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Experimental evidence of dietary ciguatoxin accumulation in an herbivorous coral reef fish

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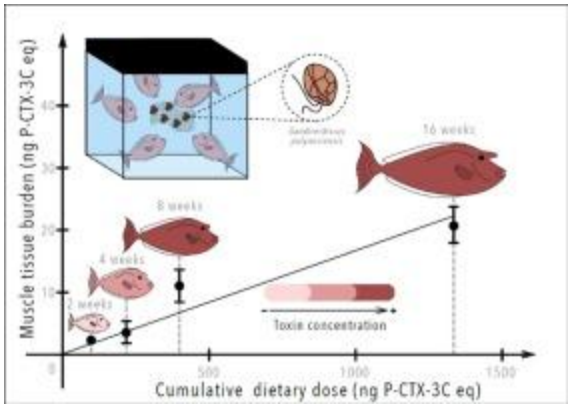
Abstract :

Ciguatoxins (CTXs) are potent algal toxins that cause widespread ciguatera poisoning and are found ubiquitously in coral reef food webs. Here we developed an environmentally-relevant, experimental model of CTX trophic transfer involving dietary exposure of herbivorous fish to the CTX-producing microalgae *Gambierdiscus polynesiensis*. Juvenile *Naso brevirostris* were fed a gel-food embedded with microalgae for 16 weeks (89 cells g.1 fish daily, 0.4 ¦Ìg CTX3C equiv kg.1 fish). CTXs in muscle tissue were detectable after 2 weeks at levels above the threshold for human intoxication (1.2 ¡À 0.2 ¦Ìg CTX3C equiv kg.1). Although tissue CTX concentrations stabilized after 8 weeks (¡«3 ¡À 0.5 ¦Ìg CTX3C equiv kg.1), muscle toxin burden (total ¦Ìg CTX in muscle tissue) continued to increase linearly through the end of the experiment (16 weeks). Toxin accumulation was therefore continuous, yet masked by somatic growth dilution. The observed CTX concentrations, accumulation rates, and general absence of behavioural signs of intoxication are consistent with field observations and indicate that this method of dietary exposure may be used to develop predictive models of tissue-specific CTX uptake, metabolism and depuration. Results also imply that slow-growing fish may accumulate higher CTX flesh concentrations than fast-growing fish, which has important implications for global seafood safety.

Graphical abstract



Highlights

► An ecologically relevant procedure of dietary ciguatoxin transfer was developed. ► Fish consuming *Gambierdiscus* cells daily showed no signs of intoxication. ► Fish flesh contained CTX above thresholds for human illness in 2 weeks. ► Fish accumulated CTX continuously at a constant rate over 16 weeks. ► Somatic growth dilution of CTX suggests slow-growing fish may be higher risk.

Keywords : Ciguatoxin, Bioaccumulation, Growth dilution, Trophic transfer, Herbivorous fish, Experimental model

42 **1. INTRODUCTION**

43 Ciguatera poisoning (CP), the most common non-bacterial seafood-borne illness globally (Fleming et al.,
44 2000; Friedman et al., 2008), is caused by the consumption of fish tissue containing ciguatoxins (CTXs;
45 Berdalet et al., 2016). CTXs are lipid-soluble, thermostable polycyclic ether molecules classed by
46 geographical origin and differences in chemical structure. They include P-CTXs (Pacific Ocean), C-CTXs
47 (Caribbean region) and I-CTXs (Indian Ocean). CTXs induce neurotoxic effects that result from binding
48 to voltage-gated sodium channels (Na_v) and potassium channels in excitable tissues (Dechraoui Bottein et
49 al., 2006; Molgó et al., 1993; Nicholson and Lewis, 2006). CTXs are produced by species of benthic
50 dinoflagellates of the genera *Gambierdiscus* (Adachi and Fukuyo, 1979; Yasumoto et al., 1977) and
51 *Fukuyoa* (Gómez et al., 2015), which are distributed throughout tropical and subtropical coastal waters
52 worldwide (Litaker et al., 2010). The presence of CTXs in the flesh of upper-trophic level fish,
53 particularly one of the most toxic analogues, CTX1B (e.g. Lewis et al., 1991), is believed to result from
54 consumption of *Gambierdiscus* cells (Bagnis et al., 1980; Yasumoto et al., 1977; Yasumoto et al., 1977)
55 by herbivorous and omnivorous fish (Satake et al., 1996) and subsequent trophic transfer and toxifying
56 biotransformation through the food web (Bienfang et al., 2013; Lehane and Lewis, 2000; Lewis and
57 Holmes, 1993; Randall, 1958). After consumption of the dinoflagellates or other ciguatoxic prey, CTXs
58 can bioaccumulate in select fish tissues such as liver (Yasumoto et al., 1977) and flesh, and
59 concentrations may then successively increase up the fish food web (Lewis and Holmes, 1993). Despite
60 the well-established threat to human health (Berdalet et al., 2016; Friedman et al., 2017), there are no
61 established means of risk-assessment or resultant regulation of the fisheries and the market.

62

63 Tissue bioaccumulation of toxins and subsequent biomagnification is a central tenet of the trophic transfer
64 of CTXs underlying CP (Bienfang et al., 2013), yet evidence is primarily limited to the sampling and
65 analysis of coral reef fish (e.g. Chan et al., 2011; Mak et al., 2013; reviewed by Yang et al., 2016) and
66 several short-term feeding studies conducted in the laboratory (e.g. Helfrich and Banner, 1963; Ledreux et
67 al., 2014; and in giant clams: Roué et al., 2016). Regional work has provided conflicting evidence, where

68 some herbivorous fish have been found to contain high concentrations of CTXs in their flesh, comparable
69 to those found in higher-trophic level fish (Chan et al., 2011; Darius et al., 2007; Gaboriau et al., 2014).
70 Work examining the relationship of CTX concentrations and trophic level (as estimated by $\partial^{15}N$) in fish
71 found weak or no correlations, depending on the CTX congener (Mak et al., 2013). An early laboratory
72 study demonstrated transfer of toxin through feeding (Helfrich and Banner, 1963), but the herbivorous
73 fish was fed ciguatoxic fish flesh, limiting the conclusions that can be drawn for natural food webs. In
74 contrast, a recent study in which the omnivorous mullet *Mugil cephalus* was given food containing toxic
75 cells of *Gambierdiscus* found that most toxin was eliminated within 24 h of feeding, and no toxin was
76 accumulated over 9 cumulative feedings in 16 days. Moreover, fish showed strong signs of intoxication
77 (Ledreux et al., 2014). Thus, until now, the mechanisms by which fish accumulate CTXs in their flesh at
78 concentrations sufficient for intoxicating humans (> 0.1 ng CTX1B g^{-1} of fish; Lewis and Holmes 1993;
79 Hossen et al. 2015) remain unclear (Ledreux et al., 2014; Yang et al., 2016) and poorly validated
80 experimentally.

81

82 Correlating the dynamics of toxic *Gambierdiscus* on the reef with seafood toxicity and risk for CP
83 involves predicting CTX concentrations in edible tissues in relation to time and environmental conditions
84 (Llewellyn, 2010; Tester et al., 2010). This capability requires an understanding of the toxicokinetics of
85 CTXs in fish and the development of mathematical models based on intake level, distribution,
86 biotransformation and elimination. However, unknown ingested doses in the wild result in unknown
87 absorption efficiencies and thus impair the ability to predict potential accumulation of flesh CTX
88 concentrations. Current models of CTX trophic transfer are limited to a conceptual nature (e.g. Cruz-
89 Rivera and Villareal, 2006; Lewis and Holmes, 1993). Consequently, algal abundances on the reef are not
90 easily associated with potential for CTX accumulation. Additional uncertainty may be introduced by high
91 spatial heterogeneity and temporal variability in *Gambierdiscus* occurrence (reviewed by Cruz-Rivera and
92 Villareal, 2006), interactions within reef trophic levels, or fish behaviour (e.g. migration and home
93 ranges). This is particularly true for CTX, as blooms of *Gambierdiscus* spp. can occur and thus be

consumed via macroalgal substrate throughout the year (Chateau-Degat et al., 2005; Chinain et al., 1999). After a disturbance, long-term (4-year) survey data indicates a ~10 month lag time before *Gamberdiscus* peak cell densities are observed (Chinain et al., 1999). The data shows an additional month passes before CTXs are detected in the flesh of herbivorous fish, and a subsequent ~3 month-lag before the maximum number of reported CP cases (Chateau-Degat et al., 2005; Clausing and Dechraoui Bottein, 2016). Thus, while short-term experiments (e.g. 9 feedings in 16 days: Ledreux et al., 2014; 48 h feeding trial: Roué et al., 2016) may provide important toxicological information, they may not reflect the true potential for toxin bioaccumulation in these species over time. Controlled experimental procedures using ecologically-relevant species and well-defined laboratory conditions are essential to better understand the processes underlying the accumulation of CTXs in fish flesh, knowledge that is required to predict the potential for CP along the food web. In this framework of seafood safety risk assessment, we developed an experimental model of CTX trophic transfer from the toxin-producing benthic dinoflagellate *Gambierdiscus polynesiensis* to an herbivorous coral reef fish, *Naso brevirostris*, over long-term dietary exposure.

2. MATERIALS AND METHODS

2.1. Study species

Cells of *Gambierdiscus polynesiensis* were obtained from mass cultures of the highly toxic strain TB92 (Tubuai, Australes archipelago, French Polynesia; Chinain et al., 2010; Chinain et al., 1999). Cultures were established in Fernbach flasks containing 1.25 L of f10k enriched natural seawater medium, inoculated at an initial cell density of 250-370 cells mL^{-1}, and grown at 26 ± 1 °C under 100 µmol photons m^{-2} s^{-1} (daylight fluorescent tubes) in a 12:12 h (light : dark) photoperiod with permanent aeration (200 L hr^{-1}). Cultures were harvested in their late exponential/early stationary growth phase and cells kept intact (as confirmed by light microscopy) at -20 °C until use for fish food preparation and toxin analysis.

119

120 *Naso brevirostris* (Acanthuridae) are herbivorous and planktivorous species (trophic level 2.2-2.79;
121 Algaebase) inhabiting coral reefs throughout the Indo-Pacific. They eat primarily benthic algae, are
122 commonly implicated in CP, and CTXs have been detected in their flesh (Gaboriau et al., 2014; Mak et
123 al., 2013). Given its ecological relevance for CTX studies, availability from a supplier, and ability to be
124 kept in aquaria, *N. brevirostris* provides a relevant model for examining the processes of CTX entry into
125 the coral reef fish food web. Wild-caught juvenile *N. brevirostris* were obtained from a fish wholesaler
126 (Tropic Nguyen, France). Fish were caught with small nets directly on the reef in the Maldives and
127 acclimatized at least 15 days to aquarium conditions (seawater 25 ± 0.5 °C and 38 psu) before shipment
128 (information provided by the supplier).

129

130 **2.2. Experimental Model**

131 To examine the processes of CTX trophic transfer from the microalgal producer into the fish food web,
132 we developed an experimental laboratory model of long-term CTX dietary exposure in fish under well-
133 defined conditions that consisted of feeding juvenile *N. brevirostris* with intact *G. polynesiensis*
134 embedded in a gelatin-based food. Toxin accumulation and resulting tissue burdens over time were
135 determined by measuring CTX levels in the flesh of fish after 2, 4, 8 and 16 weeks exposure. Although
136 species of *Gambierdiscus* may also produce maitotoxins (MTXs), evidence that these toxins can
137 accumulate in fish flesh is mixed (Kohli et al., 2014; Lewis, 2006a), and they have never been implicated
138 in human CP (Lewis, 2006b; Lewis and Holmes, 1993); thus, MTXs were not analysed in this study.

139

140 ***Fish maintenance***

141 Prior to experimentation, fish were acclimated to laboratory conditions in an open-circuit 2000-L tank
142 (150 L h^{-1} of 0.45 µm filtered seawater maintained at 25 ± 0.5 °C and 38 psu) with a 12 h light: 12 h dark
143 cycle. Fish were given a constant regimen of 10% body weight d^{-1} that initially consisted of lettuce and

144 brine shrimp (*Artemia salina*, as a nutritional supplement) and gradually incorporated the gel food (Gelly
145 Belly™ Gel Food, Florida Aqua Farms, Inc., USA, preparation detailed below). Gelly Belly™ Gel Food
146 consists of a blend (in order of proportion) of microalgae, macroalgae, fish and krill meal, and vitamins
147 and minerals mixed with gelatin. PVC tubing provided structure to minimize stress (three 10 cm x 20 cm
148 DxL tubes affixed in a pyramidal structure). After 2 months, the fish were consuming the gel food (6%
149 body weight d^{-1}) in less than 30 minutes.

150 Standards of animal welfare were carefully maintained throughout the experiment, and care was taken to
151 minimize handling to avoid physiological stress. Fish were euthanized by rapid chilling (Matthews and
152 Varga, 2012; Wilson et al., 2009).

153

154 ***Gel food preparation***

155 Gel food was prepared by mixing the Gelly Belly™ dry powder with hot seawater (~70–80 °C) at a ratio
156 of approximately 2:3 g powder to mL seawater. The resulting paste was then spread onto nylon screen (2
157 mm mesh size) that served as a feeding support and was left to solidify at -18 °C for at least 10 min
158 (Figure 1). Cell-enriched gel food was prepared by adding hot seawater to a tube of frozen *G.*
159 *polynesiensis* cells, vortexing to homogenize, and combining with the dry powder in plastic bags at the
160 same water:powder ratio as above. The seawater temperature had no obvious effect on the integrity of the
161 cells (microscopic observation, results not shown). The food was then mixed manually to ensure complete
162 homogenization. The final concentration of *G. polynesiensis* in the food was 1,333 cells g^{-1} gel food.

163

164 The stability of the *Gambierdiscus*-enriched gel food in seawater was assessed by measuring potential
165 cell or CTX release from the food into the water over a period exceeding the time fish took to consume
166 the food. Aliquots of 3 g gel food containing 1,633 cells g^{-1} *G. polynesiensis* were immersed in 5 mL of
167 seawater for 0.5, 1, 1.5, 2 and 3 h (n = 3). As gel food contained a slightly higher quantity of cells than
168 that fed to fish (1333 cells g^{-1}), results provide a conservative estimate of potential toxin release. Seawater

169 replicates were examined with light microscopy for the presence of cells and extracted using a C-18 solid
170 phase extraction (SPE) column (following Chinain et al., 2010; Darius et al., 2007, with minor
171 modification) before CTX assessment by receptor binding assay (RBA). Briefly, samples were loaded
172 onto 0.5 g, 6 mL C18 SPE columns (Agilent Technologies, France) pre-conditioned with 2 mL methanol
173 (MeOH) followed by 5 mL Milli-Q. Samples were washed with 10 mL Milli-Q to remove salts and then
174 eluted with 4 mL 90% aqueous (aq) MeOH. The column wash with 70% aq MeOH, which is used to
175 remove water soluble compounds such as MTXs, was omitted as MTXs do not cross react with CTXs.
176 Eluted samples were evaporated under nitrogen gas, resuspended in 100% MeOH, and stored at -18 °C
177 until CTX analysis with the RBA (Section 2.3).

178

179 ***Fish exposure***

180 After acclimation, juvenile *N. brevirostris* (ranging 3.05–11.6 g and 4.5–9.5 cm in length) were sorted
181 into size classes from which they were randomly distributed among five aquaria to achieve uniform size
182 distribution across tanks (n = 5; 33.2 ± 0.14 g mean total biomass ± SE per tank). Aquaria were 100-L
183 with aerated, flow-through seawater (100 L h^{-1}, all other conditions as above). To keep stress at a
184 minimum, aquaria walls were covered with an opaque plastic coating, and PVC tubes provided habitat. At
185 the time of distribution, three additional fish were euthanized, and tissue samples were collected as initial
186 controls for CTX exposure (methods described at the end of this section).

187

188 Each aquarium was randomly assigned a time point (2, 4, 8 or 16 weeks exposure or the exposure control)
189 at which to sacrifice all five fish in the aquarium (Table 1). Control fish were maintained throughout the
190 16-week duration and served both as behavioural controls for symptoms of intoxication and as analytical
191 controls. Exposed fish were given gel food embedded with *G. polynesiensis* cells at a dose of 89 cells g^{-1}
192 body weight daily, and control fish received gel food without cells. The cell concentration in the gel food
193 was chosen based on field data of natural *Gambierdiscus* spp. bloom densities (Chateau-Degat et al.,
194 2005; Darius et al., 2007) and the quantity of food required by juvenile fish (5-10% body weight d^{-1}).

195

196 Fish in all aquaria were fed five days per week at 09:00 at a constant proportion of 6% of their biomass.
197 For the 8 and 16 week treatments, 3 and 4 feedings were missed due to laboratory closure, respectively,
198 resulting in 36 and 76 feedings instead of 40 and 80 (Table 1). Food quantities were adjusted every two to
199 four weeks based on estimated increases in biomass from a growth curve created by linear regression of
200 sacrificed fish weights at each time point (see results). Fish within a tank were fed together and observed
201 during the feeding period to confirm that each fish ate and that all food was consumed within a maximum
202 period of time (<30 min). As no hierarchical behaviour (e.g. dominance or territoriality around the food)
203 was observed prior to experimentation, the fish were assumed to eat a proportion of the food according to
204 their relative size. All fish were given thawed brine shrimp in the afternoon as a food complement to
205 achieve a daily ration of 10% body weight d^{-1}.

206

207 At each time point, the individuals from the respective aquarium were collected and euthanized 8 hrs
208 post-feeding. Death was confirmed by the absence of respiratory movement. Fish were dissected
209 immediately to ensure the tissue integrity. The entirety of muscle tissue was collected, wet-weighed, and
210 stored at -18 °C until toxin analysis. Control fish were sacrificed simultaneously with the 16-week
211 treatment and were processed in an identical fashion.

212

213 **2.3 Toxin determination**

214 ***Sample Extraction***

215 Cells of *G. polynesiensis* were extracted following Chinain et al. (1999a) with modification. Briefly,
216 ~50,000 cells were extracted with 2 rounds each of 5 mL 100% MeOH and 50% aq MeOH interspersed
217 with cell lysis by probe sonication in an ice bath (20 min initially; 2 min subsequently; Branson digital
218 sonifier) and centrifugation (5 min at 2000 rpm). Supernatants from each step were combined, adjusted to
219 60% aq MeOH with Milli-Q water, and CTXs were isolated by 1:1 solvent-solvent separation with

220 dichloromethane (DCM) in which the organic phase containing CTX was collected and evaporated to
221 dryness under a stream of nitrogen.

222

223 The muscle tissue of each fish (ranging 0.87 – 12.87 g across time-points; 4.81 ± 0.65 mean ± SEM) was
224 extracted as previously described (Dechraoui et al., 2005; Ledreux et al., 2014; Yogi et al., 2011) with
225 minor modifications. Tissues were cooked in Falcon tubes in a water bath at 70 °C for 15 min,
226 homogenized with a T-25 digital Ultra-Turrax (IKA Works, Germany) and extracted three times in
227 acetone (3 mL:1 g sample) with probe sonication (2 min) and centrifugation. The combined supernatants
228 were evaporated under a stream of nitrogen. Dried extracts were resuspended in 90% aq MeOH (5 mL)
229 and lipids were removed by three rounds of solvent-solvent separation with an equal volume n-hexane. A
230 final 1:1 solvent-solvent separation (3x) between the aq MeOH phase (diluted to 60% with Milli-Q) and
231 DCM isolated the organic phase containing CTXs. The organic phase was then evaporated under a stream
232 of nitrogen, resuspended in 100% MeOH to 10 g tissue equivalent (TE) mL^{-1}, and stored at -18 °C until
233 toxin analysis. The gel food preparation was extracted following the same procedure as that for fish
234 tissues with 20 min initial probe sonication to ensure cell lysis.

235

236 ***Toxin analysis***

237 Composite CTX concentrations in *G. polynesiensis* cells and fish muscle extracts were quantified using a
238 radioligand receptor binding assay (RBA) (Dechraoui Bottein and Clausing, 2017). This method is based
239 on competitive displacement of tritiated brevetoxin ([^{3}H]PbTx-3) by the toxin standard (or CTX present
240 in the sample extract) at their shared receptor on the voltage gated sodium channel (Dechraoui et al.,
241 1999; Poli et al., 1986). The assay was carried out in a microplate format (IAEA-TECDOC-1729, 2013,
242 with some modification) using a phosphate buffered saline buffer (PBST-Tween®) with bovine serum
243 albumin (1mg mL^{-1}), a [^{3}H]PbTx-3 working solution (1 nM assay concentration), and a dilute membrane
244 preparation of porcine brain homogenate (0.8 mg protein mL^{-1}). After incubation for 1 h at 4 °C and

245 filtration with rinsing, the plate was incubated for 2 hr in the dark with liquid scintillant (Optiphase,
246 PerkinElmer) before quantification of beta emissions in a beta counter (MicroBeta Plate Counter,
247 PerkinElmer). Toxin levels in samples (dilutions from 0.1–0.6 g TE mL^{-1} in assay, in triplicate) were
248 estimated against standard curves of CTX3C (Wako-Pure Chemicals, Osaka, Japan) and PbTx-3
249 (Latoxan, Rosam, France) and expressed as CTX3C equivalents. All samples were analysed in triplicate
250 in 2 to 4 independent RBA assays. A PbTx-3 QC check was run in triplicate on every assay to assess
251 inter-assay variability, requiring that the QC fall within 30% of the stated value for assay acceptance.
252 Sample quantification was only completed on dilutions that fell on the linear portion of the curve and for
253 which the RSD of the triplicate CPM values was less than 30%. This RSD requirement also assessed
254 intra-assay variability. The QC together with additional parameters of the standard curve including the
255 EC50, slope, and 100% binding (top of the curve) were systematically compared among trials to
256 determine assay performance and acceptability.

257

258 Sample toxin concentrations were evaluated against 4 parameter-sigmoidal standard curves fit using
259 GraphPad Prism version 6.0 (San Diego, USA). Differences between the control and all exposure
260 treatments were tested using bootstrapped multiple comparisons with a Bonferroni-corrected p-value (α of
261 0.05/10 = 0.005) as the assumptions of factorial ANOVA were not met. Muscle toxin burden, the total
262 quantity of toxin contained in the organ, was calculated based on the muscle concentration and total
263 weight of the tissue collected. Regression analysis (x,y intercept set to zero) was used to assess
264 accumulation over time. All statistical analyses were performed using R software 3.0.1 (R Core Team,
265 2014).

266

267 The presence of ciguatoxin activity in the *Gambierdiscus* extract and selected fish muscle extracts was
268 also analysed using a cell based assay, the Neuro2a cytotoxicity assay (N2a-CBA) (Manger et al., 1993).
269 This assay also provides a sensitive measure of overall toxicity based on CTX properties that activate the

270 voltage-gated sodium channel (Dechraoui et al., 2005) and was employed as a secondary detection
271 method following the protocol described in Roué et al. (2016).

272

273 The gel food preparation was analysed by LC-MS/MS (method adapted from Lewis et al., 2009) using an
274 LC system (UFLC XR Nexera, Shimadzu, Japan) coupled to a hybrid triple quadrupole-linear ion trap
275 mass spectrometer (API-4000Qtrap, Sciex, CA, USA) equipped with a turboV® ion spray interface. LC-
276 MS/MS analysis was used to confirm the integrity of the CTX profile in the food as compared with the
277 cells used for its preparation. A 1.8 µm C18 Zorbax Eclipse plus column (50·2.1 mm, Agilent
278 technologies, CA, USA) was employed at 40 °C and eluted at 400 µl min^{-1} using a linear gradient of
279 eluent A (water) and eluent B (MeOH). Both eluents contained 2 mM ammonium formiate and 50 mM
280 formic acid. The elution gradient ran from 78 to 88% B over 10 min and was held for 4 min before re-
281 equilibration during 5 min. Mass spectrometry detection was operated in positive mode using Multiple
282 Reaction Monitoring (MRM). The pseudomolecular ions $[M+NH_4]^+$ and $[M+H]^+$ were selected as
283 precursor ions. The ions resulting in the successive losses of NH_4 and/or water molecules were selected as
284 product ions. Data quantification was performed from a linear calibration curve of CTX3C standard
285 (Wako-Pure Chemicals, Osaka, Japan) using Analyst software (Sciex, CA, USA). The chromatogram
286 profile was completed by injecting a mix of standards (CTX1B, 49-epiCTX3C [P-CTX3B] and CTX3C,
287 all three provided by the Institut Louis Malardé) in the sequence.

288

289 **3. RESULTS AND DISCUSSION**

290 **3.1 Rates and relevancy of experimental CTX exposure**

291 Toxin contents in *G. polynesiensis* cell extracts were estimated against CTX3C standard curves in four
292 independent assays and expressed as CTX3C equivalents (equiv). In each assay, dilutions of cell extract
293 at 7 concentrations ranging from 2 to 2100 cell mL^{-1} produced a full sigmoidal dose-response curve
294 (Figure 2A.; mean IC_{50} = 148.4 cells mL^{-1}). Using the IC_{50} (inhibitory concentration at 50%) from the

295 standard curve and that from the cell extract curve in each assay, the average toxin content per cell was
296 estimated to be 4.5 pg CTX3C equiv $cell^{-1}$. Subsequent analysis with the N2a CBA confirmed cell toxin
297 concentrations (3.6 pg CTX3C equiv $cell^{-1}$).

298

299 LC-MS/MS analysis of an extract of *Gambierdiscus*-embedded gel food (at 1333 cells g^{-1}, as prepared for
300 the dietary exposure) confirmed toxin levels and toxin stability during the preparation process (3.3 pg
301 CTX3C equiv. $cell^{-1}$). The dominant CTX analogue extracted was CTX3C, followed by 49-*epi*CTX3C
302 (Figure 2B), which matches the CTX profile previously determined for the TB92 strain of *G.*
303 *polynesiensis* (Chinain et al., 2010).

304

305 The gel food stability test showed no release of toxin into the surrounding seawater during 3 h immersion;
306 at all time points, CTX concentrations in the seawater extracts were found to be below the limit of
307 detection of the RBA (LOQ: 1.46 10^{-10} g CTX3C equiv mL^{-1} seawater, equivalent to 2% of the total toxin
308 added). Furthermore, light microscopy revealed no visible cells in the seawater. By comparison, the gel
309 food given to exposed fish contained lower cell concentrations (1,333 cells g^{-1} gel food vs 1,633 cells g^{-1}
310 in the stability assay), was immersed in a much larger volume of seawater (100 L tanks compared to 5 mL
311 in the assay), and was consumed in less than 30 min. Thus the food stability results indicate that any toxin
312 potentially released into the water of the aquaria can be considered negligible. Moreover, the fish were
313 maintained in open seawater circulation; thus, any toxin released from the food or excreted out of the
314 body during the experiment would be rapidly flushed, ensuring that fish exposure to CTX was limited to
315 the dietary pathway, not via the respiratory pathway.

316

317 Given the cell content in the food (1,333 cells *G. polynesiensis* g^{-1}) and the ration provided (0.06 g food g
318 $fish^{-1}$ d^{-1}), the fish received an estimated daily dose of 0.4 ng CTX3C equiv g $fish^{-1}$. At the onset of the
319 experiment, where the average body weight per fish across all treatments was 6.6 ± 0.4 g SEM (n = 25),

320 this corresponded to an average quantity of ~2.7 ng CTX3C equiv fish^{-1} d^{-1}, although actual quantities
321 consumed were assumed to vary with differences in size among individuals within a tank. The quantity of
322 cells that we chose for fish exposure reflects cell densities to which fish are likely exposed when feeding
323 on macroalgal substrate throughout the year. Previous field surveys have shown that bloom densities
324 commonly reach 5,000 cells g^{-1} macroalgae in French Polynesia (Chinain et al., 1999b) or 20,000-50,000
325 cells g^{-1} in the Caribbean (Cruz-Rivera and Villareal, 2006), and are frequently maintained at densities
326 greater than 1,000 cells g^{-1} throughout the year (e.g. Chateau-Degat et al., 2005; Chinain et al., 1999b).
327 Occasional cell densities as high as 1,000,000 cells g^{-1} have been reported in both the Atlantic and Pacific
328 (Litaker et al., 2010). The dietary intake of CTX in the field, however, depends on individual
329 *Gambierdiscus* species' toxicity, which varies widely both within (Chinain et al., 1999; Lewis et al.,
330 2016; Pisapia et al., 2017) and among (Dai et al., 2017; Litaker et al., 2010) ocean basins (ranging from
331 non-toxic or fg cell^{-1} up to 1.5 pg CTX3C equiv cell^{-1} for the Atlantic species *G. excentricus*: Pisapia et
332 al., 2017 and 11.9 pg CTX3C equiv cell^{-1} for the Pacific *G. polynesiensis*: Chinain et al., 2010).

333

334 **3.2 Fish behaviour**

335 Throughout the experiment, both control and dosed fish fully and rapidly (generally less than 15 minutes)
336 consumed the gel food. Over 16 weeks, fish biomass increased by roughly 400% in both exposed (133 g
337 total) and control treatments (128 g total; 4% difference), indicating a robust experimental feeding
338 procedure with no bias in nutrition with *Gambierdiscus* cell exposure. The estimated growth curve was
339 based on the initial weight of all fish and the subsequent weights of fish at the time of sacrifice (2, 4, 8 or
340 16 weeks, n = 5 at each time point). Fish gained biomass linearly (linear regression: $R^2 = 0.84$; Figure 3),
341 with a slope of 1.26 indicating that, across all tanks, each fish gained approximately 1.3 g wk^{-1} (6.3 g
342 biomass increase tank^{-1} week^{-1}).

343

344 Exposed fish exhibited no abnormal behaviour during the experiment, including signs of intoxication
345 such as rapid gill movement or erratic swimming or fin movement. Moreover, fish showed no signs of

346 stress during experimentation, either in general or in comparison with control fish. There was, however,
347 one mortality apparently unrelated to exposure (the one fish was found outside of the tank; 8 weeks
348 treatment, n = 4).

349

350 The absence of intoxication in the exposed fish indicated that the given dose was relevant to naturally
351 occurring exposure on the reef. The absence of signs of intoxication in wild fish found with high CTX
352 concentrations (e.g. from human intoxication events or from field surveys) has long confounded
353 understanding of how this potent toxin can accumulate to the high levels at which it is naturally found
354 (Lehane and Lewis, 2000). This is particularly perplexing as the toxin has been shown to bind to fish
355 brain, muscle and heart Na_v in laboratory tests (Dechraoui Bottein et al., 2006), cause fish mortality
356 through water exposure (Lewis, 1992), and result in developmental toxicity in finfish (Colman et al.,
357 2004; Yan et al., 2017). Our results provide tentative support of the hypothesis of Banner et al. (1963)
358 that naturally exposed species have developed some form of resistance, whether by physiological
359 mechanisms or genetic adaptations, such as the production of CTX storage proteins that render the toxin
360 non-bioavailable (e.g. skeletal muscle soluble proteins Hahn et al., 1992). However, early experiments
361 feeding ciguatoxic fish flesh to species potentially encountering CTX in the wild (grouper, snapper and
362 wrasse) did result in behavioural abnormalities, although the CTX dose administered was not
363 known/reported (Davin Jr. et al., 1986; Davin Jr et al., 1988). More recently, Ledreux et al. (2014) also
364 found that a mullet (*Mugil cephalus*) showed extreme signs of intoxication after exposure to similar CTX
365 doses as in this study (0.3 vs 0.4 ng g^{-1} fish) using the same strain of *G. polynesiensis* (TB92). However,
366 the concentration of cells in the food was nearly 10-fold higher (13,000 vs 1,333 cells g^{-1} food), and the
367 species of mullet used were native to streams in the Southeast of the USA where they are unlikely to be
368 exposed to CTX. Thus, toxic response to ingestion of CTXs may vary among fish; it remains unclear if
369 fish intoxication is a function of adaptations for resistance or of prey type or feed concentration, or a
370 combination of these factors.

371

372 **3.3 Toxin levels in muscle tissue**

373 Analysis of fish muscle tissue by RBA revealed that fish had accumulated significant quantities of CTXs
374 within 2 weeks (1.2 ng CTX3C equiv g^{-1} TE in 10 feedings; Figure 4A; Table 2) at values above the US-
375 FDA recommended action level of 0.01 ng g^{-1} CTX1B equiv [corresponding to a 10-fold safety margin
376 on the risk value of 0.1 ng g^{-1} proposed by Lewis (1991) after analysis by mouse bioassay of fish
377 remnants involved in ciguatera cases]. With a toxic equivalency factor (TEF) of 0.2 for CTX3C (EFSA
378 Panel on Contaminants in the Food Chain, 2010), this latter value would correspond to 0.5 ng CTX3C
379 equiv g^{-1}. After 10 additional doses of *Gambierdiscus* enriched food (4 weeks treatment), toxin
380 concentrations remained similar, but by 8 weeks (37 feedings), muscle toxin concentrations had increased
381 ~300%, where they remained through 16 weeks of exposure (Table 2). The presence of CTXs in the
382 muscle of exposed fish was confirmed by N2a CBA; however, definite quantification was limited by
383 sample availability (results not shown). No detectable amounts of toxin were found in either initial or
384 experimental controls. Overall, our results are consistent with field data indicating that CTX appears in
385 herbivorous fish less than one month after high *Gambierdiscus* densities appear on the reef (Clausing and
386 Dechraoui Bottein, 2016). Moreover, they are also in accordance with CTX concentrations found in wild-
387 caught *N. brevirostris* (1–7 ng CTX3C equiv g^{-1} flesh (Gaboriau et al., 2014), and, taken with the lack of
388 observed signs of intoxication, confirm that *N. brevirostris* is an appropriate experimental model.

389

390 As fish continued to grow throughout the duration of the experiment (~1.3 g wk^{-1}), the quantity of food
391 given was adjusted every 2 to 4 weeks to remain at a roughly constant dose by biomass. An examination
392 of toxin tissue burden in muscle revealed a linear increase with toxin intake (i.e. dose; linear regression, p
393 < 0.0001: Figure 4B; Table 2), where the rate of dietary assimilation (percent ingested toxin retained in
394 the tissue) was relatively constant at ~2%. Consequently, the rate of accumulation (~1.4 ng wk^{-1}) also
395 stayed positive and constant. However, the increase in muscle mass was not constant as the fish grew,
396 causing somatic growth dilution that masked increasing toxin tissue content and resulted in apparent
397 saturation in muscle toxin concentrations after 8 weeks (Figure 4A). Indeed, in fish sacrificed at 2 weeks,

398 muscle tissue contributed 19.8 ± 1.2 % (mean ± SEM) of the total body weight (7.9 ± 0.65 g per
399 individual). At 4 weeks, muscle tissue contribution to total body weight was still at 20.6 +/- 1.56 % but by
400 8 weeks had slightly increased to 23.9 +/- 0.70%. After 16 weeks, in contrast, muscle was 32.3 ± 4.0 % of
401 total body weight (26.7 ± 2.5 g per individual). These data suggest that fish that grow large faster may
402 have lower CTX concentrations than slow-growing fish due to somatic growth dilution (Yang et al.,
403 2016). Bio-dilution of chemical toxins (e.g. organic chemicals, mercury) is well-established in fish
404 species (Clark et al., 1990; Ward et al., 2010) and has been suggested for brevetoxins in turtles (Perrault
405 et al., 2014). Yet, fisheries regulations in regions with endemic ciguatera are often based on size (e.g.
406 Chan, 2015a; Clua et al., 2011; reviewed by Yang et al., 2016) where larger fish of the same species are
407 assumed to contain higher CTX concentrations in the flesh (Chan, 2015b; Lehane and Lewis, 2000;
408 Lewis and Holmes, 1993; Oshiro et al., 2010). Our results indicate that size alone may not be a reliable
409 indicator of ciguatoxicity. In fact, growth dilution may provide a mechanism for the contradictory
410 findings on the relationship between size and CTX concentrations in field sampled fish (Darius et al.,
411 2007; Dechraoui et al., 2005; Gaboriau et al., 2014; Mak et al., 2013; O'Toole et al., 2012), particularly as
412 growth rates are known to vary among coral reef fish families (0.2–1.0 cm yr^{-1}; McClanahan, 2015).
413 However, as fish grow more slowly after reaching maturity or during reproductive stage (Hutchings,
414 1993; Lester et al., 2004), very old fish are likely to be high risk if they are sessile in endemic areas.

415

416 The final concentration of toxins accumulated in the flesh of fish depends not only on dietary intake and
417 growth, but also on variation in physiological processes among conspecifics that may influence CTX
418 toxicokinetics. In our experimental procedure, fish were maintained and fed in treatment groups; the
419 development of a similar, but individual-based approach in which each fish is fed separately and
420 reweighed may provide a means to determine potential intraspecific variability (although the destructive
421 sampling used for tissue toxin detection precludes successive toxin measurements in individual fish). In
422 nature, both dietary exposure and fish physiology will vary significantly due to irregularity in
423 *Gambierdiscus* distribution, bloom intensity and toxicity (Chinain et al., 1999; Lewis et al., 2016; Pisapia

et al., 2017), as well as species interactions and environmental conditions. Given these known sources of variability, this laboratory procedure could be used to test scenarios and parameterize models for identified ciguatera-prone areas in which the habitat characteristics, fish populations, and *Gambierdiscus* bloom dynamics are well-known and actively monitored.

In our study, as the dose of toxin consumed and the growth rates were both known and constant over time, the roughly constant rate of accumulation found in the muscle (Figure 4B) relates to a balance between absorption and depuration efficiencies. The degree of metabolization of CTXs as they pass through digestion (Lewis et al., 1991; Murata et al., 1990) is a key factor affecting their storage in and/or elimination from fish (Lewis and Holmes, 1993). In general, the quantity of oxidized forms of CTXs increase with metabolization and thus with trophic level (Lewis, 2000; Lewis and Holmes, 1993), with an accompanying increase in potency associated with greater polarity (Dechraoui et al., 1999; Yogi et al., 2011). It has been suggested that increasing polarity with transformation in fish may be a mechanism to facilitate depuration (Lewis and Sellin, 1992). The *Gambierdiscus* species used in our study, *G. polynesiensis*, has been shown to contain primarily CTX3C (40–50%), followed by 49-*epi*CTX3C and CTX4A, and, in small quantities, CTX4B (Chinain et al., 2010). Herbivorous fish in the Pacific, however, and specifically acanthurids, may also contain more metabolized and thus more potent analogues. These CTXs include significant quantities of CTX4B (Satake et al., 1996) and lesser amounts of CTX1B, the biotransformation end-product, as well as 52-*epi*-54-*deoxy*CTX1B (P-CTX2) and 54-*deoxy*CTX1B (P-CTX3), both biotransformation intermediates (Mak et al., 2013). In herbivorous fish with acidic digestive tracts, for example, acid-catalysed spiro-isomerisation may affect the production of polar forms of CTX (Lewis and Holmes, 1993; Murata et al., 1990) and thus may affect rates of detoxification and depuration.

Overall, these processes of metabolization and depuration remain to be determined in herbivorous fish. The procedure presented here could be adapted to follow the tissue distribution of CTX among body compartments within an individual and compared among fish after varying periods of exposure and

450 depuration. Moreover, our model of CTX trophic transfer in herbivorous fish is consistent with what we
451 understand of this process in the wild; the natural means of dietary exposure to relevant doses in
452 appropriate model species, together with the resulting general health and lack of behavioural signs of
453 intoxication as well as the timeline of toxin accumulation set this experimental method apart from past
454 laboratory exposure studies (Davin Jr. et al., 1986; Davin Jr et al., 1988; Helfrich and Banner, 1963;
455 Ledreux et al., 2014). Thus, the model developed here can be useful to advance understanding of
456 ciguatoxin kinetics both within individuals and among trophic levels and can support development of
457 predictive models of risk assessment for well-characterized and regularly monitored reef systems.

458

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471

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696 **TABLES AND FIGURES**

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699 **Table 1.** Summary of experimental design

Treatment	Cumulative number of feedings with cells[a]	N	Fish collection
Control	0	3	Initial control, sacrificed 8 h post last feeding
2 weeks	10	5	Dosed fish, sacrifice 8 h post last-exposure to *G. polynesiensis*
4 weeks	20	5	
8 weeks	37[b]	5	
16 weeks	76[b]	5	
Control (16 weeks)	0	5	Experimental and terminal control, sacrificed 8 h post last feeding

700 [a]Daily feeding for exposed fish consisted of 89 cells g^{-1} corresponding to 0.4 µg CTX3C equiv kg^{-1} fish

701 [b]Treatments of 8 and 16 weeks had 3 and 4 fewer dosings than expected (40 and 80, respectively) due to
702 lab closures.

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711 **Table 2:** Comparison of the quantity of ciguatoxin (CTX) ingested by the fish and that detected in the

712 muscles

Week of experiment[a]	Cumulative number of toxic feedings	Mean total quantity of toxin ingested[b] (ng[c] $fish^{-1}$)	Mean muscle toxin concentration[d] (µg[c] kg^{-1})	Mean muscle toxin burden[e] (ng[c])	% toxins ingested accumulated in muscle[f]
2	10	71.6	1.19 ± 0.22	2.00 ± 0.43	2.5 ± 0.45
4	20	171.9	1.30 ± 0.36	3.53 ± 1.43	1.8 ± 0.68
8	37	391.9	3.24 ± 0.59	10.62 ± 3.03	2.6 ± 0.50
16	76	1268.5	2.81 ± 0.57	23.03 ± 3.86	1.9 ± 0.40

713 [a] Control fish are not included as muscle concentrations were below the limits of detection

714 [b] calculated as the total sum added to the tank divided by the number of fish in the tank (n = 5 in all tanks;
715 not weight adjusted)

716 [c] values are expressed in terms of CTX3C equiv

717 [d] determined by receptor binding assay (mean of three independent assays) and averaged within tank

718 [e] Muscle toxin burden is the CTX concentration multiplied by the weight of muscle tissue collected

719 [f] Percent ingested toxins retained were calculated per fish using weighted doses (average dose per tank
720 standardized by the proportion each fish contributed to total tank biomass) and individual muscle toxin
721 burden

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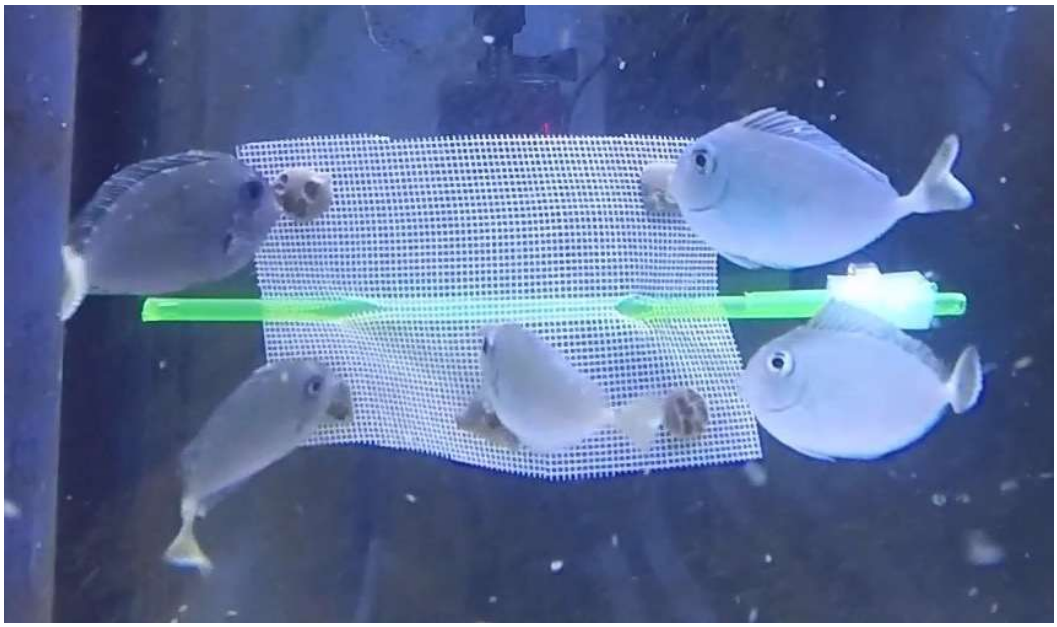

Figure 1. Experimental model for exposure to toxic microalgal cells: herbivorous fish graze on a gelatin-based food containing *Gambierdiscus* cells that has been solidified on a nylon mesh and attached to the aquarium wall with a plastic rod.

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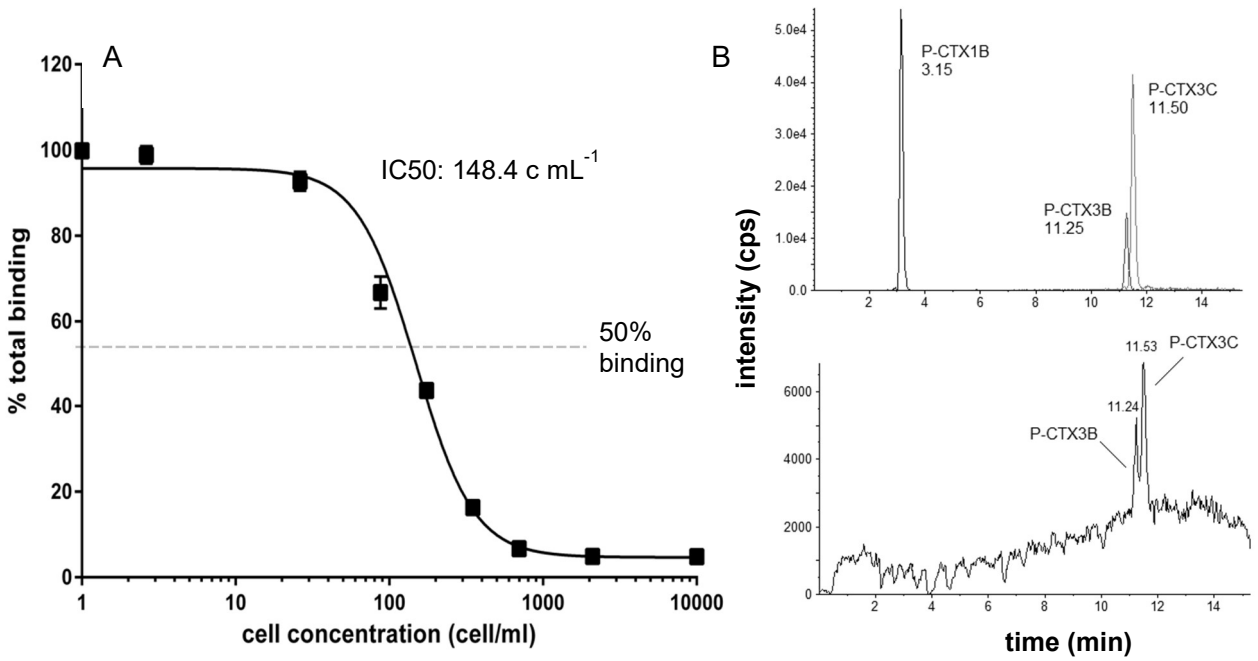

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734 **Figure 2.** Quantification of CTXs in cells of *Gambierdiscus polynesiensis* (TB92) and in gel food

735 embedded with cells: A) curve depicting inhibition of binding of radiolabelled [3H]-PbTx-3 to the

736 receptor on the voltage gated sodium channel with increasing concentrations of cells. The IC_{50} is the

737 concentration of cells at which the binding of [3H]-PbTx-3 is at 50% of its maximum; B) chromatogram

738 of food preparation extract depicting peaks of 49-*epi*CTX3C (P-CTX3B) and CTX3C, with a reference

739 chromatogram for different CTX congeners above.

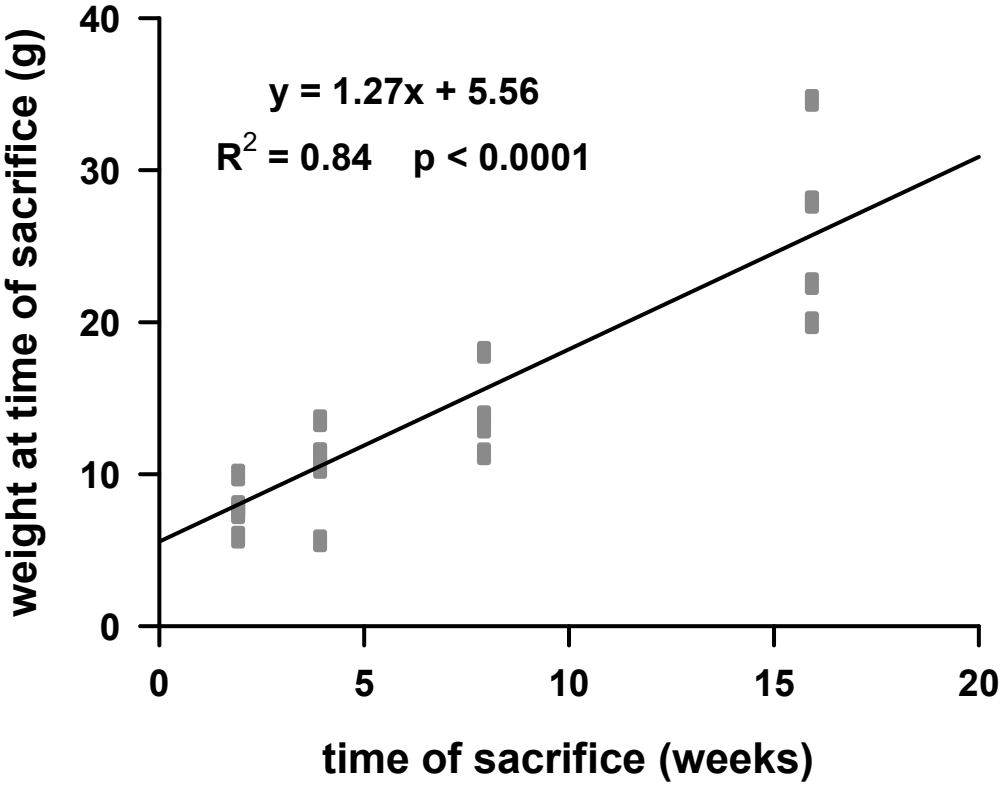
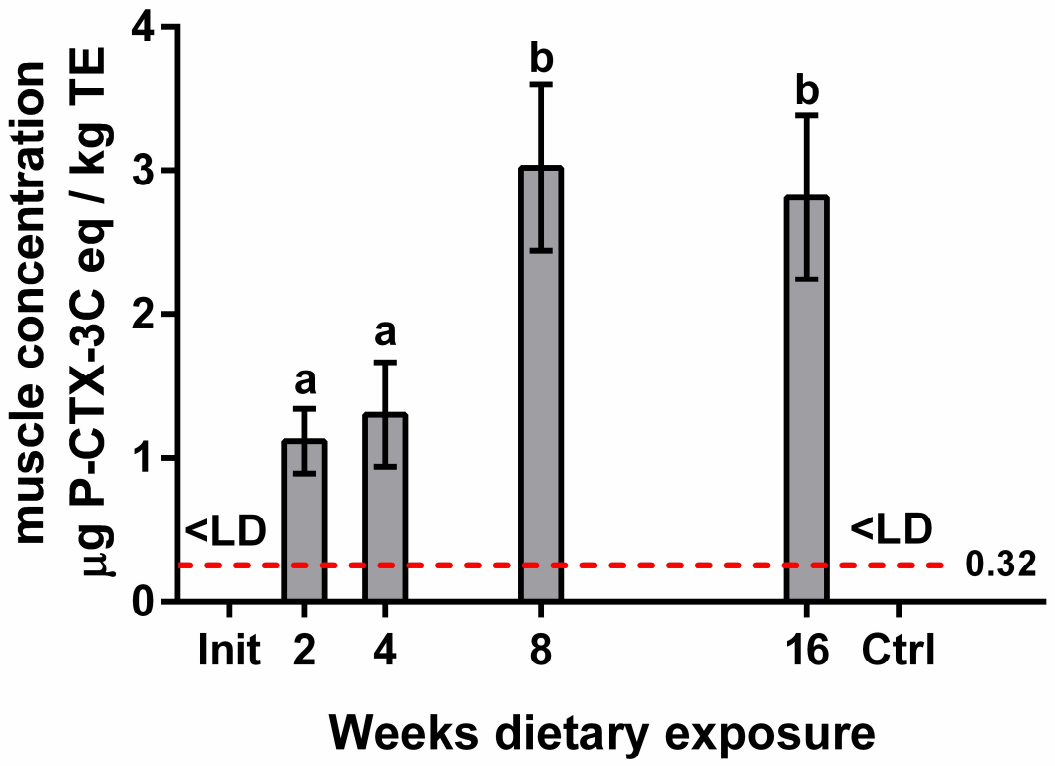


Figure 3. Growth curve of fish constructed from initial biomass (g) of all fish (n = 25) and fish weights at sacrifice for each time-point (n = 5). The slope represents the average growth rate for a fish per week in g.

744 **A**



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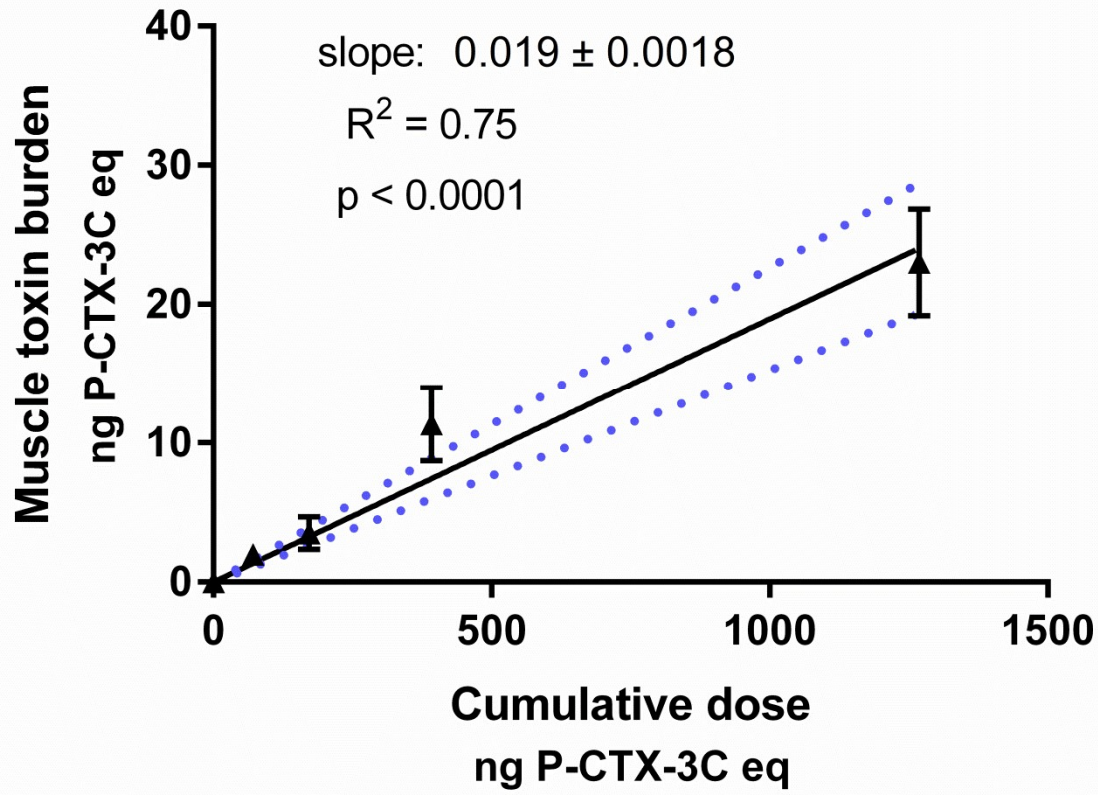
746 **B**



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748 **Figure 4.** Accumulation of CTX (CTX3C equiv) in fish muscle tissue throughout the experiment as
749 measured by RBA. A) CTX concentrations in mean µg CTX3C equiv kg^{-1} muscle ± SEM, at each time
750 point (init = initial control; 2, 4, 8 and 16 weeks exposure to *G. polynesiensis*; 16 week exposure control),
751 where concentrations in both controls were below limits of detection (0.32 µg CTX3C equiv kg^{-1}; red
752 dotted line). Differing letters indicate significant differences among treatments at $p = 0.008$ (Bonferroni
753 correction for multiple comparisons; B) CTX tissue burden (mean total ng CTX3C equiv contained in the
754 tissue ± SEM shown here) as a function of cumulative CTX dose (ng CTX3C equiv). Linear regression
755 was performed on all data and was significant at $p < 0.0001$. Blue dotted lines are 95% CIs of the slope.

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