



**UNIVERSITI PUTRA MALAYSIA**

**GROSS AND HISTOLOGICAL CHARACTERISTICS OF BELONTIA  
HASSELTII ACQUIRED FROM THE PEAT SWAMP FOREST OF NORTH  
SELANGOR, MALAYSIA.**

**MUHAMMAD FAIZ BIN HODZORI**

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FPV 2020 51**

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*HASSELTII* ACQUIRED FROM THE PEAT SWAMP FOREST OF NORTH  
SELANGOR, MALAYSIA**

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**MUHAMMAD FAIZ BIN HODZORI**

**FACULTY OF VETERINARY MEDICINE**

**UNIVERSITI PUTRA MALAYSIA**

**SERDANG, SELANGOR**

**August 2020**

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**MUHAMMAD FAIZ BIN HODZORI**

**A project paper submitted to the  
Faculty of Veterinary Medicine  
Universiti Putra Malaysia (UPM)**

**In partial fulfilment of the requirement for the  
BACHELOR OF DOCTOR OF VETERINARY MEDICINE  
UNIVERSITI PUTRA MALAYSIA  
SERDANG, SELANGOR**

## CERTIFICATION

It is hereby certified that we have read this project entitled “Gross and Histological Characteristics of *Belontia hasselti* Acquired from the Peat Swamp Forest of North Selangor, Malaysia”, by Muhammad Faiz Bin Hodzori and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course VPD 4999- Final Year Project.

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## DEDICATIONS

This project is dedicated to *ALLAH SWT*, the best of planners who had made everything possible for me and my friends completing the projects,

*PARENTS & SISTERS*

Whose encouragement and prays of day and night for make me able to finish this project

Along with all hardworking and respected

*SUPERVISOR & CO-SUPERVISORS*

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## TABLE OF CONTENTS

	PAGE
<b>MUHAMMAD.....</b>	<b>i</b>
<b>CERTIFICATION .....</b>	<b>iv</b>
<b>DEDICATIONS.....</b>	<b>v</b>
<b>ACKNOWLEDGEMENTS.....</b>	<b>vi</b>
<b>TABLE OF CONTENTS.....</b>	<b>vii</b>
<b>LIST OF FIGURES .....</b>	<b>ix</b>
<b>LIST OF TABLES .....</b>	<b>x</b>
<b>LIST OF ABBREVIATIONS .....</b>	<b>x</b>
<b>ABSTRACT .....</b>	<b>xiii</b>
<b>1.0 INTRODUCTION.....</b>	<b>1</b>
1.1 Study Background .....	1
1.1.1 Background: .....	1
1.1.2 Overall Objective .....	2
1.1.3 Justification: .....	3
1.1.4 Hypothesis.....	3
<b>2.0 LITERATURE REVIEW.....</b>	<b>4</b>
2.1 Naturally Acidic Water .....	4
2.2 Effect of Excess Hydrogen Ion in Water for Fish.....	4
2.3 Acidic Water Habitat .....	6
2.4 Characteristics of Fish from Naturally Acidic Water .....	6
2.5 Malay Combtail ( <i>Belontia hasselti</i> ).....	6
<b>3.0 MATERIALS AND METHODS .....</b>	<b>9</b>
3.1 Experimental Fish .....	9
3.2 Experimental Design .....	9
3.3 Preparation of Histological Slide .....	9
3.4 Histological Observation .....	10
3.5 Statistical Analysis .....	11
<b>4.0 RESULTS.....</b>	<b>12</b>
4.1 Water Parameter .....	12
4.2 Gross Study .....	12

4.3 Histological Analysis .....	14
4.3 Histopathological Scoring .....	17
<b>5.0 DISCUSSION .....</b>	<b>26</b>
<b>6.0 CONCLUSION AND RECOMMENDATION .....</b>	<b>32</b>
<b>REFERENCES .....</b>	<b>33</b>
<b>APPENDICES .....</b>	<b>37</b>
APPENDIX 1 .....	37



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## LIST OF FIGURES

## PAGE

<b>Figure 1</b>	pH and dissolved oxygen level of the peat swamp water	11
<b>Figure 2</b>	Gross appearance of Kepar fish ( <i>Belontia hasselti</i> )	12
<b>Figure 3</b>	Gross appearance of kidney, muscle, skin, gill and brain of the fish	12
<b>Figure 4</b>	Photomicrograph of gills of Kepar fish ( <i>Belontia hasselti</i> )	15
<b>Figure 5</b>	Photomicrograph of skin layer and muscle of Kepar fish ( <i>Belontia hasselti</i> )	16
<b>Figure 6</b>	Photomicrograph of kidney and liver of Kepar fish ( <i>Belontia hasselti</i> )	17
<b>Figure 7</b>	Photomicrograph of histopathological changes of gills of Kepar fish ( <i>Belontia hasselti</i> )	18
<b>Figure 8</b>	Histopathological lesion scoring of gills of Kepar fish ( <i>Belontia hasselti</i> )	19
<b>Figure 9</b>	Photomicrograph of histopathological changes of kidney of Kepar fish ( <i>Belontia hasselti</i> )	20
<b>Figure 10</b>	Mean rank of histopathological lesion scoring of kidneys of Kepar fish ( <i>Belontia hasselti</i> )	21
<b>Figure 11</b>	Relative length of the primary and secondary lamellae of Kepar fish ( <i>Belontia hasselti</i> )	22
<b>Figure 12</b>	Muscle density (/mm <sup>2</sup> ) of Kepar fish ( <i>Belontia hasselti</i> )	23
<b>Figure 13</b>	Relative thickness of skin of Kepar fish ( <i>Belontia hasselti</i> )	24
<b>Figure 14</b>	Relative diameter of renal tubule of Kepar fish ( <i>Belontia hasselti</i> )	25

**LIST OF TABLES****Table 1** Histological parameters for gills, kidney and liver

11

**LIST OF ABBREVIATIONS**

PO <sub>2</sub>	Partial Pressure of Oxygen
PCO <sub>2</sub>	Partial Pressure of Carbon Dioxide
IACUC	Institutional Animal Care and Use Committee
UPM	Universiti Putra Malaysia

**ABSTRAK**

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar UPM untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek Tahun Akhir.

**CIRIAN GROS DAN HISTOLOGIK *BELONTIA HASSELTII* DARI HUTAN PAYA GAMBUT SELANGOR UTARA, MALAYSIA****Oleh:****MUHAMMAD FAIZ BIN HODZORI****2020****Penyelia:** Dr Mazlina Mazlan**Penyelia Bersama:** Dr Mohd Fuad Matori, Dr Awang Hazmi Awang Junaidi

Ikan air tawar semulajadi berasid boleh dijumpai di sekitar dunia. Namun begitu, laporan mengenai spesies ini agak terhad. Hutan paya gambut mempunyai ciri seperti pH serta nutrien dan kandungan oksigen terlarut yang rendah. Hutan Paya Gambut Selangor Utara (NSPF) adalah hutan paya gambut terbesar di Semenanjung Malaysia. *Belontia hasseltii* atau dikenali tempatan sebagai kepar merupakan sejenis ikan gourami dan adalah spesies asli di Asia Tenggara and telah dikenalpasti sebagai satu daripada 47 spesies ikan air hitam yang secara semulajadi tinggal di persekitaran air tawar berasid (pH 3.24) di NSPF. Kajian ini adalah bertujuan untuk menjadi data pertama mengenai penemuan histologi untuk memahami dengan lebih baik mengenai *Belontia hasseltii* terutamanya pada ikan yang ditangkap secara liar yang tinggal di persekitaran berasid di NSPF. Sebanyak 10 ekor ikan (6 jantan dan 4 betina) telah

ditangkap dari NSPF dengan menggunakan kaedah memancing. Penilaian visual pada setiap ikan telah dijalankan dan berat serta panjang badan ikan telah direkodkan. Ikan tersebut kemudiannya dikorbankan melalui kaedah pengumbutan dan sampel insang, kulit, hati dan buah pinggang ikan tersebut telah diambil serta merta di lokasi. Penemuan kajian menunjukkan panjang ikan tersebut adalah di antara 9-14.5 cm dengan berat di antara 12-43 g. Ikan tersebut telah dibahagikan kepada Kumpulan A (<12.3 cm) dan Kumpulan B (>12.3 cm). Keputusan menunjukkan panjang relatif lamela dan ketumpatan otot mempunyai perbezaan bererti ( $p < 0.05$ ) di antara 2 kumpulan tersebut manakala diameter tubul renal untuk kedua-dua kumpulan tidak menunjukkan perbezaan bererti ( $p > 0.05$ ). Sistem skor histopatologi untuk parameter seperti hiperemia, nekrosis, hiperplasia dan keradangan pada insang, buah pinggang dan hati tidak menunjukkan perbezaan bererti ( $p > 0.05$ ). Tiada penemuan abnormal gross didapati pada semua ikan, tetapi pemerhatian secara histologik menunjukkan ikan tersebut memiliki ciri yang istimewa pada insang. Terdapat suatu struktur yang terdiri daripada otot lembut dan lapisan epitelial tebal diantara setiap lamela primer pada insang ikan. Selain itu, didapati hiperplasia dan nekrosis ringan dan tidak memudaratkan pada ikan. Kesimpulannya, ikan ini memiliki struktur yang istimewa pada insang dan hanya menunjukkan perubahan patologi ringan pada organ yang dikaji dimana ianya menunjukkan ikan dapat mengadaptasi dengan lebih baik dalam persekitaran berasid.

**Kata kunci:** *Belontia hasselti*, Hutan Paya Gambut Selangor Utara (NSPF), histologi, insang, kulit, otot, ginjal, hati.

**ABSTRACT**

An abstract of the project paper presented to the Faculty of Veterinary Medicine UPM in partial fulfillment of the course VPD 4999 – Final Year Project

**GROSS AND HISTOLOGICAL CHARACTERISTICS OF *BELONTIA HASSELTII* ACQUIRED FROM THE PEAT SWAMP FOREST OF NORTH SELANGOR, MALAYSIA****By:****MUHAMMAD FAIZ BIN HODZORI****2020****Supervisor:** Dr Mazlina Mazlan**Co-supervisors:** Dr Mohd Fuad Matori, Dr Awang Hazmi Awang Junaidi

Naturally acidic freshwater fishes can be found worldwide, yet there are limited reports regarding these species. Peat swamp forest is characterized by low pH as well as nutrient and dissolved oxygen content. North Selangor Peat Swamp Forest (NSPF) is the largest peat swamp forest in Peninsular Malaysia. *Belontia hasselti* or locally known as Kepar, is a gourami native species in Southeast Asia and was identified as one from the 47 species of blackwater fish naturally living in acidic (pH 3.24) freshwater environment of NSPF. This study aimed mainly to provide the first data on the histological finding for better understanding of *Belontia hasselti* especially of those wild caught fishes living in acidic environment of NSPF. A total of 10 fishes (6 males and 4 females) were caught from the NSPF using fishing method. Visual appraisal on each fish was done and the length and weight of the fishes were recorded.

The fishes were then sacrificed by pithing method and the gills, skin, muscle, liver and kidneys were sampled on-site. Results revealed that the length of the fishes ranged from 9-14.5 cm and the weight ranged between 12-43 g. The fishes were divided into Group A (<12.3 cm) and Group B (>12.3 cm). Results revealed that the relative length of primary lamellae and muscle density were significantly different ( $p < 0.05$ ) between two groups while relative length of secondary lamellae and the diameter of the renal tubules of both groups were not significantly different ( $p > 0.05$ ). Histopathological scoring for parameters such as hyperaemia, necrosis, hyperplasia and inflammation for the gills, kidney and liver showed no significant differences ( $p > 0.05$ ). Grossly, no abnormal finding was noted in all the fishes, but histologically, the fishes possess special features in the gills. The gills display a structure, which consists of smooth muscle and thick epithelial layer between each primary. Additionally, hyperplasia and necrosis were mild and not detrimental to the fishes. In conclusion, the fishes possess special structures in the gills and exhibited only mild pathological changes in the studied organs, which suggest the fishes are able to adapt well in the acidic environment.

**Keywords:** *Belontia hasselti*, North Selangor Peat Swamp Forest (NSPF), histology, gills, skin, muscle, kidney, liver.

## 1.0 INTRODUCTION

### 1.1 Study Background

#### 1.1.1 Background:

The Malay combtail, *Belontia hasselti* or locally known as Kepar, is a gourami species native to South-East Asia. Kepar is a source of protein for the local people population living near the peatlands. There is also a demand for Kepar to be reared as an aquarium fish. Kepar can be found in the Malay Peninsula and Greater Sunda Islands peat swamps (Kottelat, 2013). The fish originates from acidic freshwater biotopes with minimum flow of water. Generally, peat swamp can be considered as a naturally acidic habitat with pH ranging from 4.5 to 6.5. The acidic pH is due to tannins and organic acids being released into the water by plant detritus on its substratum (Ng et al., 1992; Beamish et al., 2003).

North Selangor Peat Swamp Forest (NSPSF) is a peatland located in Selangor and comprises of the Raja Musa Forest Reserve and the Sungai Karang Forest Reserve. It is the largest remaining peat swamp forest in Peninsular Malaysia, which covers approximately about 76,000 hectares. This unique wetland ecosystem comprises of soil which are very acidic with pH levels as low as 3.5. Peat swamp forest is an area having long term accumulation of sediments that remain waterlogged, with the peat forming when plant materials are inhibited from decaying by acidic condition (United Nations Development Programme, 2006). Therefore, peat swamp forest characteristically is low in nutrients and dissolved oxygen and high in acidity (Rahim et al., 2009; Yule & Gomez 2009). Interestingly, NSPSF has significantly low pH of

2.9 due to the presence of decaying plant materials which will result in low dissolved oxygen (Beamish et al., 2003; Ali Sule et al., 2015). Moreover, the peat soils contain very few nutrients. Based on published data by Ng et al. (1994), there is about 47 species of blackwater fish in the NSPSF. Based on this study and a report by Beamish et al. (2003), Kepar was identified to be one of the species which are stenotopic to acidic waters.

To date, there is no specific research on the histological study of *Belontia hasselti*. Histology is an accurate sense of study of cytoarchitectural tissue change that envisages the anatomy and provides insight into the functioning of tissues and organs. Thus, this study will help to provide the first data regarding the histological finding in *Belontia hasselti* living in a normal and yet extreme condition. The outcome of this project will furnish better understanding about the fish body mechanism in adapting in acidic water environment.

### **1.1.2 Overall Objective**

To evaluate the gross and histological characteristics of skin, liver, gills and kidney of *Belontia hasselti* living in acidic water environment acquired from the peat swamp forest of North Selangor, Malaysia.

### 1.1.3 Justification:

There is currently no specific research being done about this species especially in terms of the histological study. Hence, this study will provide the first data on the histological finding that will contribute to a better understanding of *Belontia hasselti* especially of those wild caught fishes living in acidic environment.

### 1.1.4 Hypothesis:

The hypothesis for this study is as follows:

**H<sub>0</sub>** = There is no special gross and histological characteristics of skins, liver, gills and kidney of *Belontia hasselti* living in acid water environment acquired from peat swamp forest.

**H<sub>a</sub>** = There is special gross and histological characteristics of skins, liver, gills and kidney of *Belontia hasselti* living in acid water environment acquired from peat swamp forest.

## **2.0 LITERATURE REVIEW**

### **2.1 Naturally Acidic Water**

According to Clymo (1984), around 1 to 3% of the earth's surface is categorized as acidic. Besides that, the freshwater element of these systems is lacking in numbers and species of fishes compared to a system with near neutral pH (Rahel & Magnuson, 1983). However, they also can be a habitat rich in species (Chao, 2001). Based on a paper written by Wust et al. (2007), the black water system in Southeast Asia arises from the Holocene origin as well. However, it is distinguished by slightly less species variety (Beamish et al., 2003). Peat swamp can be considered as a naturally acidic habitat with pH ranging from 4.5 to 6.5. The acidic pH is due to tannins and organic acids being released into the water by plant detritus on its substratum (Ng et al., 1992). North Selangor Peat Swamp Forest (NSPSF) is a peatland located in Selangor and comprises of the Raja Musa Forest Reserve and the Sungai Karang Forest Reserve. It is the largest remaining peat swamp forest in Peninsular Malaysia which covers approximately about 76,000 hectares. This unique wetland ecosystem comprises of soil which are very acidic with pH levels as low as 3.5 (Munawi, 2014).

### **2.2 Effect of Excess Hydrogen Ion in Water for Fish**

Water breathing freshwater fish needs to import ions from their food and environment persistently as they are hyperosmotic towards their habitat and it is believed that this mechanism helps to compensate for diffusive ion deprivation, especially from the gills with large diffusive volume to work as a respiratory organ (Dymowska et al., 2012). Wood & McDonald (1982) found that acid naive fish when exposed to heavy concentration of  $H^+$  setting, generally shows increase in mortality,

net losses of crucial monovalent ions such as  $\text{Na}^+$ ,  $\text{Cl}^-$ ,  $\text{HCO}_3^-$ , and  $\text{K}^+$  are acidotic and asphyxiate from time to time. Reid (1995) stated that the asphyxiation might be due to the excess mucous production that increases the diffusion distance for brachial oxygen uptake and normally seen at extreme low pH condition. More recent studies outlined by LeDuc et al. (2013) and Kwong et al. (2014) suggested that chronic effects of exposure to acids can be associated with reduced growth, metabolic disturbance, gill injuries, less skeletal ossification, aberrant behaviour, endocrine malfunction, weak immune system, and sensory loss. In addition, the consequences of acid exposure are delicate to a few environmental covariates such as calcium ion, sodium ion, chloride ion, total conductivity, Partial Pressure of Oxygen ( $\text{PO}_2$ ), Partial Pressure of Carbon Dioxide ( $\text{PCO}_2$ ), temperature and the presence of heavy metals (Wood & McDonald, 1982). Moreover, the environmental calcium ion is the prime covariate in controlling the survivability of fish in acidic fresh waters whereby aluminium ion was found to be the notable metal ion with synergistic toxicity. In an adult fish, the gills are the most important site of toxicity where inhibition of ion-transport proteins in gill ionocytes and disturbance of the integrity of intercellular functions are commonly reported lesions (Wood, 2001). Overall, studies in acid precipitation and fish kills reveal that fish eliminated from waters at pH levels higher were perturbing to adult fish in the laboratory. According to Sayer et al. (1993), the aluminium released into the waters of acidifying basins had a complex synergistic toxicity that depends on pH level and dissolved organic molecules. On top of that, for the majority of the species, individuals were more at risk to reduced environmental pH during reproductive stages.

### 2.3 Acidic Water Habitat

Clymo (1984) reported that naturally acidic freshwaters represent for up to 3% of the world's freshwater ecosystems. The existence of fish species endemic to ancient acidic environments (Goulding et al., 1988) and the presence of species in recently acidified waters that are more acid tolerant than conspecifics from more neutral waters (Rahel & Magnuson 1983; Turnpenney et al., 1987) implicate high environmental  $[H^+]$  as an agent of acclimatization and natural selection in fish.

### 2.4 Characteristics of Fish from Naturally Acidic Water

Based on the relative comparison of species population, animals indigenous to acidic habitats have been reported to be more tolerant of low pH levels (Dunson et al., 1977). Besides that, the animals also have less disruption of monovalent ion balance during acid exposure (Gonzalez et al., 1998; 2002). Moreover, they also exhibit greater blood oxygen transport capacity as mentioned by Wood et al. (1998). The animals also have exercise metabolism and performance that are less affected by low pH (Holeton & Stevens 1978; Nelson 1989; 1990) and they can bind  $Ca^{++}$  more tightly at their gills (McWilliams 1983; Gonzalez and Dunson 1989b; Gonzalez et al., 1998) or have  $Ca^{++}$  insensitive monovalent ion efflux (Gonzalez et al., 1998).

### 2.5 Malay Combtail (*Belontia hasselti*)

The Malay combtail, *Belontia hasselti* or locally known as Kepar, is a gourami species (Froese, 2006). Britz (1995) suggested this fish may represent the family Osphronemidae because the second external levator muscle of the second gill arch is unmodified (as in *Helostoma* and the family Anabantidae), whereas in other osphronemids it is modified into a thin layer covering the entire posterior part of the

suprabranchial chamber. However, in the molecular phylogeny of Rüber et al. (2006) *Belontia hasselti* recognized to be most closely related to the ‘giant gouramis’ of the genus *Osphronemus*, although basal relationships among osphronemids were not recovered. Based on Kottelat (2013), the fish is endemic to southern Indochina, from southern (peninsular) Thailand, throughout Peninsular Malaysia, the Greater Sunda Islands of Sumatra, Borneo, Java and also Singapore. This fish can be found in acidic freshwater biotopes with minimal water movement, specifically ancient forest peat swamps in which the water that is stained dark brown by humic acids and other chemicals released from decaying organic material. This results in a small amount of dissolved mineral content and pH can be as low as 3.0-4.0. There is very little light which can penetrate the water surface due to dense rainforest canopy above, and the substrate normally littered with fallen tree branches and leaf.

Kepar is being caught and consumed by the locals living near a peatland as an additional source of protein and being also reared as an aquarium fish. Ng et al. (1994) stated that *Belontia hasselti* can be found in North Selangor Peat Swamp Forest (NSPF) as this fish was identified as one of the 47 species of blackwater fish in NSPF which are stenotopic to acidic water. Unfortunately, the natural habitat of *Belontia hasselti* is being modified by the activities such logging due to economic reasons such as agriculture and causing declining in species diversity (Kottelat, 2013).

In Indonesia, *Belontia hasselti* are known by local people as “ikan selinca”, are small fishes commonly found in irrigation canals in the palm tree plantation and present in flood area around many rivers in Riau. This fish has economical value as it is a common aquarium fish and also consumed by the local people (Haryono, 2007;

Hasanah et al, 2019). The biological information available for this fish is very restricted to morphological characteristics and meristic of this fish (Kampai 2012). The biggest size of Kepar fish captured in the Bakuok Lake, Kampar, Riau is 20 cm with body weight of 48 grams. Hasanah et al. (2019) reported that the Kepar fishes from Bangka Belitung, Indonesia used in a study on effect of different pH had an average weight of 19.22 g and the length of 10.6 cm. This fish is an omnivorous fish that feed mainly on Chlorophyceae and Crustacean (Azwar, 2012).

### **3.0 MATERIALS AND METHODS**

#### **3.1 Experimental Fish**

This study was approved by the Institutional Animal Care and Use Committee (IACUC, UPM) with the AUP number UPM/IACUC-U051/2020. A total of 10 *Belontia hasselti* (6 males and 4 females) which were caught using fishing method from the North Selangor Peat Swamp Forest were used in this study.

#### **3.2 Experimental Design**

The pH and dissolve oxygen of the water at the peat swamp were measured using pH meter and DO meter. Both of the parameters were recorded and the temperature of the water was also noted. Following capture, visual appraisal on each fish was done and the length and weight of the fishes were recorded. The fishes were then sacrificed by pithing method, and the gills, skin, muscle, liver and kidneys were sampled on-site. Results revealed that the length of the fishes ranged from 9 to 14.5 cm and the weight ranged between 12 to 43 g. Then, the fishes were divided into two groups according to its length, Group A which is less than 12.3 cm and Group B which is more than 12.3 cm. The samples were immediately fixed into 20% neutral buffered formalin for 4 hours and kept at 6°C. Then, it was transferred into 10% neutral buffered formalin for a day and kept at 24°C before being immersed in 70% alcohol solution. Then all of the samples were routinely processed and stained with Hematoxylin & Eosin stain (H&E) for histological assessment.

#### **3.3 Preparation of Histological Slide**

Skin, liver, gills, and kidneys were processed using an automated tissue processor. The tissue samples were dehydrated using 100% alcohol and cleared with

several baths of xylene. Then, the tissue samples were infiltrated with paraffin wax at 71°C using a paraffin-embedded station (Leica EG1160, Germany). The samples were carefully placed level on the bottom of the moulds by gentle pressing from the blunt end of forceps. After cooling and trimming process, the tissue blocks were carefully trimmed (15 µm) to expose tissue and then followed by sectioning at 4 µm thickness using a microtome (Leica RM2235, Germany). The sections were dried at 48°C in drying cabinet for a minimum of 12 hours prior to staining. The 5 µm thick paraffin wax sections were then stained with H&E. Mounting of glass slide using Distyrene Plasticizer Xylene (DPX) was done after the staining process and left to dry overnight. Finally, a total of 40 histological slides were ready to be viewed.

### **3.4 Histological Observation**

All the slides were viewed under the light microscope equipped with image analyzer. For histomorphometry and histopathological scoring, each slide was viewed with 5 fields under microscope. For normal histological study we referred to Mokhtar (2017) as our guide.

For histomorphometry, the relative length of primary and secondary lamellae, muscle density, relative thickness of skin layer and relative diameter of tubule were measured. For each slide, five measurement were taken, and the results were averaged.

For histopathological scoring we adapted with modification of the parameters observed in this study from Bernet & Schmidt (1999), 5 views of region of interest were taken for scoring. The samples were evaluated for parameters listed in Table 1.

**Table 1:** Histological parameters for gills, kidney and liver

NO	PARAMETER
<b>GILLS</b>	
1	Hyperaemia & Haemorrhage
2	Structural alteration (curling of secondary lamella)
3	Hyperplasia
<b>KIDNEY</b>	
1	Hyperaemia & Haemorrhage
2	Degeneration
3	Necrosis
4	Inflammation
<b>LIVER</b>	
1	Degeneration
2	Necrosis
3	Inflammation

All parameters were graded based on a scoring system from 0-6 scale where 0 = no histopathological changes, 1-2 = mild changes, 3-4 = moderate changes, 5-6 = severe changes. The average from all 5-field view scoring from by each parameter was taken, recorded and analysed.

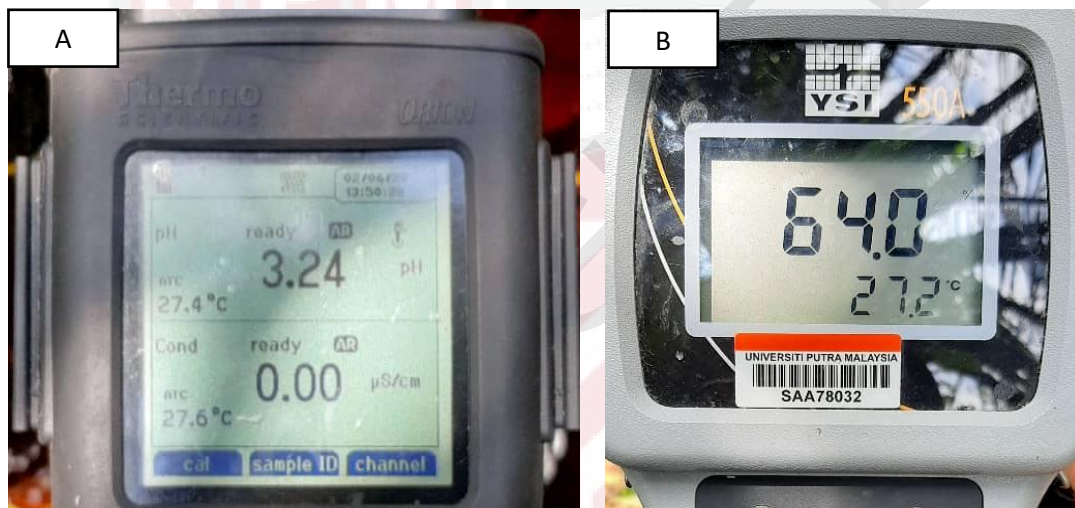
### 3.5 Statistical Analysis

For the statistical analysis, we adapted from Miki (2018) with slight modification due to number of groups that being compared in this study. There were total of a slide for each organ for each fish and a total of 4 slides for 1 fish. Altogether, for the 10 fishes, there were 40 slides. Each of the slides was viewed at 5 different regions for histomorphometry and histopathological scoring. The data was tabulated in excel worksheet and analysed using IBM SPSS statistic tool version 25 by running Independent T-test for histomorphometry and Mann Whitney U test for histopathological scoring.

## 4.0 RESULTS

### 4.1 Water Parameter

Our study revealed that the pH of the water at North Selangor Peat Swamp Forest (NSPF) was 3.24, which indicates the water is highly acidic. The temperature of the water was 27.4°C (Figure 1A). Dissolved oxygen in the water of the peat swamp was 64.0% (Figure 1B).



**Figure 1:** (A) The pH level of peat swamp water measured with pH meter was 3.24. (B) Dissolved oxygen level in the peat swamp water measured with DO meter was 64.0 mg/L.

### 4.2 Gross Study

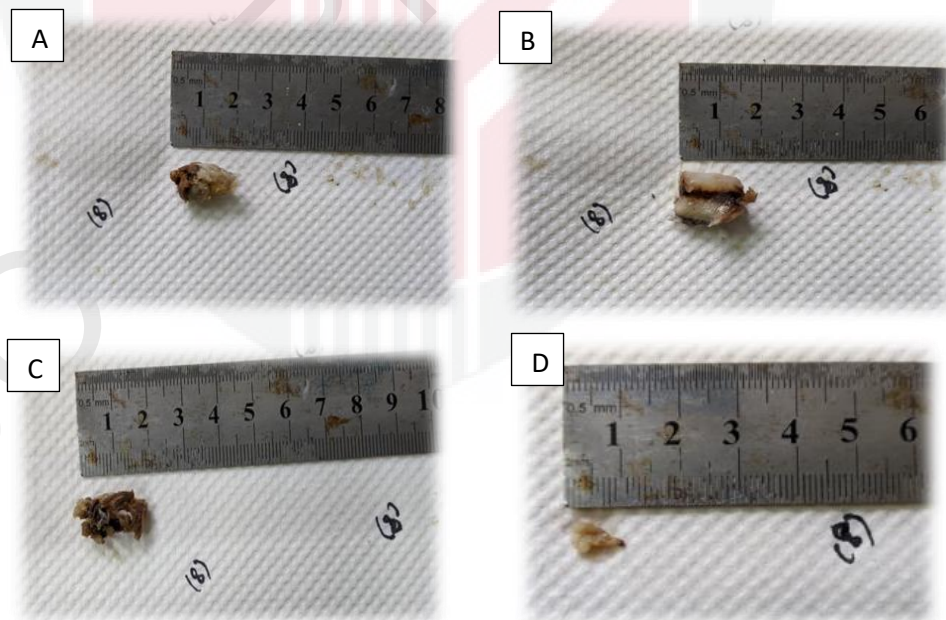
*Belontia hasselti* is one of the species which living in natural acidic water environment. It is characterised by slimy and dark coloured skin (Figure 2). The fishes caught have length between 9 cm to 14.5 cm and weight between 12 g to 43 g. The fishes showed no evidence of ectoparasites grossly.



**Figure 2:** Gross appearance of Kepar fish (*Belontia hasselti*). The fish has slimy and dark coloured skin.

The kidney, muscle, skin, gill and brain are the samples taken from the fish.

There is no presence of any special feature which can be observed grossly. (Figure 3).



**A = Gross kidney, B = Gross muscle and skin, C = Gross gill, D =Gross brain,**

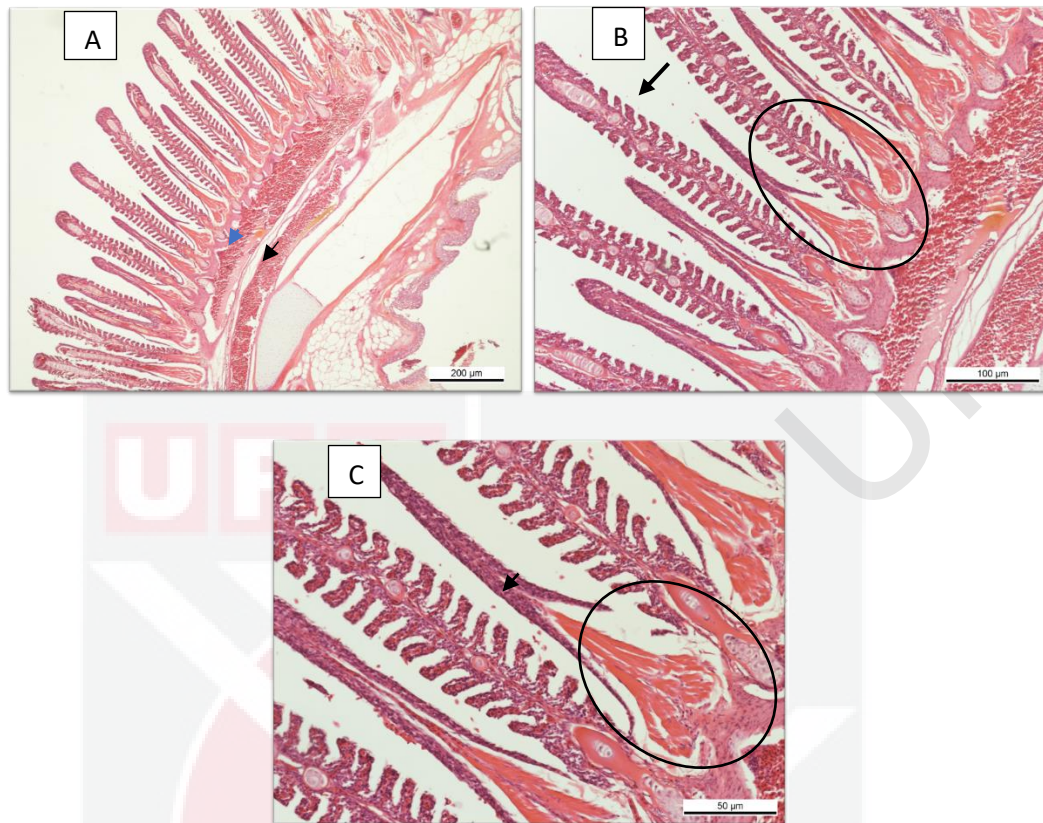
**Figure 3:** Gross appearance of kidney, muscle, skin, gill and brain of Kepar fish showing no special features that can be observed grossly.

### 4.3 Histological Analysis

The gills can be divided into gill arch and gill filaments. The gill arch is a cartilaginous structure which branches out the bony of the primary lamellae. It is covered by epidermal tissue contained large number of mucous cells. Gill filaments consists of primary and secondary lamellae. The primary lamella has a central cartilaginous support, arterioles and central venous sinus. Secondary lamella which is the sites for gas exchange contain small blood capillaries and the blood flows in the opposite direction of the water.

Kepar fish has large size of chondrocytes in the primary lamella. Interestingly, the caught Kepar Fishes all possess additional structure between the primary lamellae. This structure consists of smooth muscle. The fishes have stumpy and thick secondary lamellae (Figure 4).

Interestingly, no Kepar fish exhibited presence of any parasite infestation in the gills.

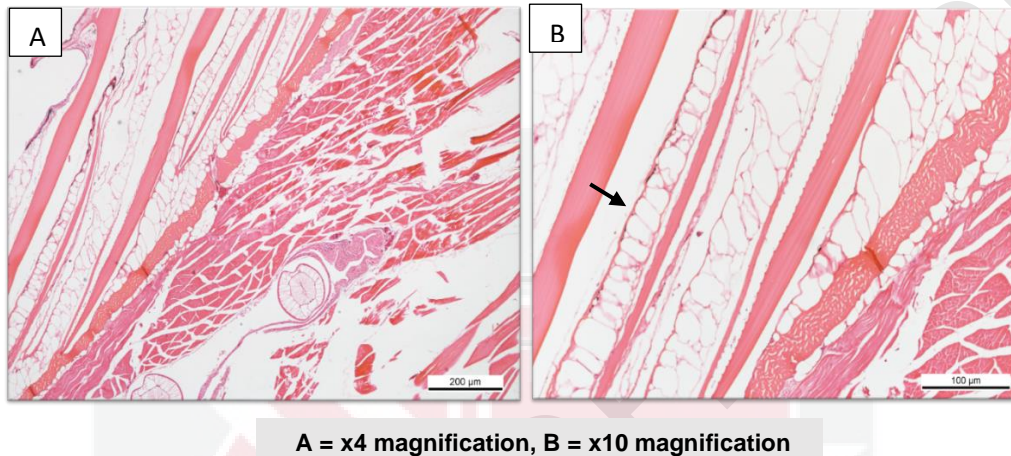


A = x4 magnification, B = x10 magnification, C = x40 magnification,

**Figure 4:** Photomicrograph of gills of fish. (A). Gills can be divided into gills filament shown by blue arrow and gill arch shown by black arrow. (B). Presence of additional structure between primary lamellae are shown in black circle. Besides, the primary lamellae have large size chondrocytes which is shown by the black arrow. (C). The additional structure consists of smooth muscle cells which is shown in the black circle. The black arrow shows the stumpy and thick secondary lamella.

Epidermis is the outermost layer of skin, which lined by stratified squamous epithelium. The dermis is located between the epidermis and the underlying body musculature. Scales will cover the superficial layer of the dermis which are arranged in scale pockets made up of loose connecting tissues. The loose connective tissue is also known as stratum spongiosum while the dense connective tissue is known as stratum compactum. The presence of the subcutaneous lipid layer was observed in this

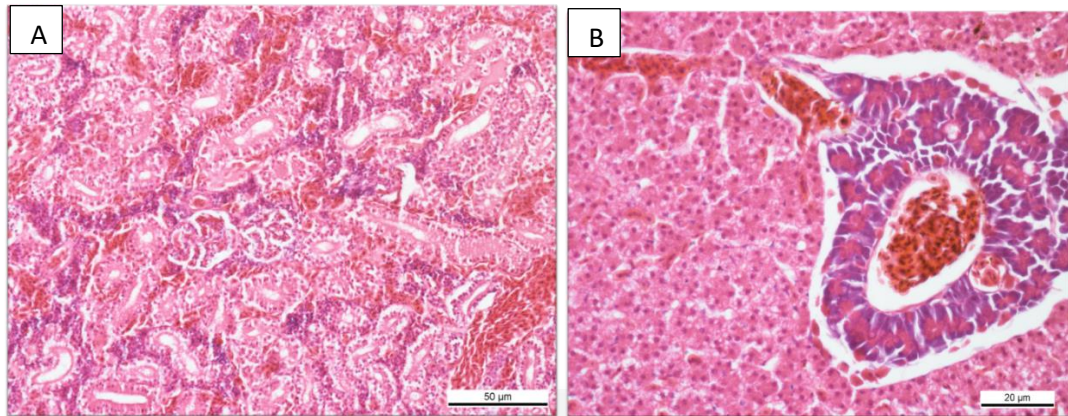
fish (Figure 5). No evidence of parasite infestation on the skin and in the muscle in any of the Kepar fish.



**Figure 5:** Photomicrograph of skin layer and muscle of fish. (A). No special features found from the histology as it is the same with other normal fish histology (B). Presence of subcutaneous adipose tissue layer shown by black arrow.

Glomerulus and renal tubules are the main components of the kidney tissues. The kidney can be divided into anterior which involved in hematopoietic and posterior which function as filtration organ. From the Figure 6A, there is no special feature observed in the kidney. Histologically the kidney consists of renal tubules and glomerulus.

Liver of *Belontia hasselti* is the compound organ in the form of hepatopancreas. The endothelial cells which lining the sinusoids are distributed irregularly between the polygonal hepatocytes. Based on the Figure 6B, no special feature was observed.

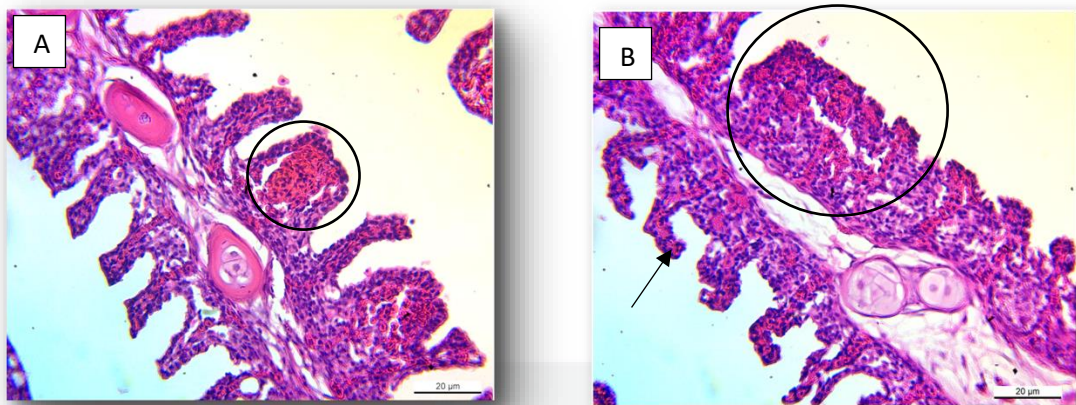


**A = Histology of kidney on x20 magnification, B = Histology of liver on x40 magnification**

**Figure 6:** Photomicrograph of kidney and liver. No special features found in the histology of kidney (A) and liver (B).

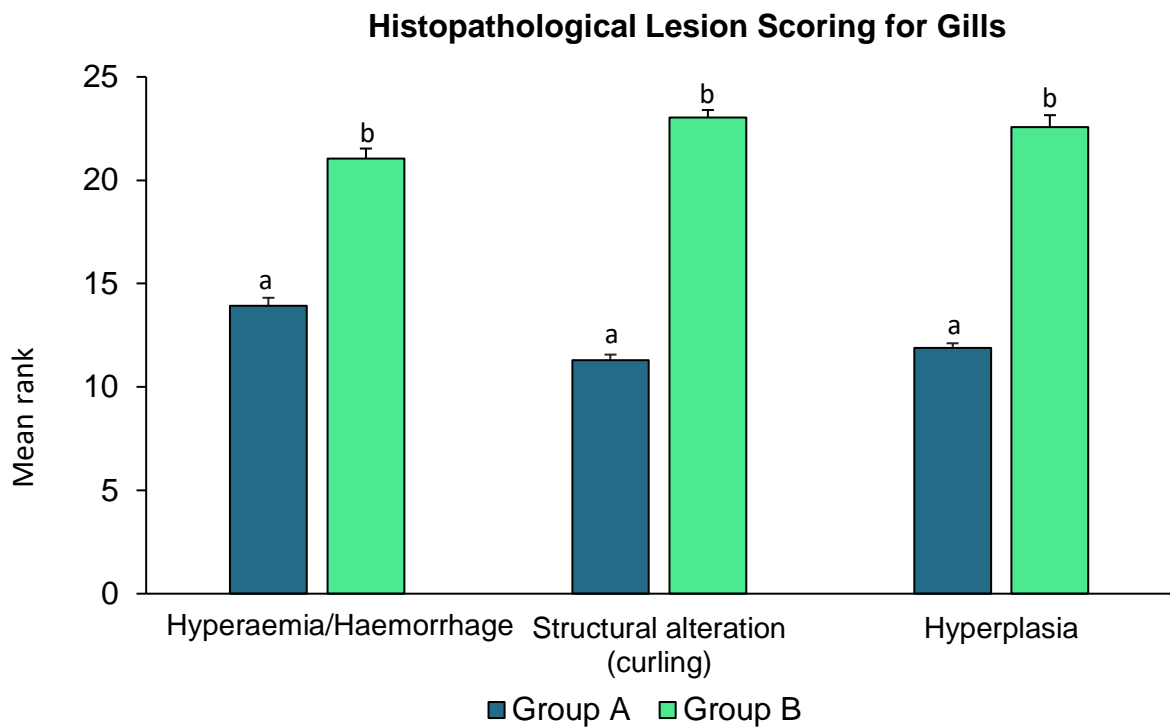
#### 4.3 Histopathological Scoring

Hyperaemia/haemorrhage, structural alteration particularly the curling of the secondary lamella and cell hyperplasia were chosen as the parameters of histopathological scoring for the gills in this study as suggested by Bernet & Schmidt (1999). There was abnormal increase in the number of red blood cell in the capillary of secondary lamella as shown by the black circle (Figure 7A). On the other hand, thickening of the primary lamella epithelium due to increase in number of epithelial cells indicate hyperplasia as shown by the black circle (Figure 7B). Curling of the secondary lamella shows by the black arrow (Figure 7B).



**Figure 7:** Photomicrograph of histopathological changes of gills of fish. (A). Hyperaemia in the secondary lamella is shown in black circle. (B). The black circle shows hyperplasia of the primary lamella. Curling of secondary lamellae is shown by black arrow.

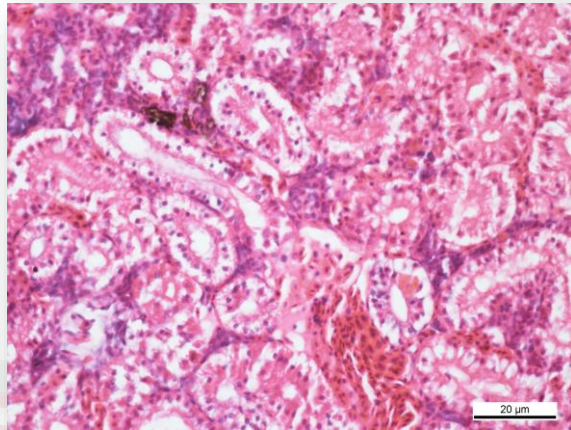
The mean rank of histopathological lesion scoring of the gills of Kepar fish caught from the NSPF is shown in Figure 8. Results show that the mean rank of histopathological lesion scoring for the gills of fishes in Group A is significantly different ( $P < 0.05$ ,  $p=0.000$ ) than group B. Fish in group B has higher lesion scoring for all of the parameters which are hyperaemia/haemorrhage, structural alteration (curling of secondary lamella) and cell hyperplasia.



Group A: Fish with length <12.3 cm and Group B: Fish length >12.3 cm. Data analysed using Mann Whitney U test. Data with different letter (a or b) between group differ significantly ( $P < 0.05$ ).

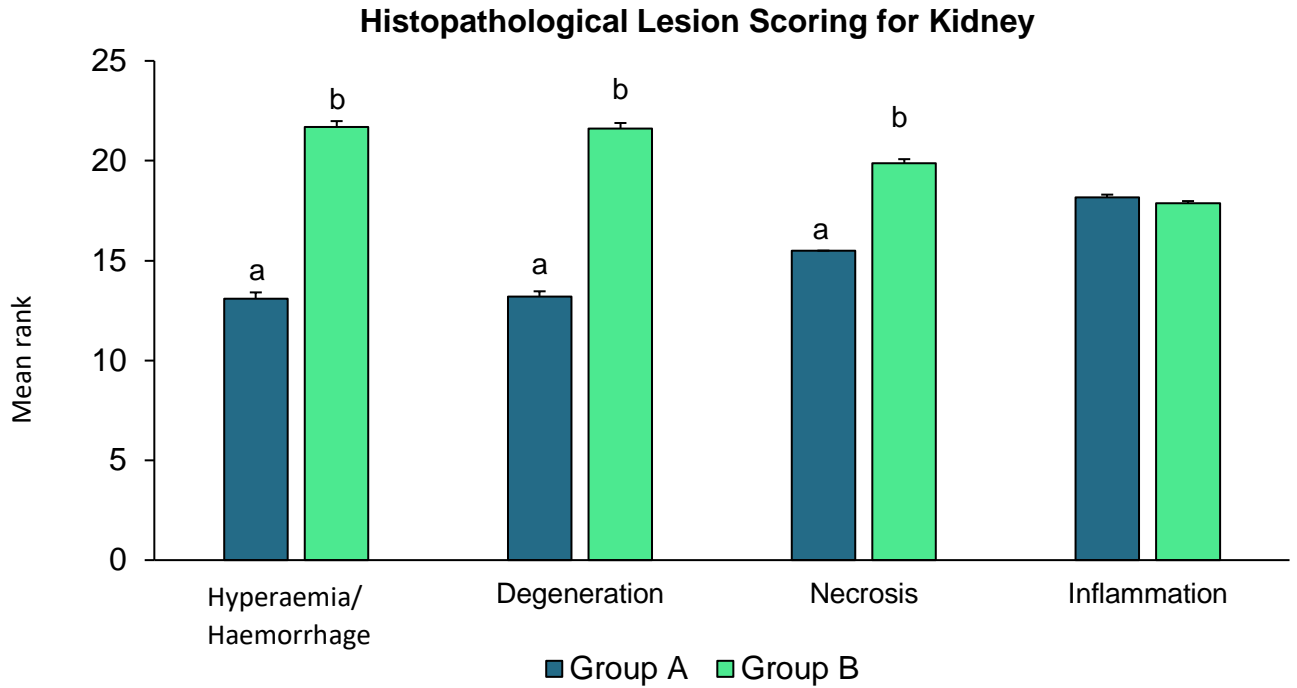
**Figure 8:** Histopathological lesion scoring (hyperaemia/haemorrhage, structural alteration (curling of secondary lamellae) and hyperplasia) of gills of Kepar fish (*Belontia hasselti*).

For kidney, hyperaemia/haemorrhage, degeneration, necrosis and inflammation of the kidney cells has been used as the parameters of histopathological scoring of this organ in the study. There is accumulation of water in the renal tubular cells making the cells to swell and lose their conformation and thus indicates hydropic degeneration of the tubules shown by black arrows. Pooling of red blood cells between the renal tubules outside the blood vessel which indicate renal haemorrhage shown by black circle (Figure 9).



**Figure 9:** Photomicrograph of histopathological changes of kidney of fish. Hyperaemia between the tubules is shown in black circle. Black arrow shows hydropic degeneration of the tubule.

Mean rank of fish for histopathological lesion scoring of the kidney of fish in Group A significantly different ( $P < 0.05$ ,  $p=0.000$ ) than group B in only three parameters which are hyperaemia/haemorrhage, cell degeneration and necrosis. Fish in group B has higher lesion scoring in these three parameters. Meanwhile there is no significant different ( $P > 0.05$ ,  $p=0.000$ ) between fish in Group A and fish in Group B in inflammation parameter.



Group A: Fish with length <12.3 cm and Group B: Fish length >12.3 cm. Data analysed using Mann Whitney U test. Data with different letter (a or b) between group differ significantly ( $P < 0.05$ ).

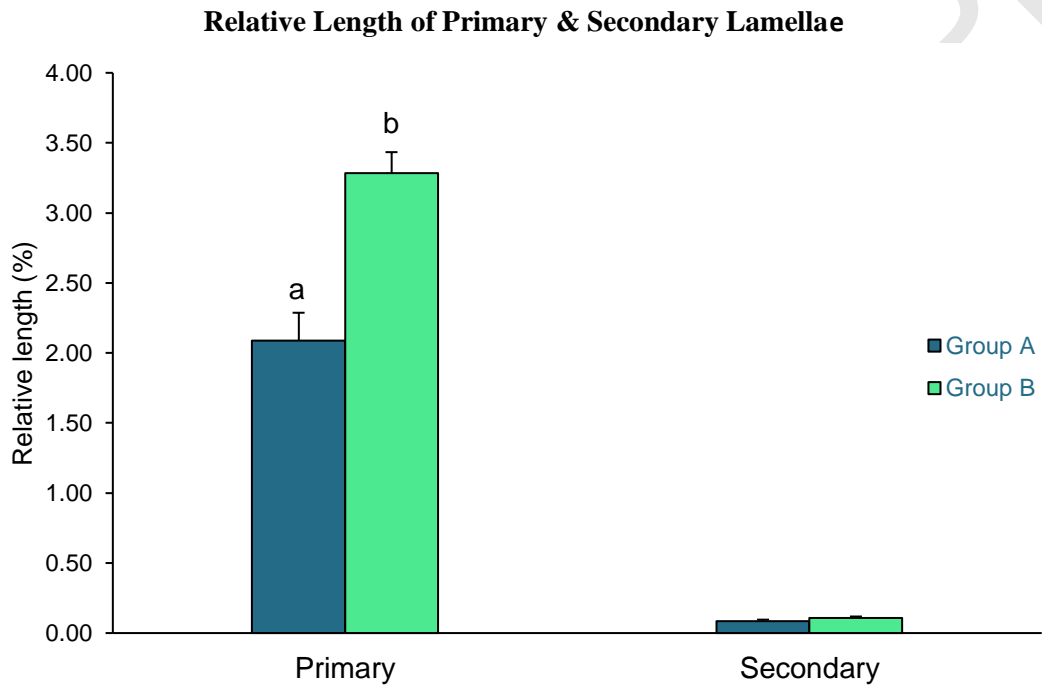
**Figure 10:** Mean rank of histopathological lesion scoring (hyperaemia/haemorrhage, cell degeneration, necrosis and inflammation) of kidneys of Kepar fish (*Belontia hasselti*).

#### 4.4 Histomorphometric evaluation

This study is used to measure the length and diameter of certain tissue of the organs. Four parameters were chosen which are relative length of primary and secondary lamellae, muscle density, relative thickness of the skin and relative diameter of tubule of the kidney.

The relative length of primary lamellae of fish in group A (relative length:  $2.08 \pm 0.2$  cm, range: 0.092 – 0.44 cm) significantly different ( $P < 0.05$ ,  $p=0.000$ ) than group B (mean relative length:  $3.28 \pm 0.2$  cm, range: 0.329- 0.598 cm). Meanwhile,

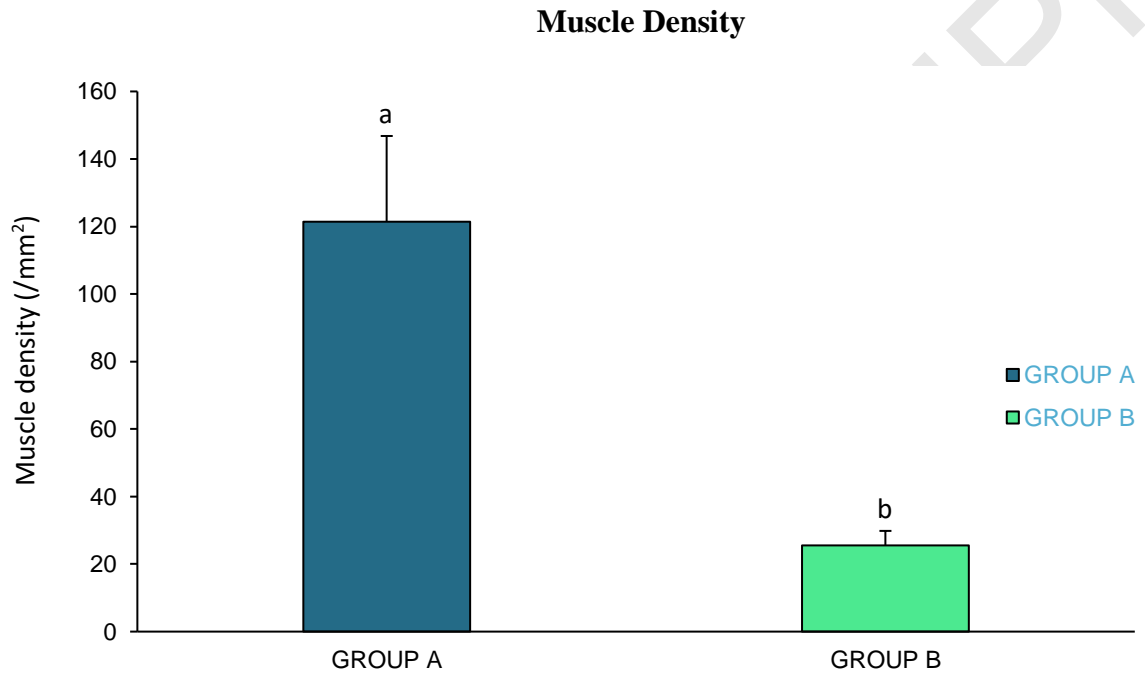
the relative length of the secondary lamellae of the two groups (Group A; relative length:  $0.086 \pm 0.01$  cm and range: 0.002 – 0.014 cm, Group B; relative length:  $0.108 \pm 0.01$  and range: 0.007- 0.022 cm) was not significant ( $P > 0.05$ ) (Figure 11).



Group A: Fish with length <12.3 cm and Group B: Fish length >12.3 cm. Data are mean  $\pm$  SEM and analysed using independent T test. Data with different letter (a or b) between group differ significantly ( $P < 0.05$ ).

**Figure 11** Relative length of the primary and secondary lamellae of Kepar fish (*Belontia hasselti*), expressed as the ratio (%) to their body length.

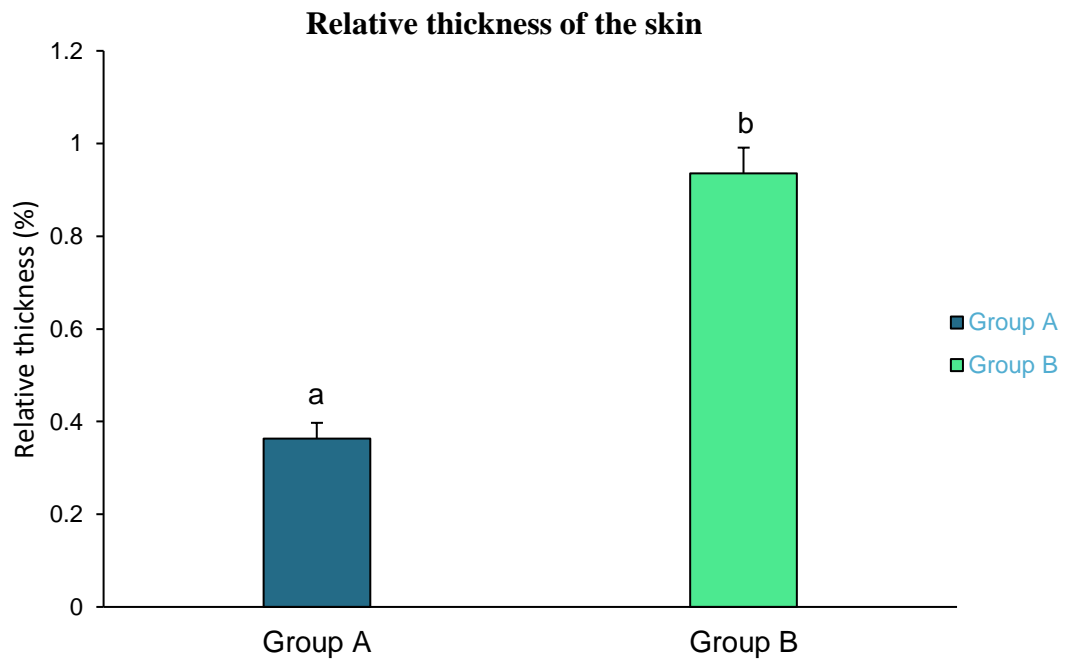
The muscle density of fish in group A (density:  $121.41 \pm 25.4 \text{ mm}^2$ , range:  $65.303 - 295.492 \text{ mm}^2$ ) was significantly different ( $P < 0.05$ ,  $p=0.000$ ) than group B (mean relative density:  $25.53 \pm 4.3 \text{ mm}^2$ , range:  $7.98 - 65.656 \text{ mm}^2$ ) (Figure 12).



Group A: Fish with length  $< 12.3 \text{ cm}$  and Group B: Fish length  $> 12.3 \text{ cm}$ . Data are mean  $\pm$  SEM and analysed using independent T test. Data with different letter (a or b) between group differ significantly ( $P < 0.05$ ).

**Figure 12:** muscle density (/mm<sup>2</sup>) of Kepar fish (*Belontia hasselti*).

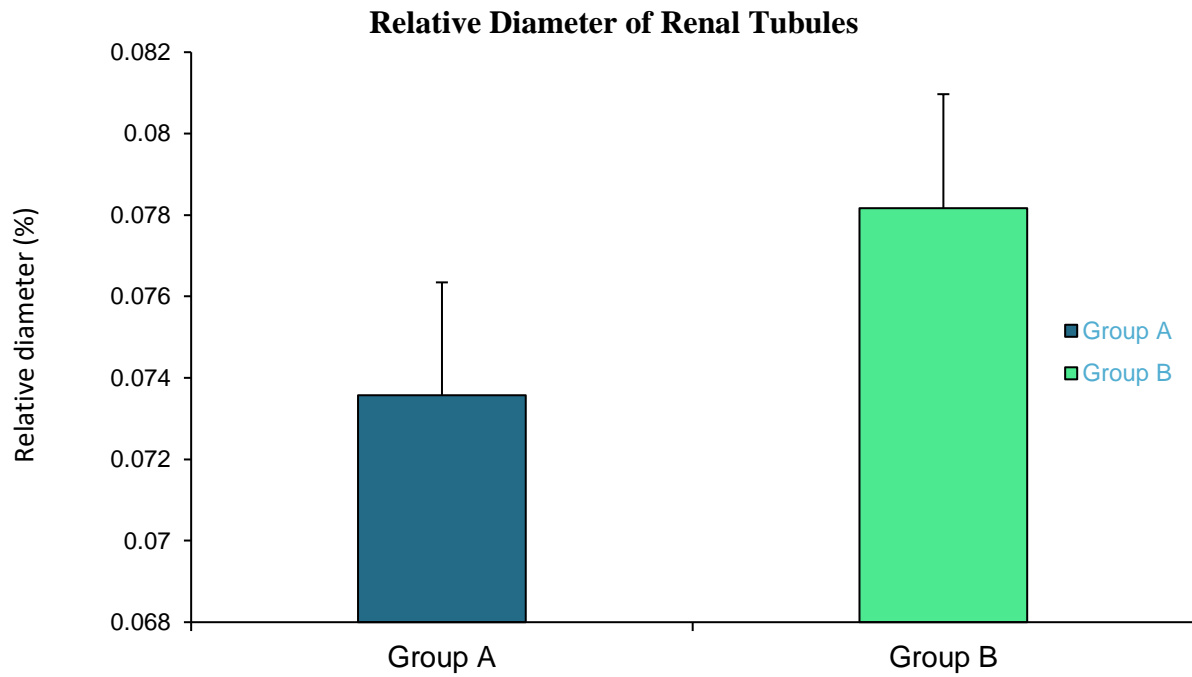
The relative thickness of the skin layer of fish in group A (mean relative thickness:  $0.363 \pm 0.03 \text{ cm}$ , range:  $0.023 - 0.079 \text{ cm}$ ) significantly different ( $P < 0.05$ ,  $p=0.000$ ) than group B (mean relative thickness:  $0.935 \pm 0.06 \text{ cm}$ , range:  $0.081 - 0.188 \text{ cm}$ ) (Figure 13).



Group A: Fish with length <12.3 cm and Group B: Fish length >12.3 cm. Data are mean  $\pm$  SEM and analysed using independent T test. Data with different letter (a or b) between group differ significantly ( $P < 0.05$ ).

**Figure 13:** Relative thickness of skin of Kepar fish (*Belontia hasselti*), expressed as the ratio (%) to their body length.

The relative diameter of the renal tubules of fish in group A (relative diameter:  $0.0736 \pm 0.002$  cm, range: 0.003 – 0.011cm) was not significantly different ( $P > 0.05$ ,  $p=0.000$ ) than group B (mean relative diameter:  $0.0782 \pm 0.003$ cm, range: 0.005-0.02cm) (Figure 14).



Group A: Fish with length 9-12cm and Group B: Fish length 13-14.5cm. Data are mean  $\pm$  SEM and analysed using independent T test.

**Figure 14:** Relative diameter of tubule of kidney of Kepar fish (*Belontia hasselti*), expressed as the ratio (%) to their body length.

## 5.0 DISCUSSION

*Belontia hasselti* is one of the native species of the natural acidic water environment. This statement was supported by Ng et al., (1994) that the habitat of *Belontia hasselti* occurred at acidic freshwater biotopes (pH ranging 3.0-4.0) with little water movement stated and this fish can be found in North Selangor Peat Swamp Forest (NSPF) as this fish was identified as one from the 47 species of blackwater fish in NSPF which are stenotopic to acidic water. Hasanah (2019) mentioned that the fish can live well at pH 5 and below in comparison to higher pH. Kepar fish can survive well at the environment with low pH because it has accessory breathing organ and hard scale on the skin. The habitat of *Belontia hasselti* have low dissolve oxygen capacity, which is inconducive to other species, however the species of this study can survive well. Nelson (1994) have pointed out that species of *Belontiidae* family are equipped with suprabranchial organ which functions as an auxillary breathing apparatus that helps in oxygen uptake in oxygen-deficient water. Moreover, the temperature of peat swamp was 27.3°C which supported by Handajani & Samsundari (2005) on their study which mentioned that the immune response of the fish depends on the temperature for example *Belontia hasselti* cannot produce antibody at temperature below 10°C. According to Froese (2006), *Belontia hasselti* can reach a length of 20 cm. Hasanah et al. (2019) also reported the Kepar fishes used in a study conducted in Bangka Belitung in Indonesia had average length of 10.6 cm. Our study revealed that the Kepar fishes caught were between 9 to 14.5 cm, which is below the mentioned value stated by Froese (2006) but this finding is comparable to the average length reported by Hasanah et al. (2019). As the sample size in this study is small (N=10), there is a possibility that the fishes caught were not representative of the fish

population in NSPF. We could only assume that either we could have missed catching larger fishes or the fishes in that locality are actually smaller. The weight of the fishes caught were between 12 g to 43 g but due to the lack of research on this species, the weight could not be compared. Nevertheless, Hasanah et al. (2019) reported that the average weight of the Kepar fishes from Bangka Belitung, Indonesia used in a study was 19.22 g, which is within the range of the fishes caught in our study.

There are several gross characteristics that can be found on the fishes living in this environment which also can be found on Kepar. Firstly, Kepar fishes have dark skin colour and this finding is similar as stated by Ng et al. (1994) and Beamish et al. (2003) that most of the fishes of the peat swamp are dark tea- or coffee-coloured. The dark skin colour may act as a protective mechanism and help the fish to blend with its environment. Camouflage is when a fish is attempting to blend into its surroundings. Some of the major types of camouflage in fish include protective resemblance, disruptive colouration, countershading, mirror-siding, and transparency (Randall, 2005). Next, the texture of the fish skin is slimy. As we all know, mucous acts as a dynamic physical and biochemical barrier, displaying biological and ecological roles such as osmoregulation and protection from toxicity (Coello, 1996). Moreover, based on the study by Fletcher & Grant in 1968, lubricating mucous provides protection against inorganic particles and biological organisms.

No ectoparasites were found grossly and histologically in the skin, muscle and gills of *Belontia hasselti* caught at the North Selangor Peat Swamp Forest (NSPF). Ectoparasites are not the only factor for infection but instead it is based on the relationship between the environment, fish and pathogen. When the environment is

not suitable, it will cause the fish to become stressful which can lead to immune suppression and thus enabling the parasite to infect the fish (Handajani & Samsundari, 2005). Besides, the fishes caught did not show any clinical signs as mentioned by Noga (2010) that some of the clinical symptoms of parasite infection are fish appears weak, loss of appetite, slow growth, abnormal swimming and excess mucous production, often floats on the water surface, pale and swollen gills until the operculum appears open.

There is no presence of special features and pathological changes that can be observed grossly on gills, kidney, and liver samples of all the caught Kepear fishes. This indicates that the pathological lesions in the organs are not severe enough to cause any changes that can be observed grossly on these selected organs. However, the changes and the special features of the organs can be observed histologically.

Most of the histopathological lesions can be observed in gills and kidney. It is because both of these organs involved directly to maintain osmoregulation process of the fish. Gills involved in regulating the exchange of salt and water and play an important role in the excretion of the nitrogenous waste products. Besides, kidney can be divided into anterior which is haematopoietic and posterior kidney that contains lesser amount of interstitial haematopoietic and lymphoid tissues but more renal tubules which functions as an osmoregulatory organ (Mokhtar, 2017). From the histopathological scoring results, fishes in Group B has higher score than fishes in group A. It is because fishes in Group B has been exposed to the extreme environment longer than the fishes in Group A. From the study, it is believed the fishes could be suffering to maintain the homeostasis but still within their biological limit. For

inflammation parameter of the kidneys, both groups of fishes have approximately similar scoring. This can be due to the infection or other factors despite of environmental stressors. As documented by Cotran et al. (1999), inflammation from a pathologic perspective is a process caused by inflammatory cells responding to signals and reaction of blood vessels leading to the accumulation of fluid and leukocytes in extravascular tissues. There are several factors that can cause chronic inflammation, which include untreated causes of acute inflammation, such as an infection or injury, an autoimmune disorder that involves immune system that mistakenly attacking healthy tissue (Adrienne, 2018).

Histology is the microscopic study of animal and plant cell and tissues through staining and sectioning and examining them under a microscope (electron or light microscope) which is mainly used in forensic investigations, autopsy, diagnosis and in education (Black, 2012). For this research, histological study allows for observation of the cellular structures and composition of gills, kidney, liver, skin and muscle to find the differences from other species of fish.

The most interesting finding of this study is the presence of a unique structure between the primary lamellae which is made up of smooth muscle and covered by thick epithelial layer. Agnihotri (2010) claims that this structure is a growing lamella. However, in our opinion, the structure does not resemble the lamella itself as the base of this structure consist of smooth muscle and there is no presence of chondrocytes in this structure. This structure may help to hold the gills from collapsing and thus, provides extra space for water to flow between the lamella as mentioned by Laurie (2013) as water is required to support the gills which will allow all the surfaces to

spread open for better gas exchange. Moreover, gills of *Belontia hasselti* has stumpy secondary lamella that are covered by thick epithelial layer. Annas (2019) suggested that gills of air-breathing fish have thick epithelial layer with short and stumpy secondary lamellae. His findings matched with this study as the fish use an air breathing organ as their primary gas exchange organ. In addition, Johansen (1970) mentioned in his study that some species rely on air-breathing organ to supplement gas exchange when adverse respiratory qualities in the water make aquatic breathing insufficient or too costly for extraction of the required oxygen. Furthermore, the gills have larger size of chondrocyte on the primary lamella. Chondrocytes are the main component cells of the cartilage which will form compact mass on the cartilaginous tissue (Mokhtar, 2017).

There is no special feature observed in the histology of skin, muscle, kidney and liver samples. However, the presence of fat cells lining indicates that these fishes have good body condition and still can survive well in this extreme environment. This finding follows the statement made by Claudine (2012) that mentioned lipids are the predominant source of energy for fish and are stored in fat depots in visceral, subcutaneous and intramuscular adipose tissues.

Histomorphometry can be defined as the measurement of the shape or form of a tissue (Frank, 2014). For this study, there are four parameters were chosen which are relative length of primary and secondary lamellae, muscle density, relative thickness of the skin and relative diameter of tubule of the kidney of *Belontia hasselti*. Results revealed that fishes from Group B have longer relative length of primary and secondary lamellae and larger diameter of tubule of kidney. This is because the organs

of fishes in Group B have fully developed and grown. However, the muscle density in fishes of Group A seem to be denser than the fishes in Group B. The older fishes appeared to have more adipose tissues stored in their intramuscular spaces which will make their muscle become less dense.



## 6.0 CONCLUSION AND RECOMMENDATION

The study shows the gill has a special additional structure found between primary lamellae in which its function remains unclear till date. Besides, histopathological changes can be observed mostly at the gills and kidneys as these two organs are involved in osmoregulation of the fish. Finally, conservation of peat swamp environment is crucial as it is the natural habitat for many species of fish and other animals. Particularly so for these extraordinary Kepar fishes which can only be found thriving only in these harsh environments.

As for the recommendation, the study can include other species from the same study area so comparison of the findings between the species from same habitat environment can be achieved. Besides, larger number of sample size can also be applied for more precise data collection and analysis. Other than that, other peat swamp can be suggested to be involved in the future study to investigate the effect of other peat swamps to the *Belontia hasselti* fishes. As part of a conservation effort, since not much is known about the Kepar fishes and owing to the fact that this fish is unique to the peat swamp environment, further study should also include the investigation of other organs like the gastrointestinal and reproductive systems. This will allow for better understanding of the biology and reproductive status of this special fish resulting in its adaptability and ability to thrive in such harsh living conditions.

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## APPENDICES

### APPENDIX 1

#### 1. Hematoxin and Eosin staining standard protocol in Veterinary

Histopathology Laboratory, Faculty of Veterinary Medicine, UPM.

Step	Description	Duration
1.	Submerge slides in xylene	5 min
2.	Submerge slides in 100% alcohol	5 min
3.	Submerge slides in 70% alcohol	5 min
4.	Rinse in tap water	1 min
5.	Submerge slides in hematoxylin	5 min
6.	Rinse in tap water (1-3 times)	1-3 min
7.	Dip slides in buffer blue	3 sec
8.	Rinse in running tap water	5 min
9.	Submerge slides in eosin	1 min
10.	Spray slides with 95% alcohol	-
11.	Rinse slides in running tap water	5-10 sec
12.	Spray slides with 95% alcohol, clean, leave to dry	Overnight

(retrieved from Basic Histology and Histopathology of fish, Annas Salleh

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