

Zeitschrift: Acta Tropica
Herausgeber: Schweizerisches Tropeninstitut (Basel)
Band: 42 (1985)
Heft: 3

Artikel: A study of the distribution of ciguatoxin in individual Caribbean fish
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DOI: <https://doi.org/10.5169/seals-313473>

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A study of the distribution of ciguatoxin in individual Caribbean fish

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Summary

Ciguatera toxins were extracted from the tissues of 36 poisonous fishes including 9 dangerous species collected in the Caribbean. Toxicity assays were carried out in mice and the distinctive symptoms of ciguatera poisoning were observed. In a single fish, ciguatoxin was found in the blood, flesh, gonads, gills, heart, skin and bones. The concentration was highest in the viscera and in particular in the liver, kidney and spleen. The ratios of the toxin concentrations of the liver or viscera to that of the flesh were high and varied with the species suggesting that the toxin is stored in different ways in different fish. Subcellular fractionation of liver cells revealed that most of the ciguatoxin was attached to cytoplasmic proteins and that some toxin was probably bound to the membranes.

Key words: ciguatoxin distribution; Caribbean fish; mouse bioassay.

Introduction

Ciguatera is caused by the ingestion of a variety of tropical reef fishes either in the Pacific (Bagnis et al., 1979), the Caribbean (Lawrence et al., 1980; Morris et al., 1982) or the Indian Ocean (Lebeau and Telmar, 1978). The main causative toxin, ciguatoxin has been isolated in the Pacific (Scheuer et al., 1967; Nukina et al., 1984) and is found in the Caribbean (Vernoux et al., 1982). Maitotoxin (Yasumoto et al., 1976) and scaritoxin (Chungue et al., 1977) also occur in some toxic fish. A benthic dinoflagellate *Gambierdiscus toxicus* has been suggested to produce ciguatoxin and maitotoxin in the Pacific (Bagnis et al.,

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1980) and in the Caribbean (Bergman and Alam, 1981). Ciguatoxin is usually detected by toxicity tests in the experimental animal and mice, cats and mongooses are commonly used (Banner, 1976). Recently, immunological assays have been attempted by several authors (Hokama et al., 1977; Berger and Berger, 1979; Chanteau et al., 1981; Kimura et al., 1982) though these need further examination and evaluation (Hokama et al., 1983). Compared to cats and mongooses, mice offer a more reliable bioassay model (Hoffman et al., 1983). In this study we used a mouse bioassay with extracted lipids to investigate the distribution of ciguatoxin in individual Caribbean fish and locate the site of toxin concentration in order to discover how the fish store and metabolize the toxin.

Material and Methods

Fish

Fishes were caught all the year round from 1980 to 1983 in fish-pots or by hook and line on the island of St. Barthelemy (French Caribbean). The species assayed were ichthyophages belonging to the following families: Muraenidae (*Gymnothorax funebris*, *Gymnothorax moringa*), Serranidae (*Mycteroperca venenosa*, *Epinephelus morio*), Scombridae (*Scomberomorus cavalla*), Carangidae (*Caranx bartholomaei*, *Caranx latus*, *Seriola dumerili*), and Sphyraenidae (*Sphyraena barracuda*) (for a complete description of these species, see Stokes, 1980). All specimens were kept frozen until use. Before testing the anatomical distribution of toxins, specimens were first divided into raw fillets and viscera. The liver, spleen, gonads and kidney were removed from the viscera and tested separately after cooking. The remaining flesh was removed from the bones by cooking for 30 min in a pressure cooker. Pooled blood samples were obtained from the combined heart contents. The waste material mentioned in the text is composed of skin, gills and fishbones.

Preparation of toxic extracts

Toxin was extracted by one of the two following methods. The first, called the "acetone method", is a modification of the technique employed by Chungue et al. (1977). The minced tissue (1v; flesh, liver, gonads, heart, spleen, or kidney) was extracted with two portions of acetone (3v each) at room temperature (a third extraction yields <1% of total toxicity). Acetone (or other solvents) were removed in a rotatory evaporator under reduced pressure. Ethanol was added to the remaining aqueous phase to give a 25% solution which was twice extracted with the same volume of diethyl ether at room temperature (a third partition yields <1% of total toxicity). The diethyl ether residue was further partitioned between 80% methanol and light petroleum (40–60°, Merck no 909) two times (1:2 then 1:1 v/v), at room temperature. This step yields toxin in the methanol soluble extract called "lipid-soluble residue A" (LR_A).

The second, or "methanol method", is a modification of the procedure of Yasumoto and Kanno (1976). The raw tissue (1v; viscera¹, gills, fishbones or skin) was roughly chopped or minced and then extracted twice with boiling methanol (3v then 2v; a third extraction yields <5% of total toxicity). The methanolic extracts were filtered through a Buchner funnel at 40°C and the filtrate evaporated to dryness. The dry residue was partitioned between methanol 80% and light petroleum as in the previous method. The methanol soluble material was further partitioned between diethyl ether and 25% ethanol as above. The ether extracts were evaporated to dryness, yielding an extract called "lipid-soluble residue M" (LR_M).

¹ Though LR_A was faster to prepare, it was not suitable for the viscera as it contained too many toxic impurities. These were probably fatty acids (see Vernoux and Bagnis, 1976).

Mouse bioassay

LR_A or LR_M was emulsified in 1% Tween 60 saline (at 37°C) and injected i.p. at lethal and sublethal doses into male or female mice weighing 20–25 g (two mice per dose). A series of three or four dilutions which differed by a constant ratio of 1.193 (and were chosen in the series of numbers including 10^n $n = 0$ or $n = -1$) were assayed. The minimum lethal dose (MLD) was the lowest dose (in mg of residue per gram of animal) capable of killing the two mice in 24 h. The toxicity of the residue was the maximum weight in grams of mice killed by the whole residue (i.e. the number of MLD found in this residue) expressed in terms of Mouse Units (MU), where 1 MU is 1 g of mouse killed by the MLD. The toxin concentration (TCC) is the amount of toxin in 1 g of the original tissue and is expressed in mouse units per gram of tissue (MU/g). The toxin content (TCT) is the amount of toxin in the whole tissue (TCC X weight tissue).

Subcellular fractionation

The liver, suspended (10% W/V) in 0.25 M sucrose and 10 mM Tris (pH 7.4) was homogenized in a Dounce tissue grinder and centrifuged for 10 min at 3000 g to sediment nuclei and plasma membranes. The supernatant was centrifuged for 60 min at 100,000 g to sediment mitochondria and microsomes. Pellets were collected in succession and the final supernatant was retained. In some experiments, the supernatant was divided into three parts: the first was used as a reference sample, the second was clarified by centrifugation after addition of 0.8 M lead (II) acetate (10% v/v) or solid ammonium sulfate (the solution was brought to 55% saturation) to precipitate the proteins, and the third was fractionated by chromatography on 100 g of Sephadex G 25 using Tris 10 mM (pH 7.4) as eluant. Ciguatoxin was extracted separately from each fraction by the acetone method.

Results

Study of extraction methods

The efficiency of the two ciguatoxin extraction methods was studied by comparing the results obtained with the fillets of the same fish, knowing that the toxicity level is uniform throughout the flesh (Vernoux et al., 1982). Toxin content in one fillet determined by the acetone method was identical to that obtained on the other fillet with the methanol method. Purity of the respective residues were different, however, LR_M being 2 to 5 times more pure than LR_A. The amount of extracted ciguatoxin is therefore not dependent on the method.

The yield ranged from 0.1 to 2.0 g of residue per 1000 g of tissue except for the liver (5 to 15) or gonads (2 to 6). The MLD did not exceed 2 mg/g and the most specific ciguatera symptoms (frequency 50–100%) observed at lethal doses in mice were: loss of activity, diarrhea, gasping, penile cyanosis and/or transitory and incomplete erection (sometimes even reaching priapism, i.e. complete and permanent erection), ataxia and very labored choking-gasping breathing. Respiratory failure was the cause of death since the heart was beating forcefully at the time of respiratory arrest.

Anatomical distribution of ciguatoxin

Ciguatoxin was detectable in all tissues of *C. latus*, *C. bartholomaei* and *S. dumerili*. The highest concentrations were found in the liver and the lowest in

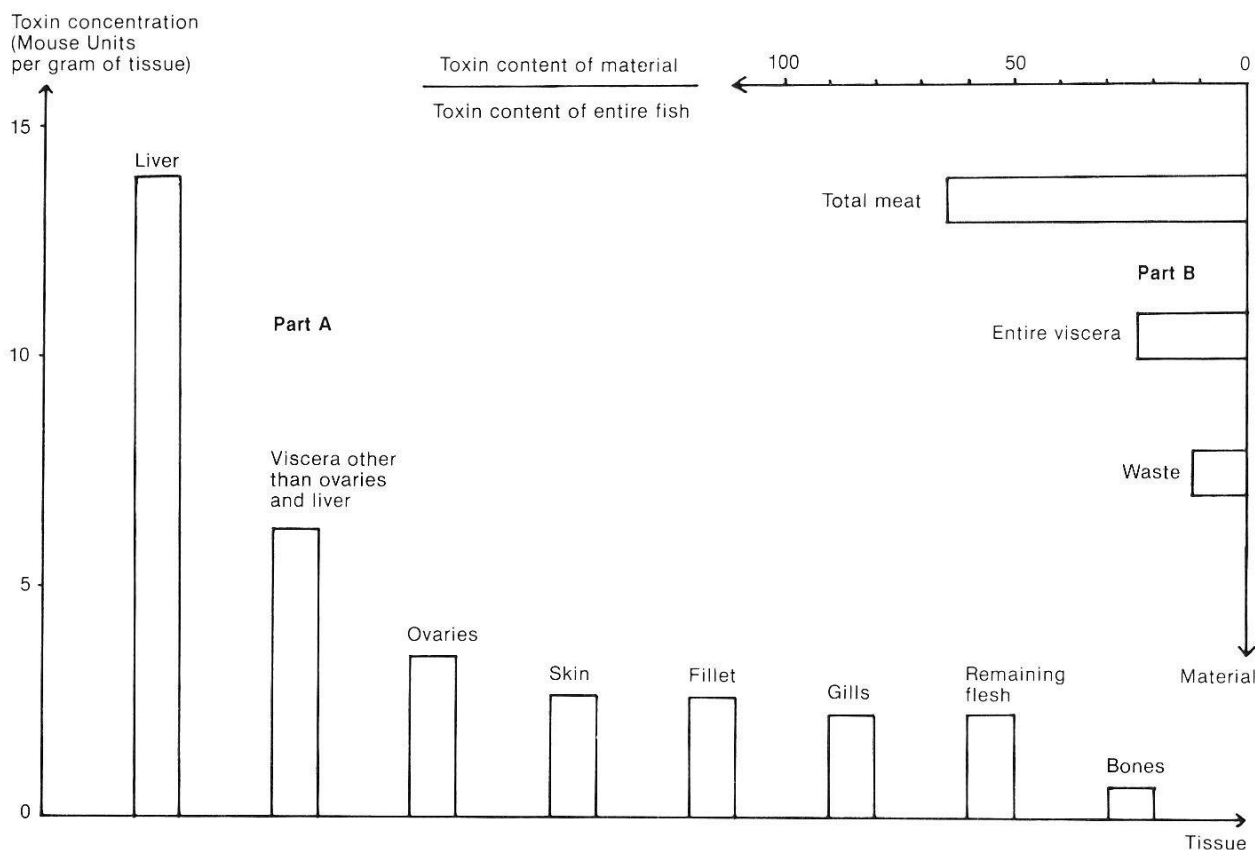


Fig. 1. Anatomical distribution of ciguatoxin in a *Caranx latus* specimen (3.6 kg). Part A: Toxin concentration (TCC) of different tissues. Part B: Toxin content (TCT) of three different materials expressed as a percentage of the total fish TCT.

the bones. An example of this distribution is given in Fig. 1 (Part 1) for a *C. latus* specimen.

Pooled tissues of other small organs were also tested. The spleen (from *C. bartholomaei*) and the kidney (from *G. funebris*) had a high toxin concentration (TCC) similar to that of the homologous livers. The TCC of the heart was similar to that of viscera and ten times higher than that of homologous blood (whose TCC was therefore similar to that of bones). No toxicity was found in residues prepared from the *G. funebris* gall-bladder bile.

Comparison between toxin content of meat, viscera and waste

The toxin content (TCT) of fish fractionated into three parts, namely meat viscera and waste, was studied in different species. An example is given in Fig. 1 (Part B). Some species (*C. latus*, *C. bartholomaei*, *S. dumerili*, *S. barracuda* and *S. cavalla*) accumulate ciguatoxin mainly in their flesh, since meat TCT was 2 to 3 times greater than viscera TCT, whereas other species (*E. morio*, *M. venenosa*, *G. moringa* and *G. funebris*) also store ciguatoxin in their viscera; in these latter species viscera TCT was similar to that of the meat or higher. In all experiments, the waste TCT was lower though never negative.

Table 1. Relation between toxin concentration (TCC) in flesh, entire viscera and liver obtained from individual fish

Fish species	Toxin concentration (TCC) of the flesh (MU/g)	Ratio of viscera*-to flesh-TCC	Ratio of liver-to flesh-TCC
<i>G. bartholomaei</i> (2–5 kg; n = 8)	0.92 ± 0.34 (0.39–1.56)	3.66 ± 0.60 (2.68–4.80)	6.21 ± 0.52 (5.44–7.41)
<i>C. latus</i> (1.3–4 kg; n = 7)	1.21 ± 0.46 (0.60–2.66)	2.40 ± 0.37 (1.81–3.06)	4.86 ± 1.36 (3.00–6.78)
<i>S. dumerili</i> (6 and 29 kg; n = 2)	2.07 ± 1.20 (0.87–3.28)	4.27 ± 0.55 (3.72–4.82)	16.03 ± 5.43 (10.60–21.47)
<i>E. morio</i> (6–8 kg; n = 3)	0.38 ± 0.20 (0.18–0.68)	9.92 ± 3.12 (6.16–14.61)	11.75 ± 3.11 (7.84–16.41)
<i>M. venenosa</i> (3–5 kg; n = 3)	0.14 ± 0.06 (0.07–0.23)	8.92 ± 1.15 (7.57–10.65)	14.86 (**)
<i>G. funebris</i> (3–15 kg; n = 5)	0.51 ± 0.17 (0.29–0.92)	6.89 ± 1.76 (3.51–10.40)	43.73 ± 28.14 (13.50–114.08)
<i>G. moringa</i> (1.5 and 2 kg; n = 2)	0.21 ± 0.09 (0.12–0.30)	10.85 ± 2.48 (8.37–13.33)	Not tested
<i>S. barracuda</i> (3–9 kg; n = 3)	0.69 ± 0.21 (0.37–0.91)	3.25 ± 1.31 (1.80–5.22)	6.65 (**)
<i>S. cavalla</i> (15–20 kg; n = 3)	1.28 ± 0.45 (0.60–1.75)	2.18 ± 0.10 (2.04–2.32)	8.69 ± 4.16 (5.39–14.93)

* including liver

** Liver TCC was studied for one specimen only.

Results are presented as mean ± standard deviation for each species (n specimens). Minimum and maximum values are quoted in brackets.

Comparison between toxin concentration of flesh, viscera and liver

Toxin concentration (TCT) of flesh, viscera (including liver) and liver obtained from specimens weighing 1.3 to 29 kg are presented in Table 1.

Flesh TCC ranged from 0.07 to 3.28 MU/g, thus reflecting the different feeding history of each fish (depending on either species or specimen). Mean values of the ratios of the toxin concentration of entire viscera or liver alone to that of the flesh were high (>2 or >3, respectively) and depended on the fish specimens and species. The values were higher (>5 or >10, respectively) in those species that were shown above to accumulate toxin in their viscera. On the other hand, where there was no visceral ciguatoxicity, the results for the flesh were negative.

Table 2. Distribution of ciguatoxin after subcellular fractionation of liver into two fractions: the nucleus and membrane fraction (3000 g pellet) and the supernatant

Fish species	Toxin content of the		
	unfractionated liver (in MU)	3000 g pellet (in %)	supernatant (in %)
<i>G. funebris</i>	2825	34.16	65.84
<i>G. funebris</i>	837	32.50	67.5
<i>S. dumerili</i>	964	36.31	63.69

Subcellular distribution of ciguatoxin in liver

The following results were obtained: (1) 30 to 40% of the ciguatoxin was collected in the 3000 g pellet mainly composed of nuclei and plasma membranes (Table 2), (2) the 100,000 g pellet (mitochondria and microsomes) was nontoxic, (3) the supernatant contained the remaining toxicity (Table 2). The lipidic cream which sometimes appeared at the top of the supernatant contained only low amounts of ciguatoxin (16% of the supernatant toxicity). When the supernatant was treated either with lead acetate or ammonium sulfate, the bulk of ciguatoxin was found in the protein precipitate; when supernatant was subjected to gel filtration through Sephadex G 25 ciguatoxin was found in the protein fraction. Ciguatoxin therefore seems closely associated with proteins in liver cells.

Discussion and Conclusion

This is the first quantitative study of the anatomical distribution of ciguatoxin in individual fish (the presence of ciguatoxin was confirmed for the same fishes by chromatographic methods as well, Abbad El Andaloussi, 1984). The most detailed previous study on this subject was published by Helfrich et al. (1968) who examined the toxicity of selected and pooled tissues from 103 specimens of *Lutjanus bohar*. These authors showed that the liver sample was the most toxic, and that the viscera were strongly toxic whereas the testes, ovaries and flesh contained lower levels of toxin. Scheuer (1977) reported that the skin, fat and bones of the toxic moray *Gymnothorax javanicus* were nontoxic. Since the bones of Caribbean fish were shown to be ciguatoxic they were carefully examined for the fish-bone lesions described by Morice (1964) at Saint Barthelemy in ciguatoxic fish. However, when these lesions were present there was no correlation with ciguatoxin concentration.

The toxin concentration of the liver or viscera alone was greater than that of the flesh for all the Caribbean specimens studied and these results are similar to those reported in the Pacific for pooled *L. bohar* (Helfrich et al., 1968).

G. javanicus (Yasumoto and Scheuer, 1969), pooled *Ctenochaetus striatus* (Yasumoto et al., 1971) and pooled *Scarus gibbus* (Chungue and Bagnis, 1976). Randall (1980) reported that the liver or viscera of individual toxic fish gave a stronger reaction than muscle in the mongoose feeding test. Therefore one can reasonably assume that liver or viscera are the most ciguatoxic organs in fishes of either Pacific or Caribbean origin. As a consequence, these organs could be used to detect ciguatoxic fishes. However, the flesh TCC cannot be accurately extrapolated on the basis of the liver or viscera results, since the ratios of liver or viscera to flesh TCC vary with specimen and species.

Since ciguatoxin is distributed in all fish tissues, it is of particular importance to determine the basis of this repartition. It is not correlated with fat since we have remarked that the skin of *G. funebris* contained much more fat than the flesh but was less toxic. Though blood cannot retain high ciguatoxin levels, it is certainly involved in the distribution of ciguatoxin to other tissues. This may explain why highly vascularized organs such as the liver, spleen and kidney, retain the highest quantity of ciguatoxin per unit weight. Nevertheless in these tissues, detoxification or elimination, if they occur at all (ciguatoxin is harmless to the fish), proceed very slowly since Banner et al. (1966) showed that ciguatoxic fish maintained in nontoxic water for up to 30 months on a nontoxic diet retained their toxicity. This is consistent with our finding that the bile is nontoxic. In liver cells the affinity of ciguatoxin for cytoplasmic proteins was demonstrated and it is reminiscent of the spontaneous formation of the ciguatoxin-protein complexes observed in vitro by Parc et al. (1979) or Emerson et al. (1983). This may explain how the fish liver can store such enormous amounts of ciguatoxin (up to 1000 MU/g. in Yasumoto et al., 1977) without metabolic disturbances.

To conclude we would like to point out the interest of the mouse bioassay. It is not only a simple and reliable quantitative test, but it also allows ciguatoxin identification by simple observation of clinical symptoms in injected mice. Among others, the penile symptom seems the most characteristic as it is always observed independently of the origin of ciguatoxic extracts, whether they come from Pacific fish (Vernoux and Bagnis, 1976), or Caribbean fish (Vernoux et al., 1982), or from *Gambierdiscus toxicus* collected around Gambier islands (Vernoux, 1975, 1981) and independently of the way of administration to the mouse (Vernoux, 1981). In addition the penile symptom does not occur with other related toxins such as maitotoxin (Yasumoto et al., 1976) or brevetoxins (Baden, 1983). The mouse bioassay is therefore a practical laboratory tool until routine immunological methods are developed.

Acknowledgments

The authors thank Professor Abdeslam Srairi, Dean of the Faculty of Medicine in Casablanca, Mr. Mokhtar El Hnot, Secretary General, Mr. and Mrs Chraibi, Miss Venise Greaux, Mr. Jean

Baptiste Greaux, Mr. Jean Marc Greaux, Mr. Denis Dufau, Mr. Marc Vol, Mr. and Mrs Desforges, Mrs Latifa Falahi, Miss Aicha Moussafr and Mrs Anita Vernoux for their assistance in the production of this work. We also thank Dr. Abderrahim Tazi, Dr. Paul Basset, Dr. Jean Louis Gallis, Mr. Jean Pascal Quod and Mr. Michel Lambœuf for discussion and advice on the matter of this work.

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