UNIVERSITI PUTRA MALAYSIA

OCCURRENCE OF ENDOPARASITIC INFECTION AND HISTOPATHOLOGICAL CHANGES IN SPOTTED GREEN PUFFERFISH, Dichotomyctere nigroviridis (Marion de Procé, 1822) IN BAGAN LALANG, SELANGOR

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FACULTY OF VETERINARY MEDICINE
UNIVERSITI PUTRA MALAYSIA
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2023/2024

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MUHAMMAD FAIZ BIN MOHD. FIKRI

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CERTIFICATIONS

It is hereby certified that we have read this project paper entitled "Occurrence of endoparasitic infection and histopathological changes in spotted green pufferfish, *Dichotomyctere nigroviridis* in Bagan Lalang, Selangor." by Muhammad Faiz bin Mohd. Fikri and in our opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999 – Project.

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DEDICATIONS

Dedicated to the harmony of health for both humans and animals, with a special nod to the often-overlooked aquatic species. In gratitude to my cherished family, supervisors, colleagues, and the entire community of the Faculty of Veterinary Medicine, UPM. May this thesis contribute to the well-being of all beings and prove beneficial to those who engage with it.

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I extend my highest gratitude to Almighty Allah S.W.T for His boundless blessings, abundant bliss, and enduring mercy and grace. Alhamdulillah for the overflowing blessings, robust health, and unwavering strength bestowed upon me throughout this study endeavor.

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ABBREVIATIONS

TTX	Tetrodotoxin
MMC	Melanomacrophage Centers
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterianar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek

KEJADIAN ENDOPARASIT DI DALAM BUNTAL GIGI KUPANG, *Dichotomyctere nigroviridis* (Hamilton,1822) DI BAGAN LALANG, SELANGOR

Oleh

Muhammad Faiz Bin Mohd. Fikri

2023

Penyelia: Dr Nur Diyana Binti Mohamad Tahir

Penyelia Bersama: Dr Nor Azlina Binti Abdul Aziz & Dr Mohd Fuad Bin Matori

Dichotomyctere nigroviridis (Marion de Procé, 1822) atau juga dikenali sebagai buntal gigi kupang tergolong dalam keluarga Tetraodontinae. Di Malaysia, *Dichotomyctere nigroviridis* dapat ditemui secara meluas di air tawar dan paya garam, contohnya di Bagan Lalang, Tanjung Sepat, Selangor. Ikan ini cenderung memakan krustasea dan moluska yang biasanya berperanan sebagai hos perantara kepada sesetengah parasit, yang boleh menyebabkan risiko kepada kesihatan orang awam. Oleh kerana jangkitan endoparasit ikan buntal ini belum pernah didokumentasikan di Malaysia, kajian ini bertujuan untuk menilai kejadian jangkitan endoparasit dan perubahan histopatologi dalam ikan buntal gigi kupang juvenil dan dewasa. Kajian ini dilakukan pada bulan September 2023 di Bagan Lalang, Tanjung Sepat, Selangor.

Sebanyak 16 ekor ikan terdiri daripada 8 ekor ikan juvenil dan 8 ekor ikan dewasa telah diambil sampel endoparasit semasa nekropsi. Kajian ini mendapati banyak parasit terdapat di dalam ginjal yang terdiri daripada myxozoan, yang memiliki prevalens 100% di kalangan dewasa dan juvenil. Tiada parasit diperhatikan di dalam otak, hati, saluran gastrousus, limpa, jantung, otot, dan gonad. Ujian Fisher's Exact yang dilakukan menggunakan perisian statistik SPSS v27 menunjukkan perbezaan yang signifikan ($p>0.05$) antara ikan buntal gigi kupang dewasa dan juvenil dari segi beban endoparasit. Penilaian histopatologi pada pelbagai organ dalaman melibatkan penilaian lesi berdasarkan kriteria tertentu, termasuk konjesi, kehadiran pigmen interstisial melanomakrofaj (MMC), dan infiltrasi limfosit. Dalam histopatologi otak, konjesi sederhana diperhatikan pada 60% dewasa tetapi tiada pada yang ikan juvenil. Histopatologi ginjal menunjukkan pengecualian MMC yang teruk pada 75% semua individu. Histopatologi usus menunjukkan 33.33% dewasa dengan infiltrasi limfosit sederhana, manakala tiada yang diperhatikan pada ikan juvenil. Pada limpa, 75% dewasa menunjukkan kehadiran MMC yang teruk, tanpa diperhatikan pada yang ikan juvenil. Histopatologi jantung menunjukkan degenerasi sel pada 37.5% semua individu, dan dalam gonad, 50% semua individu menunjukkan konjesi sederhana. Keputusan penjujukan PCR mendapati kehadiran endoparasit jenis *Triactinomyxon type* sp. dan myxozoan *Ortholinea auratae* pada ikan buntal gigi kupang. Temuan kajian ini memberikan sumbangan kepada penubuhan inventori awal parasit di kawasan ini dan membuka jalan untuk penyelidikan masa depan mengenai konsekuensi potensi mengonsumsi spesies ini.

Kata kunci: *Dichotomyctere nigroviridis*; Bagan Lalang; endoparasit; prevalens; histopathologi

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4999- Project.

OCCURRENCE OF ENDOPARASITIC INFECTION AND HISTOPATHOLOGICAL CHANGES PUFFERFISH, *Dichotomyctere nigroviridis* IN BAGAN LALANG, SELANGOR

By

Muhammad Faiz Bin Mohd. Fikri

2023

Supervisor: Dr Nur Diyana Binti Mohamad Tahir

Co-Supervisor: Dr Nor Azlina Binti Abdul Aziz & Dr Mohd Fuad Bin Matori

Dichotomyctere nigroviridis (Marion de Procé, 1822) or also known as spotted green pufferfish belongs to the family tetraodontinae. In Malaysia, the *Dichotomyctere nigroviridis* can be found widely in fresh and brackish waters and one example is the. Due to its preference for eating crustaceans and molluscs that usually play a role as intermediate hosts of certain parasites, the fish may become infected with parasites, which could pose a risk to the public's health. Since the endoparasite infection of this pufferfish has not yet been documented in Malaysia, the present study aims to evaluate the occurrence of endoparasite infection and histopathological changes in juvenile and adult spotted green pufferfish. This study was carried out in September 2023 in Bagan Lalang, Tanjong Sepat, Selangor. Sixteen fish consisting of 8 juveniles and 8 adults were sampled for endoparasites during necropsy. Numerous

amounts of parasites were observed from the kidney consisting of myxozoans, which had a prevalence of 100% in both adults and juveniles. No parasites were observed from the brain, liver, gastrointestinal tract, spleen, heart, muscle and gonad. The Fisher's Exact Test performed using SPSS v27 statistical software revealed a significant difference ($p>0.05$) between the adult and juvenile spotted green pufferfish in terms of endoparasite burden. The histological scoring system identified structural changes in the kidney, including the presence of the interstitial pigment (melanomacrophages) and tubular epithelial degeneration or necrosis. The histopathological assessment of various internal organs involved evaluating lesions based on specific criteria, including congestion, the presence of interstitial pigment melanomacrophage (MMC), and lymphocyte infiltration. In brain histopathology, moderate congestion was observed in 60% of adults but none in juveniles. Kidney histopathology revealed severe MMC deposition in 75% of all individuals. Intestinal histopathology showed 33.33% of adults with moderate lymphocyte infiltration, while none was observed in juveniles. In the spleen, 75% of adults exhibited severe MMC deposition, with none observed in juveniles. Heart histopathology revealed cellular degeneration in 37.5% of all individuals, and in the gonad, 50% of all individuals displayed mild congestion. For the PCR sequencing results presence of endoparasite *Triactinomyxon type sp*. and *Ortholinea auratae* myxozoan in the green spotted pufferfish was detected. This study's findings contribute to the establishment of a preliminary parasite inventory in the region and lay the foundation for future investigations into the potential consequences of consuming this species.

Keywords: *Dichotomyctere nigroviridis*; Bagan Lalang; endoparasite; prevalence; histopathology

1. INTRODUCTION

1.1 Background

The spotted green pufferfish, scientifically known as *Dichotomyctere nigroviridis* (Marion de Procé, 1822), is a member of the Tetraodontidae family and is widespread throughout Southeast Asia, including Malaysia. It exhibits a habitat preference ranging from freshwater environments like streams, rivers, and floodplains to brackish water environments such as estuaries, mangroves, and coastal areas (Froese et al., 2022). This species is often captured for ornamental purposes in the aquarium trade and serves as fish bait (Froese et al., 2022). Bagan Lalang in Selangor, Malaysia, a coastal town known for its ecotourism, plays a vital role in both the international tourist industry and the local ecosystem, particularly benefiting the fishing community (Perumal et al., 2017). Besides that, the Bagan Lalang waters are also sites for aquaculture activity including floating fish cages. Growing aquaculture activities' impact on wild fish is uncertain which also raises the concern of collateral diseases such as endoparasite infestation (Bouwmeester et al., 2021).

The increasing demand in the ornamental fish trade, coupled with the potential transmission of diseases, especially endoparasites, presents a need for further exploration (Cahyanto et al., 2023). In Southeast Asia, most freshwater pufferfish specimens in the aquarium trade are captured from the wild (Doi et al., 2022). Tripathi (2014) reported that this trade, known for its role in biological invasion pathways, lacks proper quarantine procedures, with wastewater often disposed of directly into sewer systems. Lowry and Smith (2007) stated that the interaction of pathogens between humans and aquatic species is complex due to multiple routes of transmission

for diseases to spread and multiple zoonotic infections have caused no apparent disease in aquatic organisms as these unaffected carriers appear healthy but can spread infections to people. It is also possible for commensal microorganisms, which are normally harmless to aquatic species, to develop into a human zoonotic pathogen. Besides, Lewin et al. (2019) emphasized the use of live bait species that belong to different water bodies causing loss or released live bait species may have a consequence on ecosystem diversity in terms of genetic diversity and species diversity.

This study aims to investigate the endoparasitic infestations in *Dichotomyctere nigroviridis*, examining associated histopathological changes and potential public health implications. Anticipated findings include the presence of various endoparasite species causing histological alterations in analyzed organs. The research, conducted at Bagan Lalang, may contribute valuable insights into the effects of parasite infections on fish in the context of aquaculture and the aquarium trade. *Dichotomyctere nigroviridis* could serve as a sentinel model, addressing information gaps regarding collateral disease impact and the consequences of consuming cultured fish. Wild fish is known to be a definitive, intermediate or parenthetic host for numerous parasite life cycles, including those of protozoan, metazoan, and crustacean parasites (Okulewicz , 2008). Histopathological alterations induced by parasites can negatively impact fish health (Timur et al., 2005), thus making the assessment of such changes a crucial technique in understanding the impact of parasites on fish tissue. Feist and Longshaw, (2008) emphasized parasites commonly induce various impacts on fish, such as acute or ongoing inflammation, degenerative changes, necrosis, and

hyperplasia. By evaluating the endoparasitic infestation and its histopathological changes, this study contributes to our understanding of aquaculture effects and serves as an initial parasite inventory, potentially expanding as a disease surveillance tool, particularly in nearby farms.

1.2 Hypothesis

The hypotheses for this study are:

$1H_o$: There is no occurrence of endoparasite infection in abdominal cavity, and visceral organs of the juvenile and adult *Dichotomyctere nigroviridis*.

$1H_a$: There is occurrence of endoparasite infection in abdominal cavity, and visceral organs of the juvenile and adult *Dichotomyctere nigroviridis*.

$2H_o$: Endoparasite infection will not cause significant histopathological changes in the abdominal cavity, and visceral organs of the juvenile and adult *Dichotomyctere nigroviridis*.

$2H_a$: Endoparasite infection will cause significant histopathological changes in the abdominal cavity, and visceral organs of the juvenile and adult *Dichotomyctere nigroviridis*.

1.3 Objectives

The objectives of this study are:

1. To evaluate the occurrence of endoparasites on the abdominal cavity, and visceral organs in juveniles and adults *Dichotomyctere nigroviridis*.

2. To evaluate the histopathological changes caused by endoparasites on the abdominal cavity, and visceral organs in juveniles and adults *Dichotomyctere nigroviridis* and the difference in juveniles and adults.

2. LITERITURE REVIEW

2.1 Spotted green pufferfish

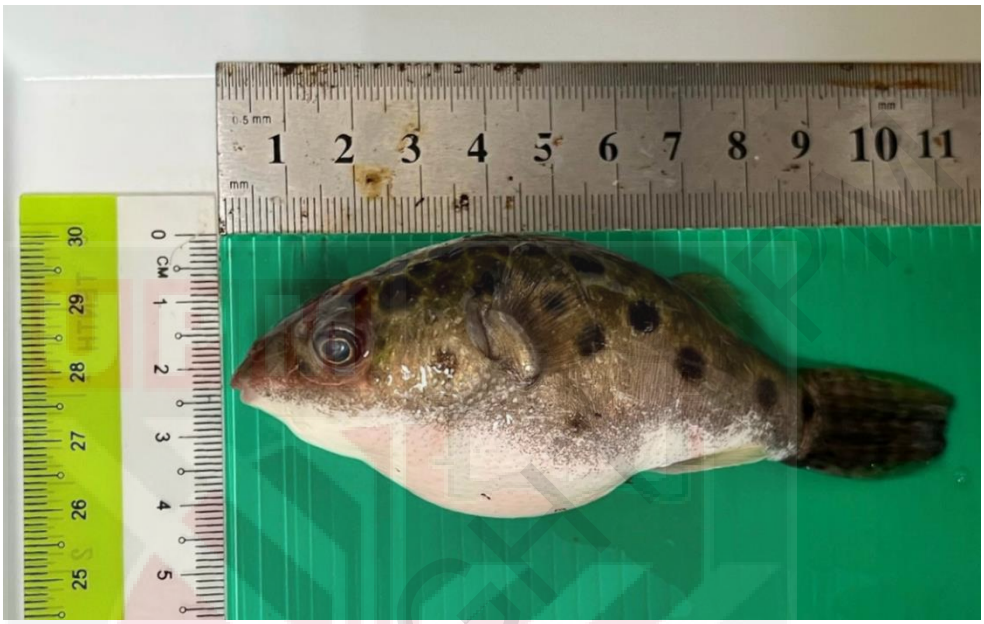

Figure 1: Spotted green pufferfish (*Dichotomyctere nigroviridis*)

Dichotomyctere nigroviridis (Marion de Procé, 1822), commonly known as the spotted green pufferfish or locally called buntal gigi kupang, belongs to the Tetraodontidae family (Kuala Lumpur: Dewan Bahasa dan Pustaka, 2010., n.d.). Synonymously named *Tetraodon nigroviridis* (Kottelat, 2013), this species showcases a versatile habitat encompassing freshwater, brackish, and saltwater environments, with distribution spanning Southeast Asia, including Malaysia. Exhibiting a diverse diet comprising mollusks, crustaceans, shrimps, worms, and other finfish, the average body size ranges from 10 to 17 cm, reaching a maximum total length of 17 cm. The distinctive coloration features a yellowish body with black spots, predominantly covering half of the body. The upper parts are tan ground, while the lower parts display a yellowish-white hue. The back and sides are embellished with numerous dark spots

and rounded blotches, varying in size. The belly maintains a uniform whitish tone, justifying the Latin nomenclature "nigroviridis," where "nigro" signifies black, referring to the spots, and "viridis" denotes a greenish hue.

Morphologically, the body posteriorly of the eye is compressed and covered by spinules. A tentacle is the nasal organ, with at least half divided into two flattened lobes. The dorsal fin boasts 12–14 rays with small dark spots near the base, while the anal fin comprises 10–12 rays, its origin situated slightly anterior to the dorsal fin or beneath its anterior half. The caudal fin typically exhibits 1–8 dark transverse bands. The body shape is oblong, with the posterior part compressed laterally, and the dorsal profile arched, reaching its highest point at the midsection of the back. Small body spines, often concealed under the skin, are not papillose. The face features a convex interorbital, devoid of a groove, and a terminal mouth directed forward. The nostril, functioning as a tentacle, is divided into two flattened lobes, with apposed surfaces often possessing spongy tissue. With a count of four teeth, the species gets its name "tetraodon" directly translated from Greek. The lateral line system is mostly indistinct, adding to the comprehensive morphological description of this intriguing species (Santhanam, 2017).

Ayub et al. (2014) highlighted the results of toxicity studies conducted on the spotted green pufferfish in Malaysian waters. The investigation revealed that the concentrations of Tetrodotoxin (TTX) in the liver, muscle, and skin were 49.8 µg/g, 33.5 µg/g, and 1.64 µg/g, respectively. These concentrations indicate a potential risk for human consumption. It is noteworthy that Santhanam (2017) highlighted a safe level of TTX is less than 10 MU/g tissue, equivalent to 2 µg/g tissue. Therefore, the

results of this study suggest that the consumption of the spotted green pufferfish from Malaysian waters may pose safety concerns for humans. Recently in April 2023, there is a reported case of Malaysian elderly couple who died after eating pufferfish unknown to be poisonous purchased from an unknown fishmonger. This emphasizes the necessity for careful handling of pufferfish, including the use of gloves (preferably double-layered) and thorough handwashing, particularly before meals. In contrast, Takifugu rubripes which is famous for as delicacy in japan have high demand as the muscle of this species has been reported to contain Tetrodotoxin (TTX) at 0.25 µg/g, falling within safe levels, while the liver and ovaries exhibit high toxicity (Froese et al., 2022). This emphasizes the need for adequate training for the delicacy preparation by a professional chef.

Spotted green pufferfish plays an important role in the aquarium trade, primarily sourced from the wild due to challenges in commercial breeding. This reliance underscores the need for a thorough study, as understanding the health and dynamics of this species is crucial for sustainable practices in the aquarium industry. Its compact genome positions it as a valuable asset in evolutionary research, serving as an ideal model organism for efficiently identifying human genes in comparative genomics (Tetraodon Nigroviridis - Spotted Green Pufferfish - Taxo4254 - Wiki.nus, n.d.). Beyond its role in captivity, *Dichotomyctere nigroviridis* emerges as a sentinel model, providing insights into the impact of aquaculture on endoparasite infections, both in captive and wild settings Notably, there is a significant gap in the literature regarding histopathological changes due to ectoparasite infections in *Dichotomyctere nigroviridis*, particularly in Malaysia. Addressing this gap is imperative, as it not only

contributes to our understanding of fish health but also sheds light on the unique challenges faced by this species in its native habitat. In essence, studying *Dichotomyctere nigroviridis* holds promise for advancing both the aquarium trade and our broader knowledge of aquatic ecosystems.

2.2 Bagan Lalang

Bagan Lalang in Selangor, Malaysia is a coastal town with ecotourism attracting international tourists and benefiting the local ecosystem and community, especially the fishing community (Perumal et al., 2017). Besides that, the Bagan Lalang waters are also sites for aquaculture activity including floating fish cages. Growing aquaculture activities' impact on wild fish is uncertain which also raises the concern of collateral diseases such as endoparasite infestation (Bouwmeester et al., 2021). According to local fisherman the wild spotted green pufferfish is also available in this area along with several other reported species namely the *Dichotomyctere fluviatis*, *Dichotomyctere ocellatus* and *Takifugu oblongus.* These pufferfish are typically caught during fishing or netting, especially at the jetty platform of the Tanjung Sepat estuary near the port of local fishermen. Although they are often considered a bycatch and are usually released back into the water, some fishermen have reported using them as a termite deterrent.

2.3 Endoparasites of Pufferfish

Endoparasites are parasites that live in the tissue, blood, and organs. In the wild population, the parasites and the fish host maintain a kind of equilibrium until something happens that disturb the equilibrium balance (Edeh and Solomon, 2017). The wild fish have been reported to have larger parasite species diversity than those

raised in hatcheries (Hoffman, 1998). It is anticipated that there is presence of various endoparasite species in wild pufferfish populations, as documented by Santhanam (2017). Examples include trematodes such as *Lintonium vibex*, *Bucephalus sp.*, *Prosorhynchus sp.*, and *Rohdella amazonica n. sp*. Additionally, from the myxozoan group, species like *Myxidium leei*, *Kudoa dianae*, *Sinuolinea tetraodoni*, and *Myxidium fugu* are expected.

2.4 Parasite of Public Health Concern

In general, consumption of raw fish harboring zoonotic parasites has been associated with a spectrum of clinical manifestations in humans, varying from mild and transient symptoms to chronic, severe, and, on rare occasions, life-threatening conditions (Bao et al., 2017). The public health implications of endoparasites, particularly myxozoan species, underscore the urgency of comprehensive studies in this field. As highlighted by Hallett et al. (2015), myxozoan infestations manifest in a spectrum of clinical presentations, ranging from asymptomatic cases, such as those caused by *Henneguya spp.*, to more severe outcomes. Notably, some myxozoans, like *Kudoa sp.*, evoke allergic responses, while others, like *Kudoa septempunctata*, have been associated with acute gastroenteritis. The endoparasitic activity of myxozoans has the potential to result in significant economic losses for both aquaculture and fisheries, given their capacity to inflict harm on both wild and farmed fish (Okamura et al. 2015). The gravity of these health impacts, coupled with the current lack of specific treatments for myxozoan infections, raises concerns not only for aquaculture and food security but also for potential zoonotic transmission. With reported cases exhibiting diverse clinical outcomes, understanding the intricacies of myxozoan

infections becomes paramount. This study addresses a critical knowledge gap, aiming to unravel the complexities of myxozoan-induced diseases and assess their implications for both aquatic ecosystems and human health.

3. MATERIALS AND METHODS

3.1 Sample Collection

A total of 16 spotted green pufferfish (*Dichotomyctere nigroviridis*) were collected for this study. The specimen was obtained from Bagan Lalang, Selangor on 2 dates which are on 4 September 2023 and 8 September 2023. The date was chosen following the lunar calendar where the 4 September 2023 falls under the full moon phenomenon in which Reis-Filho et al. (2010) demonstrated a significant rise in the mean number and mean weight of captured fish in the full moon, especially at ebb tides. Alternatively, 8 September 2023 was chosen as a repeated sampling date as suggested by local fishermen suggesting the high frequency of pufferfish yield during "*air mati*" where the changes in water level during the transition of low tide to high tide and vice versa was minimal. The fish was caught using a fishing rod by the fishing team consisting of 8 people on the first date and 6 people on the second date. The bait used for fishing was live bait such as blood cockles and prawns purchased from the nearest fish market. The fish were kept in an oxygen-supplied container during the necropsy process. Water analysis was done using equipment ProQuatro Portable (YSI Inc., USA) multiparameter water quality meter.

3.2 Morphometric Measurements

The body morphometric measurements were done according to the method described by Mohamed (2003). The total length (TL), and standard length (SL) were measured to the nearest 0.1 cm. Descriptive statistics for the body weight, TL, SL, and body width were done using SPSS v27 to obtain the minimum, maximum, mean,

standard deviation, and range. The juvenile or adult stage classification of the spotted green pufferfish was conducted via observation of underdeveloped gonads and maximum body weight with underdeveloped gonads as the cutoff point for the juvenile stage (Figure 1). The spotted green pufferfish species identification and confirmation were done according to the morphological description and Polymerase Chain Reaction (PCR) sequencing.

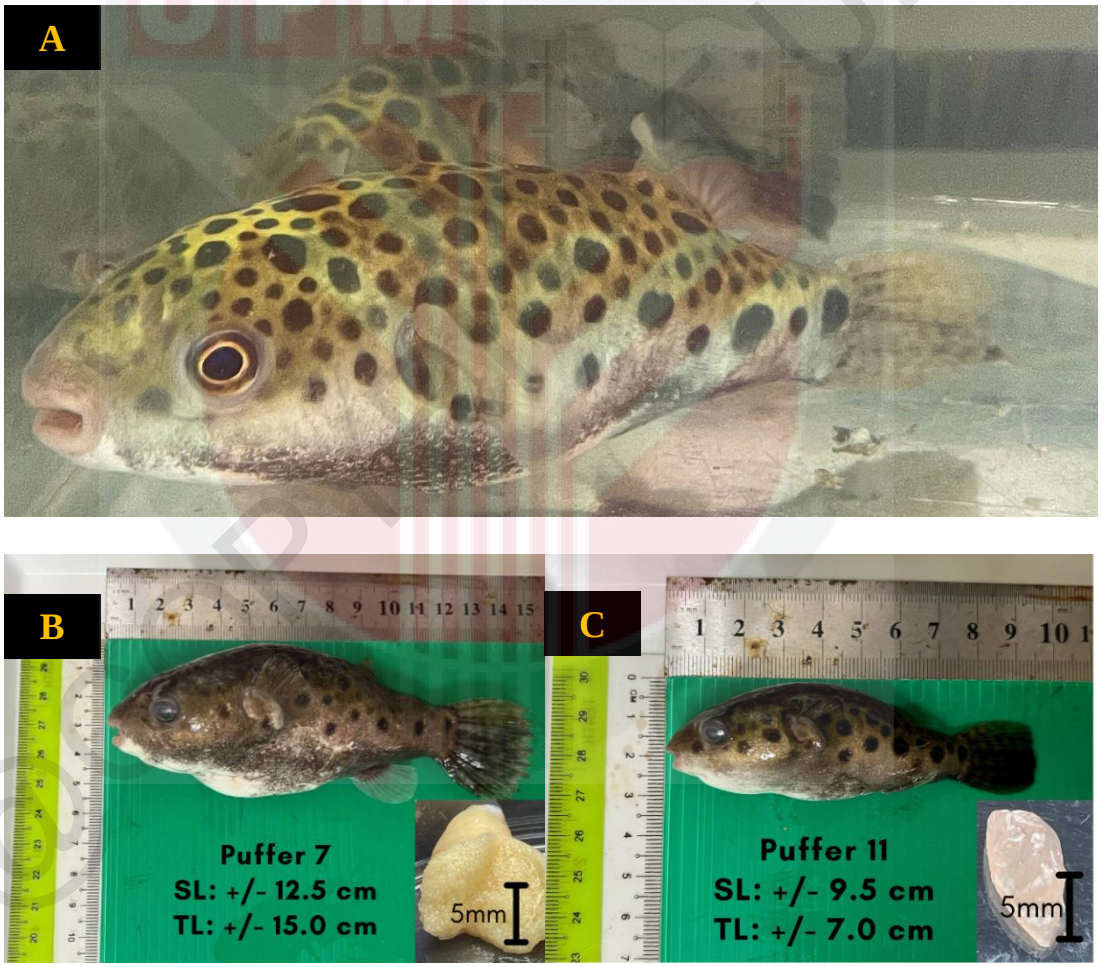

Figure 2: Live spotted green pufferfish (*Dichotomyctere nigroviridis*) swimming in collection tank (A), Example of adult spotted green pufferfish (*Dichotomyctere nigroviridis*) (B), Example of juvenile spotted green pufferfish (*Dichotomyctere nigroviridis*) (C).

3.3 Examination of the Specimen

The fish was examined for the presence of parasites at the Aquatic Animal health Unit (AAHU), UPM under IACUC approval (UPM/IACUC/AUP-U008/2023). Fish was handled cautiously using a damp cloth to reduce stress and injuries to the skin surface and to prevent the skin from drying out. As a precautious step, the handler should refrain from placing their hand in front of the fish's mouth to prevent potential finger nipping, given the fish's formidable bite power. Additionally, handlers must consistently wear gloves during the pufferfish handling process, as the skin is reported to contain a certain level of tetrodotoxin (TTX), which, if mishandled, may lead to fatal consequences. The fish were then immobilized by decapitation and pithing of the central nervous system before further examination. Parasitological analysis for the endoparasite of the spotted green pufferfish was initiated with blood collection via intracardiac puncture or bleeding of the gills. From the blood collected, a thin blood smear was done and let blot dry for about 10 minutes before continuing with May Grunwald-Geimsa (MGG) staining. Prepared MGG-stained thin blood smear slides were then observed under compound microscopy under 4x, 10x, 40x and 100x magnification for blood parasites. Then, the dissection of the spotted green pufferfish was done by an incision along the ventral side of the fish using a scalpel and mayo scissor. The incision began beneath the operculum, between the pectoral fins, and extended along the ventral midline towards the anus opening. The abdominal flap was reflected dorsolateral to expose the internal organs of the abdominal cavity. Upon exposing the internal organ of the spotted green pufferfish, organs in-situ gross examination was done to examine the presence of endoparasite and any gross

pathological lesion on the body cavity and external surface of the internal organ. Additionally, the muscle of the visceral wall was cut using a scalpel blade to examine any presence of parasite cyst. For the brain, the fish skull in between the eyes was cut open carefully using heavy-duty scissors. The brain, gastrointestinal tract (GIT), liver, spleen, gonad, heart, and kidney were carefully taken out and put on a petri dish.

On the petri dish organs ex-situ gross examination was done to examine the presence of endoparasite and any gross pathological lesion on the internal organs more extensively using the naked eye and stereomicroscope (RaxVision., USA). Additionally, the tubular organs such as GIT were cut open vertically to examine the internal surface under a stereomicroscope and the internal wall was scrapped using a scalpel blade. The scrapings were placed onto a clean and clear glass slide and one drop of methylene blue was placed on the slide. These were carefully examined under a microscope for the attachment of the parasites using 4x, 10x and 40x magnification. Simultaneously, the organs, especially the kidney, were then put in a collection tube with buffered formalin 10% for histopathological study preparation and a collection tube with alcohol 10% for molecular study preparation. Squash smear was done for the kidney and brain stained with MGG stain and for muscle stained with methylene blue and was observed under compound microscope M837 Series Trinocular Lab Compound Microscope (OMAX, USA) under 4x, 10x, and 40x magnification for the presence of parasite cyst. The squash smear was prepared by cutting small piece of the organs and putting them on the clean and clear glass slide and another clean and clear glass slide was put onto the organs piece perpendicularly and the organ were

squashed in between and then the slides was twisted 90° to parallel to each other before spreading outwards which produced two slides of squash smear.

3.4 Histology Sample Processing & Scoring

For histological studies, the target organs (brain, gastrointestinal tract (GIT), liver, spleen, gonad, heart, and kidney) were carefully removed and fixed in 10% Buffered Formalin for 24 hours. Tissues were dehydrated and processed at Histopathology Laboratory (FPV) for paraffin wax embedding. The sections were cut by a rotary microtome and stained with hematoxylin and eosin. Tissues were examined for histopathological lesions under a light microscope. Scoring was done according to the fish organ histopathological changes scoring suggested by Jovanovic *et al.*, (2014).

3.5 Histology Parasite Distribution

Additionally, for histological studies, the kidney histology slides were examined under a compound microscope and the distribution of renal tubules and renal collecting duct infested with myxozoan parasites was quantified using a cell counter (Blumenreich, 1990). In 40x magnification, all the renal tubules and renal collecting duct was counted and a total number of myxozoan-infested renal tubules and renal collecting duct was divided by total renal tubules and renal collecting duct examined (Myxozoan-infested renal tubules and renal collecting duct + myxozoan-free renal tubules and renal collecting duct).

$$\text{Percentage infested tubules} = \frac{\textbf{Total no of infested renal tubules and renal collecting duct}}{\textbf{Total renal tubules and renal collecting duct examined}} \times \mathbf{100}\%$$

3.6 Parasite Identification

Conventional PCR and nested PCR was done for species identification using Zymo DNA (Zymo Research, USA) extraction & purification kit for DNA extraction from the kidney, heart, brain, and gills sample. For the first step ZR BashingBead™ Lysis Tubes (0.1 & 0.5 mm) were employed, and the sample was added to them. Additionally, 750 µl ZymoBIOMICS™ Lysis Solution was added to the tube, and it was tightly capped. Second step, the tube was then secured in a bead beater fitted with a 2 ml tube holder assembly and processed using optimized beat beating conditions for the device. Subsequently in the third step, 95 µl of water, 95 µl of solid tissue buffer, and 10 µl of proteinase K were added, followed by incubation at 55 degrees Celsius for a minimum of 1 hour and 30 minutes. Afterward, vortexing was performed for 40 minutes until the cell tissue was completely lysed. Incubator () and Mini vortex (Biosan, Latvia) were used.

The ZR BashingBead™ Lysis Tubes (0.1 & 0.5 mm) were centrifuged in a microcentrifuge at ≥ 10,000 x g for 1 minute. In step 4, up to 400 µl of the supernatant was transferred to the Zymo-Spin™ III-F Filter in a Collection Tube and centrifuged at 8,000 x g for 1 minute in Avanti J-26s XPI centrifuge (Beckman Coulter, USA). In step 5 The Zymo-Spin™ III-F Filter was then discarded. To the filtrate in the Collection Tube from Step 4, 1,200 µl of ZymoBIOMICS™ DNA Binding Buffer was added and mixed well.

In the step 6, 800 µl of the mixture from Step 5 was transferred to a Zymo-Spin™ IICR Column in a Collection Tube and centrifuged at 10,000 x g for 1 minute. In step 7 the flow-through from the Collection Tube was discarded, and Step 6 was

repeated. Subsequently in step 8, 400 µl of ZymoBIOMICS™ DNA Wash Buffer 1 was added to the ZymoSpin™ IICR Column in a new Collection Tube and centrifuged at 10,000 x g for 1 minute. The flow-through was discarded, and in step 9, 700 µl of ZymoBIOMICS™ DNA Wash Buffer 2 was added to the ZymoSpin™ IICR Column in a Collection Tube and centrifuged at 10,000 x g for 1 minute. Again, the flow-through was discarded. Additionally in step 10, 200 µl of ZymoBIOMICS™ DNA Wash Buffer 2 was added to ZymoSpin™ IICR Column in Collection Tube and centrifuging at 10,000 x g for 1 minute.,

In Step 11, the Zymo-Spin™ IICR Column was transferred to a clean 1.5 ml microcentrifuge tube, and 100 µl (50 µl minimum) of ZymoBIOMICS™ DNase/RNase Free Water was added directly to the column matrix. The mixture was incubated for 1 minute and then centrifuged at 10,000 x g for 1 minute to elute the DNA.

Next in step 12, a Zymo-Spin™ III-HRC Filter was placed in a new Collection Tube, and 600 µl ZymoBIOMICS™ HRC Prep Solution was added. Centrifugation at 8,000 x g for 3 minutes was carried out. In step 13, the eluted DNA from Step 11 was then transferred to a prepared Zymo-Spin™ III-HRC Filter in a clean 1.5 ml microcentrifuge tube and centrifuged at exactly 16,000 x g for 3 minutes. The filtered DNA obtained is now suitable for PCR and other downstream applications. The forward and reverse primers used were 18e and 18g and SphF and SphR (Table 1) with the thermocycler set up following Székely et al., 2009b (Table 2).

Table 1: Primers used for PCR or sequencing

Primer	Sequence	Source
18e	5'-CTG GTT GAT TCT GCC AGT-3'	Hillis & Dixon (1991)
18g'	5'-CGG TAC TAG CGA CGG GCG GTG TG-3'	Hillis & Dixon (1991)
SphF	5'-ACT CGT TGG TAA GGT AGT GGC T-3'	Eszterbauer & Székely (2004)
SphR	5'-GTT ACC ATT GTA GCG CGC GT-3'	Eszterbauer & Székely (2004)

Table 2: PCR Thermocycler settings

Primer	18e & 18g	SphF & SphR
Initial denaturation	94.0°C for 240s	94.0°C for 240s
Denaturation	94.0°C for 50s	94.0°C for 50s
Annealing	56.0°C for 50s	56.0°C for 50s
Extension	72.0°C for 80s	72.0°C for 60s
Final Extension	72.0°C for 420s	72.0°C for 600s
Number of cycles	35	35

3.7 Statistical Analysis

The prevalence of parasites was calculated through the suggested formula (Shafiq et al., 2023). The parasite distribution and histopathological scoring data were analyzed using Fisher's Exact Test performed using SPSS v27 statical software to observe the significant difference ($p<0.05$) between the adult and juvenile as well as between male and female spotted green pufferfish in terms of the percentage infested tubules and histopathological lesion scoring. Microsoft Excel was used for data tabulation.

$$\text{Parasites Prevalence} = \frac{\textbf{No of infected hosts}}{\textbf{Total no.of host examined}} \times \mathbf{100}\%$$

4. RESULTS

4.1 Morphometric Measurements

The below tables show the morphometric measurement (Table 3) and morphometric measurement descriptive statistic (Table 4) of *Dichotomyctere nigroviridis* pufferfish from Bagan Lalang, Selangor.

Table 3: Morphometric measurement of endoparasites in adult spotted green pufferfish from Bagan Lalang, Selangor

Pufferfish (ID)	Body Weight (g)	Total Length, TL (cm)	Standard Length, SL (cm)	Width (cm)	Sex	Life stage
Pufferfish 1 (Adult 1)	50	13.0	11.0	4.9	Male	Adult
Pufferfish 2 (Adult 2)	52	13.0	11.5	4.7	Male	Adult
Pufferfish 3 (Adult 3)	52	13.0	11.0	4.5	Male	Adult
Pufferfish 4 (Juvenile 1)	36	9.0	7.50	3.2	Male	Juvenile
Pufferfish 5 (Juvenile 2)	38	11.4	9.00	3.7	Male	Juvenile
Pufferfish 6 (Juvenile 3)	41	11.2	8.90	4.5	Male	Juvenile
Pufferfish 7 (Adult 4)	91	15.0	12.5	5.5	Female	Adult
Pufferfish 8 (Adult 5)	53	11.5	9.4	4.2	Male	Adult
Pufferfish 9 (Juvenile 4)	41	11.0	9.3	4.1	Male	Juvenile
Pufferfish 10 (Adult 6)	45	11.5	9.7	4.0	Female	Adult
Pufferfish 11 (Juvenile 5)	22	9.5	7.7	3.0	Female	Juvenile
Pufferfish 12 (Adult 7)	44	11.0	9.6	3.5	Female	Adult
Pufferfish 13 (Juvenile 6)	25	9.0	7.5	4.0	Female	Juvenile
Pufferfish 14 (Adult 8)	60	12.7	10.5	5.0	Male	Adult
Pufferfish 15 (Juvenile 7)	44	10.2	8.4	3.2	Female	Juvenile
Pufferfish 16 (Juvenile 8)	42	11.5	9.9	3.6	Female	Juvenile

Table 4: Morphometric measurement descriptive statistic of spotted green pufferfish from Bagan Lalang, Selangor

Parameters	Minimum	Maximum	Mean	Standard Deviation
Body Weight (g)	22.00	91.00	46.0000	15.52203
Total Length (cm)	9.00	15.00	11.4688	1.61688
Standard Length (cm)	7.50	12.50	9.5875	1.45367
Body Width/ Depth (cm)	3.00	5.50	4.1000	0.71740

4.2 Prevalence of Endoparasites

The prevalence calculation was done manually according to the total number of parasites that was observed. The below tables show the prevalence of endoparasites found on the adult (Table 4) and juvenile (Table 5) of *Dichotomyctere nigroviridis* pufferfish from Bagan Lalang, Selangor.

Table 5: Prevalence of endoparasites in adult spotted green pufferfish from Bagan Lalang, Selangor

Common Parasites	Adult			Prevalence	Organ
	No. of Fish Examined	**No. of Fish Infected**	**No. of Parasite**		
Cestode (Tapeworms)	8	0	0	0.00%	-
Trematodes (Flukes)	8	0	0	0.00%	-
Nematodes (Roundworms)	8	0	0	0.00%	-
Acanthocephala (Thorny-headed worms)	8	0	0	0.00%	-
Myxozoan	8	8	abundant	100.00%	Kidney
Unidentified Encysted Metacercariae (EMC)	8	0	0	0.00%	-

Table 6: Prevalence of endoparasites in juvenile spotted green pufferfish from Bagan Lalang, Selangor

Common Parasites	Juvenile			Prevalence	Organ
	No. of Fish Examined	**No. of Fish Infected**	**No. of Parasites**		
Cestode (Tapeworms)	8	0	0	0.00%	-
Trematodes (Flukes)	8	0	0	0.00%	-
Nematodes (Roundworms)	8	0	0	0.00%	-
Acanthocephala (Thorny-headed worms)	8	0	0	0.00%	-
Myxozoan	8	8	abundant	100.00%	Kidney
Unidentified Encysted Metacercariae (EMC)	8	0	0	0.00%	-

Table 7: Percentage myxozoan-infested renal tubules & renal collecting duct in juvenile spotted green pufferfish from Bagan Lalang, Selangor

Pufferfish (ID)	**Total Myxozoan-infested renal tubules & renal collecting duct**	**Total Myxozoan-free renal tubules & renal collecting duct**	**Total renal tubules & renal collecting duct examined**	**Percentage Myxozoan-infested renal tubules & renal collecting duct (%)**	**Percentage Myxozoan-free renal tubules & renal collecting duct (%)**
Pufferfish 4 (Juvenile 1)	439	61	500	87.80	12.20
Pufferfish 5 (Juvenile 2)	452	48	500	90.40	9.60
Pufferfish 6 (Juvenile 3)	386	114	500	77.20	22.80
Pufferfish 9 (Juvenile 4)	431	69	500	86.20	13.80
Pufferfish 11 (Juvenile 5)	374	126	500	74.80	25.20
Pufferfish 13 (Juvenile 6)	404	96	500	80.80	19.20
Pufferfish 15 (Juvenile 7)	414	86	500	82.80	17.20
Pufferfish 16 (Juvenile 8)	443	57	500	88.60	11.40

Table 8: Percentage of myxozoan-infested renal tubules & renal collecting duct in adult spotted green pufferfish from Bagan Lalang, Selangor

Pufferfish (ID)	Total myxozoan-infested renal tubules & renal collecting duct	Total myxozoan-free renal tubules & renal collecting duct	Total renal tubules & renal collecting duct examined	Percentage myxozoan-infested renal tubules & renal collecting duct (%)	Percentage myxozoan-free renal tubules & renal collecting duct (%)
Pufferfish 1 (Adult 1)	466	34	500	93.20	6.80
Pufferfish 2 (Adult 2)	471	29	500	94.20	5.80
Pufferfish 3 (Adult 3)	480	20	500	96.00	4.00
Pufferfish 7 (Adult 4)	499	1	500	99.80	0.20
Pufferfish 8 (Adult 5)	447	53	500	89.40	10.60
Pufferfish 10 (Adult 6)	447	53	500	89.40	10.60
Pufferfish 12 (Adult 7)	349	29	378	92.30	7.70
Pufferfish 14 (Adult 8)	400	100	500	80.00	20.00

Eight individual *Dichotomyctere nigroviridis* juveniles were identified thus 8 spotted green pufferfish for each juvenile and adult, were used in total for the prevalence study. The only parasite observed was the myxozoan (Figure 2) which was found inside the kidney from the histology and squash smear slides prepared. No other parasites were observed in the liver, stomach, spleen, heart, gonad, and brain from squash smear, blood smear, gross examination, and histology preparation.

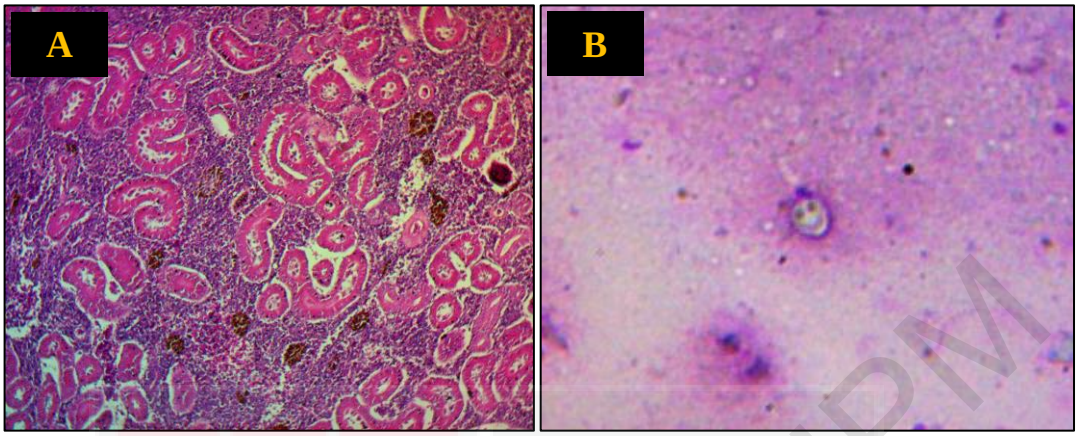

Figure 3: Presence of Myxozoan from histopathology slides (A), Presence of Myxozoan from squash smear slides (B) Inside the Kidney of *Dichotomyctere nigroviridis.*

Based on the prevalence results for endoparasites in adult (Table 3) and juvenile (Table 4) *Dichotomyctere nigroviridis*, it is evident that myxozoans exhibit a high prevalence of 100% in both adults and juveniles. Furthermore, the distribution of myxozoan parasites in the renal tubules and renal collecting ducts of adult (Table 6) and juvenile (Table 7) *Dichotomyctere nigroviridis* kidneys ranges from 74.80% to 99.80%. The adult and juvenile spotted green pufferfish had significant difference of myxozoan distribution in kidney, there is significant difference ($p<0.05$) between the adult and juvenile spotted green pufferfish in terms of endoparasites burden according to the Fisher's Exact Test run using SPSS v27 statistical software.

4.3 Histopathological Changes

The organ histopathological changes scoring was done according to the scoring guide suggested by Jovanovic et al., (2014). The scoring was done using a scale which consisted of none (0), mild (1), moderate (2) and severe (3). There were

histopathological changes that were noticed on the kidney, brain, spleen, intestine, liver, heart and gonad of the fish.

Table 9: Histopathological changes scoring for brain of the sampled spotted green pufferfish

Histopathological Changes	Adult								Juvenile							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Congestion	-	-	-	2	2	1	1	2	0	0	1	0	0	0	1	1
Hemorrhage	-	-	-	0	0	0	0	0	0	0	0	0	0	0	0	0
Inflammation of Meninges	-	-	-	0	0	0	1	1	0	0	0	0	0	0	1	0
Total	-	-	-	2	2	1	2	3	0	0	1	0	0	0	2	1

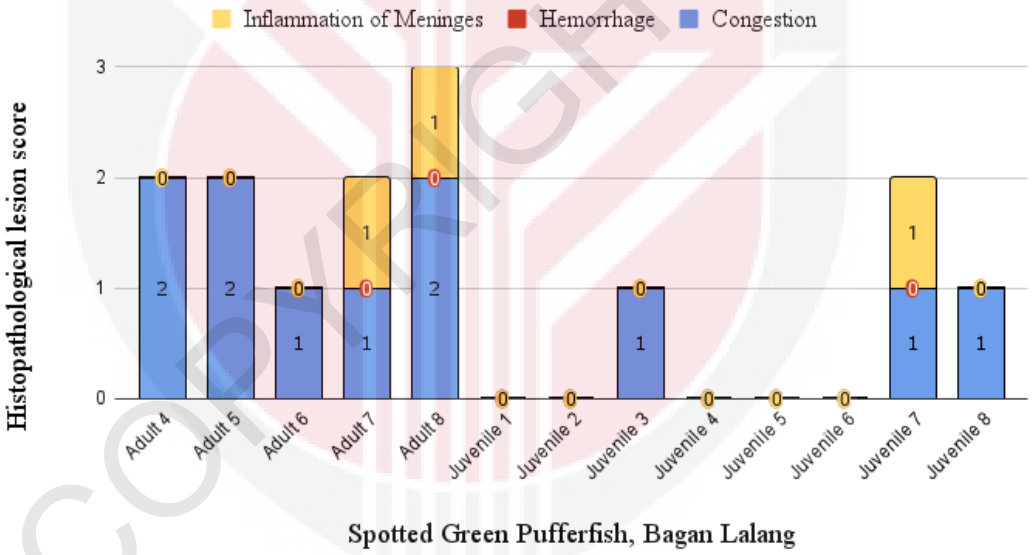

Figure 4: Brain histopathological changes scoring of *Dichotomyctere nigroviridis*, Bagan Lalang

The brain histopathological changes in each fish were documented and presented in Table 8. The scoring results for brain histopathological changes (Figure 4) revealed congestion in adult fish, with scores ranging from mild (1) to moderate (2). Among adults, 60% scored moderate (2), and 40% scored mild (1). In juvenile fish, observations included none (0) and mild (1) scores, with none (0) representing

62.5% and mild (1) representing 37.5%. Inflammation of meninges in adult fish ranged from none (0) to mild (1), with none (0) at 60% and mild (1) at 40%. In juvenile fish, the scores ranged from none (0) to mild (1), with none (0) at 87.5% and mild (1) at 12.5%. Notably, all fish scored none (0) for hemorrhage.

Table 10: Histopathological changes scoring for liver of the sampled spotted green pufferfish

Histopathological Changes	Adult								Juvenile							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Congestion	0	0	1	0	0	1	0	0	1	1	1	1	0	0	0	0
Hepatocyte Glycogen Accumulation	2	1	1	1	1	1	2	1	0	0	0	1	0	1	0	0
Lipid Accumulation	2	0	1	2	1	1	2	2	0	1	2	1	0	1	1	1
Hepatocyte degeneration, necrosis, or apoptosis	0	0	0	0	0	0	0	0	2	1	1	0	0	0	0	1
Mononuclear cell infiltration	0	0	0	2	1	0	1	0	0	0	0	0	0	0	0	1
Total	4	1	3	5	3	3	5	3	3	3	4	3	0	2	1	3

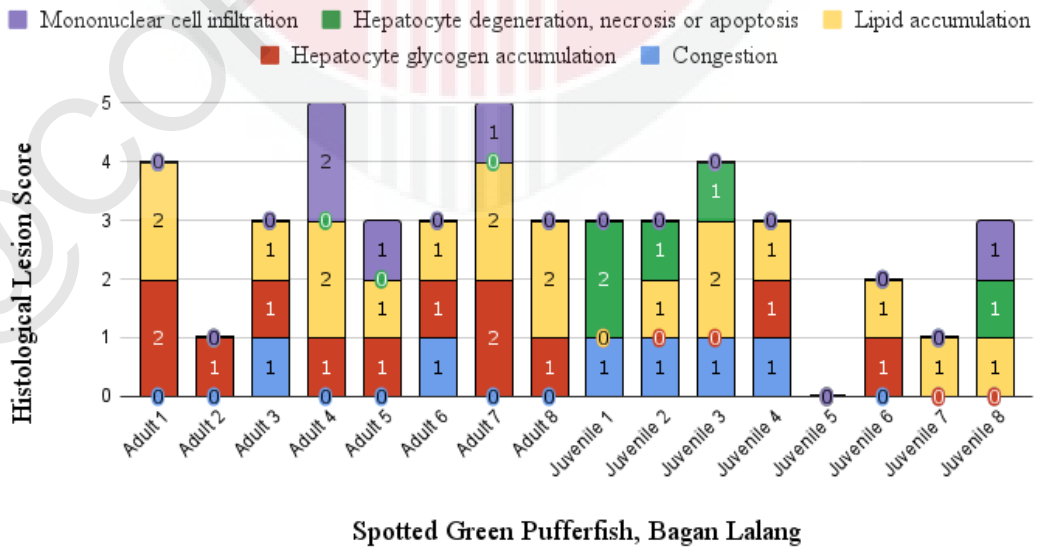

Figure 5: Liver Histopathological Changes Scoring of *Dichotomyctere nigroviridis*, Bagan Lalang

The liver histopathological changes in each fish were organized in Table 9. According to the liver histopathological changes scoring results (Figure 5), mononuclear cell infiltration in adults scored none (0) at 62.5%, mild (1) at 25%, and moderate (2) at 12.5%. In juveniles, the scores were none (0) at 87.5% and mild (1) at 12.5%. Hepatocyte degeneration, necrosis, or apoptosis in adults scored none (0), while in juveniles, 50% scored none (0), 37.5% scored mild (1), and 12.5% scored moderate (2). Lipid accumulation in liver parenchyma of both adults and juveniles ranged from none (0) to moderate (2). In adults, 12.5% had none (0), 37.5% had mild (1), and 50% had moderate (2), while in juveniles, 25% had none (0), 62.5% had mild (1), and 12.5% had moderate (2). For hepatocyte glycogen accumulation, 75% of adults scored mild (1), and the remaining 25% scored moderate (2), while 75% of juveniles scored none (0), and the remaining 25% scored mild (1). Regarding congestion, both adults and juveniles scored none (0) and mild (1). In adults, 75% scored none (0), and the rest scored mild (1), while juveniles, 50% scored none (0), and others 50% scored mild (1).

Table 11: Histopathological changes scoring for kidney of the sampled spotted green pufferfish

Histopathological Changes	Adult								Juvenile							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Congestion	1	1	1	1	0	0	0	1	0	0	0	0	0	0	1	1
Tubular Epithelial Degeneration/Necrosis	0	0	0	0	0	0	0	0	3	1	0	0	0	0	0	1
Interstitial Pigment (Melanomacrophages)	3	3	3	3	3	3	3	3	3	3	3	3	2	2	2	2
Total	4	4	4	4	3	3	3	4	6	4	3	3	2	2	3	4

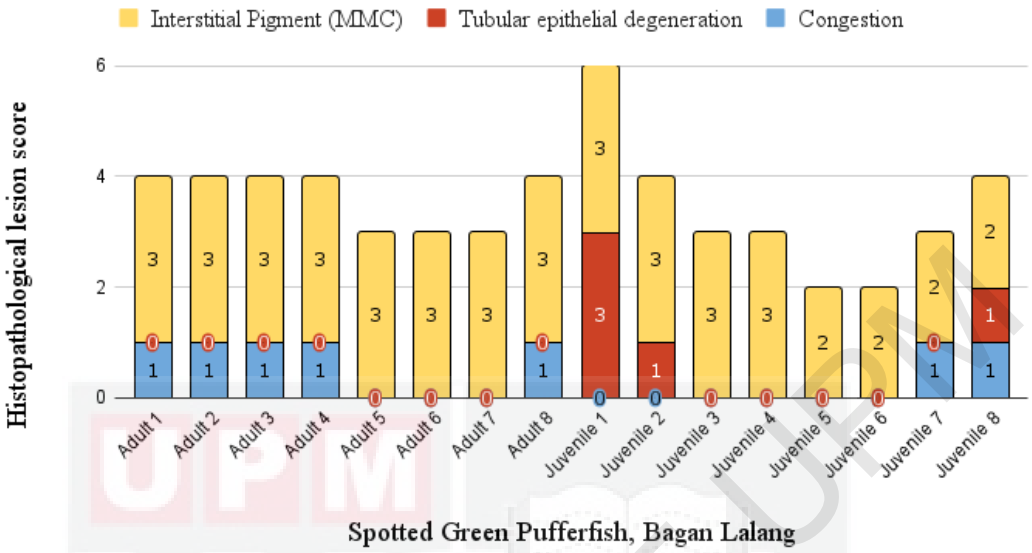

Figure 6: Kidney histopathological changes scoring of *Dichotomyctere nigroviridis*, Bagan Lalang

The kidney histopathological changes for each fish were documented in Table 10. According to the kidney histopathological changes scoring results (Figure 6), interstitial pigment melanomacrophage (MMC) was observed with a severe (3) score in all adult fish. In juveniles, 50% scored a moderate (2), and the other 50% scored severe (3). In adults, tubular epithelial degeneration was not observed (none - 0), while in juveniles, 62.5% scored none (0), 25% scored mild (1), and 12.5% scored severe (3). Regarding congestion, 62.5% of adults and 25% of juveniles scored mild (1), and the remainder scored none (0).

Table 12: Histopathological changes scoring for intestine of the sampled spotted green pufferfish

Histopathological Changes	Adult								Juvenile							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Congestion	1	1	1	1	2	1	1	0	1	1	1	0	0	0	1	1
Hemorrhage	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Infiltration of Lymphocyte in Lamina Propria	1	2	1	2	1	1	1	1	0	1	0	1	1	0	1	0
Interstitial Pigment (Melanomacrophages)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	2	3	2	3	3	2	2	1	1	2	1	1	1	0	2	1

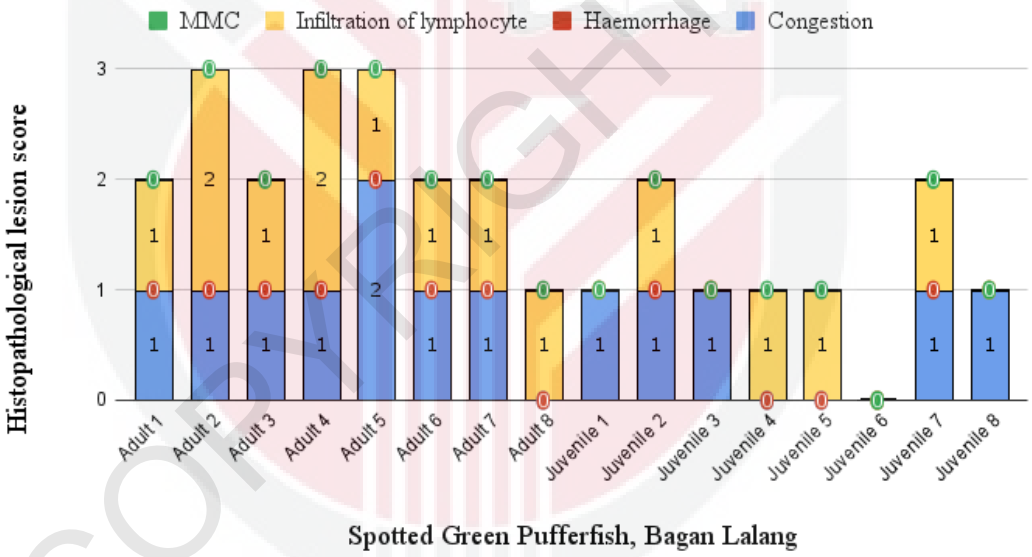

Figure 7: Intestine histopathological changes scoring of *Dichotomyctere nigroviridis*, Bagan Lalang

The intestinal histopathological changes for each fish were recorded and are presented in (Table 11). According to the scoring results for intestinal histopathological changes (Figure 7), both adult and juvenile fish exhibited scores for the infiltration of lymphocytes and congestion. In the case of lymphocyte infiltration, 75% of adults scored mild (1), with 12.5% of adults and 37.5% of juveniles scoring moderate (2) and the remaining 62.5% of juveniles shows none (0) in lymphocyte

infiltration. Hemorrhage and interstitial pigment melanomacrophage (MMC) were scored none (0) in both juveniles and adult pufferfish.

Table 13: Histopathological changes scoring for spleen of the sampled spotted green pufferfish

Histopathological Changes	Adult								Juvenile							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Congestion	2	3	1	1	1	1	1	1	0	0	0	1	1	0	0	0
Infiltration of Lymphocyte	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Granulomas Formation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Interstitial Pigment (Melanomacrophages)	3	3	3	3	1	3	1	3	2	0	2	1	0	2	1	2
Total	5	6	4	4	2	4	2	4	2	0	2	2	1	2	1	2

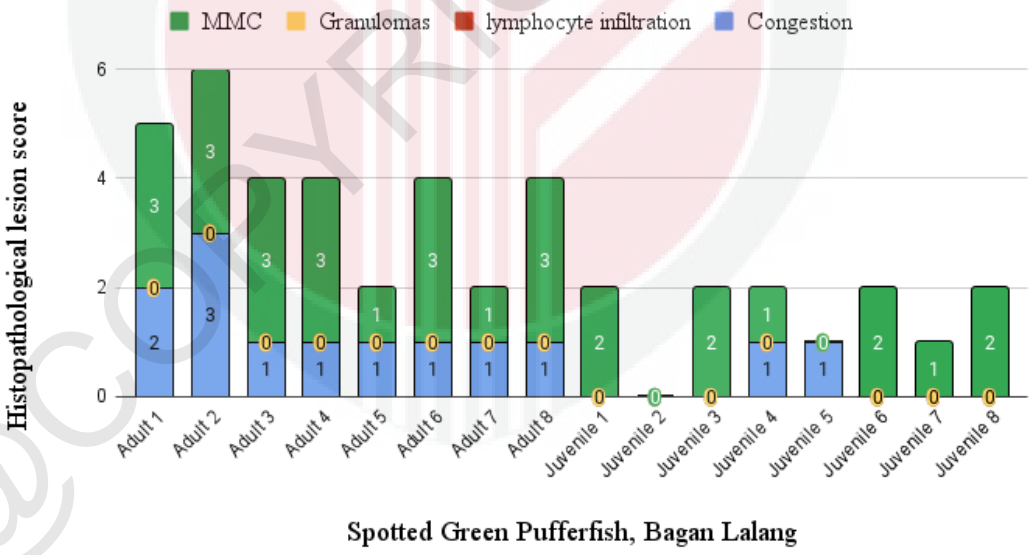

Figure 8: Spleen histopathological changes scoring of *Dichotomyctere nigroviridis*, Bagan Lalang

The spleen's histopathological changes for each fish were documented and are presented in (Table 12). According to the scoring results for spleen histopathological changes (Figure 8), congestion was observed in all adult fish, with 75% having a mild

(1) score, 12.5% having a moderate (2) score, and the remaining 12.5% having a severe (3) score. In contrast, only 25% of juveniles had a mild (1) score for congestion. Melanomacrophage (MMC) deposition was observed in 100% of adults, with varying severity, either mild (1) or severe (3), and with occurrence percentages of 25% and 75%, respectively. In juveniles, MMC deposition appeared as none (0), mild (1), and moderate (2), with occurrence percentages of 25%, 25%, and 50%, respectively. The presence of granulomas and lymphocyte infiltration in the spleen parenchyma for both adult and juvenile fish scored none (0).

Table 14: Histopathological changes scoring for heart of the sampled spotted green pufferfish

Histopathological Changes	Adult								Juvenile							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Presence of Cyst	-	-	-	0	0	0	0	0	-	0	0	0	0	0	0	0
Cellular degeneration	-	-	-	1	0	1	1	1	-	1	0	0	0	0	0	1
Interstitial Pigment (Melanomacrophages)	-	-	-	0	0	0	0	0	-	0	0	0	0	0	0	0
Total	-	-	-	0	0	1	1	1	-	1	0	0	0	0	0	1

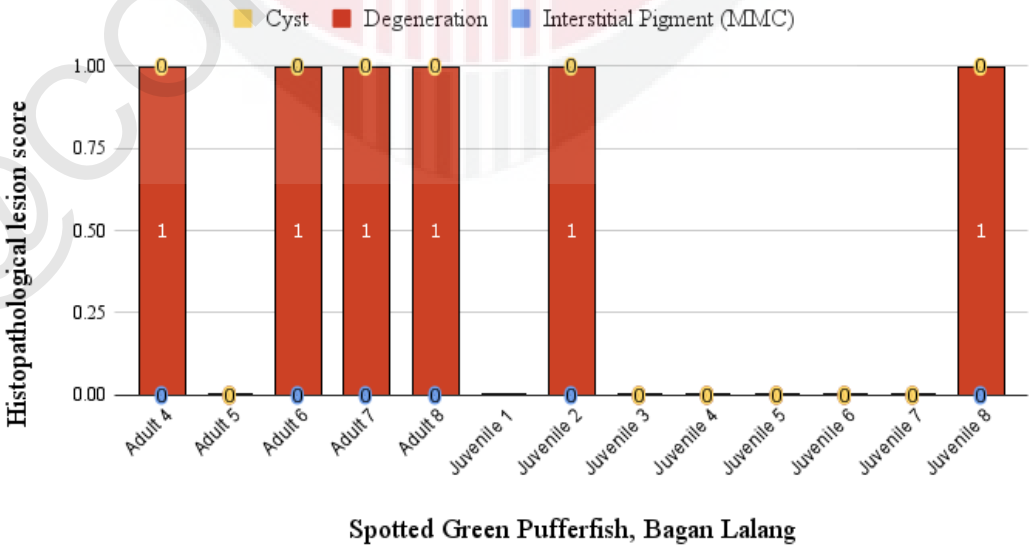

Figure 9: Heart Histopathological Changes Scoring of *Dichotomyctere nigroviridis*, Bagan Lalang

Histopathological changes in the heart of each fish were documented and are presented in Table 13. According to the scoring results for heart histopathological changes (Figure 9), only the degeneration of myocytes was observed in both adult and juvenile fish, with a mild (1) score and the remaining showed none (0) score. In adults, this was observed in 50%, while in juveniles, it was observed in only 25%. The presence of cysts and MMC in both adult and juvenile fish scored none (0).

Table 15: Histopathological changes scoring for gonad of the sampled spotted green pufferfish

Histopathological Changes	Adult								Juvenile							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Presence of Cyst	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Congestion	0	1	1	1	1	1	0	1	0	1	1	0	0	0	0	0
Cellular Degeneration	1	1	1	0	0	1	0	0	1	1	0	0	0	0	0	1
Total	1	2	2	1	0	2	0	1	1	2	1	0	0	0	0	1

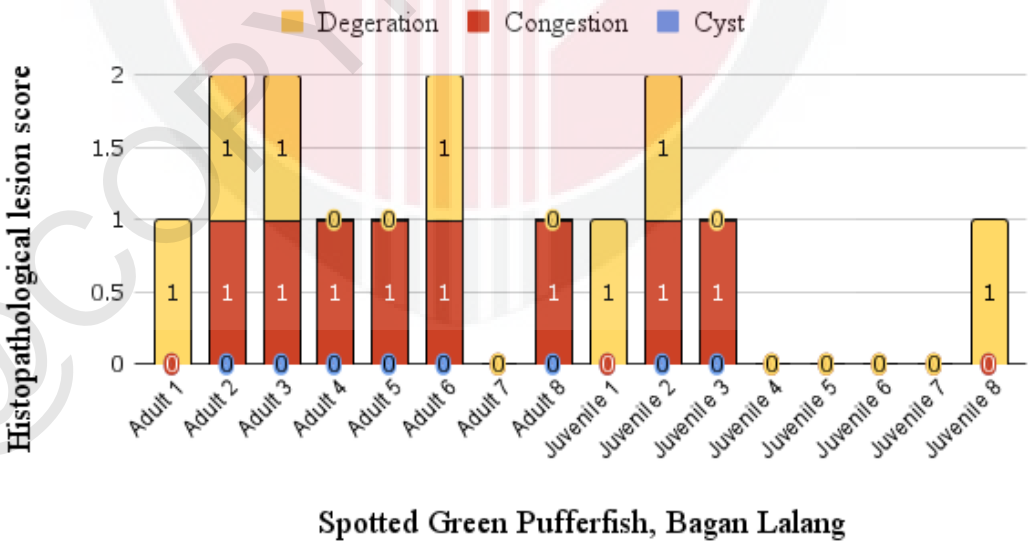

Figure 10: Gonad histopathological changes scoring of *Dichotomyctere nigroviridis*, Bagan Lalang

Histopathological changes in the gonad of each fish were documented and are presented in Table 14. According to the scoring results for gonad histopathological changes (Figure 10), mild (1) degeneration of the tissue was observed in 50% of adults and 37.5% of juveniles, while others showed none (0). Regarding congestion, 75% of adults and 25% of juveniles scored mild (1), with others showing none (0). Lastly, the presence of cysts in both adult and juvenile fish scored none (0).

4.4 Parasite Species Identifications

Aside from confirmation of the *Dichotomyctere nigroviridis* via sequencing myxozoan samples from the species *Triactinomyxon type sp.* & *Ortholinea auratae* was confirmed through the nested PCR. The PCR products yields and amplification of >1000 base pairs fragments which were possible for the *Sphaerospora sp.* (AY735410) 1349bp or *Sphaerospora renicola* (AY735411) 1413bp for primer SphF and SphR while for primer 18e and 18d PCR products yields and amplification of >500 base pairs fragments which may indicate *Henneguya shaharini* with 1033bp as suggested by Székely et al., 2009b. The accession no. was KF263540.1 for Triactinomyxon type sp. with the percentage of identity is 96.08 %, 93.12%, and 96.27% identical to the one found in the University of Munich, Germany (Hallett et al., 2004). While the accession no. was KR025869.1 for *Ortholinea auratae* with percentage of identity is 88.4%, identical to the one found in the fish farm in Algarve, Portugal by Rangel et al. (2014)

4.5 Water Quality Analysis

The water quality analysis shows that all parameters as listed below (Table 5) for the seawater at Bagan Lalang were within the normal range with slight low salinity showing the brackish condition of the estuary at the sampling location.

Table 16: Water quality analysis of Bagan Lalang, Selangor

Parameter	Sampling site at Bagan Lalang, Selangor
Temperature	28.8℃
pH	7.35
Ammonia (NH3-N)	3.34 mg/L
Ammonium (NH4-N)	224.35 mg/L
Salinity	25.13 ppt
Dissolve Oxygen (DO)	4.42 mg/L

5. DISCUSSION

5.1 Criteria for Categorization of Spotted Green Pufferfish

The categorization of spotted green pufferfish into juveniles and adults was determined by observing the underdeveloped gonad (ovary) in the heaviest pufferfish. Specifically, an underdeveloped gonad (ovary) was noted in a 44g female pufferfish, serving as the baseline weight. Pufferfish, whether male or female, falling below this baseline weight were consequently categorized into the juvenile stage group. This approach provided a practical and observable criterion for distinguishing between juvenile and adult stages based on gonadal development, particularly in relation to the noted underdevelopment in the 44g female pufferfish. This method was derived from the assumption that animals, in general, attain sexual maturity (adult stage) upon reaching 65% of their total body weight (M.P. Davis & R.P. Wettemann, 2009). It's noteworthy that literature on this specific issue is scarce, underlining the need for practical criteria such as observation of gonadal development in our categorization approach.

5.2 Parasite Prevalence

The observed 100% prevalence of myxozoan parasites in the kidneys of all 16 fish subjects presents a noteworthy and unprecedented result. This finding underscores the significance of myxozoans in this specific population and prompts further investigation into the ecological dynamics and potential implications for fish health. Contrary to some prior studies suggesting varying prevalence rates of myxozoans and other parasites in other fish populations, our results indicate a unique scenario with

100% prevalence in kidney tissues. While literature may present contrasting findings, the variations could be attributed to differences in geographic locations, host species, or methodological approaches such as smaller sample size.

The selection of 16 subjects as our sample size is a conscious choice, supported by the reasoned approach advocated by Jovani and Tella (2006) where the sample size of 15 proved to be a rational and acceptable compromise, striking a balance that prevented excessive data loss in analyses while maintaining a satisfactory level of uncertainty. On the contrary, Shvydka et al. (2018) demonstrated that for parasite data showing a highly aggregated pattern, having a sample size of 80 or more host individuals is crucial for accurate and precise mean abundance estimates. When the host sample size is between 25 and 40 individuals, median estimates are minimally biased, but the distribution tends to skew toward lower values. Thus, a larger sample size is essential for reliable estimations in datasets with highly aggregated parasite patterns. In the current study, small sample size was chosen due to limitations in time, funding, and resources, especially considering the spotted green pufferfish's wildlife status, the precise role of the fish in the local ecosystem was not fully understood, raising concerns about potential adverse effects of removing a large number of the spotted green pufferfish. Additionally, catching the fish was challenging due to its limited availability, specific time window, and the need for a high level of angler expertise.

Santhanam (2017) reported several endoparasite occurrences in pufferfish worldwide including trematode and myxozoan group which contradict with this current study findings of 0% trematode occurrence. In concurrence with this current

study findings, El-Matbouli and Hoffmann (1994) observed a 100% prevalence of Sinuolinea tetraodoni in lumen of kidney tubules and renal corpuscles of Tetraodon palembangensis, a species widespread throughout Southeast Asia. It's noteworthy that while their findings pertained to a freshwater context the current study findings observed the occurrence in a saltwater to brackish water environment. Additionally, the absence of trematode parasites, as well as the of other endoparasites like nematodes, acanthocephalans, and blood protozoans that were reported in other literature such as (Scheifler et al., 2019b) and (Arthur & Shariff, 2015) may be attributed to potential contamination from waste, possibly containing antimicrobial drugs or heavy metals in which these contaminants could cause antiparasitic effects. Additionally, the presence of tetrodotoxin in the tissue of spotted green pufferfish may contribute to the observed lack of parasites, given its known deterrent and parasiticidal effects on various parasites as reported by Calhoun et al. (2017). In conclusion, our findings shed light on a high prevalence of myxozoans in the kidneys of our fish population. While this result is unique to our study, it contributes to the broader conversation on myxozoan ecology and the intricate relationships between parasites and their hosts.

5.3 Histopathological Scoring

The presence of the myxozoan parasite in the lumen of renal tubules, and renal corpuscles shows an intriguing observation, particularly considering the minimal histopathological lesions related to tubular epithelial degeneration but vice versa in interstitial pigment melanomacrophage (MMC) deposition as occurred in the kidney (figure 2) in this study. Remarkably, only one juvenile spotted green pufferfish

exhibited severe (score 3) tubular epithelial degeneration, while two other juveniles showed mild (score 1) degeneration, and the rest exhibited none (score 0). The observed minimal tubular epithelial degeneration/necrosis in the kidney stands contradictory to the results reported by Maftuch et al. (2018) where investigation on Myxobolus sp. infected Koi carp reported significant kidney cell destruction and necrosis.

On the other hand, a notable and opposing pattern concerning interstitial pigment MMC deposition. Where severe (score 3) interstitial pigment MMC deposition evident in 75% of all individuals, while the remaining 25%, exclusively juveniles, displayed a moderate (score 2) deposition. This underscores the unknown and intricate relationship between the presence of the myxozoan in the lumen of renal tubules, and renal corpuscle with histopathological lesion tubular epithelial degeneration, and interstitial pigment MMC deposition. Statistically, in current study, the histopathological lesion scoring revealed a notable distinction between juveniles and adults, with statistical significance confirmed through Fisher's exact test. This observed difference suggests age-related variations in susceptibility or response towards endoparasite infestation. Interestingly, this finding was supported but in contradicting pattern to the conclusions drawn by Akinsanya et al., (2007), whose research suggests that juvenile specimens are more prone to endoparasite infections compared to their adult counterparts.

Interestingly, when examining gender-related differences, results of the analysis did not yield statistically significant findings between males and females. This implies that, in contrast to age-based distinctions, the histopathological characteristics appear

comparable across genders within the studied population. These non-significant in gender-based differences of endoparasite burden was in agreement with Tadiri et al. (2016) who reported although the sex of the host plays a role in the dynamics of isolated fish, male and female fish show similar levels of parasite infestation. On a different page, findings by Karvonen and Lindström (2018) reported endoparasite infestation was higher in females compared to in males. These findings underscore the importance of considering both age and gender factors when evaluating histopathological variations in the Spotted Green Pufferfish.

5.4 Molecular Identification of Myxozoan Parasite

From histology slides the presence of the myxozoan parasites was concluded with limited knowledge of the species infesting lumen of renal tubules, and renal corpuscles of the spotted green puffer fish thus, Sphaerospora renicola, or Sinuolinea tetraodoni could be the possible species who have been reported by Molnár (2007) and El-Matbouli and Hoffmann (1994) respectively in which evident in the infesting lumen of renal tubules, and renal corpuscles. Particularly, Sinuolinea tetraodoni myxozoan demonstrate having few similarities such as having 100% prevalence in pufferfish Tetraodontidae family and distribution across Southeast Asia. However, both species were only reported in freshwater hosts from freshwater environments unlike the current subject from brackish to saltwater settings.

From the conventional and nested PCR alone the gel electrophoresis result came out inconclusive due to a lack of positive control thus PCR sequencing was needed. From the PCR sequencing result, it was determined that the myxozoa obtained was of

the Triactinomyxon type sp. and Ortholinea auratae species. The PCR sequencing results revealed the highest percentage of identity to Triactinomyxon type sp. myxozoan, a finding that raises both confusion and intrigue. This confusion was because the Triactinomyxon type has only been reported in samples of oligochaetes, and there is no evidence of contamination or the presence of oligochaetes in our study. For instance, Székely et al. (2014) reported Triactinomyxon actinospore released from the oligochaetes host instead of the fish host. Therefore, a comprehensive investigation is crucial to clarify the precise myxozoan species in the present study. It is possible that similarly to what was reported by Székely et al. (2014) the Triactinomyxon actinospore such as the Triactinomyxon type 1, Triactinomyxon type 2, Triactinomyxon type 3 proved to be identical to Myxobolus fundamentalis, 99.9 %. This suggests, the identified species in this current study may fall under the category of myxobolus group myxozoan, necessitating a more detailed exploration to ascertain its accurate classification.

6. CONCLUSION

In conclusion, the study mentioned above found that there is the occurrence of myxozoan endoparasite infection in the kidney of the juvenile and adult *Dichotomyctere nigroviridis* with 100% prevalence. Besides, endoparasite infection will cause significant histopathological changes in the abdominal cavity, and visceral organs between the juvenile and adult *Dichotomyctere nigroviridis* but regardless of the gender. The presence of endoparasite *Triactinomyxon type sp.* and *Ortholinea auratae* myxozoan in the fish raises concerns about potential zoonotic transmission, prompting questions regarding public health implications.

7. RECOMMENDATION

To enhance the robustness of future studies, several improvements and recommendations can be considered. Firstly, extending the data collection period to cover both rainy and dry seasons is essential for a comprehensive understanding of parasite invasion dynamics. Additionally, diversifying sampling locations across various sites or states would provide a more detailed view of parasite prevalence variations. Increasing sample sizes is crucial for minimizing statistical errors and ensuring more representative findings. Selecting optimal sampling dates requires consideration of factors such as weather forecasts, tide schedules that influence fish availability, moon phases affecting fish behaviour, fishing techniques, and specific sampling locations. Collaborating with local fishermen with valuable insights, can greatly contribute to effective study planning. For processing improvements, prompt sample processing upon collection is advisable. Adequate storage and clear labelling are essential for maintaining sample integrity. In molecular work, meticulous planning of DNA extraction procedures is crucial. Collaboration with laboratory technicians is recommended, considering that certain steps may need continuous execution and machine settings can vary.

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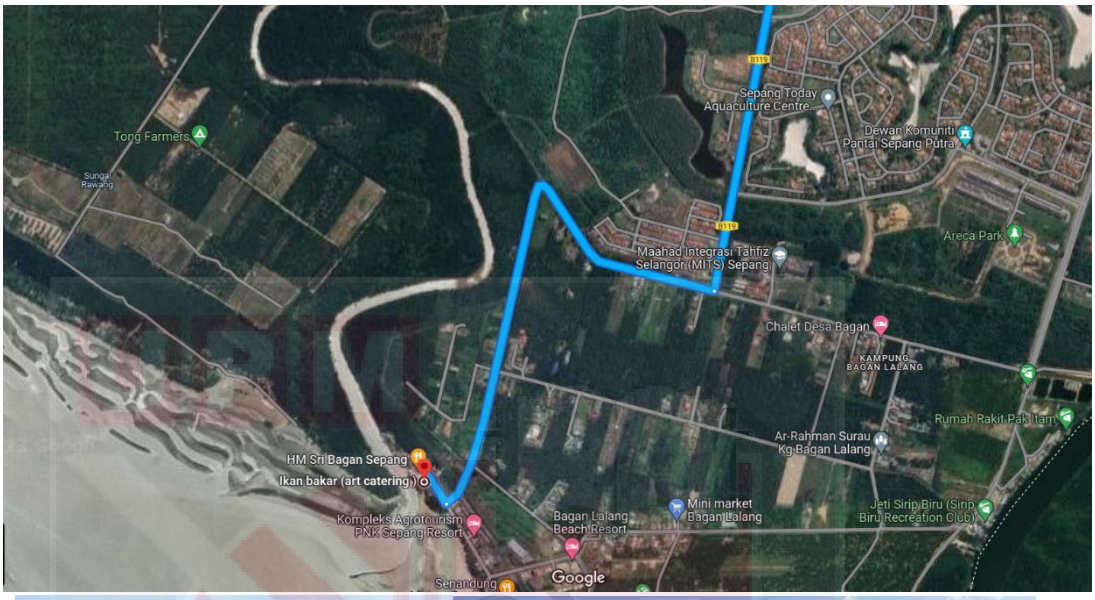
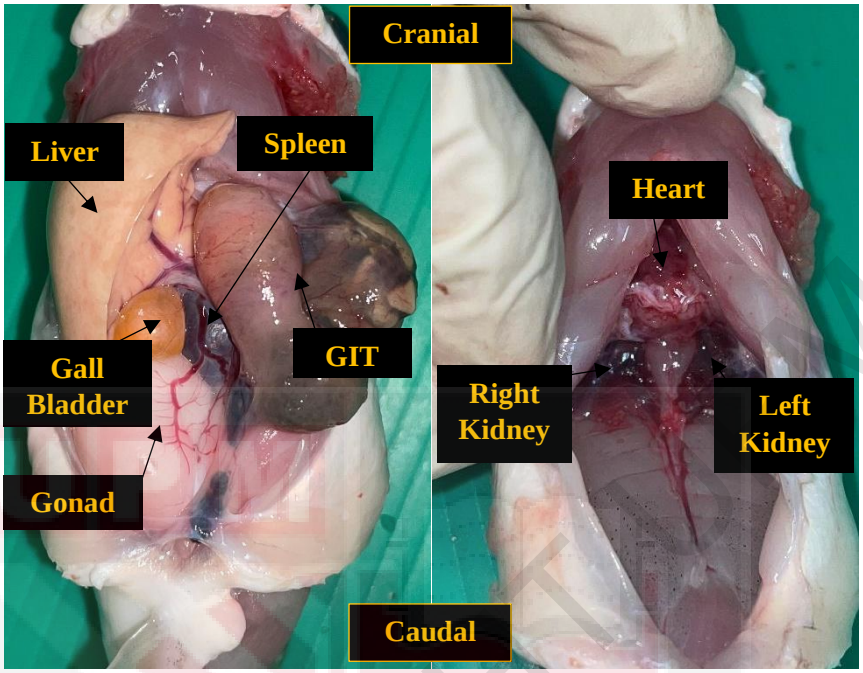
9. APPENDIX



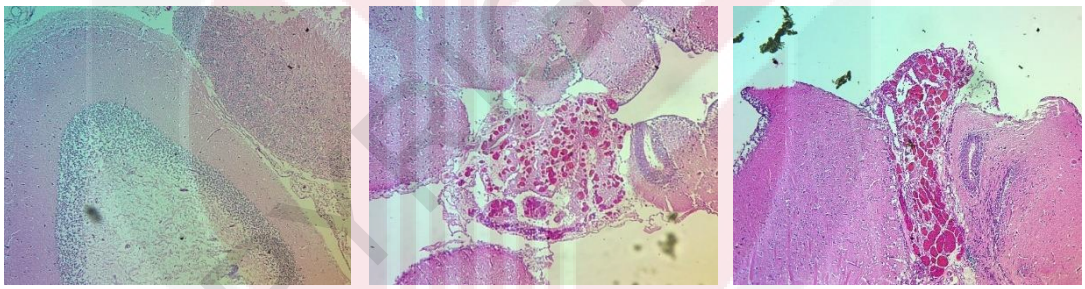
Appendix 1: Site of sample collection at the estuary in Bagan Lalang with coordinate 2°36'35.3"N 101°41'09.9"E

Appendix 2: Sampling team with fishing rod setup

Appendix 3: Each morphometric measurement of green spotted pufferfish



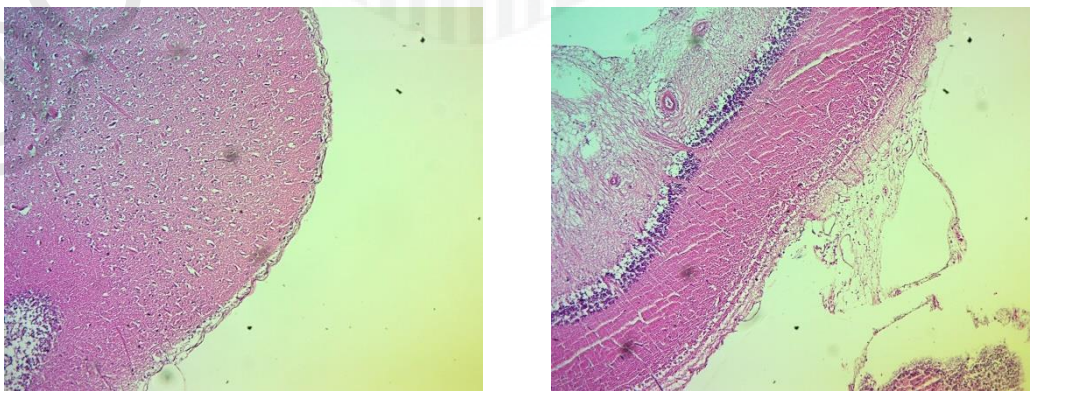
Appendix 4: Pufferfish Internal Organs



P9 Brain 10X Congestion score (0)

P10 Brain 12X Congestion score (1)

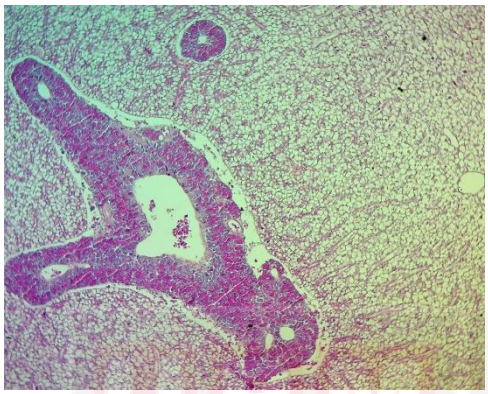
P8 Brain 10X Congestion score (2)



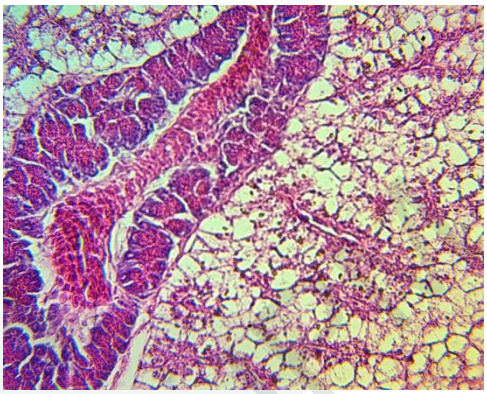
P10 Brain X10 Inflammation of Meninges Score 0

P12 Brain X10 Inflammation of Meninges Score 1

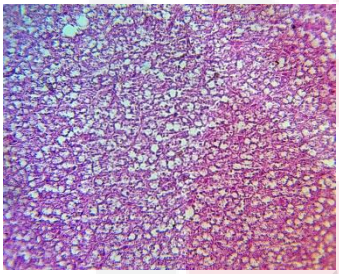
Appendix 5: Brain histopathology: Congestion, Inflammation of Meninges



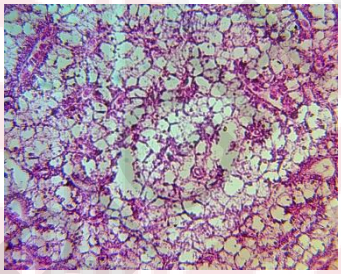
P15 Liver x10 Congestion Score 0



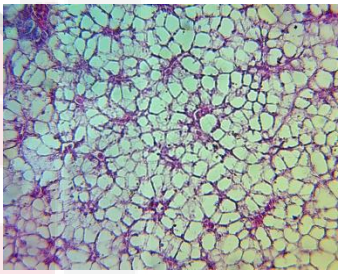
P3 Liver x10 Congestion Score 1



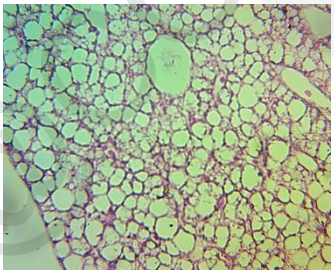
P11 Liver 40X Hepatocyte Glycogen Accumulation Score 0, Lipid Accumulation Score 0



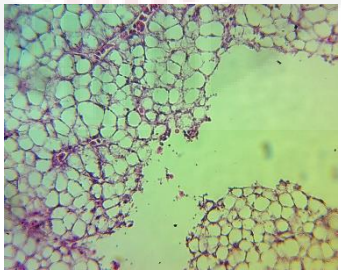
P10 Liver 40X Hepatocyte Glycogen Accumulation Score 1, Lipid Accumulation Score 1



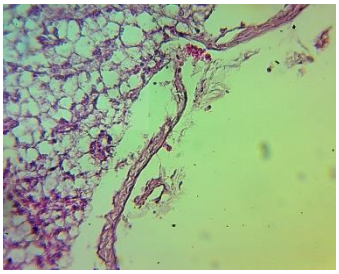
P12 Liver 40X Hepatocyte Glycogen Accumulation Score 2, Lipid Accumulation Score 2



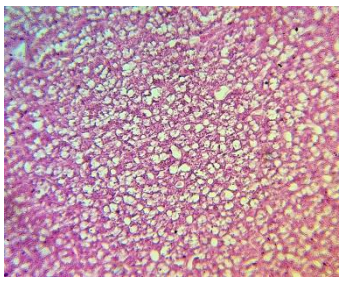
P1 Liver x40 Hepatocyte Degeneration Score 0



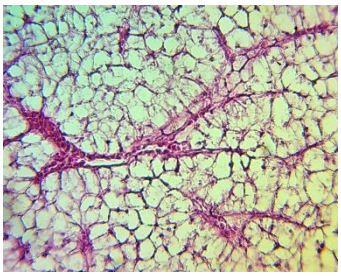
P6 Liver x40 Hepatocyte Degeneration Score 1



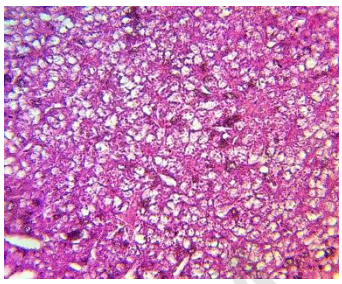
P4 Liver x40 Hepatocyte Degeneration Score 2



P11 Liver x40 Monocellular Cell Infiltration Score 0

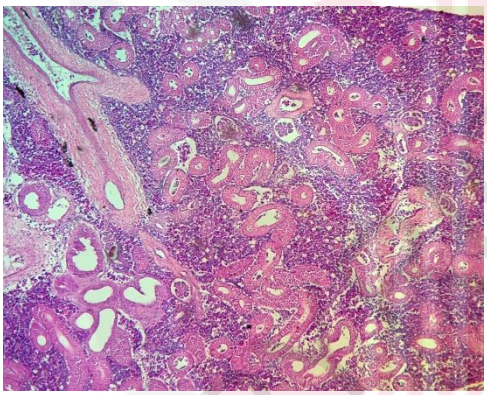


P8 Liver x40 Monocellular Cell Infiltration Score 1

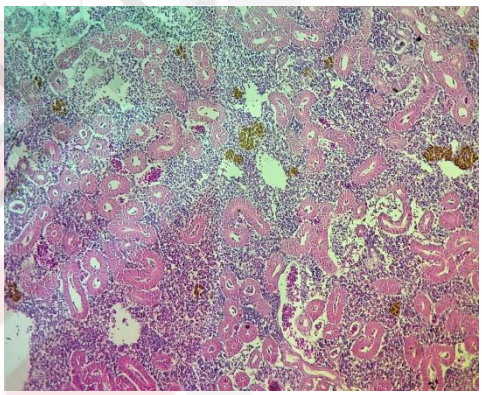


P7 Liver x40 Monocellular Cell Infiltration Score 2

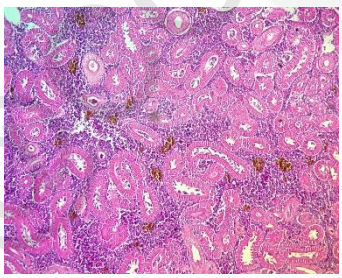
Appendix 4: Liver histopathology: Congestion, Hepatocyte Glycogen Accumulation, Lipid Accumulation, Hepatocyte Degeneration, Necrosis, or Apoptosis, Mononuclear Cell Infiltration



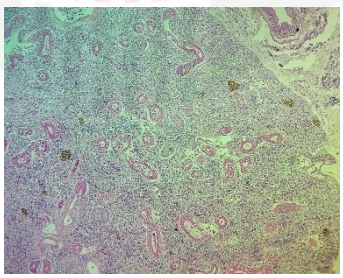
P11 Kidney X10 Congestion Score 0



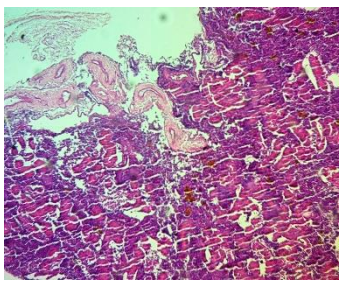
P2 Kidney X10 Congestion Score 1



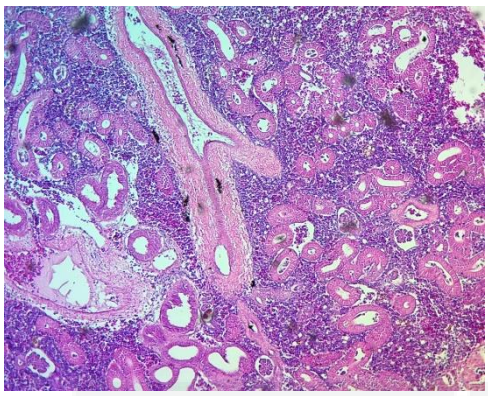
P1 Kidney X10 Tubular Epithelial Degeneration Score 0



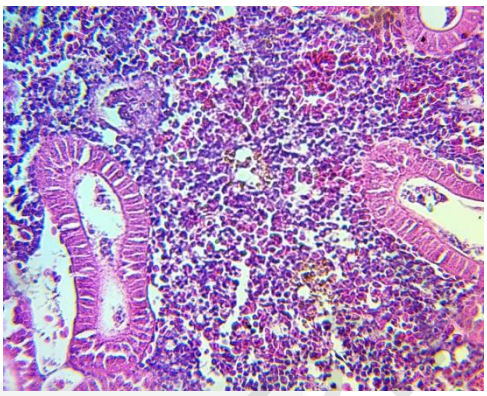
P5 Kidney X10 Tubular Epithelial Degeneration Score 1



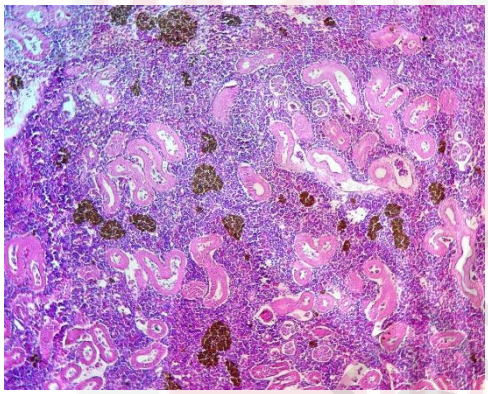
P4 Kidney X10 Tubular Epithelial Degeneration Score 3



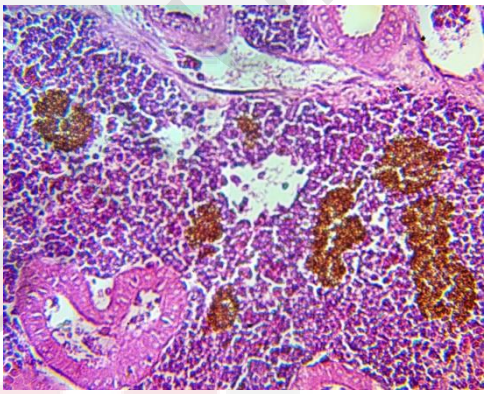
P11 Kidney X10 MMC Score 2



P11 Kidney X40 MMC Score 2

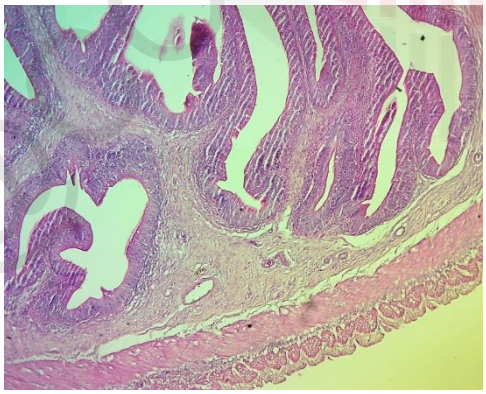


P7 Kidney X10 MMC Score 3

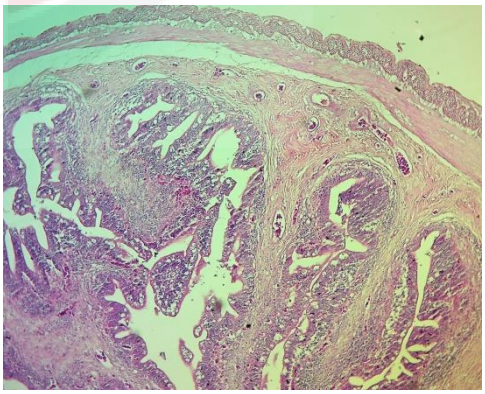


P7 Kidney X40 MMC Score 3

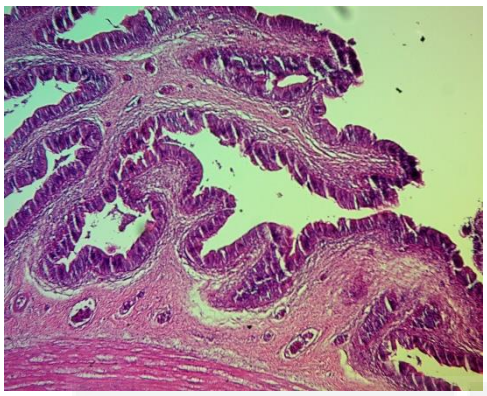
Appendix 5: Kidney histopathology: Congestion, Tubular Epithelial Degeneration, MMC



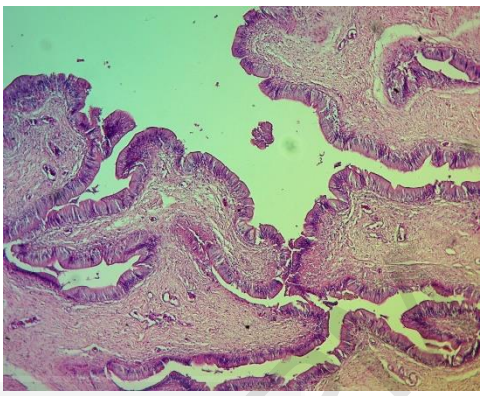
P13 GIT X10 Lymphocyte Infiltration Score 0, Congestion Score 0



P15 GIT X10 Lymphocyte Infiltration Score 1, Congestion Score 1

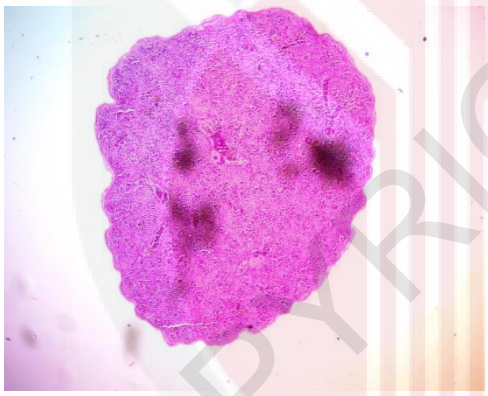


P8 GIT X10 Lymphocyte Infiltration Score 1, Congestion Score 2

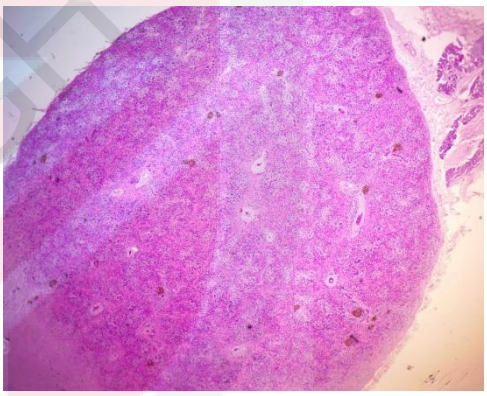


P2 GIT X10 Lymphocyte Infiltration Score 2, Congestion Score 1

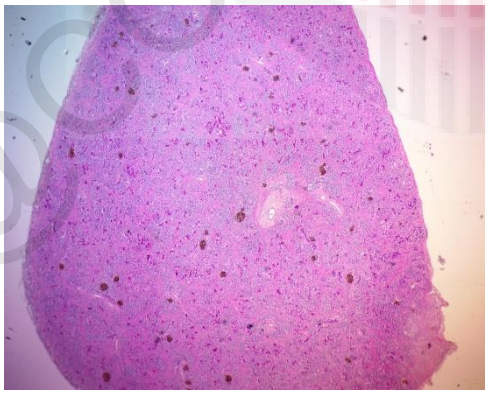
Appendix 6: Intestine histopathology: Infiltration of Lymphocytes, Congestion



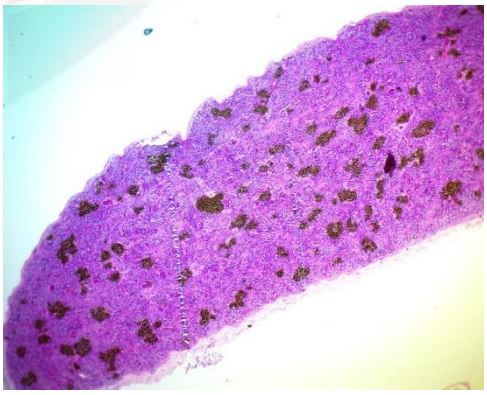
Spleen 10X MMC Score 0



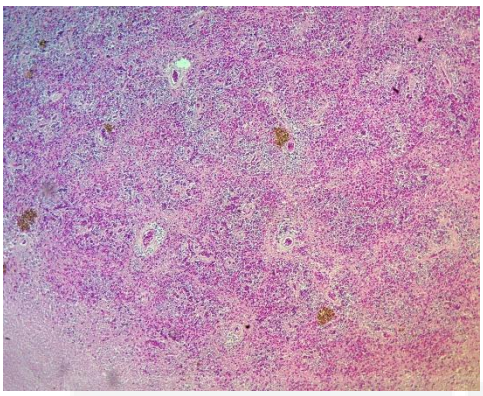
Spleen 10X MMC Score 1



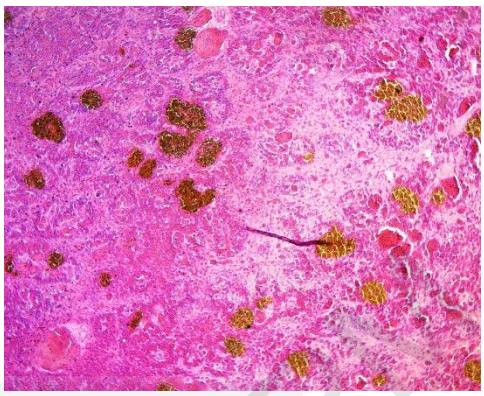
Spleen 10X MMC Score 2



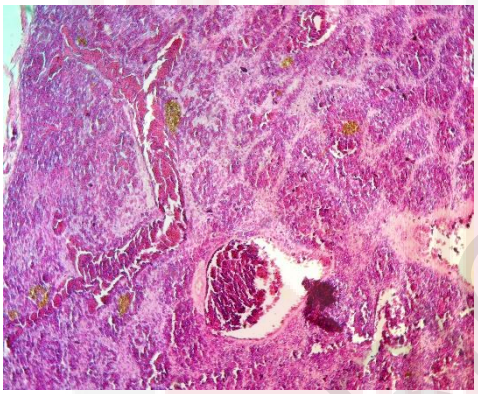
Spleen 10X MMC Score 3



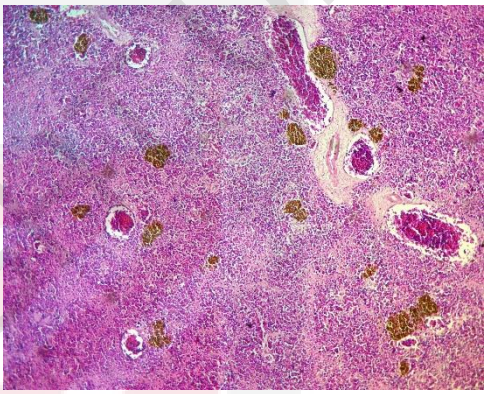
P5 Spleen X10 Congestion Score 0



P3 Spleen X10 Congestion Score 1

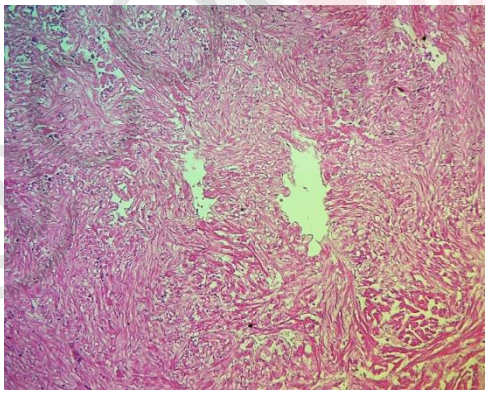


P1 Spleen X10 Congestion Score 2

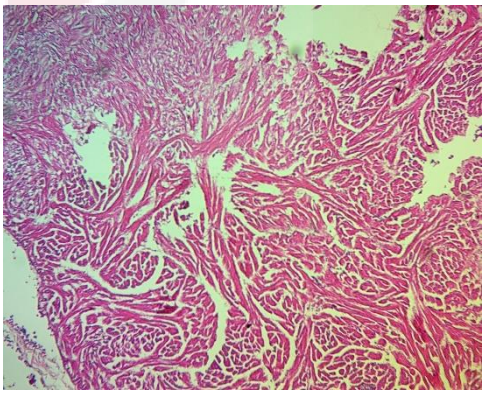


P2 Spleen X10 Congestion Score 3

Appendix 7: Spleen histopathology: mmc, congestion

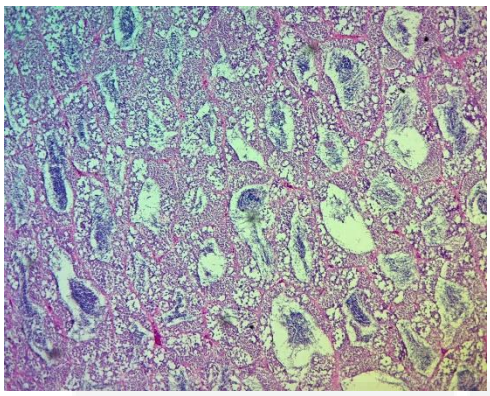


P6 Heart 10X Degeneration Score 0

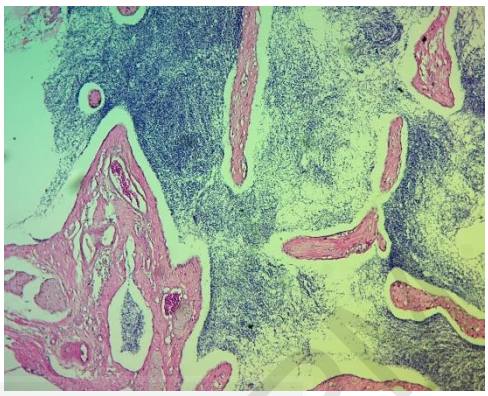


P12 Heart X10 Degeneration Score 1

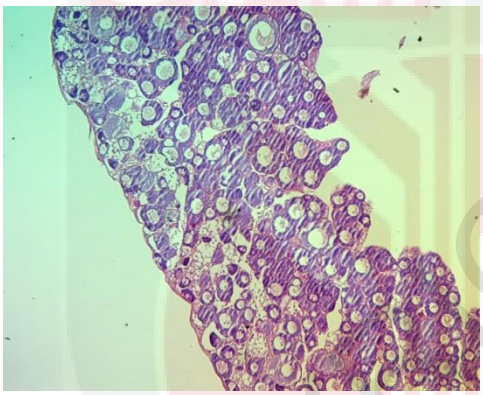
Appendix 8: Heart histopathology: degeneration



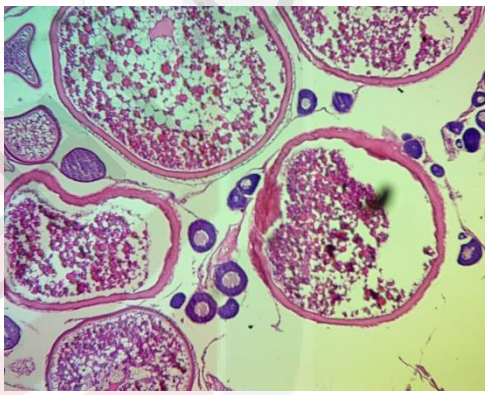
P6 (Young Male) Gonad X10 Congestion Score 0, Degeneration Score 0



P2 (Adult Male) Gonad X10 Congestion Score 1, Degeneration Score 1

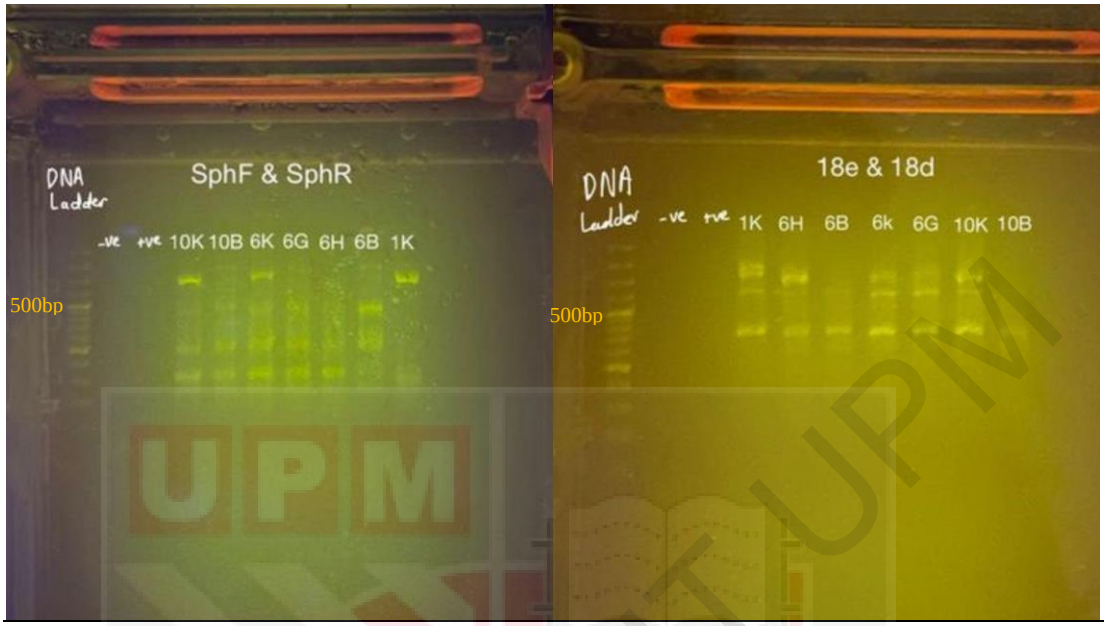


P13 (Young Female) Gonad X10 Congestion Score 0, Degeneration Score 0



P7 (Adult Female) Gonad X10 Congestion Score 1, Degeneration Score 1

Appendix 9: Gonad histopathology: degeneration, congestion



10K	10B	6K	6B	6G	6H	1K
P10 Kidney Sample	P10 Brain Sample	P6 Kidney Sample	P6 Brain Sample	P6 Gill Sample	P6 Heart Sample	P1 Kidney Sample

Appendix 10: PCR result (Conventional)

Sample code	Primer	Sequencing	Accession no.	Species (Top hit accession)	Organism common name	Strain (If applicable)	% Percentage of identity (Top hit accession)
16K	18e	18s rDNA sequencing	CR703298.2	Tetraodon nigroviridis	Spotted green puffer fish		99.54
16K	18g	18s rDNA sequencing	CR703696.2	Tetraodon nigroviridis	Spotted green puffer fish		98.09
10K	18e	18s rDNA sequencing	CR681937.2	Tetraodon nigroviridis	Spotted green puffer fish		99.73
10K	18g	18s rDNA sequencing	CR703696.2	Tetraodon nigroviridis	Spotted green puffer fish		96.98
6K	SphF	18s rDNA sequencing	KR025869.1	Ortholinea auratae	Myxozoa: Ortholineidae		88.4
6K	SphR	18s rDNA sequencing	KF263540.1	Triactinomyxon type sp.	Myxozoan	TGR-2014	96.08
1K	SphF	18s rDNA sequencing	KF263540.1	Triactinomyxon type sp.	Myxozoan	TGR-2014	93.12
1K	SphR	18s rDNA sequencing	KF263540.1	Triactinomyxon type sp.	Myxozoan	TGR-2014	96.27

Appendix 11: **PCR result (sequencing)**