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Ultrastructure of Hepatocyte and Liver Ontogeny of the Indo-Pacific Seahorse *Hippocampus barbouri* Jordan & Richardson 1908

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ABSTRACT

The Indo-Pacific seahorse, *Hippocampus barbouri*, is one of the most important marine fish and a potential target of aquaculture, but basic biological information on this fish is largely missing. In this study, we described the hepatocyte ultrastructure and the ontogeny of the liver in *H. barbouri* during the 1st and the 35th day after birth (DAB). The histological observation of the liver structure identified that hepatocytes have a centrally placed oblong to round nucleus surrounded by the basophilic cytoplasm. Extensive accumulation of lipid droplets and glycogen was observed in the cytoplasm of the hepatocytes. Transmission electron microscope observation confirmed that the hepatocytes contained cisternae of granular endoplasmic reticulum associated with mitochondria of various shapes. Secretory granules of uniform density were scatted in hepatocytes, which were considered as the glycogen storage granules. According to the liver ontogeny analysis, the basic liver structure such as a network of the hepatocyte and sinusoid capillary was present at the 1st DAB and a large amount of collagen accumulation was observed by the 14th DAB. These results increase the knowledge about the initial development of *H. barbouri*, which will be useful to assess nutritional status of this species during aquaculture development.

Introduction

Seahorses are actively traded for aquarium purpose and as an important ingredient of the traditional Chinese medicine. In addition to the fishing pressure, accidental capture by non-selective fishing gear and the habitat loss have raised a serious concern about their long-term persistence in nature (Lourie *et al*., 2004; Vincent *et al*., 2011). For all the above reasons, all seahorse species are included in the Appendix II list of endangered species of the Convention for the International Trade in Endangered Species. This situation limits the legal import and export of dead and alive seahorses. The establishment of aquaculture thus not only contributes to the conservation of seahorses, but also to the sustainable development of aquarium and seafood markets.

The liver is one of the largest internal organs in body cavity of seahorses and is the center of metabolism. This organ is essential for glycogen storage, control of metabolism, detoxification, and homeostasis (Genten *et al*., 2009). Histological and ultrastructural characterizations of the liver are hence continuously explored in several teleosts (Hugla and Thome, 1999; Yang and Chen; 2003; Vicentini *et al*., 2005; Ribeiro *et al*., 2006). The liver ontogeny has also been reported in many teleosts (Falk-Petersen, 2005; Pradhan *et al*., 2012) including seahorses such as spotted seahorse, *Hippocampus kuda* (Mi *et al*., 1998), long-snouted seahorse, *H. reidi* (Novelli *et al*., 2015), and more recently long-snouted seahorse, *H. guttalatus* (Ofelio *et al*., 2018). All results suggested that the liver is an accurate indicator of the physiological status in fish especially that of the digestive system to ingest, digest and absorb foods.

The Indo-Pacific seahorse *H. barbouri* Jordan & Richardson 1908 is an important coastal seahorse species that inhabits sea grass beds, and its distribution has been reported in Indonesia, Malaysia, and Philippines (Lourie *et al*., 2004; 2005). Recent published data confirmed the presence of *H. barbouri*

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in Thailand, raising the possibility that this species will be a target for aquaculture in Thailand and other Asian countries; however, basic biological information of this species including morphology and ontogeny is still scarce. The objective of this study, therefore, was to describe the hepatocyte ultrastructure and liver ontogeny of *H. barbouri* during the 1st and the 35th day after birth (DAB). The present study could provide the basis of liver structure throughout its development, which would be applied to fulfill for the nutritional requirements as well as aquaculture development of this seahorse.

Materials and methods

Seahorse samples and hatchery area

We used voucher specimens of healthy captive *H. barbouri* at the 1st, 7th, 14th, 24th and 35th DAB in this study. All fish (n = 3 in each DAB) were collected during October to December 2017 as reported in Kamnurdnin (2017). All fish were reared in closed seawater systems at the Phuket Marine Biological Center (PMBC), Phuket Province, Thailand. Environmental parameters include a temperature range from 26 to 28˚C, a salinity level of 31-33 ppt and a photoperiod of 12:12 h light-dark, all of which were favorable to this species. The experimental protocol was approved by the Animal Care and Use Committee of Faculty of Science in accordance with the guide for the care and use of laboratory animal prepared by Chulalongkorn University (Protocol Code. 1623004). Histological structures of other organs have been reported elsewhere (Senarat *et al*., 2020; 2021).

Histological observation of the liver

All seahorse samples were fixed in aqueous Davidson's solution for 24 h and then kept in 70% ethanol. Pieces of fixed liver tissues were processed using a standard histological technique (Presnell and Schreibman, 1997; Suvarna *et al*., 2013). The paraffin blocks were sectioned at 4 µm thickness and then stained with Harris's hematoxylin and eosin (H&E) for observation of the basic structure, Masson's trichrome (MT) for the detection of fiber and Periodic acid–Schiff (PAS) for the detection of glycogen. The structure and chemical composition of the liver were observed throughout its ontogeny. Photomicrographs were taken under the light microscope coupled with a camera for photography (TE750-Ua).

Ultrastructural observation

Small pieces of *H. barbouri* liver (1x1 mm^3, n = 3, at the 35th DAB) were fixed by immersion in 2.5% glutaraldehyde, 0.1 M phosphate buffer, pH 7.4 at 4°C and subsequently post-fixed in 1% osmium tetroxide. The plastic blocks were cut at 500 nm thicknesses, stained with toluidine blue, and examined for accurate localization of hepatocytes under the light microscopy. Ultrathin sections of 80 nm thicknesses were stained with uranyl acetate and lead citrate and examined with transmission electron microscopy (TEM, Philips, TECNAI 20).

Results

Structure and ultrastructure of hepatocytes

Histological observation of the healthy liver of *H. barbouri* showed that the hepatocytes were distributed as anastomotic cords, making two cellular layers. Each cell had a round polygon shape with a spherical nucleus, which was arranged in distinct cords (Figs. 1A-1B). Extensive intracellular accumulation of lipid droplets was found in hepatocytes, which was observed as the vacuolization in the H&E staining. Glycogen accumulation was observed as magenta pink in PAS staining (Fig. 1C). The sinusoids were observed between hepatic cords, but they were not easily identified under the light microscope (Figs. 1A-1B).

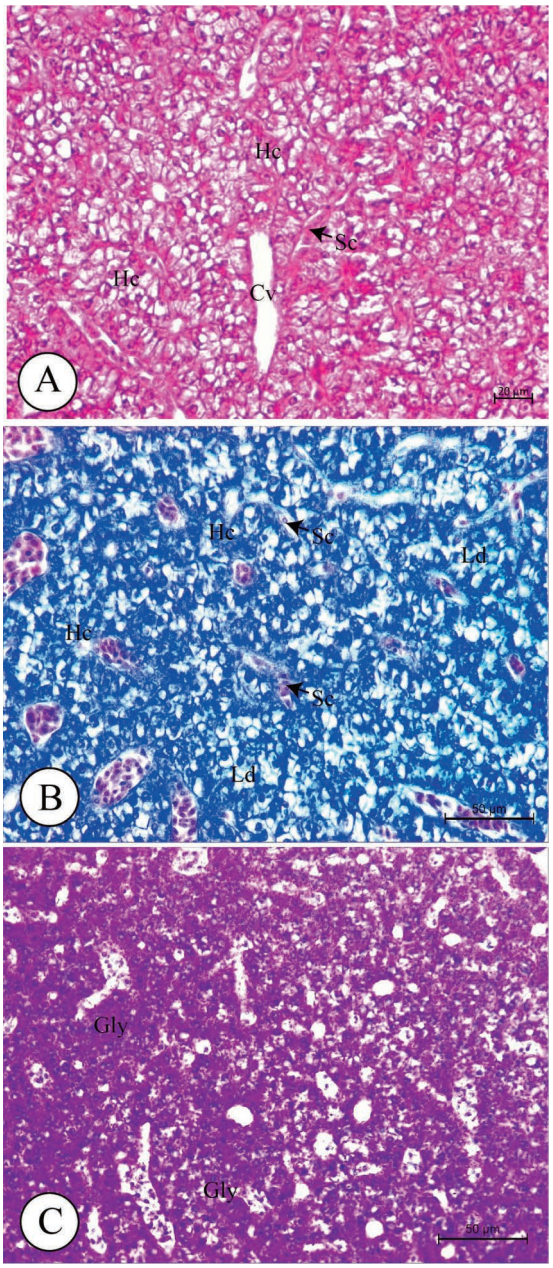

Fig. 1. Light microscope showing the liver parenchyma of *Hippocampus barbouri*. It composed of several hepatocytes (Hc) contain lipid droplet (Ld) and glycogen (Gly). (A) Harris's hematoxylin and eosin (H&E), (B) Masson's trichrome (MT) and (C) Periodic acid–Schiff (PAS). Abbreviations: Cv = central vein, Sc = sinusoid.

TEM clearly visualized hepatocytes and sinusoids containing erythrocytes (Fig. 2A). The hepatocytes were characterized by a centrally located nucleus with condensed heterochromatin (Fig. 2B), which was seen at the periphery of the nucleus (Fig. 2C). Electron-dense cytoplasm was relatively poor in organelles, but the flat cisternae of rough endoplasmic reticulum were observed along the plasma membrane (Fig. 2C). A few mitochondria were observed, and their shapes varied from round to elongated shapes (Fig. 2C). The oval lipid droplets were easily identified by the presence of electron dense granules (Fig. 2B). In contrast to the cytoplasm that had low electron density, glycogen granules were observed as a uniform homogenous deposit of high electron density (Fig. 2C).

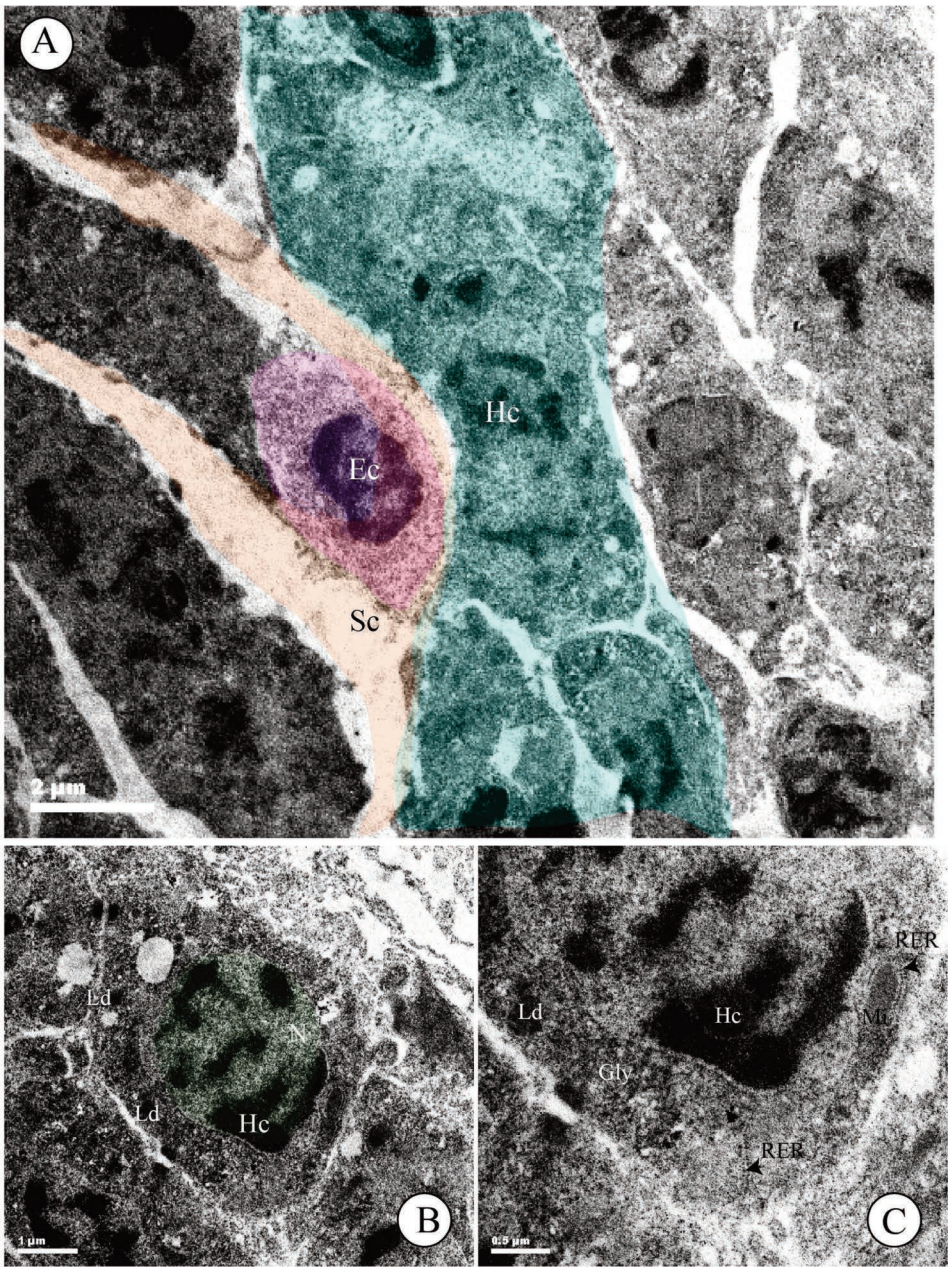

Fig. 2. Electron microscope showing the hepatocyte (Hc) of *Hippocampus barbouri*. Erythrocyte (Ec) was clearly existed in the sinusoid (Sc). High magnification showed few rough endoplasmic reticulum (RER) and mitochondria (Mi) in the cytoplasm of hepatocyte.
Abbreviations: Gly = glycogen, Ld = lipid droplet, N = nucleus

Liver ontogeny

At the 1st DAB, hepatocytes with a centrally located nuclei were packed in parenchyma and a network of sinusoid capillary was developing (Figs. 3A-3B). By the 7th to the 35th DAB, the number of hepatocytes were substantially increased, and elongated lobes were formed. In parallel to the formation of liver parenchyma, a significant increase in vacuolization, which indicates the accumulation of lipid droplets and glycogen, was observed continuously from the 1st to the 35th DAB (Figs. 3A-3F). Note that a great amount of glycogen was observed at the 14th DAB (PAS method, Figs. 3B-3D).

Discussion

The liver of *H. barbouri* was composed of numerous hepatocytes, each of which contained a centrally placed nucleus. Histological and ultrastructural observations of seahorse from this study were highly consistent with those of previous studies (Hugla and Thome, 1999; Yang and Chen, 2003; Vicentini *et al*., 2005; Ribeiro *et al*., 2006; Blanchard *et al*., 2008). Interesting features of the *H. barbouri* liver found in this study include the cisternae of rough endoplasmic reticulum, homogenous deposits of low-density materials, and few mitochondria varying in shapes from circular to elongate. A previous study pointed out the low synthetic activity for secretory proteins is associated with these features (Gonzalez *et al*., 1993). Additionally, granular endoplasmic reticulum and mitochondria are essential organelles for oxidizing, reducing, and conjugating various xenobiotics (Gonzalez *et al*., 1993). In this regard, it might be suggested that the hepatocyte of *H. barbouri* is the center of detoxification activities but of low synthetic activity for secretory proteins.

Glycogen was found in the cytoplasm of hepatocytes in *H. barbouri* as commonly seen in many fish such as *Ictalurus punctatus* (Hinton and Pool, 1976) and *Oreochromis niloticus* (Vicentini *et al*., 2005). Glycogen acts as an energy reserve. The biosynthesis and breakdown of the deposited glycogen are

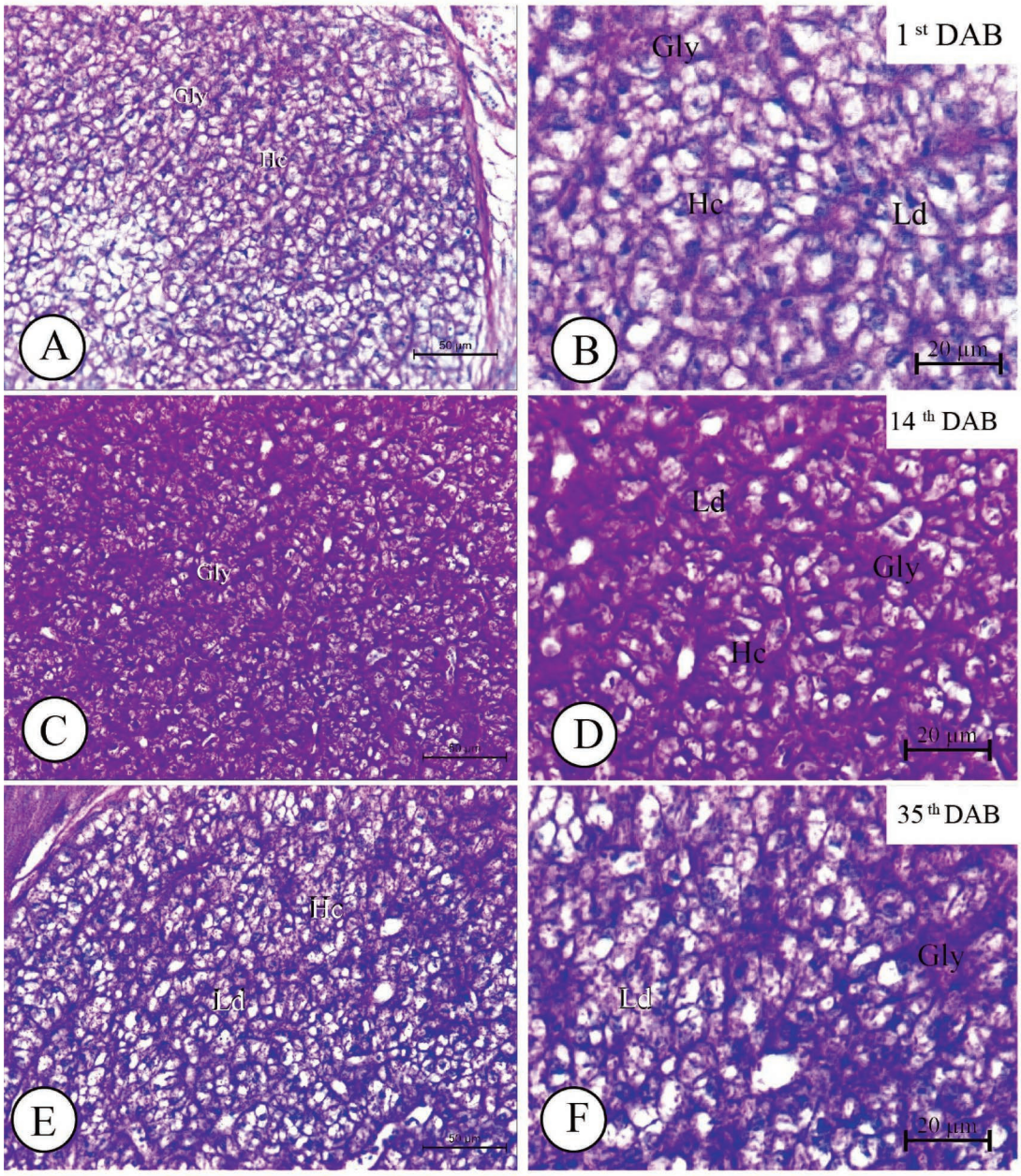

Fig. 3. Light microscope showing the liver ontogeny of *Hippocampus barbouri* during development. Representative Figs. from the 1st DAB, the 14th DAB and the 35th DAB. Abbreviations: Gly = glycogen, Hc = hepatocytes, Ld = lipid droplet

important in fish development, and the degree of accumulation may be a biomarker for healthy development.

The liver parenchyma was easily identified and contained both lipids and glycogen at the 1st DAB, indicating the specific roles of the liver, including the regulation of digestive process and synthesis and storage of macromolecule, already takes place in the 1st DAB. This is in consistent with the data from other teleosts (Luizi *et al*., 1999; Liu *et al*., 2013; Novelli *et al*., 2015). Several former documents showed that the accumulation of lipoproteins increases in association with growth rate (Diaz *et al*., 1989; Santos and Vinagre, 1991; Luizi *et al*., 1999; Liu *et al*., 2013). We also found a large glycogen accumulation in the *H. barbouri* liver at the 14th DAB, which is consistent with that at the 15th DAB in *H. guttulatus* (Ofelio *et al*., 2018) and *Gadus morhua* (Kjorsvik and Reiersen, 1992). Although there are seasonal variations in glycogen accumulation (Hyvariner *et al*., 1985), these results suggest that this is a critical period to complete glycogen storage for the growth during the following larval stage. It is thus possible that the nutrition and the activity of digestive enzymes during this period is important for the subsequent growth. This crucial question needs to be explored in further investigations for the future establishment of aquaculture.

The hepatic lipid accumulation found in this study did not reach the level of hepatic steatosis, but it needs to be determined whether wild heathy specimens have the similar level of lipid accumulation. If this feature was associated with the imbalanced diet, deficiency in dietary fatty acids, and/or inadequate culture conditions (Robaina *et al*., 1989; Caballero *et al*., 1999), the lipid accumulation might eventually cause hepatic steatosis. This feature could also be one of multiple parameters to assess the condition of this species.

Conclusion

This study demonstrated the hepatic structure of *H. barbouri* at the histological and ultrastructural levels. The obtained data supports the vital roles of the liver such as storing macromolecules and detoxification activities. Since a large amount of liver glycogen was recorded at the 14th DAB, nutri-

tional states before this period might be related to the developmental success. This study contributes to a better understanding of the digestive biology and can help to improve the larval rearing techniques.

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Conflict of interest

The authors declare that they have no conflict of interest.

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