サザナミハギの毒性

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Toxicity of the Surgeonfishes

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Specimens of surgeonfishes, *Ctenochaetus striatus* and *Acanthurus lineatus*, collected in Tahiti contained both fat-soluble and water-soluble toxins. The fat-soluble toxin was purified by column chromatography and compared with reference ciguatoxin by thin layer chromatography. The results clearly indicated that it is chromatographically identical with ciguatoxin. It was also present in the stomach contents strongly supporting the previous hypothesis that the surgeonfishes obtain ciguatoxin from their diet and transfer it to the carnivores through food chain. The water-soluble toxin found in the stomach contents was non-diffusible through a cellophane membrane, extractable with 1-butanol from aqueous solution, positive to both the Dragendorff and ninhydrin reagents, and showed hemolytic and ichthyotoxic properties.

Ciguatoxin, named and characterized by SCHEUER *et al.*[1] is the most important toxin in ciguatera poisoning. It has been proposed that the toxin is produced by a benthic organism, possibly an alga of fine structure, which is ingested by herbivorous fishes. These, in turn, are eaten by carnivores and the toxin is passed through the food chain to fishes of higher trophic level[2]. A surgeonfish *Ctenochaetus striatus* (QUOY and GAIMARD) has been predicted to play an important role in the pathway of ciguatoxin, and much evidence supporting this idea has been compiled by both biological and ecological studies of HELFRICH and BANNER[3], BANNER *et al.*[4], and HELFRICH *et al.*[5] BANNER[6] also reported preliminary studies on a water-soluble toxin from acanthurids collected in the Society, Line, and Wake Islands. Little has been learned about the chemical and pharmacological properties of the toxins in this species, although this information is required to establish the validity of this food chain hypothesis.

The toxicity of this species is also of medical importance. BAGNIS *et al.*[7] have shown in Tahiti that *C. striatus* is responsible for 61% of the cases of ciguatera which they in-

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vestigated. The high incidence of posioning by this surgeonfish may be because it is the most abundant fish of edible size on reefs in the Society Islands, is highly esteemed by the Tahitian people as food and the symptoms of ciguatera resulting from the ingestion of toxic fish of this species are usually mild.

The present report deals with the occurrence of ciguatoxin, or a closely related toxin, in the tissues and stomach contents of *C. striatus*, with some properties of a water-soluble toxin found in the stomach contents and liver, and with the result of toxicity tests on the fractionated stomach contents. The results of studies on the toxicity of another surgeonfish *Acanthurus lineatus* from Tahiti and *C. striatus* from Palau and Okinawa are also included.

Materials and Methods

Specimens: Two batches of *C. striatus* were collected at Popote Bay, Tahiti in October 1968 (CS–A) and in February 1969 (CS–B). A few specimens were also obtained from Ishigaki Island, Okinawa in May 1968 and from Palau in January 1969. Specimens of *A. lineatus* were obtained at the time of collection of CS–B from the same area. All specimens were kept frozen until used.

Testing method for toxicity: For testing the anatomical distribution of toxins, the specimens were divided first into the flesh and viscera. The liver was removed from the viscera to be tested separately. The digestive tract was also removed and the contents of the stomach and of the intestine were separated from the tract. The cleaned stomach was returned to the lot containing the viscera. The intestine, on the other hand, was not combined with the other viscera in order to avoid possible contamination from the contents which could not be removed completely. The viscera excluding the liver and intestine was named the combined viscera sample. In toxicity tests of the ingested materials the stomach contents were used in batch CS–B. However, as the stomach contents were not obtained in enough quantities from the specimens in CS–A and of *A. lineatus*, the gut contents alone were used as the test material. The batch CS–B contained about 400 specimens and only 100 of them were used for testing the anatomical distribution of toxins. The viscera from the remaining 300 specimens were combined after removal of the stomach contents. This combined sample was named viscera with gut contents and used for a large scale extraction of toxins.

From these samples the water-soluble and fat-soluble fractions were prepared by the routine method adopted by HASHIMOTO *et al.*[8] and assayed by *i.p.* injection of serial dilutions of each sample in mice weighing about 20 g each: two mice were used for each dilution. The fat-soluble fractions were administered after being emulsified in 1% solution of Tween 60. The dilution that kills mice in between 4 and 24 hours was sought and the toxicity of the extracts is expressed in μg per g of mouse (μg/g). For ready comparison, on the other hand, a total amount of toxin in the tissue is expressed in term of mouse units (MU), with one mouse unit being defined as the total weight in g of mice which would be killed by the extracts.

Chromatography of the fat-soluble toxin: The fat-soluble toxin was purified by column chromatography as detailed in Fig. 2. The procedure parallels that employed for purification of ciguatoxin from the moray eel liver*.

The purified fat-soluble toxin was compared by thin layer chromatography with ciguatoxin** from the flesh of the toxic moray eel *Gymnothorax javanicus* using aluminum oxide G in a solvent system of chloroform-methanol-6N-ammonium hydroxide (90:9.5:0.5 v/v). The chromatograms were visualized either by exposing the plate to iodine vapor or by spraying with Dragendorff reagent. To locate the toxic spot, a portion of the toxin that would kill four mice was taken from each sample, applied to an aluminum oxide G layer 1 mm thick (10×20 cm), and developed with the same solvent system to a height of 10 cm. The plates were then divided into five equal bands. Each band was scraped off and extracted with chloroform containing 20% methanol. The extracts were suspended in Tween 60 solution and injected into two mice.

Fractionation of the stomach contents: The stomach contents of CS–B contained a large amount of sand and coral fragments, and an attempt was made to separate them from the presumably algal residue by a procedure shown in Fig. 3. The stomach contents were suspended in sucrose solution ($d_4^{20}=1.23$) with vigorous stirring. The suspension was left for one min., decanted, and centrifuged. The supernatant was recombined with the sediment obtained in the first step and the procedure was repeated. The sediment which precipitated within one min. was named Fr. I and the precipitate obtained by centrifugation Fr. II. The supernatant was freed from sucrose by dialysis and concentrated to a syrup under reduced pressure (Fr. III). Each fraction was tested for toxicity.

Purification of the water-soluble toxin: Purification of the water-soluble toxin from the combined sample of the viscera and gut contents of CS–B is illustrated in Fig. 4. The toxin was separated from substances of small molecular size by dialysis in a cellophane bag, extracted from the retentate with 1-butanol, and then forced into water by adding an equal volume of diethyl ether to the organic phase. The aqueous solution was concentrated under reduced pressure and the toxin was precipitated by adding 7 times its volume of acetone. The precipitate was dissolved in a small volume of water, placed on a Sephadex G–100 column, and eluted with distilled water. Fractions appearing soon after blue dextran were combined and evaporated. The hemolytic activity of this semi-purified

* T. Yasumoto and P. J. Scheuer: Unpublished data

** Supplied by P. J. Scheuer, University of Hawaii preparation was compared with that of commercial saponin (Merck, B6, Darmstadt) by the previously reported method used for asterosaponins[9]. Ichthyotoxicity was tested by putting five guppies *Lebistes reticulatus* in a beaker maintained at 25°C and containing 50 ml of 0.0044% solution of the semi-purified toxin.

Results

The specimens of *C. striatus* and *A. lineatus* from Tahiti contained both water-soluble and fat-soluble toxins, while the specimens of *C. striatus* from Ishigaki and Palau were nontoxic. Fig. 1 shows the anatomical distribution of the toxins. The two batches of *C. striatus* showed a similar pattern of toxin distribution.

The fat-soluble toxin was detectable in all tissues tested of *C. striatus*, the concentration being highest in the liver and lowest in the flesh. On the other hand, the water-soluble

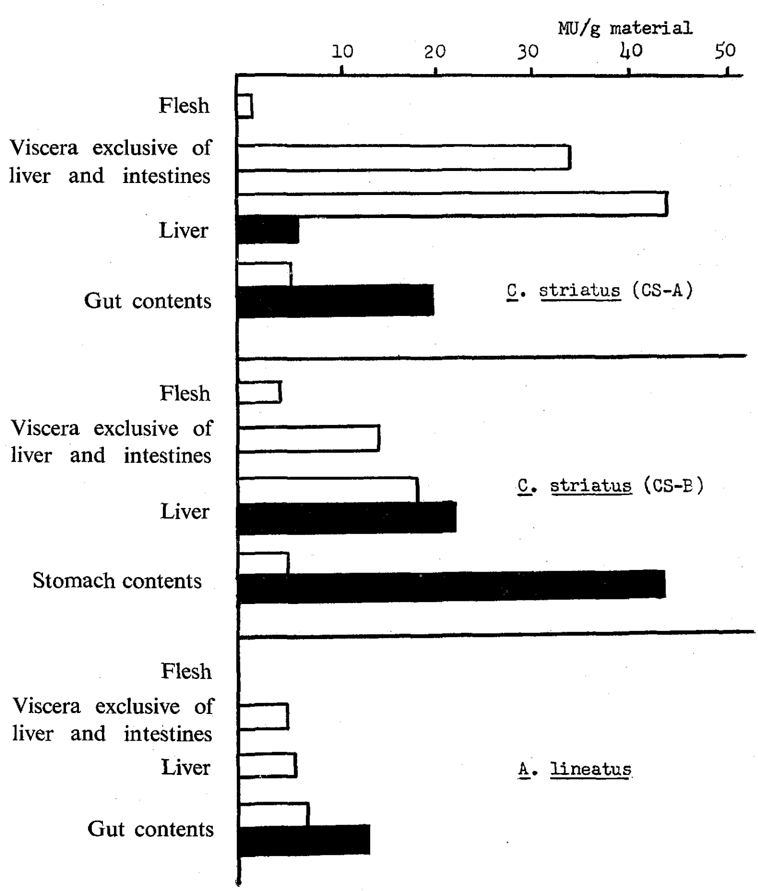

Fig. 1. Distribution of toxins in surgeonifishes
□ Fat-soluble toxin ■ Water-soluble toxin

toxin was found only in the liver and ingested materials with the latter being the more toxic. In the specimens of *A. lineatus* the fat-soluble toxin was detected in the liver, viscera exclusive of the liver and intestines, and gut contents but not in the flesh. The water-soluble toxin was found only in the gut contents. The fat-soluble fractions from the gut contents of CS–A and from the stomach contents of CS–B were lethal to mice in doses of 300 and 910 μg/g, respectively, while similar preparations from other parts of fish were lethal in doses ranging from 1,000 to 4,000 μg/g.

Results of purification of the fat-soluble toxin from the viscera with gut contents of CS–B are presented in Fig. 2. The behavior of the fat-soluble toxin paralleled well that of ciguatoxin and the final preparation showed a toxicity of 5.0 μg/g. The fat-soluble fraction from the flesh of CS–B purified similarly had a toxicity of 30 μg/g. Purification of the stomach contents of CS–B and of the gut contents of *A. lineatus* was carried out in the similar manner except that chromatography using a Florisil column was omitted because the samples were of insufficient size. The final preparations killed mice in doses of 40 and 80 μg/g, respectively.

The viscera with gut contents (2.2 kg)
Extract with 70 and 96% ethanol
Concentrate

Ether-soluble (67.113 g, 3,900 μg/g) | Water-soluble
Add methanol
Extract with hexane

Methanol-soluble (14.768 g, 1,200 μg/g) | Hexane-soluble
Silicic acid column

Chloroform-methanol eluate (9:1) (9.13 g, 500 μg/g)
Florisil column

Acetone-methanol eluate (95:5) (0.753 g, 60 μg/g)
DEAE-cellulose column

Chloroform-methanol eluate (1:1) (0.043 g, 8.0 μg/g)
Sephadex LH-20 column

Toxic fraction (0.021 g, 5.0 μg/g)

Fig. 2. Purification of the fat-soluble toxin from *C. striatus* (CS–B)

In mice the fat-soluble toxin caused loss of activity, severe diarrhea, lacrymation, dyspnea, excessive salivation, convulsive spasms, paralysis of hind limbs, and death. These symptoms were quite similar to those reported for ciguatoxin[1].

Thin layer chromatography of reference ciguatoxin and the fat-soluble toxin derived respectively from the flesh, viscera with gut contents, and the stomach contents of CS–B indicated that none of these samples was homogeneous, but they all showed a spot of Rf 0.5. When the plates were eluted and assayed with mice, only the component of Rf 0.5 was found to be toxic.

Fractionation of the stomach contents yielded 91 g of Fr. I, 18 g of Fr. II, and 5 g of Fr. III, as shown in Fig. 3. Microscopic examination revealed that sand and coral fragments were dominant in Fr. I, fragments of algae in Fr. II, and unidentifiable particles

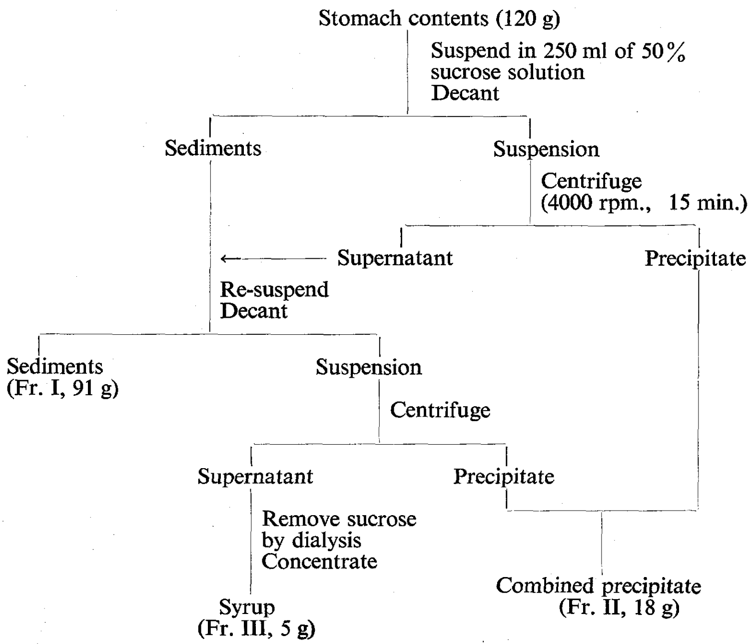

Fig. 3. Fractionation of the stomach contents of *C. striatus* (CS–B)

in Fr. III. Results of the toxicity tests are summarized in Table 1. The concentration of both the water-soluble and fat-soluble toxins was lowest in Fr. I. Fraction III consisting of the lightest materials showed the highest toxin concentration and Fr. II was intermediate.

Yield and toxicity of the water-soluble toxin at each step in purification are presented in Fig. 4.

From 2.2 kg of the viscera with gut contents of CS–B, 1.36 g of a partially purified toxin (80 μg/g) was obtained. This preparation reacted to both the ninhydrin and Dragendorff reagents and the toxin was considered to have a large molecular size from the elution volume in Sephadex G–

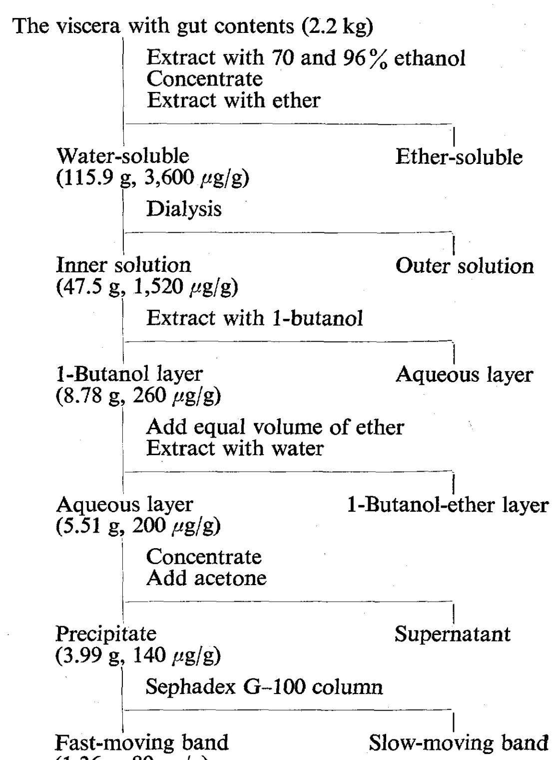

Fig. 4. Purification of the water-soluble toxin from *C. striatus* (CS–B)

Table 1. Toxicity of the fractionated stomach contents of *C. striatus* (CS–B)

Material	Fraction	Yield (g)	Toxicity (μg/g)	MU/g material	Total MU
Fr. I	Fat-soluble	0.171	Nontoxic	0	0
(91 g)	Water-soluble	3.170	3900	9	819
Fr. II	Fat-soluble	0.056	1050	3	54
(18 g)	Water-soluble	1.523	1430	59	1062
Fr. III	Fat-soluble	0.030	390	15	75
(5 g)	Water-soluble	0.725	1430	81	405

100 column chromatography. Injection (*i. p.*) in mice caused a remarkable loss of activity, weakness in limbs, and convulsion before death which occurred after several hours or as late as 3 days depending on dose. The guppies put in a 0.0044% solution of the toxin died in 26–34 min. Hemolytic activity of the toxic preparation corresponded to 80% that of the standard saponin.

Discussion

The fat-soluble toxin found in Tahitian surgeonfishes was not distinguishable from ciguatoxin chromatographically. Dr. M. D. RAYNER[10], Department of Physiology, School of Medicine, University of Hawaii, who carried out a pharmacological screening of our final preparation of fat-soluble toxin (5.0 μg/g) found that it was indistinguishable from ciguatoxin. These results clearly indicate that the fat-soluble toxin in the present specimens is similar to or identical with ciguatoxin.

It should be noted that the lighter fractions of the ingested materials contained ciguatoxin or a ciguatoxin-like substance in high concentration. This finding supports the previous hypothesis that fish obtain the toxin through their diet. In addition, it suggests that toxin may be present in the diet as such rather than as a nontoxic precursor. Thus, it seems possible to locate the primary organism which produces the toxin by a more extensive examination of the diet of toxic surgeonfishes.

Knowledge of the dentition and feeding habits of *Ctenochaetus* also supports the concept that the basic toxic organism(s) is fine in structure and relatively soft. RANDALL[11] reported that the teeth of *Ctenochaetus* are numerous, small, and very elongate with expanded incurved tips. They are loosely attached in contrast to the teeth of other genera of surgeonfishes which are firmly fixed in the jaws. In an aquarium in Hawaii *C. strigosus*, a closely related species, was observed to feed from a near vertical position, pecking head downward. Detrital material, including fine inorganic sediment, was removed from the bottom, thus indicating that a suction mechanism is involved in feeding as well as a scraping by the teeth. Comparable observations were made of *C. striatus* in its natural habitat in the Society Islands. Each feeding movement resulted in an oval area well cleaned of soft growth and fine sediment; but, when tough algal turf material was present, it appeared unaffected. It is believed that any of the coarser algal materials found as fragments in the stomachs of *Ctenochaetus* were ingested as detrital material and not directly grazed. When individuals of *C. strigosus* in an aquarium were presented with the relatively fine filamentous alga *Polysiphonia* sp., they tried to feed on it but were not able to effectively bite off pieces. When the alga was broken into fine particles and allowed to settle to the bottom of the tank, it was readily eaten. The stomach contents of seven *C. stigosus* from different localities in Hawaii were analyzed. All but one fish contained a large amount (up to 90%) of inorganic sediment; the remaining detrital material was mostly algal, but 1–2% was unidentified soft organic matter. The stomach of one specimen consisted mainly of the fine red alga *Ceramium* sp., though there was still a great deal of sediment. WALTERS* studying the food habits of *C. striatus* in the Papara District of Tahiti, with many specimens coming from Popote Bay itself, reports that during a full year cycle Rhodophyta consituted about 50% of the identifiable algae, but that the other algal groups (Cyanophyta, Chrysophyta, Chlorophyta, Phaeophyta) were present in the 18 genera recognized in the gut contents. He also reports that there was a considerable (but unspecified) amount of other debris, including sand, small invertebrates, and "unidentifiable detritus" which was found "binding the algae and other particulate matter". Similar reports on the food habits of *C. striatus* were those of HIATT and STRASBURG[12] from the Marshall Islands and HELFRICH *et al.*[5] from Palmyra. An indication of the nature of the other detritus is the yet unpublished studies of Dr. K. R. GUNDERSEN, Department of Microbiology, University of Hawaii**who has found many strains of viable yeasts and bacteria in the gut contents of *C. striatus* from Popote Bay, some of which when raised in mass culture are highly toxic to test animals. The source of the toxin in the food, therefore, may be from either the algae or the "other debris", the latter possibly helping to make up the toxic Fr. III of this study.

The stomach of *Ctenochaetus* is thick-walled and gizzard-like. This structure is shared by some species of *Acanthurus* (but not *A. lineatus*). RANDALL[13] noted that those species with thick-walled stomachs ingest much more fine sand than those with thin-walled stomachs. He suggested that the sand probably serves the purpose of grinding of the algal food into finer particles. *A. lineatus* grazes algae of many different species. Its teeth, which number about 14 in the jaws of adults, are rigid, close-set, spatulate, and denticulate on the edges, thus well suited for feeding on the filamentous algae[14]. Although this fish may ingest soft material incidentally with its algal food, it obviously takes in much less than the detrital-feeding *Ctenochaetus*. In Fig. 1 the lower yield of

* C. K. WALTERS: Personal communication.
** K. R. GUNDERSEN: Personal communication.

water-soluble toxins of *A. lineatus* is readily apparent compared to that of *C. striatus* (CS–C) taken at the same locality and at the same time.

Anatomical distribution of ciguatoxin or ciguatoxin-like substance in *C. striatus* resembled that reported on a red snapper *Lutjanus bohar*[5]. Interestingly, there was a significant difference between *C. striatus* and *A. lineatus* in toxicity of the tissues. Since toxicity of the fat-soluble toxin level of the ingested materials was similar in both species, the above difference may indicate that uptake of the toxin from the diet varies from species to species. It may be interesting to compare the toxicity level of the herbivores with that of the carnivores from the same area. At present, available data for such a comparison are limited to those on the moray eel liver[15]. As shown in Fig. 1, concentration of the fat-soluble toxin in the livers of two batches of *C. striatus* are 20 and 45 MU/g, respectively. On the other hand, the livers of the moray eel *G. javanicus* from the same district of Tahiti showed much higher toxicity levels, ranging from 80 to as high as 500 MU/g. It is evident that there may be concentration of the toxin as the trophic levels ascend[2], possibly parallel to the reported concentration of chlorinated hydrocarbon insecticides in birds of prey.

Fractionation of the stomach contents into three groups by specific gravity did not give clear-cut separation of toxic constituents from the nontoxic. It is, however, presumed from toxicity tests that toxin is produced by organism(s) both small in size and low in specific gravity. More sophisticated methods of fractionation are necessary to obtain more positive information on the nature of the biota that may elaborate the toxin. The water-soluble toxin found in the present study resembled aluterin, a toxin found in the ingested materials of a filefish[16], in being both non-diffusible in dialysis and extractable with 1-butanol from the aqueous solution, but distinctly differed from it in being hemolytic and ichthyotoxic. On the other hand, it shared many of its properties with the water-soluble toxin recently found in a marine snail, *Turbo argyrostoma*[17].

During the course of this study, a few other specimens of *C. striatus* were obtained from Tahiti which contained a fat-soluble toxin obviously different from ciguatoxin. The water-soluble toxin of this lot was also different from the one described above. The limited availability of specimens, however, did not allow us to characterize these toxins more precisely. It is most likely from these results that the surgeonfish can bear various toxins depending upon their previous history of feeding.

This apparent multiplicity of toxins has a clear relationship to the variability reported in the symptoms found in human cases of ciguatera. In his clinical analysis of 350 cases of ciguatera in French Polynesia over a period of three years, BAGNIS[18] has shown a considerable variation in symptoms. He noted that digestive and neurologic symptoms predominated in patients who became ill following the eating of surgeonfishes whereas the syndrome involves other symptoms, including cardiovascular disorders, when carnivorous fishes were consumed. When it is considered that the toxins differ both in their pharmacology and their relative concentration, it is somewhat surprising to observe even a parallel grouping of symptoms.

Indeed these results may demand a new and more precise definition of ciguatera. At present all illnesses that result from eating fresh coral reef fish except for puffers are called ciguatera in spite of the varying symptoms, and all fishes producing the variable "disease" are labelled as ciguatoxic. As our knowledge of the differing toxins and the symptoms they produce increases, it would be well to apply more specific designations to the various illnesses.

Since the water-soluble toxin appears confined to the liver and gut contents, illness from this could be avoided by removing the viscera and washing the flesh well before cooking.

Although ciguatera is well known in Okinawa, the specimens of *C. striatus* from this area did not show any recognizable toxicity. The simplest explanation for this phenomenon may be that toxic organism(s) do not grow in the sampling area. It was observed, however, that these specimens had ripe gonads but practically no ingested materials in the stomach. WALTERS* had noted that the feeding activity during spawining is "less intensive". The absence of toxins, especially the water-soluble one, in the specimens may be related to this phenomenon. Failure to detect toxins in specimens from Palau seems to be in accordance with the previous report that ciguatera is unknown in that archipelago[19].

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* C. K. WALTERS: Personal communication.

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