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THE SEQUENTIAL DIFFERENTIATION AND FORMATION OF HEPATIC AND PANCREATIC STRUCTURES IN ASP (*Aspius aspius* L.) LARVAE

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ABSTRACT

The study focussed on differentiation of hepatic and pancreatic cells in asp (*Aspius aspius* L.) larvae during early ontogeny (1–21 days post hatch). Histological evaluations and histochemical assays showed the asp liver and pancreas to commence functioning almost simulatenously, between the third and the fifth day of larval life.

Key words: fish, asp, larvae, liver, pancreas.

INTRODUCTION

The recent years witnessed a growing interest in breeding and production of stocking material of rheophilous fish. The species studied in this respect include the asp (*Aspius aspius* L.), a cyprinid that occurs, in Europe, in drainage basins of the North, Baltic, Black, and Caspian Seas. In Poland, the species inhabits mostly larger rivers, lagoons, and dam reservoirs. Due to the small size of its populations, the asp has a small commercial potential, but it is an attractive target for anglers [15]. Breeding and grow–out of newly hatched larvae under controlled conditions is one of the ways the abundance of the species can be increased. Studies on artificial reproduction of asp were carried by Kaukoranta and Pennanen [10], Śliwiński *et al.* [17], and Kujawa *et al.* [11].

Śliwiński [16] successfully grew out the summer larvae of asp in monoculture in carp ponds; equally successful was his culture of the asp autumn larvae in a polyculture with carp.

From the fish culturist's point of view, it is extremely important to be able to pinpoint the moment in time when the larvae should be fed, i.e., when the endo–exogenous nutrition begins. If supplementary feeding starts too late, the fish will starve, while if it starts too early, the water will become polluted and the culture costs will increase. According to Margulies [12], changes in the hepatocyte structure brought about by restricting the hepatocyte lipid accumulation are one of the symptoms of improper feeding or starving. The formation of the liver and the pancreas during early organogenesis was studied in a number of species, i.a., *Salmo gairdneri* [19], *Dicentrarchus labrax* [6], *Solea solea* [5], *Acipenser baeri* [7], and *Melanogrammus aeglefinus* [9].

The present work was aimed at conducting histological observations on differentiation of hepatic and pancreatic cells during the larval period of asp. The results may contribute to our ability of selecting suitable time when supplementary feeding of the larvae should commence; they may also be helpful in determining the appropriate quality of food such that it meets the demands of the asp larvae in culture.

MATERIALS AND METHODS

The study was carried out at the Warsaw Agricultural University's (WAU) Laboratory of Ichthyobiology and Fisheries (LIF) at Brwinów and involved newly hatched asp larvae obtained from the WAU Fisheries Research Station (FRS) hatchery at Łąki Jaktorowskie. The larvae were kept in three 20 dm^3 no–flow water tanks. The tanks were stocked with larvae kept at a density of 70 ind. dm^{-3}. The larvae were allowed to develop at 18–20°C. The water was collected from the FRS hatchery. Table 1 summarises the chemical and physical parameters of the tank water. The larvae were kept until 21 days post hatch. As from day 3, they were fed *ad libitum* with *Artemia salina* nauplii (about 100 nauplii per larva) offered twice a day (at 9.00 and 19.00 hours). After 11 days of feeding, the diet was supplemented with LANSY A2 pelleted feed (Inve Aquaculture, Belgium).

Table 1. Chemical and physical parameters of tank water

Temperature	18–20°C
pH	7.3–7.9
Dissolved oxygen content	7.5–9.2 mg dm^{-3}
Ammonia	< 0.005 mg $N\text{-}NH_4$ dm^{-3}
Nitrates	0.01–0.04 mg N – NO_3 dm^{-3}
Nitrites	< 0.01 mgN –NO_2 dm^{-3}

Throughout the culture period, the tank water was filtered and aerated. The tanks were cleaned every 24 h, whereupon 3/4 of the water volume were changed. Water temperature, dissolved oxygen content, and pH were measured prior to each water change, while the nitrate, nitrite, and ammonia contents were determined at 7–day intervals.

During the period of study, from hatch until 21 days post hatch, 20 larvae were harvested every 48 h and preserved for subsequent histological evaluations and histochemical assays. The larvae were anaesthetized (MS 222), fixed, and subjected to a standard histological procedure. Following fixation, the larvae were dehydrated in the ethanol series and embedded in paraffin. The 5 µm thick paraffin serial sections were obtained with a MicroTec CUT 4050 microtome; some of the larvae were sectioned transversely, while others were cut longitudinally.

Larval morphology was identified on the haematoxylin–VOF (H–VOF; light green, orange G, acidic fuxin)–stained sections, following Gutierrez [8]. Various mucines (glycogen–related compounds) and proteins were identified histochemically. The staining techniques used were those proposed by Martoja and Martoja–Pierson [13] and Pearse [14] (Table 2).

Table 2. Histochemical reactions used to detect and identify carbohydrates and proteins, following Martoja and Martoja-Pierson [13] and Pearse [14]

Staining technique		Compounds identified
Carbohydrates	PAS (periodic acid Schiff) procedure	glycogen, neutral mucopolysaccharides; magenta
	diastase (amylase) PAS	glycogen
	alcian blue (AB), pH 2.5	glycogen-like compounds with carboxyl and sulphate groups; turquoise-blue
	chloric acid-hydrolysed alcian blue (AB), pH 2.5	sialic acid
	AB; pH 1.0 AB; pH 0.5	sulphate glycogen-like compounds; dark blue
Proteins	bromophenol blue	all proteins; red
	Schiff ninhydrin	lysine-rich proteins; pink-red
	potassium ferrocyanide	cystine-rich proteins; red
	1,2 naphthylchinone-4-sulphonic acid, sodium salt	arginine-rich proteins; orange-yellow
	Hg sulphate-sulphuric acidic sodium nitrate	tyrosine-rich proteins; purple
	Ferrous ferricyanide (Fe III)	cysteine-rich proteins; blue
	p-dimethylaminebenzaldehyde	tryptophan-rich proteins; blue

The sections were made and pictures taken at LIF. Hepatocytes were measured under a Nikon–Alphaphot–2YX2 microscope coupled with a Mintron camera and the MicroScan for Windows (v. 1.5) image analysis computer software. The hepatocyte mean length and width were calculated from lengths and widths measured in 20 cells from various parts of the liver of each of the 20 larvae from an age group.
The length of the yolk sac and the total length (l.t.) of larvae at various developmental stages were measured under a light microscope. The larvae were measured to 0.01 mm. The yolk sac volume (V; mm^3) was calculated from the elongated ellipsoid volume formula:

$$V = 0.5236\ l\ h^2$$

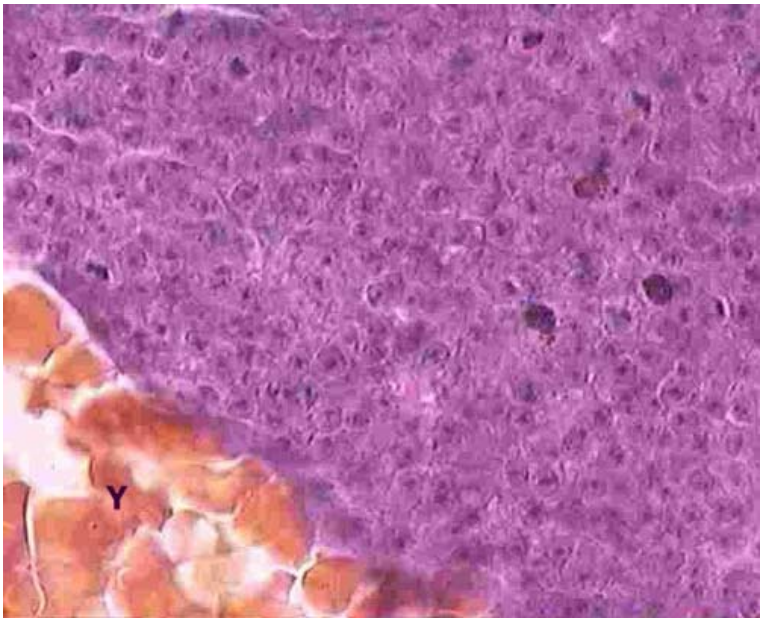
where: l, yolk sac length (mm); h, yolk sac height (mm).

The mean total length of larvae after 21 days of culture was 17.23 mm (n = 100), the overall (across 3 tanks) mean survival rate being 97.6%.

RESULTS

On the first day post hatch, the asp larvae (the nomenclature used follows that proposed by Balon [2]) measured 6.2 mm in mean total length, their pear–shaped yolk sacs being about 0.20 mm^3 in mean volume. At this developmental stage, the mouth cavity, oesophagus, and the anterior section of the intestine were blind. The terminal part of the intestine showed a narrow lumen lined with irregular cubic cells. Almost the entire length of the intestine rested on the yolk sac. Densely clumped and undifferentiated cells were observed between the anterior part of the intestine and the yolk sac. Those cells, 6 µm in mean length and width, were precursors of the liver and of the pancreas. They contained homogenous, basophilic cytoplasm and centrally located, rounded nuclei (Fig. 1).

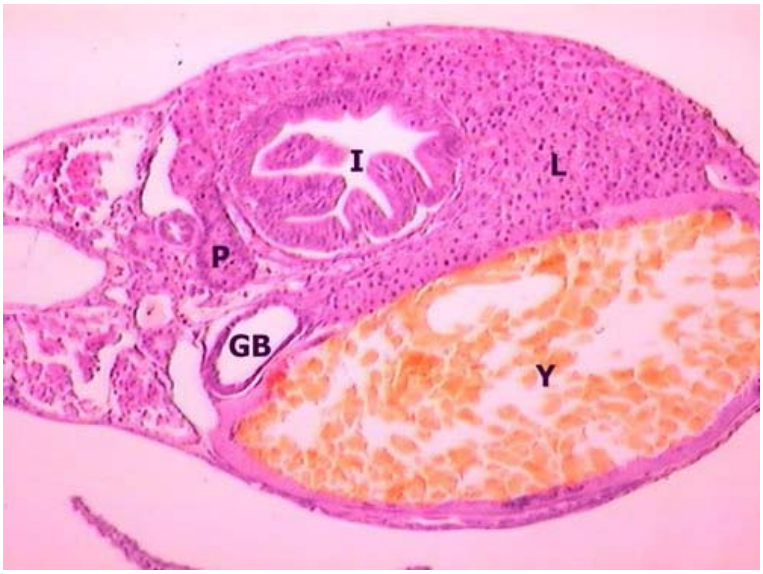
Fig. 1. Day 1 post hatch. Longitudinal section through undifferentiated cells of liver and pancreas; Y, yolk sac; AB/PAS staining; × 100



The mouth cavity became passable and the oesophagus connected with the intestine between day 2 and 3 post hatch. At that time, the *Artemia* nauplii were first given to the asp larvae which began feeding on them when their yolk sacs were still rather large (0.0816 mm^3 mean volume).

The hepatocytes were observed to increase in number and in size. No glycogen deposits were observed. As a result of hepatocyte proliferation, the hepatic gland increased in size. The intestine was surrounded by undifferentiated cells of the primordial liver (Fig. 2).

Fig. 2. Day 3 post hatch. Transverse section through an asp larva. Intestine (I) is surrounded by liver (L). Y, yolk sac; P, pancreas; GB, gill bladder; H-VOF statining; × 25

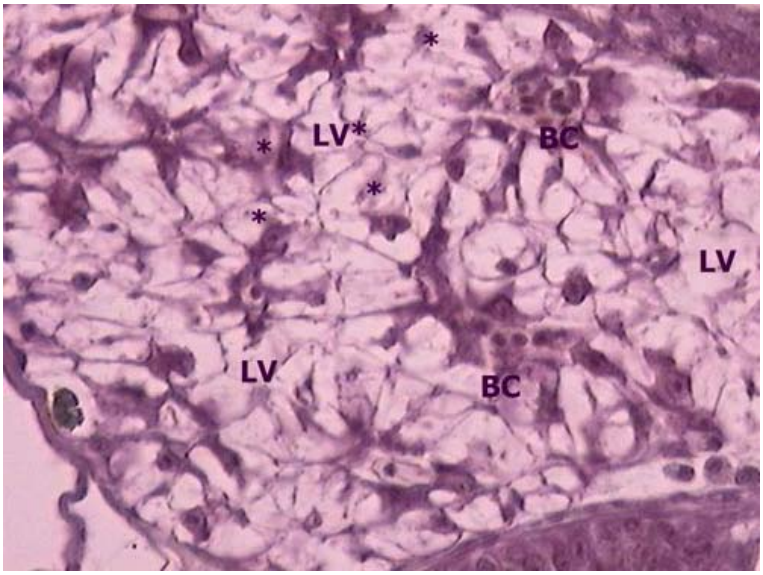


Hepatocytes began differentiating between day 3 and 5, whereupon the liver histology was observed to change markedly. Blood cells–filled sinusoids were visible between groups of hepatocytes. The hepatocyte cytoplasm showed PAS–positive and amylase–PAS–negative granules, indicative of glycogen storage. No lipid deposits were found.

During the period of endo–exogenous nutrition (day 3 to 11 post hatch), the hepatocytes were observed to gradually increase in number and volume. Their nuclei and cytoplasm were pushed out to the cell's outskirts by

the increasing amounts of glycogen (PAS–positive areas) and lipids (light areas). The largest hepatocytes (averaging 13 µm x 12 µm) were observed on days 8 – 10 post hatch when copious amounts of glycogen and lipids were being rapidly accumulated (Fig. 3).

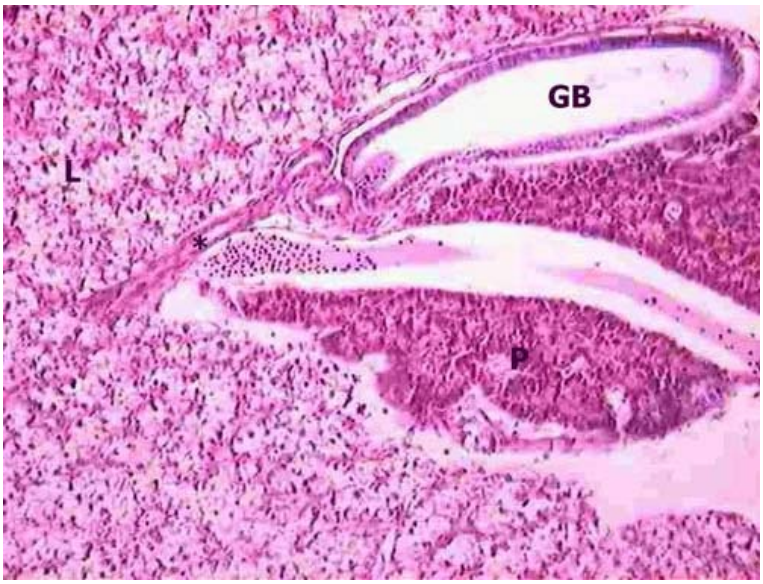
Fig. 3. Day 10 post hatch. Transverse section through liver. Lipids (LV) and glycogen (G) accumulated in liver; sinusoids with blood cells (BC); H-VOF staining; × 100



On day 11, when the larvae averaged 10.17 mm in total length, the yolk sac was completely resorbed and the period of exogenous feeding began. The hepatocytes were observed to accumulate more glycogen than lipids. At the same time, the number of sinusoids and hepatocytes surrounding them increased markedly. Subsequently, until the termination of the study, the histology of the liver underwent no change. On day 21, the hepatocytes averaged 18 × 16 µm in size.

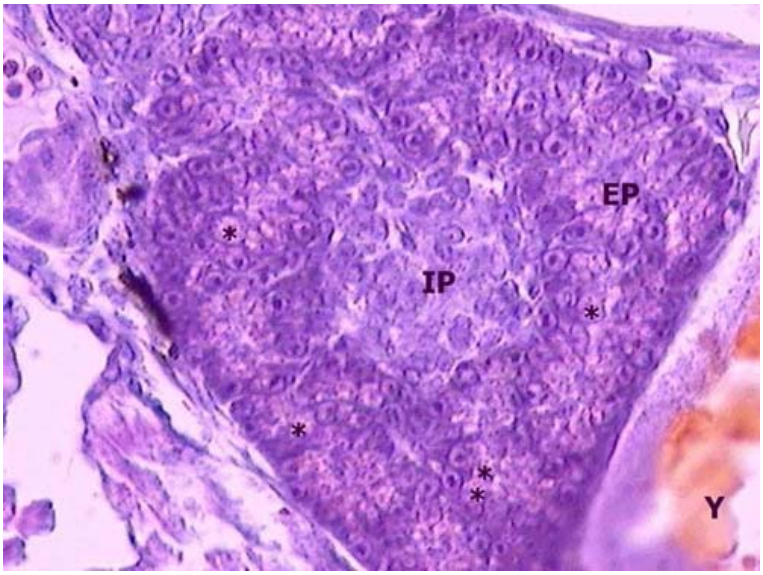
The larval pancreas began to differentiate 3 days post hatch, as evidenced by the emergence, in the central part of the organ, of a pancreatic islet, once called the Langerhans islet (the endocrine part of the pancreas), surrounded from the outside by cells of the secretory (exocrine) part. At that time, the gall bladder, located between the liver and the pancreas, was clearly visible. The bill duct, originating in the liver and connecting with the gall bladder, could be observed as well. The gall bladder and bill duct mucosa consisted of a single layer of cubic epithelial cells (Fig. 4).

Fig. 4. Day 17 post hatch. Longitudinal section through liver and pacreas. The main bill duct (DC) discharging into gall bladder (GB); L, liver; P, pancreas. AB/PAS staining; × 25



Concurrent with the onset of the hepatic gland differentiation (days 3–5 post hatch), the pancreatic endocrine cells were aggregating to form clusters, their cytoplasm showing the first zymogen granules. The pancreatic cell cytoplasm was strongly basophilic, thus differing markedly from acidophilic zymogen granules (Fig. 5).

Fig. 5. Day 5 post hatch. Transverse section of pancreas. Proenzyme granules (ZG) in cells of exocrine pancreas (EP). IP, endocrine pancreas; y, yolk sac. AB/PAS staining; × 100



The pancreatic cells which stained positively with bromophenol blue and showed affinity to orange G and H–VOF, formed vesicular structures. The vesicles were observed to house the first zymogen granules, the granules containing tryptophan–, cystine–, tyrosine–, and lysine–rich proteins. The exocrine pancreas cells were interspersed with blood vessels.

As the larval development progressed, the pancreas was increasing in size to become situated between the swim bladder and the anterior and middle section of the intestine; the major part of the gland, however, was located in the anterior part of the body cavity, to the right of the intestine.

DISCUSSION

As a result of numerous roles the liver plays in the body and due to its diverse activities, the organ indispensable for any animal, including fish, to function properly. Among other things, the liver is a storage site for numerous nutrients, primarily glycogen, and to some extent lipids [18]. Detoxication and – during the larval development – production of antibodies is another important hepatic function.

As in all other vertebrates, the asp liver and pancreas are endodermal in origin. The organs emerge as undifferentiated embryonic cells located between the anterior part of the intestine and the yolk sac. During the larval development, hepatocytes differentiated rapidly and, as a result of morphogenetic translocation, they surrounded the intestine as early as on 3 days after hatch.

The first signs of the hepatic and pancreatic function in the larvae studied were detected between days 3 and 5. Similar observations on the beginnings of the hepatic and pancreatic function during organogenesis of larval *Solea solea* and *Paralichthys dentatus* were described by Boulhic and Gabaudan [5] and by Bisbal and Bengston [3], respectively. Diaz and Connes [6] reported the glycogen appearance to coincide with hepatocyte differentiation. Glycogen accumulation begins when an animal still depends on either a maternal organisms (mammals) or yolk reserves (birds and fish) for nutrition.

Histological observations allowed to conclude that development of the digestive tract, involving the opening of the mouth cavity, formation of the connection between the oesophagus and the intestine, and the functioning of the liver and the pancreas, made it possible for a larva to ingest, digest, and assimilate the first exogenous food even before the yolk sac was completely resorbed. A comparison of the results obtained in this study with observations on the development of hepatocytes and pancreatic cells in *Salmo gairdneri* [19], *Dicentrarchus labrax* [6], and *Solea solea* [5] shows a similar sequence of the development and differentiation of the glands

during the period of endo–exogenous feeding in the asp larvae. The zymogen granules appearing in the exocrine pancreas vesicles during the early larval development were reported also by Bisbal and Bengston [3] in *Paralichthys dentatus* and by Gisbert *et al*. [7] in *Acipenser baeri*. On the other hand, Hamlin et al. [9] observed the zymogen granules in *Melanogrammus aeglefinus* larvae to appear in the exocrine pancreatic vesicles earlier, at hatching.

The presence of both the gall bladder and the bill duct connecting the liver with the anterior part of the intestine was also evidence of hepatic activity and bill production. The appearance of lipid reserves in the larval asp hepatocytes, observed 8–9 days post hatch, was a result of the larvae feeding on natural food. A similar observation was earlier reported by Diaz and Connes [9] from hepatocytes of larval *Dicentrarchus labrax* fed *Artemia*. According to Appelbaum *et al*. [1], the liver histology reflects accurately the fish feeding regime. Glycogen accumulated in the hepatocytes is a primary source of energy and can be released when the fish starve [4, 12]. During the period of exogenous nutrition, once the yolk sac reserves had been completely resorbed, the larval asp liver histology did not change, the larval hepatocytes resembling in appearance those of the adults.

CONCLUSIONS

Histological observations on development of the liver and the pancreas of asp larvae allowed to conclude that the changes taking place on the formation of the glands could be divided into three groups:

1. From hatch until day 3: hepatocytes and pancreatic cells were morphologically undifferentiated, the larvae obtaining nutrition from substances stored in the yolk sac.
2. Between day 3 and 11: hepatocytes and pancreatic cells were undergoing differentiation and the glands were beginning to function; that was the stage at which the larvae started endo–exogenous feeding.
3. As from day 11, following the complete resorption of the yolk sac, the liver and the pancrease were fully formed morphologically.

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