

Original article

Life-stage dependent Histological Characterization of Liver HIF-1 α Expression in the Indonesian Shortfin Eel (*Anguilla bicolor bicolor* McClelland, 1884)

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Abstract

The Indonesian shortfin eel (*Anguilla bicolor bicolor* McClelland, 1884) is catadromous fish. The developmental phase of the fish is influenced by the availability and type of feed, and dissolved oxygen levels (DO). Oxygen level fluctuations during migration from sea to fresh waters can cause hypoxia and trigger activation of the Hypoxia-inducible factor 1 alpha gene (HIF-1 α). Therefore, understanding HIF-1 α expression is very important in understanding the life cycle and physiology of this organism. This study aimed to perform a histological comparison between the liver structure of eel in the elver eel phase to silver eel and compared the expression of HIF-1 α in the liver. The eels were collected from Kulon Progo Regency, Segara Anakan in Cilacap, and Cangkringan in Sleman, respectively. The eel livers were prepared with standard paraffin method. The samples were stained with Hematoxylin-Eosin, Mallory Acid Fuchsin and Periodic Acid-Schiff Alcian Blue for histological observation. The HIF-1 α protein expression was detected using immunohistochemistry. The results showed that the structure of liver elver, yellow, and silver eel shared similar tubular hepatocytes. Moreover, there was no significant differences in the parenchymal structure of liver of all stage of eel. Mucin levels were different in each life cycle phase. HIF-1 α expression varied by eel phase, with the elver eel showing weak expression, yellow stage showing moderate expression, and silver eels displaying the highest levels of expression. We conclude that the highest expression of HIF-1 α was found in the silver eel, which resides in freshwater environments and is found in freshwater while returning to the sea for reproduction.

Keywords: Eel life cycle, oxygen level, HIF-1 α , liver histology

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Introduction

The Indonesian shortfin eel (*Anguilla bicolor bicolor*) is catadromous fish, which exhibits migration from seawater at early stages to fresh water during maturation. During its life cycle the fish has 5 stages of development: leptocephalus, glass eel, elver eel, yellow eel, and silver eel. Leptocephalus and early stages of glass eel reside in the ocean, late-stage glass eels exist at the transition between ocean and fresh water, and the elver, yellow and silver eel occupy the freshwater habitat. The environmental changes greatly impact fish biology. A particularly vital organ for eel environmental adaptation is liver (McMenamin and Parichy, 2013). The fish liver is a key organ which controls many physiological functions, including carbohydrate, fat, and protein metabolism; detoxification; filtering and recycling of blood components; the forming of gall; vitamin and ferrum metabolism; immunity; and vitellogenesis. Therefore, as a consequence of environmental and habitat changes, environmental changes result in physiological and structural changes to fish liver (Brusle and Anadon, 1996). Thus, proper liver development and structural changes are critical to metamorphosis between each eel stage and are a key component of fish adaptation and survival in the new habitat.

The liver structure at each stage of eel development

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can be examined with histological and histochemical analysis. The histochemical profile of the liver can be determined with protein expression, in both quality and quantity. Catadromous fish differ in metabolic levels depending on whether they reside in ocean or freshwater habitats. The sea and inland waters have several different characteristics, including salinity and dissolved oxygen (DO) levels. Seawaters in general have DO levels of 20% lower compared to their freshwater counterpart. Low oxygen level can lead to hypoxic stress in fish, which could trigger activation of Hypoxia-inducible factor (HIF).

HIF is a central transcription factor which provides an adaptive response to hypoxia stress, in normal or in abnormal conditions (Poellinger and Johnson, 2003). One among them is HIF-1, forming transcription complex of HIF-1 protein which, via its DNA-binding site activates transcription of erythropoietin in hypoxic condition. HIF-1 consists of an alpha and beta sub-units (Wang and Semenza, 1995). HIF-1 α is recognized as a factor which plays important role in the adaptation of eel to hypoxia along the development phase, healing phase, regeneration, and the growth of tumors (Iyer et al., 1997, Elson et al., 2000, Maeno et al., 2005).

HIF-1 α contributes to metabolic adaptation by controlling enzymes that are responsible for shifting metabolism toward anaerobic glycolysis. Moreover, mitochondrial respiration is also influenced by HIF-1 α . HIF-1 α modulates the key path of metabolism in optimizing the use of glucose and oxygen in hypoxic conditions to create enough ATP without producing excessive ROS by preventing the tricarboxylic-acid (TCA) cycle and mitochondrial respiration (Weidemann

and Johnson, 2008). It was reported that inactivation of HIF α s and HIF related gene also causes embryonic lethality (Semenza, 2009).

The aim of the research is to compare the liver histological structure in elver eel, yellow eel, and silver eel and characterize HIF-1 α expression levels. These results will increase our understanding of eel development and the physiological adaptations necessary for them to complete their life cycle.

Methods

This research was conducted at the Laboratory of Structure and Development of Animals, Faculty of Biology, Gadjah Mada University.

Eel fish Samples

Eels used in these experiments were the Indonesian shortfin eel (*Anguilla bicolor bicolor*) during developmental phases of glass, elver, yellow and silver eels. The eels were obtained from the Progo River in Kulon Progo Regency, Segara Anakan in Cilacap Regency, and Cangkringan in Sleman Regency, in the Special Region of Yogyakarta on the island of Java.

Liver histology preparation

Liver histological sections were prepared following using the paraffin standard method. Eels were

anesthetized and sacrificed by dissection. Livers were dissected, collected, and then washed using isotonic solution (NaCl 0.9%) and were fixed with Bouin's solution for 24 h; washed with 70% alcohol, dehydrated with series of ethanol concentration. Organ clearing and infiltration were conducted by immersion in toluene solution and then paraffin at 60°C. The liver sections were cut 6 μ m thick and were stained with *Hematoxylin Ehrlich Eosin* (HE) and *Periodic Acid Schiff Alcian Blue* (PAS-AB).

Immunohistochemistry preparation

Immunohistochemistry was prepared with a kit (Universal HRP Detection System) produced by Biocare Medical, including blocking background Sniper, Trekkie Universal Link, Trek-Avidin-HRP label, Betazoid DAB substrat buffer, Betazoid DAB Chromogen, and color frosted slide premium coated (produced by Biogear). Primary antibody of anti-HIF-1 α monoclonal antibody (produced by Thermo Fisher Scientific) was used for detection. Citrate buffer (pH 6), phosphate buffer saline (pH 7,4), phosphate buffer saline Tween, hydrogen peroxide 3%, hydrogen peroxide 0,3% in 60% methanol, Bovine Serume Albumin (BSA), were prepared and used during the experiment. ImageJ software was used for detection.

Results and discussion

Histological Structure of Eel Liver and cellular analysis (stained with HE)

A histological comparison and cellular analysis was conducted on eel liver on the stage of elver, yellow and silver eel. The analysis on elver eel was conducted on the anterior and posterior part of liver because the liver size was still very small. Both regions (anterior and posterior) of Elver eel liver showed similar patterns and consisted of liver cells (hepatocytes) in polyhedral shapes, with a rounded nucleus. The hepatocytes were arranged as one layer of hepatocytes. Moreover, liver sinusoid showed irregular shape, which exist in between hepatic plate (Figure 1).

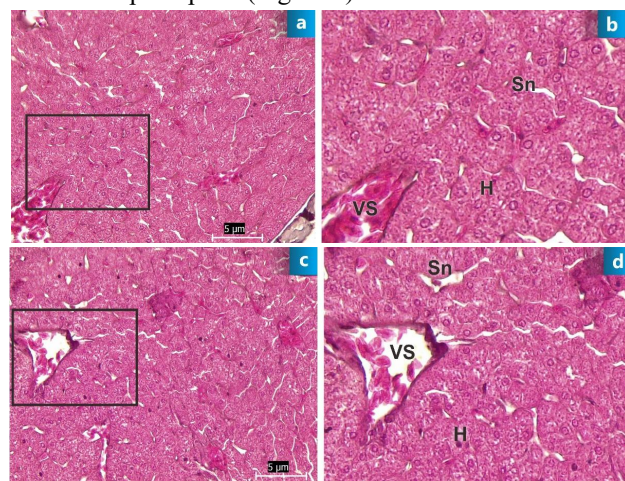


Figure 1. Liver histological structure of: (a) Elver eel anterior, (b) Elver eel anterior (increased magnification), (c) Elver eel posterior, (d) Elver eel posterior liver (increased magnification). Showing: central vein (VS), hepatocyte (H), sinusoid (Sn). HE stained, 400x magnification.

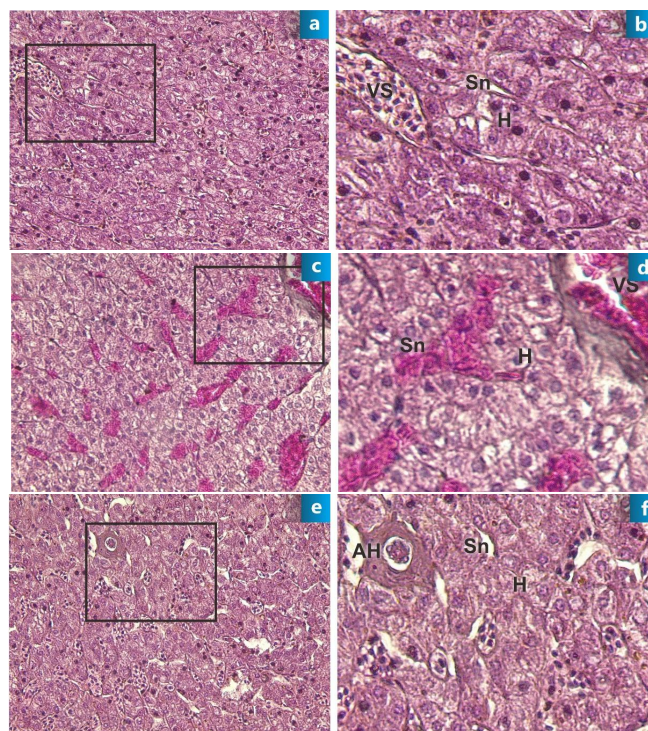


Figure 2. Yellow eel liver histological structure of, (a) anterior liver (b) anterior liver (increased magnification), (c) middle liver, (d) middle liver (increased magnification), (e) posterior liver, (f) posterior liver (increased magnification). Showing: central vein (VS), hepatocyte (H), sinusoid (Sn), hepatic artery (AH). HE stained, 400x magnification

The analysis on yellow eel was conducted on the anterior, middle, and posterior liver, in order to cover representative areas. Results showed that all parts of the liver shared a similar pattern and consisted of polyhedral hepatocytes, with a rounded nucleus, similar to those on

elver eel. However, the yellow eel hepatocytes were arranged in a double-layered configuration (Figure 2).

The analysis on silver eel and yellow eel was conducted on the anterior, middle, and posterior liver. Results showed that all parts of the liver shared a similar pattern and consists of polyhedral hepatocytes, with a rounded nucleus, identical to those on elver and yellow eels. The Silver eel hepatocytes were arranged on a form double-layered configuration. Sinusoid were positioned between the hepatic plate and has irregular shape (Figure 3).

The histological structure side to side comparison of elver, yellow, and silver eel showed similar hepatocyte

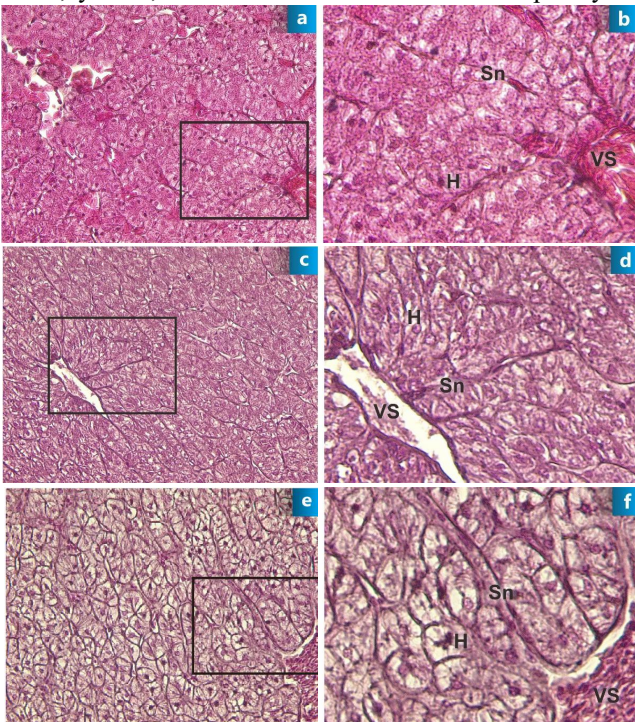


Figure 3. Silver eel liver histological structure of, (a) anterior liver (b) anterior liver (increased magnification), (c) middle liver, (d) middle liver (increased magnification), (e) posterior liver, (f) posterior liver (increased magnification). Showing: central vein (VS), hepatocyte (H), sinusoid (Sn). HE stained, 400x magnification

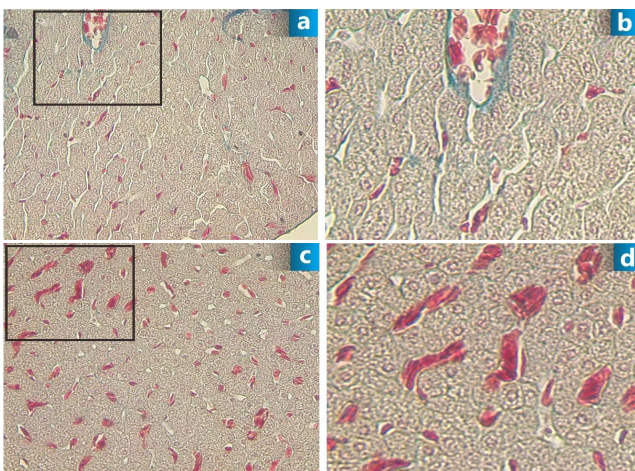


Figure 5. Liver histological structure of: (a) Elver eel anterior liver, (b) anterior liver (increased magnification), (c) Elver eel posterior liver, (d) posterior elver eel liver (increased magnification). Showing: central vein, connective tissues layer (arrow). MAF stained, 400x magnification

shapes and cellular arrangements in a regular pattern. However, the sinusoid of yellow eel was more dynamic in width size compared to elver and silver eel. Moreover, the elver and silver eel sinusoid were more consistent in shape compared to yellow eel (Figure 4).

Tubular shaped hepatocyte-sinusoidal histological structure that were observed on elver yellow and silver eel is in line with the report by Akiyoshi and Inoue (2004), on the Anguiliformes, the Japanese eel (*A. Japonica*) which also has a tubular hepatocyte-sinusoidal structure.

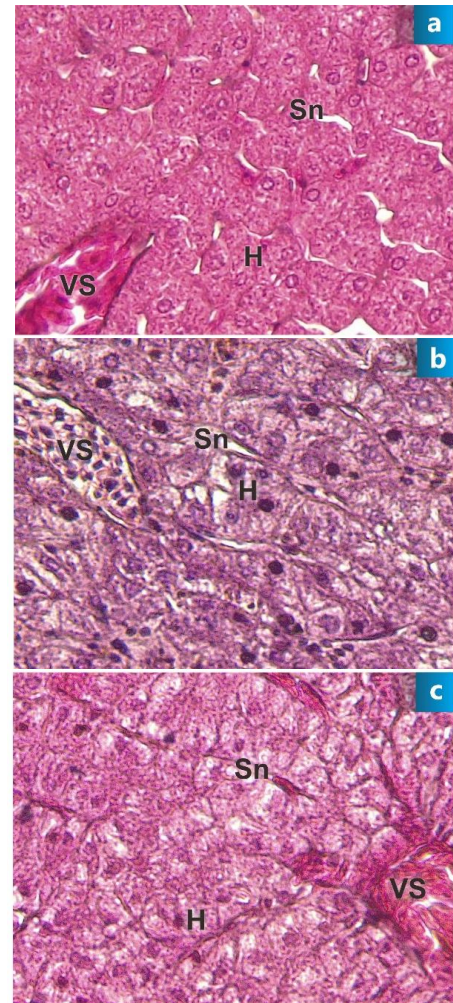


Figure 4. Hepatic structure (a) Eel anterior elver, (b) Yellow eel anterior, (c) Silver eel anterior. Showing side-side comparison of each stage eel liver structure. central vein (VS), hepatocyte (H), sinusoid (Sn). HE stained, 400x magnification

Histological Structure of Eel Liver for matrix non-cellular analysis (stained with MAF)

Matrix non-cellular analysis on liver histological structure, stained with MAF, showed matrix in the form of connective tissue lining on the sinusoid edge. The connective tissues consisted of collagen fiber, which stained green in color.

The histological structure examination showed the existence of sinusoid in the area of conjunction with pre- and post-sinusoidal sites between hepatocyte plates and the central vein. Sinusoid shape and diameter were uniform in each eel stage. However, interspecies side to

side comparisons of elver, yellow, and silver eel showed variation in connective tissue thickness levels of collagen fibers and connective tissue structure.

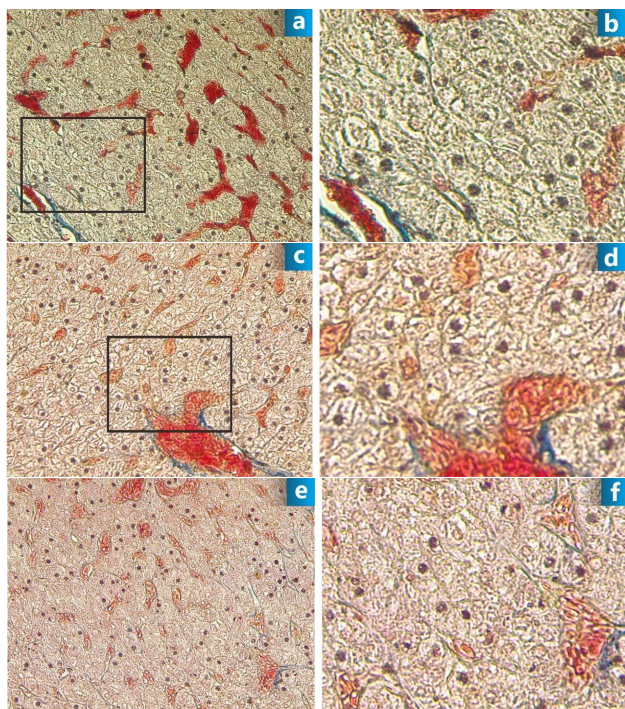


Figure 6. Yellow eel liver histological structure of, (a) anterior liver (b) anterior liver (increased magnification), (c) middle liver, (d) middle liver (increased magnification), (e) posterior liver, (f) posterior eel liver (increased magnification). Showing: central vein, and connective tissues layer (arrow). MAF stained, 400x magnification

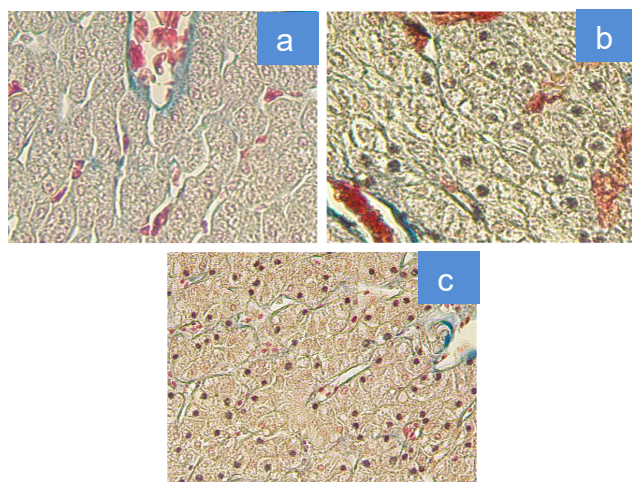


Figure 8. Hepatic structure (a) Eel anterior elver, (b) Yellow eel anterior, (c) Silver eel anterior. Showing side-side comparison of each stage eel liver structure. central vein, hepatocyte, sinusoid (arrow). MAF stained, 400x magnification

Histochemical analysis of Eel Liver for cellular product (stained with PAS-AB)

The histochemical analysis of glycogen accumulation in hepatocytes with PAS-AB staining showed that the anterior part of the liver was stained more strongly than the posterior part of the elver, and yellow eel, which suggest a heterogeneity of glycogen distribution in the liver of elver, and yellow eel, respectively. However, in the silver eel, the PAS hepatocyte-stained intensity tends to be evenly distributed in the anterior and

The connective tissues of elver eel were thinner compared to yellow and silver eel. The thin layer of connective tissue causes the border between each hepatocyte on elver were not clearly seen, compared to yellow and silver eel (Figure 5, 8). In yellow and silver, thick connective tissues were observed. The border of each cell, the hepatocyte plates and the central vein were clearly observed (Figures 6, 8). In the silver eel, the cell boundary was more pronounced than in the elver and yellow eel, respectively (Figure 7, 8).

Results showed that all part of the liver share a similar pattern and consist of polyhedral hepatocytes, with a rounded nucleus, identical to those on elver and yellow eel. The Silver eel hepatocytes were arranged in a double-layered configuration. Sinusoid were positioned between the hepatic plate and have irregular shapes (Figure 3).

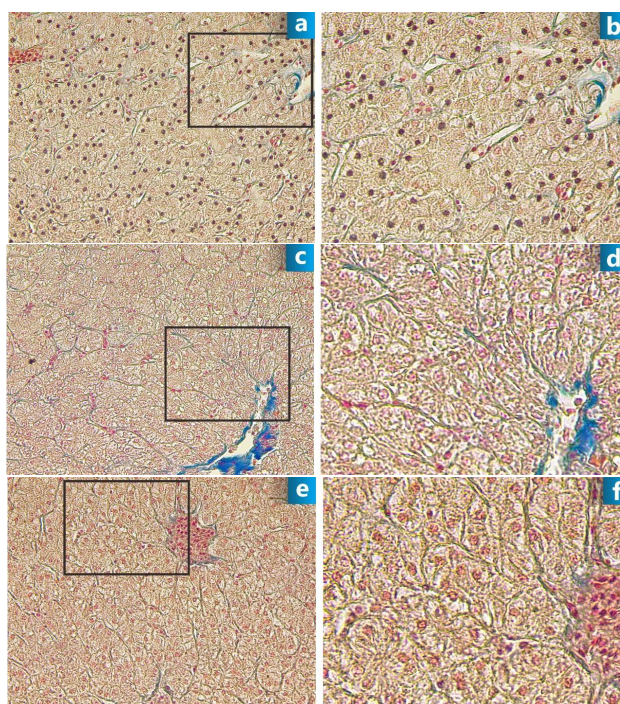


Figure 7. Silver eel liver histological structure of, (a) anterior liver (b) anterior liver (increased magnification), (c) middle liver, (d) middle liver (increased magnification), (e) posterior liver, (f) posterior of eel liver (increased magnification). Showing: central vein, hepatocyte, and connective tissues of sinusoid (arrow). MAF stained, 400x magnification

posterior parts. This suggests highly saturated glycogen accumulation in the silver eel phase hepatocyte, compared to elver eel and yellow eel. The different glycogen levels in each eel stage suggests the possibility of shifting the prey target which provides different glycogen supply level, or a metabolic strategy that stores lower amounts of glycogen for reserve since in elver and yellow the eel is developing rapidly. In contrast to the silver stage where the eel stored as much as possible

glycogen for reserve energy during the fresh water-open-sea migration for reproduction.

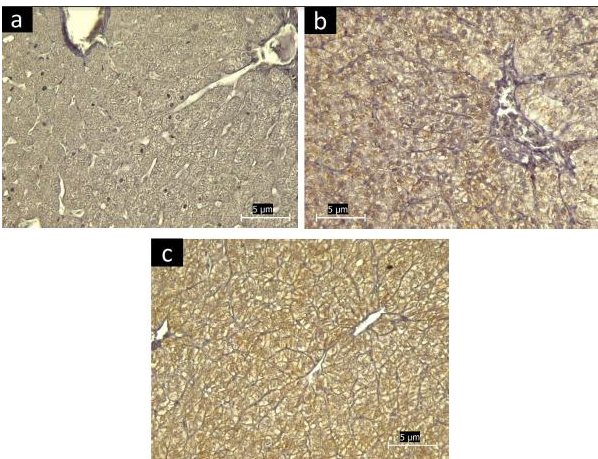


Figure 9. Expression of HIF-1 α in the liver, (a) Elver eel, (b) Yellow eel, (c) Silver eel. Note: brownish stain indicates the presence of HIF-1 α expression. 400x, IHC preparation.

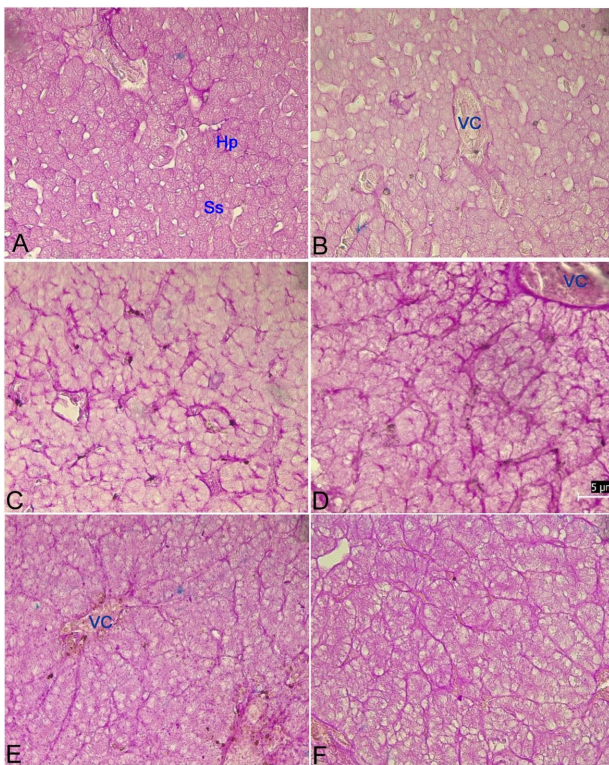


Figure 10. Liver Histological structure of; (a) Elver eel anterior, (b) Elver eel Posterior, (c) Yellow eel anterior, (d) Yellow eel posterior, (e) Silver eel anterior, (f) Silver eel posterior, Showing: deposition of glycogen on hepatocyte and lipid vacuole, VC: Central vein, Hp: Hepatocyte, Ss: Sinusoid. PAS AB staining, magnification 400x

The possibility of food preference shifts also was reported by Rupashinge and Attygale (2006), where eels experience a change of diet together with the growth of its body. Eels that have a body length of 300 mm prey on crustaceans and insects, while eels 300-500 mm long prey on annelida, mollusks and fish, and less frequently on crustaceans and insects. For eel with a body length of more than 500-600 mm, the main prey is crustaceans and fish. Glycogen deposits play an important role with regard to feed and the life cycle of eel. Glycogen serves as a source of glucose released

when fasting, or as a response to acute stressors (Viegas, 2012). In the silver eel phase, there were more lipid droplets that appeared than the elver eel and yellow eel phases. The number of fat droplets is related to reserve energy in the silver stage, as previously reported Lewander, *et al.*, (1974), where fat accumulation has been shown to be used when migrating back to the sea for spawn.

HIF-1 α protein expression

Immunohistochemical detection of HIF-1 α in elver, yellow, and silver eel liver showed that elver eel expressed the lowest levels of HIF-1 α , followed by yellow eel, and the highest level were observed in silver eel (Figure 9 and Table 1).

The high expression of HIF-1 α in the silver eel liver possibly were influenced by several factors, such as body metabolism rate, behavior, and also habitat type. Silver eel has a larger body size and exhibit active movement so that the oxygen demand is greater when compared to elver and yellow eel (Deelder, 1970; Egginton, 1986). Moreover, if oxygen needs are greater than those available in the environment, fish can experience hypoxia.

Hypoxia also triggers glycogen accumulation. The results showed that the silver eel had the most glycogen accumulation compared to elver eel and yellow eel (Figure 10). Glycogen synthase (GYS1) is a HIF-dependent gene that induces mediation of glycogen accumulation when oxygen is limited. HIF-dependent increases glycogen stores in hypoxic adaptation, which prepares cells to protect themselves when oxygen conditions are limited. This is done to ensure that the substrate is sufficient for anaerobic glycolysis supply (Pescador *et al.*, 2010).

Regulation of HIF-1 α expression has several paths. Under normal conditions, HIF-1 α is transcribed and synthesized together with signals that involve other growth factors or molecular signaling genes. In normoxia conditions, HIF-1 α is easily degraded, with a life span of 5 minutes. Under hypoxic conditions, there are several pathways to control the stability of HIF-1 α and transcriptional activity. Pathways for regulation of HIF-1 α also may involve post-translational modification, which involves hydroxylation, acetylation, ubiquitination, and phosphorylation (Masoud and Li, 2015).

When oxygen is present, hydroxylation prolyl catalyzed by Fe (II) -, oxygen- and PHD 2-oxoglutarate-dependent. The hydroxylated prolyl residue can cause the capture of HIF-1 α by the von Hippel-Lindau (pVHL) protein, which leads to ubiquitination and subsequent proteasomal degradation. Asparaginil hydroxylation catalyzed by an enzyme is termed a factor-inhibiting HIF (FIH) on one site in C-TAD. The hydroxylation prevents the recruitment of cofactors. In the absence of hydroxylation due to hypoxia or PHD inhibition, HIF-1 α translocates to the nucleus, with heterodimerization of HIF-1b and binds to hypoxic response elements (HRE) in the regulatory region of the target gene (Weidemann and Johnson, 2008).

Expression levels of HIF-1 α is as a form of adaptation to hypoxic conditions, which activates the uptake of glucose, glycolysis, and pyruvate be converted to lactate, in where production ATP persist although in hypoxic condition. HIF also reduces the use of oxygen by mitochondria with how to prevent mitochondrial biogenesis and activation of mitophagy (Goda and Kanai,2012).

In conclusion, The liver cellular structure of elver, yellow, and silver eel contains tubular hepatocytes. There is no difference in parenchyma general structure except the thickness level of connective tissues in each eel stage. The level of glycogen stores is different in each eel phase. There are differences in the HIF-1 α expression levels in eel liver, with the lowest expression in elver eel, followed by increased expression in yellow eels and the most in silver eels.

Table 1. Expression of HIF-1 α in eel liver

Phase	High Positive	Positive	Low positive	Negative	Score
Elver eel	0.2772	4.7780	87.0884	7.8565	Low positive
Yellow eel	0.3552	24,5957	64.7113	9.3673	Low positive
Silver eel	0.3676	34.3304	58.7821	6.5198	Positive

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