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Article

Analytical Studies on Ciguateric Fish in Okinawa, Japan (II): The Grouper *Variola albimarginata*

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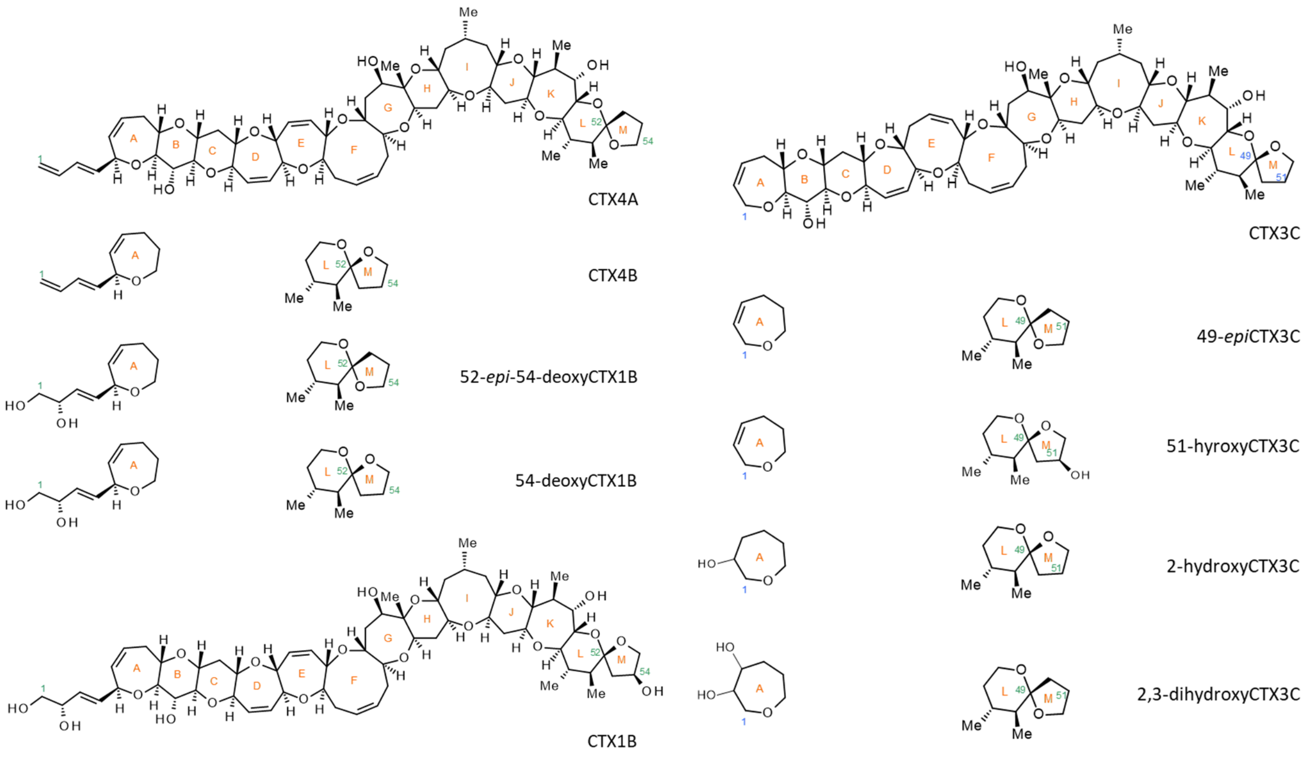
Abstract: Ciguatera fish poisoning (CFP) refers to an illness caused by ingesting fish that have accumulated ciguatoxins (CTXs). CFP frequently occurs in the tropical and subtropical Indo-Pacific Ocean and the Caribbean Sea. In Japan, CFP occurs sporadically but constantly in Okinawa and the Amami Islands. The grouper *Variola albimarginata* is regarded to be safe for consumption. To assess the real risk of *V. albimarginata*, we analyzed 133 specimens of the fish in Okinawa using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Ciguatoxin-1B, 54-deoxyciguatoxin-1B, and 52-*epi*-54-deoxyciguatoxin-1B were detected in 28 specimens (21%). In 11 of these specimens (8%), the CTX levels exceeded the US FDA guidance level (0.01 µg/kg CTX1B equivalent). However, only one fish (<1%) was found to have levels above the recommended level in Japan (0.175 µg/kg CTX1B equivalent). The amount of CTXs in the flesh (280 g) of the most toxic specimen (0.225 µg/kg) did not reach the level needed to cause illness. The CFP risk due to the consumption of this species was thus considered to be low in Okinawa, supporting local belief. The CTX levels in the flesh were positively correlated with standard length, body weight, and age. The total CTX levels significantly fluctuated between the male and the female of the species. The estimated annual catch of *V. albimarginata* in Okinawa and Yaeyama Islands was 4909 kg or 13,636 fish. As many as 1227 fish had levels over the US FDA guidance level, but only 136 fish had levels above the Japanese recommendation. Risk management based on the Japanese recommendation level seems to be effective in protecting public health and enabling appropriate exploitation of fishery resources.

Keywords: ciguatera; ciguatoxin; *Variola albimarginata*; risk management; LC–MS/MS; Okinawa

1. Introduction

Ciguatera fish poisoning (CFP) is the most prevalent food poisoning arising from the consumption of seafood contaminated with natural toxins. The estimated number of patients amounts to tens of thousands annually [1–3]. The main areas of CFP occurrence are the tropical and subtropical areas of the Pacific and Indian Oceans and the Caribbean Sea [3], with the recent addition of Macaronesia, which includes Madeira (Portugal) and the Canary (Spain) Islands, located in the eastern Atlantic Ocean and west of Morocco in northwest Africa [4], leading to concerns that the CFP area may be expanding [3]. In the Pacific, the causative agents, ciguatoxins (CTXs), produced by the benthic dinoflagellates *Gambierdiscus* spp. and *Fukuyoa* spp., accumulate in fish and other grazing animals and then move to carnivorous fish through the food chain [5–8]. The chemical structures are characterized by contiguous cyclic ether rings A through L and a terminal spiroketal ring M, all aligned in a ladder shape. There are two types of CTXs, CTX4A/4B and 49-*epi*CTX3C/CTX3C (Figure 1) [6,9]. These CTXs undergo a metabolic change in fish to nearly 20 analogs [10], sometimes with enhanced toxicity, as exemplified by CTX1B and 51-hydroxyCTX3C (Figure 1). Thus, careful structural identification and toxicity assessment are needed. In this study, two criteria were used to assess the CFP risk from a fish: (1) the guidance level proposed by the US FDA (0.01 µg/kg CTX1B equivalent) [11] and (2) the recommended level in Japan (0.175 µg/kg CTX1B equivalent) as described in the book *The Standard Methods of Analysis in Food Safety Regulation*, edited by a government agency [12]. No legal enforcement was involved.



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

Figure 1. The chemical structures of the representative ciguatoxins (CTXs) reported from the Pacific. CTX1B group toxins derived from CTX4A and 4B (**a**) and CTX3C group toxins derived from 49-*epi*CTX3C and CTX3C (**b**).

The genus *Variola* consists of two species: *V. louti* and *V. albimarginata.* They inhabit coral reefs in the Indo-Pacific tropical to temperate zones, feeding mainly on fish and crustaceans [13,14]. The two species are morphologically similar, with a reddish ground color, numerous light-colored spots on the body surface, and a crescent-shaped caudal fin. They are distinguishable by the yellowish margins of the caudal, dorsal, anal, and pectoral fins in *V. louti* (Figure 2a) and the white margin of the caudal fin of *V. albimarginata* (Figure 2b) [14]. *V. albimarginata* has a high market value because of its bright coloration [15]. Both species are prohibited from being imported into Japan, but the fate of the fish captured in Japanese waters is left to the judgment of the local governments where the fish were caught.

(**a**)

(**b**)

Figure 2. The groupers, *Variola louti* (**a**) and *Variola albimarginata* (**b**).

Over a period of 22 years, from 1989 to 2011, while *V. louti* was the primary cause of CFP in Japan (16 incidents, 21%), *V. albimarginata* was implicated in only two incidents [16]. The ratio of toxic flesh estimated in Okinawa using mouse bioassays (MBA) was 14% (7 out of 49) for *V. louti* and 2.8% (1 out of 36) for *V. albimarginata* [17]. Contrastingly, out of 33 CFP events confirmed by MBA between 2004 and 2013 in Hong Kong, *V. albimarginata* was reported to be responsible for 2 cases, whereas *V. louti* was responsible for none [18]. In addition, in some areas, such as New Caledonia, people avoid consuming *V. albimarginata* because of the potential risk [13]. In a survey in the Philippines, two out of twelve (17%) specimens of *V. albimarginata* were judged as toxic by MBA [19]. The contradictory information on the toxicity of the *Variola* fish needs clarification based on an accurate analysis of the implicated toxins and identification of fish species. New analytical data may reinforce or replace the previous data that were obtained by distributing questionnaires or animal experiments and instrumental analysis of limited scales [17,20–23].

Currently, consumers are advised about the CFP risks on the basis of the epidemiological reports on its occurrence rather than on the analytical data of fish. To lay a solid basis to discuss the issue, we have developed a precise analytical method using the LC–MS/MS technique [24,25]. First, because a large number of fish have to be tested, we prepared a secondary standard solution, dubbed NIHS-CTX-Mix, composed of major CTXs purified from fish and dinoflagellate in order to save the CTX reference toxins quantified by qNMR in the Japan Food Research Laboratories (JFRL-RM-CTX) [26]. The concentration of each analog in NIHS-CTX-Mix was quantified with the help of JFRL-RM-CTX. Second, we established analytical methods and investigated the toxin profiles of various fish using the new standard mix [27–31]. We also demonstrated that flesh samples taken from different parts of fish possess nearly homogeneous toxicity, validating the credibility of our data and those in literature, even though the location of the tissues was unspecified [32]. However, the tissue surrounding the eyeballs had exceptionally high levels of toxins and thus was not included in this study. Third, with the collaboration of biologists, we ensured the correct identification of fish species and measured ages and condition factors (an index similar to the body mass index, BMI, in humans) to define biological traits. Fourth, to lay a foundation for discussing the difference between two similar-looking species, we searched for information on diet and metabolic mechanisms [10].

Prior to this study, we had analyzed 154 flesh samples of *V. louti* using LC–MS/MS and detected CTXs in 99 samples (64%). The CTX levels in 65 samples (43%) exceeded the US FDA guidance level (0.01 µg/kg CTX1B equivalent) [11], but the CTX levels in only four samples (2.6%) exceeded the recommended level in Japan (0.025 MU/g = 0.175 µg/kg CTX1B equivalent) [29]. We plan to continue surveys on major species in Okinawa and report the results as a series titled "Toxicity study series on fish in Okinawa." The preceding report on *V. louti* will be considered the first one, and this study the second one in the series [29].

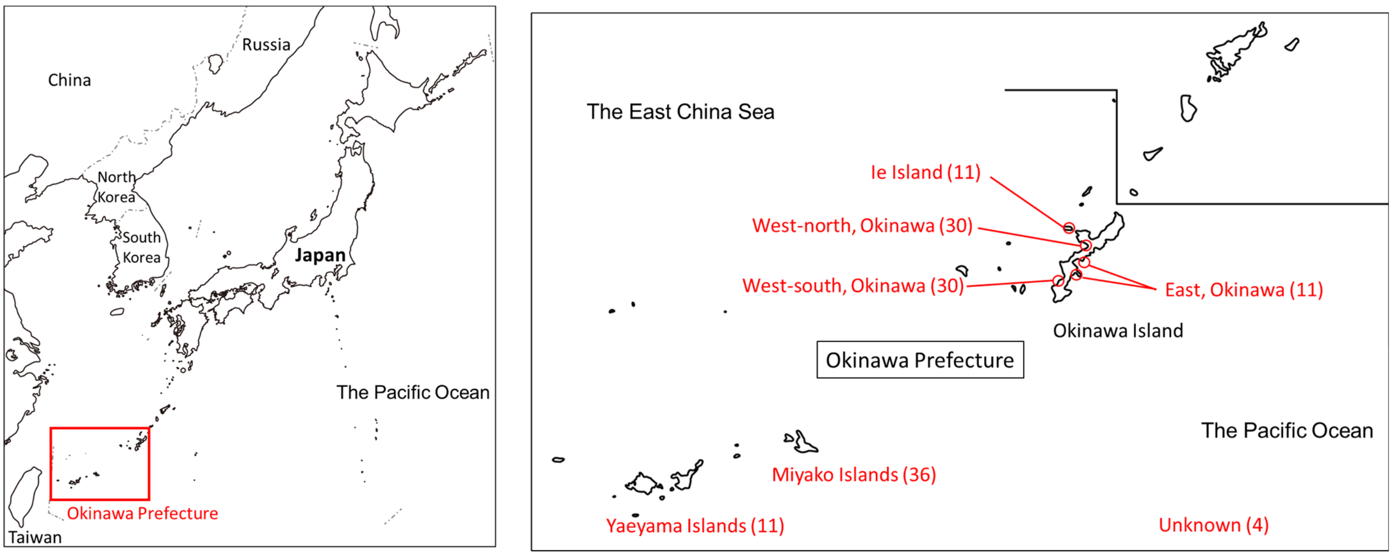
Here, we report the levels and profiles of CTXs in the flesh of the grouper *V. albimarginata* determined by an LC–MS/MS method. The fish were captured within the waters of Okinawa. We also collected the biological data of the fish.

2. Materials and Methods

2.1. Fish Specimens

Specimens of fresh fish (133) were purchased or collected at the local wholesale fish markets located in Okinawa, Ie, Miyako, and the Ishigaki islands between July 2013 and September 2016 (Table S1; Figure 3). Ice-cooled specimens were brought to the University of the Ryukyus, and the species were identified on the basis of their morphological characteristics. The specimens were measured for the standard length (SL) and body weight (BW). The following equation calculated the specimens' condition factors (CFs) [33,34].

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



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

Figure 3. The location of collection sites of *Variola albimarginata* specimens from the Okinawan Islands, Japan. (**a**) Location of Okinawa; (**b**) Expansion of the red rectangle in (**a**). Numbers in parentheses indicate the number of specimens collected from each area.

To estimate the age of each fish, thin sections of approximately 0.55 mm were prepared from otoliths (squamosal stones) taken from the fish head, and the opaque bands of the thin sections of the otoliths were counted under a stereomicroscope [35–37]. The sex of the individual fish was determined by observing tissue sections prepared from the gonads under an optical microscope.

Portions of the flesh (ca. 100 g each) of the specimens were stored at −20 °C in a plastic bag and transported under a frozen condition to the National Institute of Health Sciences (NIHS) for LC–MS/MS analysis.

2.2. Reference Toxins and Reagents

The CTX-Mix solution prepared at NIHS contained eight CTX congeners (NIHS-CTX-Mix), CTX1B; 52-*epi*-54-deoxyCTX1B; 54-deoxyCTX1B; CTX4A; CTX4B; 2,3-dihydroxyCTX3C; 51-hydroxyCTX3C; and CTX3C, isolated from natural sources [29,32]. The concentrations of the former three congeners (CTX1B, 52-*epi*-54-deoxyCTX1B, and 54-deoxyCTX1B) in the NIHS-CTX-Mix solution were determined by comparing with the NMR-quantified standard of Ciguatoxin-1B (43.3 $\pm$ 1.3 ng) provided by the JFRL (JFRL-RM-CTX) [26].

Acetone (Primepure), diethyl ether (special grade), methanol (LC–MS grade), hexane (Primepure), and acetonitrile (LC–MS grade) were purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). Ethyl acetate, ammonium formate solution (1 mol/L), and formic acid of HPLC grade were the products of Fujifilm Wako Pure Chemical Industry, Ltd., Osaka, Japan. Ultrapure water (Q-POD) was produced by Milli-Q® Integral Water Purification System (Millipore, Bedford, MA, USA).

2.3. Analysis by LC–MS/MS System

The methods we followed for preparing sample solutions from fish flesh (Figure S1) and the parameters for LC–MS/MS measurements (Table S2) were the same as those in our previous papers [29,30,32]. The limit of detection (LOD, S/N > 5) and the lower limit of quantification (LOQ, S/N > 10) for each CTX using this method were estimated to be 0.001 µg/kg and 0.005 µg/kg, respectively [29,30,32].

2.4. Toxicity Evaluation

The toxicity equivalency factors (TEFs) of CTX1B, 52-*epi*-54-deoxyCTX1B, and 54-deoxyCTX1B were proposed by the European Food Safety Authority (EFSA) as 1, 0.3, and 0.3, respectively, based on intraperitoneal (i.p.) administration to mice [38]. However, an FAO/WHO expert meeting concluded that due to limited data from in vivo oral studies, it was impossible to derive TEFs [1]. Therefore, we evaluated fish toxicity mainly on the basis of the total CTX level, as we did in previous papers [27–29,31].

2.5. Statistical Analysis

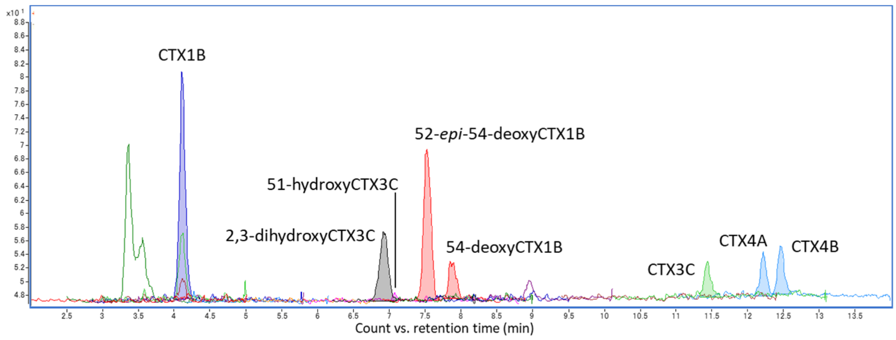
The obtained data were evaluated using the statistical analysis software EZR ver. 1.55 provided by the Saitama Medical Center, Jichi Medical University (https://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmed.html (accessed on 12 January 2023)) [39]. Spearman's rank correlation test helped evaluate the correlation, and the statistical significance was analyzed by the non-parametric Mann–Whitney U test.

3. Results

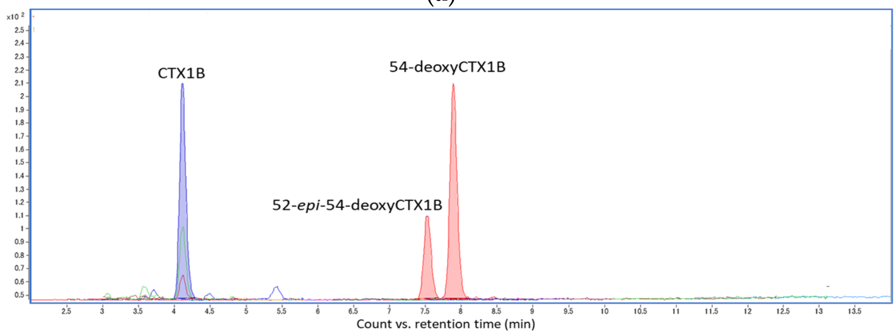
CTXs were detected in 28 (21%) of the 133 specimens of *V. albimarginata* captured in Okinawan waters (Table 1, Table S1). Only three toxins, CTX1B, 52-*epi*-54-deoxyCTX1B, and 54-deoxyCTX1B, were detected (Figure 4). Toxins from a low trophic level, CTX4A, CTX4B, and CTX3C, were not detected, in accordance with our previous observation on carnivorous fish, represented by the grouper *V. louti* [29,32] and snappers *L. bohar* and *L. monostigma* [24,25].

Table 1. The CTX levels in the flesh samples of grouper *V. albimarginata* collected off Okinawa, Japan.

Area	Total CTX Levels (μg/kg)								
	Total	**<0.003**		**0.003–0.010**		**0.011–0.17**		**≥0.18**	
Ie	11	7	64%	2	18%	2	18%		
West-north, Okinawa	30	26	87%	3	10%	1	3%		
West-south, Okinawa	30	26	87%	2	7%	1	3%	1	3%
East, Okinawa	11	10	91%	1	9%				
Miyako	36	32	89%	2	6%	2	6%		
Yaeyama	11	3	27%	6	55%	2	18%		
Unknown	4	1	25%	1	25%	2	50%		
Total	133	105	79%	17	13%	10	8%	1	1%



(**a**)



(**b**)

Figure 4. LC–MS/MS chromatograms of reference CTX-Mix solution (**a**) and *V. albimarginata* specimen, NIHS-Cig-153063 (**b**).

Of 28 specimens positive for CTXs, in 11 specimens (9%), the CTX levels exceeded the FDA guidance level (0.01 μg/kg CTX1B equivalent), and in 1 specimen (9%), the CTX level exceeded the recommended level (0.175 μg/kg CTX1B equivalent) in Japan (Table 1, Table S1). This specimen (NIHS-Cig-153065), purchased from the southwest coast of Okinawa Island, showed a total CTX level of 0.225 μg/kg (Table 1, Table S2).

Significant differences in total ciguatoxins were observed among the specimens collected from Yaeyama and the other locations using the non-parametric Mann-Whitney U test: the northwest coast of Okinawa Island (p = 0.0092), the southwest coast of Okinawa Island (p = 0.0229), the east coast of Okinawa Island (p = 0.0449), and Miyako (p = 0.0022) (Figure 5a).

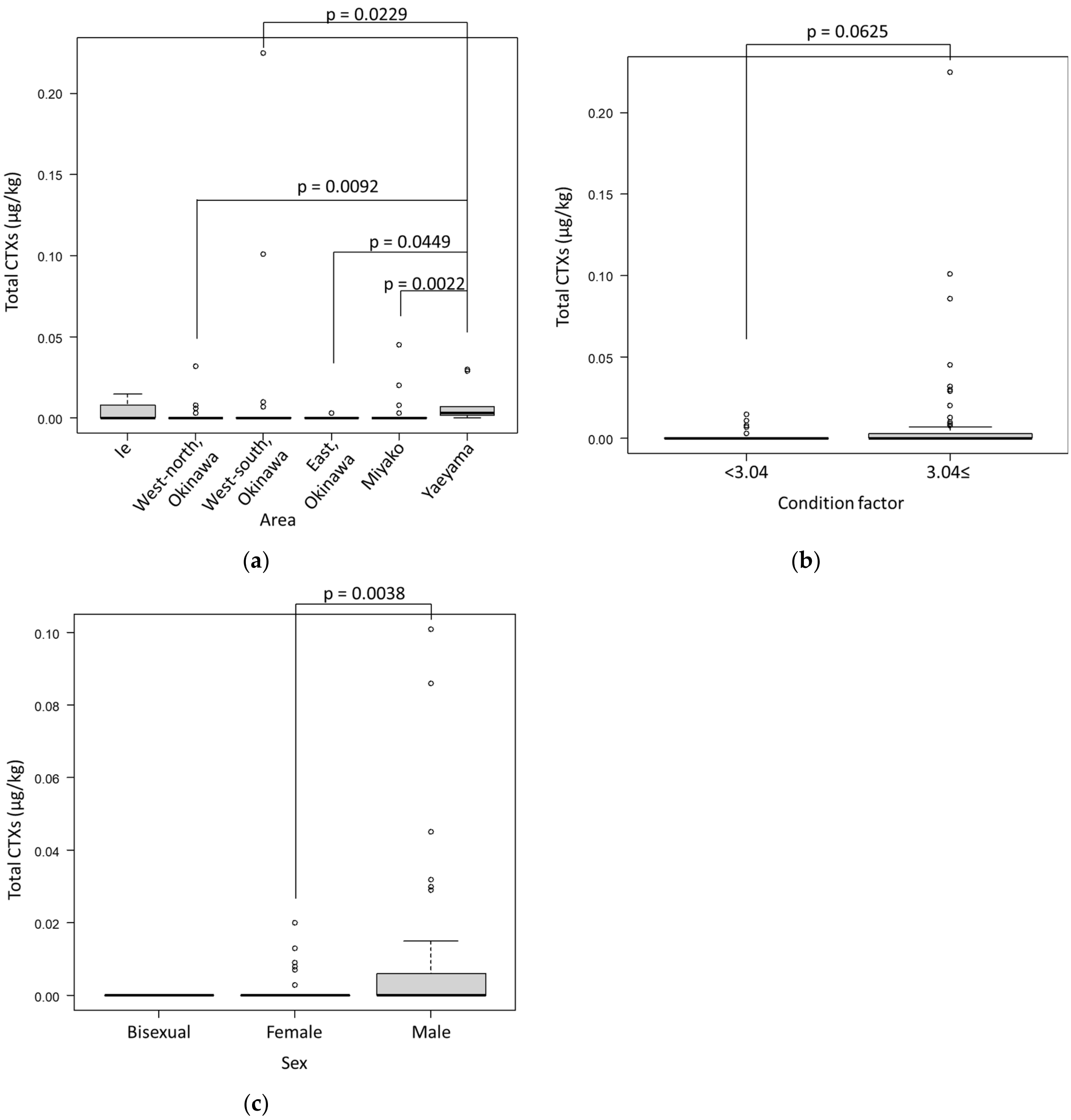


Figure 5. Distribution of total CTXS levels in the flesh of *V. albimarginata* captured from Okinawa, Japan, by (**a**) collected area, (**b**) condition factor (the median (3.04) was used as a criterion to divide the population into two groups), and (**c**) sex. The significances ($p < 0.05$) were found and indicated with a *p*-value in (**a**,**c**). No significance was found, but the *p*-value was indicated in (**b**).

The SL ranged from 13.0 to 33.7 cm (mean, 22.8 cm; median, 22.9 cm) and was positively correlated with the total CTX level (r = 0.488, $p = 2.61 \times 10^{-9}$; Figure 6a). The BW ranged from 61 to 1251 g (average, 398 g; median, 360 g) and was positively correlated with the total CTX level (r = 0.491, $p = 2.01 \times 10^{-9}$; Figure 6b). The CF ranged from 2.45 to 3.64 (mean, 3.03; median, 3.04) and showed no correlation with the total CTX contents (r = 0.13, $p = 0.135$; Figure 6c). When the median CF was used as a criterion to divide the population into thin (<3.04) and fat (3.04≤), there was no significant difference in the total CTX content between the two groups ($p = 0.0625$). Still, the individuals with the highest total CTX levels were included in the fat group (Figure 5b). The 130 individuals whose ages could be estimated ranged from 1 to 15 years old (mean, 5.3 years; median, 5 years), and the age was positively correlated with total CTX levels (r = 0.403, $p = 2.03 \times 10^{-6}$; Figure 6d). The

123 sex-discriminated individuals consisted of 76 females, 45 males, and 2 bisexuals, and there was a significant difference in the total CTX content between the sexes ($p = 0.0038$; Figure 5c).

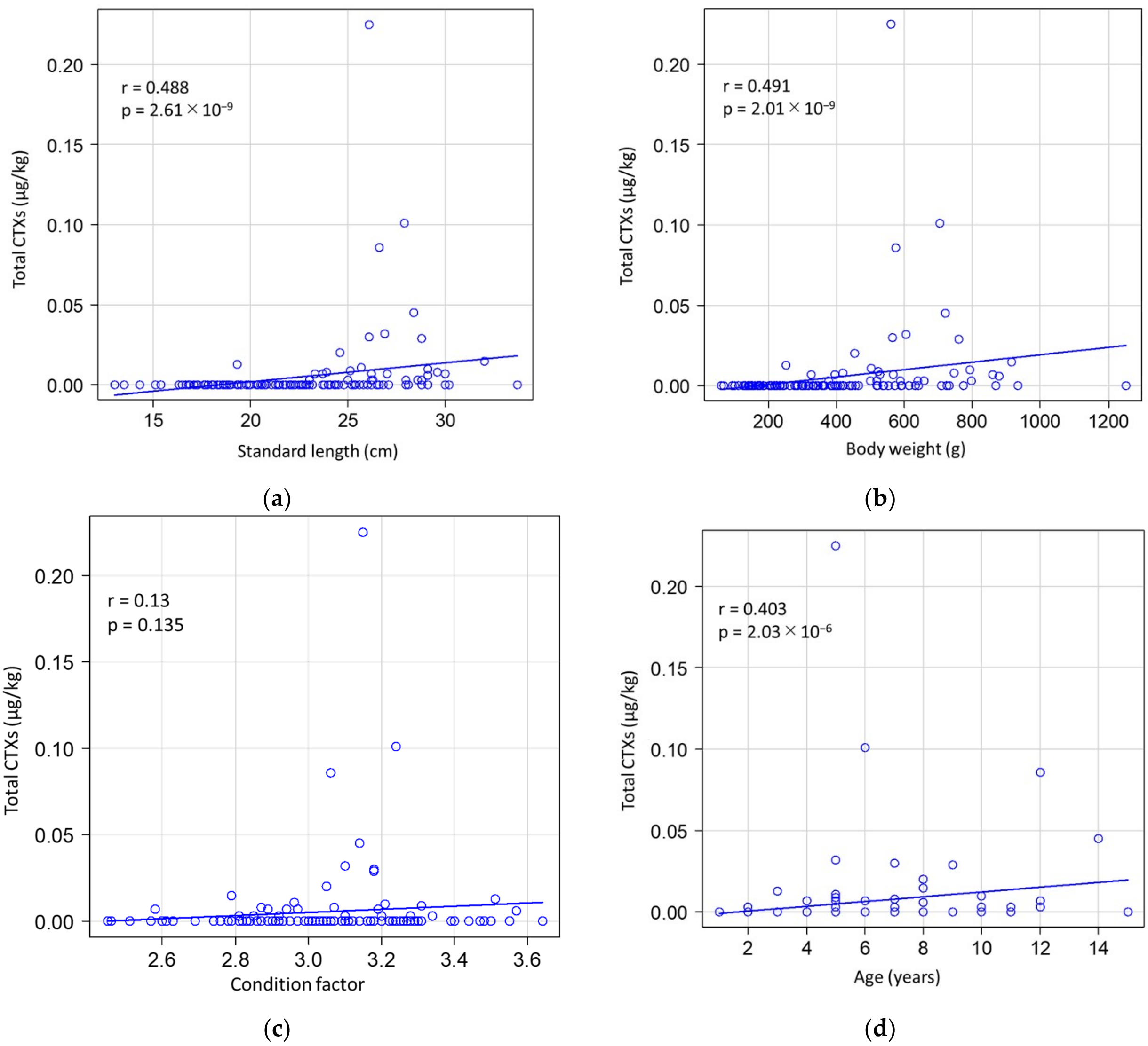


Figure 6. Relationship between biological data and total CTX levels in the flesh of *V. albimarginata* captured from Okinawa, Japan. (**a**) Standard length, (**b**) body weight, (**c**) age, and (**d**) condition factor.

4. Discussion

Of the 133 specimens of *V. albimarginata* from Okinawa, 28 specimens contained CTXs (21%), and 11 of them had CTX levels above the FDA guidance level (0.01 µg/kg CTX1B equivalent). Using the recommended level in Japan (0.175 µg/kg CTX1B equivalent), however, only one specimen was judged risky (NIHS-Cig-153065). This fish was relatively large, having all the indices above the median (Table S1). Nevertheless, the total CTX amount in this fish did not reach 10 MU (70 ng for CTX1B), the level thought to cause illness. This fish, with the highest CTX level, would likely have caused illness had more than 311 g of its flesh been consumed. However, as the weight of fillets available from one fish normally is between 1/3 and 1/2 of the BW, the maximum weight of flesh from this fish would be between 180 g and 280 g. Assuming that the entire filet was consumed by one person, the total CTX level consumed by that person would be below the level that could induce symptoms of CFP (70 ng for CTX1B). Thus, the risk of CFP by this species from Okinawa is probably low.

The correlation coefficients between the total CTX level and SL, BW, and age were low (0.403–0.491; Figure 6). In addition, many fish with low CTX levels were larger or older than the one with the highest CTX level. The CTX level of the largest and oldest individual was <LOD.

The total CTX levels in male specimens were significantly higher than those in female specimens (Figure 5C). In addition, the SLs, BWs, and ages of males were higher than those of females (Figure 7). In other words, the males were longer, heavier, and older than the females, suggesting the transfer of sexual features, resulting in female fish turning into male fish in *V. albimarginata* with growth similar to that in *V. louti* [29].

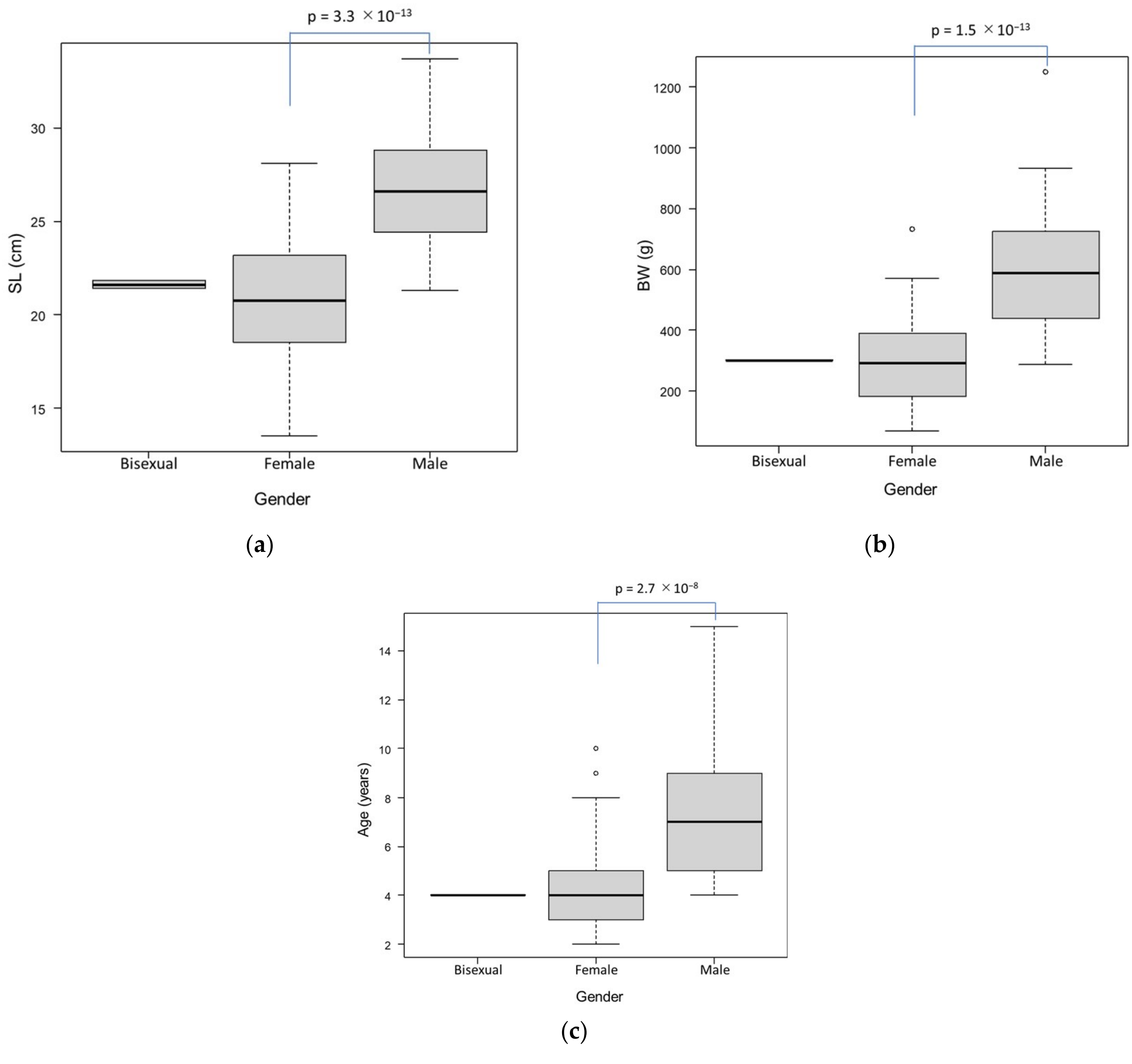


Figure 7. Distributions of (**a**) the standard length (SL), (**b**) body weight (BW), (**c**) and age relationship to the gender of the specimens of *V. albimarginata* captured from Okinawa. B: bisexual, F: female, M: male.

V. louti, another grouper, is a representative CFP-causing species in the Ryukyu Islands. However, the incidents caused by *V. albimarginata* are few (two out of 78 incidents from 1989 to 2011) [16]. Of the 154 individuals of *V. louti* captured from the Ryukyu Islands, 63% had CTX levels higher than the FDA guidance levels, of which three percent had CTX levels above the Japanese recommendation level [29]. The levels of CTXs in two species from Okinawa were significantly different (p = 3.78 $\times$ 10^{-14}), indicating the higher CTX levels in the *V. louti* specimens (Figure 8). The maximum CTX content (0.376 µg/kg) in *V. louti* is 1.67 times higher than that in *V. albimarginata* (0.225 µg/kg) [29]. In addition, since *V. louti* grows to a larger size than *V. albimarginata* [14], the amount of flesh available for a meal is also larger than that of *V. albimarginata*, leading to higher amounts of CTXs consumed by humans. The risk of CFP from consuming *V. albimarginata* was estimated to be low because of the low CTX level and the small amount available at a time for human consumption.

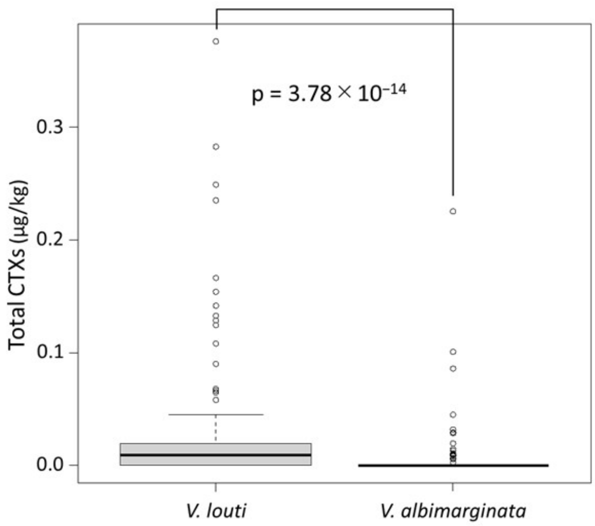


Figure 8. Distribution of total CTX levels in the flesh of *V. louti* [29] and *V. albimarginata* captured from Okinawa Islands and Amami Islands, respectively.

Ecologically, both species feed on fish and crustaceans and inhabit the inner, marginal, and outer coral reefs, with little relationship to coral coverage [40]. The larger the individual, the more likely it is to be found on the open ocean side of the reef. In addition, *V. albimarginata* inhabits deeper waters than *V. louti* [15]. Therefore, the depth of distribution of the two species may be one of the factors causing the difference in CTX levels.

Reports from Okinawa Prefecture estimate the annual catch of *V. albimarginata* to be 2475.5 kg from around Okinawa Island [41] and 2433.5 kg from around Yaeyama Islands [42]. The total catch, 4909 kg, corresponds to 13,636 fish, based on the median weight of 360 g. *V. albimarginata* has been implicated in only two incidents in the 22 years spanning 1989–2011 [16]. However, the number of reported CFPs is assumed to represent only 2–10% of the actual occurrences [2]. Therefore, the actual number of incidents due to *V. albimarginata* is between 20 and 100 (0.9 to 4.5 incidents/year). The number of fish with CTX levels exceeding the guidance level of the FDA or the level recommended for safety in Japan would be 1227 and 136 fish/year, respectively. Considering the frequency of the incidents and the detected levels of CTXs in *V. albimarginata*, the FDA guideline level (0.01 µg/kg CTX1B equivalent) could be too severe to manage the risk of *V. albimarginata*, and risk management based on the recommendation in Japan (0.175 µg/kg CTX1B equivalent) would be more realistic, as we have previously described for *V. louti* [29]. Regarding the contradictory information from other areas, standardization of the methods seems preferable: use of standard toxins, such as the JFRL-RM-CTX and NIHS-CTX-Mix, in LC–MS/MS analysis, and accurate identification of fish species supplemented by DNA analysis.

5. Conclusions

The flesh of 133 specimens of *V. albimarginata* captured in Okinawa was analyzed using LC–MS/MS. CTXs were detected in 28 specimens (21%). Of those, in 11 specimens, the CTX levels exceeded the FDA guidance level (0.01 µg/kg CTX1B equivalent), and in one specimen, the CTX level exceeded the recommended level in Japan (0.175 µg/kg CTX1B equivalent). The highest CTX level was 0.225 μg/kg CTX1B equivalent. However, it was calculated that the total CTXs in this specimen would not induce illness in a human. The CFP risk due to *V. albimarginata* was considered to be low, agreeing with the general belief in Okinawa. The level of the total CTXs in the flesh of *V. albimarginata* was lower than that in the flesh of *V. louti*.

Considering the annual consumption (estimated at 13,636 fish), the frequency of the occurrence of CFP (estimated as 0.9 to 4.5 incidents/year), and the detected levels of CTXs in the specimens of *V. albimarginata* from Okinawa, the Japanese level recommended for safe consumption (0.175 μg/kg CTX1B equivalent, 136 individuals/year) appears to be more realistic for managing CFP than the FDA guidance level (0.01 μg/kg CTX1B equivalent, 1227 individuals/year).

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jmse11020242/s1: Table S1: The specimens of Variola albimarginata analyzed in this study and their CTXs levels in the flesh, Table S2: LC-MS/MS condition for CTX analysis, Figure S1: Sample preparation from flesh for LC-MS/MS.

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Conflicts of Interest: The authors declare no conflict of interest.

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