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Tetrodotoxin in Various Tissues of Yellow Puffer Fish, *Xenopterus naritus* (Richardson 1848) from Betong, Sarawak, Malaysia

MOHD NOR AZMAN, A. [1,2*], SAMSUR, M.[1], MOHAMMED, M.[3], OTHMAN, M.[2], IMELDA, R. R.[3], SHABDIN, M. L.[1] and FASIHUDDIN, B.A.[1]

[1]Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia
[2]Fisheries Research Institute, 11960 Batu Maung, Penang, Malaysia
[3]Fisheries Research Institute Sarawak, Bintawa, 93744 Kuching, Sarawak, Malaysia

Abstract

Tetrodotoxin (TTX) content of the yellow puffer fish, *Xenopterus naritus* (Richardson 1848) collected from Kg. Manggut, Betong, Sarawak, Malaysia was studied from August 2011 to June 2012. A total sample of 276 fish (141 males, 135 females) with total lengths ranging from 12.5 to 31.0 cm and body weights from 38.9 to 711g were used in the study. The amount of TTX in different tissues (liver, skin, muscle, gonad and stomach) was analysed using liquid chromatography-mass spectrometry mass spectrometry (LC-MS/MS). Results showed that the concentrations of TTX in all tissues between the months were significantly different throughout the year ($p<0.05$). The highest mean of TTX was detected in the ovary (330 $\mu g g^{-1}$), followed by the stomach (22.1 $\mu g g^{-1}$), liver (17.8 $\mu g g^{-1}$), skin (17.1 $\mu g g^{-1}$), muscle (11.1 $\mu g g^{-1}$) and testis (7.88 $\mu g g^{-1}$). The highest amount of TTX was recorded in June 2012 in all the tissues. The highest mean gonadosomatic index (GSI) was recorded in August 2011. All the tissues in the present study contained TTX that exceeded the regulatory limit of 2 µg.g-1 (10 $MU \cdot g^{-1}$) established as a safe level for human consumption by the Japan Food Hygiene Association (2005). The information gained from this study indicates the importance removing TTX to ensure the safe consumption of puffer fish.

Introduction

Puffer fish from the Tetraodontidae family is probably the most common fish known to possess a neurotoxin or tetrodotoxin (TTX) which can cause puffer fish poisoning. Almost all puffer fish are poisonous due to the poison (TTX) present in many parts of their body. In the family of Tetraodontidae, there are 28 genera and at least 185 species of puffer fishes (Oliveira et al. 2006). Puffer fishes are commonly found in Malaysian waters and the common species are *Lagocephalus lunaris* (Bloch & Schneider 1801), *L. sceleratus* (Gmelin 1789) and *L. spadiceus* (Richardson 1845) (Kan et al. 1987). However, *Xenopterus naritus* (Richardson 1848) or yellow puffer fish belonging to the family Tetraodontidae is a well-known species amongst the local people in Sarawak. It is a migratory species that inhabits the South China Sea and returns to the river to spawn. *Xenopterus naritus* is widely distributed in China, Thailand, Vietnam, Indonesia and Malaysia. In Malaysia, it is

[*]Corresponding author. E-mail address: mohayo01@yahoo.com
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abundant only in Sarawak and the fish is found in the coastal waters especially in areas fringing the mangroves particularly along the Batang (River) Saribas in Betong, Sarawak from the estuary until the middle stretch of the river or until the water salinity reaches zero (Gambang and Lim 2004). It can be easily identified by the prominent yellowish or golden coloration especially at the lower part of the body. Locally known as 'ikan buntal kuning', it is considered a delicacy by the local community particularly in the Manggut village area. It has become a tourist attraction in Sarawak through the 'yellow puffer fish festival' celebrated every year in August at Manggut Village, Betong, Sarawak.

Yellow puffer fish has been regularly consumed especially by the people living in the middle regions of Batang Saribas, Betong, Sarawak. Although the locals are aware of the poisonous effect of yellow puffer fish, this fish and its products (salted eggs/roes) are easily available and much sought after by the locals. Dried salted puffer fish eggs in Sarawak can fetch up to RM 30-40 per kilogram (USD 10-14) (Muliadi and Muhammad Raduan 2008). Since it is difficult to remove the toxin from the puffer fish, the consumption of the fish and its product can be harmful. The preparation of yellow puffer fish requires a skill and knowledge to discard the internal organs thoroughly without leaving any traces to ensure it safe to eat.

Although puffer fish consumption is rare in other parts of Malaysia, cases of food poisoning from this fish have been reported to occur. Food poisoning has been reported from different geographical regions due to ingestion of puffer fish and the lethality depended on the concentration of TTX present in the consumed fish tissues (Chou et al. 1994). There is limited information on the toxicity of yellow puffer fish and its products except for the reports on *X. naritus* of Andaman Sea (Kungsuwan 1994), Batang Saribas, Sarawak (Bojo et al. 2006; Mohamad, S., pers. comm.), and Batang Sadong, Sarawak (Che Nin et al. 2010). This study was conducted to determine the amount of TTX from different tissues of yellow puffer fish collected from Kg. Manggut, Betong, Sarawak.

Materials and Methods

Specimen collection and identification

Specimens of the puffer fish *X. naritus* (141 males, 135 females) were caught using trammel net by local fishermen from Kg. Manggut, Betong, Sarawak, Malaysia between August 2011 and June 2012. The specimens were kept in ice, stored at -20 ˚C and transported to the laboratory of the Fisheries Research Institute Sarawak, Bintawa, Sarawak, and subsequently kept frozen at -20˚C. The specimens were analysed within one month after collection.

Sample extraction for toxin analysis

The puffer fish was identified based on the morphological characteristics (Froese and Pauly 2011). The samples were weighed and measured for total length individually. Each specimen was dissected to remove tissue from the liver, skin, muscle, gonad and stomach. Some of the tissues were pooled due to the small size. The tissues were collected in duplicate according to the Japanese Official Method (Japan Food Hygiene Association 2005) with slight modifications. Each tissue was minced and a small portion (2-5 g) was used and subjected to an equal volume of 0.1% acetic acid. The samples were then homogenized using an ultrasonic probe (OMNI-Ruptor 4000, Georgia, USA) for 1-3 min except the skin which was diluted 3-5 times with 0.1% acetic acid in order to liquefy the mixture. The mixture was heated in a boiling water bath (BUCHI B-480, Germany) for 10 min and then cooled in slurry ice and centrifuged at 5,000 rpm for 15 min (Eppendorf 5430, Hamburg, Germany). The supernatant obtained was filtered through a 0.45 µm nylon membrane filter before analysed using liquid chromatography-mass spectrometry mass spectrometry (LC-MS/MS).

Analyses by LC-MS/MS

Mass spectrometry was performed using a TSQ Quantum Discovery MAX model from Thermo Electron, USA consisting of an MS Surveyor pump with autosampler coupled to a mass spectrometer equipped with an electro spray ionisation (ESI) probe. Prior to analyses, mass calibration was done using 1,3,5 polityrosine in both negative and positive modes. Compound optimisation was carried out in the positive mode for the detection of the analyte based on its ionisation using a 1 ppm tuning standard solution of TTX (Groupe Biomedix, Malaysia). Optimal ion source and interface conditions were achieved at a spray voltage of 3,800V, sheath gas flow of 10 units, auxillary gas flow of 3 units, collision energy (CE) of 18, collision gas pressure of 1.5 mTorr and capillary temperature of 300 °C. In the positive ionization, the ion transitions from the typical $[M+H]^+$ molecular ion of TTX (m/z 320) to the product ion (m/z 162) (quantifier) (Shoji et al. 2001; Jen et al. 2008; Jang et al. 2010; Yotsu-Yamashita et al. 2011) which was detected in selected reaction monitoring (SRM) mode with argon as the collision gas. Peak detection, data acquisition and the calibration graph plot were performed using the Xcalibur 2.1.0 software. TTX was separated on a 5 µm, 150 mmx2.1 mm inner diameter ZIC-HILIC column (SeQuant, Haltern, Germany) with a guard column 20x2.1 mm, 5 µm (SeQuant, Haltern, Germany). A gradient elution was used at a flow rate of 250 $\mu L \cdot min^{-1}$. LC was performed with mobile Phase A consisting of 10 mM ammonium formate and 10 mM formic acid in water. Mobile phase B contained 5 mM ammonium formate and 2 mM formic acid in acetonitrile/water (80/20, v/v). The gradient programme was applied as described by Diener et al. (2007).

Assessment of gonadosomatic index (GSI)

The spawning season was determined following the monthly changes of the gonadosomatic index (GSI), calculated from its gonad weight (GW) and body weight (BW) using the following equation: GSI = GW/BW x 100 (Anderson and Gutreuter 1983).

Statistical analysis

Data were analysed by the statistical software of SPSS (Statistical Package for the Social Sciences) version 16.0 for Windows. The data were transformed to a normal distribution prior to analysis. One way analysis of variance (ANOVA) was applied to the toxicity data to compare differences in the mean of TTX of different tissues of puffer fish and followed by Tukey's-b post hoc test analysis. Means±SD of duplicate determinations were reported and considered significantly different when $p<0.05$.

Results

Length and weight comparison

Results of mean total length and mean body weight of *X. naritus* by sex and month are shown in Table 1. The mean total length and body weight of male and female specimens were significantly different ($p<0.05$). The total length of male and female specimens was from 12.5- 22.5 cm and 13.0-31.0 cm, respectively. Their mean body weight ranged from 38.9-711 g with the mean weight of 325±130 g for female and 85.5±42.2 g for male specimens. The female specimens were more abundant in August 2011 than in November 2011 except in December 2011 and May 2012 when male and female specimens were found in equal proportion (Table 1).

Toxin analysis

TTX was confirmed by LC-MS/MS analysis, initially by a full scan identification of a parent ion m/z 320 possibly corresponding to TTX $[M + H]^+$ and then by using MS/MS daughter scan functions to compare pure TTX reference material with tissue extracts of *X. naritus* (Fig. 1). The product ion m/z 162 was monitored because it had the most abundant and stable ion. All the tissues tested gave a peak in each of the mass chromatograms. In this study, 4-epiTTX and 4,9-anhydroTTX were not examined due to lack of toxin standard. Figure 1 shows examples of selected ion mass chromatogram of the gonad, the liver and the stomach extract of *X. naritus*. The extracts gave a peak at retention times of 8.69, 8.66 and 8.65 min respectively which are almost consistent with that of the standard TTX (8.66 min). This toxin was identified as TTX, which was confirmed by comparing with the TTX standard. Extract of muscle of the same *X. naritus* also contained TTX which showed the same toxin profile as that of the gonad extract (data not shown).

All the tissues of yellow puffer fish were found to contain TTX at significantly different concentrations ($p<0.05$) (Table 2). The ovary recorded significantly ($p<0.05$) the highest mean TTX concentrations (330 $\mu g g^{-1}$) among the tissues. No significant difference was found in TTX concentration among the skins (17.1 $\mu g g^{-1}$), the muscles (11.1 $\mu g g^{-1}$) and the testis (7.88 $\mu g g^{-1}$). Overall, the puffer fish specimens were found to be toxic and significantly different in all the sampling months ($p<0.05$) (Table 2).

Among the sampling months, June 2012 was found to be the most significantly ($p<0.05$) toxic month with the highest mean TTX concentration as well as the highest toxin levels in all tissues. However, the mean TTX concentrations in all tissues in June 2012 were not significantly different from May 2012. The ovary was the most toxic tissues compared to other tissues of yellow puffer fish throughout the year. The mean TTX concentration in the muscles was less than 10 $\mu g g^{-1}$ from August to December 2011. The testis also recorded TTX concentrations of less than 10 $\mu g g^{-1}$ in all sampling months except in May 2012 (34.4 $\mu g g^{-1}$). The TTX concentrations in most of the tissues increased from November 2011 to May 2012 (Table 2).

Monthly variations in the Gonadosomatic Index (GSI)

Monthly variations in gonadosomatic index (GSI (%)) are shown in Fig. 2. There was significant difference in mean GSI (%) between the sampling months ($p<0.05$). The mean GSI (%) began to increase in November (1.8%), peaked in August (7.8%) and then decreased drastically in October (0.2%).

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Table 1. Average size of *X. naritus* by sex and month.

Sampling month	Sex	Number of specimens	Mean Total length (cm)	Range (cm)	Mean Body weight (g)	Range (g)
Aug. 2011	♂	2	15.0±0.5[a]	14.5-15.5	63.8 ± 6.9[a]	57.2-70.4
	♀	30	26.2±2.3[e]	22.0-31.0	405±92.9[d]	223-553
Sept. 2011	♂	20	14.5±0.7[a]	13.0-16.0	56.2±7.1[a]	38.9-71.3
	♀	10	25.4±2.5[de]	21.5–30.5	359±99.6[cd]	240–605
Oct. 2011	♂	34	18.4±1.9[b]	14.0–22.5	141±48.6[b]	61.2–254
	♀	-	-	-	-	-
Nov. 2011	♂	39	15.5±1.5[a]	13.0–20.5	68.9±17.7[a]	47.3– 23
	♀	7	26.6±2.0[e]	24.3–31.0	422±123.8[d]	340–711
Dec. 2011	♂	15	15.4±1.7[a]	13.5–19.0	70.5±23.1[a]	47.8–126
	♀	19	25.7±2.7[de]	21.5–29.5	394±119[d]	213–613
May 2012	♂	31	15.7±0.9[a]	12.5–18.0	72.3±12.3[a]	43.0–102
	♀	34	21.8±3.9[c]	14.5–29.0	257±138[c]	48.7–593
June 2012	♂	-	-	-	-	-
	♀	35	22.5±3.4[cd]	13.0–26.0	258±86.6[c]	44.0–420
Total	♂	141	16.1±2.0	12.5–22.5	85.5±42.2	38.9–254
	♀	135	24.0±3.7	13.0–31.0	325±131	44.0–711

Data are shown as mean±standard deviation (SD)

Different alphabetical superscripts indicate significant difference among the measured values in each column ($p<0.05$)

NA - Not available

Table 2. TTX concentration in different tissue (μg g^{-1}) of *X. naritus* by sampling month.

Sampling month	Number of specimens	Total Mean TTX (μg g^{-1})	Mean TTX (μg g^{-1})					
			Muscle	Skin	Liver	Stomach	Ovary	Testis
Aug. 2011	32	13.8±4.60[bc]	5.45±2.06	8.66±2.69	9.68±4.33	18.0±4.68	66.8±4.29	4.18±1.42
Sept. 2011	30	12.6±3.83[bc]	3.77±1.46	9.38±2.43	19.1±2.28	15.0±2.07	274±5.96	5.95±2.01
Oct. 2011	34	3.4±2.48[a]	2.78±2.28	2.23±2.42	7.01±2.49	3.12±1.86	NA	4.50±1.05
Nov. 2011	46	8.95±2.84[b]	8.87±2.76	11.0±2.69	9.14±2.91	5.35±2.35	33.6±8.89	7.83±1.95
Dec. 2011	34	15.5±8.86[c]	6.64±3.30	15.5±7.56	12.9±8.42	11.5±11.4	208±5.43	6.46±3.32
May 2012	65	72.4±5.70[d]	34.5±2.93	67.5±2.35	44.1±4.59	95.5±7.46	2170±2.78	34.4±3.65
June 2012	35	89.7±5.70[d]	48.9±2.53	69.3±5.69	41.6±3.37	82.1±3.19	2550±2.13	NA
Total Mean TTX (μg g^{-1})			11.1±4.02[a]	17.1±5.24[ab]	17.8±4.69[bc]	22.1±7.41[c]	330±8.35[d]	7.88±2.33[a]

Data are shown as mean±standard deviation (SD)
NA – not available

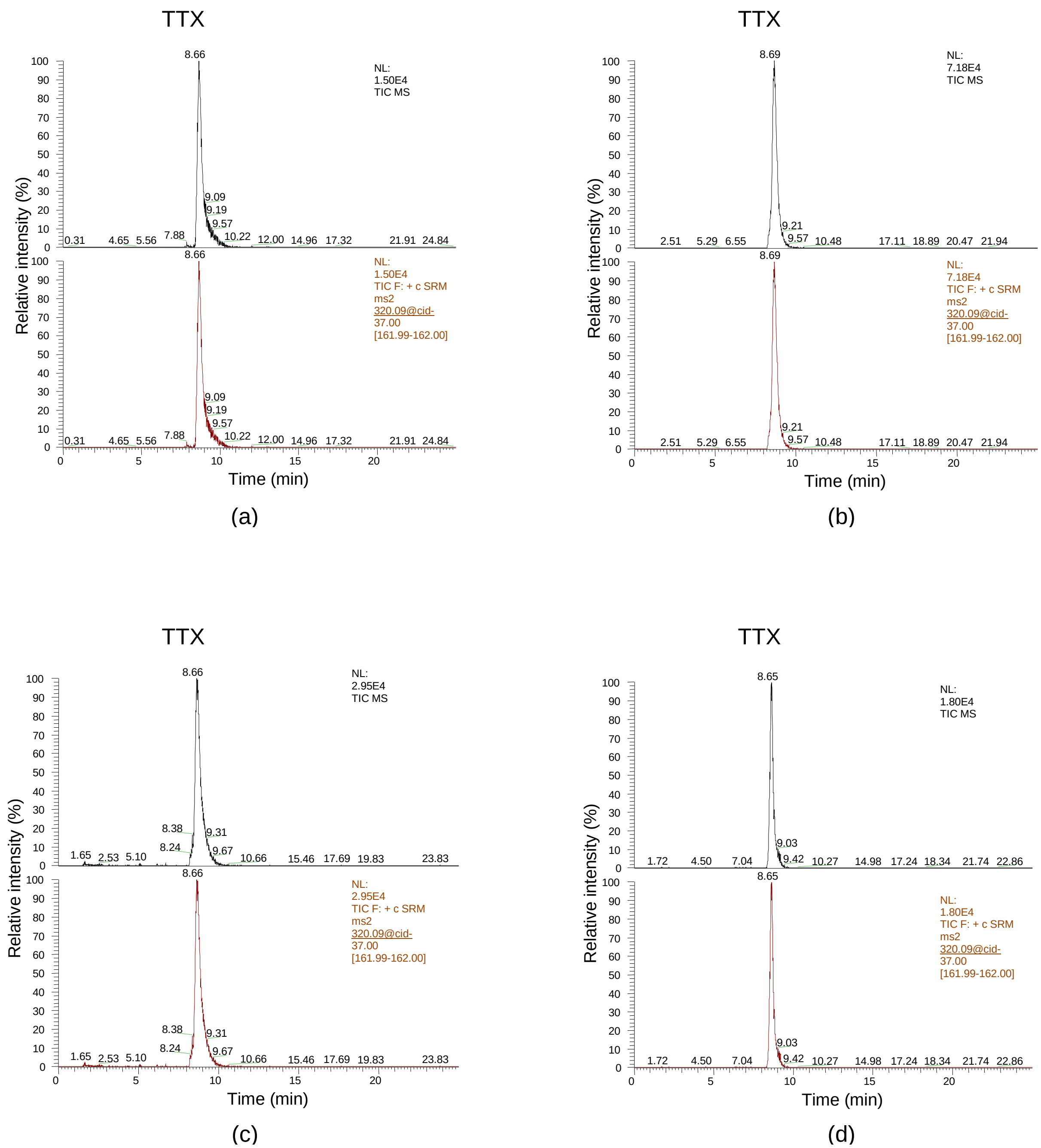

Fig. 1. Full scan Total Ion Current (TIC) and Selected Reaction Monitoring (SRM) chromatography for ion spray LC-MS/MS analysis of (a) standard of tetrodotoxin (TTX) (5 ng), (b) extract of the gonad (10 µl), (c) extract of the liver (10 µl), (d) extract of the stomach (10 µl) of *X. naritus*.

Discussion

In the current study, the female yellow puffer fish were larger and heavier than the male specimens throughout the sampling months. Imelda, R.R. (pers. comm.) also obtained similar results with female yellow puffer fish ranging from 21.6-33.9 cm and the male fish 1.3-19.8 cm. The total length observed in the six samples of yellow puffer fish studied by Mohamad, S. (pers. comm.) was between 19.6 cm and 26.9 cm. Moreover, the maximum body weight of puffer fish observed in this study (711 g) was higher than that of 533.8 g and 190.7 g reported by Mohamad, S. (pers. comm.) and Imelda, R.R. (pers. comm.) respectively. According to Aydin (2011), length and weight are regarded as important growth criteria in the ecology of fish.

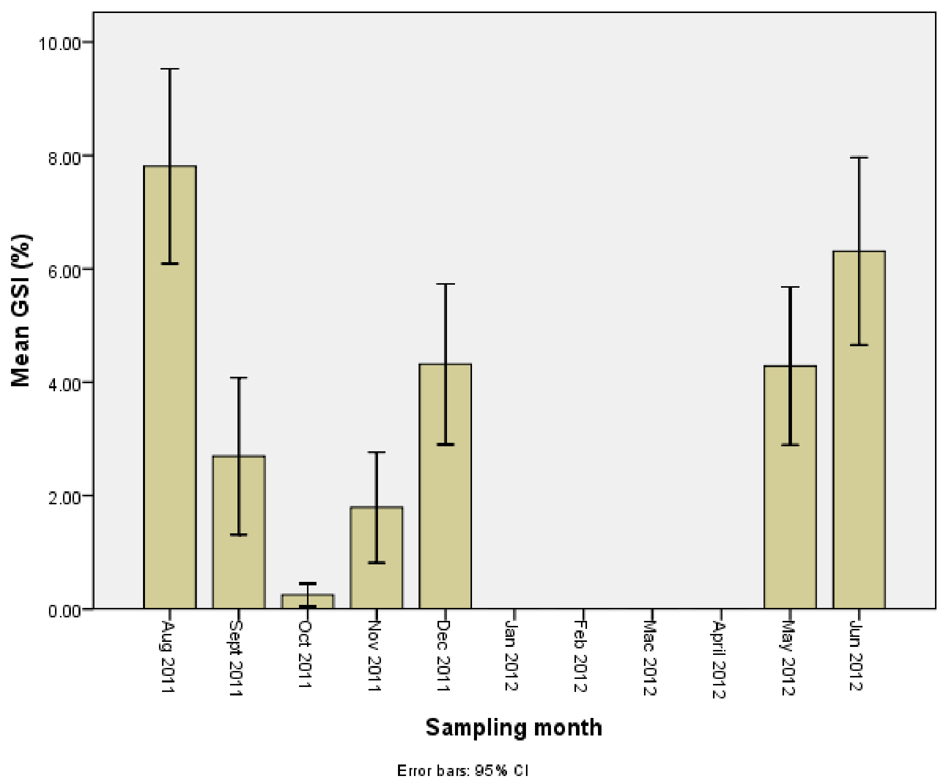

Fig. 2. Monthly variations in gonadosomatic index (GSI %) of *X. naritus.*

The female specimens predominated the catch between May and August, while the male specimens during October (Table 1). In the present study, the female and male specimens were not available during October 2011 and June 2012 respectively. The puffer fish was also not found from January 2012 to April 2012 in this study area for both sexes. The absence of the puffer fish during this period from the area suggested that the fish migrate upstream to spawn due to lower salinity condition. There was evidence that the puffer fish moved from high salinity near shore to upstream low salinity areas before ovulation (Imelda, R.R., pers. comm.).

The spawning of puffer fish seems to correlate with the spring tides. The present work is the first attempt to study the biology of the puffer fish *X. naritus* with regard to the TTX content, and there is a need to examine more samples during this period in the future.

Liquid chromatography results obtained from the puffer fish tissue extracts and reference standard TTX gave the same fragmentation products after MS/MS suggesting that the puffer tissue extracts contained TTX. Nagashima et al. (2001) demonstrated that TTX was a major toxic principle and showed the same toxin profile in the muscle and liver extracted from *L. lunaris* by using LC/ESI-MS. The retention time of puffer fish TTX sometimes was not identical to standard TTX. The difference was probably due to existence of TTX as a mixture of its derivatives in puffer fish (Nakamura and Yasumoto 1985). Generally toxins in puffer fish are thought to be TTX, but paralytic shellfish poisoning toxins have also been detected in some puffer fish including freshwater ones from tropical regions (Kungsuwan et al. 1997; Sato et al. 1997).

The results from this study indicated that all the tissues of yellow puffer fish from Kg. Manggut, Betong, Sarawak were toxic but the distribution of the toxin was unequal. The level of toxin varied among the individual puffers and the sampling months. This finding was in agreement with other studies which showed that the toxicities of puffer fish vary with seasons even within the same species in the same locations (Kungsuwan 1994; Nagashima et al. 2001; Yu and Yu 2002; El-Sayed et al. 2003). Rodriguez et al. (2012) demonstrated that toxin distribution within the tissues of six *L. sceleratus* specimens was different depending on fish size, area and season. The toxicity of the tissue also varied with time of collection and sex.

In general, TTX concentrations in the liver, stomach, and gonad were maintained at high levels throughout the year while the muscle and skin were at low levels. TTX concentrations in these tissues increased gradually during the maturation period which was between November and May and declined markedly after spawning in August. Generally, the level of TTX in female specimens was higher compared to male because ovaries actively accumulate TTX. Ikeda et al. (2010) also reported that the toxicity of the testis of the puffer fish *Takifugu poecilonotus* (Temminck & Schlegel 1850) was much lower than the ovary because the testis cannot actively accumulate TTX. The ovary was the organ with the highest concentration of toxin during maturing in an adult puffer fish (Alcock 2010). This was to protect themselves and their fertilized eggs from predators (Saito et al. 1984). In this study, the TTX concentration in the ovaries increased between November 2011 and June 2012 (maturation period) and decreased in August 2011 which coincided with the spawning period (Table 2). This result was in agreement with other studies by Yu and Yu (2002) and Brillantes et al. (2003). However, this contradicts the general belief that ovaries of puffer fish should be more toxic during spawning in order to protect their fertilized eggs (Hwang et al. 1992; Jeong et al. 1994; Ghosh et al. 2004; Sabrah et al. 2006). Kodama et al. (1983) reported that the toxicity begins to increase just before the spawning season and then decreases drastically after spawning activity.

The amount of TTX recorded in this study was higher than the previous study (Mohd Nor Azman et al. 2012) and the tissues of 48 yellow puffer fish samples from Saribas River (0.7-4.5 $MU \cdot g^{-1}$) as reported by Mohamad, S. (pers. comm.). The results obtained indicated that muscle, the edible part of fish, contained 5.45-6.64 $\mu g g^{-1}$ of TTX during August-December 2011 and 34.5- 48.9 $\mu g g^{-1}$ of TTX during May-June 2012 which can be classified as weakly toxic and moderately toxic, respectively (Noguchi et al. 2006; Sabrah et al. 2006). However, TTX in the muscle of yellow puffer fish from Sampadi, Kuching was not detected even by using the highly sensitive gas chromatography-mass spectrometry (GCMS) (Che Nin et al. 2010). This was not surprising as puffer fish in general has been reported to show large individual, regional and seasonal variations in toxicity (Noguchi et al. 2006).

Although according to Froese and Pauly (2011), *X. naritus* is harmless to humans and was categorized as safe to eat but the present study has shown relatively high levels of TTX in the tissues and could be considered unsafe for human consumption if more than 1 g is consumed. According to Katikou et al. (2009), the minimum lethal dose and minimum acute dose of TTX to humans (wt. 50 kg) were estimated to be around 2 mg (10,000 MU) and 0.2 mg (1000 MU), respectively. However, muscles in many toxic species were regarded as edible (Mahmud et al. 2001). Although the muscle of *X. naritus* in this study was classified as weakly/moderately toxic, it is safe for consumption if prepared in a proper manner. Some of the local people of Betong had skills and experiences with the preparation of yellow puffer fish as they have eaten the fish for generations. In Japan, preparation of puffer fish is strictly controlled by law and only chefs who have been trained and licensed can prepare or sell puffer fish to the public (Cohen et al. 2009).

The GSI (%) can be used as an indicator of reproductive activity of puffer fish. Based on the results, it is suggested that maturation of *X. naritus* inhabiting Kg. Manggut, Betong occurs during November-May and spawning takes place in the months of June-August. This indicates that the presence of yellow puffer fish with enlarged ovaries and the presence of eggs were abundant in August when spawning took place. Imelda, R.R. (pers. comm.) reported that the reproductive season for yellow puffer fish was between July and August. High abundance of yellow puffer fish was found in the Saribas River during the month of August, which probably coincided with the spawning season of the species (Mohamad, S., pers. comm.).

Conclusion

This study showed all tissues were found to be toxic for human consumption as the TTX level was above the regulatory limit (10 $MU \cdot g^{-1}$) (Japan Food Hygiene Association 2005). The TTX concentration in all tissues was significantly higher ($p<0.05$) during the maturation period than during the spawning period. To our knowledge, this is the first report on the TTX determination from the tissues of yellow puffer fish from Kg. Manggut, Betong, Sarawak. This study provides information which could be used as a guide on the amount of yellow puffer fish that can be consumed safely if prepared in a proper manner. The local population of Sarawak should be advised and educated on the importance of consuming carefully prepared yellow puffer fish which would otherwise pose a potential health risk. To ensure the safety of consumers in Malaysia, puffer fish must be closely monitored as the TTX levels may vary during the different months as well as the sex of fish.

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