae were anaesthetized by cooling (2–4 °C) the water and then treated by double fixation with 2.5% glutaraldehyde and 1% osmium tetroxide in cacodylate buffer at pH 7.2 (450 mOsm). The larvae were then embedded in Epon 812, and ultrathin sections were contrasted with uranyl acetate and bismuth subnitrate.

Lipid detection was carried out on ultrathin sections with thiocarbohydrazide and osmium tetroxide; the staining method was OTO (Seligman et al., 1966).

2.4. Immunoassays

Blood cannot be sampled from such small fish, and insulin was assayed using the indirect ELISA method using homogenized prelarvae and larvae in a buffer solution (phosphate-buffered saline: PBS pH 7.4; constant ratio of larva: PBS volume of 500 μg μl^{-1}). The first antibody used was a porcine anti-insulin synthesized by guinea pig (ICN 65-104), diluted to 1:200, and the second was a guinea pig anti-immunoglobulin antibody conjugated with peroxidase (GAGp/IgG(H + L)/Po-Jackson Immunoresearch). The assays were repeated three times in parallel with a porcine insulin standard (Velosuline, Nordisk). Controls were performed by omitting the antigen or the first antibody or both. Details of ELISA–Nunc Maxisorp plate handling have been previously described in the work of Guyot et al. (1998).

2.5. Statistical method

Each sample was determined three times for the ELISA assays. The results are expressed as the standard error of the mean (SEM) (Casio FX-702P).

3. Results

3.1. Variations in the insulin level in fed and fasting larvae

Insulin assay using the ELISA method (Fig. 1) showed that prelarvae produce large quantities of the hormone and that the level falls sharply after mouth opening. In the fed larvae, the level remained low and rose very slightly at the end of the endo–exotrophic period. In the fasting larvae, the fall in insulin level following mouth opening was very marked; the level then increased strongly until larval death (day 10).

3.2. Yolk vesicle and liver in fed and fasting larvae

The structure and functioning of the yolk vesicle complex and liver during the post-embryonic development of the gilt-head sea bream has been thoroughly studied by our team (Guyot et al., 1995; Mani-Ponset et al., 1996; Diaz et al., 1997b). We will, therefore, summarize the final results to allow comprehension of the present work.

The yolk syncytial layer plays a primordial role in the assimilation of yolk reserves. The endoplasmic reticulum and the Golgi apparatus are involved in the synthesis of

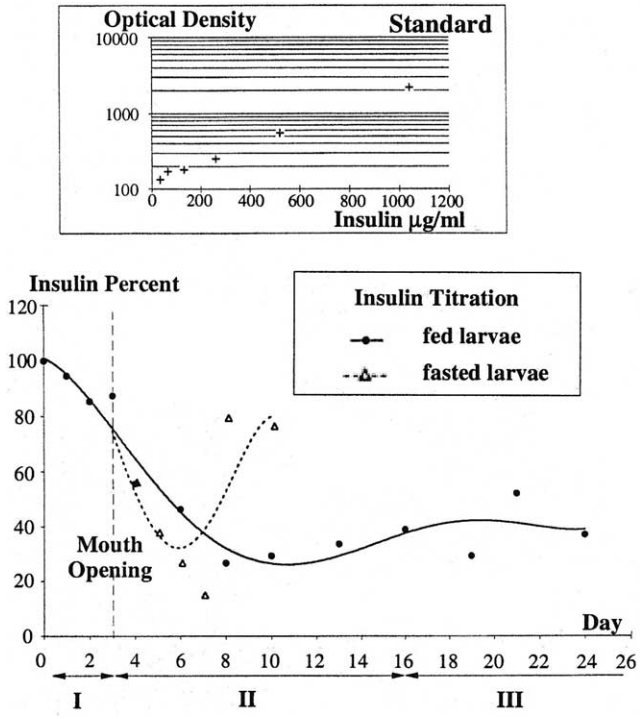

Fig. 1. Variations in insulin levels during the early development of fed and fasting gilt-head sea bream larvae (ELISA assay). Insulin is presented as percentage with homogenate levels of prelarvae at hatching taken as 100%. The standard was set with porcine insulin solution at various concentration levels. I: Endotrophic stage; II: endo–exotrophic stage; III: exotrophic stage.

lipoproteins that are subsequently released into the blood stream via the perivitelline sinus. This mechanism is active during the endotrophic period and ceases shortly after the start of feeding.

Glycogen is accumulated in the liver during the endotrophic period. The maintenance and increase of these reserves then depend on feeding.

Biliary lipid secretion begins at the end of the endotrophic period. It decreases shortly after mouth opening and then recovers and intensifies at the end of the endo–exotrophic period. Biliary lipid synthesis can be seen cytochemically in the hepatocytes through the presence of very low density lipoproteins (VLDLs) in the endoplasmic reticulum and certain Golgi vesicles and more finely granular or homogeneous lipids in other vesicles in the Golgi and pericanalicular areas. These lipids are then released into the bile ducts. Synthesis of biliary lipids is correlated with the presence of plasmatic lipoproteins. The latter are of yolk origin from hatching until shortly after mouth opening and are subsequently of food origin.

In fasting larvae, resorption of the vitelline oil globule is incomplete until death (day 10). It is accompanied (Fig. 2a) by the accumulation in cavities in the endoplasmic reticulum of lipoproteins that are no longer transferred into the blood stream. The lipoproteins produced have unusual structure and size in some larvae (Fig. 2b).

The livers of fasting larvae rapidly absorb all the glycogen accumulated during the endotrophic period and cholestasis-type histopathology develops. Substantial changes (Fig. 2c), identical to those that we have described in three teleost species (Diaz et al., 1998) including gilt-head sea bream, affect the hepatic structure and lead to hepatocyte degeneration. Golgi activity and the production of pericanalicular vesicles are very intense. They are often combined with multilamellar bodies and lead to the formation of autophagous vacuoles. The synthesis of particulate biliary lipids ceases rapidly after mouth opening, while plasmatic lipoproteins tend to disappear. Lipid droplets appeared in all the larvae at the sinusoidal pole of the hepatocytes on day 8 (Fig. 2b, c). The mitochondria are hypertrophied and pleomorphic (Fig. 2c). They are associated with membrane stacks originating at the endoplasmic reticulum. These multilamellar bodies are then released into the bile canaliculi and sinusoids. The sinusoids and intrahepatic bile ducts (Fig. 2c) are strongly dilated.

3.3. Yolk vesicle and liver in larvae reared in sea water containing glucose, with or without added insulin

The reaction of the larvae differed according to whether they were reared in the dark in sea water containing glucose, with or without insulin. In sea water not containing insulin, the synthesis of lipoproteins by the yolk syncytial layer and their release into the blood stream were continuous (Fig. 2d) until the end of the experiment (day 9). Liver development was identical to that of the fed larvae. Hepatocytes accumulated large glycogen reserves in the form of compact zones that tended to compartmentalize the cell (Fig. 3a). There was relatively little Golgi activity and production of pericanalicular vesicles (Fig. 3b). They were always associated with the presence of lipids, consisting of VLDL-like particles in the endoplasmic reticulum and dictyosomes (Fig. 3c), and more finely granular or homogeneous lipids in the Golgi and pericanalicular vesicles (Fig. 3d). Mitochondria were normal and generally spherical and around 1 µm in diameter, grouped at the cell periphery or around the nucleus (Fig. 3a). The bile canaliculi were not very distinct, being filled by numerous microvilli (Fig. 3a, d). Most of the sinusoids were very narrow, and extended endothelial cells occluded their lumina. The perisinusoidal spaces were small and contained few hepatocyte microvilli. Large quantities of lipoproteins were detected in the sinusoids and perisinusoidal spaces (Fig. 2d).

When the rearing water contained insulin, the larvae displayed strong retention of lipoproteins in the reticular cavities of the yolk syncytial layer (Fig. 4a) in the first sample taken after mouth opening. Release into the blood stream was slight or nil. Lipoproteins of unusual structure and size were sometimes produced, as in the fasting larvae.

The larvae developed hepatic cholestasis from the first sampling after mouth opening (day 4). This pathology became more marked in the following samples (Fig. 4b) and

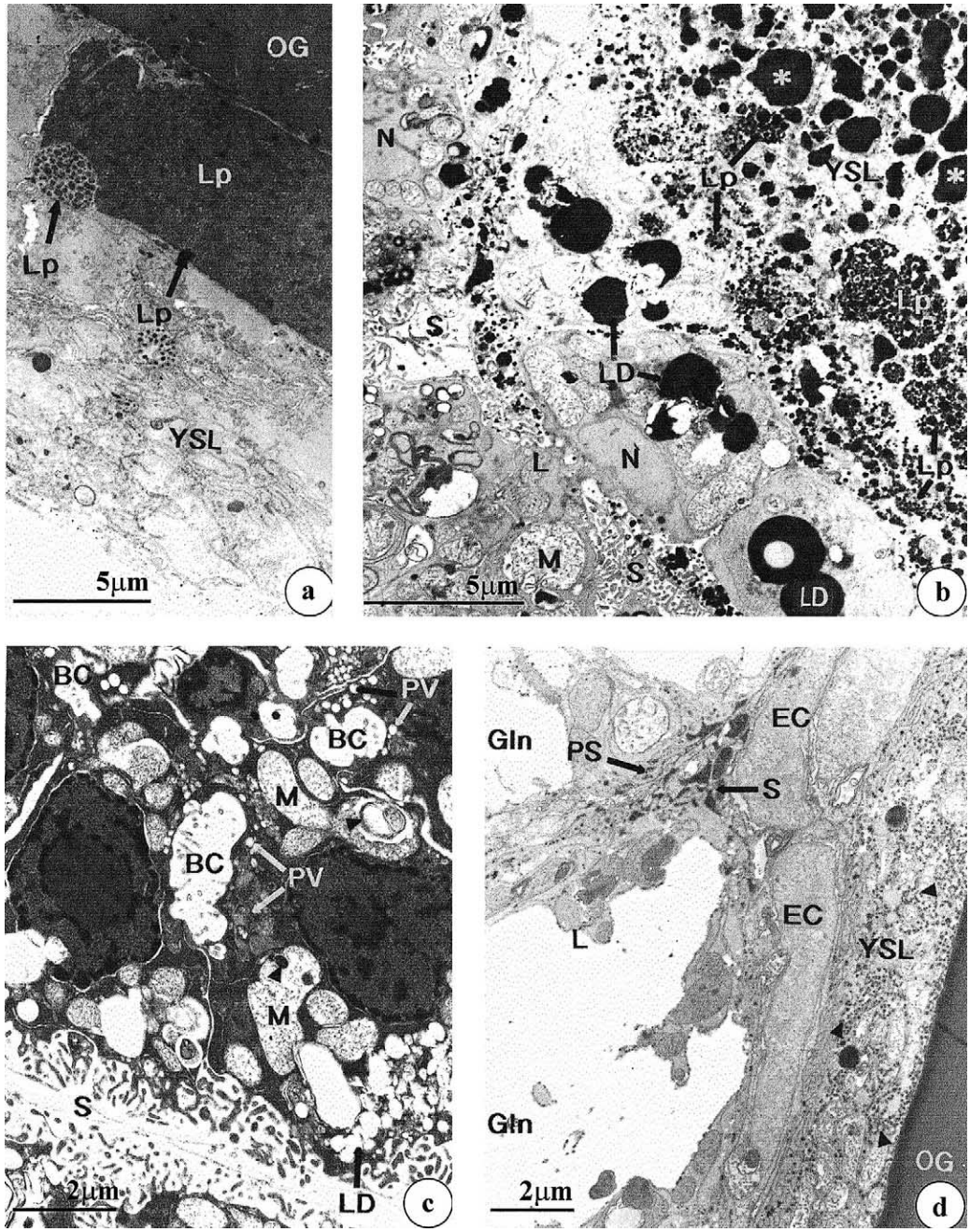

Fig. 2. Ultrastructural aspects of the yolk vesicle and the liver of fasting larvae (a, b, c) and liver of larvae reared in the dark in sea water containing glucose but without insulin (d). (a) Partial view of the yolk vesicle of a 6-d-old larva showing substantial accumulation of lipoprotein particles in the cavities of the endoplasmic reticulum of the yolk syncytial layer (staining method: OTO). Lp: Lipoprotein particles; OG: oil globule; YSL: yolk syncytial layer. (b) Yolk syncytial layer in contact with the liver in an 8-d-old larva, showing an accumulation of lipid inclusions (*) and lipoproteins of unusual structure and size in the cavities of the endoplasmic reticulum (compare with (d)). The hepatocytes contain lipid droplets (OTO method). L: Liver; LD: lipid droplets; Lp: lipoprotein particles; M: mitochondria; N: nucleus; S: sinusoid; YSL: yolk syncytial layer. (c) Changes in the hepatic structure in an 8-d-old larva. The hepatocytes contain hypertrophic and pleomorphic mitochondria, some being combined with multilamellar bodies (arrowheads) and numerous pericanalicular vesicles. Lipid droplets are present at the sinusoidal pole. The bile canaliculi are dilated. BC: bile canaliculus; M: mitochondria; LD: lipid droplets; PV: pericanalicular vesicles. (d) Yolk syncytial layer in contact with the liver in a 6-d-old larva. The endoplasmic reticulum of the yolk syncytial layer contains lipoprotein particles (arrowheads). In the liver, lipoproteins are visible in a sinusoid and in the perisinusoidal space (OTO method). EC: endothelial cell; Gln: glycogen; OG: oil globule; L: liver; PS: perisinusoidal space; S: sinusoid; YSL: yolk syncytial layer.

was accompanied by substantial cell degeneration on day 9. The symptoms (Fig. 4b–d) were very similar to those described in fasting larvae. They displayed strong Golgi activity and substantial production of pericanalicular vesicles, formation of autophagous vacuoles, hypertrophy and mitochondrial changes, formation of multilamellar bodies, dilatation of the bile ducts and sinusoids and disappearance of plasmatic lipoproteins.

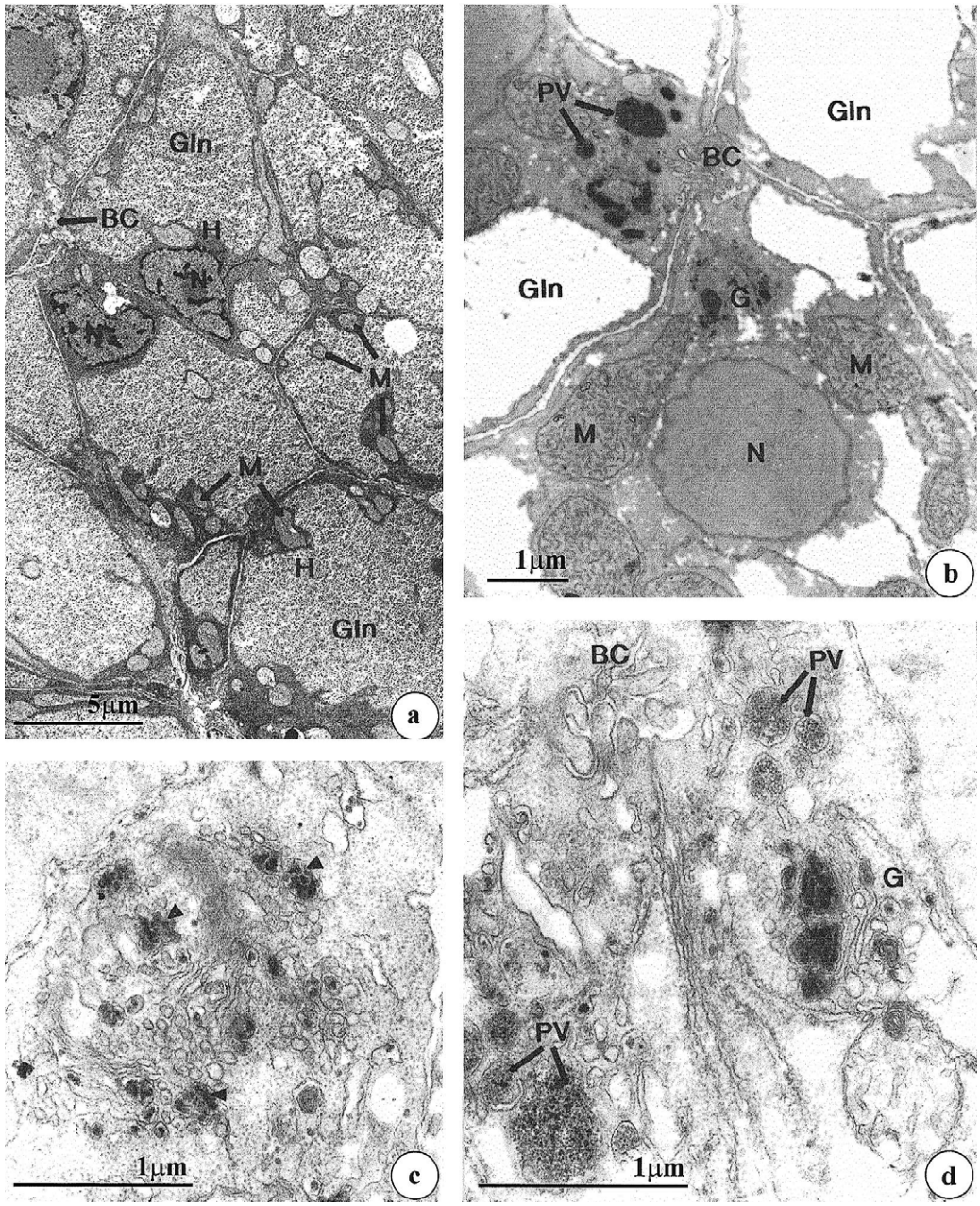

Fig. 3. Ultrastructural aspects of the liver of larvae reared in the dark in sea water containing glucose but without insulin. (a) Hepatic parenchyma in a 6-d-old larva. The hepatocytes contain large glycogen areas that displace the organelles to the edge of the cell and around the nucleus. The mitochondria are normal. The bile canaliculi are not very distinct. BC: bile canaliculus; Gln: glycogen; H: hepatocyte; M: mitochondria; N: nucleus. (b) Hepatocyte in a 9-d-old larva. Lipids are present in the Golgi apparatus and the pericanalicular vesicles (OTO method). BC: bile canaliculus; G: Golgi apparatus; Gln: glycogen; M: mitochondria; N: nucleus; PV: pericanalicular vesicles. (c) Golgi apparatus containing very low density lipoprotein, VLDL-like particles (arrowheads) in a 4-d-old larva (OTO method). (d) Very low density lipoprotein [VLDL]-like particles in a dictyosome and more finely granular lipids in the pericanalicular vesicles in a 6-d-old larva, OTO method. BC: bile canaliculus; G: Golgi apparatus; PV: pericanalicular vesicles.

However, a number of features should be stressed. Throughout the experience, the hepatocytes displayed small glycogen reserves in the form of scattered particles. The pericanalicular vesicles increased in size and became polymorphic, while their contents diversified (Fig. 4c, d). At the beginning of larval life (day 4), they contained mainly particulate, finely granular and homogeneous lipids. Then, while the vesicles with this type of content decreased in number and tended to disappear, other types increased (Fig. 4c, d). Some contained lamellar bodies. Others became multivesicular bodies. The contents of a certain number were formed of stacks of mixed or closely linked mem-

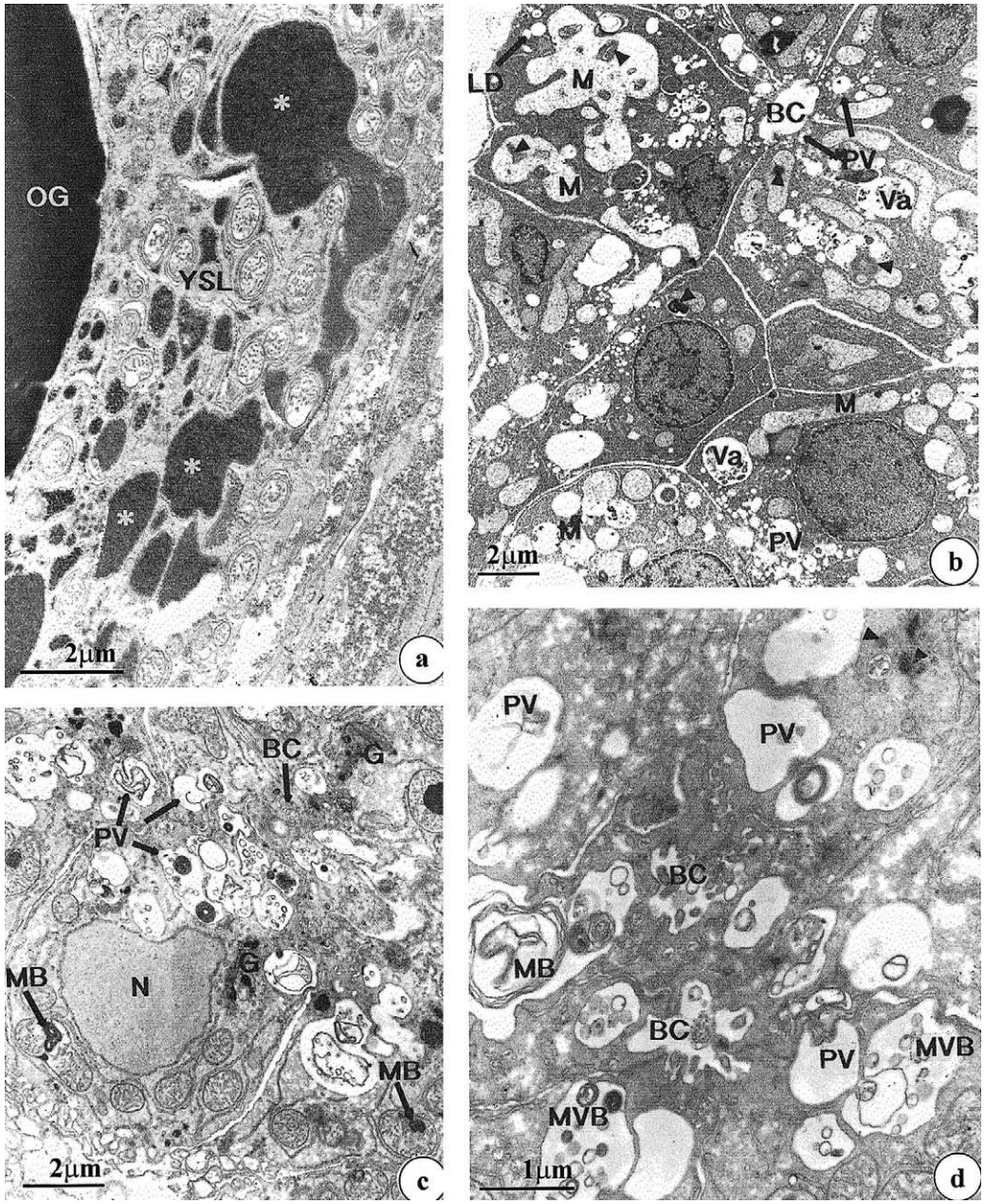

Fig. 4. Ultrastructural aspects of the liver of larvae reared in the dark in sea water containing glucose and insulin. (a) Partial view of the yolk syncytial layer in a 4-d-old. The substantial accumulation of lipoprotein particles (*) can be noted in the cavities of the endoplasmic reticulum (OTO) method. OG: oil globule; YSL: yolk syncytial layer. (b) Hepatic histopathology in a 9-d-old larva. Accumulation of pericanalicular vesicles and autophagous vesicles can be seen in the hepatocytes. The mitochondria are hypertrophic, pleomorphic and combined with multilamellar bodies (arrowheads). The bile canaliculi are dilated. Lipid droplets are visible at the sinusoidal pole of the hepatocytes. BC: bile canaliculus; LD: lipid droplets; M: mitochondria; PV: pericanalicular vesicles; Va: autophagous vesicles. (c) Hepatocyte with an accumulation of polymorphic pericanalicular vesicles with varied contents in a 6-d-old larva (OTO) method. BC: bile canaliculus; G: Golgi apparatus; MB: multilamellar body; N: nucleus; PV: pericanalicular vesicles. (d) Detail of the apical hepatocyte region of a 6-d-old larva. There are numerous polymorphic pericanalicular vesicles. They contain mainly multilamellar bodies and microvesicular bodies and more rarely particulate lipids (arrowheads) (OTO) method. BC: bile canaliculus; MB: multilamellar bodies; MVB: microvesicular bodies; PV: pericanalicular vesicles.

branes and microvesicles. Finally, a few vesicles contained biliary lipids and lamellar formations that were together or sometimes closely associated.

4. Discussion

The ontogenetic study of the fish endocrine pancreas (Epple and Brinn, 1986; Youson and Al-Mahrouki, 1999),

supported by immunocytochemical, ultrastructural or molecular biology methods, allowed the detection of early appearance of various hormone producing cell types (Rombout et al., 1979; Beccaria et al., 1990; Garcia Hernandez and Agulleiro, 1992; Agulleiro et al., 1994; Reinecke, 1998; Argenton et al., 1999). In *S. aurata*, insulin producing cells were detected 3–4 d after hatching by Sarasquete et al. (1993), and from the first day of free life by our team (Guyot et al., 1998). Using immunoassays for the first time in fish, we confirmed the early and important presence of insulin during the post-embryonic development. Recent studies in development genetics (Argenton et al., 1999; Biemar et al., 2001; Huang et al., 2001) proved that the pancreatic hormonal genes express early during ontogenesis.

We have shown (Diaz et al., 1998) that starvation, as well unsuitable diet, induces, in fish larvae, dysfunction that leads to hepatic cholestasis. Even in sea bream larvae fed with standard diet (*Brachionus plicatilis*), which allows the best survival rates (25%), drastic changes in hepatic structure occur in a great number (Diaz et al., 1998). As it was essential to dispose of control larvae without hepatic histopathology in order to test the effects of insulin on the liver, our experiments were conducted on larvae reared in the dark, in sea water containing glucose, at the beginning of the larval period. Indeed, we have shown that all larvae reared in these conditions remain healthy during the experimental time (Diaz et al., 1994).

4.1. Variations in the insulin level in fasting larvae

The fall in the insulin level at mouth opening in fasting gilt-head sea bream larvae was more marked than in fed larvae. However, in contrast with the latter, the increase following the fall is rapid and substantial until the death of the larvae (day 10). To the best of our knowledge, this is the first time that such an increase in insulin level has been reported in fasting animals. The increase is in contradiction with data showing variation in insulin levels during fasting in both fish and mammals. In fish, where only juveniles and adults were investigated (reviewed by Plisetskaya, 1989, and by Mommsen and Plisetskaya, 1991), the insulin levels decreased during fasting in salmon (Plisetskaya et al., 1986; Larsen et al., 2001), cod (Thorpe and Ince, 1976; Hemre et al., 1990), carp (Blasco et al., 1991, 1992) and trout (Thorpe and Ince, 1976; Navarro et al., 1992). In mammals, there were no significant differences between fed and fasting piglets for 24–48 h after birth (Flecknell et al., 1988).

The increase of insulin level that occurs simultaneously with acute cholestasis and precedes the death of fasting gilt-head sea bream larvae corresponds most probably to a pathological response.

4.2. Insulin and cholestasis

It is known that hormones taken by mouth conserve their biological activity. This is the case of bovine growth hormone given to trout (Le Bail et al., 1989) and insulin given to carp (Hertz et al., 1992) and European eel (Degani and Abraham, 1992). In carp, insulin administered orally has the same effects on the hepatic structure as insulin administered intravenously (Vera et al., 1993). Comparison of gilt-head sea bream larvae reared with or without insulin clearly shows the influence of the hormone on the liver. This results in cholestasis for larvae reared in water containing insulin. Our results suggest that the insulin added to rearing water is well absorbed by the larvae and that it conserves the same biological activity as in fasting larvae.

To the best of our knowledge, no correlation between cholestasis and a high insulin level has been reported. Nevertheless, as in mammals and man, hepatic cholestasis occurs during total parenteral nutrition (Dahms and Halpin, 1981; Zahavi et al., 1985; Das et al., 1993); it is possible that this results from an increase in insulin secretion following venous transfusion of glucose, amino acids and fatty acids.

The cholestasis symptoms (Diaz et al., 1998) are reminiscent of some hepatic alterations of toxic origin found in several fish species (Braunbeck, 1998; Braunbeck and Appelbaum, 1999). Among these symptoms, the intensity of Golgi activity and the formation of pericanalicular vesicles deserve to be stressed. Indeed, the vesicle contents are astonishingly varied, as they contain finely granular and homogeneous particulate lipids, lamellar formations, membrane stacks and microvesicles. The structure and location of the particulate, finely granular and homogeneous lipids correspond to those of the biliary lipids that can be displayed cytochemically in fish larvae (Diaz et al., 1997b). Secretion of these lipids continued throughout the duration of the experiment in gilt-head sea bream larvae reared in the dark and in water containing glucose and in which plasmatic lipoproteins were observed. In contrast, it decreased and then stopped shortly after mouth opening in the fasting larvae and in those reared in medium containing insulin and in which there were no plasmatic lipoproteins and where cholestatic development was observed. In this case, biliary lipids were replaced in the Golgi and pericanalicular vesicles by lamellar bodies and microvesicles. Plasmatic lipoproteins form one of the main possible sources of biliary lipids (Coleman and Rahman, 1992). As it has been suggested that cholestasis is linked with the depletion of cell phospholipids (Yousef et al., 1987; Baumgartner et al., 1992), it is possible that the disappearance of the plasmatic lipoproteins causes dysfunction or failure of the synthesis of biliary lipids, resulting in the pathology observed in gilt-head sea bream larvae. In this hypothesis, insulin would have an effect at plasmatic lipoprotein synthesis level.

Lipoproteins originated from yolk reserves, due to yolk syncytial layer activity (Mani-Ponset et al., 1996), and then from feeding (Diaz et al., 1997a). The secretion of lipoproteins by the yolk syncytial layer continued until the end of the experiment (day 9) in the larvae reared without insulin.

In contrast, larvae reared in the presence of insulin and fasting larvae, with a substantial increase in insulin level, accumulated lipoproteins in the yolk syncytial layer and, according to the individual, stopped release into the blood stream. In addition, it is known that insulin expresses effects in lipid metabolism (Mommsen and Plisetskaya, 1991), and it has been shown that the injection of insulin causes a decrease in the free fatty acid, phospholipid and cholesterol levels in serum in several teleost species (Leibson et al., 1968; Plisetskaya and Mazina, 1969; Minick and Chavin, 1972; Ince and Thorpe, 1974; Lewander et al., 1976; Perez et al., 1989) and lamprey (Kao et al., 1999).

The disappearance of plasmatic lipoproteins in gilt-head sea bream larvae reared in the presence of insulin and fasting larvae can be seen as the result of dysfunction of the yolk syncytial layer, and linked in turn to the presence of high insulin level. There are no other data today on the influence of insulin on lipoprotein secretion by the yolk syncytial layer in fish larvae. However, much work has been devoted to its inhibitory effect on VLDL secretion by the liver in man (Pietri et al., 1983; Cummings et al., 1995), rat (Durrington et al., 1982; Patsch et al., 1983; Pullinger and Gibbons, 1985; Bartlett and Gibbons, 1988; Duerden et al., 1989; Duerden and Gibbons, 1990) and fish such as eel (Fu-Gong et al., 1992). It has also been shown that insulin inhibits Apo B secretion (Sparks et al., 1986; Jackson et al., 1990; Salhanick et al., 1991), suggesting that it is involved in the regulation of Apo B and triglycerides and, hence, in VLDL secretion. These data show that it is highly probable that insulin affects the mechanisms of secretion of lipoproteins by the yolk syncytial layer in gilt-head sea bream.

During cholestasis, gilt-head sea bream larvae accumulated lipid droplets at the sinusoidal pole of hepatocytes. This may be caused by the effects of insulin. Indeed, injection of this hormone in fish causes an increase in liver lipids (Ablett et al., 1981; Perez et al., 1989). These data were confirmed by the increase in the stock of triglycerides in rat hepatocytes cultured in the presence of insulin (Duerden et al., 1989; Duerden and Gibbons, 1990).

In conclusion, our study reveals the influence of insulin in the triggering of hepatic cholestasis. This effect is exerted at the level of lipoprotein synthesis by the yolk syncytial layer. The resulting disappearance of plasmatic lipoproteins would, therefore, cause dysfunction of the biliary lipid secretion mechanisms and, hence, hepatic histopathology.

Acknowledgements

The authors thank Mrs C. Blasco and Mr T. Noëll, and the staff of the Electron Microscopy Center of the Université Montpellier II, for their excellent technical collaboration.

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