Presence of CTXs in moray eels and dusky groupers in the marine environment of the Canary Islands

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ABSTRACT

Local population frequently consumes moray eels and dusky groupers from the Canary Islands. These species are top predators and the interactions between them include predation but also, in some cases, collaborative hunting. These fish are well known to cause ciguatera (CFP) outbreaks in several marine areas such as Japan, Hawaii, French Polynesia and Caribe. Groupers have been involved in CFP events in the Canary Islands, however, moray eels have not yet been well studied in this regard. The present research seeks to describe the finding of a black moray in the stomach of a positive dusky grouper during its necropsy, and to clarify the implication of groupers and moray eels in the food webs, accumulating CTXs in the Canarian environment. The study also updates statistics on the presence of toxic groupers in this archipelago. For these purposes, 248 grouper samples from the CFP official control in the Canary Islands (2018-2019) were analysed and 36 moray eels (5 species) were collected under the EuroCigua project and one was obtained during a dusky grouper necropsy. All samples were analysed with the Neuro-2a cell-based assay (CBA) to evidence CTX-like toxicity. Regarding the necropsied grouper and the moray eel found in its stomach content, the LC-MS/MS method allowed the identification and quantification of C-CTX1 in both fish at similar levels while none of the P-CTXs for which standards were available were detected. Among groupers, 25.4% displayed CTX-like toxicity with differences between islands. For moray eels 38.9% showed toxicity, involving 4 species. Black moray exhibited a high proportion of positives (9/12) and a positive correlation was found between CTX-like toxicity quantification and the black moray weight. Regarding the grouper, and the moray eel found in its stomach, the LC-MS/MS method allowed the identification and quantification of C-CTX1 in both fish at similar levels. This found suggests a trophic interaction between these species and their role in maintaining CTXs in the Canary waters where local population commonly demand those species for consumption. The island of El Hierro stands out above all the other Canary Islands with the concerning percentage of positive grouper samples and the high CTX toxicity levels obtained in moray eel specimens analysed in this marine area.

This is the first report of CTX-like toxicity in flesh of moray eels fished in the Canary archipelago and the confirmation of the presence of C-CTX1 by LC-MS/MS in a black moray from this marine area.

Keywords: ciguatoxin; moray eel; dusky grouper; Canary Islands;

1. INTRODUCTION

Ciguatera Fish Poisoning (CFP) caused by consumption of fish contaminated with ciguatoxins (CTX) (Dickey and Plakas, 2010), is one of the most common seafood-borne illness worldwide associated with biotoxins, previously considered a tropical and subtropical disease (Fraga et al., 2011; Friedman et al., 2008; Lewis, 2006). Gastrointestinal, neurological and cardiovascular symptoms usually appear. In 2004, the first CFP record was reported in the Canary Islands (Pérez-Arellano et al., 2005), and since 2015, CFP has been categorized in the Canary Islands by the local authorities as a notifiable disease.

The main species of fish involved in ciguatera outbreaks are typically large and apex predators (Chan, 2017), although herbivorous fish may also be at risk, (Gaboriau et al., 2014), since CTX's analogues had been found in parrotfish *Scarus gibbus* (Chungue et al., 1997; Satake et al., 1996); and the *Scaridae* family was involved or suspected in CFP outbreaks (Lewis, 1996; Rongo and Wan Voesik, 2011). Moray eels and groupers are the species with the greatest CTX concentrations, among other carnivorous fish (Chan et al., 2011; Mak et al., 2013), and are responsible for numerous outbreaks in the Indo-Pacific and Atlantic Ocean regions (Lehane and Lewis, 2000).

The Canary Islands (NE Atlantic, Spain) are an archipelago off the northwest coast of Africa with a strong fishing tradition (Bas et al., 1995). The groupers of the family *Serranidae* as dusky grouper (*Epinephelus marginatus*) are highly appreciated for fisheries and recreational fishing (Craig et al., 2011; Espino et al., 2018). Among the 10 fish species of the family *Muraenidae* described in the Canary Islands archipelago, up to five of them, the black moray (*Muraena augusti*), the mediterranean moray (*Muraena helena*), the fangtooth moray (*Enchelycore anatina*), the brown moray (*Gymnothorax unicolor*) and the polygon moray (*Gymnothorax polygonius*) are of interest to commercial and artisanal fishery (Espino et al., 2018).

Groupers and moray eels are sedentary and high order carnivores (Almada et al., 2009; Ebner et al., 2016; Espino et al., 2018) which share preferences for prey when it comes to feeding (large crustaceans, fish and mollusks) (Brito et al., 2002; Condini et al., 2015; Espino et al., 2018; Machado et al., 2008). Groupers have also been reported to prey on moray eels (Linde et al., 2004). A collaborative hunting between both families has been described for mutual benefit due to their natural complementary hunting tactics (Bshary et al., 2006). Grouper is proved to have a high burst of speed, which helps catching the prey in open waters and, in contrast, the moray sneaks through reef cracks or crevices in search for hidden preys thanks to its elongated thin body (Bshary et al., 2006; Steinegger et al., 2018).

Up to date, the official records of CFP in the Canary Islands involve dusky grouper in 4 out of 19 CFP outbreaks from 2008 to 2018 affecting a total of 32 people. Other fish species involved in CFP outbreaks are: amberjack, island grouper, bluefish and ocean triggerfish. Nonetheless, until now, none of the ciguatera cases recorded by the Canarian Epidemiological Surveillance Network has been linked to moray eel consumption (Canary Government, 2019). The average annual consumption per species in the Canary Islands is, during the last three years, about 32 and 77 tons for dusky groupers and moray eel species respectively (DG of Canary Government, 2019).

There are limited regulatory measures preventing the sale of toxic fish in same places in the world, but several countries have imposed a ban or recommended avoiding certain species for consumption (Chan, 2015; Clua et al., 2011; Laurent et al., 2005). Several countries analyse CTX toxicity in fish in order to better understand the risks according to species, weight, time of the year and geographical area among others. Nonetheless, to the best of our knowledge, the Canary Islands, through the DG Fisheries of the Canary Government, are the only region in the world taking an official preventive action against CFP outbreaks based on the analyses of the presence of CTX-like toxicity in flesh, in all the fish within a detailed list of species and weights before being commercialised. This official routine control has been implemented since 2011 and the Institute of Animal Health and Food Safety (IUSA) is responsible for this monitoring. The protocol of this official monitoring provides a list of certain fish species and weights to be sampled at the authorized first sale points and submitted to the laboratory for CTX detection. This list, which is periodically revised, includes dusky groupers (*E. marginatus*) from 16 kg occurring in Canary waters, but none of the moray eels are currently considered within this list (DG of Fisheries of the Canary Government, 2018).

Under the official control program, 27% of the *Epinephelus* spp. samples, analysed in 2016 and 2017, exhibited measurable CTX-like toxicity with the implementation of a CBA. Within the archipelago, El Hierro Island is of great concern, particularly for the high percentage of positive grouper samples obtained through this official monitoring (Sanchez-Henao et al., 2019).

Based on the function of dusky groupers and moray eels as species of concern for human consumption worldwide, and also considering the interactions between both fish (collaborative hunting and predatory relation among them), this work has focused in dusky groupers and moray eels as two groups of fish that may contribute to accumulate CTXs in the Canarian marine environment.

The objective of this research deepens the activity of dusky groupers (*E. marginatus*) and the black morays (*M. augusti*) to hold CTXs in marine environment, showing clear evidences of this interaction, regardless other trophic relations that may exist with other fish within the food webs. Furthermore, this study updates statistics on the presence of toxic groupers in this archipelago and describes, for the first time, the presence of CTX-like toxicity in moray eels caught in the Canary Islands.

2. MATERIALS AND METHODS

2.1. Study area

The Canary Islands are an archipelago located in the northeast of the Atlantic Ocean near Europe and north of Africa (about 100 km from the Moroccan coast) composed by a group of 7 main islands, which are the following from east to west: Lanzarote, Fuerteventura, Gran Canaria, Tenerife, La Gomera, La Palma and El Hierro. This archipelago has a strong fishery tradition (Bas et al., 1995), and constitutes FAO Major Fishing Area 34 in the subdivision 1.2 (FAO, 2004).

2.2. Fish species studied

Grouper samples

The dusky groupers flesh samples analysed (n = 248) in this study were provided by the DG Fisheries of the Canary Government through the official control of CFP in the period going from January 2018 to May 2019. The information on the geographical area of sampling is given in table 2. These samples were sent to the Institute of Animal Health and Food Safety (IUSA) laboratory for analysis. All specimens were over the weight limit (16 kg) established by the Canary Government above which all groupers need to follow CTX analysis (DG of Fisheries of the

Canary Government, 2018). Additionally, a necropsy was conducted with one of the positive groupers reclaimed by EuroCigua project to the Canary Government.

Moray eel samples

Specimens of the *Muraenidae* family studied in the present research (n = 36) were collected from local markets or authorized first sale points from the Canary Islands (n = 35) except one of the individuals tested found, during the necropsy, in the stomach of a toxic dusky grouper, sampled from Lanzarote, which resulted to present CTX-like toxicity (Table 2). All these samples were studied in the framework of the EuroCigua project (GP/EFSA/AFSCO/2015/03).

The moray eels analysed belong to five species, black moray (*M. augusti*) (n = 12), polygon moray (*G. polygonius*) (n = 2), fangtooth moray (*E. anatina*) (n = 3), mediterranean moray (*M. helena*) (n = 13) and brown moray (*G. unicolor*) (n = 6).

2.3. Sample preparation and extraction of CTX

The extraction of CTX was conducted according to the protocol described in the literature (Lewis, 2003; Sanchez-Henao et al., 2019) with minor modifications. Briefly, 10 g of fish flesh samples were cooked at 70 °C during 10 minutes. Each sample was extracted with 30 ml of acetone and homogenized with an Ultraturrax blender. The supernatant was recovered by centrifugation at 3000 g during 5 minutes at 4 °C. This last step was repeated and both supernatants were pooled and filtered through a 0.45 µm PTFE filter and evaporated to dry extract with a rotary evaporator at 55 °C. Liquid/liquid partition was conducted twice with a mix of water and Diethyl-Ether (DEE) (1:4, v-v). The two DEE fractions were pooled and evaporated to dryness. This dried residue was dissolved for subsequent partition (twice) in methanol:water (8:2) and n-Hexane (1:2, v-v). The n-hexane upper phases were discarded and the methanol phases were pooled and dried under N_2 current at 40 °C. The final residue was re-dissolved in 4 ml of methanol and preserved at -20 °C until analysis with the cell-based assay (CBA).

2.4. Neuroblastoma (Neuro-2a) cell-based assay (CBA)

The cellular line used in this study was the Neuro-2a (Cell line: CCL131, from ATCC, LGC Standards S.L.U., Barcelona, Spain) and were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 5-10% of foetal bovine serum (FBS) at 37 °C in a 5% CO_2 atmosphere. The CTX1B standard (STD) (R.J. Lewis, The Queensland University, Australia) was used for the assessment of CTX-like toxicity.

The cytotoxicity assay was performed as previously described for this research group (Sanchez-Henao et al., 2019) with minor adaptations; cells were seeded in a 96-well flat bottom plate (200 µl/well) at a concentration of 40.000 cells/well. Ouabain (0.1 mM) and veratridine (0.01 mM) were used to evidence cell mortality in case of the presence of CTX and after incubation cells were exposed to flesh extract and the CTX1B STD at decreasing concentrations.

Finally, cell viability was assessed with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] and DMSO solutions. Absorbances were read at 570 nm with a multi-well spectrophotometer scanner and the dose-response curves were evaluated with the Microsoft Office Excel 2016 and GraphPad Prism 7 softwares (GraphPad, San Diego, California, USA).

Being specific to this study, the moray eel samples and the muscle of the dusky grouper necropsied containing the moray eel in its stomach were analysed in duplicate, reporting their toxicity according to a reference standard curve of CTX-1B obtained the same day of the analysis. Extracts were exposed at a maximum concentration of 200 and 125 mg Tissue Equivalents (TE) of flesh/ml for moray eels and dusky groupers, respectively, in order to avoid matrix interferences on the cytotoxicity assay. A response producing less than 20% cell mortality was considered as non-toxic effect (Caillaud et al., 2012). The LOD and LOQ was set at the concentration of CTX1B STD causing 20% inhibition of cell viability (IC_{20}) considering the maximum concentration of fish extracts for cell exposure. According to the mean value of IC_{20} observed in the dose-response curve performed with the STD simultaneously with extracts from moray eel and dusky grouper samples (1.981 ± 0.303 and 1.096 ± 0.322 pg CTX1B/ml, respectively) and the maximum concentration of extracts (200 mg TE/ml with moray eels and 125 mg TE/ml with dusky groupers), the LOD/LOQ obtained were 0.0099 and 0.0088 µg CTX1B equivalents/kg flesh (ppb), respectively.

Data on the rest of groupers (n = 248) were obtained from the regular monitoring programme which discriminates between fish free of CTXs and those containing CTXs, in accordance with EU regulations, and that does not include detailed quantification due to the large number of samples that are processed on a regular basis.

Toxic content in fish analysed for CTX-like toxicity with the CBA was expressed in ppb units (µg CTX1B equivalents/kg flesh). Dose-response curves by N2a cell-base assay obtained with a dusky grouper, a black moray and CTX1B standard are depicted in Figure 1.

2.5. CTX identification and quantification by LC-MS/MS

LC-MS/MS analysis was recently published (Estévez et al., 2019) and performed based on the methodological approach of Yogi *et al.* (2011) and Abraham *et al.* (2012) with modifications in order to optimize laboratory conditions and improve CTXs detection. This method was carried out using Agilent 1290 Infinity LC system coupled to an Agilent 6495 Triple Quadrupole LC-MS (Agilent Technologies, CA) equipped with an Agilent Jet Stream electrospray ionization source (iFunnel). The following CTXs were monitored by Multiple Reaction Monitoring (MRM) comparing with the retention time of the standards available (Fig. 2): Caribbean CTX (C-CTX1) and Pacific CTXs (CTX1B, 2,3-dihydroxyCTX3C, 51-hydroxyCTX3C, 52-epi-54-deoxyCTX1B/54-deoxyCTX1B, CTX3C, CTX4A/CTX4B).

2.6. Necropsy procedure

A necropsy of a dusky grouper (*E. marginatus*) from Lanzarote (Canary Islands) was performed at the IUSA laboratory. The fish was found positive to CTX-like toxicity during the routine official control. The necropsy was conducted following the Meyers protocol proposed in 2009 (Meyers, 2009). A partially digested black moray (*M. augusti*) was found in the stomach content of this grouper, which was also necropsied and its flesh was subsequently analysed with the CBA. In addition, flesh samples of both animals were sent to the University of Vigo, Spain, to confirm and quantify the results by the LC-MS/MS method.

2.7. Statistical analysis

Statistical analysis was conducted using the IBM SPSS Statistics 23.0 software.

The percentages of the variables (fish species, weight, fishing island, CTX-like result and toxicity) were compared using the Chi-square ($\chi 2$) test or the exact Fisher test. As appropriate, the statistical significance between other categories was assessed using non-parametric tests as the mann-whitney U-test and the Kruskal-Wallis test for independent groups. Spearman's correlation test was carried out to determine a possible relationship between variables. The statistical significance was set at a *p-value* < 0.05.

3. RESULTS

3.1. Black moray found in the stomach content of a CTX positive dusky grouper

A necropsy of a dusky grouper (*E. marginatus*) of 17.4 kg and 93 cm of total length (Fig. 3a), captured in August 2017 by a professional fishermen in La Santa (North coast of Lanzarote), was performed.

During necropsy, a partially digested body of a black moray (*M. augusti*) was found in the stomach content, weighing 1.03 kg and presenting 82 cm of total length (Fig. 3b). In addition, other non-identified moray eel rests were also observed; these were not studied.

Both specimens were analysed twice for CTX-like toxicity with the CBA and toxicity expressed in ppb units (µg CTX1B equivalents/kg flesh) with a maximum Relative Standard Deviation (RSD) of 8 % calculated with the tripled absorbance values of the CTX1B STD dose-response curve (Fig.1). Average values obtained from duplicate analyses of both fish were quite similar (0.032 ppb for dusky grouper and 0.037 ppb for black moray) (Table 1).

The analysis conducted by LC-MS/MS method in the University of Vigo identified the presence of C-CTX1 in both samples, with a quantification of 0.03 ppb and 0.05 ppb in *E. marginatus* and *M. augusti*, respectively (Table 1).

3.2. Evaluation of the presence of CTX-like activity by CBA in dusky groupers

From the official control program, 248 muscle samples of groupers (*E. marginatus*) were analysed with the CBA from January 2018 to May 2019, and 63 of them showed CTX-like toxicity above the detection limit of 0.0088 µg CTX1B equivalents/kg flesh (ppb), resulting in 25.4% of positive fish (Table 2).

Positive results of dusky grouper samples showed different distribution among islands ($p < 0.001$) (Table 2). In addition, the estimated toxicity for groupers also showed differences regarding islands ($p < 0.001$) (Fig 4).

El Hierro island showed the highest contamination rate in grouper samples (77.8%), approximately two fold greater than the values obtained in samples from Tenerife, La Palma and Lanzarote (38.5%, 30.0% and 27.4%, respectively); this island showed statistical difference as a unique category compared to the rest of the islands ($p < 0.001$). In a deep analysis of these dusky groupers from the Official Control screening, the toxicity of each positive sample was estimated (as approximated value) using the cell viability obtained with doses 1 (D1) by CBA and the corresponding CTX1B STD curve performed. Additionally, a statistical analysis was conducted with the estimated toxicities and the island of capture; El Hierro also stands out against the rest of the islands with the highest estimated toxicity in groupers ($p < 0.001$). This latter difference was also observed for Tenerife ($p = 0.025$) among the rest of the islands, excluding El Hierro (Fig. 4). A low number of toxic groupers were obtained from Fuerteventura (6.1%) and the 3 samples received from Gran Canaria resulted negative.

The weight of groupers were 20.6 ± 3.2 kg (mean ± SD) and 20.2 kg (16.0-37.0) median (min-max) for negative samples, and 21.4 ± 3.4 kg (mean ± SD) and 21.1 kg (16-30.0) median (min-max) for positive samples and no significance was observed between this variable and CTX-like result, Island of fishing category or toxicity level.

3.3. Evaluation of the presence of CTX-like activity by CBA in moray eels

CTX-like toxicity was found in 14 moray eels (38.9%) out of the 36 individuals analysed (Table 2).

Out of the 5 moray eel species, 4 showed CTX-like activity by CBA (Table 2). A significant difference in the presence of toxicity among species was observed ($p = 0.001$). The 75% (9 out of 12) of black moray (*M. augusti*) samples were positive and also the 3 fangtooth moray (*E. anatina*) tested. However the small number of samples of this latter (n = 3) precludes any conclusion (Table 2). In contrast, the mediterranean moray (*M. helena*) represents the largest category according to number of individuals, but only one sample was toxic (Table 2).

However, the sample size of each moray species was insufficient to perform a proper statistical analysis by fishing island or weight.

The CTX-like toxicity of each moray eel sample was quantified (duplicate CBA) based on the toxicity displayed by the CTX1B standard, enabling the analysis of the CTX-quantification by CBA of the flesh samples depending on the species and fishing island (Table 3).

In this research, a positive relationship was also observed in black moray between the CTX-like toxicity quantification (ppb) and the weight of the specimens (Fig. 5), based on 9 results. Thus, the results showed that increasing toxicity level was associated with increasing weight of black moray. Spearman's correlation coefficient (rs) exhibited a no significant but positive association with a coefficient of determination (R^2) for lineal regression of 0.416.

4. DISCUSSION

Although bibliographic references have described predation from groupers on moray eels (Linde et al., 2004), the black moray (*M. augusti*) found in the stomach of a dusky grouper (*E. marginatus*) would be the first case described in the Canary Islands and also represents the first reported evidence of the presence of CTX in grouper and moray eel, as predator and prey respectively, in this particular marine area. The cytotoxicity assay and LC-MS/MS methodology performed in the flesh of each specimen (table 1), showed similar toxicity levels for both individuals. The presence of CTX in these specimens was showed by CBA and by LC-MS/MS with a quite similar quantification but not strictly comparable since these two methods rely on different principles of analysis (Diogène et al., 2017). It is important to emphasize that LC-MS/MS not involving non-targeted analysis identifies specific CTX analogues already described, and CBA measures the toxicity of an extract which may be caused by a complex mixture of CTX analogues.

Taking into account the Toxicity Equivalence Factors (TEF's) for this group of toxins collected in the EFSA report (EFSA, 2010), C-CTX1 is 10 times less potent than CTX1B; however, this CTX-Like potency could be by sum of the action of non-targeted CTX's analogues by LC-MS/MS analysis.

Our results are similar to those described in other areas, like the Pacific Ocean, where moray eels and groupers caught from the same locations displayed similar CTX-like toxicity (Chan et al., 2011). It has been suggested that this pattern is likely due to the high trophic level of these fish, and their common food source (Bshary et al., 2006), contributing to reach great levels of toxicity.

Moray eel and grouper species are sedentary and also share habitat, the same prey preferences and they even perform collaborative hunting (a beneficial synergic effect for both species) (Bshary et al., 2006; Steinegger et al., 2018; Strübin et al., 2011; Vail et al., 2013). Furthermore, in some cases, the interaction of these species could end in predation between them; in fact, some authors suggest moray eels as the best bait to catch groupers (Gaspare et al., 2015).

Considering the last study aforementioned, it is necessary to deepen the contribution that dusky groupers have on transfer or sharing the CTXs in marine environment and their relationship with moray eels in the Canary Islands. Comparing with recently published results from the official control monitoring, carried out in the period 2016-2017 in the Canary Islands (Sanchez-Henao et al., 2019) that reported an incidence of 26.9% in dusky grouper samples, the percentage of positive CTX-like activity for this species found in the current study (25.4%) shows a stable rate over the last years. Additionally, in comparison with other fish species also considered in the official control protocol, like amberjack and wahoo, dusky grouper exhibits a high positive rate, which confirms the importance of this species (*E. marginatus*) to present and maintain CTX levels of some particular areas being a risk for fish consumers.

It should be stressed that these grouper samples correspond to specimens with certain weights considered at risk for human health by the Canary Government (over 16 kg) (DG of Fisheries of the Canary Government, 2018) and that groupers below this weight (16 kg) were not considered.

Thus, it was not possible to analyse dusky groupers under 16 kg. This circumstance must be taken into account before any further conclusion at this respect can be drawn.

Additionally, the CTX-like toxicity of 2 groupers linked to CFP human cases were confirmed in our laboratory, both of them caught in the Canary Islands waters. One of the samples corresponded to a specimen weighing 7 kg, less than half of the limit established by the official control (Sanchez-Henao et al., 2019; Canary Government, 2019). This results may be indicative that the presently weight limit for groupers set by the administration should be reviewed.

As previously reported by this research group, differences in the percentage of CTX-positive groupers were observed among islands ($p < 0.001$). El Hierro showed the highest contamination rate (77.8%) followed by Tenerife (38.5%), La Palma (30.0%) and Lanzarote (27.4%). Although Fuerteventura provided the largest number of grouper samples, it showed a low percentage of positivity (6.1%). There were no positive groupers from Gran Canaria but the low number of samples from this island (n = 3) precludes any conclusion.

These results suggest that the island of El Hierro may be considered a geographical area at high risk for CTXs in dusky groupers, and probably other species within their food web, and the consequent risk for possible emergence of CFP cases in this area. The significant difference between islands found in relation to the percentage of positive groupers, may be due to the sedentary behaviour of grouper species linked to the areas of *Gambierdiscus* occurrence (Chan, 2015). The presence of *Gambierdiscus* may be sporadic and limited to one specific zone within a larger area (Dickey and Plakas, 2010), thus a CTX free area may be located only a few kilometres away from a place with CFP cases (Chan et al., 2011; O'Toole et al., 2012).

Moray eels are well known to cause CFP outbreaks in Pacific and Indian Ocean and Caribbean Sea (Chan et al., 2015; Laurent et al., 2005; Lehane and Lewis, 2000). However, in the Canary Islands these fish species have not been associated with any CFP human outbreak (Canary Government, 2019). Given the concentration obtained by CBA in CTX1B equivalents all moray eels are over 0.01 ppb (the threshold established by EFSA and FDA) and some of them exceed it more than ten times, this group of fish represents a latent risk of CFP to humans. This fact must be properly taken into account in further studies. In fact, the present finding, with 38.5% of moray eel flesh samples presenting CTX-like toxicity, represents the first evidence of CTX content in moray eels fished in the Canary Islands waters (Table 2). The sampling design of the moray eel species included in the present work was set according to consumption and fishing habits common in the archipelago. It is important to note that the study objective was not to statistically analyse moray eels but to highlight the presence of CTXs in these fish species and hence their role as reservoirs of CTXs in the marine food chain in Canarian waters.

The most frequent moray eel species fished in the Canary Islands, the black moray and the mediterranean moray, showed high difference in the proportion of positive results found in this research, 9 out of 12 and 1 out of 13, respectively (Table 2). The black moray clearly stands out from the other species because of its large number of toxic samples.

Although black moray constitutes a significant fraction of catches in the Canary Islands (Espino et al., 2018), there is no known CFP associated with its consumption. On the other hand, the two polygon morays studied here were negative to CTX-like toxicity, but a greater sample size would be required to evaluate the actual presence of CTX in this species. Thus, it is not possible to conclude that polygon moray does not accumulate CTX, especially since this species has similar feeding behaviour to that of the rest moray eel species analysed (Espino et al., 2018), and also it belongs to the same genus *Gymnothorax* as the giant moray (*Gymnothorax javanicus*) and the yellow-edged moray (*Gymnothorax flavimarginatus*), two of the most toxic species of the family Muraenidae, due to their high CTX levels and large sizes showed (Chan, 2017). To the best of our knowledge, the maximum concentrations of CTXs reported in the literature for moray eels and groupers are 39.20 and 81.14 ppb (P-CTXs) for the yellow-edged moray and Giant moray respectively (Chan et al., 2011; Mak et al., 2013), and 5.60 and 12.43 ppb for the groupers

Cephalopodis miniata and *Epinephelus spilotoceps* respectively (Chan et al., 2011; Soliño and Reis, 2018).

Our results confirm the presence of positive moray eels throughout all the islands in the Canary Islands, except for Gran Canaria (Table 2). Among the archipelago, El Hierro clearly stands out as the island with the highest percentage of positives and most toxic dusky groupers and also with the most toxic moray eel obtained in this research (Fig. 4 and Table 3), which stresses the need of a more efficient control in the prevention of CFP in this particular area.

In the literature consulted, a positive correlation was described between CTX concentration in muscle and size or weight in *Gymnothorax* spp. (Chan et al., 2011) and in giant moray eel (*G. javanicus*) from the Kiribati Islands (Mak et al., 2013). In this research, we did not found a statistical influence of the fish weight on the presence or absence of toxicity, but a relationship was observed in positive black morays (n = 9) between the CTX-like toxicity quantification (ppb) and the weight of the specimens (Fig. 5). Thus, the results showed that increasing toxicity level was associated with increasing weight of black moray, although in the assessment of the linear regression, the correlation coefficient obtained indicates a positive correlation with a moderate strength of association (R^2 = 0.416). This association is undoubtedly impacted with the input of a black moray sample, 2.81 kg weight from El Hierro (Table 3), which showed the highest CTX content (0.232 ppb CTX1B equivalents) for moray eels in this study.

Regarding dusky groupers, a relationship between the weight of the fish and the CTX result was not found, but it is important to stress that all specimens studied weight over 16 kg, which is the limit established by the official control protocol, and therefore a population survey regarding CTX content should be required including fish below 16 kg. The consulted bibliography provides controversial results in this respect. Dierking and Campora (2009) found a positive association between CTX concentration and the standard length of peacock grouper (*Cephalopholis argus*) in Hawaii, and Oshiro et al., in 2010, described a relationship between the ratio of ciguatoxic individuals and the body weight of lyretail grouper (*Variola louti*) and Brown-marbled grouper (*Epinephelus fuscoguttatus*) in Japan. In contrast, Latter Bienfang et al. (2012) found no size influence on CTX concentration in the peacock grouper in Hawaii, supported by Gaboriau et al. (2014) who also did not find that correlation in groupers species sampled in French Polynesia. Interestingly, in the same study, the proportion of toxic individuals was even reported to decrease with increasing size of camouflage grouper (*Epinephelus polyphekadion*). Chan (2015) concluded that P-CTX level in flesh and size dependency varies with its geographical origin and so the risk of ciguatera after *E. fuscoguttatus* eating.

A previous work stated that the changes observed in the liver metabolism of ciguatoxic individuals of undulate moray (*G. undulatus*) and peacock grouper (*C. argus*) could explain the different concentration of CTXs in the fish tissue (Jiang et al., 2012). Gaboriau et al. (2014) also observed differences in ciguateric toxicity between families of similar trophic level (*Muraenidae* and *Serranidae* among others), concluding that CTX concentration in fish tissues may be the result of biological and physiological processes more complex than the merely positive relationship with the size or weight of individuals.

In summary, from the human health prospect this work emphasizes the relationship between dusky groupers and moray eels, which could represent a prominent behaviour as fish with high CTX levels to imply real risk in particular marine areas.

This work provides data on contamination of dusky groupers and moray eels by CTXs, species with high interest for human consumption in the Canary Islands.

5. CONCLUSIONS

This research, for the first time, describes the presence of a positive CTX black moray (*Muraena augusti*) present in the stomach content of a ciguateric dusky grouper (*Epinephelus marginatus*) from the Canary Islands, which confirms a trophic interaction between these species and their relevance in maintaining CTXs in this marine environment.

The presence of CTX-like toxicity was observed in the fish flesh of both specimens with the CBA. C-CTX1 (Caribbean CTX) was identified by LC/MS-MS analysis with similar concentrations in these two specimens. To the best of our knowledge, this is the first identification of CTXs in a black moray from this archipelago.

In the present work we studied five species of moray eels caught in the Canary Islands, and four of them were found naturally contaminated with CTXs. Further research is needed to assess the risk these species would represent to human health according to their consumption levels.

The rate of contamination by CTX in the population of dusky groupers in the Canary Islands seems to be stable over the study period, highlighting El Hierro as the island of special concern due to the high percentage of toxic groupers obtained through the official control program.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

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Table 1. CTX quantification in flesh from the grouper necropsied and the moray eel found in its stomach.

Fish species	CTX-like toxicity[a]	LC-MS/MS[b]
Dusky grouper (*E. marginatus*)	0.032 ± 0.009 ppb	0.03 ppb C-CTX1
Black moray (*M. augusti*)	0.037 ± 0.015 ppb	0.05 ppb C-CTX1

a. Quantification of CTX (µg CTX1B equivalents/kg matrix, ppb) obtained by CBA in the IUSA laboratory. Average of duplicate results ± standard deviation (sd).

b. Identification and CTX quantification (ppb) obtained by the University of Vigo using LC-MS/MS method.

Table 2. Number of grouper and moray eel flesh samples analysed according to species and fishing island.

Fish species	Fishing Island							TOTAL
	LZ	FU	GC	TF	LG	LP	HI	
Dusky grouper (*E. marginatus*)	84 (23)	98 (6)	3 (0)	26 (10)	-	10 (3)	27 (21)	248 (63)
Moray eels species (Total)	13 (2)	2 (1)	1 (0)	12 (5)	3 (1)	3 (3)	2 (2)	36 (14)
Black moray (*M. augusti*)	5 (2)	-	-	3 (3)	1 (1)	2 (2)	1 (1)	12 (9)
Polygon moray (*G. polygonius*)	-	-	-	2 (0)	-	-	-	2 (0)
Fangtooth moray (*E. anatina*)	-	-	-	2 (2)	-	1 (1)	-	3 (3)
Mediterranean moray (*M. helena*)	7 (0)	2 (1)	1 (0)	2 (0)	1 (0)	-	-	13 (1)
Brown moray (*G. unicolor*)	1 (0)	-	-	3 (0)	1 (0)	-	1 (1)	6 (1)

Note: The number in brackets indicates the CTX-Like toxicity positive samples obtained in each category; Fishing island: LZ, Lanzarote; FU, Fuerteventura; GC, Gran Canaria; TF, Tenerife; LG, La Gomera; LP, La Palma; HI, El Hierro.

Table 3. Estimated CTX content in moray eels from the Canary Islands according to CTX-like toxicity obtained by CBA (µg CTX1B equivalents/kg matrix, ppb) conducted in duplicate

Fishing Island and moray eel species	Weight (kg)	[a]CTX content
Lanzarote		
Black moray (*M. augusti*)	1.03	0.037 ± 0.015
Black moray (*M. augusti*)	1.65	0.191 ± 0.016
Fuerteventura		
Mediterranean moray (*M. helena)*	1.14	0.026 ± 0.008
Tenerife		
Black moray (*M. augusti*)	0.40	0.029 ± 0.005
Black moray (*M. augusti*)	0.87	0.025 ± 0.009
Black moray (*M. augusti*)	1.74	0.028 ± 0.003
Fangtooth moray (*E. anatina*)	0.63	0.021 ± 0.006
Fangtooth moray (*E. anatina*)	1.44	0.039 ± 0.018
La Gomera		
Black moray (*M. augusti*)	1.47	0.088 ± 0.044
La Palma		
Black moray (*M. augusti*)	0,41	0.066 ± 0.017
Black moray (*M. augusti*)	0,51	0.126 ± 0.029
Fangtooth moray (*E. anatina*)	0,82	0.025 ± 0.016
El Hierro		
Black moray (*M. augusti*)	2,81	0.232 ± 0.174
Brown moray (*G. unicolor*)	2,72	0.175 ± 0.037

a. CTX1B equivalents (ppb) quantified by the N2a cell assay (CBA), average value ± standard deviation (sd).

FIGURE CAPTIONS

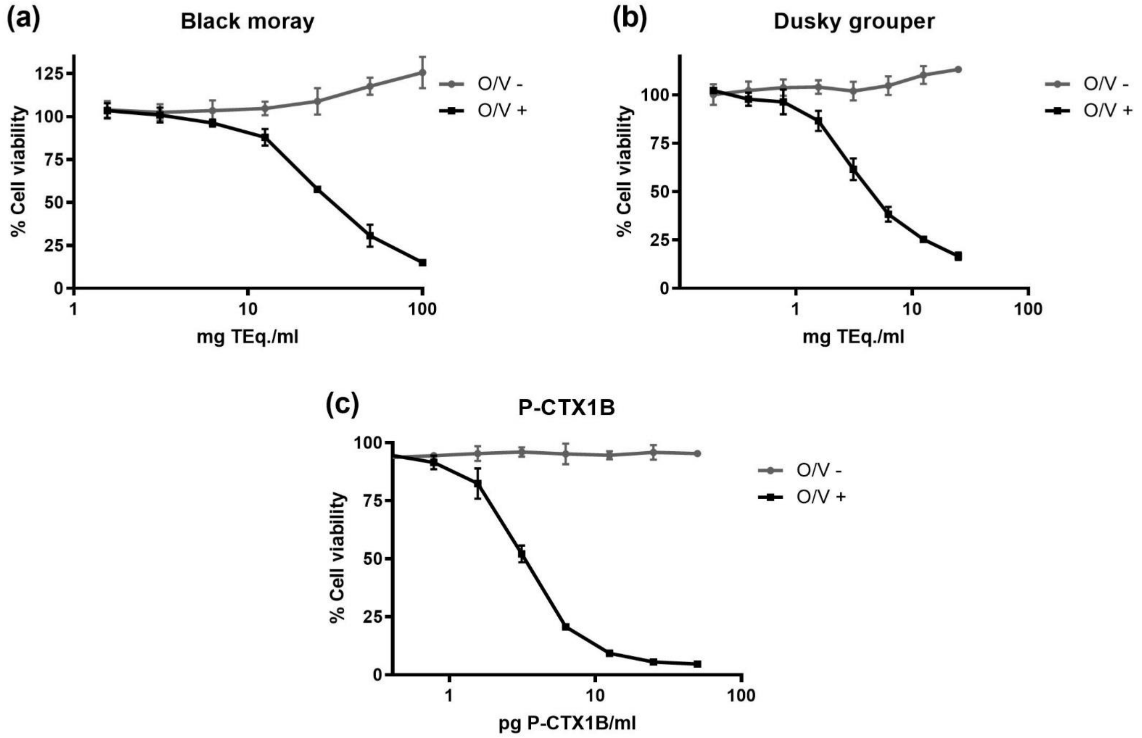
Figure 1. Representative dose-response curves obtained by N2a cell-base assay with (a) a dusky grouper flesh extract from Lanzarote island, (b) a black moray flesh extract from El Hierro island and (c) with CTX1B standard. Error bars represent the standard deviation (sd) from three well replicates.

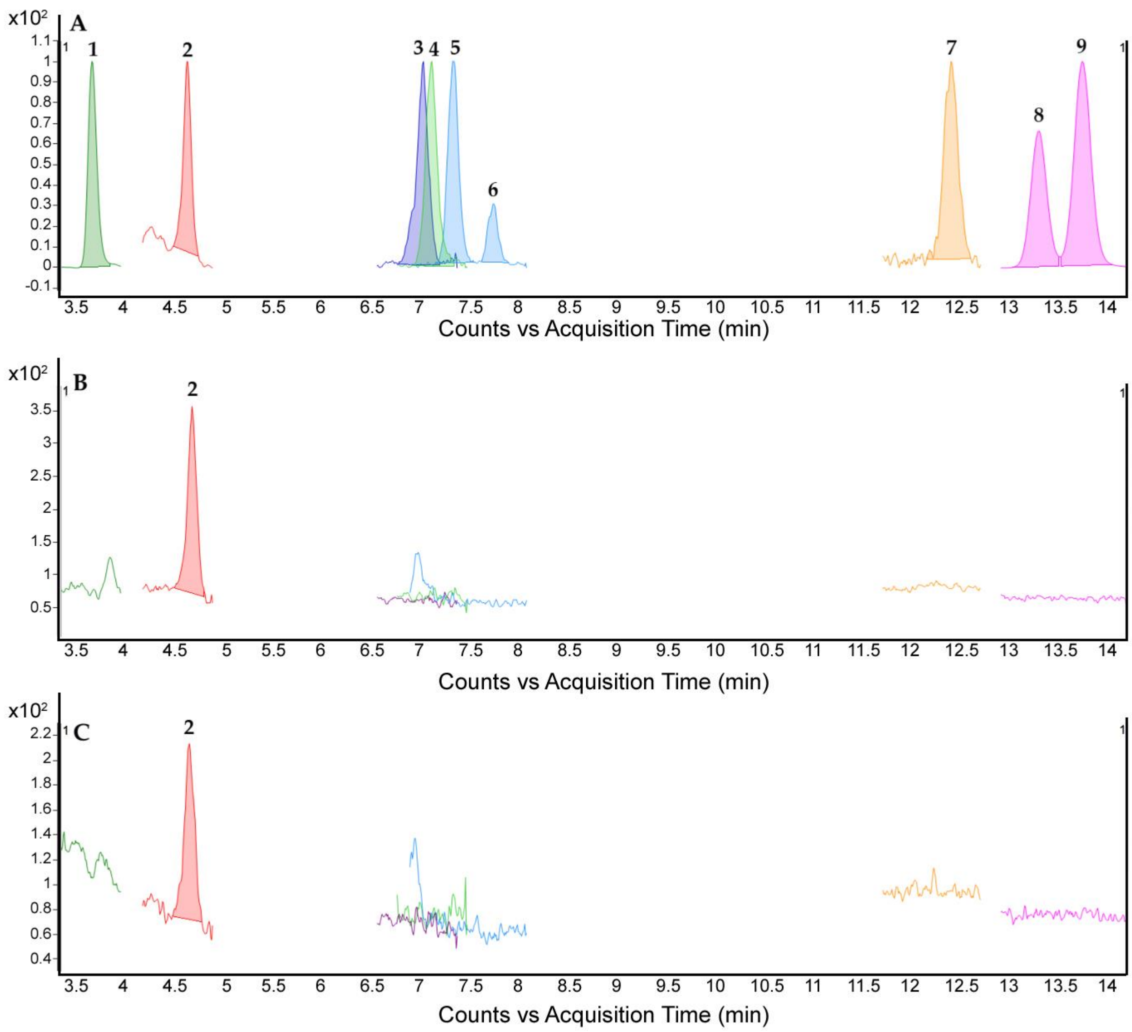
Figure 2. LC-MS/MS chromatogram of: (A) Standard mixture containing: CTX1B (1), C-CTX1 (2), 2,3-dihydroxyCTX3C (3), 51-hydroxyCTX3C (4), 52-epi-54-deoxyCTX1B (5), 54-deoxyCTX1B (6), CTX3C (7), CTX4A (8) and CTX4B (9); (B) C-CTX1 detected at a concentration of 0.05 ng/g in *M. augusti*; (C) C-CTX1 detected at a concentration of 0.03 ng/g in *E. marginatus*.

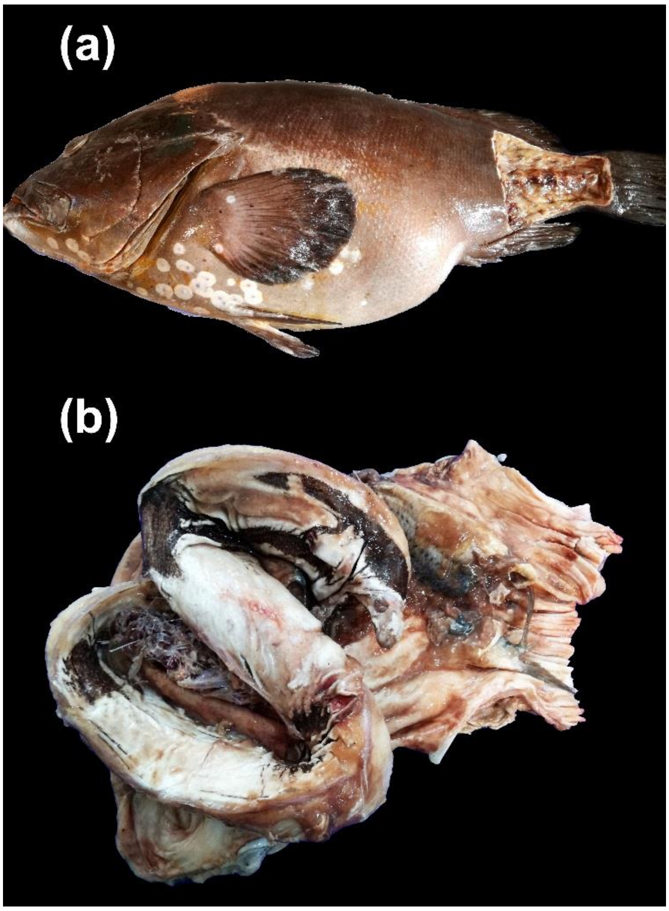
Figure 3. (a) Lateral view of a dusky grouper from the Canary Islands before necropsy. (b) Stomach content of the specimen with a partially digested black moray eel.

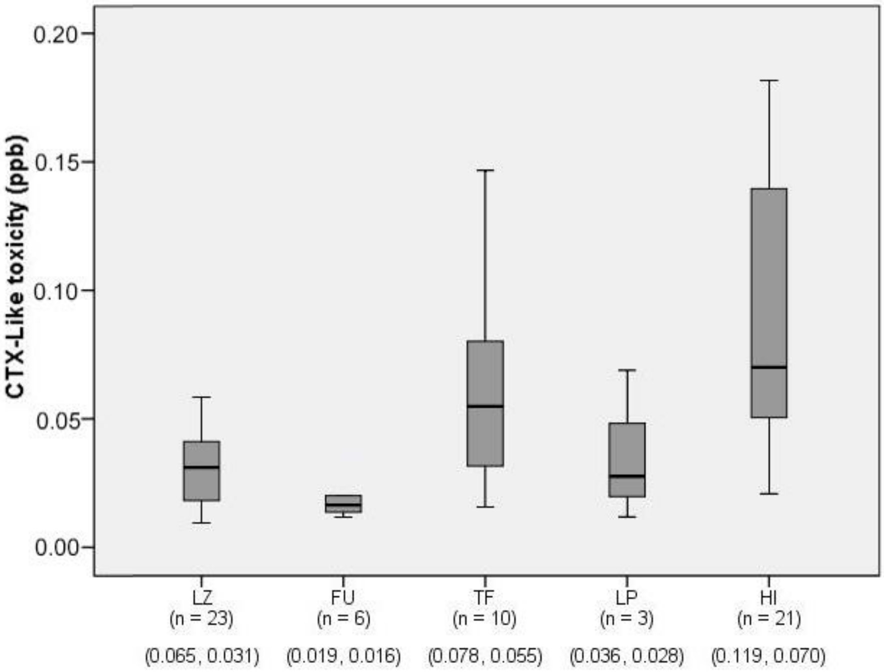
Figure 4. Distribution of CTX-like toxicity estimated for grouper samples (ppb P-CTX1B Equivalents) by location of fishing ($p < 0.001$). In brackets are indicated mean and median toxicity values for each island of capture. The Canary Islands: LZ, Lanzarote; FU, Fuerteventura; TF, Tenerife; LP, La Palma; HI, El Hierro.

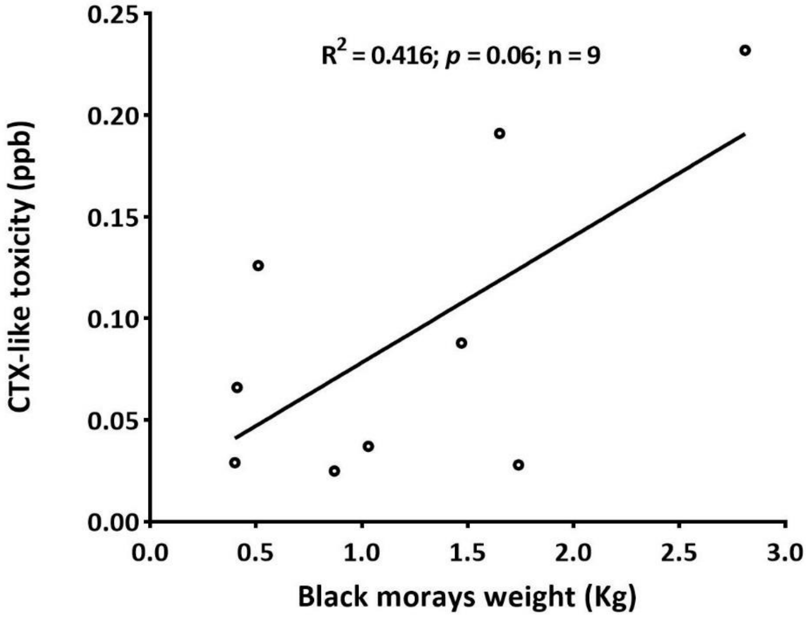
Figure 5. Correlation between ppb of CTX-like toxicity obtained by CBA in positive black morays (n = 9) and their corresponding weight.


(a)
Black moray
% Cell viability
mg TEq./ml
O/V -
O/V +
(b)
Dusky grouper
% Cell viability
mg TEq./ml
O/V -
O/V +
(c)
P-CTX1B
% Cell viability
pg P-CTX1B/ml
O/V -
O/V +


A
x10²
Counts vs Acquisition Time (min)
B
x10²
Counts vs Acquisition Time (min)
C
x10²
Counts vs Acquisition Time (min)


(a)
(b)


CTX-Like toxicity (ppb)
0.20
0.15
0.10
0.05
0.00
LZ
(n = 23)
(0.065, 0.031)
FU
(n = 6)
(0.019, 0.016)
TF
(n = 10)
(0.078, 0.055)
LP
(n = 3)
(0.036, 0.028)
HI
(n = 21)
(0.119, 0.070)



R^2 = 0.416; *p* = 0.06; n = 9
CTX-like toxicity (ppb)
0.25
0.20
0.15
0.10
0.05
0.00
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Black morays weight (Kg)