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An Extensive Survey of Ciguatoxins on Grouper *Variola louti* from the Ryukyu Islands, Japan, Using Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS)

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Abstract: Ingesting fish contaminated with ciguatoxins (CTXs) originating from epibenthic dinoflagellates causes ciguatera fish poisoning (CFP). CFP occurs mainly in the tropical and subtropical Indo–Pacific region and the Caribbean Sea. Furthermore, it occurs sporadically in Japan, especially in the Ryukyu Islands between Taiwan and Kyushu, Japan. *Variola louti* is the most frequently implicated fish with a suggested toxin profile, consisting of ciguatoxin-1B and two deoxy congeners. Therefore, using the liquid chromatography–tandem mass spectrometry (LC-MS/MS), we analyzed CTXs in the flesh of 154 individuals from various locations and detected CTXs in 99 specimens (64%). In 65 fish (43%), CTX levels exceeded the Food and Drug Administration (FDA) guidance level (0.01 µg/kg). Furthermore, in four specimens (3%), the guideline level in Japan (>0.18 µg/kg) was met. Additionally, although the highest total CTX level was 0.376 µg/kg, the consumption of 180 g of this specimen was assumed to cause CFP. Moreover, only CTX1B, 52-*epi*-54-deoxyCTX1B, and 54-deoxyCTX1B were detected, with the relative contribution of the three CTX1B analogs to the total toxin content (35 ± 7.7 (SD)%, 27 ± 8.1%, and 38 ± 5.6%, respectively) being similar to those reported in this region in a decade ago. Subsequently, the consistency of the toxin profile in *V. louti* was confirmed using many specimens from a wide area. As observed, total CTX levels were correlated with fish sizes, including standard length (r = 0.503, $p = 3.08 \times 10^{-11}$), body weight (r = 0.503, $p = 3.01 \times 10^{-11}$), and estimated age (r = 0.439, $p = 3.81 \times 10^{-7}$) of the specimens. Besides, although no correlation was observed between condition factor (CF) and total CTX levels, a significance difference was observed ($p = 0.039$) between the groups of skinnier and fattier fish, separated by the median CF (3.04). Results also showed that the CF of four specimens with the highest CTX level (>0.18 µg/kg) ranged between 2.49 and 2.87, and they were skinnier than the average (3.03) and median of all specimens.

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1. Introduction

Ciguatera fish poisoning (CFP) is a frequent seafood poisoning obtained from the consumption of marine finfish contaminated with ciguatoxins (CTXs) [1,2]. CTXs are potent neurotoxins that bind to the voltage gated sodium channel and cause hyperexcitation of the nerve membrane [3]. Hence, their clinical manifestations are diverse and are associated with gastrointestinal, neurological, and cardiovascular symptoms. The most characteristic symptom is dysesthesia, induced by cold material contact [3]. When the patients touch cold materials, such as metallic items, cold water, and cold winds, they feel an electrical stimulus. This symptom is also referred to as a "dry ice sensation" in Japan [4,5]. The mortality is low, and patients recover within a few days in mild cases. However, in severe cases, despite the severity of symptoms being low, the symptoms may last for several months or years [3,6–8].

The causative toxins, CTXs, are ladder-shaped cyclic polyethers, classified into four groups based on their skeletal structures and the place of occurrence. The CTX1B and CTX3C groups were first isolated and later detected in vast areas of the Pacific Ocean (Figure 1), the Caribbean CTXs (C-CTXs) from the Caribbean Sea, and the Indian Ocean CTXs (I-CTXs) from the Indian Ocean [1]. The structure of I-CTX remains unknown. In the Pacific, epiphytic dinoflagellates, belonging to the genera *Gambierdiscus* and *Fukuyoa*, produce CTX4A, CTX4B, CTX3C, and 49-*epi*CTX3C as major toxins (Figure 1) [9–11]. Besides, although the CTXs produced by dinoflagellates are of low polarity, they are oxidized in fish while moving up the food chain from herbivorous or grazing animals to carnivorous fish, diversifying in structure (Figure 1) [10,12,13]. Due to the lipophilic features and low levels of CTXs in fish, the structural diversity and vast difference in polarity pose great difficulties during analysis, especially when the CTX1B and CTX3C groups coexist. Moreover, despite the minor modifications added by fish metabolism, backbone structures remain unchanged, difference in the type of dinoflagellates that produce these two CTX groups.

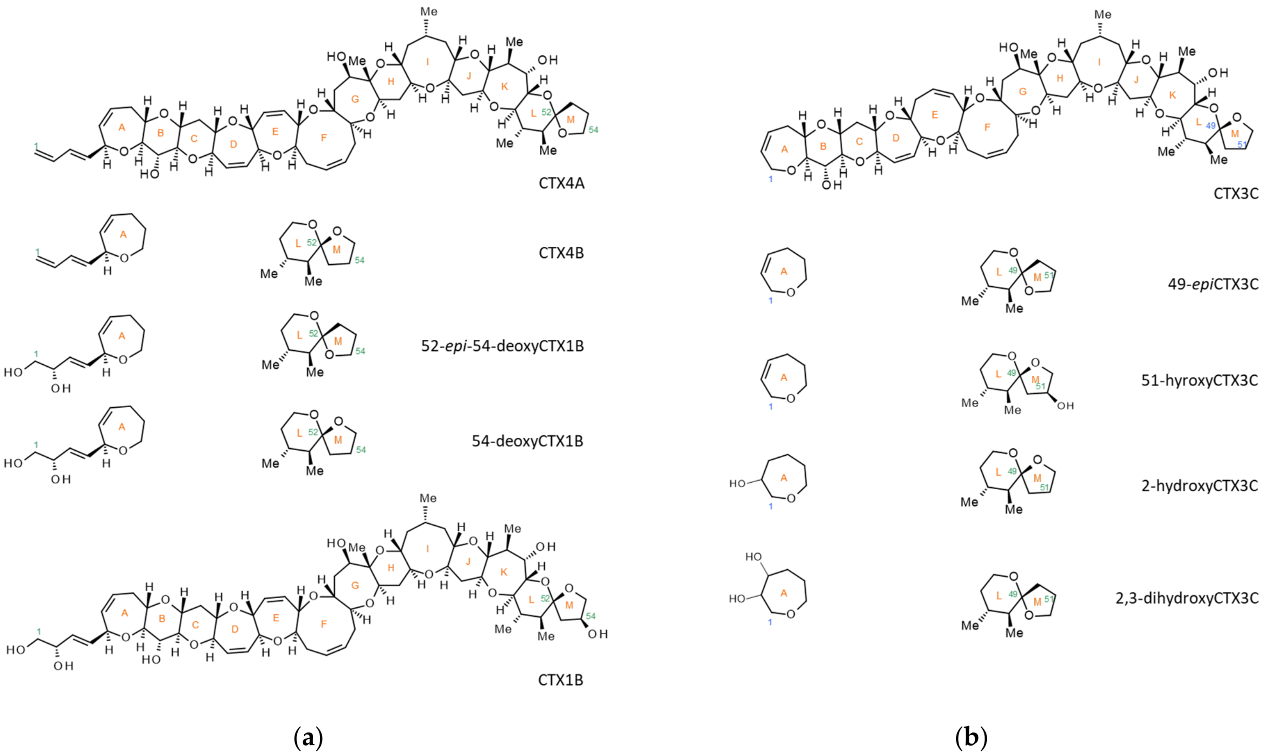
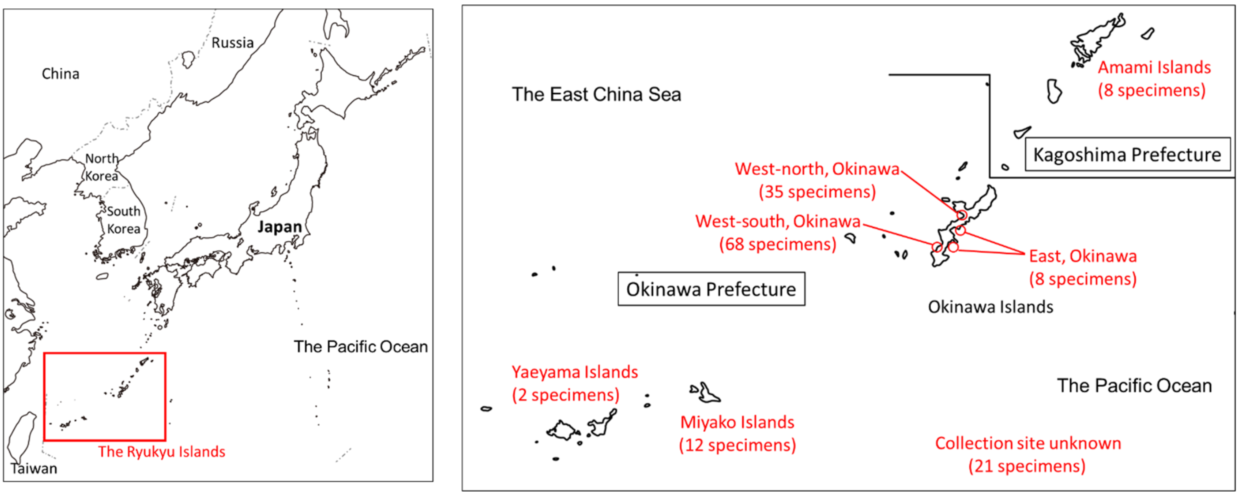


Figure 1. Structures of the representative ciguatoxins (CTXs) reported from the Pacific. (**a**) CTX1B group toxins; (**b**) CTX3C toxins.

Previous studies indicated that ciguateric carnivores in the Ryukyu Islands bore only CTX1B and its two 54-deoxy congeners, no toxin of the CTX3C group was detected [14–16]. As observed, the rather simple toxin profile showed only three analogs of CTX1B, proposing fortunate fact for analytical chemists because the narrow polarity range of the toxins would simplify the analytical process, including sample preparation and detection. Therefore, in the present study, we confirmed the consistency of the toxin profile by testing more fish specimens compared to previous studies that were conducted a decade ago [14–16]. These specimens were collected from the vast areas of the Ryukyu Islands that presently extend approximately 1000 km from a region near Taiwan to Kyushu Island, Japan (Figure 2). The grouper, *Variola louti* (Figure 3), was chosen for analysis because the fish is the most frequently implicated species in CFP in this region [17,18]. Therefore, to understand toxin accumulation in fish flesh, we examined the relationship between toxin levels and various biological factors related to the fish size. Since the early days of the ciguatera study, it was reported that large fish were more toxic than small ones [19,20]. Furthermore, in many countries, fish size restrictions and removing large fish from markets prevent CFP [1]. Thus, to define the "large fish," we measured standard lengths (SL) in addition to the body weights (BW). We also measured sagittal otolith to define the precise age of analyzed fish. Additionally, condition factors (CF) were calculated to test the possibility frequently discussed among fishers that toxic fish were lean because of the harmful effects of toxin accumulation [21].



(**a**) (**b**)

Figure 2. Location of the Ryukyu Islands, Japan, where *Variola louti* specimens were collected. (**a**) Location of the Ryukyu Islands; (**b**) Expansion of the red rectangle in (**a**). Numbers in parentheses indicate the number of specimens collected from each area.

Figure 3. The grouper, *Variola louti* (Serranidae).

Finally, by comparison with reference toxins calibrated with quantitative nuclear magnetic resonance spectroscopy (qNMR) [22], precise levels and types of toxins were analyzed and quantified. Hence, this study analyzed CTXs in the flesh of *V. louti* captured off the Ryukyu Islands, Japan, using a liquid chromatograph–tandem mass spectrometer (LC-MS/MS).

2. Materials and Methods

2.1. Fish Specimens

First, 154 individuals of *V. louti* were purchased at fish markets, from retailers or fishers in the Ryukyu Islands, Japan, between August 2013 and October 2016 (Figures 2 and 3, Tables 1 and S1). Most of them (147 specimens) were kept in ice and transferred to the University of the Ryukyus (UR) laboratory, after which they were identified based on their morphological characteristics. Subsequently, biological data were recorded, including standard lengths (SL), fork lengths, total lengths, and body weights (BW). Then, part (e.g., 100 g) of the flesh samples obtained were frozen and sent to the National Institute of Health Sciences (NIHS) laboratory to analyze CTXs. Prof. Tsuyoshi Ikehara, National Fisheries University, provided three frozen flesh samples with their SL and BW data. Four specimens were directly sent to NIHS from fish retailers or fishers to have their SL and BW measured at NIHS. The frozen flesh samples were stored at −30 °C until use. The condition factors (CF, a fitness related trait) of the specimens were calculated from the following equation to evaluate the emaciation or fatness of the fish [23,24].

$$\text{Condition factor (CF)} = 100 \times \text{BW (g)}/[\text{SL (cm)}]^3$$

Table 1. CTX levels in the flesh of *V. louti* specimens collected off the Ryukyu Islands, Japan.

Area	Total CTX Levels (μg/kg)								
	Total	**<0.003**		**0.003–0.010**		**0.011–0.17**		**≥0.18**	
Amami	8		0%	2	25%	6	75%		0%
West-north, Okinawa	35	16	46%	8	23%	11	31%		0%
West-south, Okinawa	68	21	31%	19	28%	26	38%	2	3%
East, Okinawa	8	2	25%	1	13%	5	63%		0%
Miyako	12	6	50%		0%	4	33%	2	17%
Yaeyama	2		0%		0%	2	100%		0%
Unknown	21	10	48%	6	29%	5	24%		0%
Total	154	55	36%	36	23%	59	38%	4	3%

From the heads of the specimens that arrived at UR, the sagittal otoliths were taken for age estimation. First, the otoliths were washed with water, dried, embedded in epoxy resin, and dried again for one day. Next the samples were sectioned transversely into 0.275 mm thick sections using an IsoMet™ Low Speed diamond saw (Buehler, Lake Bluff, IL, USA). Finally, the sectioned otolith samples were observed under a light microscope to count the number of opaque rings based on the description in previous studies [25–27].

2.2. Reference Toxins and Reagents

The reference mixture solution containing various CTXs was prepared at NIHS and comprised CTX1B, 52-*epi*-54-deoxyCTX1B, 54-deoxyCTX1B, CTX4A, CTX4B, 2,3-dihydroxyCTX3C, 51-hydroxyCTX3C, and CTX3C. These CTXs were purified or semi-purified from natural sources and characterized by spectroscopic methods in the previous studies [28–33]. However, during this study, only two reference materials, Ciguatoxin-1B (43.3 ± 1.3 ng) and Ciguatoxin-3C (38.5 ± 2.6 ng), quantified by quantitative nuclear magnetic resonance spectrometry (qNMR) were provided by Japan Food Research Laboratories (JFRL). With these two reference materials, the concentrations of each CTX analogs in the reference solution were determined: Ciguatoxin-1B used for high polar analogs of CTX1B, 52-*epi*-54-deoxyCTX1B, 54-deoxyCTX1B, 2,3-dihydroxyCTX3C, and 51-hydoxyCTX3C and Ciguatoxin-3C used for low polar analogs 49-epiCTX3C, CTX3C, CTX4A, and CTX4B).

Acetone, hexane, diethyl ether, and methanol used for extraction, were of analytical grades and were purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). Ethyl acetate, methanol, and acetonitrile used for sample preparation and the mobile phase in LC-MS/MS were of high-performance liquid chromatography (HPLC) or LC-MS grade (Kanto Chemical Co. Inc., Tokyo, Japan). Ammonium formate solution (1 mol/L, HPLC grade) and formic acid (HPLC grade) were purchased from the Wako Chemical Industry, Ltd. (Osaka, Japan). Ultra-pure water was obtained from the Milli-Q® Integral Water Purification System (Millipore, Bedford, MA, USA).

2.3. Extraction and Sample Preparation of the Fish Flesh

The preparation of flesh extracts for LC-MS/MS analysis was conducted as described previously [34–36]. The outline of the cleanup procedure is shown in Figure S1. As shown, the flesh sample (5 g) was first extracted with acetone (15 mL, twice), after which combined extracts were condensed to a syrup under a nitrogen stream at 40 °C to remove acetone. Next, organic contents were extracted from syrup with diethyl ether (5 mL, twice). Then, the combined organic layers were completely dried under nitrogen stream at 40 °C. Subsequently, the residue was suspended with 90% methanol (*v*/*v*, 1.5 mL) and defatted with hexane (3 mL, twice). After removing hexane, the methanolic solution was completely dried under a nitrogen stream at 40 °C, resulting in the crude extract that was later dissolved with an ethyl acetate/methanol mixture (9:1, v/v, 5 mL) and passed through a Florisil cartridge column (500 mg, GL Sciences Inc., Tokyo, Japan). Afterward, the eluate was completely dried under a nitrogen stream at 40 °C. Then, the resulting residue was dissolved using acetonitrile (5 mL) and applied to a primary and secondary amine cartridge column (PSA, 200 mg, GL Sciences Inc., Tokyo, Japan). The low polar analogs including CTX4A, CTX4B, 51-hydroxyCTX3C, 49-epiCTX3C, and CTX3C were passed through with acetonitrile (ACN eluate). Subsequently, hydroxylated analogs, CTX1B, 52-*epi*-54-deoxyCTX1B, 54-deoxyCTX1B, 2,3-dihydroxyCTX3C, and 2,3,51-trihydroxyCTX3C, were eluted with methanol (3 mL, MeOH eluate). Both, ACN and MeOH eluates were dried, respectively, and dissolved with methanol (1 mL) to apply LC-MS/MS. A 1 mL portion of the solution to be applied to LC-MS/MS was equivalent to 5 g of the flesh. The limit of detection (LOD) and the limit of quantification (LOQ) for all CTX analogs were estimated to be 0.001 (S/N > 5) and 0.005 µg/kg (S/N > 10), respectively. Moreover, levels between LOD and LOQ when the total CTXs were defined as 0.03 µg/kg (the average of LOD and LOQ).

2.4. LC-MS/MS Analysis

The equipment used comprised an Agilent 1290 HPLC system (Agilent Technologies, Santa Clara, CA, USA) and an Agilent 6460 Triple Quadrupole MS instrument (Agilent Technologies, Santa Clara, CA, USA). Besides, the HPLC and MS conditions were described in our previous papers (Table S2) [34–36].

2.5. Toxicity Evaluation

The European Food Safety Authority (EFSA) proposed the toxicity equivalency factor (TEF) values for some analogs based on intraperitoneal (i.p.) administration to mice [37]. However, an FAO/WHO expert meeting concluded that due to limited data from oral in vivo studies, it was impossible to derive TEFs [1]. Thus, this study discussed total CTXs, the sum of each CTX analog level.

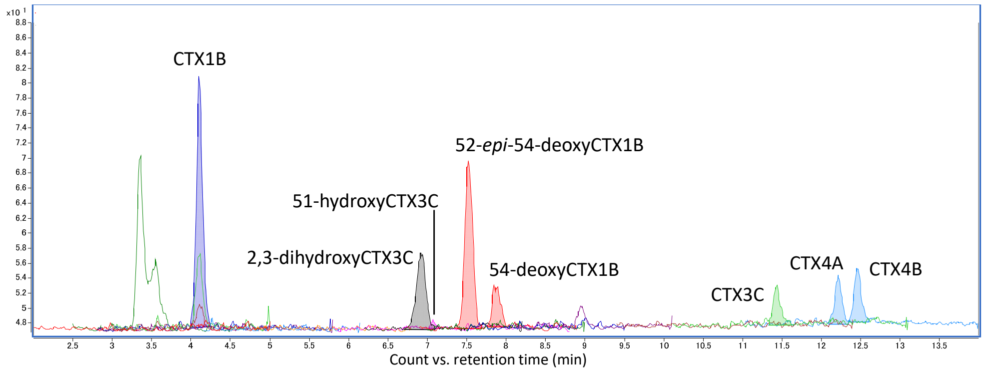
2.6. Statistical Analysis

Data were statistically evaluated using the EZR software for Windows (ver. 1.55) (https://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmedEN.html (accessed on 11 March 2022)) [38]. The Spearman's rank correlation test was used to evaluate the correlation between fish data (TL, BW, CF, and age) and total CTXs levels. The statistical significances were assessed using a non-parametric test, Mann–Whitney U test.

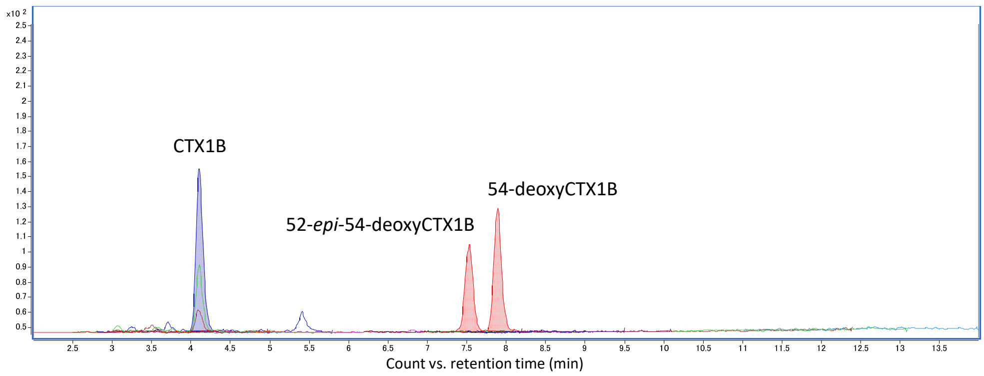
3. Results

Of the 154 *V. louti* specimens collected from the Ryukyu Islands, although CTXs were detected in 99 specimens (64%), they were below LOD in the remaining 55 specimens (36%) (Table 1). The identified analogs were CTX1B, 52-*epi*-54-deoxyCTX1B, and 54-deoxyCTX1B. However, CTX4A, CTX4B, and CTX3C derivatives were undetected (Figure 4).The results also showed that 65 fish (43%) contained CTXs above the FDA guidance level (0.01 µg/kg) [39]. In contrast, when judged based on the Japanese guideline [40], only four fish (3%, ID: 160024, 160136, 163065, and 163077) were above the warning level (>0.18 µg/kg), indicating that the potential risk to be intoxicated was by consuming ~400 g of flesh (Table 1 and Table S1). The highest level of the total CTXs was at 0.376 µg/kg. Additionally, of the four specimens above the Japanese guideline, two were from the West–south coast of Okinawa Island, and the other two were from Miyako Island (Table S1, Figure 5). The occurrence of the specimens whose CTXs were above the FDA guidance levels was as follows: Amami, 75% (6 of 8 specimens); West-north, Okinawa Island, 31% (11 of 35), West-south, Okinawa Island, 41% (28 of 68), East, Okinawa 63% (5 of 8), Miyako Island 50% (6 12), and Yaeyama Islands, 100% (2 specimens) (Table 1, Figure 5). Furthermore, in all CTXs detected specimens from Miyako and Yaeyama, the levels were above the FDA guideline (Table 1, Figure 5). Though the Mann–Whitney U test, no significance difference

($p > 0.05$) was observed in the proportion of toxic fish based on the collection area when judged as CTX levels of >0.003, >0.01, and >0.18 µg/kg, respectively.



(**a**)



(**b**)

Figure 4. Representative LC-MS/MS chromatograms of (**a**) the reference CTX mixture and (**b**) the *V. louti* specimen (NIHS-Cig-153063).

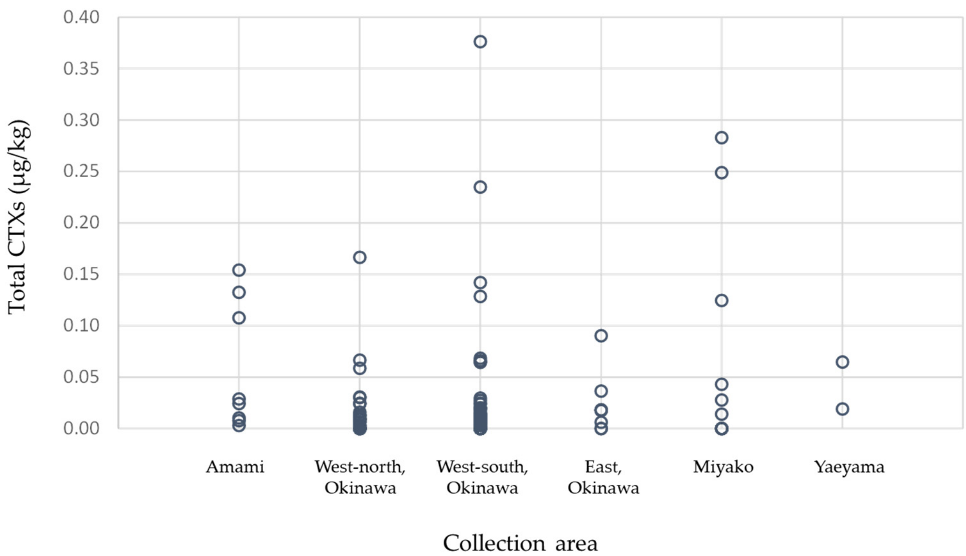


Figure 5. Total CTX levels in the flesh of *V. louti* collected at various sites as shown in Figure 2.

We also observed that SL ranged between 13.5 cm and 53.0 cm (average: 34.3 cm; median: 34.0 cm, Table S1) and was positively correlated with CTX levels as per the Spearman's rank correlation test (r = 0.503, $p = 3.08 \times 10^{-11}$, Figure 6a).

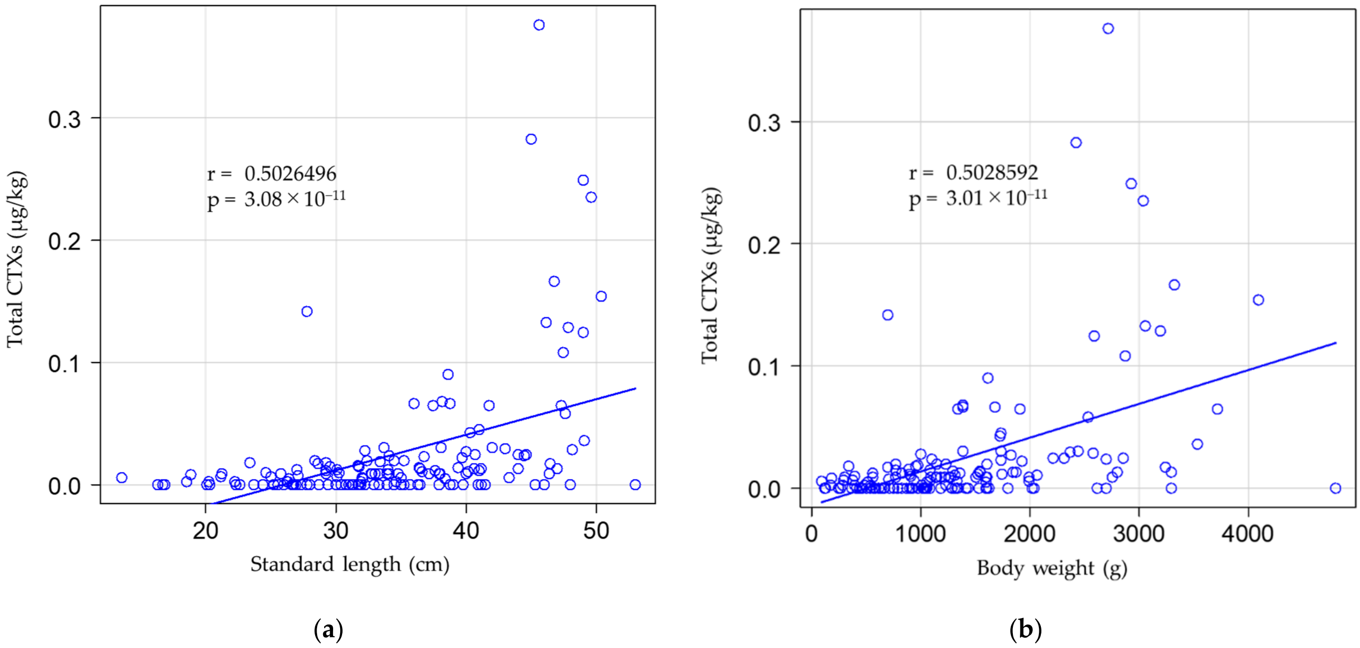


Figure 6. The relationship between fish size ((**a**): SL, (**b**): BW) and total CTX levels in the flesh of *V. louti* specimens collected from Ryukyu Island, Japan.

Moreover, BW ranged between 90 g and 4800 g (average: 1399 g; median: 1209 g), and the Spearman's rank correlation test found a positive correlation of BW with the total CTXs levels (r = 0.503, $p = 3.01 \times 10^{-11}$, Figure 6b).

CF ranged from 1.87 to 4.03, with an average of 3.03 and a median of 3.04 (Table S1). Furthermore, no correlation was observed between CF and total CTX levels in the specimens' flesh (r = 0.175, $p = 0.0298$, Figure S2). When the specimens were divided into two groups, the skinnier and fattier, using the median CF value at 3.04, a significance difference was detected using the Mann–Whitney U test ($p = 0.039$, Figure 7). Thus, the total CTX levels in the skinner specimens were higher than those in the fattier ones.

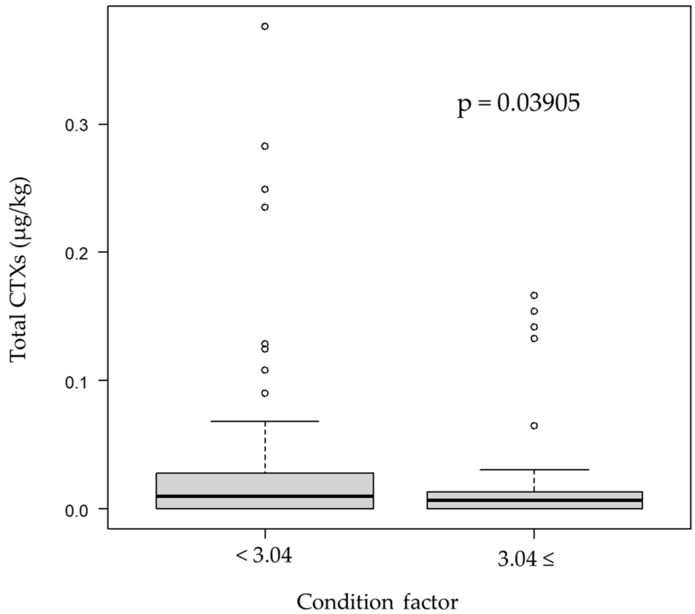


Figure 7. Total CTX levels in the flesh of *V. louti* specimens collected from Ryukyu Island, Japan, classified into two groups using the median of the condition factor (3.04).

Of the 154 *V. louti* specimens, we estimated the ages of 124 individuals by counting the number of rings in their sagittal otoliths. Their ages ranged from 1 to 18 years (average: 4.8 years, median: 4, Tables S1 and S3). Likewise, a positive correlation with total CTX levels was observed (r = 0.439, $p = 3.81 \times 10^{-7}$, Figure 8).

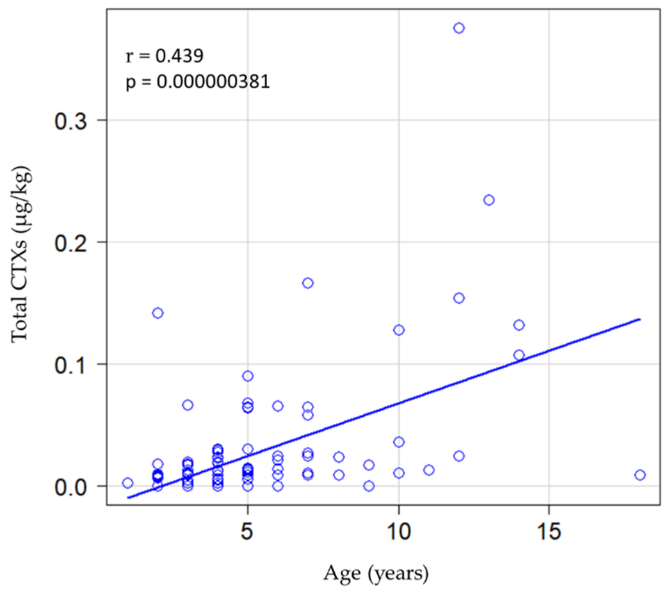


Figure 8. The relationship between age and total CTX levels in the flesh of *V. louti* specimens collected from Ryukyu Island, Japan.

Additionally, 135 specimens' gender were identified (females: 113, bisexuals: 3, males: 13, Table S1). Although, the number of specimens were very different between female and male, CTX levels in the male specimens were higher than those in the female specimens ($p = 0.0001$, Figure 9). Furthermore, the differences between sex (male and female) and other biological data (SL, BW, and age) were observed (Figure S3a–c). According to this observation, the male specimens tended to be longer, heavier, and older than females. Moreover, *V. louti* appeared transgender from female to male as they grew.

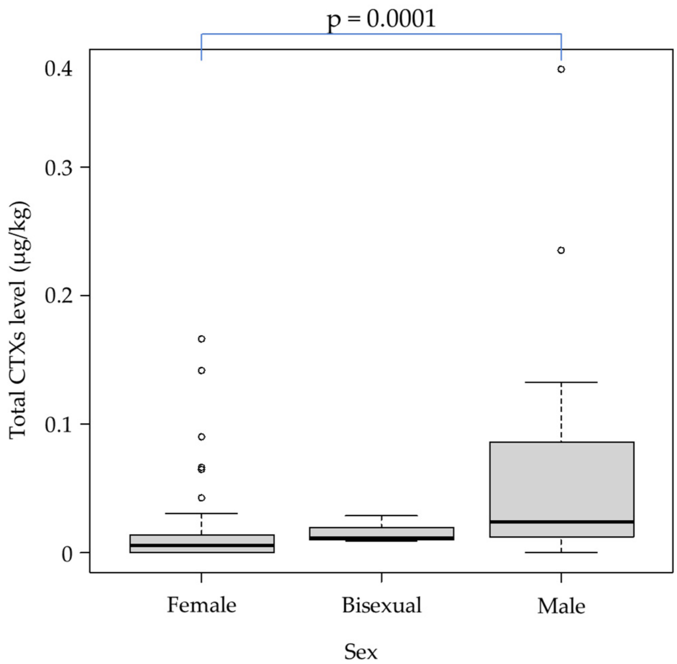


Figure 9. Compared total CTX levels in the flesh of *V. louti* specimens collected from Ryukyu Island, Japan, based on gender.

We also observed that the CTX profiles were composed of CTX1B, 52-*epi*-54-deoxyCTX1B, and 54-deoxyCTX1B, and the relative ratios of the three analogs were as 35 ± 7.7 (SD)%, 27 ± 8.1 (SD)%, and 38 ± 5.6 (SD)%, respectively (Table 2, Figure S4).

Table 2. The ratios of three analogs, CTX1B, 52-*epi*-54-deoxyC TX1B, and 54-deoxyCTX1B in the flesh of *V. louti* specimens from which quantifiable levels of CTXS were detected (n = 36).

CTX Analogs	Average (%)	Standard Deviation
CTX1B	35	7.7
52-*epi*-54-deoxyCTX1B	27	8.1
54-deoxyCTX1B	38	5.6

4. Discussion

This study collected 154 *V. louti* specimens from the Ryukyu Islands spread from Okinawa Prefecture to Amami Island, Kagoshima Prefecture, Japan. Then, we measured the profiles and levels of CTXs in the specimen's flesh using LC-MS/MS. Positive correlations were observed between the total CTX levels and fish size, including SL (r = 0.503, $p = 3.08 \times 10^{-11}$), BW (r = 0.503, $p = 3.01 \times 10^{-11}$), and age (r = 0.439, $p = 3.81 \times 10^{-7}$).

Size restriction (the elimination of large fish from the market), imposed as one of the risk management strategies for CFP in many countries, as well as the relationship between the toxicity of ciguateric fish and size has been discussed [1]. In addition, investigations that the positive correlations between toxicity and fish size in certain carnivorous species exist, including moray eels (*Gymnothorax javanicus* [41] and *Muraena augusti* [42]), snapper (*Lutjanus bohar*) [43], peacock grouper (*Cephalopholis argus*) [44], Amberjack (*Seriola* spp.), and the dusky grouper (*Epinephelus marginatus*) [45]. Similar results were observed in our previous study in Okinawa [17]. The toxicities of ciguateric fishes were evaluated using the mouse bioassay (LOD: 0.025 MU/g or 0.18 μg CTX1B equivalent/kg), and toxicity of individuals was proposed to be related to fish size. For example, among the 49 of *V. louti* specimens, 7 (14.3%) were judged as toxic and weighed over 1.8 kg. A clear result was observed in the snapper (*L. bohar*). Among the 168 individuals, 20 (11.9%) were toxic, and no toxic specimens were found in individuals weighing less than 4 kg. As reported, the toxic ratio rose to 37.7% among specimens above 4 kg and jumped to 61.1% in fish over 7 kg. Alternatively, an opposite observation (no correlation between toxicity and fish size) has also been reported in Spanish Mackerel (*Scomberomorus commerson*) [46] and barracuda (*Sphyraena barracuda*) [47]. Similarly, Gaboriau et al. reported that the toxicity and fish size do not always matter based on their observations with 856 specimens from 59 species sampled at six islands in French Polynesia [43]. Thus, the size-toxicity relationship is proposed to be species-specific.

This study observed no relationship between CF and total CTX levels. However, a significance difference was discovered (p = 0.039) when the specimens were divided into two groups, the skinnier and fattier, using the median CF (3.04). Interestingly, the CF of four specimens containing the highest levels (>0.18 μg/kg) of CTXs, ranged between 2.49 and 2.87, indicating that they were skinnier than the average and median of all specimens. This result followed the fishermen's folklore in the Ryukyu Islands that skinny fish are more toxic than fatty [21]. Furthermore, although the advertising effect of CTXs in some fish species other than *V. louti* has been demonstrated previously [48–51], many of them are not "ciguateric" fish. Therefore, considering the species-specific characteristics of ciguatera toxicity, further investigations on the toxicological effect on *V. louti* and other representative ciguateric species will be needed to understand the ethology of CFP further.

Results also showed the relative contents of three CTX1B analogues (CTX1B, 52-*epi*-54-deoxyCTX1B, and 54-deoxyCTX1B) in total CTXs were 35 ± 7.7 (SD) %, 27 ± 8.1 (SD) %, 38 ± 5.6 (SD) %, respectively. A similar tendency was obtained in the previous study in Okinawa decades ago [14] and has also been reported in Kiribati [41], and Fiji [35]. Hence, this CTX1B analog profile is proposed to be characteristic of *V. louti* species.

V. louti has been well recognized as ciguatoxic and has been implicated in CFP cases not only in Ryukyu Islands but also in other regions including Queensland, Australia [52], Marshall Islands [53], Cook Islands [54], Fiji in the Pacific [55], and Mauritius [56,57], and Réunion Islands in the Indian Ocean [58]. Despite this fact, *V. louti* is an important species in commercial fishery in the Maldives [59], Saudi Arabia [60], Egypt in the Indian Ocean [60], and Indonesia [61,62], American Samoa [63,64], Guam [65], Papua New Guinea [66], Northern Mariana Islands [64], Philippines [67], Okinawa, Japan [68,69], and other South Pacific islands [70]. Our previous study in Ogasawara (Bonin) Islands was located approximately at the same latitude as Okinawa and around 1000 km south of Tokyo. Based on the results, no CTXs were detected in 65 *V. louti* specimens, even though their weights ranged from 2170 to 7000 g [71]. They were also heavier than the average and median weight in this study. In addition to this observation, the residents in this area recognized the fish as "safe," and no CFP occurred due to consumption of this fish [71]. Furthermore, the mean annual catch of *V. louti* from 2010 to 2014 in Okinawa was estimated to be 2886.9 kg and most of them might be sold for human consumption. For example, when this value was divided by the median BW (1209 g) of specimens in this study, ca. 2390 individuals were consumed. However, reported CFPs due to the consumption of *V. louti* were only eight incidents in 1997–2006 or 0.8 incident/year, and the toxicities of implicated fishes were above 0.025 MU/g (0.18 μg CTX1B equivalent/kg) [17]. When considering this fact, the current FDA guideline is considered as too strict and should be re-evaluated based on the human CFP cases. To achieve the needed results, further investigation and more data accumulation related to human CFP cases and fish toxicities should be conducted.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jmse10030423/s1, Table S1. The *V. louti* specimens analyzed in this study and their CTXs levels, Table S2. LC-MS/MS condition for CTX analysis, Table S3. The CTXs levels in the flesh of *V. louti* by age collected from the Ryukyu Islands, Japan, Figure S1. Sample preparation from flesh for LC-MS/MS, Figure S2. The relationship between the condition factors and the total CTX levels in the flesh of *V. louti* specimens collected from the Ryukyu Island, Japan, Figure S3. Relationship between the genders and other biological data (a: total length, b: body weight, and c: age) of *V. louti* specimens collected from the Ryukyu Islands, Japan, and Figure S4. CTXs profiles of *V. louti* specimens collected from Ryukyu Island, Japan, from which quantifiable levels of CTXS were detected (n = 36).

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