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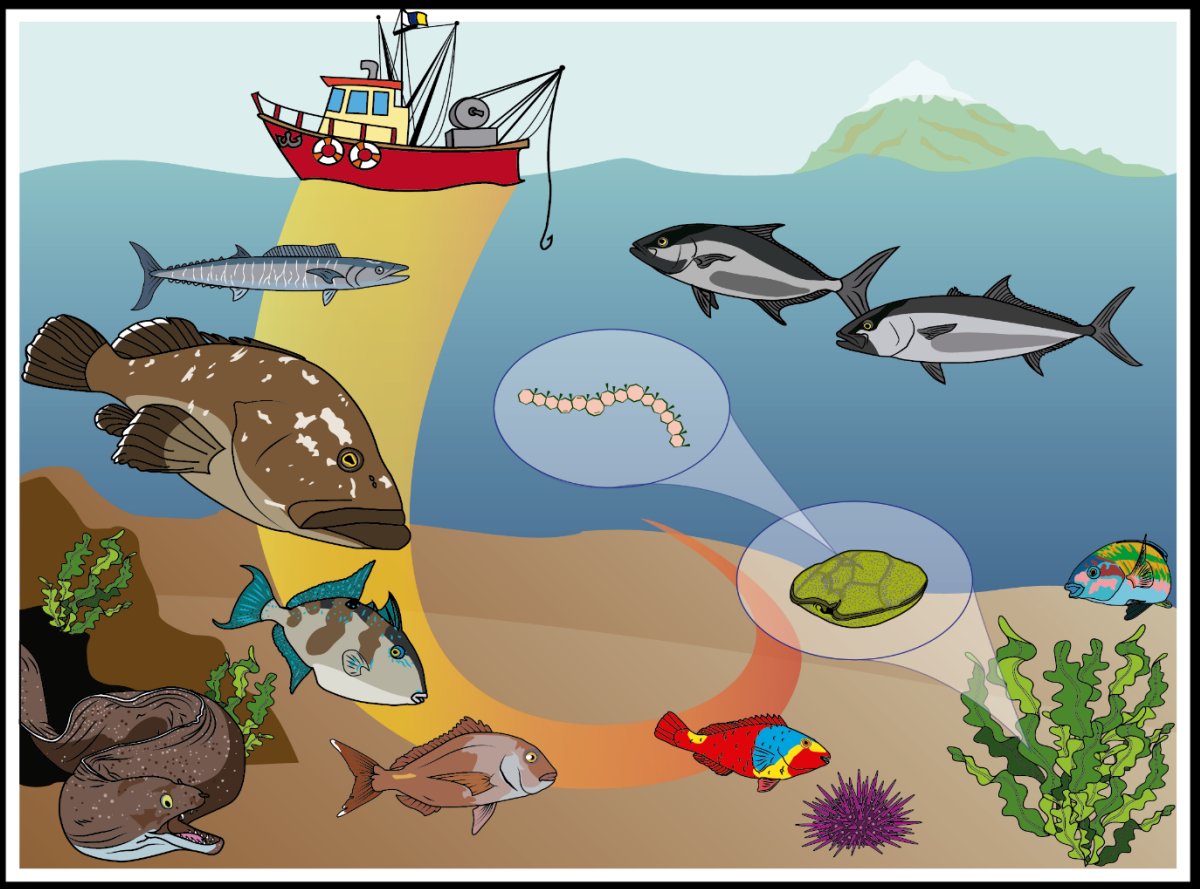

J. Andres Sanchez Henao
Doctorado en Sanidad Animal y Seguridad Alimentaria

Las Palmas de Gran Canaria
Marzo 2021

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TESIS DOCTORAL

PRESENCIA DE CIGUATOXINA EN PECES CAPTURADOS EN CANARIAS Y SU ACUMULACIÓN BASADA EN UN ESTUDIO EXPERIMENTAL CON *CARASSIUS AURATUS*

JULIAN ANDRES SANCHEZ HENAO

DOCTORADO EN SANIDAD ANIMAL Y SEGURIDAD ALIMENTARIA

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D. ANTONIO FERNÁNDEZ RODRÍGUEZ, COORDINADOR DEL PROGRAMA DE DOCTORADO DE SANIDAD ANIMAL Y SEGURIDAD ALIMENTARIA DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA,

INFORMA,

Que la Comisión Académica del Programa de Doctorado, en su sesión de fecha / / , tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral titulada "**Presencia de ciguatoxina en peces capturados en Canarias y su acumulación basada en un estudio experimental con *Carassius auratus*)**" presentada por la doctorando **D J. Andres Sanchez Henao** y dirigida por el **Doctor Fernando Real Valcárcel** y la **Doctora Natalia García Álvarez**.

Y para que así conste, y a efectos de lo previsto en el Artº 11 del Reglamento de Estudios de Doctorado (BOULPGC 7/10/2016) de la Universidad de Las Palmas de Gran Canaria, firmo la presente en Las Palmas de Gran Canaria, a de de 2021.

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ABREVIATURAS

Bomba Na/K	Bomba sodio/potasio
C-CTXs	Ciguatoxinas del Caribe
CBA	Ensayo Celular (por sus siglas en inglés: *Celular Bassed Assay*)
CP	Ciguatera (por sus siglas en inglés: *Ciguatera Poisoning*)
CTXs	Ciguatoxinas
EFSA	Autoridad Europea de Seguridad Alimentaria (por sus siglas en inglés: *European Food Safety Authority*)
FDA	Administración de Medicamentos y Alimentos de Estados Unidos (por sus siglas en inglés: *Food and Drug Administration*)
I-CTXs	Ciguatoxinas del Índico
IUSA	Instituto Universitario de Sanidad Animal y Seguridad Alimentaria de la ULPGC
LC-HRMS	Cromatografía Líquida de Alta Resolución (por sus siglas en inglés: *Liquid chromatography - high resolution accurate mass*)
LC-MS/MS	Cromatografía Líquida Acoplada a la Espectrometría de Masas (por sus siglas en inglés: *Liquid chromatography–mass spectrometry*)
LOAEL	Nivel mínimo de efecto adverso observado (por sus siglas en inglés: *Lowest Observed Adverse Effect Level*)
Na_V	Canal de sodio voltaje dependiente
N2a	Neuroblastoma 2a de ratón
MBA	Ensayo en ratones (por sus siglas en inglés: *Mouse Bio-Assay*)

MTT	3-[4,5-dimethylthiazole-2-yl]-2,5 diphenyltetrazolium bromide
P-CTXs	Ciguatoxinas del Pacífico
RBA	Ensayo de unión a receptor (por sus siglas en inglés: *Receptor Binding Assay*)
SVEICC	Sistema de Vigilancia Epidemiológica de la Intoxicación por Ciguatera en Canarias

INTRODUCCIÓN

La intoxicación por ciguatoxinas (CTXs), también conocida como ciguatera (CP), es la intoxicación por toxinas marinas naturales más comúnmente asociada al consumo de pescado a nivel mundial (Friedman et al., 2008). Dichas CTXs son producidas por microalgas de los géneros *Gambierdiscus* y *Fukuyoa*.

La enfermedad cursa con una gran variedad de síntomas gastrointestinales, cardiovasculares e incluso nerviosos (Friedman et al., 2017; Vlamis et Katikou, 2014). La CP ha sido asociada históricamente a zonas tropicales, como el océano Pacífico, el océano Índico y el mar Caribe (Lewis, 2006), pues los registros más antiguos conocidos de intoxicaciones con sintomatología similar a la descrita datan de 618 y 917 d. C. en China, además de describirse numerosos casos de intoxicación en tripulaciones de barcos de famosas expediciones realizadas entre los siglos XVII y XIX (Russell et Egen, 1991). Curiosamente, a pesar de ser una enfermedad producida por consumo de pescado tóxico, su nombre proviene del gasterópodo (*Cittarium pica*), conocido como Cigua por los aborígenes cubanos (Gudger, 1930), que al consumirlo podía provocar indigestiones con síntomas similares a la ciguatera; las personas que sufrían estas indigestiones eran conocidas como "ciguatos" o "enciguatotodos". Desde entonces, el término ciguatera siguió usándose para indicar intoxicaciones con síntomas gastrointestinales y neurológicos por consumo de cualquier tipo de animal marino, pero durante el siglo XX se usó para referirse únicamente a las intoxicaciones por consumo de pescado coralino o semipelágico (Guzmán-Pérez et Park, 2000; Russell et Egen, 1991).

ASPECTOS RELEVANTES DE LA CIGUATERA

DISTRIBUCIÓN Y REGULACIÓN DE LA CIGUATERA

Esta enfermedad endémica del cinturón circumtropical abarca las latitudes 35ºN y 35ºS (FAO, 2020) donde se registran numerosos casos anualmente (entre 10000 a 50000) (EFSA, 2010). Sin embargo, se estima que sólo el 20% de las intoxicaciones producidas mundialmente se registran adecuadamente, ya sea por la levedad del cuadro asociado o por la falta de seguimiento epidemiológico necesario en las áreas donde ocurren. Dichas latitudes, donde la CP es común, incluyen numerosas islas remotas con un sistema sanitario limitado que dependen en gran parte de la pesca para su subsistencia (Azziz-Baumgartner et al., 2012; Friedman et al., 2008; Laurent et al., 2005). En las últimas décadas la CP se ha extendido a áreas más templadas, incluso se han registrado nuevas especies de *Gambierdiscus* en áreas no endémicas (Rodríguez et al., 2017). A pesar de ello, el consumo de pescado importado contaminado y el turismo en zonas donde la enfermedad es endémica son las principales causas de los casos en zonas tradicionalmente libres de la enfermedad, como los registrados en países de Europa como Francia, España continental, Portugal continental, Alemania, Italia y Holanda (Caillaud et al., 2010; FAO, 2020).

En España se tienen datos anecdóticos de intoxicaciones de personas que viajaron a países del Caribe (Gascón et al., 2003), hasta el año 2004, cuando se presentó el primer brote de CP en las islas Canarias con 5 personas afectadas tras consumir un pescado capturado en aguas entre el archipiélago canario y Madeira (Pérez-Arellano et al., 2005). Más tarde, en 2008, se reportaron 2 brotes más en el archipiélago con aproximadamente 30 individuos afectados (Boada et al., 2010). Mientras, entre 2007 y 2008, se registraron los primeros brotes en las islas portuguesas del atlántico este, el primero afectó a

6 guardas de las islas Salvajes (islas vírgenes al sur de Madeira, Portugal), y el segundo en Madeira afectando a 11 personas. Ambos brotes fueron provocados por el consumo de pescado local (Otero et al., 2010). Desde entonces, los brotes de CP en el archipiélago canario no han dejado de sucederse, hasta alcanzar un total de 20 brotes, el último registrado en diciembre de 2019 con un total de 6 personas afectadas por consumo de un medregal proveniente de pesca deportiva (AESAN, 2021; Dirección General de Salud Pública, 2021).

Aunque la CP es una enfermedad de amplia distribución, existe muy poca regulación enfocada a evitar la comercialización de pescado contaminado con CTXs en algunos lugares donde es endémica. Algunos países prohíben la comercialización e importación de ciertas especies de pescado, otros sugieren a la población evitar su consumo y otros realizan análisis en muestras representativas de pescado en función de la especie, peso, periodo del año y área geográfica (Chan, 2015; Clua et al., 2011; EFSA, 2010; Laurent et al., 2005).

En España existen los Reglamentos (UE) 853/2004 del Parlamento y del Consejo, de 29 de abril de 2004, por el que se establecen normas específicas de higiene de los alimentos de origen animal y 854/2004 del Parlamento y del Consejo, de 29 de abril de 2004, por el que se establecen normas específicas para la organización de controles oficiales de los productos de origen animal destinados al consumo humano, donde se especifica que: "no se pondrán en el mercado los productos de la pesca que contengan biotoxinas tales como la ciguatoxina y otras toxinas peligrosas para la salud humana"; pero no existía una herramienta específica que ayudase a prevenir dicha comercialización. Fue a raíz de los casos ocurridos en 2008 en la región Macaronésica cuando el Gobierno de Canarias desarrolló un Sistema de Vigilancia Epidemiológica de la Intoxicación por Ciguatera en Canarias (SVEICC) (Dirección General de Salud Pública, 2021), donde se estableció un protocolo de actuación en caso de presentarse un paciente con un cuadro clínico

compatible y antecedentes de haber consumido pescado de algunas de las especies consideradas de riesgo que se capturan en Canarias (medregal, abade, mero, pejerrey, bicuda, morena, peto y sierra) (Anexo I). Más tarde, en el año 2011, y ante el continuo suceso de brotes de CP en Canarias, la Dirección General de Pesca del Gobierno de Canarias estableció un protocolo de prevención de la enfermedad con el objetivo de muestrear ciertas especies de peces, principalmente aquellas que se han visto involucradas en casos de CP autóctonos y otras que podrían representar un riesgo, todas dentro de un peso establecido (medregal, mero, abade, pejerrey, peto, picudo y pez espada) (Anexo II). Con este protocolo ya se cumplía con el Reglamento de Ejecución (UE) 2019/627 de 15 de marzo de 2019, por el que se establecen disposiciones prácticas uniformes para la realización de controles oficiales de los productos de origen animal destinados al consumo humano donde se indica qué: "no se comercializarán productos de la pesca que contengan biotoxinas tales como la ciguatoxina". Este muestreo se realiza en los puntos de primera venta con el fin de analizar la presencia de CTXs en el músculo de cada ejemplar considerado de riesgo antes de comercializarlos, realizándose dicho análisis en el Instituto Universitario de Sanidad Animal y Seguridad Alimentaria de la Universidad de Las Palmas de Gran Canaria (IUSA) (Dirección General de Pesca, 2021). Desde entonces, los brotes registrados de CP han sido asociados a pesca deportiva o al consumo de pescado no controlado de venta ilegal, en algunos casos de peso inferior a lo establecido en dicho protocolo oficial, lo que ha llevado a una continua actualización de los pesos mínimos de riesgo indicados para realizar el muestreo (Anexo II). Además, en agosto del 2015 la CP pasó a ser considerada como una enfermedad de declaración obligatoria en las islas Canarias (Dirección General de Salud Pública, 2021).

MICROALGAS Y PRODUCCIÓN DE CTXs

La CP está provocada por la ingestión de CTXs presentes en la carne de pescado. Sin embargo, estas toxinas son producidas inicialmente por microalgas de los géneros *Gambierdiscus* y *Fukuyoa* (Vlamis et Katikou, 2014). Actualmente se conocen un total de 16 especies de *Gambierdiscus* y 3 de *Fukuyoa*, y es probable que este número aumente gracias a las técnicas moleculares de identificación (Kretzschmar et al., 2019; FAO, 2020). Estos dinoflagelados prefieren aguas con poca corriente y lugares específicos protegidos donde fijarse a la superficie de macroalgas y corales (Dickey et Plakas, 2010). Por esta razón, pueden coexistir zonas libres de CTX a pocos kilómetros de zonas con presencia de casos de CP (Chan et al., 2011; O'toole et al., 2012). Las alteraciones de los corales, producidas de forma natural o por la actividad humana, se han visto relacionadas con un aumento de brotes de CP, probablemente por la diseminación de dichas microalgas (Bagnis, 1994; Rongo et Woesik, 2013; Chateau et al., 2007).

Estas microalgas producen las CTXs y otros metabolitos tóxicos como la maitotoxina (Soliño et Costa, 2018), presentando diferentes perfiles tóxicos dependiendo de la especie. Estas toxinas son incorporadas a la cadena alimentaria por los peces herbívoros que se alimentan de las macroalgas o los corales donde se han asentado los dinoflagelados. Una vez en el pez, estos compuestos pueden bioacumularse y sufrir transformaciones a su paso por los eslabones de la cadena trófica llegando a alcanzar altos niveles tóxicos en los grandes peces carnívoros (Dickey et Plakas, 2010; Mak et al., 2013).

Las CTXs son grandes moléculas conformadas por una sucesión de 13 o 14 anillos de éter, con diferencias en su estructura, número de carbonos y mecanismo de acción (Soliño et Costa, 2018). Tradicionalmente, las CTXs se clasificaban en función de la región donde eran producidas como CTXs del océano Pacífico (P-CTX), del océano Índico (I-CTX) y

del mar Caribe (C-CTX) (Vernoux et Lewis, 1997). Actualmente se conocen más de 30 análogos de CTXs pertenecientes a los grupos de las P-CTXs, C-CTXs y de las I-CTXs (Diogène etal., 2017; Kryuchkov et al., 2020). Sin embargo, atendiendo a sus diferencias en el número de carbonos se pueden dividir en 3 grupos:

- P-CTX-I (60 carbonos), es el grupo más estudiado cuyas CTXs son más tóxicas cuanto más polares son. Este proceso podría producirse tras su paso por el metabolismo del pez (Ikehara et al., 2017), siendo la más tóxica la P-CTX1B (también conocida como CTX1B) que a su vez se encuentra más frecuentemente en grandes peces carnívoros y no se ha detectado en las microalgas (Lewis, 1991; Mak et al., 2013; Oshiro et al., 2010; Soliño et Costa, 2018).
- P-CTX-II (57 carbonos) al que pertenece la P-CTX3C (también conocida como CTX3C). Estas toxinas se encuentran tanto en microalgas como en peces carnívoros y al igual que las P-CTX-I también sufren las mismas transformaciones (Yasumoto, 2000). El análogo 51-OH-CTX3C presenta una toxicidad similar a la CTX1B. Sin embargo, a diferencia del grupo anterior, la molécula más oxidada no es la más tóxica (Yogi et al., 2014).
- Grupo de las CTXs del Caribe y el Índico (C-CTX e I-CTX) (62 carbonos), ambas similar en masa y con muchos congéneres aún por elucidar. Las C-CTXs comparten ciertas características químicas estructurales con las P-CTX-II (Lewis et al., 1998). Además, sus productos más oxidados no son los más tóxicos (Soliño et Costa, 2018). Sin embargo, a diferencia de las CTXs del Pacífico, los microorganismos productores de estas CTXs no están claros aún y es necesario determinar mejor sus características químicas y su potencial tóxico (Caillaud et al., 2011; Soliño et Costa, 2018).

Sin embargo, un informe reciente de la FAO realizado por un comité de expertos en ciguatera (FAO, 2020), considera más apropiado clasificar las CTXs según su estructura química en 4 grupos principales: CTX4A, CTX3C, C-CTX e I-CTX.

En la región Macaronésica cabría esperar encontrar únicamente C-CTXs, no obstante, se han encontrado en numerosos organismos (microalgas del género *Gambierdiscus* y peces) diferentes análogos de los tres grupos de CTX: CTX4A/B, CTX1B pertenecientes al grupo P-CTX-I; CTX3C y sus congéneres más tóxicos del grupo P-CTX-II; además de la C-CTX1 (Boada et al., 2010; Estévez et al., 2019; Fraga et Rodríguez, 2014; Pérez-Arellano et al., 2005; Paz et al., 2011).

INTERACCIÓN DE LAS CTXs EN LA CADENA TRÓFICA MARINA

Actualmente, más de 400 especies de peces se han visto involucradas en brotes de CP (Tester et al., 2010), siendo los peces carnívoros de arrecife los más comúnmente implicados (Lehane et Lewis, 2000). Los peces carnívoros más comunes son: barracudas, medregales, meros, pargos, carángidos y morenas. Los peces herbívoros pueden acumular niveles de CTXs suficientes para causar intoxicaciones en humanos (Gaboriau et al., 2014; Satake et al., 1996) y los más frecuentemente implicados son: lábridos, escáridos o peces loro y acantúridos o peces cirujano (FAO, 2020).

Aunque la CP es una enfermedad producida principalmente por consumo de pescado, las CTXs han sido encontradas de forma muy poco frecuente en algunos invertebrados marinos como pulpos, langostas, almejas, caracoles, erizos y estrellas de mar, en estos últimos, probablemente, por su comportamiento roedor sobre la superficie de los corales (Darius et al., 2018; Mak et al., 2013; Silva et al., 2015; Roué et al., 2016; Yasumoto et Kanno, 1976). Sin embargo, son pocos los casos de CP que se han registrado

a partir del consumo de invertebrados, a pesar de observarse una transferencia de CTXs en este grupo de animales (Chinain, 2020).

MECANISMO DE ACCIÓN DE LAS CTXs

La CTX, una vez entra en el pez, es absorbida rápidamente a través del tracto gastrointestinal y distribuida por el organismo (Davin et al., 1988; Ledreaux et al., 2014). Sin embargo, la retención de las CTXs en los tejidos de los peces parece variar ampliamente entre especies (Ledreux et al., 2014; Li et al., 2020). En algunas especies frecuentemente involucradas en brotes de la enfermedad se ha demostrado que la toxina puede permanecer en el tejido muscular amplios periodos de tiempo (Banner et al., 1966; Laurent et al., 2005; Li et al., 2020).

Esta toxina tiene la capacidad de unirse a los canales de sodio voltaje dependientes (Na_v), tanto de mamíferos como de peces (Dechraoui et al., 1999; Dechraoui et al., 2006). Cuando se une, activa dichos canales presentes en el exterior de los axones, que se encuentran normalmente cerrados, induciendo una apertura que favorece la entrada de moléculas de sodio al interior celular, que de forma normal desencadena despolarizaciones y potenciales de acción. Este influjo de sodio normalmente está acompañado por una salida de potasio hacia el exterior celular que evita la entrada de agua a través de la membrana celular. Sin embargo, cuando la CTX altera este sistema se produce una inflamación de los axones, lo que ralentiza la conducción de las señales sensoriales y motoras, provocando una sintomatología en función del sistema afectado (Benoit et al., 2002; Cameron et al., 1991; Mattei et al., 2014). En los peces aún no se conoce el mecanismo por el cual algunas especies (herbívoras y carnívoras) pueden acumular grandes cantidades de CTXs en sus tejidos sin desarrollar sintomatología (Soliño et Costa, 2020).

SINTOMATOLOGÍA DE LA CIGUATERA Y TRATAMIENTO

En humanos, los síntomas de la CP aparecen normalmente en las primeras 12 - 24 h tras la ingestión de pescado contaminado. Los primeros en aparecer, aunque no siempre presentes, son los gastrointestinales, tales como vómito, dolor abdominal y diarrea. Más tarde, dentro de los primeros 2 días, aparecen síntomas neurológicos. Los más comunes son parestesias en pies, manos o región oral, sabor metálico y sensación de pérdida de dientes, prurito, dolores musculares y articulares, dolor de cabeza, mareos y el más representativo: la alodinia al frío, que se define como una alteración de la percepción de la temperatura que al tocar superficies frías provoca una sensación de ardor. Menos comunes en general son las alteraciones del sistema nervioso central como alucinaciones o coma. Los síntomas cardiovasculares, como hipotensión junto con irregulares subidas de tensión arterial, bradicardias y taquicardia son poco frecuentes y pueden aparecer en los primeros días de la enfermedad (Friedman et al., 2017; Lehane, 2000; Lehane et Lewis, 2000; Quod et Turquet, 1996). Muchos de estos síntomas pueden persistir durante días o meses, describiéndose en algunos casos una persistencia de años (Friedman et al., 2017; Lawrence et al., 1980). Aunque la sintomatología es aparatosa, la tasa de mortalidad es realmente baja, y sucede debido al resultado de una suma de condiciones como deshidratación, fallo vascular o fallo respiratorio por parálisis de los músculos respiratorios; situaciones que se ven agravadas en zonas donde el acceso a los cuidados médicos de emergencia es limitado (Friedman et al., 2017; Hamilton et al., 2010; Lewis, 2006).

Los síntomas pueden repetirse semanas o meses después de superada la intoxicación, tras la ingestión de alimentos como pollo, cerdo, café, nueces y pescado no marino, inclusive tras actividad física o deshidratación (Gillespie et al., 1986; Lehane et Lewis, 2000).

A pesar de los avances científicos, actualmente no existe un tratamiento efectivo. Hasta la fecha se recomienda la administración de manitol intravenoso dentro de las primeras 72 h tras la ingestión de pescado tóxico, cuya función como diurético osmótico es la de reducir el edema neuronal producido por la CTX. Este tratamiento debe administrarse con precaución en caso de pacientes con síntomas cardiovasculares (Friedman et al., 2008; Neves et al., 2019; Pearn, 2001; Darius et al., 2013). Aparte del manitol, solo quedaría suministrar al paciente tratamiento sintomático usando antieméticos, antidiarreicos, antihistamínicos, analgésicos, psicofármacos o el uso de carbón activado (Davis et Villar, 1986; Darius et al., 2013; Lewis et Endean, 1984; Neves et al., 2019).

DIAGNÓSTICO DE LA CIGUATERA Y DETECCIÓN DE CTX

Aún no existe un método que diagnostique la intoxicación por CTXs en personas, por lo que el diagnóstico se basa en la sintomatología y el antecedente epidemiológico de haber consumido pescado de alguna de las especies consideradas de riesgo y se confirma en caso de detectarse CTX en el remanente del pescado involucrado en el brote (Wang et al., 2020; Anexo II).

Otra característica de esta enfermedad, que aumenta su complejidad, es la dificultad para detectar el pescado contaminado, al no verse afectadas con la presencia de CTXs sus características organolépticas: olor, sabor y color. Además, sus propiedades químicas facilitan la permanencia de la toxina en la carne de pescado, ante determinadas acciones como: la congelación, el cocinado y la exposición a medios ácidos y básicos (Dickey et Plakas, 2010; Friedman, 2008; Lehane et Lewis, 2000).

Las CTXs son, por tanto, casi imposibles de evitar por el consumidor. Sin embargo, en las áreas históricamente afectadas por la CP, la población local implementa métodos caseros para evitar consumir pescado contaminado como la observación de la presencia o no de

rigor mortis, cocinar la pieza de pescado con una moneda, observar signos hemorrágicos en el pescado o darle un trozo de pescado a un animal doméstico; estos métodos carecen de evidencia científica y de implicación ética en el caso del uso de animales (Darius et al., 2013; Lehane et Lewis, 2000). La existencia en el mercado de un test rápido (inmunológico) de detección de CTXs en pescado ofrecía el resultado en menos de una hora. Sin embargo, dado el alto número de falsos negativos y positivos, no supuso una protección eficiente (Bienfang et al., 2011).

Los ensayos de detección de CTXs en laboratorio requieren técnicas y material específico, así como personal cualificado para la realización de los mismos. Estos ensayos requieren un primer paso donde se obtiene un extracto de la muestra a analizar que, en función de la metodología a aplicar, podría requerir una fase más de purificación. Dichos ensayos se dividen en:

- Ensayo *in vivo* en ratones (MBA), fue ampliamente realizado desde 1961 hasta finales del siglo XX. Este ensayo se basa en la inyección por vía intraperitoneal del extracto de pescado en ratones para después observar y medir su reacción dentro las 24 h siguientes. A pesar de conllevar una serie de desventajas éticas y económicas, aún se utiliza al no requerir un equipamiento analítico complejo. Este ensayo no está recomendado por la Autoridad Europea de Seguridad Alimentaria (EFSA) y cada vez es menos frecuente su uso (Leonardo et al., 2020; Pasinski et al., 2020).

- Ensayos *in vitro*, son los métodos de elección para la detección de CTXs. Los principales son:

    - Ensayo N2a-MTT: ensayo celular (CBA) donde se emplea normalmente un cultivo de células de neuroblastoma de ratón (N2a) para detectar

toxinas marinas con capacidad de activar los Na_V. La CTX carece de efecto citotóxico en estas células, por ello esta técnica emplea el uso de ouabaína (inhibidor de la bomba Na/K) y veratridina (activador del Na_V al igual que las CTXs, pero en diferente sitio de unión) cuyos efectos combinados en presencia de CTXs generan un efecto citotóxico. Es por esto que este ensayo se basa en la detección colorimétrica de células N2a metabólicamente activas tras ser tratadas con un extracto de CTX en presencia de ouabaína/veratridina, usando el reactivo MTT, el cual sufre en el interior de las células vivas una reducción química que produce un cambio de color evidenciando las células supervivientes (Berridge et al., 2005). Este ensayo detecta la presencia de bajas cantidades de CTX, dado su bajo límite de detección (0,01 ppb P-CTX1B cuando el método está bien optimizado), y se obtienen resultados en 3 días. Por ello, es el más utilizado para analizar el potencial tóxico de una muestra y es la técnica empleada en el protocolo de control oficial que se lleva a cabo en Canarias. Este método considera una muestra como positiva cuando se observa toxicidad tipo CTX a partir de un 80% de viabilidad celular y permite la cuantificación de la toxicidad con respecto a un estándar. Sin embargo, no permite identificar ni cuantificar el análogo o cóctel de análogos de CTXs presente en la muestra (Caillaud et al., 2012; Manger, 1993, 1995; Pasinszki et al., 2020).

- Ensayo de unión a receptores (RBA): esta técnica se basa en la capacidad de la CTX para desplazar a la brevetoxina, una toxina producida por microalgas que se une al Na_V en el mismo sitio que la CTX. Las CTXs tienen una afinidad 50 veces mayor al sitio de unión del Na_V que la brevetoxina. Esta metodología emplea una membrana cerebral de rata

con brevetoxina marcada, que tras la exposición a un extracto de CTX se ve reducida su radioactividad o fluorescencia proporcionalmente, según el método de marcado utilizado, permitiendo la cuantificación de la toxina presente en el extracto. Esta metodología genera resultados en unas horas, pero es menos sensible para detectar la presencia de CTXs que el CBA (FAO, 2020; Pasinszki et al., 2020).

- Ensayos inmunológicos: han estado presentes desde hace 40 años, llegando alguno incluso a usarse como método de determinación de CTX en pescado antes de su venta en Hawaii, pero debido a los costes generados dejó de emplearse (Kimura et. al., 1982). La principal característica de estas técnicas es el uso de anticuerpos específicos para las moléculas buscadas. Las CTXs son una extensa familia de moléculas, lo que implica fabricar anticuerpos específicos para cada molécula e incluirlos en cada ensayo o fabricar un anticuerpo que se una a un sitio común de los diferentes análogos de CTX. Esto podría generar reacciones cruzadas con componentes que tengan una estructura molecular similar o la detección de moléculas de CTXs de baja potencia que no reflejen el potencial tóxico de la muestra (Bienfang et al., 2011; Lehane et Lewis, 2000; Pasinszki et al., 2020).

- La cromatografía líquida acoplada a la espectrometría de masas (LC-MS/MS) y la cromatografía líquida de alta resolución (LC-HRMS), son técnicas complementarias a la CBA y RBA ya que, a diferencia de estas, permiten confirmar y analizar el perfil tóxico de una muestra, detectando y cuantificando los análogos de CTXs presentes. Sin embargo, son técnicas más costosas que emplean instrumental

complejo, difícilmente disponible en cualquier laboratorio. Estos motivos las hacen, de momento, inviables como protocolo de rutina para la detección de CTXs en pescado previo a su comercialización, sobre todo cuando es necesario analizar un número destacado de muestras (Diogène, 2017; FAO, 2020; Pasinski et al., 2020).

TOXICIDAD EN HUMANOS Y LÍMITES DE RIESGO

La Administración de Medicamentos y Alimentos de Estados Unidos (FDA) recomienda 0,01 ppb equivalentes de P-CTX1B como nivel de seguridad y la opinión científica de la EFSA también lo recoge en sus textos (EFSA, 2010). No obstante, estas son recomendaciones no consideradas a nivel legislativo (FAO, 2020), pues los datos de donde se desprende este valor provienen de estudios realizados a finales de los años 90 donde la concentración más baja de CTX registrada hasta ese momento, en pescado asociado a brote, era de 0,1 µg P-CTX1B/Kg de carne. A este dato se le aplicó un valor de incertidumbre de 10 (EFSA, 2010). Además, recientemente se han registrado valores de 0,02 ppb equivalentes de CTX1B en carne de pescado asociado a brotes de CP (Farrell et al., 2017; Hossen et al., 2015), dejando el anterior valor de nivel de seguridad (0,01 ppb equivalentes de P-CTX1B) justo por debajo de los nuevos datos.

Todo lo descrito sumado a los pocos estudios que aportan información completa de los brotes como: la dosis ingerida y la concentración de toxina en el pescado implicado, impiden una evaluación adecuada del riesgo de exposición a CTX. En consecuencia, el informe de la Reunión de Expertos sobre la Intoxicación por Ciguatera (FAO, 2020) propone una estimación del riesgo de intoxicación basada en escenarios de exposición alimentaria aguda a CTX en función de la cantidad de pescado ingerido y la concentración de toxina presente en pescado, basados en los datos publicados del estudio más completo hasta el momento de un brote de CP (Hossen et al., 2015).

La comunidad científica precisa encontrar material de referencia y estándares de CTX certificados que permitan la validación de los métodos de detección, así como una precisa estimación del riesgo de intoxicación por CP en las diferentes áreas del mundo y una regulación de la misma.

JUSTIFICACIÓN Y OBJETIVOS

En 2004, se informó de la primera intoxicación por CP en las islas Canarias (Pérez-Arellano et al., 2005) y desde 2015 la enfermedad ha sido incluida en este archipiélago en la lista de enfermedades de declaración obligatoria.

En algunos lugares del mundo existen limitadas medidas reguladoras frente a la enfermedad. Varios países han impuesto una prohibición o han recomendado evitar el consumo de ciertas especies (Chan, 2015; Clua et al., 2011; Laurent et al., 2005) y otros analizan la toxicidad en el pescado para conocer mejor los riesgos. En este sentido, las islas Canarias, a través de la Dirección General de Pesca del Gobierno de Canarias, es la única región del mundo que está tomando una acción preventiva oficial contra los brotes de CP basada en el análisis de la presencia de CTXs mediante el ensayo N2a-MTT en la carne de pescado de ciertas especies y pesos considerados de riesgo, previo a su comercialización. Este control oficial de rutina se aplica desde 2011 y se realiza en el laboratorio del IUSA que, a su vez, colabora activamente en proyectos de marco europeo, como es el caso del proyecto EuroCigua, cofinanciado por la EFSA, o el proyecto RASPA, de la convocatoria Interreg, que incluye financiación FEDER.

Esto permite contar con bancos de muestras y extractos únicos para el estudio de CTX en peces capturados en este archipiélago. Entre ellos se cuenta con extractos de morenas y meros que, además de ser de gran interés pesquero en canarias (Espino et al., 2018), son las especies con mayores concentraciones de CTXs descritas en la literatura, entre otros peces carnívoros (Chan et al., 2011; Mak et al., 2013). También son las especies responsables de numerosos brotes en las regiones del océano Indo-Pacífico y del Atlántico (Lehane et Lewis, 2000). Los meros en las islas Canarias han estado implicados en intoxicaciones por CP, sin embargo, las morenas no se han asociado a ningún brote, a pesar de observarse toxicidad en muchas de ellas y del frecuente consumo de esta especie por parte de la población canaria.

En Canarias se han aislado diferentes especies de *Gambierdiscus* productoras de CTXs en muestras de agua y macroalgas (Aligizaki et al., 2008; Rodríguez et al., 2017; Tudó et al., 2020). Estos autores destacaron el alto impacto socioeconómico de la CP sobre la actividad pesquera y la salud pública en el archipiélago canario, que requiere investigar de forma multidisciplinar la presencia de CTXs en los peces y conocer el ciclo de vida y la toxicidad de *Gambierdiscus* spp. Se sabe que estas toxinas se bioacumulan y se transforman en los peces pudiendo causar intoxicación en los seres humanos, pero se han realizado pocos ensayos para comprender cómo se incorporan las CTXs a los peces y su posible efecto tóxico (Davin et al., 1986, 1988; Ledreux et al., 2014; Clausing et al., 2018; Li et al., 2020).

Todo ello justifica el desarrollo de la presente tesis doctoral, cuyo objetivo general es el de ampliar el conocimiento de la presencia de CTXs en el pescado de consumo del archipiélago canario y su acumulación en el tejido muscular de los peces mediante el desarrollo de un modelo experimental.

Para alcanzar este objetivo general se propusieron los siguientes objetivos específicos:

Identificar y analizar las variables que puedan influir en la probabilidad de capturar pescado contaminado con CTXs de las especies de riesgo consideradas en el protocolo de control oficial del pescado, realizado en los puntos de primera venta autorizados del archipiélago canario.

- Profundizar en el estudio de especies pesqueras de especial importancia para la población canaria como portadoras de CTXs en el medio marino, particularmente meros y morenas.

- Desarrollar un modelo experimental con una especie de pez de fácil manejo en el laboratorio: carpa dorada (*Carassius auratus*), alimentada con carne de medregal contaminado con C-CTX1, para describir posible sintomatología asociada a altos niveles de CTX y estimar el tiempo necesario para que sea detectable en su tejido muscular.

Los objetivos se pueden ver resumidos en el siguiente esquema.

PROYECTO DE TESIS

FUENTES DE MUESTREO/FINANCIACIÓN

Protocolo del control oficial del Gobierno de Canarias

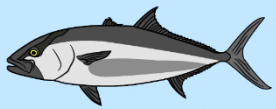
Proyecto EuroCigua (GP/EFSA/AFSCO/2015/03)

RASPA, Programa de Cooperación Interreg-V-a MAC 2014-2020 (MAC2/1.1a/305)

CIGUARISK-CIGUAFOOD, Plan Estatal de Proyectos I+D+i (PID2019-108781RR-C22)

ESPECIES ESTUDIADAS

Petos
(*Acanthocybium solandri*)

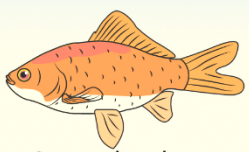


Medregales
(*Seriola* spp.)

Meros
(*Epinephelus marginatus*)

Meros
(*Epinephelus marginatus*)

Morenas
(Familia *Muraenidae*)



Carpas doradas
(*Carassius auratus*)
Libres de CTX

OBJETO DE ESTUDIO

CTXs
en especies del medio natural

Alimentación con **CTX**

OBJETIVOS ESPECÍFICOS

Valor predictivo de CTX en Medregal

Factores asociados a la presencia de CTX en pescado (peso, estación del año e isla de captura)

Análisis de la presencia de CTXs en morenas

Descripción de la interacción trófica entre meros y morenas en Canarias

Descripción de la sintomatología en peces asociada a CTXs

Análisis de la bioacumulación de CTXs en los peces

PUBLICACIONES

VALOR PREDICTIVO Y PROBABILIDAD DE TOXICIDAD TIPO CTX EN MUESTRAS DE PECES DEL CONTROL OFICIAL DE LA CIGUATERA EN LAS ISLAS CANARIAS

Sanchez-Henao, J.A., García-Álvarez, N., Fernández, A., Saavedra, P., Silva Sergent, F., Padilla, D., Acosta-Hernández, B. (2019). **Predictive score and probability of CTX-like toxicity in fish samples from the official control of ciguatera in the Canary Islands**. *Science of The Total Environment*. 673: p. 576-584. DOI: 10.1016/j.scitotenv.2019.03.445

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Predictive score and probability of CTX-like toxicity in fish samples from the official control of ciguatera in the Canary Islands

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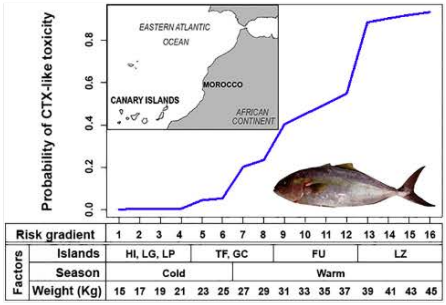
HIGHLIGHTS

- A predictive score of CTX-like toxicity in amberjack fish samples from the Canary Islands was obtained.
- Factors associated with the CTX-like toxicity of fish from the Canary Archipelago were identified.
- The minimum weight limits established by the official control of ciguatera in the Canary Islands need to be revised.
- CTX-like toxicity in fish in the Canary Islands is confirmed to be an endemic problem.

GRAPHICAL ABSTRACT



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ABSTRACT

This research identifies factors associated with the contamination by ciguatoxins (CTXs) in a population of fish and proposes a predictive score of the presence of CTX-like toxicity in amberjack samples from the official control program of ciguatera in the Canary Islands of the Directorate-General (DG) Fisheries (Canary Government). Out of the 970 samples of fish studied, 177 (18.2%) samples showed CTX-like toxicity. The fish were classified according to the species, amberjack (*Seriola dumerili* and *S. rivoliana*) (n = 793), dusky grouper (*Epinephelus marginatus*) (n = 145) and wahoo (*Acanthocybium solandri*) (n = 32). The data were separated by species category and statistically examined, resulting in 137 (17.3%) amberjack and 39 (26.9%) grouper samples showing CTX-like toxicity; regarding wahoo species, only 1 toxic sample (3.1%) was found. According to fishing location the contamination rates suggested grouping the islands in four clusters; namely: {El Hierro: HI; La Gomera: LG; La Palma: LP}, {Gran Canaria: GC; Tenerife: TF}, {Fuerteventura: FU} and {Lanzarote: LZ}. For the amberjack species, the multivariate logistic regression showed the factors that maintained independent association with the outcome, which were the warm season (OR = 3.617; 95% CI = 1.249–10.474), the weight (per kg, 1.102; 95% CI = 1.069–1.136) and the island of fish catching. A prediction score was obtained for the probability of contamination by CTX in amberjack fish samples. The area under de curve (AUC) obtained using the validation data was 0.747 (95% CI = 0.662–0.833). Regarding grouper species, the island of fishing was the only factor that

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showed significant differences associated with the presence of CTX-like toxicity. We provide herein data for a better management and prediction of ciguatera in the Canary Islands, suggesting a review of the minimum limits of fish weight established by the Canary Government for the control program.

1. Introduction

Ciguatera Fish Poisoning (CFP) is one of the most relevant seafood-borne illnesses worldwide and the most commonly reported human food poisoning related to natural marine toxins (Friedman et al., 2008; Suzuki et al., 2017). It consists of a debilitating human neuro-intoxication caused by consumption of varieties of fish species from tropical and subtropical waters, contaminated with bioaccumulated ciguatoxins (CTXs) (Meyer et al., 2016). CFP is characterized by causing gastrointestinal, neurological, and cardiovascular symptoms (Friedman et al., 2017). A range of 10 to 50 thousand people suffering from CFP annually worldwide has been estimated (EFSA, 2010). However, epidemiological data remain unreliable, given that it has been estimated that only ~10–20% of cases are properly diagnosed and reported (Azziz-Baumgartner et al., 2012; Laurent et al., 2005).

CFP is found endemically in tropical and subtropical waters such as the Caribbean Sea, the Indian and the Pacific Oceans (Lewis, 2006). In the 40 years that followed the discovery of CTX (Yasumoto et al., 1977), more than 400 fish species have been implicated in poisoning incidents (Tester et al., 2010), most of which are high-order carnivores (Lehane and Lewis, 2000; Lewis, 2006). In Europe, CFP and CTXs have been gaining interest in recent years due to several reported cases in European countries (e.g., France, Spain, the Netherlands, Germany, and Italy), mostly related to consumption of imported ciguateric fish or people who visited endemic areas of CFP (Caillaud et al., 2010). However, none of the current methods of analysis to determine CTX-group toxins in fish have been formally validated (EFSA, 2010).

Regarding the East Atlantic Ocean, in the Canary Archipelago CFP had not been described until 2004, when 5 people became poisoned (Perez-Arellano et al., 2005). In 2008 two more outbreaks were reported in the Canary Islands (Boada et al., 2010) and 11 people were also affected by CFP in Madeira Archipelago (Otero et al., 2010). Therefore, for some species collected from authorized first sale points considered a risk factor in the Canary Islands, an action protocol with the objective of making the detection of CTX prior to sale and human consumption has been implemented since 2011 by the DG Fisheries of the Canary Government (DG of Fisheries of the Canary Government, 2018). In the last decade, several outbreaks of CFP affecting 113 people (Canary Government, 2017b) have been confirmed in the Canary Archipelago, following the consumption of subsistence and recreational harvested fish and not related to controlled fish. Additionally, since 2015, CFP has been designated a notifiable disease in the Canary Islands.

Regarding the marine biotoxins, precursors of CTXs are produced by benthic dinoflagellates of the genus *Gambierdiscus* (Rodríguez et al., 2017). These precursors are transferred and metabolized through the food web, as *Gambierdiscus* cells are ingested by herbivorous fish, which are then taken by piscivorous fish, both of which are finally consumed by humans. It is believed that CTXs are bioaccumulated through the trophic webs, thus, fish higher in the food web tend to contain the highest CTX concentrations (Banner et al., 1966; Dickey and Plakas, 2010). In addition, CTXs are tasteless, colourless and odourless, which increases the risk of poisoning (Friedman et al., 2008). To date, more than 29 CTX congeners have been identified and grouped according to geographic distribution: Indian CTXs (I-CTX), Caribbean CTXs (C-CTX) and most investigated, Pacific CTXs (P-CTX) according to the presence in the waters where CFP is endemic (Hamilton et al., 2002; EFSA, 2010).

Different species of *Gambierdiscus* have been isolated from water samples collected in the Canary Islands during a spatial study (Aligizaki et al., 2008; Rodríguez et al., 2017). These authors highlighted that socioeconomic impact of ciguatera on fisheries activity and public health in the Canary Islands requires further efforts to implement a faster analytical response to detect CTXs in fish samples, and multidisciplinary research to know life cycle, distribution and toxicity of *Gambierdiscus* spp.

The major goal of the present research was to study several factors which may be associated with the probability to find CTX-like toxicity in fish obtained from first sale points in the Canary Islands in order to describe the statistical significance of these associations or achieve a predictive score, if possible.

2. Materials and methods

2.1. Study area

The Canary Archipelago is located in the Northeastern Atlantic Ocean near Europe (2000 km SW from the Iberian Peninsula) and North Africa (100 km W from the Moroccan coast), FAO Major Fishing Area 34 in the subdivision 1.2 (FAO, 2004).

Two co-occurring amberjack species present in this area were analyzed (*Seriola dumerili* and *S. rivoliana*). These predators can reach large sizes living up to 15 years (Murie and Parkyn, 2008); when the fish are mature, they migrate to coastal areas to spawn. Amberjack species are important fish stocks in the Canary Islands being the fourth and sixth most caught species in La Gomera (*Seriola dumerili*) and El Hierro (*Seriola rivoliana*), respectively (Canary Government, 2017a). Dusky grouper (*Epinephelus marginatus*) is a benthonic species that lives on the coast within 5 to 45 m depth; it is considered a solitary fish, very territorial and sedentary (Göthel, 1992) which predates other fish, crustaceans and cephalopods (Smale, 1986); although it is a fish of relevance to human consumption in the Canary Archipelago, dusky grouper is not among the 10 most caught species in the islands (mainly represented by fish shoals) (Canary Government, 2017a), possibly due to its solitary behaviour. Wahoo is a pelagic animal and a seasonal migratory species, relatively new in the Canary Islands waters where it has settled. Normally it feeds on pelagic prey (Espino et al., 2006).

2.2. Fish sample collection

The official control protocol for CFP implemented by the Canary Government (DG of Fisheries of the Canary Government, 2018) establishes a list of certain species and weights of fish considered a risk factor. This list, based on the local experience, shows the limit weights for fish to be sampled at the authorized first sale points and investigated in the Institute of Animal Health and Food Safety (IUSA) laboratory for CTX detection (Table 1). This list has been updated in 2018.

Table 1
Fish species included in the present work, and weight limits established for CTX analysis by the Canary Government through the official control protocol.

Species	Latin name	Weight[a] (kg)
Amberjack	*Seriola* spp.	15/14[b]
Wahoo	*Acanthocybium solandri*	35
Dusky grouper	*Epinephelus spp.*	17

[a] A particular fish is sampled if weight is equal to or greater than this value.
[b] The minimum weight regarding amberjack was decreased from 15 to 14 kg in 2017 by DG Fisheries of the Canary Government, to better adjust the risk of CTX detection for this species.

The fish samples used in the present study were obtained between April 2016 and December 2017 from the official monitoring of CFP (DG Fisheries of the Canary Islands). Through the mentioned programme, fish samples from professional fishermen associations were sent to the IUSA laboratory of the University of Las Palmas de Gran Canaria (ULPGC) in order to determine the CTX presence in fish flesh. The laboratory received 1538 samples in the study period. However, this research only included 970 fish samples selected from the total, based on the availability of relevant information, such as fish species, weight of the specimen and the island of fishing. Fish species and weights were consistent to those established by the official control protocol, particularly amberjack (*Seriola* spp.) (n = 793), dusky grouper (*Epinephelus marginatus*) (n = 145) and wahoo (*Acantocybium solandri*) (n = 32). Thus, the samples were classified according to the species and different data such as the year of fish catching (2016/2017), period (cold/warm), weight and fishing island.

2.3. Preparation and extraction of CTX

Toxin extraction was performed following the protocol proposed by Lewis in 2003 and carried out by Hossen et al., in 2015 with slight modifications according to the laboratory needs. Fish samples were first homogenized and then a portion of 10 g of flesh was cooked at 70 °C during 10 min. When samples reached room temperature, 30 ml of acetone was added, mixed with ultraturrax and centrifuged at 3000 ×g during 5 min at 4 °C; this last step was repeated twice and both supernatants were pooled. The resulting acetone was filtered through a 0.45 μm of PTFE filter and evaporated with a rotary evaporator at 55 °C. The dried extract was re-suspended in methanol: water (9:1) and N-Hexane for phase separation. The upper phase of N-Hexane was discarded and the methanol phase was dried under N_2 current at 40 °C for a subsequent partition with ethanol:water (1:3) and Diethyl-Ether (DE). The DE was reserved and evaporated under N_2 current to dryness. The final residue was resuspended in 4 ml of methanol and kept at −20 °C to be used in the cytotoxicity assay.

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

Neuro-2a cells (Cell line: CCL131, from ATCC, LGC Standards S.L.U., Barcelona, Spain) 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 P-CTX-1 standard (STD) (R.J. Lewis, The Queensland University, Australia) was used as a reference toxin for the assessment of CTX-like toxicity, as C-CTX reference material is not commercially available (Soliño et al., 2015).

For the cytotoxicity assay, cells were seeded in a 96-well flat bottom microplate (200 μl/well) following the procedure of Caillaud et al. in 2012 using RPMI medium supplemented with 5% of FBS at a density of 70,000 cells per well. Cells were incubated 24 h in the same mentioned conditions. Ouabain (0.1 mM) and veratridine (0.01 mM) were added to half of the seeded wells to favour cell mortality in case of the presence of CTX. In parallel to fish sample extracts testing by Neuro-2a assay, a dose-response curve with P-CTX-1 STD was always performed as an internal control, for cell response evaluation and limit of detection and quantification (LOD/LOQ) establishment. Thus, fish extracts and the STD were evaporated and resuspended in RPMI medium supplied with 5% FBS. Then, cells were exposed to flesh extract and the P-CTX-1 STD at decreasing concentrations in order to ensure the cells sensibility to CTX. Every sample extract and the STD were assayed in triplicate. After a 24 h-period of exposure to fish extracts and to P-CTX-1 standard solution, cell viability was evaluated with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] and DMSO (dimethyl sulfoxide) solutions. The corresponding absorbances were read at 570 nm by a multiwell spectrophotometer scanner and plotted into the Microsoft Office Excel 2016 and GraphPad Prism 7 softwares (GraphPad, San Diego, California, USA). For the interpretation of the dose-response curves, cell viabilities were related to the cell viability of the control column (cells with and without pre-treatment with ouabain/veratridine, O/V).

The assessment of fish matrix effect on the Neuro-2a assay was performed using different concentrations of several fish extracts to expose the cells with or without O/V (50–200 mg Tissue Equivalents (TE)/ml). Several muscle samples from grouper and amberjack showed interference with the assay with concentrations higher than 100 mg TE/ml; on the contrary extracts from muscle tissue of Wahoo species displayed interference above 80 mg TE/ml. For that reason and to unify methodology, 80 mg TE/ml was set as the maximum concentration for testing with the Neuro-2a assay.

Due to the large amount of samples used in this surveillance study, the 50% inhibition concentration (IC50, with O/V) was only assessed with the P-CTX-1 STD in ppb units (pg P-CTX-1/ml) in order to evaluate the cell response and limits. Samples extracts were tested using 1 to 4 columns (serial dilutions) of the 96-well microplate, depending on the number of samples received every week; thus, semi-quantitative estimation of the content in P-CTX-1 equivalents in fish extracts was not always possible to determine. Therefore, in the present study, a response producing less than 20% cell mortality was considered as a non-toxic effect, as other authors suggested (Caillaud et al., 2012), being the concentration of P-CTX-1 STD causing 20% inhibition of cell viability (IC20) set as the LOD and LOQ according to this concentration of fish extract used for testing. Thus, a "positive sample" was considered when the corresponding extract showed an inhibition of cell viability over this LOD value. According to the mean value of IC20 (1.359 pg P-CTX-1/ml) obtained from all dose-response curves performed with the STD in the study period and the maximum concentration of extracts set to avoid matrix effect (80 mg TE/ml), the LOD/LOQ was 0.017 ppb.

2.5. Statistical analysis

The statistical analysis was performed using the R package, version 3.3.1 (R Development Core Team, 2016) and IBM SPSS Statistics 23.0 software.

Univariate analysis: Categorical variables are expressed as frequencies and percentages and continuous as medians and interquartile ranges (IQR = 25th–75th percentiles). The percentages were compared, as appropriate, using the Chi-square (χ^2) test or the exact Fisher test, the means by the t-test and the medians by the Wilcoxon test for independent data. As usual, the statistical significance was set at p-value < 0.05 and the rates of contamination by the CTX were estimated by means of confidence intervals (CI) at 95% using a bootstrap method.

Multivariate analysis: In order to identify those factors that maintain independent association with the outcome (contamination by CTX), a multivariate logistic regression analysis were performed. All variables of the study were entered into the analysis and a selection based on complete enumeration algorithm (Morgan and Tatar, 1972) and Bayes information criterion (BIC) was carried out. For each one of these regressions, we evaluate the lack of fit according the BIC criteria (Schwarz, 1978). The models were summarized as coefficients (SE), p-values (likelihood ratio test), BIC values (for the residual models) and odds-ratio, which were estimated by 95% CI.

Receiver operating characteristics: The discriminant power of the score deduced from the logistic model was evaluated from a receiver-operating characteristic analysis (ROC). The area under the ROC curve was estimated by means of the 95% CI. Statistical significance was set at $p < 0.05$.

3. Results and discussion

To identify the factors associated with the contamination by CTX in the population of fish under study, data were analyzed according to the following variables:

Table 2
Percentages of CTX positive and negative samples according to fish species and the corresponding fish weights, expressed as the mean and median values (kg).

Species		Presence of CTX-like toxicity		Total
		Negative	Positive	
Amberjack (14 kg)[a]	Number of samples	656	137	793
	% of samples	82.7%	17.3%	
	Mean weight ± SD	22.77 ± 7.8	28.9 ± 10.3	
	Median weight (min-max)	20.0 (14–54)	27.0 (14.5–54.9)	
Grouper (17 kg)[a]	Number of samples	106	39	141
	% (Species samples)	73.1%	26.9%	
	Mean weight ± SD	21.4 ± 3.2	22.7 ± 3.3	
	Median weight (min-max)	21.1 (17.0–34.1)	22.5 (17.4–33.0)	
Wahoo (35 kg)[a]	Number of samples	31	1	32
	% (Species samples)	96.9%	3.1%	
	Mean weight ± SD	39.5 ± 6.0	40.0	
	Median weight (min-max)	37 (28.0–58.0)	40.0	
Total samples	Number of samples	793	177	970
	% (Total samples)	81.8%	18.2%	

SD, standard deviation. Min, minimum weight. Max, maximum weight.
[a] Minimum weight limits established for CTX analysis by the Canary Government through the official control protocol.

3.1. Influence of fish species in the presence of CTX-like toxicity

Out of the 970 fish samples included in this research, 793 (81.8%) belonged to both amberjack species, 145 (14.9%) to the grouper and 32 (3.3%) to the wahoo. Overall, 228 (18.2%) of all samples exhibited measurable CTX-like toxicity. Comparing species categories, Pearson's Chi-squared (χ^2) test revealed a significant difference in CTX prevalence between species of the samples tested ($p = 0.002$). Although most of the samples correspond to amberjack species, the grouper displayed the highest percentage of positive samples (26.9%), see Table 2. This observation may indicate that the chance for catching a positive grouper is two-fold higher than fishing a positive amberjack in the Canary Islands waters possibly explained by the sedentary behaviour of grouper species (Göthel, 1992; Espino et al., 2006) which may continually feed in areas where *Gambierdiscus* are more abundant, allowing a continuous accumulation of the toxin. However, it must be highlighted that the fish analyzed in this study correspond to certain weights considered as risk factors for human health (Table 1) and therefore, this limitation must be taken into account before raising any conclusion. In addition, the high percentage of toxic grouper samples obtained may support the review of the lower limit of weights previously suggested for this species (see Table 1 and Fig. 1). Additionally, 32 samples of wahoo species were analyzed corresponding to the 3.3% of all flesh fish studied and note that only 1 of them showed a CTX-like toxicity. Thus, this species was not considered in this analysis.

3.2. Influence of the fish weight

Bioaccumulation of CTXs in amberjack has been reported to be highly dependent on the weight of the specimen (Bravo et al., 2015). Thus, an analysis of the presence of CTX in fish against this variable is essential to fully understand the accumulation of this biotoxins through the lifetime of these animals. Accordingly, in comparison with the last mentioned reference, the present study has tripled the number of samples under investigation.

Due to the biological difference between species (Reid et al., 2016; Šegvić-Bubić et al., 2016), each species weight data were analyzed separately using Mann-Whitney (M-W)/Wilcoxon non-parametric tests (Table 2), demonstrating that the median value of weight for fish which showed CTX-like toxicity was significantly higher than the median weight of negative fish, both in amberjack (27 kg vs. 20 kg; $p < 0.001$) and grouper (22.5 kg vs. 21.1 kg; $p = 0.013$, respectively). In both species, CTX toxicity was more frequently observed in larger specimens. The descriptive statistics of the weight of the CTX positive and negative samples according to fish species (interquartile range-IQR, median and minimum-maximum range) are summarized in Table 2. The distribution of weight data between species and CTX results is represented in the box plot diagrams below (Fig. 1). Regarding wahoo samples, only one positive result was obtained, what makes the statistical analysis impossible to be performed.

It is remarkable to note the presence of positive fish with a weight close to the minimum limit established by the government of the Canary Islands (see Table 2 and Fig. 1). The smallest positives amberjack and grouper species weighed 14.5 kg and 17.4 kg respectively, see Fig. 1. This observation may justify an extension of the minimum weight proposed for analysis in these two species, mainly in grouper fish, as mentioned before. Additionally, a CFP outbreak occurring in 2016 also supports this suggestion, when two people in Tenerife island were poisoned by the consumption of 7 kg grouper, and the presence of CTX-like toxicity confirmed in our laboratory (Canary Government, 2017b).

3.3. Influence of the fishing island

The information of the island where the fish were caught is important to analyze due to the nature of the studied species and the possible risk of poisoning related to the location. From the selected samples, significant difference in CTX result was found between islands of fishing ($p < 0.001$). Lanzarote showed the highest contamination rate (52.9%) which was more than two fold greater than the value obtained in samples from Fuerteventura (21.0%) and Gran Canaria (17.8%) and more than three times higher than the CTX positivity showed from El Hierro (15%) and Tenerife (13.5%). The number of positive samples obtained from these Canary Islands far exceeded those resulted in the islands of La Palma (5.1%) and La Gomera (2.4%) where the lowest number of positive fish was observed (Fig. 2). However, it must be considered that, under the official control program of ciguatera during the studied period, more than 300 samples received from Lanzarote were not accompanied by the necessary information, and thus, were not included in this research, which could limit accurate result from this island. These results must be considered with caution.

An in depth analysis by species, both amberjack and grouper also showed a statistical difference in CTX results between islands of capture ($p < 0.001$). For amberjack species, the number of positive samples seems to decrease from the Eastern islands to the Western islands (see Table 3 and Fig. 2), thus, these rates suggested grouping the island category in four cluster; namely: {HI; LG; LP}, {GC; TF}, {FU} and {LZ}. Table 3 displays the contamination rates corresponding to each cluster.

On the contrary, the profile mentioned above was not found regarding grouper, which showed positive results more likely linked to certain islands in particular, possibly due to its sedentary behaviour (Göthel, 1992; Espino et al., 2006). Thus, the highest number of ciguatoxic grouper was obtained in El Hierro, with a remarkable percentage of toxic samples (10 samples, 90.9%) and Lanzarote (19 samples, 41.3%). Although Fuerteventura provided the highest number of samples, only 3 were found positive to CTX (5.6%). Additionally, the low number of results from Gran Canaria and La Palma precluded any conclusion.

Despite of these findings, other confounding variables should be taken into consideration before any conclusion can be drawn (see Section 3.4.).

In addition, it is important to emphasise that results from each island presented one or more positive individuals weighted close to the minimum control limit except for those fish caught in La Gomera.

Furthermore, the only positive wahoo obtained in this study was fished in El Hierro, representing the 4.5% of all samples analyzed from this island.

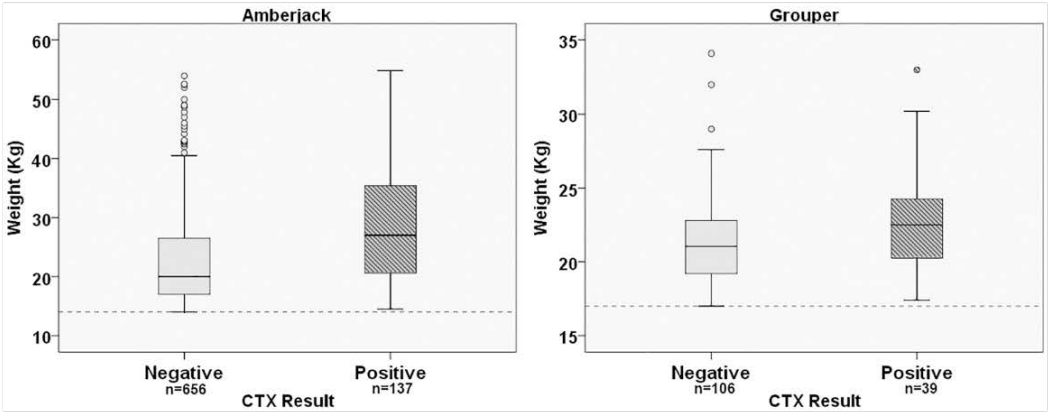

Fig. 1. CTX results by the weight variable in amberjack (box plot graph on the left) and grouper (box plot graph on the right). Line indicates the minimum limit of weight established for CTX analysis by the DG Fisheries (14 kg and 17 kg for amberjack and grouper species respectively). The plot represents the interquartile range (Q_3–Q_1). Sample size is shown below the corresponding group category.

3.4. Influence of the period of fishing

Fish samples studied in the present research were received in the period from April 2016 to December 2017 and analyzed for the presence of CTX-like toxicity.

Considering the results obtained by Rodríguez et al. (2017), peaks of *Gambierdiscus* spp. cells densities were observed in the Canary Islands associated to temperatures higher than 20 °C. For this reason, time frame was divided in "cold period" (January to April) and "warm period" (May to December) in accordance with the surface seawater temperature registered in both years (NOAA, 2017), with a difference of 3 °C between both periods. Samples available for this research only allowed comparison of data in the warm period between both years of study. Thus, considering species separately, a significant decrease ($p < 0.001$) was observed in the percentage of positive samples of amberjack species caught within the warm period between 2016 and 2017 (31.4% and 12.1%, respectively). This finding could be explained due to the modification in the official protocol of the lower weight limit in amberjack species from 15 kg in 2016 to 14 kg in 2017 (Table 1) and the increasing demand for analysis with the consequent rise of samples received in the laboratory in 2017 over 2016 (573 and 220 samples, respectively, see Table 3). In contrast, grouper species maintained a similar rate of toxicity in the warm periods of both evaluated years.

Considering both years in conjunction, the amberjack species showed a rate of CTX toxicity of 12.5% (95% CI = 5.6–20.8) and 17.8% (95% CI = 15.0–20.5) in the cold and warm period, respectively, what seems to be an increase in the number of positive samples from the cold to the warm season, but no statistically significant difference ($p = 0.261$) was found (Table 3). In this regard, it is important to stress that in the warm period the laboratory received a considerably greater amount of samples (see Table 3) what could therefore partially explain the difference of CTX toxicity rates found between seasons. Regarding grouper fish, the number of CTX-positive samples were quite similar, being 26.3% (95% CI = 10.5–47.4) in the cold period and 27.0% (95% CI = 19.8–34.9) in the warm period.

3.5. Risk gradient assessment: predictive score of the presence of CTX-toxicity in a population of amberjack fish

For the statistical analysis of results of samples from the amberjack species, using the training data, the multivariate logistic regression showed that the factors that maintained independent association with the outcome were the warm season (OR = 3.617; 95% CI = 1.249–10.474), the weight (per kg, 1.102; 95% CI = 1.069–1.136) and the island of fishing (grouping according gradient, see Table 3). It should be noted that the season did not show statistical significance in the

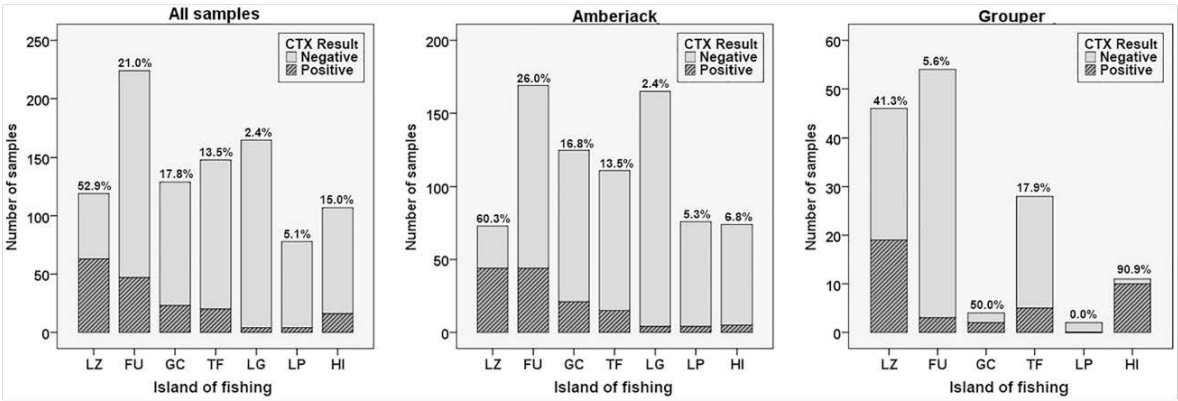

Fig. 2. CTX results in all samples included in this study (left), in amberjack (middle) and grouper (right) species by the location of capture. Percentages of positive samples from the different Canary Islands are indicated in each bar graph. The Canary Islands: LZ, Lanzarote; FU, Fuerteventura; GC, Gran Canaria; TF, Tenerife; LG, La Gomera; LP, La Palma; HI, El Hierro.

Table 3
Rates of contamination by CTX according to the considered factors.

Factor	Species					
	Amberjack (n = 793)			Dusky grouper (n = 145)		
	N	Crude rate[a] (95% CI)	P	N	Crude rate[a] (95% CI)	P
Year			<0.001			0.555
2016	220	30.5 (24.5–36.8)		54	24.1 (13.0–37.0)	
2017	573	12.2 (9.6–15.0)		91	28.6 (19.8–37.4)	
Period			0.261			0.951
Cold	72	12.5 (5.6–20.8)		19	26.3 (10.5–47.4)	
Warm	721	17.8 (15.0–20.5)		126	27.0 (19.8–34.9)	
Fishing Island			<0.001			< 0.001
LZ	73	60.3 (49.3–71.2)		46	41.3 (27.1–55.5)	
FU	169	26.0 (19.5–32.5)		54	5.6 (−0.6–11.7)	
GC	125	16.8 (10.4–23.2)		4	50 (1–99)	
TF	111	13.5 (7.2–19.8)		28	17.9 (3.7–32.0)	
LG	165	2.4 (0.6–4.8)		0	–	
LP	76	5.3 (1.3–10.5)		2	–	
HI	74	6.8 (2.7–13.5)		11	90.9 (73.9–107.9)	
Gradient			<0.001			< 0.001
HI; LG; LP	315	4.1 (2.2–6.3)		13	76.9 (53.8–100)	
GC; TF	236	15.3 (11.0–19.9)		32	21.9 (9.4–37.5)	
FU	169	26.0 (19.5–33.1)		54	5.6 (0—13)	
LZ	73	60.3 (47.9–71.2)		46	41.3 (28.3–54.3)	
Island Orientation			<0.001			0.099
Eastern	367	29.7 (25.1–34.6)		104	23.1 (15.4–31.7)	
Western	426	6.6 (4.2–9.2)		41	36.6 (19.5–51.2)	

Fishing Island: LZ, Lanzarote; FU, Fuerteventura; GC, Gran Canaria; TF, Tenerife; LG, La Gomera; LP, La Palma; HI, El Hierro.
[a] Toxin.

univariate analysis (see Section 3.4) but did so in the multivariate testing. This is attributable to the confounding effect of the weight, since in the warm season the weight of the fish was significantly lower than in the cold period ($p < 0.001$), as it is shown in Fig. 3.

For the amberjack species, a predictive score of contamination by CTX was obtained. For this purpose, the data were randomly divided into a training data set (n = 510) and a validation data set (n = 283). The predictive score was obtained by means of the multivariate logistic analysis using the training dataset. Its discriminant power was evaluated by means of the ROC analysis using the validation dataset and was summarized as the estimated area under the ROC curve (AUC-ROC, Fig. 4).

The next prediction score was then obtained from this logistic analysis:

$$Score = 1.286 \times Warm + 0.097 \times Weight + 1.962 \times D_1 + 2.555 \times D_2 + 4.191 \times D_3$$

Here, the season is a binary variable (1 = Warm; 0 = Cold) and D_1, D_2 and D_3 are the dummies variables associated with the island of fish catching (clusters) according to the design shown in Table 4. Four clusters were considered: El Hierro-La Gomera-La Palma; Gran Canaria-Tenerife; Fuerteventura and Lanzarote.

The AUC obtained using the validation data was 0.747 (95% CI = 0.662–0.833) (Fig. 4). Table 5 displayed the increasing probabilities of the contamination by the CTX according to a gradient from the cluster of western islands, in the cold season and fish with low weight to the eastern islands, in warm season and fish with high weight. Therefore, the probability of finding a positive result in a sample from amberjack is less than 1% if the sample comes from a small specimen (less than 22 kg), from the western cluster islands {HI; LG; LP} and fished in the cold season. Additionally, as can be seen in Table 5, the change to the warm season leads to a strong increase in the probability of contamination, being over 50% when

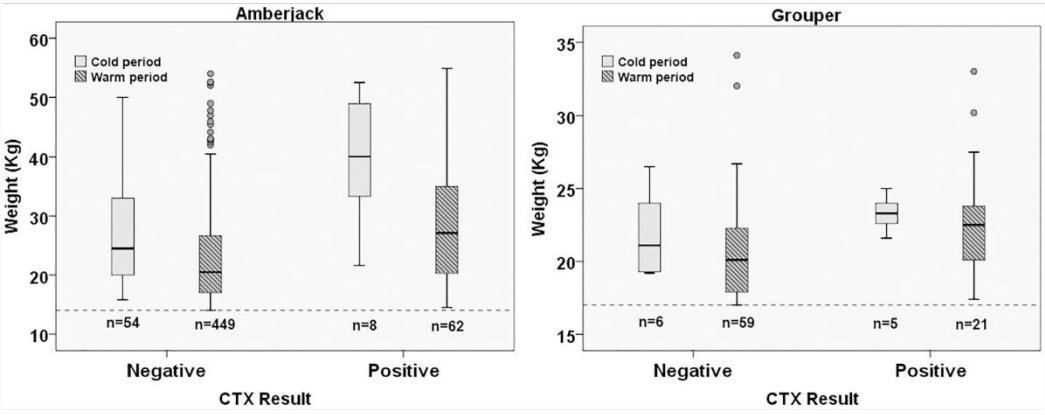

Fig. 3. CTX results by the weight variable in cold and warm periods for amberjack (box plot graph on the left) and grouper (box plot graph on the right). Line indicates the minimum limit of weight established for CTX analysis by the DG Fisheries (14 kg and 17 kg for amberjack and grouper species respectively).

Corrección: en la fórmula del *predictive score* el nombre correcto de la primera variable es *Period,* que contiene dos opciones: *warm period* o *cold*

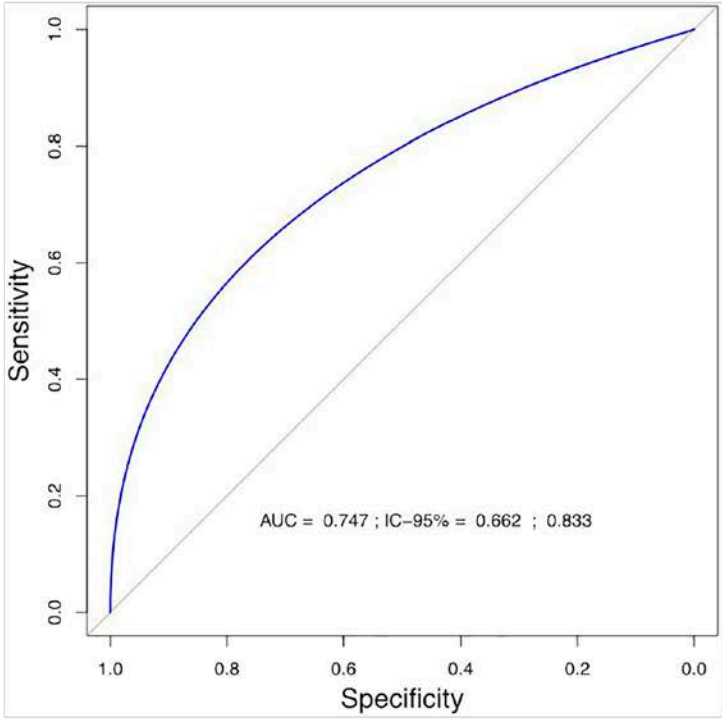

Fig. 4. Receiver operating characteristics for the score obtained from the logistic regression. The score obtained with the *data training* was validated using the *data validation*.

fish weighing more than 35 kg are caught in Fuerteventura. And, in contrast, this probability reaches higher values (more than 90%) in fish caught in Lanzarote, weighting more than 41 kg.

The decreasing gradient of presence of CTX in amberjack species observed from the eastern to the western islands is strongly consistent with the results obtained by Rodríguez et al. (2017). These authors measured higher density of *Gambierdiscus* spp. in Lanzarote and Fuerteventura islands, and they also reported that *G. exentricus*, one of the most toxic *Gambierdiscus* found in the Canary Islands, was more abundant in the eastern islands compared to the western ones. This finding could be explained by the conjunction of Northwestern African upwelling and the Cold Canary Current (Sangil et al., 2012) at the bottom of these islands, along with the shallow waters of these eastern islands which provides abundance of nutrients for the growth of plankton and seaweeds (Rodríguez et al., 2017).

Regarding results obtained from grouper, the only factor that showed significant differences associated with the presence of CTX was the island of fishing ($p < 0.001$), mentioned before in Section 3.3. These results are in accordance with Dierking and Campora (2009) who analyzed samples of *Cephalopholis argus* from the Hawaiian Islands in 2009 and found no geographic patterns in toxicity between or within islands.

Table 4
Design of the dummies variables associated with the island of fishing (clusters).

Cluster	D_1	D_2	D_3
El Hierro, La Gomera, La Palma	0	0	0
Gran Canaria, Tenerife	1	0	0
Fuerteventura	0	1	0
Lanzarote	0	0	1

1 = warm season; 0 = cold season.

Ciguateric groupers showed a different profile of distribution, as exposed above, which may be explained for a sedentary and very territorial behaviour of these fish (Reid et al., 2016). Furthermore, it must be considered the presence of an algae bloom of *G. caribaeus* occurred in October 2016 in El Hierro (Soler-Onís et al., 2016) and the rate of CTX bioaccumulation in fish tissue (Lehane and Lewis, 2000; Banner et al., 1966), what could explain that this certain island represents the highest percentage of CTX positive groupers in the Canary Archipelago (90%) in this period of study (Table 3).

Even so, the aim of the present study was not to calculate the real prevalence of CTX in fish from the Canary Islands, but to propose a predictive value of finding a positive sample according to different associated factors, such as fish species, weight, season or fishing island. Although results showed different probabilities of contamination by CTX-like toxicity between islands, none of them is free of ciguateric fish. Therefore, official monitoring should continue throughout the archipelago to ensure the food safety.

4. Conclusions

This study confirms the Canary Islands as an area of expansion of CFP endemicity and contains the first reported predictive score for the presence of CTX-like toxicity in amberjack fish samples from this area.

This work identifies the several factors associated with the probability of contamination by CTX-like toxicity of fish caught in the Canary Archipelago.

A risk gradient was obtained for amberjack, considering weight of fish, season and island of fishing, this latter being the only factor significantly associated with grouper. The risk of contamination by CTX could not be adequately assessed for wahoo due to small sample size of this species.

Table 5
Probabilities of contamination by CTX in amberjack species according to a gradient of risk (from the western islands in the cold season with low weight of fish to eastern islands in warm season and specimens with high weight). Study period (2016–2017).

Gradient	Islands	Period	Weight (Kg)	Probability-CTX (95% CI)*
1	HI, LG, LP	Cold	15	0.31 (0.07 ; 1.31)
2	HI, LG, LP	Cold	17	0.37 (0.09 ; 1.55)
3	HI, LG, LP	Cold	19	0.45 (0.11 ; 1.83)
4	HI, LG, LP	Cold	21	0.55 (0.14 ; 2.17)
5	TF, GC	Cold	23	4.55 (1.48 ; 13.12)
6	TF, GC	Cold	25	5.47 (1.82 ; 15.31)
7	TF, GC	Warm	27	20.3 (13.8 ; 28.8)**
8	TF, GC	Warm	29	23.6 (16.1 ; 33.2)
9	FU	Warm	31	40.4 (29.0 ; 52.9)
10	FU	Warm	33	45.2 (32.8 ; 58.2)
11	FU	Warm	35	50.0 (36.6 ; 63.4)
12	FU	Warm	37	54.8 (40.5 ; 68.4)
13	LZ	Warm	39	88.3 (77.7 ; 94.3)
14	LZ	Warm	41	90.2 (80.3 ; 95.4)
15	LZ	Warm	43	91.8 (82.6 ; 96.3)
16	LZ	Warm	45	93.1 (84.7 ; 97.1)

The Canary Islands: LZ, Lanzarote; FU, Fuerteventura; GC, Gran Canaria; TF, Tenerife; LG, La Gomera; LP, La Palma; HI, El Hierro.
*The probabilities of the presence of CTX-like toxicity are expressed as percentages.
**The change to the warm season leads to a strong increase in the probability of contamination.

Presence of CTX in amberjack from some areas seems to be highly related to the season of the year which may be related to the abundance of the most toxic *Gambierdiscus* found in the Canary Islands.

The minimum weight limits established by the official control of ciguatera in the Canary Islands for amberjack and dusky grouper need to be reviewed to safeguard consumer health.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

CRediT authorship contribution statement

J. Andres Sanchez-Henao: Formal analysis, Investigation, Methodology, Software, Writing - original draft. **Natalia García-Álvarez:** Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Writing - review & editing. **Antonio Fernández:** Conceptualization, Data curation, Funding acquisition, Project administration, Resources. **Pedro Saavedra:** Formal analysis, Methodology, Software, Validation, Writing - review & editing. **Freddy Silva Sergent:** Investigation, Methodology. **Daniel Padilla:** Investigation, Methodology. **Begoña Acosta-Hernández:** Investigation, Methodology. **Manuela Martel Suárez:** Investigation. **Jorge Diogène:** Methodology, Validation, Writing - review & editing. **Fernando Real:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing - review & editing.

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PRESENCIA DE CTXS EN MORENAS Y MEROS EN EL MEDIO AMBIENTE MARINO DE LAS ISLAS CANARIAS

Sanchez-Henao, A. García-Álvarez, N., Silva Sergent, F., Estévez, P., Gago-Martínez, A., Martín, F., Ramos-Sosa, M., Fernández, A., Diogène, J., Real, F. (2020). **Presence of CTXs in moray eels and dusky groupers in the marine environment of the Canary Islands**. *Aquatic toxicology*. 221, 105427. DOI: 10.1016/j.aquatox.2020.105427

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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 CCTX1 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.

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

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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., 1977; 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 some 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 (Food and Agriculture Organization of the United Nations (FAO, 2004).

2.2. Fish species studied

2.2.1. 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 Euro-Cigua project to the Canary Government.

2.2.2. 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 min. 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 min 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.

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 Fig. 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 $p\text{-value} < 0.05$.

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

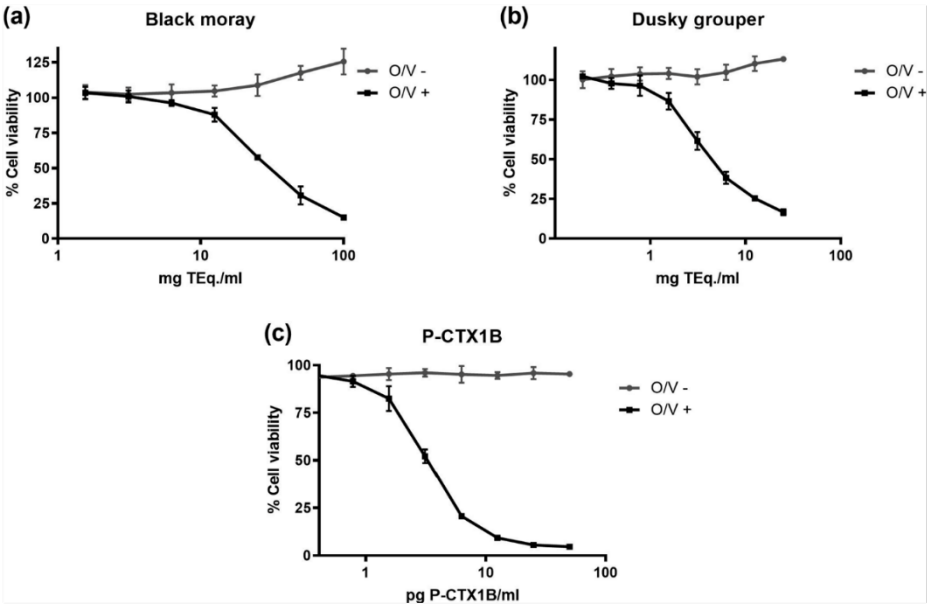

Fig. 1. Representative dose-response curves obtained by N2a cell-base assay with (a) a dusky grouper flesh extract from Lanzarote island, (b) a black moray flesh extract from El Hierro island and (c) with CTX1B standard. Error bars represent the standard deviation (sd) from three well replicates.

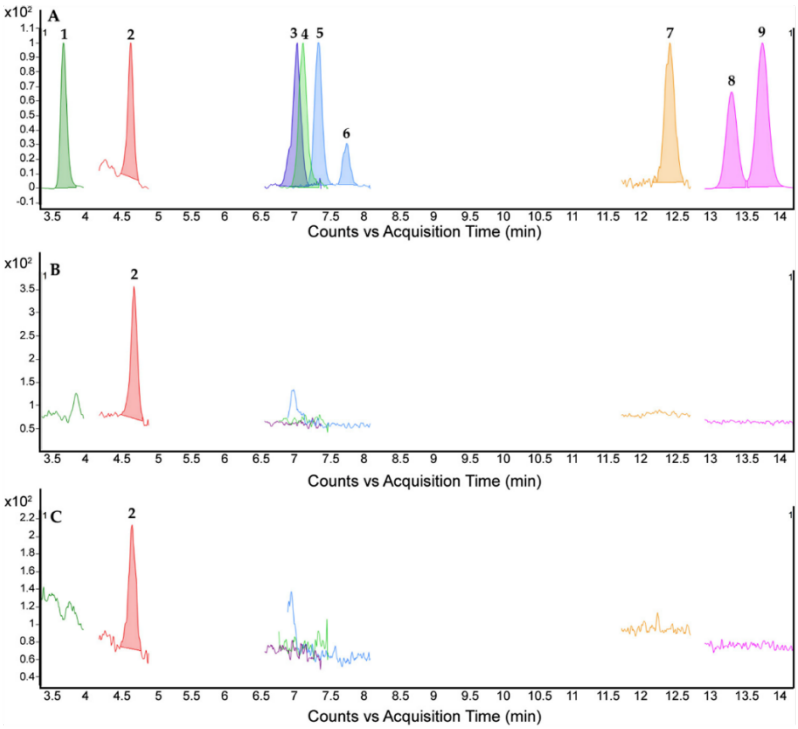

Fig. 2. LC–MS/MS chromatogram of: (A) Standard mixture containing: CTX1B (1), C-CTX1 (2), 2,3-dihydroxyCTX3C (3), 51-hydroxyCTX3C (4), 52-epi-54-deoxyCTX1B (5), 54-deoxyCTX1B (6), CTX3C (7), CTX4A (8) and CTX4B (9); (B) C-CTX1 detected at a concentration of 0.05 ng/g in M. augusti; (C) C-CTX1 detected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

(*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

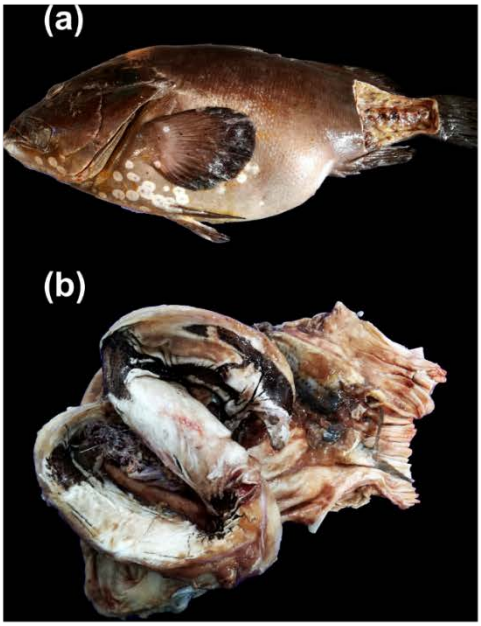

Fig. 3. (a) Lateral view of a dusky grouper from the Canary Islands before necropsy. (b) Stomach content of the specimen with a partially digested black moray eel.

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.

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

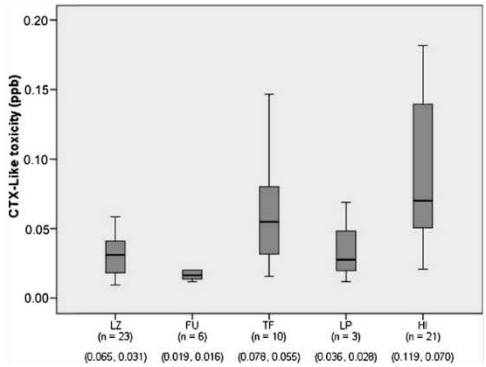

Fig. 4. Distribution of CTX-like toxicity estimated for grouper samples (ppb P-CTX1B Equivalents) by location of fishing ($p < 0.001$). In brackets are indicated mean and median toxicity values for each island of capture. The Canary Islands: LZ, Lanzarote; FU, Fuerteventura; TF, Tenerife; LP, La Palma;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

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 (16.0–30.0) 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

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

Fish species	Fishing				Island			
	LZ	FU	GC	TF	LG	LP	HI	TOTAL
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)	CTX content content[a]
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).

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, 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.

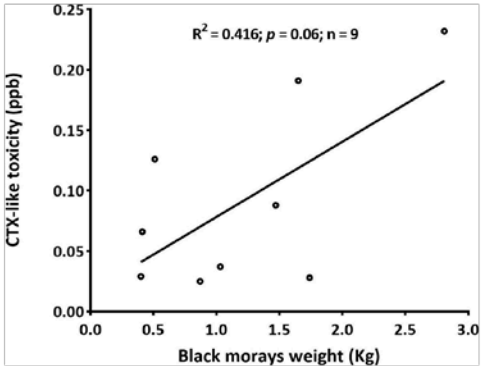

Fig. 5. Correlation between ppb of CTX-like toxicity obtained by CBA in positive black morays (n = 9) and their corresponding weight.

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; 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, 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 Costa, 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.

CRediT authorship contribution statement

Andres Sanchez-Henao: Formal analysis, Investigation, Methodology, Software, Writing - original draft. **Natalia García-Álvarez:** Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Writing - review & editing. **Freddy Silva Sergent:** Investigation, Methodology. **Pablo Estévez:** Investigation, Methodology. **Ana Gago-Martínez:** Methodology, Validation. **Francisco Martín:** Funding acquisition, Project administration, Resources. **María Ramos-Sosa:** Investigation, Methodology. **Antonio Fernández:** Conceptualization, Data curation, Funding acquisition, Project administration, Resources. **Jorge Diogène:** Methodology, Validation, Writing - review & editing. **Fernando Real:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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ACUMULACIÓN DE C-CTX1 EN EL TEJIDO MUSCULAR DE CARPA DORADA (*CARASSIUS AURATUS*) MEDIANTE EXPERIENCIA ALIMENTARIA

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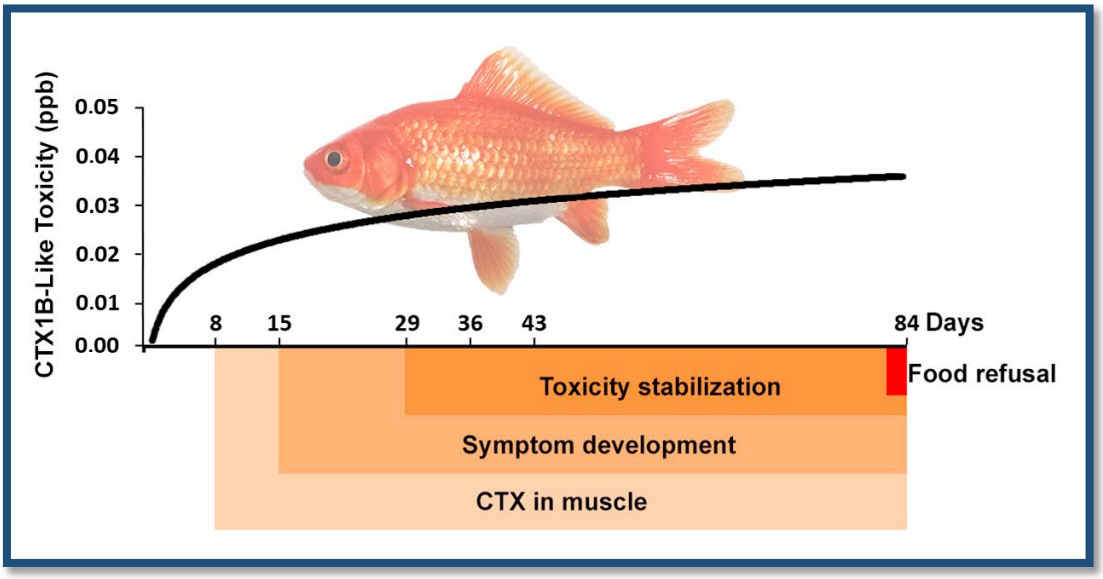
GRAPHICAL ABSTRACT



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Article

Accumulation of C-CTX1 in Muscle Tissue of Goldfish (*Carassius auratus*) by Dietary Experience

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Simple Summary: Some marine microalgae usually present in warm waters can produce ciguatoxins (CTXs); these toxins can accumulate in fish through the trophic chain, causing the food poisoning known as ciguatera in humans. It is important to understand how these compounds could be incorporated into fish muscle. For this purpose, this study was conducted using goldfish, an omnivorous freshwater species, daily fed raw fish flesh contaminated with a known toxicity concentration of CTX, seeking the accumulation profile in muscle and any signs of intoxication. Toxicity was detectable from day eight of the toxic diet and reached its maximum after two weeks. Signs of poisoning were observed after two weeks in all treated fish. However, two individuals developed strong symptoms, and one of them was separated and fed non-toxic food for 60 days; it showed recovery signs after the first week, and no toxicity was observed at the end of that non-toxic period. These results demonstrate that this toxin can accumulate in the muscle tissue of goldfish and produce associated symptomatology. Moreover, goldfish can recover and eliminate the CTX from its muscle if the toxin source is not available.

Abstract: Ciguatoxins (CTXs) are produced by dinoflagellates usually present in tropical and subtropical waters. These toxins are bioaccumulated and transformed in fish causing ciguatera fish poisoning (CFP) in humans. Few trials have been performed to understand how CTXs are incorporated into fish. This study developed an experimental model of goldfish (*Carassius auratus*) fed flesh contaminated with Caribbean ciguatoxin (C-CTX1). Fourteen goldfish were fed 0.014 ng CTX1B (Eq. g^{-1} of body weight) daily, and control goldfish received non-toxic flesh. CTX presence was determined by a cell-based assay on days 1, 8, 15, 29, 36, 43, and 84. Toxicity was detected in muscle from the second sampling and then seemed to stabilize at ~0.03 ng CTX1B Eq. g^{-1}. After two weeks, all experimental goldfish developed lethargy and loss of brightness, but only two of them displayed erratic swimming and jerking movements near the sixth sampling. One of these fish had its toxic diet replaced by commercial food for 60 more days; the fish showed recovery signs within the first weeks and no CTX activity was detected. These results indicate that C-CTX1 could accumulate in goldfish muscle tissue and produce toxic symptoms, but also remarked on the detoxification and recovery capacity of this species.

Keywords: ciguatera; Caribbean ciguatoxin; muscle bioaccumulation; cytotoxicity assay; goldfish; omnivorous fish; experimental model; detoxification

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1. Introduction

Ciguatoxins (CTX) are a group of polyether compounds responsible for causing a worldwide illness known as ciguatera fish poisoning (CFP), which produces gastrointestinal, neurological, and cardiovascular symptoms [1]. CTXs have been traditionally divided into three groups according to the region where they were produced: P-CTX (Pacific Ocean), I-CTX (Indian Ocean), and C-CTX (Caribbean Sea) [2]. However, current nomenclature reflects the chemical diversity that divides them into three groups: oxopene (P-CTXs from group I to which CTX1B belongs), oxocene (P-CTXs from group II, such as P-CTX3C), and the Caribbean/Indian CTXs [3,4]. CFP was considered a tropical and subtropical water disease; however, recently, it has occurred in new areas such as the Canary Islands (Spain) and Madeira (Portugal) [5–9].

CTXs are the result of chemical transformation of their toxin precursors, which are initially produced by benthic dinoflagellates of the genera *Gambierdiscus* and *Fukuyoa* that settle on macroalgae [10]. These initial toxins are ingested mainly by herbivorous fish when feeding on macroalgae; then, these fish could be predated by carnivorous fish, which subsequently would be predated, and so on, climbing the food chain up to high-order carnivores [11,12]. During this process, the toxin may be biotransformed and accumulated through the food web, reaching risk levels considered hazardous to humans. Some authors have suggested that fish metabolism increases the toxic potential of CTXs, which are changed into other toxic analogues, as supported by direct toxicological evidence on purified P-CTX congeners in mice [13–15]. The pathway of this process could implicate p450 enzymes of fish metabolism to eliminate toxins from their tissues by oxidation. However, during this process, more potent congeners of CTXs could result [16] or increase their toxicity after exposure to an acidic environment, such as that of the intestinal tract by acid-catalyzed spiroisomerization [13]. However, this tendency, in which the most toxic CTX is the most oxidized, seems to occur in the P-CTXs from group I (oxopenes) and not in those from group II (oxocenes) [4]. Regardless of the bioaccumulation process through the food web, small fish and herbivorous fish could represent a real risk for human consumption [17,18].

CTXs are rapidly absorbed from the gastrointestinal tract and distributed throughout the body, as animal laboratory research shows [19]. CTXs can link to voltage-gated sodium channels (Na_v) in fish cells [20], but the physiological mechanisms that allow some fish species to tolerate the toxin effects remain unclear [21]. Moreover, CTX-sensitive Na_v have been found in all organs and systems affected by CFP (i.e., brain, skeletal muscle, heart, peripheral nervous system, sensory neurons), as these channels may mediate the symptomatology of CFP [22,23].

Only a few trials have been conducted to understand the transmission, biotransformation, and bioaccumulation of CTXs in fish with large differences in the results. This is probably due to the fish species examined and their ability to tolerate CTX effects [21].

Some initial studies were performed with methodologies that prevented a good comparison, as the CTX used and their concentrations were not well determined. Helfrich and Banner [24], in 1963, fed toxic flesh to the herbivorous fish *Acanthurus xanthopterus*, which was capable of accumulating the toxin, albeit the fish showed no signs of intoxication. Davin et al., in 1986 and 1988 [25,26], fed four species of herbivorous and carnivorous fish different toxic substrates and demonstrated that fish could suffer the toxic effects of CTX, in addition to reporting bioaccumulation. In 1992 [27], Lewis exposed *Gambusia affinis*, a freshwater fish species, to P-CTXs added to water; the fish accumulated CTX and developed strong symptoms preceding death. In 1993, a shoal of *Serranus cabrilla* was fed *Gambierdiscus* cells, and changes in liver function and density were observed [28]. Recent trials have reproduced some of those studies results (regarding symptomatology and toxin burden) and provided additional details about the origin of CTXs, the congeners, and doses used [29–32]. Some experiments have been performed injecting CTX1B and C-CTX1 in fish larvae to observe toxic effects and fish developmental interference [33–35]. According to the

literature, no feeding trials have been performed using C-CTXs to emulate its transmission in the food web.

Goldfish is an easy species to maintain and handle in captivity. Goldfish also show a major advantage, as they do not live in the natural marine environment where these toxic microalgae are found. Therefore, all the experimental animals were free of CTXs at the beginning of the experimental design.

For the reasons given previously, this study aimed to feed adult omnivorous goldfish (*Carassius auratus*), of the *Cypriniudae* family, flesh from an amberjack containing C-CTX1 confirmed by LC–MS/MS, with two objectives: to describe whether high levels of toxin could cause any associated symptomatology in the experimental fish receiving the toxin and to estimate the time required for CTX to accumulate in fish tissues.

2. Material and Methods

2.1. Experimental Fish Species

Goldfish originally from Asia is a resistant, omnivorous, and brightly colored species, frequently used as ornamental fish kept in domestic tanks [36,37]. This species can resist cold temperatures (close to 2 °C) and hypoxic conditions, also can tolerate brackish water [38].

Goldfish of the common variety were obtained from a commercial fish distributor from Las Palmas de Gran Canaria, Spain. All specimens were >2.5 years of age and had an average body weight of 48.05 ± 11.7 g.

2.2. Maintenance of Goldfish

The experimental protocol was approved by the Committee for Animal Welfare of the University of Las Palmas de Gran Canaria and by the Department of Agriculture, Livestock, Fisheries and Water of the Canary Islands Government (code no. OEBA-ULPGC 28/2018).

Fish specimens (n = 28) were randomly separated into two groups (experimental and control) after 30 days of acclimatization in our laboratory in 150-L tanks equipped with two filters and an air stone each. Two replicate tanks were used for each group to correctly set the sampling experiment.

Fish were exposed to a natural photoperiod (11 h light, 13 h darkness) and approximately 23 °C temperature. Water parameters, such as pH, oxygen, ammonia (NH_3), nitrogen molecules (NO_2, NO_3), and dissolved oxygen were measured every three days.

2.3. Experimental Model

The omnivorous preference and resistance of *C. auratus* makes this species adequate to be used for analyzing the CTX accumulation in intermediate organisms of the food webs under laboratory conditions; however, the lack of any evolutionary compensatory adaptations should be considered.

The experimental model (Supplementary Data S2) consisted of a dietary exposure of goldfish to *Seriola* sp. raw flesh. *Seriola* sp. was captured in the Canary Islands, naturally contaminated with C-CTX1, as confirmed by LC–MS/MS (0.27 ppb). These data were obtained from the University of Vigo, Spain (methodology described in Supplementary Data S1) [39].

The presence of CTX on this raw flesh was determined by a N2a cell-based assay (CBA), which allowed the quantification of CTX-like toxicity, and may be caused by the action of multiple CTXs congeners [1.1 ng CTX1B equivalents (Eq.) g^{-1} raw flesh, ppb]. Toxin concentration and burden in the muscle of the experimental goldfish group were measured after 1, 8, 15, 29, 36, 43, and 84 days of daily toxic feeding (Table 1). Two fish per group were analyzed (one from each tank) (n = 4), except on sampling days 43 and 84, when only one fish from the experimental group (fish no. 11 and 13, respectively) and two from the control group were sampled.

Table 1. Individualized fish data from the experimental group, collected on each sampling day.

Experimental Fish (No)	Sampling Day [a]	Consecutive Dose Intakes [b]	Body Weight [c]	Muscle Weight [d]	Standard Length [e]	Observations
1	1	1	53.7	13.1	11.7	Normal behavior
2	1	1	46.9	11.4	11.5	Normal behavior
3	8	8	46.0	11.3	11.0	Normal behavior
4	8	8	43.7	11.6	11.0	Normal behavior
5	15	15	55.5	15.1	11.8	Lethargy and loss of brightness
6	15	15	59.4	17.8	12.5	Lethargy and loss of brightness
7	29	29	42.8	11.2	11.0	Lethargy and loss of brightness
8	29	29	63.3	15.8	12.5	Lethargy and loss of brightness
9	36	36	81.2	21.4	13.5	Lethargy and loss of brightness
10	36	36	41.2	12.0	10.9	Lethargy and loss of brightness
11	43	43	38.3	10.6	10.2	Sideway swimming, drifting, jerking movements and difficulty on feeding
12	60	57	38.3	10.0	10.0	Starvation for 3 days and discarded after observing a tumor lesion in the cerebellum
13	84	82	45.4	10.6	11.0	It began to increase feeding time and then consciously refused toxic feeding on the last 2 days
14	102	43	45.6	10.5	10.9	Same symptomatology than fish no. 11 on day 43, then, it was separated and sampled after 60 days of commercial food feeding

[a] Sampling performed >24 h after last feeding. [b] Total daily doses ingested by each goldfish before sampling. [c] Total body weight (g). [d] Total weight of muscle tissue collected (g). [e] Measure taken from mouth up-the peduncle (cm).

In addition, one fish (no. 14) was separated from the group on day 43 for a depuration process. The fish was then fed commercial food up to day 102 (S2).

During the experiment, the control goldfish were fed non-toxic *Seriola* sp. raw flesh with CTX concentration < limit of detection/limit of quantification (LOD/LOQ) observed by CBA. These control goldfish were sampled along with experimental fish. The control group was also used as a reference for behavioral assessment and appearance of any symptoms.

2.4. Food Preparation

Raw flesh used for experimental dietary exposure was homogenized and supplied to the goldfish in agarose gel (35%) to ensure total consumption. This compound does not add nutrients that may interfere with the interpretation of the results.

Agarose gel (Agarose D-1 Medium EEO, Condalab, Madrid, Spain) was prepared at 3% using deionized water at room temperature and heated in a microwave until it completely melted. The mixture was cooled to approximately 45 °C and mixed with homogenized raw flesh before solidification. The resulting gel was cut into cube-shaped 3–5-mm pieces and stored at −20 °C until use.

The resulting CTX concentration in the prepared food obtained by CBA was 0.714 ppb Eq. of CTX1B.

2.5. Toxic Dietary Exposure

In aquaculture, bred goldfish are fed a diet corresponding to 2% of the live weight [40]. In the present study, both groups were daily fed 1.9% of their live weight to accommodate their natural behavior; thus, experimental fish received 0.014 ng CTX1B Eq. g^{-1} of fish weight in their food daily. Each feeding was timed as part of the trial to monitor any variations in their foraging habits. In addition, the fish were observed during the next two hours after exposure to search for possible regurgitations or behavioral changes.

At sixth sampling (day 43), fish no. 11 and 14 showed severe symptoms while feeding; one was then sampled and analyzed (no. 11), and the other (no. 14) was isolated to assess a possible recovery after returning to commercial food and identified as "Detox Fish no. 14".

During this study, each sampling was performed 24 h post feeding and before any new exposure (Table 1). When a total absence of life signs was confirmed, fish were immediately dissected and flesh was collected for the CTX-like toxicity assay. The extraction was conducted on the sampling day, and extracts were stored at −20 °C until toxin analysis.

2.6. Extraction of CTX from Experimental Fish Flesh

The extraction of CTX was performed according to the protocol proposed by Lewis [41], with minor modifications based on our laboratory needs. Briefly, 10 g of fish flesh was cooked at 70 °C for 10 min. Each sample was extracted twice with 20 mL of acetone and homogenized with an Ultra Turrax blender at 17,500× *g*. The supernatant was recovered by centrifugation at 3000× *g* for 10 min at 4 °C. Both supernatants were pooled and filtered through a 0.45 µm PTFE filter and evaporated with a rotary evaporator at 55 °C until dried residue. Liquid/liquid partition was conducted twice with a mix of water and diethyl ether (DEE) (1:4). The DEE fractions were pooled and evaporated to dryness under a N_2 current. The resulting residue was dissolved for subsequent partitioning in methanol:water (8:2) and N-hexane (1:2). The N-hexane upper phase was discarded, and 4 mL of N-hexane was added to the methanolic fraction for extra cleaning. The methanol phase was collected 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 CBA.

2.7. Determination

A mammalian CBA was the selected method for this study because it enables the estimation of the activity caused by multiple CTX congeners, which may be present in the sample from the *Seriola* sp. raw fish diet or resulting from the goldfish metabolism. This method of analysis also allows the quantification of this toxic activity [42].

The cellular line used in this study was the Neuro-2a (Cell line: CCL131, from ATCC, LGC Standards SLU, Barcelona, Spain) and cells were maintained in Roswell Park Memorial Institute medium (RPMI)-1640 supplemented with 5–10% of fetal bovine serum at 37 °C in a 5% CO_2 atmosphere. The Pacific type 1 CTX standard (STD) (named P-CTX-1) was provided by Pr. Richard J. Lewis (Queensland University, Australia) [43] and used for the assessment of CTX-like toxicity by CBA.

The cytotoxicity assay was conducted as previously described by Caillaud et al. [44] 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 assess cell mortality in the presence of CTX. After incubation, cells were exposed to goldfish flesh extract and the CTX1B STD at decreasing concentrations. Each sample extract and the STD were assayed in triplicate wells, and each value was taken from the average of these three absorbances.

Cell viability was evaluated using 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; dose–response curves were evaluated with Microsoft Office Excel 2016 and GraphPad Prism 7 software (GraphPad, San Diego, CA, USA).

CTX-toxicity levels in goldfish flesh were determined twice by comparison with the standard curve of CTX1B (IC_{50} = 3.257 ± 0.149 pg CTX1B mL^{-1}), obtained the same day as the corresponding assay. A response producing less than 20% cell mortality was considered a non-toxic effect [44]. The LOD and LOQ were 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} (1.528 ± 0.045 pg CTX1B mL^{-1}) observed in the dose–response curve obtained with the STD and the maximum concentration of extracts (150 mg tissue Eq. mL^{-1}), the LOD/LOQ obtained was 0.010 ng CTX1B Eq. g^{-1} of goldfish flesh (ppb).

CTX-1B was used as the reference standard to evaluate toxicity, as our laboratory regularly analyses all fish by using this standard. As in all toxicological studies, it is important to use a reliable standard to compare toxicity in different samples, regardless of the toxins they may contain.

3. Results

3.1. Symptomatology and Fish Behavior

Experimental goldfish (n = 14) were exposed to a constant daily level of CTX during this experiment (84 days). Within the first two weeks, experimental fish displayed no symptoms or abnormal behavior (Table 1). However, after that period, all the exposed specimens progressively developed symptomatology and some behavioral disturbances, such as loss of brightness and lethargy (which disappeared during feeding). Two goldfish, no. 11 and 14, showed signs of intoxication two weeks after lethargy onset, with rapid evolution and deterioration. Thus, the symptoms that appeared during feeding time included some loss of equilibrium (sideway swimming) and reduced stability (drifting in the water column). After two days, symptomatology evolved to hyperactive signs, such as intermittent erratic swimming with jerking movements, disorientation, and difficulty on feeding. On day 43, goldfish no. 11 was sampled and analyzed by CBA, and Detox Fish no. 14 was maintained alive to evaluate a possible recovery of signs after returning to commercial food.

On day 58, one of the two last fish (no. 12) started to show signs similar to those described for the two goldfish mentioned previously. In 24 h, fish no. 12 developed an inability to feed, which was concomitant to continuous excitation and loss of control to avoid fixed objects, such as filters, air stones, or thermometers. After two days without feeding and no changes in symptomatology, the fish was sampled. The necropsy revealed a compressive tumor lesion in the cerebellum, which, along its variable and deficient feeding behavior, makes the estimation of the CTX toxicity difficult to assess; thus, this result was not included in the present survey.

The last experimental fish (no. 13) failed to show strong symptoms; however, it showed the same symptoms as previous specimens (some lethargy and loss of brightness). This fish was fed a toxic diet until day 84, at which time it started to refuse food. To evaluate an appropriate eating capacity, the fish was fed commercial granulated food (2 mm larger than toxic food), which was correctly ingested; but the toxic diet offered the next day was

refused again. The CTX-intake experimental trial was then considered to be completed, and the level of CTX in that specimen was evaluated.

Detox Fish no. 14, which did not receive toxic food after day 43 to evaluate CTX recovery, was fed commercial food for 60 more days. The strong symptomatology appeared during feeding time in the first two days after returning to commercial food. This condition started to fade, but lethargy remained. Nevertheless, it was not until day seven of regular feeding that lethargy began to disappear and, on day 20, that color and behavior was completely recovered to normal.

Control goldfish fed non-toxic food did not show atypical behavior. Additionally, several fish sampled from this group showed gonadal development, and one female was near spawning. Although experimental goldfish fed a toxic diet were exposed to the same environmental conditions, they never showed any reproductive behavior.

3.2. CTX Accumulation Profile in Goldfish Muscle

CTX presence in muscle tissue from the goldfish was evidenced by the CBA and estimated by comparing it with a CTX1B STD [45]. The CTX-like toxicity in the flesh was established with the assay 24 h after the first feeding with a LOD/LOQ of 0.010 ng CTX1B Eq. g^{-1} of goldfish flesh and reached quantifiable levels over this limit from the second sampling, performed on day eight, with a greater value than the feeding rate (0.020 ± 0.001 ng CTX1B Eq. g^{-1} of fish flesh; Figure 1). Levels of CTX in muscle reached the risk value specified by the European Food Safety Authority (EFSA) (0.01 ng CTX1B Eq. g^{-1}; Table 2) [46], in eight days of daily intake with toxic food. The CTX levels in fish muscle increased at a slow rate during the following weeks until day 29, when the concentration seemed to stabilize until day 84 (~0.03 ng CTX1B Eq. g^{-1}), showing a positive correlation between days of dietary exposure and CTX-like toxicity, with a coefficient of determination (R^2) of 0.815 for a logarithmic regression (Figure 1). This includes fish no. 11, which presented difficulties in feeding due to its symptoms, and was sampled on day 43, resulting in 0.022 ng CTX1B Eq. g^{-1} (Figure 1).

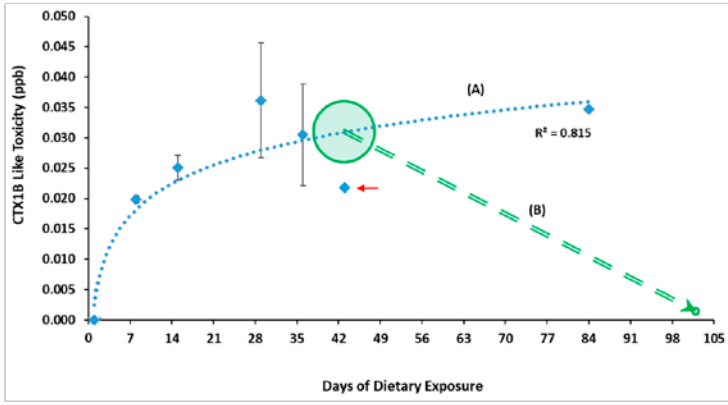

Figure 1. The mean ciguatoxins CTX-like toxicity level (expressed in CTX1B ppb) in fish muscle per day of sampling (1, 8, 15, 29, 36, 43, and 84) measured by cell-based assay CBA. (**A**) Logarithm regression with coefficient $R^2 = 0.815$; arrow shows fish no. 11, which displayed hyperactive symptomatology with difficulty in feeding, days before sampling. (**B**) Expectable CTX concentration for Detox Fish no. 14 from the moment of suspending toxic feeding on day 43 (highlighted circle), and the CBA result obtained on day 102, after returning to commercial food feeding.

Table 2. Comparison of the Ciguatoxin (CTX) values ingested by goldfish and detected in muscle tissue during sampling.

Sampling Day	Experimental Fish Sampled (No.)	Mean Total Quantity of Toxin Ingested [a] (pg CTX)	Mean Muscle CTX-Like Toxicity Level [b] (ppb CTX)	Mean Muscle Toxin Burden [c] (pg CTX)	% Toxins Ingested Accumulated in Muscle [d]
1	1, 2	681.82	0.000 ± 0.000	0.00 ± 0.00	0.0%
8	3, 4	4864.55	0.020 ± 0.001	226.92 ± 10.90	4.7%
15	5, 6	1168.13	0.025 ± 0.002	411.09 ± 13.81	3.5%
29	7, 8	20,848.21	0.036 ± 0.010	504.36 ± 246.93	2.3%
36	9, 10	29,877.93	0.031 ± 0.008	482.46 ± 63.09	1.8%
43	11	22,334.22 *	0.022	231.59	1.0%
84	13	51,726.92 *	0.035	368.17	0.7%

[a] Calculated as the total sum of CTX ingested by fish until sampling time, divided by the number of fish sampled on that day. [b] The average CTX-like toxicity level per sampling day determined by CBA was conducted twice in each fish sample. [c] Calculated by multiplying the CTX concentration by the weight of muscle tissue collected per fish. [d] Calculated using total quantity of toxin ingested and muscle toxin burden per sampling day. * Only one data point is available within the sampling day.

Fish no. 14 allowed the study of possible CTX detoxification. The fish was sampled 60 days after suspending the toxic feed on day 43 and did not show any CTX-like toxicity (Figure 1).

The percentage of ingested toxins accumulated in muscle was calculated for each individual and adjusted by the days of daily intake and the toxin concentrations in the muscle. This percentage was not constant, showing a progressive decrease from 4.7% of the total toxin intake accumulation (day 8) to 0.7% of the total toxin ingested at the end of the experiment (day 84). These data displayed a positive correlation between days of exposure and percentage of CTX accumulated in muscle with an R^2 of 0.9624 (Figure 2 and Table 2).

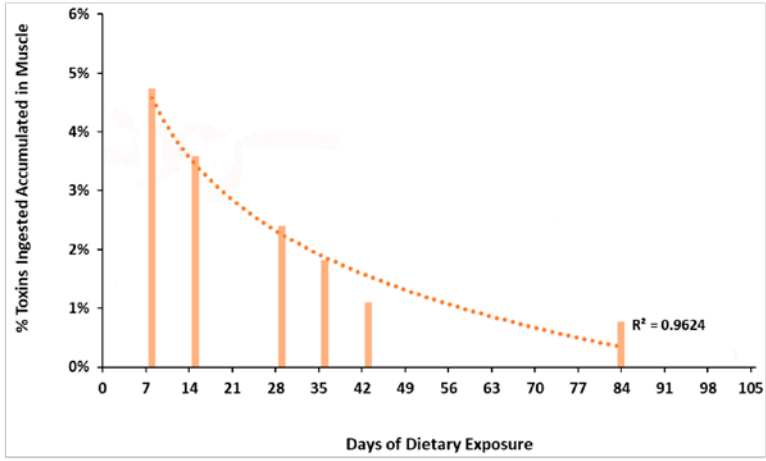

Figure 2. Percentage of CTX muscle burden (%) compared to the total toxin ingested over the experiment per day of sampling (logarithmic regression with a coefficient $R^2 = 0.9624$).

4. Discussion

4.1. Behavioral Disturbances and Signs of Intoxication

Experimental goldfish were asymptomatic to CTX until day 15 of daily toxic feeding (0.014 ng CTX1B Eq. g^{-1} of fish flesh), when they started to show lethargy and color brightness alteration. These hypoactive behaviors after continuous toxic intake in fish have also been reported in previous studies [25–27,29]. Ledreux et al. [29] fed herbivorous *Mugil cephalus* (improbably exposed to CTX in its natural habitat) *Gambierdiscus polyniensis*

cells (0.3 ng CTX3C Eq. g^{-1} of fish); in that study, behavioral disturbance was more evident on the second consecutive day of feeding (6 ng CTX3C $fish^{-1}$ of cumulative dose intake). Clausing et al. [30] fed an *Acanthuridae* fish (*Naso brevirostris*, an herbivorous fish naturally present in CFP endemic areas), similar toxin daily doses (0.4 ng CTX3C Eq. g^{-1} of fish), but the fish did not show any symptoms or behavioral disturbances. As evidenced by an earlier survey, in 1958, another *Acanthuridae* fish fed toxic flesh from *Lutjanus bohar* did not show any symptoms [24]. The present study suggests that goldfish may be particularly sensitive to dietary CTX.

Lethargy and alteration in skin color were the first and main intoxication effects displayed by all the experimental goldfish until the end of the present trial. Davin et al. [25] observed these two symptoms at the beginning of their experiment with *Thalassoma bifasciatum* fed *Gambierdiscus* sp. cells collected in the Caribbean Sea, which were also observed by Lewis in 1992 [27]. Additionally, in the present study, two goldfish (no. 11 and 14) displayed hyperactive symptomatology, such as erratic swimming episodes and jerking. Goldfish no. 11 was sampled on day 43, and a lower CTX concentration was displayed in muscle (0.022 ppb CTX1B Eq.) than those measured previously, day 36 (0.031 ppb CTX1B Eq.) and subsequent day 84 (0.035 ppb CTX1B Eq.; Table 2, Figure 1). This unexpected value may be associated with difficulty on food intake during the days before sampling and ability of detoxification, as it was proved with Detox Fish no. 14 after 43 days of cumulative toxic feeds and subsequent 60 days of regular feeding. However, this suggestion does not overlook individual susceptibility and a possible chronic effect on the nervous system in these fish, unrelated to the actual amount of CTXs in muscle tissue, as this toxin could bind to the fish brain Na_v [20]. Detox Fish no. 14 showed hyperactive symptoms during the two days after returning to commercial feeding and, seven days later, these symptoms started to disappear. This fish completely recovered at day 20. Similar findings were obtained by Davin et al. [26], studying *Lutjanus apodus* fed barracuda ether extract after a short period of toxic intake. The symptoms displayed (disequilibrium, erratic behavior, hitting the sides and bottom of the tank while swimming) disappeared after 94 h of the last exposure, proving the capacity of fish to recover from ciguatoxic effects. Detox Fish no. 14 maintained normal behaviur and healthy appearance as the control goldfish until sampling (60 days after toxic food was suppressed). This fact seems to indicate that goldfish takes less than 60 days to remove CTXs from the muscle tissue, reaching non-detectable levels.

Experimental goldfish, in addition to showing signs of intoxication, also showed differences in breeding behavior and gonadal development compared with control fish. Several experiments performed in *Oryzias* spp. larvae or embryos exposed to P-CTX and C-CTX demonstrated that CTX effects interfere with species survival due to abnormalities and malfunction in fish larvae [33–35,47]. In the case of the reproductive effects of CTX in adult fish, a recent study performed with *Oryzias melastigma* showed that the ingestion of CTX1B can cause, apart from various symptoms, a decline in egg production, hatching failure, and delay of hatching, which results in a decrease in reproductive success [31]. This would explain the difference in gonadal development between the experimental and control groups. However, further investigation is needed.

The fact that the last experimental goldfish (no. 13; day 84) gradually increased feeding time (offering the same amount of food) and consciously rejecting the toxic food might suggest that the fish could recognize the harmfulness of this diet. Thus, this behavior could be a survival strategy in some fish species naturally exposed to CTXs as a conditioned response, similar to observations described for other animal species with foodborne diseases [48] and, particularly in goldfish, with a similar progression [49]. If this occurs in nature, as other authors suggest [26], it will allow the total recovery of the fish species, thereby allowing them to hide from their predators for survival. However, this hypothesis needs further studies to be refuted or confirmed.

4.2. CTX Accumulation in Muscle Tissue

Some aspects of C-CTXs are not yet well known, such as their producer, *Gambierdiscus* sp. [4]. However, attending to some of the chemical similarities with P-CTXs of group II, C-CTX could accumulate in the goldfish tissue and be transferred to a possible predator with no major chemical modifications [29]. Interestingly, goldfish are stomatchless fish with a stable pH (6.6–8.4) throughout its gut when exposed to a carnivorous diet [50]. Acid digestive tracts could promote the production of polar forms of CTX, increasing their toxicity by acid-catalyzed spiroisomerization [13]. A recently published report [3] suggests that reliable toxicity equivalency factors for CTX-group toxins could not be derived because of the limited data from in vivo assays in mice (MBA) [43,46,51]. However, another recent report [52] provides a comparison of the toxicity by CBA of C-CTX1 and CTX3C in relation to CTX1B, which is two times more potent than the other two. This approach theoretically allows comparison of our results with the few trials performed with fish fed CTX3C congeners (P-CTX group II) [29,30].

Thus, experimental goldfish ingested an average of 0.68 ng CTX1B Eq. per individual (0.014 ng CTX1B, Eq. g^{-1} of fish) in the first intake, and neither was CTX-like toxicity evidenced by CBA (LOD/LOQ = 0.0104 ppb), nor were signs of intoxication observed. However, Ledreux et al. [29] fed their experimental fish 0.3 ng CTX3C Eq. g^{-1} of fish, and the authors observed CTX activity at 24 h post exposure. In addition, their experimental fish never showed an increase in CTX retention after consecutive doses; nevertheless, the fish showed signs that were more evident on the second day of feeding, similar to those presented by goldfish since day 15. These differences could be associated with the metabolism of each fish species to clean exogenous molecules from their tissues, the different susceptibility, and food sources. Additionally, goldfish could store the toxins in other organs, such as liver or gonads, at a higher rate than in muscle, as observed in other fish species [29,32,53,54], although this fact was not demonstrated in this work.

Since the second measurement on day 8, goldfish muscle started to show CTX-like toxicity (0.020 ng CTX1B Eq. g^{-1} of muscle), which represents 4.7% of the total toxin ingested, but no signs of intoxication were observed. The symptomatology occurred from day 15, with an initial concentration of 0.025 ng CTX1B Eq. g^{-1} of muscle (after the intake of 1.17 ng CTX1B Eq. per fish and 0.41 ng CTX1B accumulated in the muscle tissue). This CTX ingestion could represent a threshold below which the goldfish remain asymptomatic. The symptomatology presented by the goldfish beyond that level of CTX may favor predation, as Davin et al [25] suggested. This initial resistance may allow wild fish to accumulate high amounts of CTX in their muscle before reaching large carnivores. This finding is in accordance with the *N. brevirostris* experiment, in which CTXs reached large amounts in the fish tissues, without clinical signs [30].

In 2018, Clausing et al. [30] found a linear increase in CTX burden in muscles from *N. brevirostris* juveniles during their experiment. However, the toxin concentration in muscle increased during the first eight weeks and then remained stable, according to the different growth rates of muscle assessed during their experiment. The goldfish used in this study were adults between two and three years old with a small growth rate and no great variations in muscle volume during the experiment. Thus, the different toxin evaluations observed in goldfish could be explained by an increase in the toxin depuration rate by continuous exposure or the impossibility of fixing more CTX in muscle tissue due to the saturation limit (Figures 1 and 2).

Regarding Detox Fish no 14, due to the destructive character of the sampling, the exact CTX amount in the muscle tissue was not possible to be determined at the time of returning to commercial food (day 43); however, it was expected to be 0.022–0.03 ng CTX1B Eq. mL^{-1} (Figure 1, line B). Additionally, the fish did not show any CTX-like toxicity 60 days after suspending the toxic diet when sampling was performed. This result provides evidence of a depuration process in the goldfish muscle tissue.

Depuration of the assimilated CTX in goldfish muscle and recovery from symptomatology is evident from our results, as other authors [26,32] observed in other fish species.

However, further studies should be performed to clarify the rapid metabolic process involved in detoxification and the mechanisms to avoid toxic effects in fish species that can store high concentrations of CTXs in their tissues for long periods [11,27].

5. Conclusions

For the first time, goldfish (*C. auratus*) has been described as a fish species with the capability to accumulate C-CTX1 in its muscle tissue and suffer its toxic effects.

These results suggest that goldfish could recover from the toxic effects caused by CTXs and eliminate them from muscle tissue.

The data obtained in this study propose goldfish as an efficient model to better study CTX accumulation and depuration processes in fish, even though goldfish is not a marine species.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-2615/11/1/242/s1. Data—Chromatogram obtained after the LC-MS/MS analysis in MRM mode and for Supplementary data S2—Outlined research design.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare that there are no conflict of interest.

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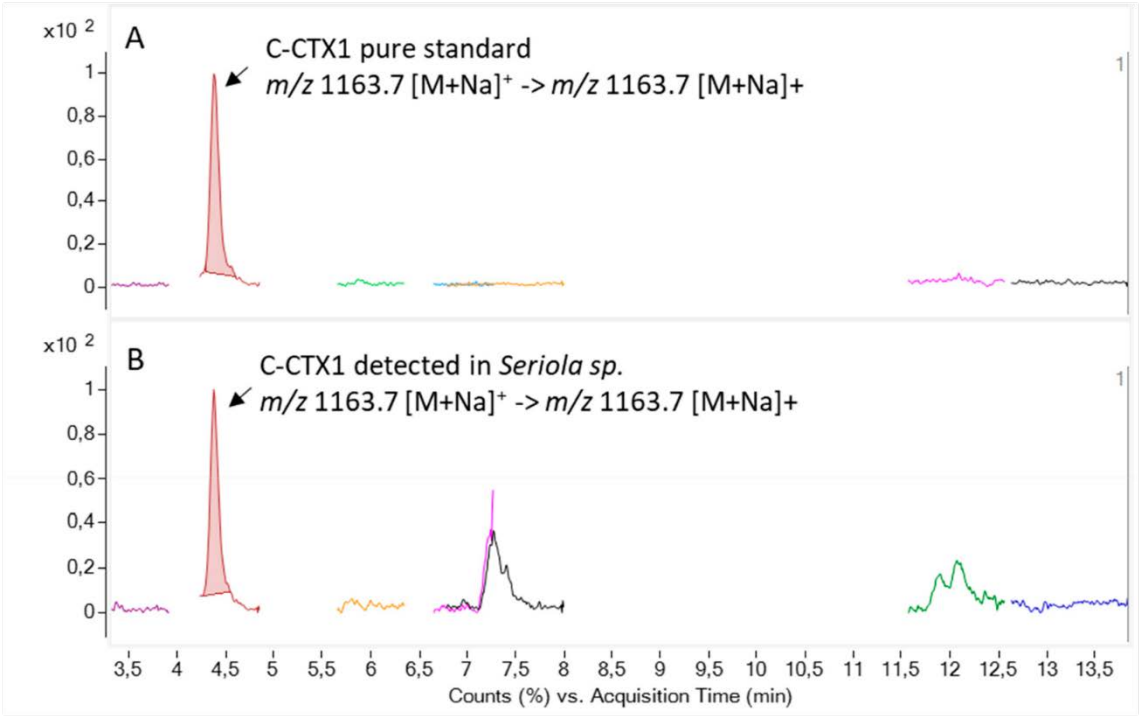
Supplementary data S1

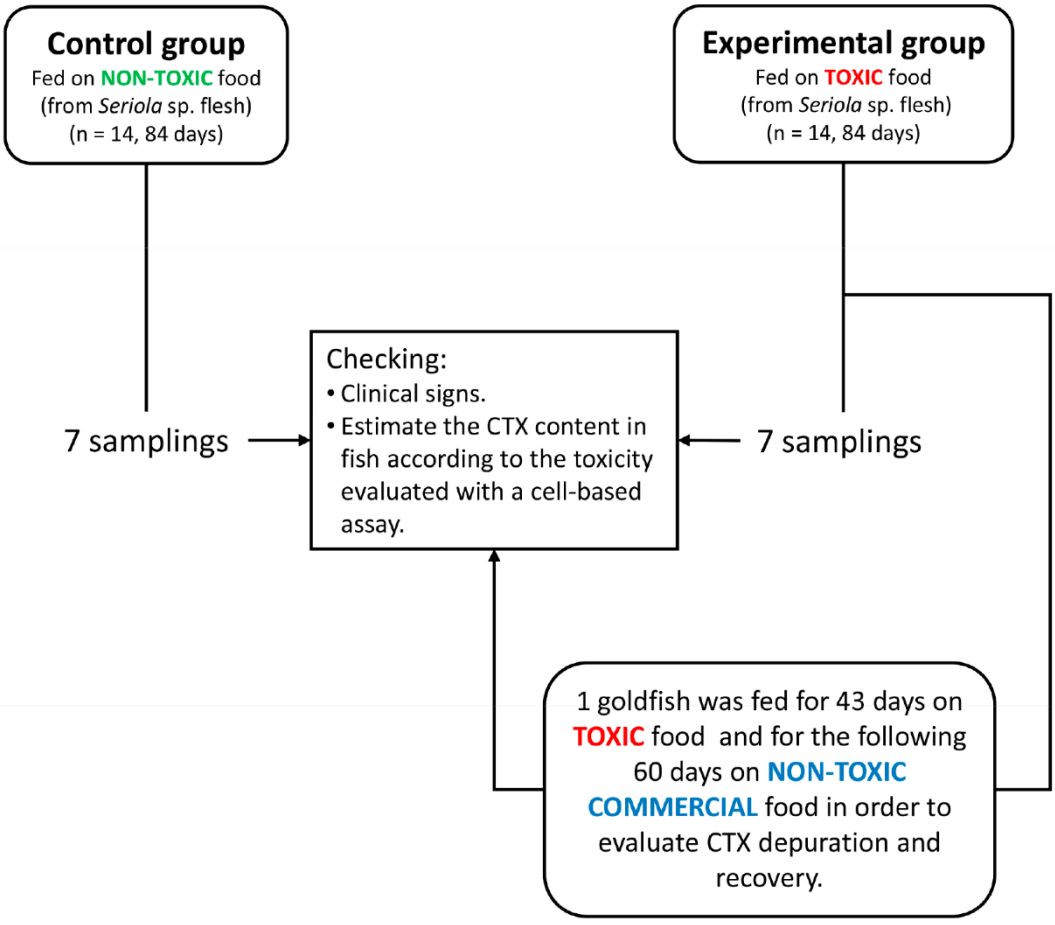
Chromatogram obtained after the LC-MS/MS analysis [1] in MRM mode of **A)** C-CTX1 pure standard (5 ng / mL); **B)** C-CTX1 detected in the *Seriola* sp. Along with the C-CTX1 peak, some traces of C-CTX1-Me and interferences, which could be erroneously identified as other CTXs from the P-CTXs family, were found.



Supplementary data S2

Outlined research design.

Objectives	To describe the symptomatology in goldfish (*Carassius auratus*) associated to ciguatoxin (CTX) ingestion.
	To estimate the time required for CTXs to be accumulated in fish muscle.
Fish species	Goldfish which lives in a naturally CTX free environment.



CONCLUSIONES FINALES

1. Se confirma el endemismo de la CP en las islas Canarias y se presenta el primer valor predictivo publicado para la presencia de toxicidad tipo-CTX en muestras de medregal capturado en esta área marina.
2. Se han identificado los factores asociados con la probabilidad de contaminación con CTX de los peces capturados en el archipiélago canario en el marco del protocolo del control oficial.
3. Se ha obtenido un gradiente de riesgo para el caso del medregal, considerando el peso, la estación y la isla de captura, siendo este último el único factor asociado en el mero, que mostró diferencias estadísticamente significativas respecto a la presencia de CTX.
4. La presencia de CTXs en el medregal de ciertas áreas parece estar altamente relacionada con la estación del año, lo que podría estar a su vez vinculado a la abundancia de los *Gambierdiscus* spp. más tóxicos encontrados en Canarias.
5. El peso mínimo de riesgo establecido por el protocolo de control oficial de CP de Canarias para el mero y el medregal requiere revisarse para salvaguardar la salud de los consumidores.
6. Se describe por primera vez la presencia de CTXs en una morena negra (*Muraena augusti*) hallada en el contenido estomacal de un mero (*Epinephelus marginatus*) contaminado con CTXs, lo cual confirma la interacción trófica entre estas especies y su relevancia en el mantenimiento de las CTXs en el medio marino.
7. La presencia de toxicidad tipo-CTX fue observada en la carne de ambas especies (mero y morena negra) señaladas en el punto anterior, mediante ensayo celular N2a-MTT. La C-CTX1 fue identificada mediante análisis LC/MS-MS con concentraciones similares en esos dos especímenes. Este estudio representa la primera descripción de la presencia de CTXs en una especie de morena capturada en el archipiélago canario.

8. Se han estudiado cinco especies de morena capturadas en las islas Canarias y se observó la presencia de CTX en individuos de cuatro de ellas. Aunque, hasta la actualidad, estas especies no han estado asociadas a brotes de CP en el archipiélago, es necesario seguir investigando para evaluar el riesgo que estas especies representan para la salud humana.
9. La tasa de contaminación por CTXs en la población de meros de las islas Canarias parece ser estable a lo largo del periodo de estudio, destacando El Hierro como la isla de especial interés, debido al alto porcentaje de meros tóxicos obtenidos a través del programa de control oficial.
10. Se describe, por primera vez, la carpa dorada (*Carassius auratus*) como una especie de pez capaz de acumular C-CTX1 en su tejido muscular y sufrir sus efectos tóxicos.
11. Nuestros resultados sugieren que las carpas doradas podrían recuperarse de los efectos tóxicos causados por las CTXs y eliminarlas del tejido muscular.
12. Los datos obtenidos proponen a la carpa dorada como un modelo eficaz para profundizar en el estudio de los procesos de acumulación y depuración de CTXs en los peces, a pesar de no tratarse de una especie marina.

ANEXOS

ANEXO I. PROTOCOLO DE ACTUACIÓN PARA LA VIGILANCIA EPIDEMIOLÓGICA DE LA INTOXICACIÓN POR CIGUATERA EN CANARIAS

Servicio
Canario de la Salud
DIRECCIÓN GENERAL DE SALUD PÚBLICA

Gobierno de Canarias

PROTOCOLO DE ACTUACIÓN PARA LA VIGILANCIA EPIDEMIOLÓGICA DE LA INTOXICACIÓN POR CIGUATERA EN CANARIAS

1. Introducción:

La ciguatera es un tipo de intoxicación alimentaria producida por el consumo de peces que contienen ciguatoxina. Para que se produzca la intoxicación en el hombre es necesario que el pescado consumido haya acumulado la toxina en suficiente cantidad. Se trata de especies grandes, depredadoras, que han ido acumulando toxina aportada por otras especies herbívoras que se alimentan de algas y dinoflagelados tóxicos (*Gambierdiscus toxicus*), propios de los arrecifes de coral. La toxicidad comienza a ser manifiesta a medida que la toxina se va concentrando a través de la cadena alimenticia, llegando en última instancia al hombre. Los peces más grandes, de más edad, son más tóxicos.

Se conocen más de 400 especies de peces, todas de aguas tropicales o cálidas, que pueden haber producido casos de ciguatera. Algunas de las especies más frecuentemente afectadas son: mero, pargo, cuna, barracuda, jurel, morena, medregal, abade, etc.

Se considera una incidencia mundial de 50.000 casos/año, principalmente en zonas donde es común el consumo de peces de arrecife: Australia, el Caribe, sur de Florida y el Pacífico Meridional. En Europa se han descrito casos relacionados con viajes a países caribeños o con el consumo de peces exóticos en restaurantes étnicos. En España no se han descrito casos autóctonos y en Canarias existe el antecedente de un brote que afectó a 5 personas en Fuerteventura, publicado en 2005.

En los últimos meses se han detectado en Tenerife dos brotes de **intoxicación alimentaria por ciguatera** asociados al consumo de pescado de la especie medregal capturado en aguas cercanas a Canarias.

Ante esta situación, la Dirección General de Salud Pública ha puesto en marcha una serie de actuaciones dirigidas a evaluar si estos procesos pudieran estar vinculados con la presencia de ciguatoxinas en el medio marino de Canarias (en cuyo caso es posible esperar la aparición de mas casos), o si por el contrario se trata de sucesos puntuales relacionados con peces migratorios procedentes de zonas de riesgo y que de forma ocasional fueron capturados a su paso por nuestras aguas.

Con base a la normativa vigente[1], que recoge en su artículo 25, punto 1, que la Comunidad Autónoma de Canarias, a través de la Dirección General de Salud Pública del Servicio Canario de la Salud, puede establecer sistemas específicos de vigilancia epidemiológica, se

[1] Decreto 165/1998, de 24 de septiembre, por el que se crea la Red Canaria de Vigilancia Epidemiológica y se establecen las normas para regular su funcionamiento, publicado en el BOC de Miércoles 7 de Octubre de 1998.

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establece el **Sistema de Vigilancia Epidemiológica de la Intoxicación por Ciguatera en Canarias** para recoger datos de los casos que lleguen a nuestro sistema sanitario, con el objetivo de conocer la incidencia y características epidemiológicas de presentación de estos procesos en nuestro medio.

2. Definición de caso de "Intoxicación por Ciguatera":

Paciente con **antecedentes de haber consumido pescado de alguna de las especies consideradas de riesgo*** y que presenta un cuadro clínico con:

- **Síntomas neurológicos:** pueden presentarse un amplio abanico de síntomas, aunque los mas frecuentes son: parestesias (en labios, manos y extremidades), prurito, inversión de la temperatura (los objetos fríos dan sensación de estar calientes y los calientes se perciben como fríos), dolor y debilidad en extremidades inferiores.

- Estos síntomas pueden cursar simultáneamente o aparecer días después de un cuadro digestivo, caracterizado por uno o varios de los siguientes **síntomas gastrointestinales:** vómito, diarrea, nauseas y dolor abdominal, que suelen presentarse en las primeras 48 horas (más frecuentemente entre 2 a 8 horas) posteriores a la ingesta,

* Las especies consideradas de riesgo y que se capturan en Canarias son: medregal, abade, mero, pejerrey, bicuda, morena, peto y sierra.

No existe ninguna prueba analítica que confirme el diagnóstico en el paciente. La confirmación solo es posible si se detecta presencia de ciguatoxina en el análisis del pescado consumido por los afectados. **Es importante indicar al paciente que si tiene algún resto del pescado (crudo ó cocinado) se abstenga de consumirlo, lo coloque en una bolsa de plástico limpia y lo conserve en el congelador de su nevera hasta que sea recogido y trasladado al laboratorio por personal de la Dirección General de Salud Pública.**

Sintomatología de la intoxicación por ciguatera:

La ciguatera es un síndrome con manifestaciones digestivas y neurológicas características, que puede presentarse en el término de una hora después de haber consumido pescado tóxico. Primero aparecen los síntomas digestivos (vómitos, diarreas, nauseas y dolor abdominal) que suelen durar entre 24 y 48 h. después de la ingestión de pescado.

Los síntomas neurológicos, generalmente se manifiestan uno o dos días después, y consisten en: parestesias (hormigueo en labios, manos y pies), prurito intenso localizado en la piel, trastornos inusuales en la percepción de la temperatura (inversión de la temperatura: los objetos fríos dan sensación de estar calientes y los calientes se perciben como fríos), dolor y debilidad en miembros inferiores. Son frecuentes la sensación de fatiga, dolores musculares, articulares y de dientes, y en menor frecuencia depresión y ansiedad. También se han descrito casos con dolor intenso en pene y coitalgia.

Pueden presentarse síntomas cardíacos como hipotensión y bradicardia.

En los casos muy graves (son raros) los síntomas neurológicos pueden evolucionar hasta el coma y paro cardíaco.

Las alteraciones neurológicas comúnmente se resuelven en semanas, aunque el prurito, la artralgia y la fatiga pueden persistir durante meses o años.

3. Declaración de caso y recogida de información:

Cuando se detecte un **caso sospechoso** el médico que atiende al paciente deberá:

- Cumplimentar la **encuesta epidemiológica de intoxicación alimentaria por ciguatera.**
- **Realizar la notificación urgente** del caso por teléfono ó fax.

4. Fuentes de información:

El Sistema de Vigilancia Epidemiológica de la Intoxicación por Ciguatera en Canarias, afecta a **todos los médicos que realizan su trabajo en la red Atención Primaria y Atención Especializada**, tanto del sector público como del privado de la Comunidad Autónoma Canaria, y que son fuentes de información del mismo**.**

5. Circuitos de notificación:

El circuito de transmisión de información que se establece para los casos atendidos en la Atención Especializada y Atención Primaria, es el siguiente:

1. El médico que diagnostica el caso, deberá realizar la notificación del mismo al Servicio de Epidemiología y Prevención de la Dirección General de Salud Pública de **forma urgente** por teléfono o fax. En el plazo máximo de 24 horas enviará por fax la encuesta epidemiológica, debidamente cumplimentada.

2. Si existen restos del alimento, la Dirección General de Salud Pública se encargará de organizar su recogida y envío al laboratorio correspondiente.

3. En caso de brote, corresponde al Servicio de Epidemiología y Prevención de la Dirección General de Salud Pública iniciar la investigación epidemiológica correspondiente, así como establecer el protocolo de actuación en función de las características de la situación detectada.

4. Cuando el caso se diagnostique en **fin de semana ó festivo, la comunicación se realizará al 112.**

5. Las direcciones, teléfonos y fax para realizar la comunicación son los que se indican a continuación:

En Las Palmas

C/ Alfonso XIII, nº4.

35003. Las Palmas de Gran Canaria

Tfno: 928-45 22 66/ 06

Fax: 928- 45 22 60

En S/C de Tenerife

Rambla de Santa Cruz, nº 53

38006. S/C de Tenerife

Tfno: 922-47 42 32/ 33 /44

Fax: 922-47 42 36

ANEXO II. PROTOCOLO DE ACTUACIÓN PARA EL CONTROL OFICIAL DE LA CIGUATOXINA

Gobierno de Canarias

Consejería de Agricultura, Ganadería y Pesca
Dirección General de Pesca

ANEXO

PROTOCOLO DE ACTUACIÓN PARA EL CONTROL OFICIAL DE LA "CIGUATOXINA", EN LOS PRODUCTOS DE LA PESCA EXTRACTIVA EN LOS PUNTOS DE PRIMERA VENTA AUTORIZADOS. (NOVIEMBRE 2020)

Trámites a seguir para la campaña de determinación de la presencia de ciguatoxinas en pescados procedentes de la pesca extractiva en Canarias, cuando se capturan piezas de pescados con pesos iguales o superiores a los indicados en la siguiente tabla de perfiles:

Medregales	*"Seriola spp":*	= 13 Kg.
Peto	*"Acanthoocybium solandri"*	= 35 Kg.
Pejerrey	*"Pomatomus saltatrix"*	= 9 Kg.
Abade	*"Mycteroperca fusca"*	= 12 Kg.
Mero	*"Epinephelus marginatus"*	= 13 Kg.
Picudo	*"Makaira nigricans"*	= 320 Kg.
Pez espada	*"Xiphias glaudius "*	= 320 Kg.

PRIMERO.- Se prohíbe la comercialización de las especies de pescados con pesos iguales o superiores a los indicados en la tabla de perfiles, a los que **no** se les haya realizado las pruebas diagnósticas de detección de la ciguatoxina.

SEGUNDO.- Cuando los profesionales capturen ejemplares con pesos recogidos en la tabla anteriormente expuesta, se llevarán a cabo los siguientes pasos:

1 La pieza de pescado se dejará en depósito en la Cofradía o Punto de Primera Venta, previa **emisión de la Declaración de Recogida** a través del Programa Oficial de Primera Venta de Canarias que comunicará a la Dirección General de Pesca, la existencia de la pieza. Posteriormente, se le asignará una clave (ejemplo: FUMJ201805001), con el que quedará identificada tanto la pieza como la muestra que se tome de la misma.

2 El personal encargado tomará la muestra, que consistirá en un trozo de carne de la zona caudal, de aproximadamente **300 gramos, que se partirá en dos,** y se introducirán en dos envases alimentarios, limpios e identificados correctamente con la clave de muestra (ejemplo: FUMJ201805001), y se guardarán en congelación. La pieza se conservará envuelta en film o bolsa de plástico alimentario, en hielo y congelada, bien identificada con la clave de la muestra. (ejemplo: FUMJ201805001) y la Declaración de Recogida.

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Si fuera necesario tomar muestras a más de una pieza de pescado, se procederá a la limpieza del instrumento de corte antes de la nueva toma.

3 Para proceder a la retirada de la muestra, la Cofradía o Punto de Primera Venta informará a la Dirección General de Pesca de la existencia de la misma, a través de los siguientes números de teléfonos: **618798347,** 928117598, 928117601, enviando a su vez la ficha técnica y la declaración de recogida por correo electrónico a la siguiente dirección: eborrod@gobiernodecanarias.org

4 La Dirección General de Pesca contactará con la empresa de mensajería y comunicará al laboratorio el envío de la muestra.

5 El personal encargado de la Cofradía o Punto de Primera Venta **entregará una de las muestras**, al personal de la agencia de transporte**. La otra se mantendrá en sus instalaciones debidamente congeladas, hasta que se les indique en qué momento se envían (normalmente, después de obtener el resultado).** El transporte al laboratorio se realizará manteniendo las condiciones en las que se encuentra la muestra (congelada), introduciendo la misma en una nevera (corcho blanco o semejante), con mantenedor de frío, para que llegue en condiciones óptimas.

6 **El laboratorio enviará los resultados a la Dirección General de Pesca a través de la plataforma creada para ello (CIGUARED)** y por correo electrónico.

7 La Dirección General de Pesca informará a la Cofradía o Punto de Primera Venta sobre dichos resultados para que proceda a actuar según sea positivo o negativo:

- Si el resultado es **positivo,** la Cofradía o Punto de Primera Venta, mantendrá las piezas congeladas hasta que sean retiradas por agente autorizado en la destrucción de los SANDACH (residuos o subproductos de origen animal, no destinados al consumo humano). **Mensualmente, (los día 26) contactará con la Dirección General de Pesca a la dirección eborrod@gobiernodecanarias.org** comunicando el número de piezas positivas que guardan en depósito para gestionar su retirada.

- Si el resultado es **negativo**, la pieza será librada al consumo, e irá acompañada de l**a nota de primera venta y** deberá estar identificada con bridas plásticas (latiguillo) en las que se incluye en una cara el logo de la Consejería de Agricultura, Ganadería y Pesca y en la otra la numeración necesaria para la distinción de los ejemplares, y etiquetada, sin cuyos requisitos no podrá introducirse en el circuito comercial. Cada brida (latiguillo) tiene impresa una numeración que permite la trazabilidad del producto una vez realizada la prueba diagnóstica de la ciguatoxina.

8- Cada Cofradía o PPV rellenará, en la ***Libreta para control oficial de la ciguatoxina***, el correspondiente registro donde se detalle el número de la brida (latiguillo), el de la pieza del pescado que se le ha asignado a la muestra y el número de primera venta (lote). Se hará una copia de la página donde conste el registro y se enviará por correo electrónico a la Dirección General de Pesca. Con una frecuencia de 15 días, si ha habido incidencias.

TERCERO.- Las piezas de medregal *"Seriola spp"* inferiores a los 13 Kg, mero *"Epinephelus spp"* inferiores a los 13 Kg , peto *"Acanthoocybium solandri"* inferiores a los 35 Kg y pejerrey *"Pomatomus saltatrix"* inferiores a los 9 Kg, que son librados al circuito comercial deberán de ir acompañadas, además de la Nota de Primera Venta de procedencia, número de lote (nº de Nota de la 1ª Venta) y su etiqueta.

Excepcionalmente los ejemplares de las especies de medregales "*Seriola fasciata"* , conocidos como **"loquillos"**, inferiores a **dos kilogramos (2Kg) podrán ser agrupados en un único lote**, siempre y cuando se indique el número de piezas que lo compone (el lote). Cada ejemplar irá acompañado de una copia de la etiqueta donde se determina el numero total de piezas que conforman el lote, especificando el número de orden del lote ejemplo: 1/50, 2/50 .... .

CUARTO.- Para la comercialización de los pescados medregal *"Seriola spp"* , mero *"Epinephelus spp"* , peto *"Acanthoocybium solandri" y* pejerrey *"Pomatomus saltatrix",* por existir riesgo para la salud de los consumidores, se tiene que añadir a la etiqueta y al envase **la leyenda de advertencia:**

"RIESGO"
"NO PONER A LA VENTA AL CONSUMIDOR FINAL, LAS VÍSCERAS DE ESTA *ESPECIE"*

QUINTA.- Este protocolo será de obligado conocimiento por parte del personal de la Cofradía o Punto de Primera Venta y deberá estar expuesto al público, permitiendo así su consulta por los usuarios, en todo momento.

3

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