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## DEVELOPMENT OF HEPATOCYTES IN NASE (*CHONDROSTOMA NASUS* (L.)) LARVAE FOLLOWING HATCH

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**ABSTRACT.** The development of the nase liver was examined under light and electron microscopes from the moment of hatching until the juvenile stage. Three phases of hepatocyte differentiation were observed during the organogenesis of nase livers. In the first phase, from hatching until day 4, the hepatoblasts of the primordial liver are morphologically undifferentiated and divided by sinus vessels. They also store glycogen. In the second phase, from the moment when the mouth cavity becomes passable until the resorption of the yolk sac, organelles typical of the structure of hepatocytes appear and begin to function. At the end of this phase signs of bile lipid synthesis and secretion become visible. The third phase is when exogenous nutrition begins and is characterized by the increased activity of the significant organelles engaged in protein synthesis and secretion, such as the endoplasmic reticulum and the Golgi apparatus.

Key words: NASE (*CHONDROSTOMA NASUS*), LIVER, HEPATOCYTES

## INTRODUCTION

The liver is necessary for life due to the various functions it performs. It is both an endocrine and exocrine gland. The liver is classified as an exocrine organ due to the synthesis of bile, which is transported by ducts to the duodenum, and in fish to the anterior intestine. Various other products of this gland are released directly into the blood stream (*i.e.* endocrine secretion). It functions as a storage center for many substances, mainly glycogen and, to a lesser degree, lipids (Takashima and Hibiya 1995). An important liver function is the coupling and chemical decomposition of toxins and the deamination of amino acids. Ultrastructural investigations of the liver have been done primarily in the higher vertebrates. Few publications to date have addressed the differentiation of hepatocytes in fish. The organogenesis of the liver was described in the rainbow trout, *Oncorhynchus mykiss* (Wal.) (Byczkowska-Smyk 1967) in European seabass, *Dicentrarchus labrax* (L.) (Diaz and Connes 1991) in sea bream, *Sparus aurata* L. (Guyot et al. 1995), and in carp, *Cyprinus carpio* L. (Fishelson and Becker 2001).

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The aim of this research was to identify the morphology and follow the differentiation process and functioning of the liver during organogenesis in the nase, *Chondrostoma nasus* (L.), during the period from hatching until the juvenile stage.

## MATERIAL AND METHODS

The materials used in the research were nase larvae from the Hatchery of the Experimental Fisheries Station in Łąki Jaktorowskie. Post-embryonic development was conducted in water at a temperature of 18-20°C. Rearing was conducted from the moment of hatching (total length (TL)  $8.22 \pm 0.7$  mm) until day 60 of development (TL  $36.8 \pm 0.7$  mm). From day 4 after hatching the larvae were fed four times daily *ad libitum* with *Artemia* sp. nauplii (approximately 100 per specimen). During development, samples were taken every 24 hours and preserved in Bouin's solution for histological analysis. The larvae were then dehydrated in increasing concentrations of ethanol and embedded in paraffin. Paraffin serial sections 5  $\mu$ m thick were cut with a MicroTec CUT 4050 microtome.

The morphology of the larvae was evaluated with sections that had been stained with hematoxylin and eosin. The material used in the analyses of the ultrastructure was preserved in 2.5% glutaraldehyde buffered with 0.1 M cacodylate-HCl to a pH of 7.4. It was then rinsed in the same buffer. Samples were fixed in 1% osmium tetroxide buffered with 0.1 M cacodylate-HCl to a pH of 7.4. The preparations were embedded in Epon 812 (Serva). Ultrathin sections were cut with an Ultratom III ultramicrotome manufactured by LKB (Sweden). The sections were contrasted with uranyl acetate and lead citrate. Observations and electronograms were done with a JOEL JEM 100 C transmission microscope (Japan). Measurements of cell structure sizes were taken to the nearest 0.1  $\mu$ m on an electrogram.

## RESULTS

At the moment of hatching, the nase larvae had an average total length of  $8.22 \pm 0.7$  mm. The yolk sacs were pear-shaped. The mouth opening at this developmental stage was not passable. Nearly the full length of the intestines was on the yolk sac. A thick clump of undifferentiated cells was observed between the anterior section of the intestines and the yolk sac. These were the primordial liver and pancreas. The hepatoblasts (undifferentiated liver cells) were of an irregular, polygonal shape with a homogeneous, basophilic cytoplasm containing a round nucleus that had a visible

nuclear membrane. A small amount of cytoplasm surrounded the nucleus. A centrally located nucleolus with dispersed chromatin was noted in each nucleus. The average length and width of these cells was  $9.4 \times 6.9 \mu\text{m}$ , and the diameter of the nucleus was  $5.1 \mu\text{m}$  (Table 1).

TABLE 1

Size of hepatocytes and other cellular organelles (mean  $\pm$  Se, (N = 100)) at significant stages of the post-embryonic development of nase

Size ( $\mu\text{m}$ )	Day after hatching (1 DAH*)	Mouth cavity passable (4 DAH)	Exogenous feeding begins (9 DAH)	Beginning of juvenile stage (21 DAH)	Juvenile stage (60 DAH)
Hepatocytes	$9.4 \pm 0.14$ $\times 6.7 \pm 0.11$	$18 \pm 0.04$ $\times 12.3 \pm 0.08$	$29.3 \pm 0.03$ $\times 18.1 \pm 0.06$	$18 \pm 0.14$ $\times 15.0 \pm 0.11$	$20.6 \pm 0.05$ $\times 16.3 \pm 0.09$
Nucleus	$5.1 \pm 0.04$	$4.8 \pm 0.13$	$4.8 \pm 0.08$	$4.5 \pm 0.06$	$5.3 \pm 0.10$
Mitochondria	$2.4 \pm 0.08$	$2.3 \pm 0.12$	$2.4 \pm 0.06$	$2.3 \pm 0.11$	$2.4 \pm 0.11$

\*DAH- days after hatching

Densely clumped, undifferentiated hepatic cells divided by a few sinusoids were also found. The hepatic cells were separated from the yolk sac by a thin layer of the perivitelline syncytium (Photo 1). The liver cells, hepatocytes, developed and outnumbered them. The reproduction of hepatoblasts led to the increasing size of the liver gland. On day 4 of post-embryonic development, the undifferentiated cells of the primordial liver surrounded intestines as a result of morphogenetic movement. At this developmental stage (day 4 after hatching,  $10.9 \pm 0.6 \text{ mm TL}$ ) two types of hepatoblasts were observed: pale, with a transparent cytoplasm; and dark, with a thick, non-transparent cytoplasm (Photo 2). The cytoplasm of both cell types contained several round mitochondria with a few short cristae. Some of them contained pseudo myelin structures (Photo 3). A few rough endoplasmic reticulum (RER) cisternae were visible in the cytoplasm (Photo 3). The most visible character that indicated hepatocyte differentiation was the accumulation of glycogen during the end of the endogenous nutrition phase (day 4 after hatching). Single lipid droplets were visible in some of the hepatoblasts in the cytoplasm. Sinusoids were observed during this period. The Disse space between the surface of the hepatocyte sinus and the cell endothelium was limited by the narrow sinusoid lumen. The endothelial cells had an elongated nucleus with strips of compact chromatin and a small amount of cytoplasm seeping through the pores. These were characterized by the absence of a nucleolus. Lymphocytes were observed in the sinusoid lumen (Photo 4).

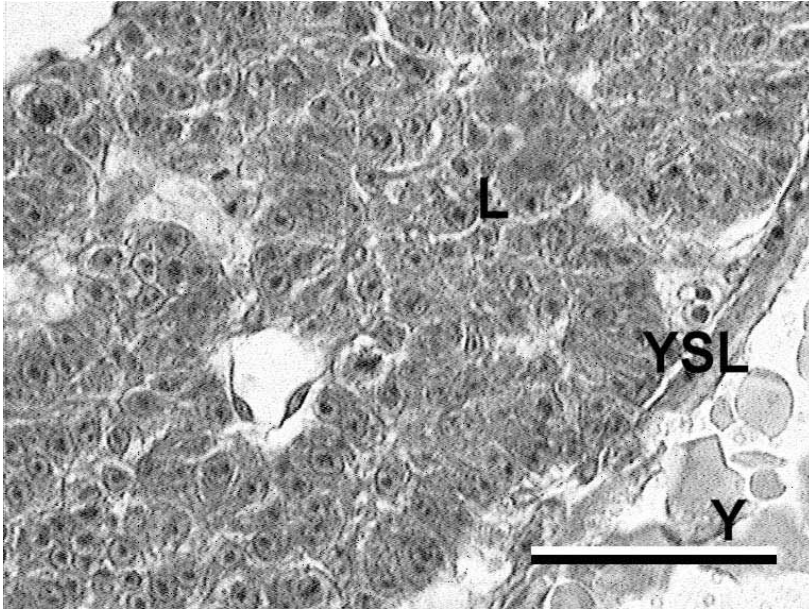


Photo 1. Primordial liver (L) on the day of hatching separated by the perivitelline syncytium (YSL) from the yolk sac (Y); (H-E). Bar 50  $\mu$ m.

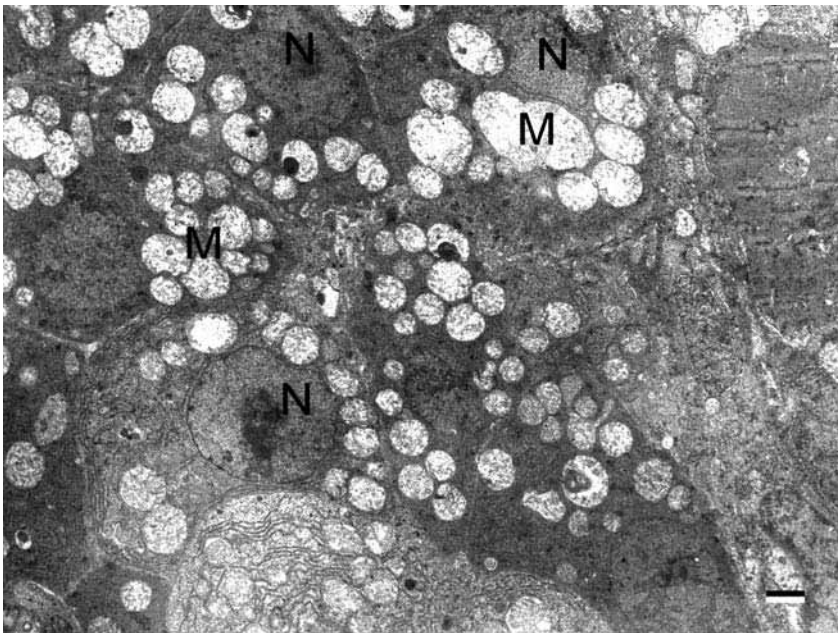


Photo 2. Undifferentiated hepatocytes on day 4 after hatching. Numerous rounded mitochondria. High nucleus/cytoplasm ratio; N – nucleus, M – mitochondria. Bar 2  $\mu$ m.

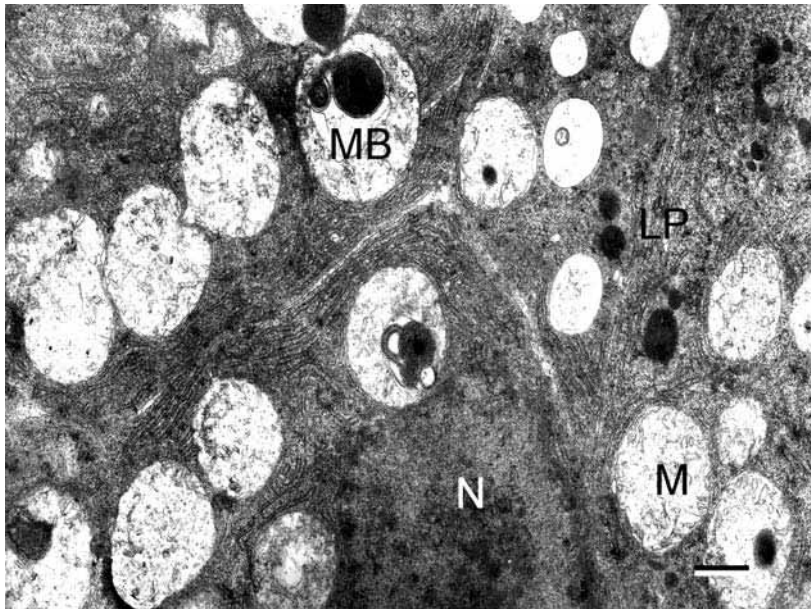


Photo 3. Multilamellar bodies (MB) in larva mitochondria on day 4 after hatch. RER cisternae around the nucleus hepatoblasts are not clearly visible. Lipoprotein droplet (LP), M – mitochondria, N – nucleus. Bar 1 $\mu$ m.

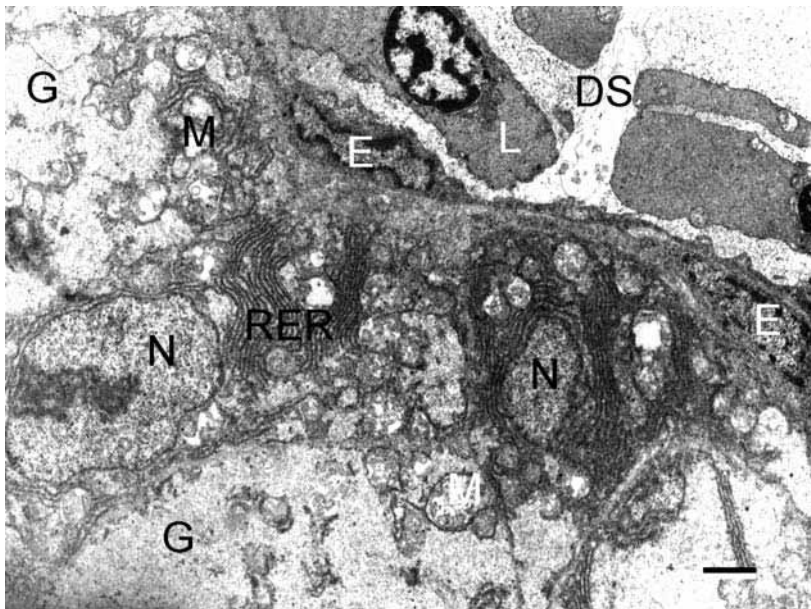


Photo 4. Liver section day 6 after hatching. RER cisternae around hepatocyte nucleus. Liver Disse space (DS) lined with endothelial cells (E). Light and dark hepatocytes. M – mitochondria, N – nucleus, G – glycogen rosettes, L – lymphocyte. Bar 2  $\mu$ m.

The formation of the gall bladder was observed from day 2 after hatching. The mouth cavity became passable on day 4 after hatching. The period of endo-exogenous nutrition began at this point and lasted until day 9 of life. Nase larvae began to collect exogenous food (*Artemia* sp. nauplii) although they still had significant stores in their yolk sacs. At this stage of embryonic development between days 4 and 9 further changes occurred in the differentiation of the liver occurred. The liver grew and occupied the space of the diminishing yolk sac. The size of the hepatocytes was  $18.0 \times 12.3$   $\mu\text{m}$ . Their shape changed and became rounder. The RER cisternae occurred very abundantly around the hepatocyte nuclei and surrounded groups of mitochondria (Photo 4). The mitochondria at this stage of development did not exhibit pseudo myelin structures (Photo 4). Intercellular bile canaliculi appeared where three liver cells adjoined. In the final phase of the resorption of yolk sac stores, large areas occupied by glycogen were noted in the hepatocyte cytoplasm. The functioning of the Golgi apparatus intensified; this was not observed during the endogenous nutrition period. The Golgi apparatus occupied the space between the nucleus and the bile canaliculi. Two types of lipoprotein vesicles created in the Golgi areas appeared near the bile canaliculi. Small vesicles with a diameter from 30 to 70 nm collected in the Golgi areas; they were also observed in the endoplasmic reticulum. Other larger ones contained small homogeneous granules (Photo 5). These lipoprotein vesicles were also observed in the bile canaliculi.

The total resorption of the yolk sacs occurred on day 9 after hatching ( $14.4 \pm 0.4$  mm TL). At the same time the larvae began to feed only exogeneously. In the hepatocytes, intracellular bile canaliculi formed, lined by long, irregular microvilli. As the fish grew, so did the number of hepatocytes. The size of the hepatocytes also grew as a result of the increase in the cytoplasm volume and the accompanying cell organelles, especially due to the storage of glycogen and lipids.

In the juvenile stage from day 20 ( $20.5 \pm 0.8$  mm TL) until the end of the research at day 60 ( $36.8 \pm 0.7$  mm TL) of fish life the liver still exhibited two types of hepatocytes – light and dark. The light cells occurred less often. Irregular microvilli penetrated from the surface of both types of cells into the Disse space (Photo 6). Distinct pores were observed in the endothelium separating the Disse space from the sinusoid passage. The Disse spaces were accompanied by Kupffer cells, or macrophages, with an irregular surface, numerous lysosomes and heterophagosome. Additionally, there were erythrocytes, lymphocytes, and reticulocytes in the vessel lumen. The cisternae of the

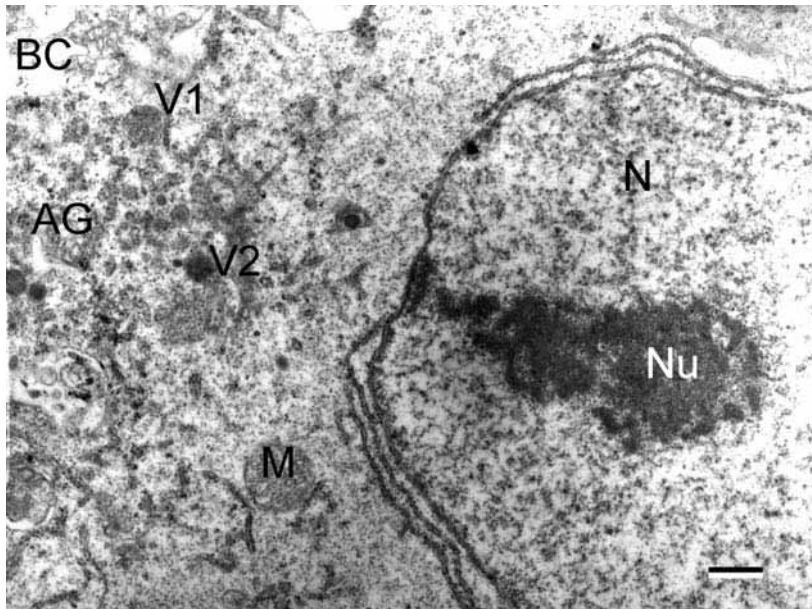


Photo 5. Liver section 8 days after hatching. Golgi apparatus (AG) near the bile canaliculi (BC). Two types of lipoprotein vesicles (V1 and V2) formed in the Golgi apparatus. M – mitochondria, N – nucleus, Nu – nucleolus. Bar 1  $\mu$ m.

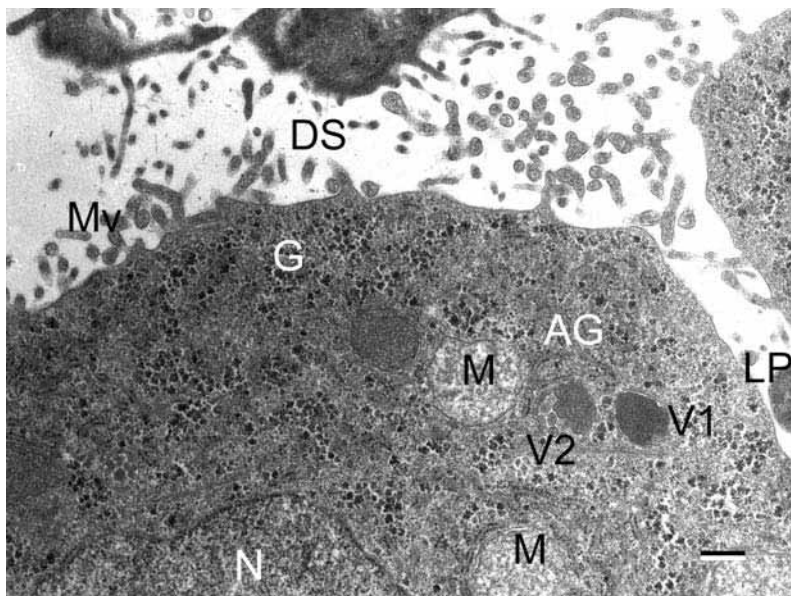


Photo 6. Juvenile stage. Microvilli (Mv) in the Disse space (DS). Lipoproteins in the intercellular spaces. V1, V2 – lipoprotein vesicles, AG – Golgi apparatus, LP – lipoprotein droplet, M – mitochondria, N – nucleus. Bar 0.5  $\mu$ m.

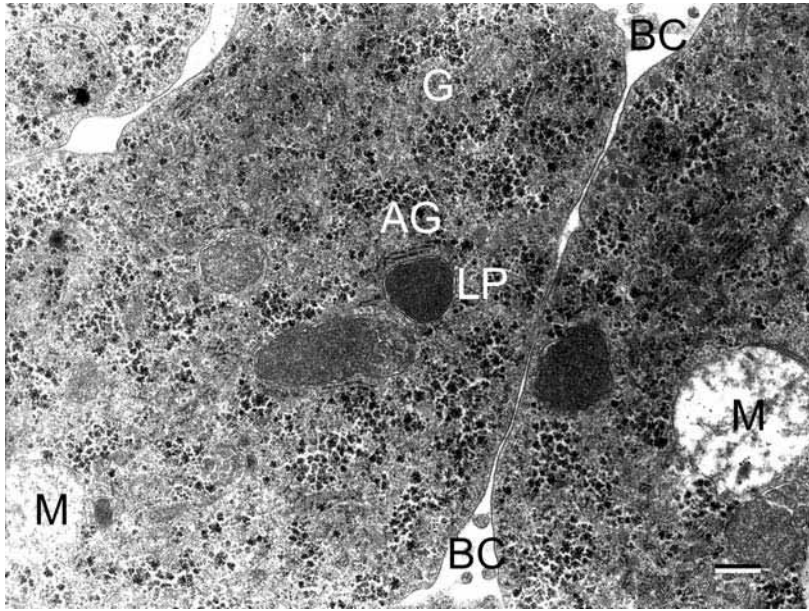


Photo 7. Juvenile stage. Intercellular bile canaliculi (BC). Glycogen rosettes (G) distributed in the cytoplasm. AG – Golgi apparatus, LP – lipoprotein droplet, M – mitochondria. Bar 0.5  $\mu$ m.

Golgi apparatus visible in the hepatocyte cytoplasm occurred more numerous than in earlier stages of development and were more robustly formed (Photo 6, 7). Biliary lipid secretion continued in the juvenile stage (Photo 6). The number of cristae in the mitochondria also increased. Many of the liver cells did not contain lipid droplets, although all of the liver cells stored glycogen, which formed rosettes in the cytoplasm (Photo 6, 7). Lipid droplets were located close to the glycogen zone. In this phase the hepatocyte lobules were formed in a line that was separated by the sinusoid network. The development of the hepatocytes was accompanied by that of the circulatory system of the liver.

## DISCUSSION

The differentiation of hepatocytes during the post-embryonic development of nase was similar to that of most fish species investigated to date. Undifferentiated liver cells and a small number of sinusoid vesicles were already present at the moment of hatching. The first signs of liver function were detected in the studied larvae between days 2 and 4 of development. Similar observations concerning the begin-



nings of liver function during the organogenesis phase of the larvae of sole, *Solea solea* (L.), were described by Boulhlic and Gabaudan (1992), by Bisbal and Bengtson (1995) in the larvae of summer flounder, *Paralichthys dentatus* (L.), and by Diaz and Connes (1991) in European seabass. These authors reported that the appearance of glycogen is simultaneous with hepatocyte differentiation. Glycogen stores began to be built when young are still dependent on the mother's body (in mammals) or on the yolk sac reserves (birds and fish). The RER cisternae appeared in the studied specimens on days 2 to 3 after hatching just as they did in European seabass (Diaz and Connes 1991). The development of the RER indicates the synthesizing function of the hepatocytes, which was weak at the beginning of endogenous nutrition but increased significantly at the end of the endo-exogenous nutrition phase in nase.

The Golgi apparatus in the early stages of nase hepatocyte development was identified by the presence of lipoprotein particles, while distinct Golgi apparatus cisternae were observed in the juvenile stage. Similar changes were observed in sea bream (Guyot et al. 1995) during liver differentiation.

The first lipoprotein droplets in the hepatocyte cytoplasm of nase observed before the mouth cavity became passable originated from the stores of the yolk sac. It was documented earlier by Diaz et al. (2002) and Ostaszewska (2002) that the perivitelline syncytium synthesizes lipoprotein from yolk sac stores. These are then sent to the area near the perivitelline and the circulatory system. The presence of vesicles containing lipid materials in the area of the Golgi apparatus during the endo-exogenous feeding phase in nase larvae suggests their involvement in the secretion of bile lipids. In accordance with this hypothesis (Havel and Hamilton 1988, Diaz et al. 1997), bile lipids are synthesized from blood plasma lipids taken up by the hepatocytes, transformed in the endoplasmic reticulum and Golgi apparatus and then transported through the vesicles and released by exocytosis to the bile canaliculi.

The presence of light hepatocytes in the liver parenchyma that was observed in nase was also confirmed in Atlantic salmon, *Salmo salar* L. (Spellberg et al. 1994), sea trout, *Salmo trutta* L. (Rocha et al. 1996), and carp (Fishelson and Becker (2001). Rocha et al. (1996) suggested that these cells are a type of transitional macrophage.

From the beginning of larval development of nase, the hepatocyte nuclei contained nucleoli with dispersed chromatin without nucleolonema as in channel catfish, *Ictalurus punctatus* (Raf.) (Hinton 1976) and rainbow trout (Chapman 1981). However, in carp the nucleolus was not observed until the hepatocytes had matured and they were surrounded by nucleolonema (Fishelson and Becker 2001).

In the livers of vertebrates, the bile canaliculi are located in intercellular spaces (*i.e.* among two or more hepatocytes). According to Segner and Braunbeck (1990), the livers of fish from the family Cyprinidae only intracellular canaliculi occur (single cells). In the current research and in that of Fishelson and Becker (2001) on carp, bile canaliculi occurred in the intercellular spaces, but in mature hepatocytes there were also intracellular canaliculi. However, in the bile ducts of nase no complex intercellular connections between two or three adjoining hepatocytes were noted. Intercellular connection complexes that separate the lumen of the canaliculi from the remaining surfaces of the hepatocytes occur in vertebrates as well as in fish (Hinton 1976, Chapman 1981).

In the studied specimens of nase, pores were confirmed while there was an absence of the basal lamina in the epithelial lining the sinusoids allowed blood serum metabolites easy passage through the vessel walls to the liver cells.

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## STRESZCZENIE

### ROZWÓJ HEPATOCYTÓW PODCZAS ORGANOGENEZY ŚWINKI (*CHONDROSTOMA NASUS* (L.))

Na podstawie obserwacji histologicznych i ultrastrukturalnych stwierdzono, że rozwój hepatocytów świnki ma podobny przebieg jak u innych gatunków ryb kostnoszkieletowych.

Rozwój hepatocytów świnki można podzielić na trzy okresy obejmujące kolejne fazy odżywiania się. Okres endotroficznego odżywiania, podczas którego rezerwy pęcherzyka żółtkowego są przetwarzane w syncytium okołożółtkowym i rozprowadzane przez system krążenia. Wówczas hepatocyty różnicują się, pojawiają się zapasy glikogenu i niewielka ilość lipoprotein (fot. 1, 2, 3). Drugi okres endo-egzotroficzny rozpoczyna się udrożnieniem jamy ustnej, pobraniem pierwszego pokarmu, zwiększeniem aktywności aparatu Golgiego i wytwarzaniem lipidów żółciowych (fot. 4, 5). Trzeci okres to wyłącznie odżywianie egzogenne, w wątrobie magazynowane są i odtwarzane zapasy glikogenu oraz lipidów (fot. 6, 7).