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Warm temperature acclimation impacts metabolism of paralytic shellfish toxins from *Alexandrium minutum* in commercial oysters

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Abstract

Species of *Alexandrium* produce potent neurotoxins termed paralytic shellfish toxins and are expanding their ranges worldwide, concurrent with increases in sea surface temperature. The metabolism of molluscs is temperature dependent, and increases in ocean temperature may influence both the abundance and distribution of *Alexandrium* and the dynamics of toxin uptake and depuration in shellfish. Here, we conducted a large-scale study of the effect of temperature on the uptake and depuration of paralytic shellfish toxins in three commercial oysters (*Saccostrea glomerata* and diploid and triploid *Crassostrea gigas*, $n = 252$ per species/ploidy level). Oysters were acclimated to two constant temperatures, reflecting current and predicted climate scenarios (22 and 27 °C), and fed a diet including the paralytic shellfish toxin-producing species *Alexandrium minutum*. While the oysters fed on *A. minutum* in similar quantities, concentrations of the toxin analogue GTX1,4 were significantly lower in warm-acclimated *S. glomerata* and diploid *C. gigas* after 12 days. Following exposure to *A. minutum*, toxicity of triploid *C. gigas* was not affected by temperature. Generally, detoxification rates were reduced in warm-acclimated oysters. The routine metabolism of the oysters was not affected by the toxins, but a significant effect was found at a cellular level in diploid *C. gigas*. The increasing incidences of *Alexandrium* blooms worldwide are a challenge for shellfish food safety regulation. Our findings indicate that rising ocean temperatures may reduce paralytic shellfish toxin accumulation in two of the three oyster types; however, they may persist for longer periods in oyster tissue.

Keywords: accumulation, *Alexandrium*, bivalves, depuration, metabolic enzymes, metabolism, ocean temperature increases, paralytic shellfish toxins

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Introduction

Increases in ocean temperature have widespread effects on the distribution, abundance, physiology and interactions of marine species (IPCC, 2013; Vermeer & Rahmstorf, 2009; Poloczanska *et al.*, 2012; Fig 1a). In Australia, the increase in ocean temperature at mid-latitudes of up to 2.0 °C over the past 100 years (Thompson *et al.*, 2009; Ridgway & Hill, 2012) is significantly greater than the global mean and is related to a southern range extension of the East Australian Current. The majority of animals within the world's oceans are ectotherms that are influenced directly by increases in ambient temperature. Temperature increases lead to thermodynamic changes in physiological function, and the ability of organisms to cope with these changes will depend on the thermal sensitivity of thermal performance curves and their plasticity resulting from developmental and reversible acclimation, or genetic adaptation (Seebacher *et al.*, 2010; Wilson *et al.*, 2010; Hoffmann & Sgrò, 2011; Munday *et al.*, 2012; Seebacher & Franklin, 2012). For sessile intertidal organisms, acclimation is the most feasible response to rapid ocean warming (Harley, 2011).

Ocean temperature change can also indirectly impact marine invertebrates due to its effects on phytoplankton distribution and abundance (Hobday *et al.*, 2006; Halle-

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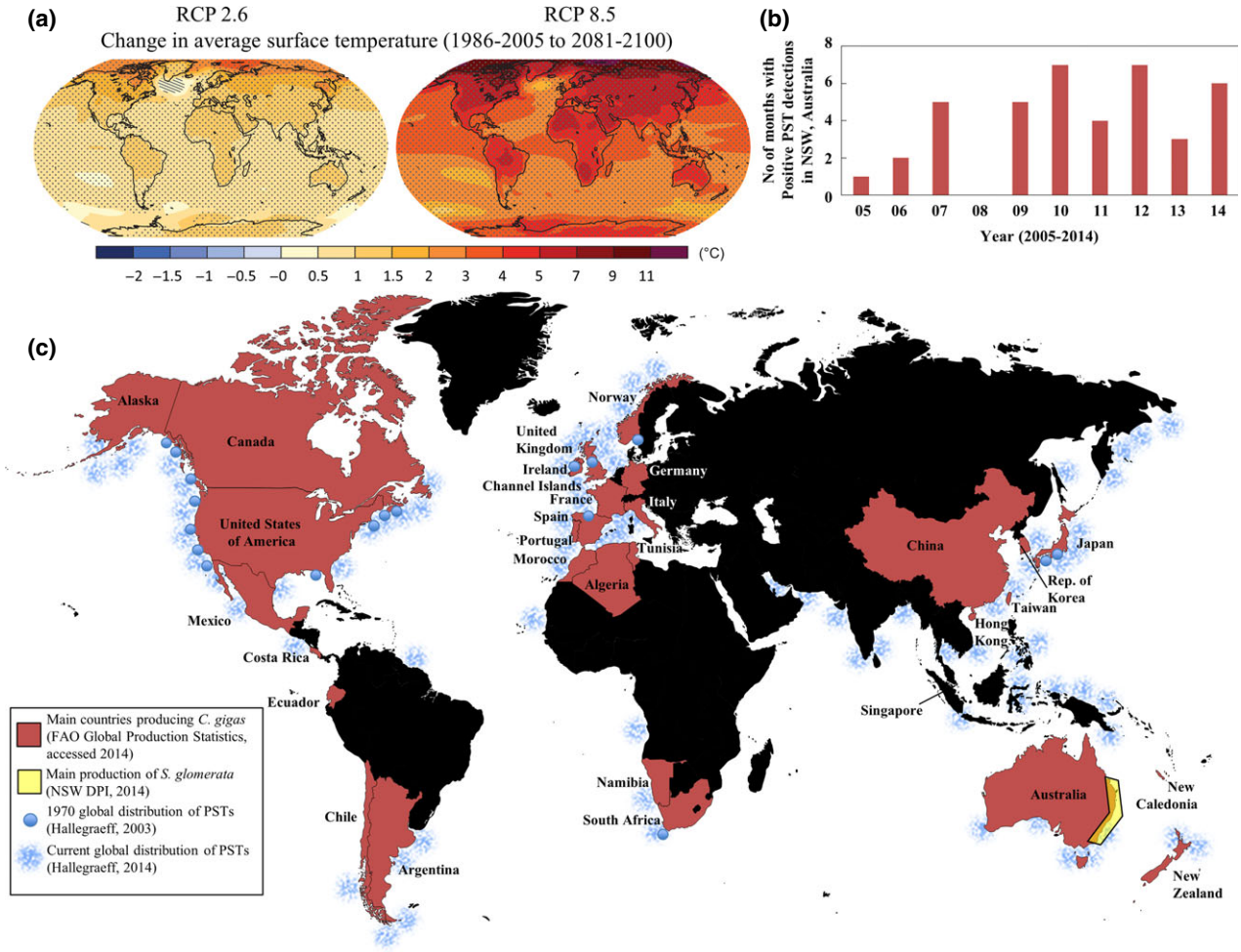

Fig. 1 (a) Estimated annual mean surface temperature change between 1986–2005 and 2081–2100 for two climatic model scenarios (RCP2.6: low forcing and RCP8.5: high emission levels) (adapted from IPCC, 2013). (b) Total number of months each year where positive *Alexandrium*-related PSTs were reported along the NSW coastline between February 2005 and December 2014 (NSW Food Authority Marine Biotoxin Monitoring data 2005 - 2014, unpublished data). (c) Global distribution of production of *C. gigas* (FAO, 2014) and *S. glomerata* (NSW DPI, 2014) overlain on reported incidence of PSTs worldwide 1970 (Hallegraeff, 2003) and current (Hallegraeff, 2014).

graeff, 2010; Thomas *et al.*, 2012), specifically, changes to the abundance and distribution of harmful algal bloom forming taxa (Glibert *et al.*, 2014). The increase in temperature in the East Australian current region is likely to cause an earlier timing of peak production and an increase in the seasonal window of species of *Alexandrium* and *Gymnodinium catenatum* (Hallegraeff, 2010), which produce paralytic shellfish toxins (PSTs). PSTs produced by species of *Alexandrium* include more than 20 known analogues, of which saxitoxin (STX), neosaxitoxin (NEO) and the gonyautoxins (GTX1, GTX2, GTX3, GTX4) are the most potent (Llewellyn *et al.*, 2006). PSTs have severe impacts on humans and a broad range of marine organisms, including mammals, birds, fish, molluscs and crustaceans, by selectively blocking voltage-gated Na+ channels in excitable cells, affecting neural impulse generation (Catterall, 1980). In the context of human well-being, negative effects of climate change on valuable food species are of particular concern. Global annual mollusc food production is approximately 2.1×10^7 *t*, of which oyster production comprises around 22% (FAO, 2014). Pacific oysters (*Crassostrea gigas*) are produced worldwide, and in Australia, the indigenous Sydney rock oyster, *Saccostrea glomerata*, is one of the main species produced (Fig. 1c). Since 2005, there has been an increase in blooms of *Alexandrium* in south-eastern Australian coastal waters (Farrell *et al.*, 2013; Murray *et al.*, 2011; Fig. 1b), which have resulted in over 50% of algal-related shellfish harvest area closures.

The thermal dependence of bivalve physiological responses to *Alexandrium*, and the way in which this impacts the total PST concentrations in species, has never been assessed. This information is crucial given

the current and predicted rates of ocean temperature increases. Based on experimental feeding studies, variation between bivalve species in the rate of uptake of PSTs, and the total uptake and depuration of PSTs, has been found (Bricelj *et al*., 1990; Sekiguchi *et al*., 2001; Chen & Chou, 2002; Blanco *et al*., 2003; Lassus *et al*., 2005; Li *et al*., 2005; Kwong *et al*., 2006; Asakawa *et al*., 2006; Hégaret *et al*., 2007; Lassus *et al*., 2007; Galimany *et al*., 2008; Murray *et al*., 2009; Haberkorn *et al*., 2011; Contreras *et al*., 2012; Fernández-Reiriz *et al*., 2013; Bricelj *et al*., 2014; Haberkorn *et al*., 2014). This was hypothesised to be due to differences in the feeding level on *Alexandrium* among bivalve species (Hégaret *et al*., 2007; Contreras *et al*., 2012). Some studies have used relatively small samples sizes or pooled samples, which may not take into account the substantial differences found between bivalve individuals in PST toxin levels (Lassus *et al*., 2005; Kodama, 2010).

Our aim was to determine the temperature dependence of the dynamics of *Alexandrium* feeding, PST accumulation and depuration, and physiological and enzyme responses of oysters (*S. glomerata* and diploid and triploid *C. gigas*), using a large-scale study, to take into account large individual variability. As the ploidy level in *C. gigas* has been shown to impact metabolic rate (Haberkorn *et al*., 2010; Guéguen *et al*., 2012), we tested both diploid and triploid *C. gigas*. We hypothesised that oysters held at a predicted higher temperature (27 °C) relative to their current range (22 °C) would not differ significantly in their rate of toxin accumulation, as they would acclimate their metabolic processes accordingly. To test these hypotheses, we temperature acclimated the oysters ($n = 252$ per species/ploidy level) and fed them with cultures of toxic *Alexandrium minutum* over a period of 12 days. We examined toxin dynamics, routine metabolic rate and metabolic enzyme activity.

Materials and methods

Study species and acclimation treatments

Adult *S. glomerata* and diploid and triploid *C. gigas* were sourced from farms in Port Stephens, NSW, during September and October 2012, and all experiments were conducted at the NSW Dept. Primary Industries, Port Stephens Fisheries Institute. Sea surface temperatures at the time of collections were 18–19 °C. Shell size and body mass were measured before the experiment to ensure that the specimens were of marketable condition (see supplementary information Table S1). Oysters were cleaned to remove fouling and held in 400 L aerated tanks (ca. 100 individuals tank^{-1}), containing 1 μm filtered sea water from their estuary of origin (salinity ~ 35 g L^{-1}). Over 5–7 days, tank water temperatures were gradually increased to either 22 or 27 °C (± 0.5 °C) and ca. 200 oysters per type or species were held in each acclimation treatment. The oysters were held at the final constant acclimation temperatures for two weeks. Sea water changes took place every two days, and no mortalities occurred during the acclimation period.

Phytoplankton culture growth and maintenance

All algal cultures were grown at 23 °C with a 12/12 h light: dark photoregime at 60 μmol m^2 s^{-1}. Nontoxic live feed comprising *Isochrysis* aff. *galbana* (CS-177), *Pavlova lutheri* (CS-182) and *Chaetoceros muelleri* (CS-176) were grown in f/2 medium (Guillard & Ryther, 1962). During the acclimation period (described above), the oysters were fed daily with a mixed algal diet of late exponential phase nontoxic feed (2 x 10^9 cells oyster^{-1} day^{-1}). *Alexandrium minutum* culture (CS-324/16) was obtained from the CSIRO National Algae Culture Collection. This strain was originally isolated from Adelaide, South Australia. The toxin profile of the strain was characterised as containing primarily gonyautoxins GTX1,4 and low levels of GTX2,3 and STX (Negri *et al*., 2003). *Alexandrium minutum* cultures were grown in GSe medium and harvested during late exponential phase. To confirm the presence of PSTs, two samples (200 mL of ~200 000 cells mL^{-1}, 400 mL of ~120 000 cells mL^{-1}) of late exponential phase *A. minutum* were collected and centrifuged at 4700 g. The supernatant was removed, and the pellet was frozen at −80 °C for later quantification of toxins.

Feeding experiments

Alexandrium minutum feeding experiments were carried out in 200 L aquaria. For each species and temperature combination, 252 oysters were distributed randomly across 12 tanks (21 oysters tank^{-1}), a total of 756 oysters. For 12 days, 6 tanks received a mixed nontoxic algal diet only. The remaining 6 tanks received the mixed algal diet, plus late exponential phase cultures of *A. minutum* (300 cells mL^{-1}) were added three times daily. *Alexandrium minutum* cell concentrations in the tanks were checked twice daily. Salinity was ~35 g L^{-1}, and water temperatures were maintained at 22 °C and 27 °C ± 0.5 °C. Sea water changes took place every two days. Water samples were collected directly from each water tank, preserved with lugol's iodine and examined, via light microscopy, to determine *A. minutum* clearance (feeding) rate by the oysters. *Alexandrium minutum* cells were added to maintain a tank concentration of 300 cells mL^{-1} to ensure maximum consumption (Bricelj *et al*., 1990; Murray *et al*., 2009). On day 0, before introduction of the *A. minutum*, and on days 6 and 12, three oysters were collected from each tank. This was equivalent to 36 individuals per time point for each oyster species/ploidy level. Following the sea water change on day 12, the remaining oysters of all treatments were fed the mixed algal diet only to allow depuration of toxins. Depuration was carried out for 7 days, and further sampling (3 oysters tank^{-1}) was carried out on days 13 and 19. At the time of sampling, body mass, shell length, breadth and height of each oyster were recorded. After euthanising, the shell and tissue wet

mass were also recorded for each oyster, and tissue was retained at −80 °C for toxin analysis.

Toxin analysis

The PST content was measured in 300 individual oysters (n = 9 oysters per toxic treatment and n = 30 nontoxic control oysters), according to Lawrence *et al.* (2005) and Harwood *et al*. (2013). Briefly, homogenised (Omni Tissue Homogeniser, Omni International, USA) oyster tissue was vortexed with 3 mL of 1% acetic acid solution. The mixture was heated at 100 °C for 5 min, re-vortexed and then cooled and incubated at 4 °C for 5 min. Following centrifugation (10 min at 3600 g), the supernatant was collected. The pellet was re-suspended with a further 3 mL of 1% acetic acid and re-centrifuged (10 min at 3600 g). The resulting supernatant was added to the original quantity and diluted with deionised water to 10 mL. The *A. minutum* cell pellet was extracted according to the same method with slight modifications. Initially, the cell pellets were freeze-thawed to ensure cell lysis. Also, after both supernatants were combined, the dilution step to gain a final volume of 10 mL was excluded to gain a higher toxin yield. Prior to analysis, a SPE C18 clean up (GracePure SPE C18-Max 500 mg/3 mL, Alltech Associates (Australia) Pty Ltd.) was carried out on 1 mL of each extract. The pH of the final 4 mL effluent was adjusted to 6.5 with 1 M NaOH.

Quantification of toxins by ultra-performance liquid chromatography (UPLC) and fluorescence detection (FD) was carried out as per Harwood *et al*. (2013). Analytical certified reference standards were sourced from the National Research Council of Canada. The UPLC chromatogram of the extract from the *A. minutum* pellet had two peaks that corresponded to the analogues GTX1,4 and a third peak that fit the retention times of both GTX1,4 and GTX2,3 (refer supplementary information; Fig. S1b). The GTX1,4 concentration was determined from the second peak (Fig. S1b), while the concentration of GTX2,3 was verified by an additional peroxide oxidation (Harwood *et al*., 2013). Extracts from oysters that had not been exposed to *A. minutum* were spiked with known quantities of GTX1,4 and GTX2,3, to estimate the method's recovery factor. Recovery factors were incorporated into the final estimates of toxin levels.

Routine metabolic rate

On day 0 and day 12, the routine metabolic rate (RMR) of individual oysters (3 oysters tank^{-1}) was measured using a closed respirometry system according to Parker *et al.* (2012). In total, the measurement procedure was carried out on 216 individual oysters (n = 9 oysters treatment^{-1} sampling point^{-1}). Measurements were carried out at either 22 °C or 27 °C, depending on the treatment temperature. During RMR measurements, one control (nontoxic diet) diploid *C. gigas* (27 °C) and one replicate (toxic diet) *S. glomerata* (27 °C) failed to respire during the analysis. RMR represents the level of metabolism for normal, unrestricted activity. In this case, the valve of the oysters was unhindered, and the shells could open freely. Digestion was ongoing, evidenced by the production of faecal pellets during the measurement process. This differed from measurements of standard or resting metabolic rate, where minimal activity, independent of digestion, is quantified (Willmer *et al*., 2009). A fibre-optic probe (PreSens dipping probe DP-PSt3, AS1 Ltd, Palmerston North, New Zealand) was fitted to an airtight 500 mL chamber. Individual oysters (displacement volume < 50 mL) were submerged gently in sea water within the darkened chamber. Estimates of RMR were based on the time taken for the percentage oxygen saturation of sea water in the chamber to reduce from 100 to 80%, because of respiration by the oyster. The oxygen probe was calibrated based on a two-point calibration (0% and 100%). Values for RMR (mg O_2 g^{-1} DTM h^{-1}) were calculated as

$$\mathrm{RMR} = \frac{V_r \times \Delta C_w O_2}{\Delta t \times \mathrm{DTM}} \quad (1)$$

where V_r (L) is the volume of the chamber minus the displacement volume of the oyster $\Delta C_w O_2$ (mg O_2 L^{-1}) is the measured change in oxygen concentration over time Δt (h), and values were normalised to 1 g of dry tissue mass (DTM, g).

Metabolic enzyme activities

After RMR measurements, the 216 individual oysters (n = 9 oysters treatment^{-1} sampling point^{-1}) were euthanised and 50 mg of tissue from the adductor muscle and the digestive gland was dissected, placed in 1.5-ml Eppendorf tubes and flash frozen with liquid nitrogen. All samples were stored at -80 °C until further enzyme analysis. The remaining oyster tissue was freeze-dried for 48 h (Alpha 2-4 LSC plus, Martin Christ Gefriertrocknungsanlagen GmbH, Germany) to determine dry tissue mass (DTM).

Activities of citrate synthase (CS), cytochrome c oxidase (COX) and the combined activity of lactate, glycine and β-alanine dehydrogenase (LDH) were measured as indicators of tricarboxylic acid cycle, mitochondrial electron transfer and anaerobic ATP production, respectively. All assays were conducted according to published protocols (Seebacher *et al*., 2003; Sinclair *et al*., 2006). Briefly, digestive gland or adductor muscle tissue (0.05 g) was homogenised in nine volumes of extraction buffer (50 mmol L^{-1} imidazole/HCl, 2 mmol L^{-1} $MgCl_2$, 5 mmol L^{-1} ethylene diamine tetra-acetic acid (EDTA), 1 mmol L^{-1} reduced glutathione and 1% Triton X-100). All samples were kept on ice during homogenisation. For COX and CS assays, homogenates from digestive gland tissue were further diluted by a factor of 10. All assays were measured spectrophotometrically in an UV/visible spectrophotometer (Ultrospec2100 pro, Biochrom, UK) with a temperature-controlled cuvette holder (Seebacher *et al*., 2003). Each assay was performed in duplicate at two temperatures, which coincided with acclimation temperatures (22 and 27 °C).

Statistical analyses

Clearance rate (CR) of *A. minutum* was calculated according to

$$\mathrm{CR} = \frac{(\ln C_o - \ln C) \times V}{t} \quad (2)$$

where C_o and C are the initial and final *A. minutum* cell concentrations, respectively, V is the volume of suspension (holding tank volume) and t is time (Coughlin, 1969).

On selected days (n = 5 oyster species/ploidy level^{-1} treatment^{-1}), the final cell concentration for each replicated was estimated over 24 h periods, following tank sea water changes. Clearance rates were normalised to 1 g oyster tissue wet weight based on the mean values in Table S1 (Bricelj *et al.*, 1990). To determine any significant difference between oyster species/ploidy level and acclimation temperature on clearance rates, data were analysed by a two-way permutation analysis of variance (ANOVA).

To determine the effect of temperature on the accumulation and depuration of PSTs (GTX1,4 and GTX2,3) in oysters, we examined toxicity on day 12, the period of maximum exposure to *A. minutum*, and day 13, following 24 h of the oysters receiving a nontoxic diet only (depuration). Analysis of PSTs in each oyster species/ploidy level was by two-way permutation ANOVA with exposure treatment (days 12 and 13, as above) and acclimation temperature as factors.

Also on day 12, RMR data for each oyster species/ploidy level (n = 9 oysters treatment^{-1}) were analysed by a two-way permutation ANOVA with diet (toxic or nontoxic) and acclimation temperature as factors. Enzyme activities were analysed (n = 9 oysters treatment^{-1}) with a three-way permutation ANOVA with acclimation temperature and diet as factors and test temperature as a repeated measure.

All analyses were carried out in R (R.app GUI 1.63, 2012). Permutation analysis was carried out using the lmPerm package (Wheeler, 2014). Results are expressed as mean ± standard error mean (SEM). For all statistical analyses, the significance level was set at the $P \leq 0.05$ alpha-level. For each multifactorial analysis, the highest significant interaction was examined. Significant main effects were only examined when no significant interactions were reported. *Post hoc* analysis of means was by Tukey's honest significant difference (HSD).

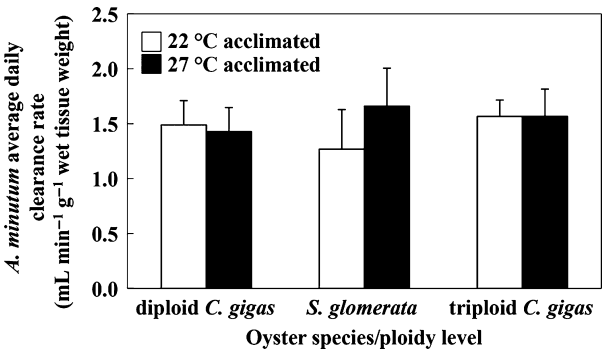

Fig. 2 Average daily clearance rate (mL min^{-1} g^{-1} wet tissue) of *A. minutum* for each oyster type and temperature treatment across the feeding trial. Results are shown from oysters acclimated at current mean summer water temperature (22 °C; white bars) and predicted warmer conditions (27 °C; black bars), and oyster species/ploidy level is shown on the *x*-axis; n = 5; bars = SEM.

Table 1 Summary of significant (interaction or main effect) responses by warm (27 °C)-acclimated oysters (diploid and triploid *C. gigas* and *S. glomerata*) to experimental measures of *A. minutum* clearance rate, paralytic shellfish toxin (PST) concentrations after 12 days of exposure to toxic diet and 24-h depuration, routine metabolic rate and metabolic enzyme activity [lactate, glycine and *β*-alanine dehydrogenase (LDH), citrate synthase (CS), cytochrome c oxidase (COX)]

		Oyster species/ploidy level (27 °C treatment)			
Experiment		Diploid *Crassostrea gigas*	*Saccostrea glomerata*	Triploid *Crassostrea gigas*	Ref.
Clearance rate of toxic *A. mintutum*		–	–	–	Fig. 2, Table S2
PSTs after 12-day exposure					
GTX1,4		↓	↓	–	Table 2, Fig. 3
GTX2,3		–	–	–	
PSTs after 24-h depuration					
GTX1,4		↓	↓	–	Table 2, Fig. 3
GTX2,3		–	–	↓	
Routine metabolic rate (irrespective of diet)		–	↑	–	Table 3, Fig. 4,
Metabolic enzymes					
Digestive gland	LDH	↑	–	↓	Table 4, Figs S2, S4, S6
	CS	–	↓	↓	
	COX	–	–	–	
Muscle	LDH	–	↑	↑	Table 5, Figs S3, S5, S7
	CS	–	–	↓	
	COX	–	–	↓	

Key: ↑ increased response; ↓ reduced response; – no significant response.

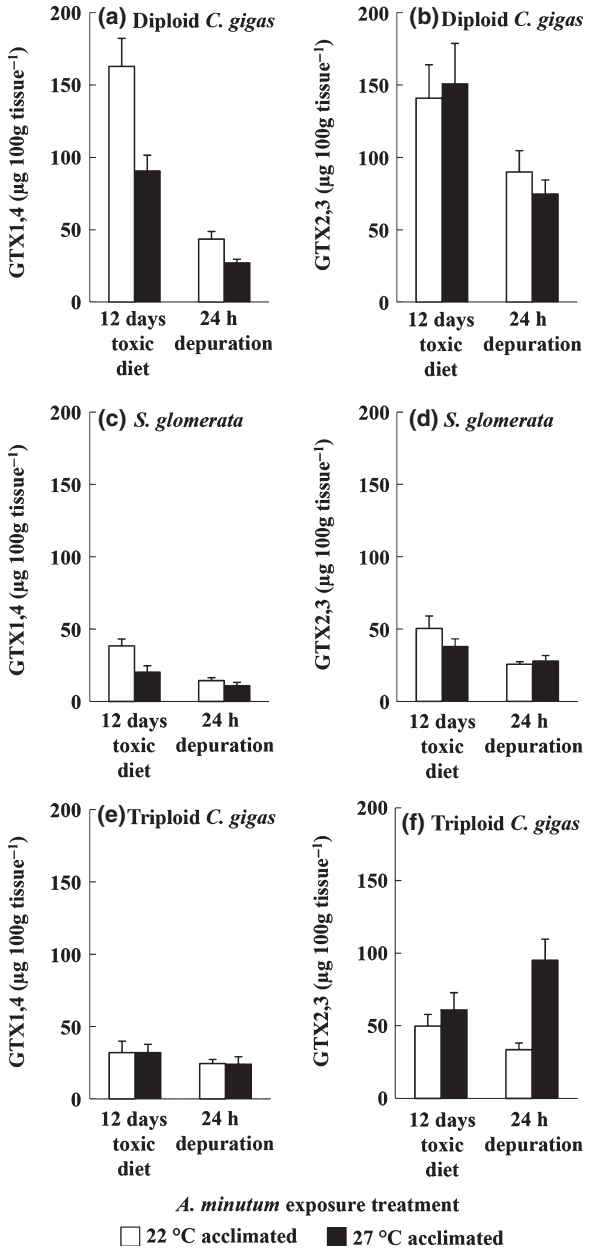

Fig. 3 Paralytic shellfish toxin content in oysters after the maximum period of exposure (day 12) to *A. minutum* and 24 h after feeding with toxic cells had ceased (day 13). Concentrations (µg 100 g tissue^{-1}) of the analogues GTX1,4 (a, c, e) and GTX2,3 (b, d, f) are shown for each oyster species/ploidy level: diploid *C. gigas* (a, b), triploid *S. glomerata* (c, d), *C. gigas* (e, f). Each panel shows results from oysters acclimated at current mean summer water temperature (22 °C; white bars) and predicted warmer conditions (27 °C; black bars), and *A. minutum* exposure treatment is shown on the *x*-axis; $n = 9$; bars = SEM. For GTX1,4, there were significant interactions between exposure treatment and acclimation temperature for diploid *C. gigas* (a) and S. glomerata (c). For GTX2,3, there was a significant interaction between exposure treatment and acclimation temperature for triploid *C. gigas* (f).

For analyses where nominal variables had only two levels, no *post hoc* analysis was carried out.

Results

Uptake of Alexandrium minutum

Temperature did not affect the rate at which the different oyster species/ploidy levels fed on toxic *A. minutum* (Fig. 2, Tables 1 and S2).

Toxicity of Alexandrium minutum and oysters

The PST analogues GTX 1,4 (1112 ± 208 ng mL^{-1}) and GTX 2,3 (22.21 ± 4.01 ng mL^{-1}) were found in the culture of *A. minutum*. These concentrations corresponded to 0.59 ± 0.08 pg GTX1,4 cell^{-1} and 0.012 ± 0.003 pg GTX2,3 cell^{-1}. All oysters that were exposed to a diet of *A. minutum* accumulated both GTX1,4 and GTX2,3 during the 12-day treatment (Fig. 3). PSTs were not found in the control (nontoxic diet) or day 0 samples (data not shown).

The GTX1,4 concentrations were lower (exposure treatment × acclimation temperature; Tables 1 and 2) in warm-acclimated diploid *C. gigas* (Fig. 3a) and *S. glomerata* (Fig. 3c) following 12 days of exposure to *A. minutum*. Following 24 h of receiving a nontoxic algal diet only, warm-acclimated diploid *C. gigas* (Fig. 3a) and *S. glomerata* (Fig. 3c) had slower detoxification of GTX1,4 (exposure treatment × acclimation temperature, Tables 1 and 2). For both diploid *C. gigas* and *S. glomerata* GTX2,3 concentrations after the 12-day exposure treatment and 24-h depuration process were unaffected by acclimation temperature (Tables 1 and 2, Fig. 3b, d).

Acclimation temperature did not affect GTX1,4 concentrations in triploid *C. gigas* after the 12-day exposure treatment and 24 h depuration period (Tables 1 and 2, Fig. 3e). Similarly, temperature did not influence concentrations of GTX2,3 in triploid *C. gigas* at the end of the 12-day exposure to a toxic diet. However, following the 24-h depuration treatment, detoxification was reduced in warm-acclimated triploid *C. gigas* (exposure treatment × acclimation temperature interaction; Tables 1 and 2, Fig. 3f).

Routine metabolic rate and metabolic enzyme activities

Diet and acclimation temperature did not have a significant influence on RMR in diploid and triploid *C. gigas* (Tables 1 and 3, diploid *C. gigas*: Fig. 4a, b; triploid *C. gigas*: Fig. 4e, f). RMR was higher in warm-acclimated *S. glomerata*, independent of diet (Table 3, Fig. 4c, d).

Table 2 Summary of two-way permutation ANOVA for accumulation of paralytic shellfish toxins (GTX1,4 and GTX2,3) in each oyster species/ploidy level, with exposure treatment (maximum exposure to *A. minutum*, and 24 hours of depuration) and acclimation temperature as factors

			Diploid *Crassostrea gigas*		Triploid *Crassostrea gigas*		*Saccostrea glomerata*	
PST analogue	Source of variation	df	*F*	*P*	*F*	*P*	*F*	*P*
GTX 1,4	Exposure	1	63.99	<0.001	1.93	0.318	21.48	<0.001
	Temperature	1	15.03	<0.002	0.00	0.902	9.02	0.002
	Exposure*Temperature	1	5.96	0.014	0.00	1.000	4.19	0.035
	Error	32						
GTX 2,3	Exposure	1	9.94	0.004	0.75	0.400	10.22	0.000
	Temperature	1	0.02	0.922	12.30	0.001	0.88	0.390
	Exposure*Temperature	1	0.39	0.554	5.88	0.016	1.79	0.121
	Error	32						

The LDH activity was elevated in digestive gland tissue from warm-acclimated diploid *C. gigas* (Tables 1 and 4, Fig. S2). Lower LDH activity was observed in adductor muscle of diploid *C. gigas* exposed to a toxic diet (Table 5, Figure S3).

Citrate synthase activity was reduced in digestive gland tissues from warm-acclimated *S. glomerata* (Tables 1 and 4, Fig. S4). The acclimation temperature × diet × test temperature interaction was significant for LDH activity in adductor muscle samples from *S. glomerata* (Table 5, Fig. S5). The interpretation of this interaction was unclear based on very little discernable differences in the graphed results. However, samples from warm-acclimated oysters had greater LDH activity (Tables 1 and 5, Fig S5).

Triploid *C. gigas* digestive gland samples had a reduced response for LDH and CS activities in warm-acclimated oysters (Tables 1 and 4, Fig. S6). Triploid *C. gigas* adductor muscle tissue had elevated LDH activity in warm-acclimated oysters at the 27 °C test temperature (acclimation temperature × test temperature interaction) (Tables 1 and 5, Fig. S7), whereas CS and COX activities were reduced in the adductor muscle of warm-acclimated triploid *C. gigas* (Table 5, Fig. S7).

Where test temperature produced a significant main effect (Tables 4 and 5), the majority of increased responses were at 27 °C (LDH: diploid *C. gigas* adductor muscle (Fig. S3); CS: all oyster species/ploidy level digestive gland (Figs. S2, S4, S6), *S. glomerata* and triploid *C. gigas* adductor muscle (Figs. S5, S7); COX: *S. glomerata* and triploid *C. gigas* adductor muscle (Figs. S5, S7)). LDH activity for triploid *C. gigas* digestive gland was elevated at the 22 °C test temperature (Table 4, Fig. S6).

Discussion

Filter-feeding bivalves face a combination of stressors from climate change, because the metabolic and physiological responses of these ectotherms are modulated by water temperature (Hawkins, 1995; Angilletta *et al.*, 2002; Clarke & Fraser, 2004; Peck *et al.*, 2004) and also because the distribution and abundance of toxin-producing dinoflagellate species are likely to change (Hallegraeff, 2010; Glibert *et al.*, 2014). South-eastern Australia in particular is considered a climate change 'hotspot' due to decadal increases in temperature of ~0.2 °C since the 1940s, accompanied by a southern range expansion of the Eastern Australian Current (Ridgway, 2007; Ridgway & Hill, 2012; Wu *et al.*, 2012). This area is also home to the vast majority of Australia's edible bivalve shellfish aquaculture industry.

We conducted the first large-scale experiment examining toxin uptake and depuration dynamics in three oyster species/ploidy levels to determine the combined impact of PSTs and temperature. While differences in the feeding efficiency on *A. minutum* by the different oyster types were not apparent (Table 1), significant differences in the concentration of PST analogues were observed between oyster types and acclimation temperatures after the 12-day exposure treatment (Table 1). In particular, diploid *C. gigas* and *S. glomerata* contained less GTX1,4, the more potent of the two PST congeners present, at warmer temperatures (Table 1). There was no apparent influence of temperature in the accumulation of GTX1,4 by triploid *C. gigas* or GTX2,3 by any of the three oyster types at the end of the 12-day exposure treatment. PST analogues, identical to that in the *Alexandrium minutum*, were detected in all individual oysters after 12 days of exposure to a toxic diet (Fig. S1). Biotransformation of PST analogues, in which the toxin analogues or their proportions in bivalves differ from that of the *Alexandrium* culture, commonly occurs in clams, scallops and mussels (Bricelj & Shumway, 1998; Sagou *et al.*, 2005; Kwong *et al.*, 2006; Bricelj *et al.*, 2014), but appears to be less commonly reported in oysters (Bricelj & Shumway, 1998; Murray *et al.*, 2009), in line with our findings.

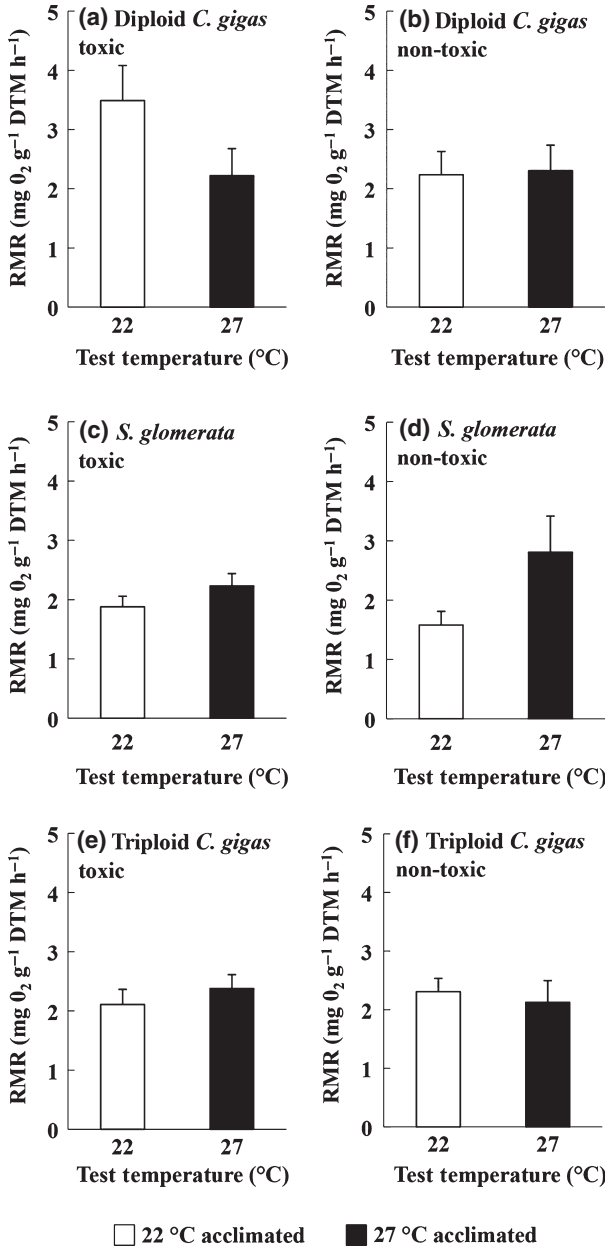

Fig. 4 Routine metabolic rate, RMR (mg 0_2 g^{-1} DTM $h^{-1}\pm$ SEM), on day 12, the maximum period of exposure in oysters that received *A. minutum* (a, c, e) and those that were fed a non-toxic diet only (b, d, f) are shown for each oyster species/ploidy level: diploid *C. gigas* (a, b), triploid *C. gigas* (c, d), *S. glomerata* (e, f). Each panel shows results from oysters acclimated at current mean summer water temperature (22 °C; white bars) and predicted warmer conditions (27 °C; black bars), and test temperature, which corresponded to the acclimation temperatures, is shown on the *x*-axis; $n = 9$, with the exception of nontoxic diploid *C. gigas* and toxic *S. glomerata* at 27 °C, where $n = 8$ (see text); bars = SEM. There was a significant main effect of temperature for *S. glomerata* (e, f).

In our study, triploid *C. gigas* and *S. glomerata* were found to be approximately 50% less toxic than diploid *C. gigas* at the end of the period of exposure to a toxic diet. Experimental feeding studies using PST-producing dinoflagellates have been conducted on mussels (Bricelj *et al.*, 1990; Blanco *et al.*, 2003; Li *et al.*, 2005; Kwong *et al.*, 2006; Galimany *et al.*, 2008), scallops, cockles and clams (Bricelj *et al.*, 1990; Sekiguchi *et al.*, 2001; Chen & Chou, 2001, 2002; Kodama, 2010; Higman and Turner, 2010; Contreras *et al.*, 2012;) and oysters (Lassus *et al.*, 2005, 2007; Murray *et al.*, 2009; Haberkorn *et al.*, 2010, 2011, 2014). Most studies analysed a single bivalve species or strain, but for those studies that compared total toxicity among species or ploidy levels, given the same experimental conditions, significant differences were generally found (Haberkorn *et al.*, 2011; Contreras *et al.*, 2012). This was attributed to differences in feeding levels among bivalve species (Hégaret *et al.*, 2007; Higman and Turner, 2010, Contreras *et al.*, 2012) or ploidy levels (Haberkorn *et al.*, 2010). In our study, differences in total PST toxicity were found among species (Table 1) and at certain temperature treatments despite feeding levels not differing significantly, suggesting that differences in PST metabolism rates may be instead contributing to these differences.

Both diploid and triploid *C. gigas* acclimated their RMR to their respective treatment temperatures independently of diet, which indicated that the exposure to *A. minutum* did not incur a metabolic cost. However, the reduced response in LDH activity suggested an increase in potential for aerobic metabolism in diploid *C. gigas* that were fed with *A. minutum*. Aerobic metabolism in oysters is associated with increased circulation and filtration of sea water (Lucas, 2012). This finding may explain how, overall, the greatest toxicity was observed in diploid *C. gigas*, at both temperatures. The extra set of chromosomes in triploid oysters, their main genetic distinction from diploid types, affords greater resources towards growth and other physiological functions largely due to a decline in reproductive effort (Allen & Downing, 1986; Nell & Perkins, 2005). Such enhanced growth may cause faster metabolism of toxins and explain the reduction in toxicity seen here for triploid *C. gigas*. However, previously, faster accumulation of PSTs has been found in triploid *C. gigas* compared to diploid strains and was considered to be correlated to their faster metabolic rates (Haberkorn *et al.*, 2010), depending on sexual maturity (Guéguen *et al.*, 2012).

We had anticipated that oysters would adjust their metabolic processes with increasing temperatures. This was not the case for *S. glomerata* on day 12, as the ele-

vated RMR, independent of diet, implied a higher metabolic maintenance cost associated with warmer conditions. Temperature-dependent responses of some metabolic enzyme activities (CS and LDH) promoted the potential for anaerobic metabolic pathways in warm-acclimated *S. glomerata*. While all three oyster types studied experienced some effects of higher temperature acclimation on metabolic enzyme activity, a

Table 3 Summary of two-way permutation ANOVA for routine metabolic rate, RMR (mg 0_2 g^{-1} DTM h^{-1} ± SEM), for each oyster species/ploidy level on day 12, the maximum period of exposure to *A. minutum*. Acclimation temperature and diet (toxic or non-toxic) were factors for each analysis

	Diploid *Crassostrea gigas*			Triploid *Crassostrea gigas*			*Saccostrea glomerata*		
Source of variation	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Diet	1	1.49	0.115	1	0.01	0.922	1	0.15	0.804
Temperature	1	1.58	0.322	1	0.03	0.863	1	4.78	0.024
Diet*Temperature	1	1.96	0.171	1	0.66	0.548	1	1.46	0.413
Error	31			32			31		

Table 4 Summary of three-way permutation ANOVA for lactate, glycine and β-alanine dehydrogenase (LDH), citrate synthase (CS), cytochrome c oxidase (COX) from diploid and triploid *Crassostrea gigas* and *S. glomerata* digestive gland. Acclimation temperature (Acc), diet (toxic vs. nontoxic) and test temperature (Test; repeated measure) were factors for analysis

			Diploid *Crassostrea gigas*		*Saccostrea glomerata*		Triploid *Crassostrea gigas*	
Assay	Source of variation	df	*F*	*P*	*F*	*P*	*F*	*P*
LDH	*Between subject effects*							
	Acc	1	5.58	0.012	1.25	0.200	3.19	0.030
	Diet	1	0.03	0.980	0.03	0.902	0.15	0.745
	Acc *Diet	1	0.04	0.784	0.12	0.726	0.60	0.391
	Error	32						
	Within subject effects							
	Test	1	6.22	0.161	1.27	0.222	28.96	0.040
	Acc*Test	1	1.82	0.162	0.06	0.667	0.20	0.961
	Diet*Test	1	0.84	0.473	0.05	0.824	0.00	0.863
	Acc*Diet*Test	1	0.05	0.961	0.82	0.273	0.30	0.527
	Error	32						
CS	*Between subject effects*							
	Acc	1	0.12	0.941	7.36	0.002	3.74	0.050
	Diet	1	1.06	0.267	0.36	0.452	1.40	0.236
	Acc *Diet	1	1.48	0.216	0.36	0.824	0.12	0.980
	Error	32						
	Within subject effects							
	Test	1	55.10	<0.001	132.88	<0.001	208.70	<0.001
	Acc*Test	1	0.09	0.922	0.08	0.824	0.16	0.745
	Diet*Test	1	2.55	0.765	0.19	0.594	0.80	0.686
	Acc*Diet*Test	1	0.01	0.922	0.09	1.000	0.32	0.882
	Error	32						
COX	*Between subject effects*							
	Acc	1	1.13	0.220	1.40	0.414	2.42	0.191
	Diet	1	0.03	0.686	2.01	0.201	3.38	0.058
	Acc *Diet	1	0.55	0.385	0.47	0.478	1.39	0.667
	Error	32						
	Within subject effects							
	Test	1	10.01	0.452	10.34	0.065	0.60	0.070
	Acc*Test	1	0.00	0.686	1.58	0.324	0.06	0.643
	Diet*Test	1	0.01	1.000	3.64	0.065	0.23	0.444
	Acc*Diet*Test	1	0.20	0.863	0.78	0.706	0.00	1.000
	Error	32						

Table 5 Summary of three-way permutation ANOVA for lactate, glycine and β-alanine dehydrogenase (LDH), citrate synthase (CS), cytochrome c oxidase (COX) from diploid and triploid *Crassostrea gigas* and *S. glomerata* adductor muscle. Acclimation temperature (Acc), diet (toxic vs. nontoxic) and test temperature (Test; repeated measure) were factors for analysis

			Diploid *Crassostrea gigas*		*Saccostrea glomerata*		Triploid *Crassostrea gigas*	
Assay	Source of variation	df	*F*	*P*	*F*	*P*	*F*	*P*
LDH	*Between subject effects*							
	Acc	1	0.02	0.941	15.32	<0.001	4.74	0.038
	Diet	1	7.81	0.014	0.27	0.500	1.09	0.236
	Acc *Diet	1	0.41	0.373	0.27	0.592	0.53	0.638
	Error	32						
	Within subject effects							
	Test	1	25.68	0.007	145.06	<0.001	89.96	<0.001
	Acc*Test	1	0.56	0.686	7.55	0.006	5.85	0.022
	Diet*Test	1	1.35	0.193	0.38	0.444	0.05	0.922
	Acc*Diet*Test	1	0.82	0.660	4.58	0.044	3.48	0.059
	Error	32						
CS	*Between subject effects*							
	Acc	1	1.13	0.295	0.44	0.368	19.19	<0.001
	Diet	1	0.01	1.000	0.97	0.258	0.47	0.411
	Acc *Diet	1	0.23	0.686	2.55	0.165	0.70	0.667
	Error	32						
	Within subject effects							
	Test	1	22.88	0.061	69.99	0.000	164.47	<0.001
	Acc*Test	1	2.54	0.082	1.15	0.396	0.32	0.633
	Diet*Test	1	0.64	0.583	0.21	0.660	1.11	0.304
	Acc*Diet*Test	1	3.67	0.069	0.01	1.000	0.12	1.000
	Error	32						
COX	*Between subject effects*							
	Acc	1	0.06	0.784	0.88	0.394	3.22	0.042
	Diet	1	0.17	0.554	0.24	0.583	0.65	0.288
	Acc *Diet	1	0.97	0.346	0.88	0.371	0.32	0.554
	Error	32						
	Within subject effects							
	Test	1	2.26	0.571	65.36	0.006	8.51	0.615
	Acc*Test	1	0.57	0.765	1.22	0.321	3.35	0.170
	Diet*Test	1	1.94	0.147	0.10	0.726	0.85	0.302
	Acc*Diet*Test	1	1.29	0.396	0.10	0.980	1.52	0.231
	Error	32						

greater number of responses were noted in triploid *C. gigas*. The majority of these suggested a greater potential for anaerobic metabolic scope (Table 1). Multiple stressors (temperature and cadmium or temperature and elevated CO_2) have been found to have inhibitive effects on the aerobic scope of *C. gigas and Crassostrea virginica* (Lannig *et al*., 2006, 2010). Our findings on *S. glomerata* and diploid *C. gigas* were similar, although the responses were fewer than in triploid *C. gigas*.

In both diploid *C. gigas* and *S. glomerata*, warm-acclimated oysters had a slower depuration of GTX1,4, following the dietary change to exclude *A. minutum*, while there was no effect of temperature on the detoxification of GTX2,3 for either of these species. For triploid *C. gigas*, the detoxification rate of GTX1,4 was unaffected by temperature; however, warm-acclimated triploid *C. gigas* experienced slower reduction in GTX2,3. Detoxification rates have been found previously to vary between species (Mons *et al*., 1998; Kwong *et al*., 2006). While exposure to a toxic diet had a significant effect at a cellular level in diploid *C. gigas*, the temperature-dependent responses of metabolic enzyme activities to warmer conditions suggested that predicted changes to ocean temperatures will influence toxin accumulation and depuration dynamics in all three oyster types.

By simulating an *Alexandrium* bloom under two temperature scenarios, using a large-scale study, we have shown differential toxin uptake and depuration in three oyster species/two ploidy levels. Our findings indicate that both *S. glomerata* and diploid *C. gigas* may have

lower GTX1,4 concentrations in warmer waters given the same density of *A. minutum* bloom, while detoxification will be slower. However, the current trend of increasing abundance and distribution of PST-producing species of *Alexandrium* (Anderson *et al.*, 2012) will add a layer of complexity to determining future risk of PSTs in commercial bivalves.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Example chromatographs showing the toxin peaks for the extracts from the *A. minutum* pellet (a) and the corresponding peaks observed in extracts from oyster tissue (b and c).

Figure S2. Activities (μmol g^{-1} min^{-1}) of lactate, glycine and β-alanine dehydrogenase (LDH; a-b), citrate synthase (CS; c-d), cytochrome c oxidase (COX; e-f) from diploid *C. gigas* digestive gland.

Figure S3. Activities (μmol g^{-1} min^{-1}) of lactate, glycine and β-alanine dehydrogenase (LDH; a-b), citrate synthase (CS; c-d), cytochrome c oxidase (COX; e-f) from diploid *C. gigas* adductor muscle.

Figure S4. Activities (μmol g^{-1} min^{-1}) of lactate, glycine and β-alanine dehydrogenase (LDH; a-b), citrate synthase (CS; c-d), cytochrome c oxidase (COX; e-f) from *S. glomerata* digestive gland.

Figure S5. Activities (μmol g^{-1} min^{-1}) of lactate, glycine and β-alanine dehydrogenase (LDH; a-b), citrate synthase (CS; c-d), cytochrome c oxidase (COX; e-f) from *S. glomerata* adductor muscle.

Figure S6. Activities (μmol g^{-1} min^{-1}) of lactate, glycine and β-alanine dehydrogenase (LDH; a-b), citrate synthase (CS; c-d), cytochrome c oxidase (COX; e-f) from triploid *C. gigas* digestive gland.

Fig. S7 Activities (μmol g^{-1} min^{-1}) of lactate, glycine and β-alanine dehydrogenase (LDH; a-b), citrate synthase (CS; c-d), cytochrome c oxidase (COX; e-f) from triploid *C. gigas* adductor muscle. Data for oysters exposed to either a toxic or non-toxic diet are shown separately.

Table S1 Summary of oyster species/ploidy level and weight ranges used for the controlled feeding experiment.

Table S2 Analysis of clearance rate of *A. minutum* by each oyster species/ploidy level (diploid and triploid *C. gigas* and *S. glomerata*) at each acclimation temperature across the 12-day exposure period.