Faculty of Resource Science and Technology

BIOLOGY, GUT CONTENT, AND TOXICITY STUDIES OF SPOTTED-GREEN PUFFER FISH COLLECTED FROM SAMPADI, KUCHING

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Bachelor of Science with Honours
(Aquatic Resource Science and Management Programme)
2010

Biology, Gut content, and Toxicity Studies of Spotted- Green Puffer fish (*T. nigroviridis*) collected from Sampadi, Kuching.

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This dissertation is submitted in partial fulfillment of the requirement for the degree of Bachelor Science with Honours in Aquatic Resource Science and Management

Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
2010

DECLARATION

No portion of the work referred to in this dissertation has been submitted in support of an application for another degree qualification of this any other university or institution of higher learning.

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LIST OF ABBREVIATIONS

AR	Anal rays
BW	Body weight
cm	centimeter
DR	Dorsal rays
g	gram
GPS	Global positioning system
GSI	Gonad somatic index
GW	Gonad weight
HL	Head length
LSI	Liver somatic index
LW	Liver weight
MU	Mouse unit
MW	Muscle weight
NKA	Na^{+}/K^{+}-ATPase
PR	Pectoral rays
SL	Standard length
SnL	Snout length
TLC	Thin Layer Chromatography
TL	Total length
TTX	Tetrodotoxin

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ACKNOWLEDGEMENTS

Without the help and support of a number of people it would not have been possible to prepare this thesis. Firstly, I would like to thank the GOD for giving me good health conditions and blessings to complete my project. I would like to express my appreciation to my beloved parents, Mr. Muthukrishnan a/l Thiruvengadam and Mrs. Kokilavani a/p Paramanandam, who had support me with their love.

I would like to express my utmost heartfelt gratitude to my supervisor, Dr. Samsur Mohamad for his valuable advice, guidance, and encouragement to complete this study. He spent hours helping me and my friends in conducting mouse bioassay. The experiment would not have been possible without the guidance of Dr. Samsur Mohamad.

I would also like to thank Dr. Ruhana Hassan (mentor), Dr. Lim Po Teen, Associate Professor Dr Lee Nyanti (examiner) and Dr. Zainab Ngaini for their assistants and guidance. Dr. Zainab Ngaini has taught me to do thin layer chromatography. Without her help, it would be impossible for me to conduct thin layer chromatography.

My sincere thanks also go to Mr. Zaidi bin Ibrahim, Mr. Nazri bin Latip, Mr. Mohamad Norazlan Bujang Belly, and Mr. Zulkifli bin Ahmad who contribute to this thesis. They helped me out in lab section and sample collection which is very important in my research.

Special thanks to the aunties who does the cleaning in Faculty of Science and Technology and Alamanda College for their information on green-spotted puffer fish. They had provided me with valuable information to proceed with the project successfully. Even though, they do not know about the toxicity in terms of science, they know which part of the fish is more toxic through their cultural believe and how to discard that particular part when cooking. This is important information for me as I am doing the project on toxicity.

Last but not least, I would like to thank aquatic science lectures, master students, course mates, and friend who contributed to this research, specially the anonymous respondents who took the time to talk to me about the spotted-green puffer fish.

The Biology, Gut content and Toxicity Studies of spotted-green puffer fish collected from Sampadi, Kuching

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ABSTRACT

The biology and toxicity of 104 samples were analyzed and 10 samples were used for gut content analysis. Samples of 104 fish with total lengths ranging from 9.90 cm to 17.30 cm were collected from Sampadi, Kuching using three layer gill nets in December, 2010. The physical appearances such as spots characteristic and colour of the body were observed by naked eyes and the numbers of dorsal, anal, and pectoral fin rays were counted using dissecting microscope. From the results, the standard length and body weight ranged from 7.60 cm to 13.00 cm and 40.66 g to 177.18 g respectively and shows significant difference in size between male and female. *T. nigroviridis* is omnivores which prey on invertebrates and leafs such as copepod, crustacean, gastropods, polychaete, small crabs and leafs. The gut content analysis supports the exogenous hypothesis in TTX accumulation through the diet. The anatomical distribution of toxicity level was tested by using mouse bioassays. Skins shows the higher toxicity score (155.80 ± 76.17) MU/g followed by eggs, gonad, liver, and muscles. Thin layer chromatography (TLC) was carried under two solvent systems. The R_f value are 0.77 and 0.22 respectively showing the existence of TTX in *T. nigroviridis*.

Keywords: *T. nigroviridis*, gut content, mouse bioassay, thin layer chromatography

ABSTRAK

Biologi dan toksin untuk 104 sampel ikan buntal hijau dianalisis dan 10 sampel ikan digunakan untuk analisis kajian pemakannan. Satu ratus empat sampel ikan buntal dari Sampadi, Kuching dengan jumlah panjang berjulat 9.90 cm hingga 17.30 cm telah ditangkap dengan menggunakan pukat insang yang berlapis tiga pada bulan Disember 2010. Morfologi fizikal ikan buntal seperti bintik badan, warna badan, dan bilangan jejari pada sirip dorsal, dubur dan pektoral turut dikira dengan menggunakan mikroskop pembedahan. Keputusan menunjukkan panjang piawai dan berat badan setiap individu adalah berjulat 7.60 cm hingga 13.00 cm dan 40.66 g hingga 177.18 g dimana terdapat variasi berat di antara ikan jantan dan ikan betina. T. nigroviridis merupakan ikan dalam kelas omnivore iaitu memakan invertebrate kecil dan daun kecil antaranya copepoda, crustacea, gastropoda, polychaeta, ketam kecil, dan ranting pokok. Kajian kandungan pemakanan menyokong hipotesis exogenus dimana kandungan racun ikan berasal dari pemakanan. Kajian terhadap tahap ketoksikan mengikut organ telah dijalankan dengan menggunakan bioesei tikus. Tisu kulit, menunjukkan jumlah ketoksikan yang paling tinggi iaitu (155.80 ± 76.17) MU/g dan diikuti oleh telur, gonad, hati, dan tisu badan. kromatografi lapisan nipis dijalankan dalam dua system pelarut. Nilai R_f untuk kedua-dua system tersebut ialah 0.77 dan 0.22 menunjukkan kehadiran TTX dalam T. nigroviridis.

Kata kunci: T. nigroviridis, kandungan pemakanan, bioessei tikus, kromatografi lapisan nipis

1.0 INTRODUCTION

Generally, puffer fish from family tetradontidae contains tetrodotoxin (TTX) which intoxification in humans (Noguchi *et al*., 2006). TTX have the molecular formulae of $C_{11}H_{17}N_3O_8$. TTX is extremely potent low molecular weight neurotoxin.TTX was originally believed to be a true ichthyosarcotoxin produced by the fish itself. TTX can also be found in fish, amphibians, arthropods, nematodes, echinoderms, mollusks, dinoflagellate, and bacteria. Some of the scientist reported that TTX is produced by bacteria. However, the origin of TTX has been a controversial subject.

TTX is toxic because it inhibits the flow of sodium ion in and out of the cells by blocking the sodium channels on the cell membranes which cause paralysis of the muscles concerned. Finally, it will lead to the respiratory failure which causes death (Noguchi and Akaeda, 1998). Puffer fish is one of the food source and most of the Japanese people consume it. Hundred annual deaths were recorded in the year of 1960 due to ingestion of puffer fish. This fish is also consumed by the local people in Sarawak.

T. nigroviridis is one of the toxin puffer fish which did not consume by human due to high toxicity of TTX in their muscle, skin, gonad, and liver. According to Mahmud *et al*. (1999a), the highest toxicity value of *T. nigroviridis* is in their skin followed by muscle, liver, and intestine. However, it is reported that the toxicity is different depending o the location, season, and food abundance. It is because TTX toxicity is depending on the metabolic rate of *T. nigroviridis.*

This study is very important to impose quarantine rule and creating public awareness after a series study of toxicity and toxin properties of puffer (Noguchi and Ebesu, 2001). It is believed that different region has different possible environment effect on puffer toxicity level (Hashimoto, 2001). Therefore, the objective of this study is to document the biological and toxicity of *T. nigroviridis* which will be collected from Sampadi by using mouse bioassay, thin layer chromatography, and gut content analysis. Additional to that, this study is also conduct to compare the previous study of *T. nigroviridis* collected from Kampung Bako. This will give us a very precise baseline studies on biological and toxicity of *T. nigroviridis* since there are previous studies which can be used as a comparison.

The objectives of this study were;

1. To document the biological and toxicity of *T. nigroviridis* collected from Sampadi, Kuching.
2. To examine the stomach content of the *T. nigroviridis* by performing gut content analysis.
3. To compare the studies with the previous research to come out with better results and discussion.

2.0 LITERATURE REVIEW

2.1 Spotted-green puffer fish, *Tetraodon nigroviridis*

The order for this species is Tetraodontiformers. In this context, ‘tetra’ means four, ‘odontos ‘means teeth, and ‘formes’ means shape. Therefore, it means this species exhibits four teeth. This is an unusual group of fishes famous for their ability to inflate their body by swallowing air or water. Their teeth are very strong which area fused and resembling a parrot’s beak with powerful jaws.

Tetraodon nigroviridis has the smallest known number vertebrate genome (Hinegardner and Rosen, 1972). Analysis of the *Tetraodon* and human genomes shows that whole-genome duplication occurred in the teleost fish heredity, consequent to its difference from mammals. This species is used in comparative analysis to clarify the human genome and vertebrate genome in general. *Tetraodon* sequences had a significant role in providing a reliable estimate of the number of genes in the human genome (Jailon *et al.,* 2004). This species have highly compact genome. Therefore, it has become an important animal model in genomic research (Grutzner, 1999).

T. nigroviridis, the spotted-green puffer is also known as ‘Buntal Hijau’ or ‘Buntal Bako’ (Atack, 2006). *T. nigroviridis* has black spots which surrounded by yellow rings on the body. According to Inger and Chin (1990), this species have dorsal fin with less than 20 rays. The body is olive green in colour at the above part and yellow or whitish at the below part of the body. The caudal fin has faint dusky spots. The shape of caudal fin is in

truncate shape. The karyotype of *T. nigroviridis* consists of 2n which is equal to 42 biarmed chromosomes.

It is quite tough to identify between the *T. nigroviridis* and *T. fluviatilis* because they have almost similar morphology and are confusing. Since, *T. nigroviridis* and *T. fluviatilis* appear to be very closely related, species definition based on morphological approach alone is not sufficient (Chang *et al.,* 1997).

2.2 Distribution of Spotted-green puffer fish, *Tetraodon nigroviridis*

The toxicity of the Japanese marine puffer fish was reported that 14 of the 21 species examined were toxic. Later, 8 puffer fish species were added to the list of toxic species, and a total of 22 species are presently listed as TTX-bearing marine puffer fish, all belonging to the Tetraodontidae family (Noguchi and Arakawa, 2008).

T. nigroviridis can be found in brackishwater (Mahmud *et al.,* 1999a). The spotted - green puffer fish is a tetraodontid teleost whose native habitats include the rivers and estuaries of South East Asia (Rainboth,1996).The sodium pump is an ATP-dependent enzyme essential for fish osmoregulation (Hwang and Lee, 2007). Spotted green puffer fish, gill Na^+/K^+-ATPase (NKA) activity was at a minimum when the salinity of blood was close to that of the medium (Lin *et al.,* 2004). This study proves that *T. nigroviridis* can be found in brackishwater. *T. nigroviridis* is also known as euryhaline species (Lee and Tang, 2007).

T. nigroviridis is found in brackishwater of Sungai Sarawak (Atack, 2006). *T. nigroviridis* is also found in Sungai Bako (Mohamad and Che Awin, 2008). Addition to that, this species also can be found in Sungai Santubong, Sungai Sarawak, Sungai Sampadi, and Sungai Bako (Zulkernine, 2006). The distribution of *T. nigroviridis* is quite vast in Sarawak.

2.3 Tetrodotoxin (TTX)

TTX is a potent neurotoxin which specifically blocks the voltage-gated sodium channels on the surface of nerve membranes (Mahmud *et al.,* 1999b). TTX is thirteen times more toxic than potassium cyanide (Mohsin and Ambak, 1992). Recent researches have provided strong evidence of the bacteriological origin of TTX. This is because puffer fish grown in culture do not produce TTX until they fed tissues from a toxin producing fish. Other than that, the blue-ringed octopus found in Australian waters accumulates TTX in special salivary glands and infuses its prey with toxin bites. This octopus contains tetrodotoxin–producing bacteria. All this information and research gave strong evidence that TTX originates from bacteria. Therefore, TTX and anhydrotetrodotoxin are synthesized by several bacterial species, including strains of the family Vibrionaceae (Yasumoto *et al.,* 1986).

TTX of a toxic puffer fish is not endogenous but is derived from the food chain itself (Noguchi *et al.,* 2006). Another possibility is that, TTX can be produced by symbiotic or parasitic bacteria that are directly accumulated in the body of the puffer itself where it is not obtained via the food chain (Noguchi and Arakawa, 2008). However, the

amount of TTX produce by the bacteria is too little in puffer fish as compare to the TTX derived from the food chain. Bioconcentration plays an important role in the accumulation of TTX in puffer fish.

Wild puffer fish like to feed on benthic organisms. Starfish, small gastropods, and skeleton shrimp which possess TTX can be found in their stomach through gut content analysis. In china, some puffer fish are reported to consume flatworms. These puffers prefer TTX-bearing organisms as their food which results in accumulating TTX in their body due to defence's mechanisms (Noguchi, 1988). This hypothesis is supported because an experiment was conduct using the non-toxic puffer fish. The non-toxic puffer fish become toxic when it is feed on TTX-containing diets (Noguchi *et al.,* 2006).

2.4 Chemistry of tetrodotoxin (TTX)

The basic molecules of TTX consist of a positively charged Guanidinium group. This group consists of three nitrogen atom which gives the name of class of neurotoxins that is guanidinium toxin. TTX also consists of pyrimidine ring with additional fused ring systems with hydroxyl groups which stabilized the TTX sodium channel binding complex at the aqueous interface. TTX-Na Channels binding site is extremely tight ($K_d = 10^{-10}\mu M$) (Tsuda *et al.,* 1964). The structure of TTX was determined by (Goto and Hirata *et al*., 1965; Tsuda *et al*., 1964; Woodward, 1964). Figure 1, shows the chemical structure of TTX.

In natural, Tetrodotoxin exists as a mixture of its analogues (TTXs) (Shoji *et al.,* 2000). TTX analogues such as 4-epiTTX; 4,9-anhydroTTX; 6-epiTTX; 11-deoxyTTX; 11-oxoTTX; 11-norTTX -6 (R)-ol; 11-norTTX-6(*S*)-ol; chiriquitoxin; 1-hydroxy-5,11-dideoxyTTX; 5,6,11-trideoxyTTX; and 5-deoxyTTX can be found in puffer fish (Shoji *et al.,* 2001).

TTX binding-protein in blood plasma of puffer (Matsui *et al.,* 2000) might participate in accumulation of TTX and its analogues in puffer (Michiko *et al.,* 2008). According to Michiko *et al.* (2008), TTX analogues in puffer depend on the diet of the puffer. The TTX analogues that accumulate in the body of puffer through diet will not transform into chemically more stable compound but remain as it is.

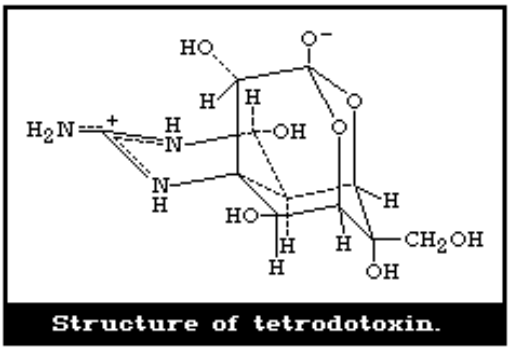

Figure 1: Structure of Tetrodotoxin (adopted from Noguchi and Arakawa, 2008).

2.5 Mechanism of Action on Sodium Channels

By using the intracellular microelectrode techniques, TTX was found to block the action potential without any effect on the resting membrane potential, the resting membrane resistance, and the delayed rectification which is indicative of the activity of potassium channels (Kao, 1966). TTX binds to the Na^+ channel which blocks the exchange of Na^+ ions in and out of the neuron. TTX binds extremely tight in the Na^+ channel because the positively charged nitrogen acts as the Na^+ ion, allowing it to dive into the channel and bind to certain peptides (Narashi, 1960).

2.6 Importance of *Tetraodon nigroviridis* in Research

T. nigroviridis possess of smallest known vertebrate genomes (Jailon *et al.,* 2004). *T. nigroviridis* are used to do genome analysis to improve the fish gene catalogues and help in identifying the key genes that are previously thought to be absents in fish. This species is also used to compare with human genome. This is important in reconstructing the evolutionary history too.

T. nigroviridis is cheap, small, and easily can be obtained. Therefore, most of the researchers prefer to choose this species to do the research on scale- sequencing of complex vertebrates genomes (Crnogorac-Jurcevic *et al*., 1997). Normally, puffer fish is relatively large, expensive, and difficult to rare in laboratory aquarium. In addition, puffer fish is widely consumed in Japan but it is not easily obtained in some part of countries. But, *T. nigroviridis* is much cheaper and available. Hence, *T. nigroviridis* is becoming more and more popular in research.

Moreover, this toxin has been used as a powerful pharmacology tool due to the high affinity and selective blocking of the voltage-gated sodium channels (Shoji *et al.,* 2001).

2.7 Method and Material used in this toxicity studies

2.7.1. Mouse Bioassay

The purpose of Mouse Bioassay is carried out to identify the level of toxicity in a sample of interest according to the organs. The mouse bioassay is performed by intraperitoneal injection (Kawabata, 1978). Toxicity of an extract is expressed in terms of mouse units (MU) (Noguchi *et al.*, 2006). The amount of TTX required to kill a 20g male mouse of ddY strain in 30 minutes is equivalent to one MU. After injection with TTX, the mice show the symptoms such as scratching of the shoulder and mouth, weakness of limbs, uncoordinated movement, and difficulties in breathing and respiratory failure (death).

2.7.2. Thin Layer Chromotography (TLC)

TLC was first referred to in 1938 by two Russian workers, Iz mailov and Shraiber where they called it drop chromatography on horizontal thin layers. Ten years later, two American chemists described the separation of terpenes in essential oils by thin layer chromatography. In 1958, Stahl came out with the equipment and efficient sorbents for the preparations of the plates (Touchstone, 1992)

TLC is a useful technique in laboratories because it is much cheaper and easier than high performance liquid chromatography (HPLC) and other costly analytical systems. In TLC analysis the R_f value for TTX are around 0.70, 0.45, and 0.20 with pyridine-ethyl acetate-acetic acid-water, 3-butanol-acetic acid-water and 1-butanol-acetic acid-water solvent. TTX and their derivatives were visualized as a pink spot by spraying the Weber

reagent, or detected as a yellow fluorescent spot under UV (365nm) after spraying 10% KOH and heating at 100°C (Noguchi *et al.*, 1981).