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Qualitative and quantitative assessment of the presence of ciguatoxin, P-CTX-1B, in Spanish Mackerel (*Scomberomorus commerson*) from waters in New South Wales (Australia)

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ABSTRACT

Ciguatera Fish Poisoning (CFP) is a tropical disease caused by the consumption of fish contaminated with ciguatoxins (CTXs). Currently, the only feasible prevention methods for CFP are to avoid the consumption of fish of certain species from some regions, avoid larger fish of certain species, or avoid all fish caught from specific regions. Here, we quantified levels of P-CTX-1B in Spanish Mackerel (*Scomberomorus commerson*), which is the main fish species that causes CFP in New South Wales and Queensland, Australia, using LC–MS detection against a toxin standard. We found detectable P-CTX-1B in both flesh and liver tissues in fish from New South Wales (n = 71, 1.4% prevalence rate, with a confidence interval of 1%–4%, and 7% prevalence, 1%–12%, in flesh and liver, respectively). In the small sample of fish from Queensland, there was a 46% prevalence (19–73%, n = 13). Toxin levels found were 0.13 μg kg^{-1} to < 0.1 μg kg^{-1} in flesh, and 1.39 μg kg^{-1} to < 0.4 μg kg^{-1} in liver, indicating that liver tissue had a significantly higher concentration (~5 fold) of P-CTX-1B. No apparent relationship was observed between the length or weight of *S. commerson* and the detection of P-CTX-1B in this study.

Footnote

1. Introduction

Ciguatera Fish Poisoning (CFP) is the most common non-bacterial illness associated with fish consumption internationally [1], impacting 50,000–500,000 people per year [2]. In Australia, CFP is recognised as one of two major safety risks linked to Australian seafood products [3]. Species of the marine dinoflagellate (single-celled microbial eukaryote) *Gambierdiscus* are the main producers of Ciguatoxins (CTXs) [4–7]. CTXs accumulate in the food web when *Gambierdiscus* cells attach to surfaces, such as macroalgae, and are consumed by herbivorous fish, which are then preyed on by carnivorous fish. The ingestion of contaminated herbivorous and carnivorous fish by humans causes CFP [8,9]. CFP can present with a range of gastrointestinal, neurological and sometimes cardiovascular (in cases of severe intoxication) symptoms [9]. Despite being significantly underreported, CFP occurrence worldwide appears to be increasing, with reports of a 60% increase in the Pacific region over the last decade [10]. CFP predominantly occurs in mid-latitude tropical and sub-tropical zones, in accordance with the distribution of *Gambierdiscus* [11] and references therein). However, CFP has also been reported in non-endemic areas because of an increase in seafood imports [12,13].

The accurate identification of the exact congeners of CTXs present in fish and their toxicity is necessary to evaluate the risks of CFP in a particular region. Structurally, CTXs are thermostable and liposoluble, cyclic polyether ladders. CTXs are classed as P-CTXs (Pacific Ocean), C-CTXs (Caribbean region) and I-CTXs (Indian Ocean), based on their origin and differences in the structure of these toxins. P-CTXs are further classes as type I and type II, due to their structural differences [14].

Abbreviations: CFP, Ciguatera Fish Poisoning; CTX, Ciguatoxin; LC–MS, Liquid chromatography mass spectrometry; MTX, Maitotoxin; NMR, Nuclear magnetic resonance; NSW, New South Wales; NT, Northern Territory; P-CTX-1B, Pacific Ciguatoxin 1B; QLD, Queensland; RLB, Radio ligand binding; SFM, Sydney Fish Market; SIMS, The Sydney Institute for Marine Science; US-FDA, United States Food and Drug Administration; WA, Western Australia

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Type I P-CTXs have 13 rings and 60 carbon atoms [15–18] and category consists of the first CTX to be fully structurally described as CTX-1B [15] (or CTX-1 as described by [16] from Moray Eels. P-CTX-1B is the principal toxin in the carnivorous fish from the Pacific [19,20]. Experimentally, CTX toxin profiles have been determined by chromatographic techniques (HPLC, UPLC and LC–MS) [21,15,16,22–24]. Confirmation of toxins by UPLC/HPLC followed by LC–MS involves the isolation and fractionation of CTX compounds, of known molecular weights.

The US Food and Drug Administration (FDA) has published guidance levels for CTXs in foods, suggesting that 0.01 ppb CTX equivalent of Pacific CTX–1 B (0.01 μg kg^{-1} CTX) in fish flesh or lower amounts are safe for human consumption (USFDA, 2011). Biological assays have been developed for the detection of the total toxicity of the fish samples, including the mouse bioassay [25], the receptor binding assay [26] and cytotoxicity assays using neuroblastoma cells (N2A) [27] or in vitro human erythrocyte cells [7]. However, these assays cannot be used to quantify with certainty the levels of specific congeners of CTXs in fish tissue, such as the levels of P-CTX-1B, as required to compare the concentration in samples to the US FDA guidance level. Given the technical issues with the available bioassays in comparison to the US FDA guidelines, in the present study, we decided to use LC–MS/MS analysis with a known and quantified standard for P-CTX-1B.

As a rapid, cost-effective and reliable screening test for CTXs does not exist, health authorities around the world have mostly provided guidelines to prevent high-risk fish from entering the commercial market to reduce the risk of CFP, including guidance on the size of fish of individual species that are more or less likely to contain CTXs [40]. This may be due to the fact that CTXs can bioaccumulate over time, and therefore older and larger fish might be considered more likely to have higher levels of CTXs. However, relatively few studies have directly examined the evidence for a relationship between fish size and CTX presence for individual fish species. A study in Japan found a positive relationship of size vs toxicity in specimens of *Lutjanus monostigma*, *Epinephelus fuscoguttatus*, *Lutjanus bohar* and *Variola louti* [28]. In another study, *Sphyraena barracuda* liver samples from the Caribbean were analysed using the neuroblastoma cell assay (N2A), and no relationship between fish size/weight and toxicity was observed among 40 samples [29].

In Australia, the main fish species that has been associated with the majority of reported CFP illnesses is Spanish Mackerel (*Scomberomorus commerson*) [3]. In the state of New South Wales (NSW), confirmed CFP cases linked to the consumption of Spanish Mackerel caught locally have been reported from Brunswick Heads in 2002, Evans Head in February 2014 (4 people), Scott's Head in March 2014 (9 people) and South West Rocks in April 2015 (4 people) [30]. All affected people were diagnosed with CFP as they suffered classic CFP symptoms [30]. Many of those involved required hospitalisation, and at least one victim was disabled for at least seven months [30]. The NSW CFP cases in 2014–2015 are the southernmost confirmed sources of CFP in Australia [31]. These cases generated significant concern among the commercial and recreational fishing communities, highlighting the need to determine appropriate management strategies to prevent CFP illnesses in Australia. Given the need to understand the distribution and abundance of fish contaminated with CTXs in NSW, Australia, the objectives of this research were to: 1) Determine if CTXs are present in Spanish Mackerel (*Scomberomorus commerson*) caught in NSW waters, and if so, generate qualitative and quantitative information; and 2) If found, analyse data on CTX presence and concentration in relation to: fish size, location of the catch, date, and water temperature.

2. Materials and methods

2.1. Fish sampling

Approximately 400 sampling packs were distributed to the Sydney Fish Market, Byron Bay Deep Sea Fishing club, Coffs Harbour Deep Sea Fishing club and the following fishing co-operatives across the Northern NSW coast: Coffs Harbour, Evans Head, Ballina and Brunswick. These clubs and locations were chosen because: 1) Recreational fishing for Spanish Mackerel is significant in northern NSW, and may represent up to 90% of the total catch; and 2) The vast majority of the Spanish Mackerel catch in NSW comes from these regions. For comparison, samples packs were also distributed to a recreational fishing group in Far Northern QLD. Fish from this region are also considered part of the east coast Spanish Mackerel stock.

The sample pack consisted of several labelled tubes, which could each contain ~10 g of liver or muscle (flesh) tissue samples. Sample packs were given out to commercial and recreational fishing groups in northern NSW during January–March 2015. Following sample collection, samples were stored at −20 °C until further analysis. The date of catch, length from head to tail, location of catch and weight of each specimen were recorded.

2.2. Toxin analysis via LC–MS/MS

Each tissue sample was chopped using a scalpel blade and 5 ± 0.1 g biomass was weighed, and placed in a 50 mL centrifuge tube. The samples were extracted using the method described in (Boundy M and Harwood T, 2017, unpublished data). The tissue samples were solvent extracted, lipids removed via solvent partition and the CTX containing fraction purified using normal phase SPE separation. The samples were dried under nitrogen stream. The dried samples were resuspended in an organic solvent, purified using normal phase SPE separation and stored at −80 °C until LC–MS/MS analysis. Analysis of the fish extracts was performed at the Cawthron Institute (New Zealand) using a triple quadrupole LC–MS/MS instrument using the method described in (Boundy M and Harwood T, 2017, unpublished data). For detection of P-CTX-1B, a target-mass of m/z 1128.6102 was extracted with a mass tolerance of 5 ppm, which is consistent with the ammoniated-adduct ($[M + NH_4]^+$) of the target molecule. For quantitation, peak areas were integrated and sample concentrations calculated from linear calibration curves generated from standards. TargetLynx software was used for the analysis (Water- Micromass, Manchester, UK).

2.3. Spike recovery

To ensure a satisfactory performance of the method, numerous flesh and liver samples were analysed in duplicate, with one of the samples spiked with a known amount of P-CTX-1B standard (11 of 168 samples). The spiking of samples with CTX was for calibration purposes only, and these results were not included in the final concentrations. Mean recoveries were calculated for each matrix and applied to the toxin concentration determined in samples. The P-CTX-1B spiking solution was provided by the Cawthron Institute, with a given concentration of 68.2 ng/mL. Additionally, for instrument calibration, the Cawthron Institute provided three standard solutions of P-CTX-1B-concentrations: 0.341 ng/mL, 1.705 ng/mL and 3.41 ng/mL. These calibration standards were analysed at the same time as the various fish samples and were used to create a calibration curve. The concentration of P-CTX-1B was calculated by comparing the peak areas observed in contaminated fish samples with the calibration curve generated at the time of analysis.

2.4. Spanish Mackerel identification via qPCR

To determine the identity of the fish specimens collected, DNA was extracted from 0.5 g of liver tissue of each specimen via the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). The manufacturer's protocol was followed and samples were stored at −20 °C until PCR amplification.

To determine whether the samples originated from the same species, *S. commerson,* a quantitative PCR was performed. All PCR reactions were performed in 20 µL reaction volumes containing 10 µL SYBR® Select Master Mix (Bioline, Eveleigh, NSW), 10 pmol each of the forward and reverse primers and between 10 and 100 ng genomic DNA. Primer-pair (TGGGCCGTCCTTATTACAGC, CTCCTCCTGCTGGGTCAAAG) specific for the cytochrome oxidase subunit I (COI) gene from *S.commerson,* were used [32]. Cycling conditions were a 95 °C holding stage for 10 min, followed by 35 cycles of 95 °C for 15 s and 60 °C for 1 min, followed by a melt curve analysis of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 30 s.

3. Results

3.1. P-CTX-1B detection using LC–MS/MS

The performance of the method established to quantify P-CTX-1B in samples was assessed and linear calibration over concentration range tested. The inter- and intra-day variability in sample quantification was evaluated. Spiking experiments (spiking P-CTX-1B standard in fish liver and flesh samples) were carried out to calculate the toxin recovery rate, variance and standard deviation to monitor potential matrix impacts.

As seen in Table 1, the recovery rates of P-CTX-1B varied between individual samples. In both liver samples (samples 8 and 10), no detectable amount of P-CTX-1B was found. Therefore, a correction of the toxin values was needed.

To obtain realistic results, the calculated concentration was corrected using the recovery rate factor, which represents the amount of implemented standard that can be detected on average after the extraction and measurement of toxin. The average recovery rate was determined to be 25.83%. Therefore, a factor of 3.87 was used to adjust the measured value in the *S. commerson* samples to the real values calculated using the equation r = 100/25.83 = 3.87, where r is the recovery rate factor. The variability of the calculated concentration was $s^2 = 0.232$, with a standard deviation of s = 0.481. The variability of the recovery rate was $s^2 = 800.28$, the standard deviation is s = 28.29. This implies considerable fluctuations of ± 0.48 ng/mL or ± 28.29% for presumed equal conditions resulting from the effect of either the extraction method or the measurement setup.

3.2. Detection of P-CTX-1B in S. commerson samples

In total, 84 samples of *S. commerson* were collected (71 from NSW and 13 from QLD). Most of the samples returned came from the recreational fishing community, who showed a relatively high level of engagement. Despite the comparatively low sample collection from sections of the commercial fishing community, we consider this a relatively good rate of return, considering that the sample collection required a high commitment from participants. We confirmed the identity of every specimen as *S. commerson*, as all fish liver samples showed amplification with the qPCR assay, specific for *S. commerson*, described in section 2.4.

Samples from recreational fishers in Coffs Harbour were the most numerous (Table 2). This reflected the fact that this is the largest and most active recreational fishing community in the area; that Spanish Mackerel are taken in greater numbers by recreational fishers as compared to commercial fishers in NSW; and that this mid-far north coast region is the region from which the vast majority of Spanish Mackerel are caught in NSW. Spanish Mackerel are infrequently caught south of Port Macquarie in NSW, such that the ~400 km stretch of coastline from Port Macquarie to the Queensland Border, in which all fish in this study were caught, represents almost the complete region for the Spanish Mackerel fishery in NSW.

From 71 fish specimen collected in NSW, liver and flesh tissues from one fish (Fig. 1, Table 2) and liver tissues from 4 other fish specimens were positive for P-CTX-1B (Fig. 2, Table 2). Whereas, from the 13 fish specimen collected in QLD, liver and flesh tissues from five fish and flesh from one other fish specimen were positive for P-CTX-1B (Table 2).

Table 1
Recovery rates as percentage from the debit.

Matrix	Retention time [min]	Peak area	Calculated concentration [ng/mL]	Percentage of measured concentration to debit [%][1]
Flesh	2.4	46.974	0.93948	55.26
Flesh	2.39	15.084	0.30168	17.75
Flesh	2.33	22.245	0.4449	26.17
Flesh	2.31	12.042	0.24084	14.17
Flesh	2.31	72.346	1.44692	85.11
Flesh	2.31	3.821	0.07642	4.50
Liver	2.38	10.417	0.20834	12.22
Liver	ND[2]	ND[2]	NC[3]	NC[3]
Liver	2.38	1.796	0.03592	2.11
Liver	ND[2]	ND[2]	NC[3]	NC[3]
Liver	2.31	57.000	1.14	66.87

[1] debit = 1.705 ng/mL.
[2] ND = not detected.
[3] NC = not calculated.

3.3. Determination of species, and size-to-toxin content ratio in toxic fish

To determine any measurable relationship between the size of *S. commerson* caught versus the level of P-CTX-1B found in liver and flesh samples, data from fish collected in NSW, QLD and from previous 2014–2015 CFP incidents in NSW were pooled together. No noticeable correlation was observed (Figs. 3 and 4). Although the levels measured in the *S. commerson* samples were quite low, they were higher than the US FDA's level considered safe for human consumption (0.01 µg kg^{-1} CTX equivalent for P-CTX-1B). Fig. 5 shows the relationship between the weight and length of toxic/non-toxic specimens of Spanish Mackerel, showing that specimens of toxic Spanish Mackerel are distributed evenly among all size categories. The trend line in the graph represents the mean of the length versus mass relationship of all Spanish Mackerel, demonstrating that toxic specimens of Spanish Mackerel tend to be lighter for their length as compared to the non-toxic specimens of Spanish Mackerel. The reason for this trend is unclear, and may require further analysis.

3.4. P-CTX-1B levels associated with CFP illness

From the literature and our own data, we have compiled information on the P-CTX-1B levels in any fish known to be associated with CFP illnesses in Australia (Table 3) and overseas (Table S1). The data shows that levels above ~0.1 µg kg^{-1} have been known to be associated with illness, with mean levels found in implicated fish flesh of 1.2 µg kg^{-1} (from 6 Australian samples) and 1.3 µg kg^{-1} (from 16 overseas samples) (Tables 3 and S1). This compares to the US FDA 'guidance level' of 0.01 µg kg^{-1}, which was established due to the consideration that levels above 0.1 µg kg^{-1} may cause illness, based on the results of the mouse bioassay [16].

4. Discussion

CFP is a well-known disease in the warmer waters of Australia along the coastline of Queensland and the Northern Territory (NT), down to Byron Bay in NSW (~28°S). We found no evidence of confirmed reports of CFP from Western Australia. Most CFP outbreaks have resulted from fish caught in QLD and the NT (eastern Arafura Sea), with most of the documented cases involving Spanish Mackerel [35,3,30]. Prior to 2014, almost all cases of CFP in NSW or Victoria have been caused by fish from QLD or the NT, or fish imported from other countries [30].

Table 2
LC–MS/MS analysis of P-CTX-1B in samples of S. commerson flesh and liver collected for this study.

Location	Date of Catch	Length (cm)	Weight (kg)	P-CTX-1B in flesh ($\mu g\ kg^{-1}$)[1]	P-CTX-1B in liver ($\mu g\ kg^{-1}$)[1]
Davies Reef, QLD	2/01/15	149	21	ND	ND
Davies Reef, QLD	2/01/15	105	6	ND	ND
Port Douglas, QLD (14°.47.88S 149°.25.18E)	12/01/15	134	13.5	< 0.1	< 0.4
Port Douglas, QLD (14°.47.88S 149°.25.18E)	–	136	16	0.13	1.39
Great Barrier Reef, Rockhampton, QLD (22°.00.48S 152°.38.85E)	23/01/15	110	6.3	< 0.1	ND
Whitsundays, QLD (Reef No: 19-138)	12/01/15	106	6.1	< 0.1	< 0.4
Whitsundays, QLD (Reef No: 19-138)	13/01/15	120	11.9	< 0.1	< 0.4
Townsville, QLD (19°.47.88S 144°.25.18E)	12/01/15	117	11.2	< 0.1	< 0.4
Whitsundays, QLD (20°.01.45S- 149°.41.02E)	13/01/15	103	5.8	ND	ND
Brunswick Heads, NSW	2/02/15	120	8	ND	ND
Mooloolaba, QLD	6/01/15	96	6	ND	ND
Port Bundaberg, QLD	18/12/14	120	9.4	ND	ND
Mooloolaba, QLD	14/01/15	149	24	ND	ND
Mooloolaba, QLD	16/01/15	133	17	ND	ND
Coffs Harbour, NSW	12/02/15	110	12	ND	ND
Split Island, Coffs Harbour, NSW	19/02/15	125	12.2	ND	ND
Lighthouse, Coffs Harbour, NSW	10/02/15	130	13.6	ND	ND
Patch, Coffs Harbour, NSW	2/03/15	131	13.3	ND	ND
Patch, Coffs Harbour, NSW	2/03/15	130	12.5	ND	ND
Lighthouse, Coffs Harbour, NSW	12/02/15	120	11.1	ND	ND
Coffs Harbour, NSW	23/01/15	110	12	ND	ND
South Solitary Island, Coffs Harbour, NSW	26/02/15	128	15.8	ND	ND
Patch, Coffs Harbour, NSW	2/03/15	124	11.2	ND	ND
South Solitary Island, Coffs Harbour, NSW	26/02/15	143	20.5	ND	ND
Coffs Harbour, NSW	28/02/15	125	11.2	ND	ND
Evans Head, NSW	5/03/15	150	23.6	ND	ND
Evans Head, NSW	28/04/15	129	13.5	ND	ND
Black Head, NSW	26/03/15	129	13.1	ND	ND
Evans Head, NSW	28/04/15	127	12.5	ND	ND
Ballina, NSW	12/03/15	128	11.2	ND	< 0.4
Evans Head, NSW	28/04/15	124	12.5	ND	ND
Ballina, NSW	12/03/15	142	19.5	ND	< 0.4
Brunswick Head, NSW	26/03/15	110	10.5	ND	ND
Brunswick Head, NSW	21/03/15	120	13	ND	ND
Brunswick Head, NSW	9/04/15	110	11	ND	ND
Brunswick Head, NSW	27/03/15	120	12	ND	ND
Brunswick Head, NSW	5/04/15	90	9	ND	ND
Brunswick Head, NSW	21/01/15	90	9	ND	ND
Brunswick Head, NSW	14/02/15	100	10	ND	ND
Brunswick Head, NSW	26/01/15	95	9	ND	ND
Brunswick Head, NSW	29/03/15	110	8	ND	ND
Byron Bay, NSW	19/04/15	80	4.5	ND	ND
Byron Bay, NSW	19/04/15	90	6	ND	ND
Byron Bay, NSW	4/03/15	120	12	ND	ND
Byron Bay, NSW	4/03/15	95	7	ND	ND
Coffs Harbour, NSW	18/04/15	124	15	ND	ND
Coffs Harbour, NSW	20/03/15	95	10	ND	ND
Coffs Harbour, NSW	20/03/15	98.5	7	ND	< 0.4
Coffs Harbour, NSW	20/03/15	100	12	ND	ND
Coffs Harbour, NSW	23/03/15	95	9	ND	ND
Coffs Harbour, NSW	26/03/15	90	8	ND	ND
Coffs Harbour, NSW	26/03/15	100	12	ND	ND
Solitary Island, Coffs Harbour, NSW	2/04/15	135	12	ND	ND
Coffs Harbour, NSW	23/04/15	110	11.5	ND	ND
Coffs Harbour, NSW	19/04/15	145	17.5	ND	ND
Coffs Harbour, NSW (30°. 17S 153°. 10E)	15/03/15	110	11	ND	< 0.4
Coffs Harbour, NSW (30°. 22S 153°. 50E)	31/03/15	120	12	ND	ND
Coffs Harbour, NSW (30°. 75S 153°. 10E)	15/03/15	115	11.5	ND	ND
Coffs Harbour, NSW (30°. 22S 153°. 50E)	31/03/15	130	19	ND	ND
Macqualies, Coffs Harbour, NSW	1/04/15	120	14.5	ND	ND
Coffs Harbour, NSW	2/04/15	129	18.7	ND	ND
Coffs Harbour, NSW	7/03/15	123	11	ND	ND
Coffs Harbour, NSW	29/03/15	140	14.7	ND	ND
Coffs Harbour, NSW	26/04/15	120	17	ND	ND
Coffs Harbour, NSW	30/05/15	110	11	ND	ND
Coffs Harbour, NSW	5/04/15	118	14.8	ND	ND
Coffs Harbour, NSW	5/04/15	127	19.8	ND	ND
Coffs Harbour, NSW	5/04/15	134	19.2	ND	ND
Coffs Harbour, NSW	19/04/15	131.5	16.2	ND	ND
Coffs Harbour, NSW	7/04/15	135	19.4	ND	ND
Coffs Harbour, NSW	3/04/15	132	18.9	ND	ND
Coffs Harbour, NSW	3/04/15	134.5	19	ND	ND

(continued on next page)

Table 2 *(continued)*

Location	Date of Catch	Length (cm)	Weight (kg)	P-CTX-1B in flesh (μg kg^{-1})[1]	P-CTX-1B in liver (μg kg^{-1})[1]
Coffs Harbour, NSW	3/04/15	117	14.2	ND	ND
Coffs Harbour, NSW	3/04/15	135	19.4	ND	ND
Coffs Harbour, NSW	4/04/15	120	14.5	ND	ND
Coffs Harbour, NSW	6/04/15	130.4	16	ND	ND
Coffs Harbour, NSW	10/04/15	117	14	ND	ND
Coffs Harbour, NSW	14/04/15	134.5	19.2	ND	ND
Coffs Harbour, NSW	12/04/15	133	18.9	ND	ND
South Solitary Island, Coffs Harbour, NSW	30/05/15	142	16	< 0.1	< 0.4
North Solitary Island, Coffs Harbour, NSW	30/05/15	145	17	ND	ND
Forster, NSW	6/04/15	125	13	ND	ND
Forster, NSW	6/04/15	120	12	ND	ND
Coffs Harbour, NSW	31/03/15	134	14.6	ND	ND

ND: Not detected; NT: Not tested.

[1] LC–MS/MS analysis was performed at the Cawthron Institute, Nelson, New Zealand.

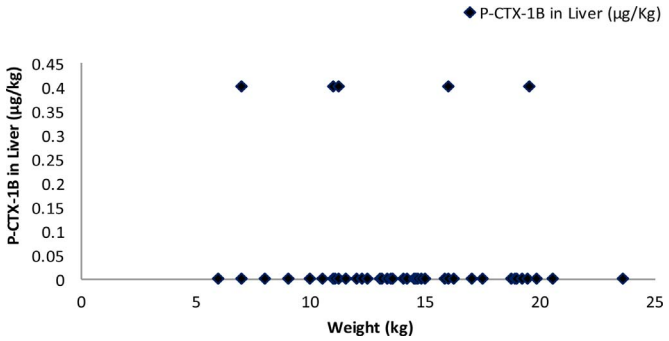

Fig. 1. Absolute quantification of P-CTX-1B in liver tissue of Spanish Mackerel from NSW in relation to the size of the fish (kg).

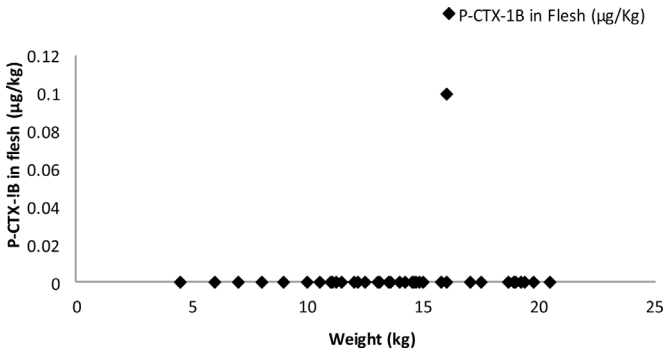

Fig. 2. Absolute quantification of P-CTX-1B in flesh tissue of Spanish Mackerel from NSW in relation to the size of the fish (kg).

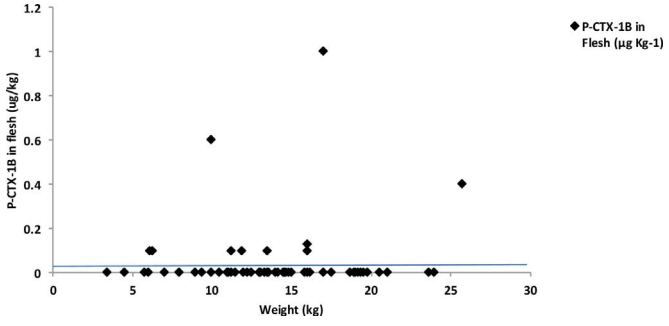

Fig. 3. Relationship between the weight and level of P-CTX-1B in flesh tissue of Spanish Mackerel caught in NSW, QLD and from previous 2014–2015 CFP incidents in NSW (n = 87). The blue line represents the US FDA level considered safe for human consumption.

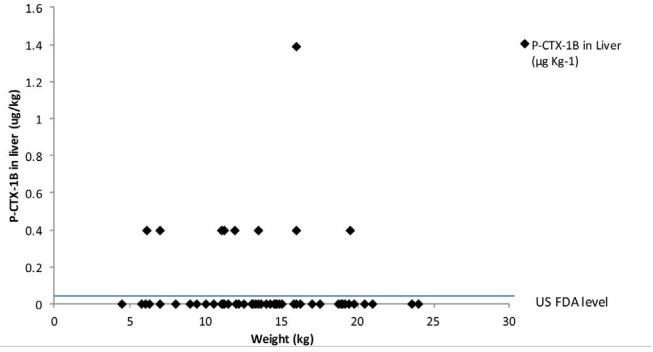

Fig. 4. Relationship between the weight and level of P-CTX-1B in liver tissue of Spanish Mackerel caught in NSW, QLD and from previous 2014–2015 CFP incedents in NSW (n = 87). The blue line represents the US FDA level considered safe for human consumption.

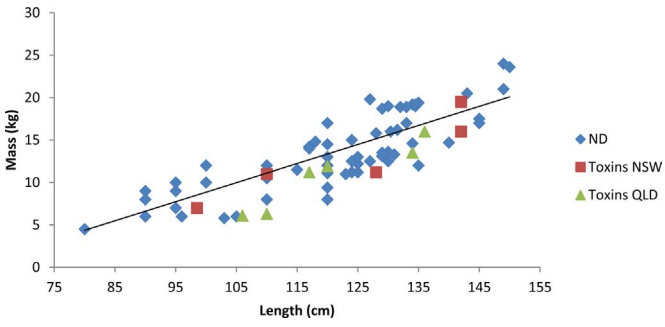

Fig. 5. Relationship between the weight and length of toxic/non-toxic Spanish Mackerel caught in NSW, QLD and from previous 2014–2015 CFP incidents in NSW (n = 87). (ND: fish specimen in which P-CTX-1B levels were not detected; Toxins NSW: fish specimen in which P-CTX-1B levels were detected in fish caught in NSW; Toxins QLD: fish specimens in which P-CTX-1B levels were detected in fish caught in QLD).

We have provided the first evidence that P-CTX-1B toxins are present in a random sample of Spanish Mackerel from NSW waters. The results indicate that these toxins occur in fish not previously known to be associated with any CFP illnesses. So far, the only Spanish Mackerel from NSW that had been tested and found to carry CTX toxins were those few individual fish that were sampled after the event, as the remains of a meal, due to their presumptive role in CFP illnesses [30]. These results of CTX toxins in Spanish Mackerel in NSW is in line with recent evidence of an increase in cases of CFP in more southerly waters along the East Australian Current region [30,31].

In Spanish Mackerel randomly sampled in this study, 1 in 71 fish were positive for P-CTX-1B in the flesh samples, which would indicate a 1.4% prevalence (1%–4%, as lower and upper values, based on 95% confidence intervals) at the sampled sites. In the liver samples, 5 in 71

Table 3
P-CTX-1B levels in fish known to be associated with illness with CFP symptoms in Australia.

Location	Fish species	P-CTX-1B in flesh ($\mu g\ kg^{-1}$)	Reference
Capel Banks, Coral Sea	Purple rock cod	0.1	SIMs Unpublished data
Scotts Head, NSW	Spanish Mackerel	0.4	[30]
Evans Head, NSW	Spanish Mackerel	0.6 – 1.0	[30]
Gove, Arnhem Land, NT	Coral Cod	3.9	[33]
Queensland	Sawtooth Barracuda	1.1	[34]

fish were positive for P-CTX-1B, which would indicate a 7% prevalence (1%–12%, as lower and upper values, based on 95% confidence intervals) at the sampled sites. We consider this to represent the Spanish Mackerel fishery in NSW relatively well, as we covered the vast majority of the region from which Spanish Mackerel are caught in NSW, with the majority sourced from the Coffs Harbour region, and also captured relatively accurately the percentage caught by the recreational community as compared to the commercial fishing community.

We also examined a small number of samples from QLD (n = 13), of which 6 were found to be positive in the flesh for P-CTX-1B (an incidence rate of 46%, but 95% confidence intervals of 19% −73%). As examining the rate of CTX in Spanish Mackerel in QLD was not the aim of this study, these data are considered exploratory. One previous quantification of the rate of P-CTX-1B in a random sample of 13 Spanish Mackerel in southern QLD (Platypus Bay) found a mean rate of 0.19 μg P-CTX-1B kg^{-1} flesh [25]. Taken together, these data show that potentially high rates of CTX may be present in Spanish Mackerel from QLD, and that further investigations and public safeguards are required.

There are several caveats associated with the results presented here. Firstly, any final prevalence rate is subject to relatively high confidence intervals, as discussed above. Secondly, this study was limited to samples that were returned to us (84 of 400 sample packs that were distributed) from the recreational and commercial fishing community. The sites at which samples were taken broadly represents the major locations (Coffs Harbour, Byron Bay) and the relative distribution of commercial compared to recreational fisheries catches of Spanish Mackerel in NSW (~1:10 ratio), however it was not designed to exactly emulate the distribution of the total NSW catch. Finally, other CTX analogs likely exist in these fish alongside P-CTX-1B [25], which we currently cannot measure accurately using LC–MS, as standards for these analogs are not readily available internationally. The presence of these additional analogs may increase the overall toxicity of a particular fish, even at low levels of P-CTX-1B [36].

In the analysis of the length/weight of the fish that were found to have significant levels of P-CTX-1B, no relationship could be seen between these variables. This is similar to what has been found in the study on CTX levels in Barracuda in the Caribbean [29], in which no correlation was found between CTX levels in liver and fish size. A recent study, which analysed CTX levels in fish using the receptor binding assay from 45 species in French Polynesia, including a total of 856 individual fish, also found that there was a positive correlation between fish size and CTX levels in only one species (*Lutjanus bohar*, Red Bass) of the 45 species assessed [37]. The others showed no clear relationship, except for two fish species, which showed a negative relationship (smaller fish had higher toxin levels). They concluded that fish size cannot be used as a universal predictor of likely fish CTX levels in French Polynesia, and that more research needs to be undertaken towards understanding the processes driving CTX bioaccumulation and depuration in individual fish species [37]. In contrast, a clear positive relationship between fish size and CTX levels was found for four fish species in Japan [28], one of which was *L. bohar*. These data indicate that a relationship between fish size and CTX levels may differ on a species-specific, and/or a regional basis, and therefore likely needs to be verified for individual fisheries.

The levels of P-CTX-1B in fish that are correlated with CFP illnesses (Table 3, Table S1) has been found to vary, due several factors. We consider that the individual Spanish Mackerel that were identified as positive for P-CTX-1B in this study had the potential to cause illness, as their levels of P-CTX-1B were > 0.1 μg kg^{-1}, which is 10 times the US FDA "guidance level", and at a similar level to that found in fish flesh known to have caused illness previously (Table 3, S1). We contacted all those who supplied fish that we detected as positive for P-CTX 1B, to question them regarding any possible illness reports. While not all those contacted responded, and it is not possible to ascertain whether the CTX positive fish in this study were consumed, no CFP-like illnesses have been reported to date caused by fish from this random study. This indicates that there is a need to understand the relationship between the levels of P-CTX-1B in Australia in relation to CFP illnesses.

Although a slightly faster method for the extraction of samples for CTX analysis has been proposed [38], acquiring purified CTX standards remains problematic due to the limited supply of purified natural CTX compounds [41]. Though artificial synthesis of CTX is possible [39], it is highly complex. Without a consistent source of reference material, the absolute quantification of CTXs and their congeners is hard to achieve. Technical issues such as co-eluting peaks of similar compounds and inhibiting/promoting matrix effects remain unresolved unless a known CTX standard is used.

As several of the fish in this study were found to contain P-CTX-1B at very low levels, it appears that further research is required to determine the appropriate safe level of P-CTX-1B in fish in Australia. In any study such as this, it would be necessary to compare fish using several methods, such as toxicity assays (bioassays, or other assays such as the receptor binding assay) as well as by LC–MS/MS.

5. Conclusion

In this study, we present the first evidence of the presence of P-CTX-1B in the flesh and liver of Spanish Mackerel from NSW waters. These data are in line with current observations of the increasing frequency of cases of CFP in more southerly waters in Australia. In the analysis of the length/weight of Spanish Mackerel that were found to have detectable levels of P-CTX-1B in this study, no relationship could be seen between these two variables. This suggests that the theory that larger fish are likely to contain higher levels of CTX toxins may be species-specific, as has also been reported recently, and may not hold for Spanish Mackerel. The current study focused on a single CTX analogue, P-CTX-1B, as it is a common analogue found in ciguatoxic fish from the Australian region. However, several other potent CTX analogs exist in fish in this region. There is a need to obtain further CTX reference material to act as standards for the testing process. The levels of P-CTX-1B that lead to illness may require more investigation, in order to determine whether the US FDA guidance level is appropriate for Australia. A more broad scale overview of the levels of all CTX analogs in Spanish Mackerel (liver and flesh) and the potential risk to consumers would be determined by expanding the geographic range and sample size in a national baseline survey.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxrep.2017.06.006.

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