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Changes in gene expression and histological injuries as a result of exposure of *Crassostrea gigas* to the toxic dinoflagellate *Gymnodinium catenatum*

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ABSTRACT

The harmful alga *Gymnodinium catenatum* is one of the species that produces the neurotoxic, hydrophilic, tetrahydropurine derivatives known as paralytic shellfish toxins (PST). Studies have shown that several classes of marine toxins have genotoxic effects on bivalves. However, there are few studies on PST and their genotoxic effects on bivalves such as oysters. Experiments were performed to assess the effects of the toxic dinoflagellate *G. catenatum* on changes in the transcription level of the genes involved in cell-cycle regulation (*p21*, *p53*, *cafp55*) and initial inflammatory (*casp1*) processes and injuries in tissues of juvenile *Crassostrea gigas*. Our results reveal an effect at the level of transcription. The high transcription levels of the *p21* and *p53* genes found in oysters exposed to dinoflagellate diets suggest an activation of the cell-cycle checkpoints by DNA damage induction. At the histological level, the oysters exposed to the toxic dinoflagellate showed exfoliation, epithelial rupture and inflammation, which correlate with the observed changes in expression level of *casp1* that is known to play a role in initial inflammatory responses. Our results provide molecular data suggesting probable DNA damage through expression modulation of the analysed key genes, in addition to histological evidence of tissue injuries due to PST dinoflagellate exposure.

INTRODUCTION

The occurrence of proliferations of toxic algae, also known as harmful algal blooms (HABs), is a worldwide problem (Shumway, 1990). The dinoflagellate *Gymnodinium catenatum* is a producer of paralytic shellfish toxin (PST), implicated in massive fish die-offs in Bahía Concepción in the southern part of the Gulf of California (Sierra-Beltrán *et al.*, 1998) and in many localities along the Mexican Pacific coast (Band-Schmidt *et al.*, 2005, 2010). Cell concentrations can reach 38 to 570 cells ml^{-1} (Band-Schmidt *et al.*, 2010). PST is a large group of neurotoxic alkaloids that include saxitoxin and analogues, causing paralytic shellfish poisoning (PSP) in humans (Llewellyn, 2006; Kodama, 2010). PST is a sodium channel blocker that interacts with potassium and calcium channels affecting the flux of these ions into vertebrate cells (Llewellyn, 2006; Kodama, 2010).

Bivalves, such as the Pacific oyster *Crassostrea gigas*, accumulate PST toxins in tissues mainly in their digestive tract (Bricelj & Shumway, 1998; Lassus *et al.*, 2000, 2004; Landsberg, 2002; Laabir *et al.*, 2007; Haberkorn *et al.*, 2014). Exposure to PST-producing algae causes negative impacts on ingestion, such as reduced filtration activity and clearance rate, pseudofaeces production (Shumway, 1990; Bardouil *et al.*, 1993; Bricelj & Shumway, 1998; Wildish *et al.*, 1998; Lassus *et al.*, 2000, 2004; Tran *et al.*, 2010; Haberkorn *et al.*, 2014) and injuries and inflammation in the oysters' tissues (Hégaret & Wikfors 2005; Tran *et al.*, 2010; Hégaret *et al.* 2012; Haberkorn *et al.*, 2014).

Little information is available about gene expression modifications in bivalves in response to the presence of the dinoflagellates producers of PST or their toxins. Recent studies have revealed changes in expression levels of the genes involved in oxidative (Mat *et al.*, 2013; Medhioub *et al.*, 2013; Mello *et al.*, 2013) and mitochondrial metabolism (Mat *et al.*, 2013; Mello *et al.*, 2013); cell detoxification, stress and immune responses (Mat *et al.*, 2013; Medhioub *et al.*, 2013; Mello *et al.*, 2013; García-Lagunas *et al.*, 2013); apoptosis and cell signalling (Medhioub *et al.*, 2013) and cell-cycle regulation and immune responses (Romero-Geraldo *et al.*, 2014) in *C. gigas* exposed to marine toxins (diarrhoeic, paralyzing and brevetoxins) and in *Mytilus galloprovincialis* exposed to a PST producer (Gerdol *et al.*, 2014).

Several studies have indicated that marine biotoxins are genotoxic (Wikfors, 2005; González-Romero *et al.*, 2012; Mirzayans *et al.*, 2012; Mat *et al.*, 2013; Romero-Geraldo *et al.*, 2014). Genotoxic stress triggers a variety of cellular responses, including dysregulation of the genes involved in critical cellular pathways (e.g. *p21*, *cafp55* and *p53*) (Mirzayans *et al.*, 2012), of which *p53* is a sequence-specific transcription factor playing a major role in

the regulation of DNA repair, apoptosis and cell-cycle progression. The *p21* (or *waf1*) gene encodes for a protein involved in cell-cycle regulation (Mirzayans *et al.*, 2012). Likewise, the *cafp*55 gene is a histone chaperone protein essential in remodelling chromatin and in DNA repair (Tyler *et al.*, 1996). The *caspase 1* (*casp1*) gene, which plays a role in inflammatory responses, has been detected in all of the examined tissues of *C. gigas*, including mantle, gills, gonads, adductor muscle, labial palps and haemolymph. This universal expression of *casp1* indicated it might be essential for most physiological functions in *C. gigas* (Qu *et al.*, 2014).

Therefore, the aim of our study was to analyse the changes in expression level of the genes involved in cell-cycle regulation (*p21*, *p53*, *cafp55*) and initial inflammatory process (*casp1*) in *C. gigas* juveniles induced by exposure to *G. catenatum* cells. Changes in gene expression and dinoflagellate cell concentration were assessed at different times of exposure in order to also observe changes in tissues, principally the digestive gland, mantle, gills and muscle of *C. gigas* juveniles fed with *G. catenatum*.

MATERIAL AND METHODS

Microalga cultivation

The planktonic dinoflagellate *Gymnodinium catenatum* GCCV 6 (obtained from the microalgal collection of Centro de Investigaciones Biológicas del Noroeste, La Paz, Baja California Sur (BCS), México) produces saxitoxin (STX) containing from 25.7 to 101 pg STXeq cell^{-1} (Band-Schmidt *et al.*, 2005; Pérez-Linares *et al.*, 2009) and four of its analogues (dcSTX, GTX2,3, NEO and GTX1,4). This species typically forms chains of up to 64 cells. Single cells range in size from 27–43 μm in width to 34–65 μm in length. Cultures were grown on modified f/2 medium in Fernbach flasks and maintained at 24 ± 1 °C, 12 h:12 h light/dark and 150 μmol photons m^{-2} s^{-1} light intensity. For the bioassays, cells were harvested during the late exponential growth phase by centrifugation at 2500 × *g* for 10 min 19 d after inoculation (Band-Schmidt *et al.*, 2005). Culture-cell density was determined by counting in Sedgwick-Rafter chambers after cell fixation with Lugol's solution (Gifford & Caron, 2000) under an optical microscope (Olympus BX41, Tokyo).

The *Isochrysis galbana* strain (ISG-1), provided by CIBNOR's Live Food Laboratory, is used especially as a primary alga in shellfish hatcheries (size 3–5 μm, spherical to pear shaped). Cultures were grown in plastic bags with F/2 medium and maintained at 20 ± 1 °C under constant illumination at 150 μmol photons m^{-2} s^{-1} light intensity.

Oysters

Diploid juvenile individuals of *Crassostrea gigas* (3 ± 1 mm, 0.022 ± 0.008 g) were obtained from the hatchery 'Acuacultura Robles' in Bahía Magdalena (Las Botellas), BCS, Mexico. They were acclimatized before the bioassay in aerated 0.22-μm filtered seawater (35 psu) at 24 ± 1.0 °C for 15 d to eliminate oysters that were too weak to show physiological defects. Afterwards, the organisms were separated into groups, kept in plastic aquariums (20 l) in the same conditions for acclimation for 10 d, and fed daily quantities of *I. galbana* according to the tables reported for their age (Helm *et al.*, 2004).

Experimental design

The bioassay consisted of *C. gigas* exposure to two mixed diets of *G. catenatum* (3 × 10^3 and 30 × 10^3 cell ml^{-1}) combined with a fixed amount of *I. galbana* (0.75 × 10^6 cell ml^{-1}) fed daily for 14 d. Two control diets were included: (1) a nontoxic control diet consisting only of *I. galbana* (0.75 × 10^6 cell ml^{-1}) to identify both normal behaviour and ingestion of microalgae; (2) a toxic control diet consisting only of *G. catenatum* (3 × 10^3 cell ml^{-1}) to identify the effect of the toxic dinoflagellate alone by ingestion and/or starvation.

Bioassays were developed over a period of 12 h/12 h light/dark cycle. During the development of the bioassays, the experimental unit was cleaned every day by removing faeces and food waste; seawater was completely changed every 24 h for 14 d. A single dose of microalgae was provided every 24 h at 09.00 h every day; aeration was used throughout the experiment to prevent cell sedimentation. Microalgal doses were provided based on field data observations of HAB events on BCS shores (Sierra-Beltrán *et al.*, 1998). Groups of 25 oysters (in triplicate) were placed in 100-ml transparent polypropylene containers and exposed to a 1:1 microalgal mixture (v:v, on a final volume of 50 ml). Throughout the bioassay, five juvenile oysters of each experimental unit were randomly sampled after 1, 7 and 14 d post-challenge. Sampled organisms in each sample time were replaced by the same number of organisms exposed to *G. catenatum* under the same conditions (mirror bioassay, only used for organism replacement). Samples (complete juvenile oysters) were transferred to Eppendorf tubes, washed twice with sterile seawater and immediately frozen at −80 °C until use. For the histological analysis, the five organisms were sampled only at the end of exposure day 14; they were fixed immediately with Davison solution for 48 h and afterwards in 70% ethanol until use.

Total RNA preparation and first-strand cDNA synthesis

Total RNA was extracted with TRIzol® (Invitrogen, USA) according to the manufacturer's instructions. Samples (a pool of five whole-body juveniles) were homogenized using a glass pestle; later, two consecutive extractions of each sample were made. RNA purity and quantity were determined by spectrophotometry (NanoDrop ND-2000, Thermo Scientific) by OD 260/280 and OD 260/230 absorbance ratios (range 1.90–2.08). To ensure complete DNA absence, a direct PCR was performed, using 1 μl (100 ng) of each RNA preparation with 28S ribosomal specific primers as a nonamplified control. After that, 0.5 μg was taken from each verified RNA sample for cDNA synthesis using the cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen). Total RNA was reverse-transcribed using oligo-dT and stored at −80 °C until used.

Real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Crassostrea gigas sequences were obtained from GenBank (Benson *et al.*, 2006) and oligonucleotide primers specific to each of the genes (Table 1) were designed with Primer3 (Rozen & Skaletsky, 2000) and investigated for primer-dimers and hairpin formation using RNAfold®. A series of standard curve primer amplifications of candidate reference genes 28S ribosomal RNA, *gapdh* (glyceraldehyde-3-phosphate dehydrogenase), *ef2 (*elongation factor 2), *act* (actin) and *β tub* (tubulin) and target genes (*cafp55*, *p21*, *p53*, *casp1*) were generated using serial dilutions (1:5) of cDNA obtained from oysters exposed to PST and appropriate controls. PCR efficiency (E) and correlation coefficient (R^2) were determined and used for gene stability analyses of a potential set of reference genes; the acceptable E value was defined as 95 to 100%.

A qPCR cocktail-mix was prepared in the laboratory containing 50 mM $MgCl_2$, 2 mM dNTP (each), 0.3 U of platinum Taq DNA polymerase (Invitrogen), 0.05 μM of each primer, 20× EvaGreen fluorescent dye (Biotium®) and 3.2 ng of cDNA in 15 μl of final volume per reaction. Amplification conditions

Table 1. Primer sequences, expected amplicon size and efficiency for target (cell-cycle regulation and initial inflammatory process) and reference genes (*) of *Crassostrea gigas* used for analysis of gene expression after challenge with toxic dinoflagellates.

Primers	Sequence 5′–3′	Gene name	Amplicon size (pb)	PCR Efficiency	GenBank
Cg- *28S*-Fw	GGAGTCGGGTTGTTTGAGAATGC	*Ribosomal subunit 28S**	114	1.97	AY632555
Cg- *28S*-Rv	GTTCTTTTCAACTTTCCCTCACGG				
Cg- *gapdh*-Fw	GTTCAAATATGATTCAACTCACGG	*Glyceraldehyde 3 phosphate dehydrogenase**	109	2.0	AB122066
Cg- *gapdh*-Rv	TGGATCCCGTTCGCAATATACG				
Cg-*tub*-Fw	AGCAGATGTCGTAGAGAGCTTC	*Tubulin β**	144	1.96	CB617442
Cg-*tub*-Rv	TGAACACATTCTCCGTTGTCCC				
Cg-*act*-Fw	TACTCTTTCACCACCACAGCCG	*Actin (GIA)**	117	1.95	AF026063
Cg-*act*-Rv	TAGAGATGAGGATGAAGCAGCAG				
Cg- *p53*-Fw	CTGTAGTTCTGGCCCGTGAA	*Tumour-suppressor protein p53*	110	1.98	AM236465
Cg- *p53*-Rv	AAACACAAGGGCACCACAAG				
Cg-*p21*-Fw	TTCCCATTCCTCCCATGTTGTTC	*Cell-cycle regulator p21 protein*	100	1.98	CB617437
Cg-*p21*-Rv	ACAGGCGACATGGATTTAGAAGC				
Cg-*cafp55*-Fw	TCGAAGATCCCACAAAGCAACAG	*Chromatin assembly factor 1 p55 subunit*	77	1.98	CB617555
Cg-*cafp55*-Rv	TGTCCTTCAACCCCTACAGCGA				
Cg-*ef2*-Fw	TTGATCACGGCAAGTCTACTCTG	*Elongation factor 2**	109	2.0	CB617558
Cg-*ef2*-Rv	GAGATGGCAGTGGACTTGATGG				
Cg-*casp 1*- Fw	ACTACAGAAAGCCGCCGAAT	*Caspase 1*	108	1.97	HQ425703
Cg-*casp 1*- Rv	GATCATTCTGTGCTACCCCC				

were: 95 °C for 5 min; 40 cycles of 95 °C (60 s), 61 °C (30 s) and 74 °C (5 s), acquiring the fluorescence at 79 °C (1 s); finally a dissociation step from 65 °C to 95 °C (at 1 °C/s). The melting curve analysis of amplification products was done at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. The qPCR reactions were conducted in triplicate in holding Strip Tubes (0.1 ml) (Qiagen, Hilden, Germany) using a Rotor gene 6000 Real-Time PCR detection system (Corbett Life Science, San Francisco).

Histological analysis

The five organisms sampled at the end of the 14-d exposure were processed in the same way as previously described in the experimental design. The oysters were fixed whole; therefore, a decalcification solution of sodium citrate/formic acid was used for shell removal. Soft tissue was dehydrated in an ascending ethanol series (70, 80, 95 and 100%), cleared with xylene and embedded in paraffin. Then, 5 μm-thick sections were cut with a rotatory microtome (Leica RM 2155 Leica Microsystems, Wetzlar, Germany) and mounted on glass slides. Each slide was stained with Harris haematoxylin for 5 min and counter-stained with eosin-phloxine for 12 min (Martoja *et al*., 1967); stained slides were covered, examined and photographed using a light microscope (Olympus BX41, Tokyo) fitted with an Evolution VF Color Cooled camera (Media Cybernetics) to observe tissue injuries.

Statistical analysis

The comparative Ct method was used to analyse gene expression levels. The Ct for the gene target amplification and the Ct for the reference gene were determined for each sample. The non-toxic control group was used as the reference sample (calibrator). The ΔCt for each sample was subtracted from the ΔCt of the calibrator and the difference was called the ΔΔCt value. The relative amount of gene transcripts was calculated according to the $2^{-\Delta\Delta Ct}$ algorithm (Livak & Schmittgen, 2001), considering the two most stable genes (*28S RNAr* and *gapdh*) preselected by geNorm (Vandesompele *et al*., 2002), Normfinder (Andersen *et al*., 2004) and BestKeeper (Pfaffl *et al*., 2004) from a group of five housekeeping genes. Expression data were analysed using a normality test; homogeneity of variances was verified by Leven's test and comparisons of the gene expression were determined by two-way ANOVA; significant differences were analysed with Fisher's least significant difference (LSD, $\alpha = 0.01$). All analyses were performed with Statistica 7 software (StatSoft, Tulsa, OK). Significant differences were set at $P < 0.05$.

RESULTS

Gene expression analysis by quantitative RT-qPCR

The three experimental groups exposed to *Gymnodinium catenatum* cells showed higher expression levels in almost all genes tested than those of the nontoxic control group (Fig. 1). Overexpression of the *cafp55* gene was observed in oysters in all treatments at 7 d, decreasing to basal values at 14 d of exposure (Fig. 1A). Also a significant increase of *p21* relative expression ($P < 0.05$) was observed in all treatments from 24 h of exposure to the dinoflagellate (Fig. 1B), but the highest expression level was observed in the treatment with 30×10^3 cells ml^{-1} at 7 d. A decrease in expression was observed in the treatments with the lowest cell concentration at 14 d, contrary to *p21* expression that significantly increased (8-fold) in the mixed *G. catenatum* diet with 30×10^3 cell ml^{-1} (Fig. 1B). The expression level for *p53* gene was from 2.5 to 3.5 times higher in the groups treated with dinoflagellate than in those of the nontoxic control group after a short-term exposure (24 h) (Fig. 1C). These differences increased significantly ($P < 0.05$) six times above the basal level (nontoxic control) after 7 d of exposure, but the expression level was repressed significantly below basal level at 14 d. The *casp1* gene expression level did not change after 24 h exposure, but it increased significantly with respect to the nontoxic control group ($P < 0.05$) in all treatments after 7 d, returning to basal level after 14 d of exposure (Fig. 1D).

The analysed genes exhibited a constitutive expression under normal conditions (unchallenged oysters), but inducible under the conditions tested (oysters exposed to *G. catenatum*). It is important to emphasize that these changes in the level of expression of the studied genes were dependent on exposure time,

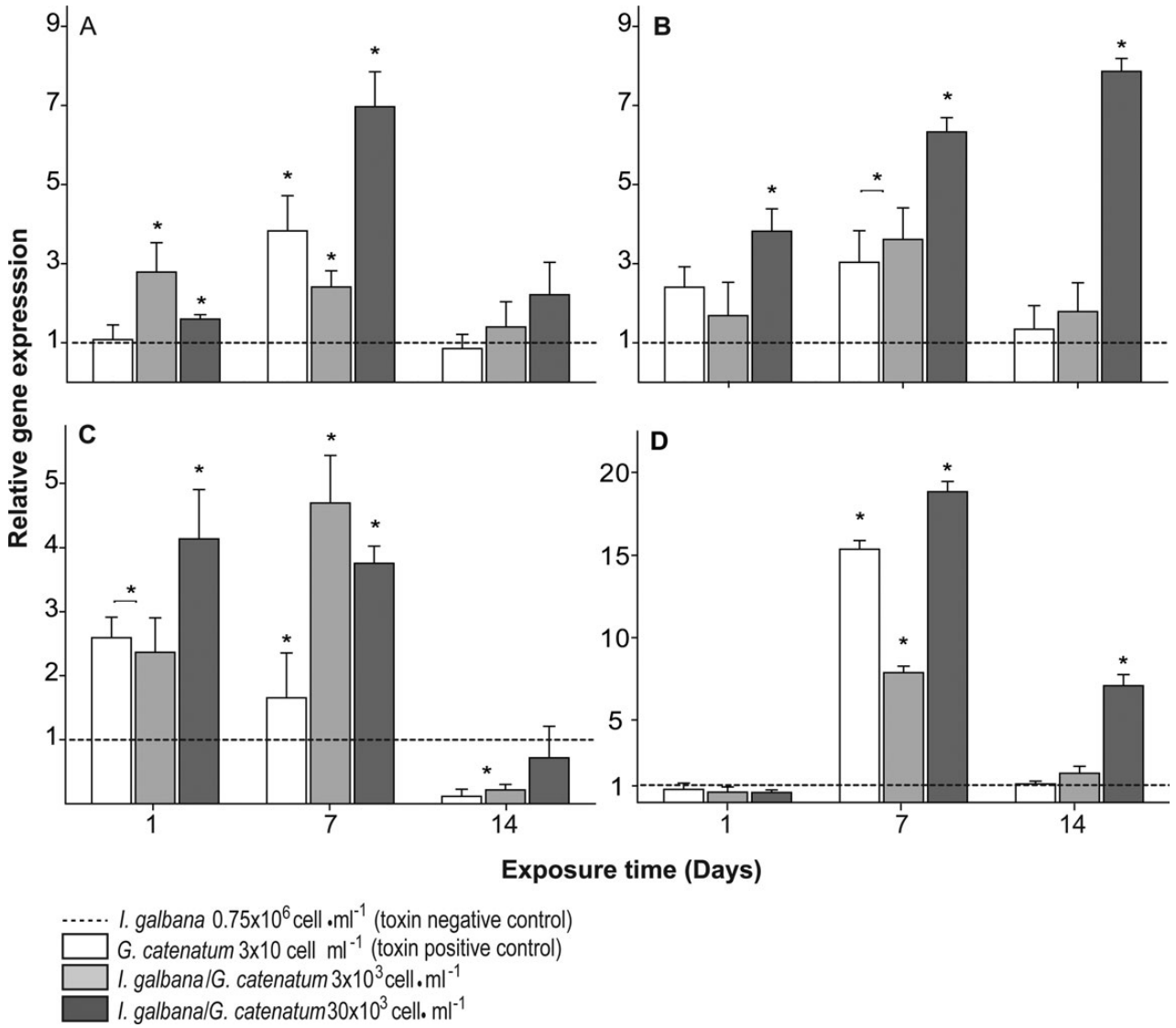

Figure 1. Expressions of genes involved in cell-cycle regulation and inflammatory process in *Crassostrea gigas* exposed to *Gymnodinium catenatum* and/or *Isochrysis galbana* cells. Each gene expression is shown as relative expression ($2^{-\Delta\Delta Ct}$) with *28S* ribosomal and *gapdh* as reference genes. Asterisk indicates significant differences between treatments and nontoxic control ($P < 0.05$ in Fisher's LSD).

dinoflagellate cell concentrations and diet type (mixed or single dinoflagellate).

Histopathology of juvenile oysters

The oysters filtered and ingested both microalgae in the bioassay, but those exposed to *G. catenatum* showed an inflammatory response (tissue damage). Oysters fed nontoxic control showed normal height of the absorption cells of the digestive gland tubules; no flattened epithelia were observed and the lumen of the tubular gland showed normal star-shaped morphology (Fig. 2A). Injuries and structural changes observed in the digestive gland of *C. gigas* fed mixed diets and toxic control for 14 d were cell flattening, inflammation, epithelial rupture, exfoliation and metaplasia (Fig. 2B–D). Oysters fed only toxic control showed digestive gland epithelial metaplasia, with a low cuboidal appearance of its cells accompanied by a marked dilation of the digestive gland lumen and epithelial rupture (as indicated in Fig. 3B by black unfilled arrow). In oysters fed with mixed *G. catenatum* diet 3×10^3 cells ml^{-1}, the epithelial cuboidal absorption cells showed intermediate height and detachment of some cells within the lumen of the tubules (Fig. 2C).

The animals fed with the mixed *G. catenatum* diet 30×10^3 cells ml^{-1} were the most severely affected, showing squamous appearance of the tubular epithelium. They also showed a marked height decrease in the absorption cells of the digestive gland tubules, with acute exfoliation and migration of cells into the gland lumen (Fig. 2D, solid black arrow) and metaplasia.

Other tissues were also affected by exposure to *G. catenatum* diets. The adductor muscle fibres of oysters fed nontoxic control did not show injuries (Fig. 3A), but changes were observed mainly in those oysters fed with the mixed diet of *I. galbana* (0.75×10^6 cells ml^{-1}) + *G. catenatum* (30×10^3 cells ml^{-1}); the primary lesion was oedema between fibres, showing a severe atrophy characterized by the reduction in cell size and increase of space between fibres (Fig. 3B, black arrow). The gills of oysters fed nontoxic control did not show injuries (Fig. 3C) whereas in oysters fed the mixed diet of *I. galbana* (0.75×10^6 cells ml^{-1}) + *G. catenatum* (30×10^3 cells ml^{-1}) these structures suffered damage such as loss of cilia, gill tissue inflammation due to

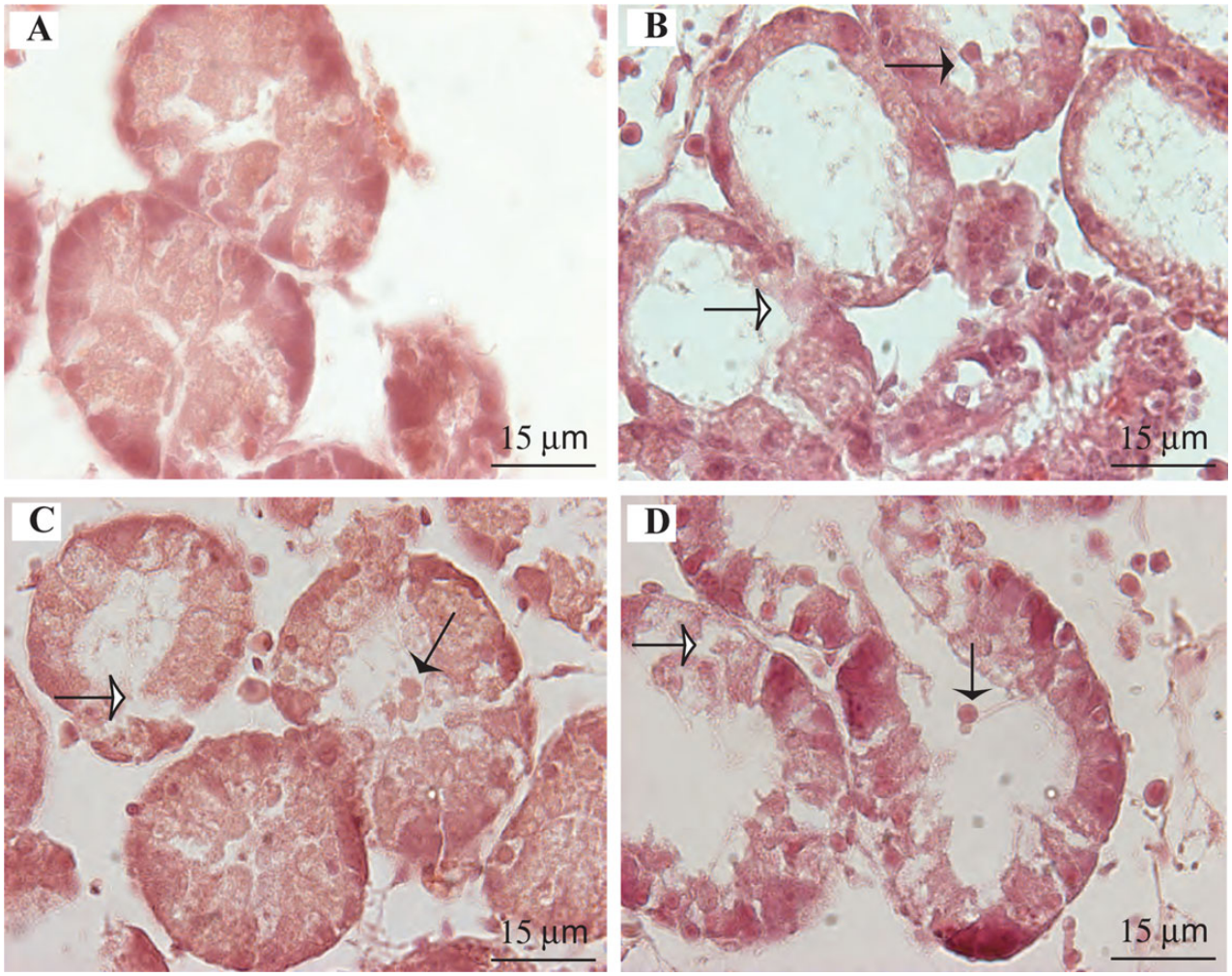

Figure 2. Transverse section of tubules of digestive gland of *Crassostrea gigas* juveniles exposed to *Gymnodinium. catenatum* (haematoxylin-eosin stain) show epithelial rupture (black unfilled arrows) and cell exfoliation (solid black arrows), respectively. **A.** Nontoxic control oysters fed with *Isochrysis galbana* (0.75×10^6 cells ml^{-1}). **B.** Toxic control fed only *G. catenatum* (3×10^3 cells ml^{-1}). **C.** Fed mixed diet of *I. galbana* (0.75×10^6 cells ml^{-1}) + *G. catenatum* (3×10^3 cells ml^{-1}). **D.** Fed mixed diet of *I. galbana* (0.75×10^6 cells ml^{-1}) + *G. catenatum* (30×10^3 cells ml^{-1}).

epithelial cell exfoliation, and disorganization of the gill arches (Fig. 3D, black arrow). Whereas the mantle of oysters fed non-toxic control did not show injuries (Fig. 3E), in oysters fed with the mixed diet of *I. galbana* (0.75×10^6 cells ml^{-1}) + *G. catenatum* (30×10^3 cells ml^{-1}), and toxic control (only *G. catenatum*), it did show damage, resulting in melanization of the mantle epithelium (Fig. 3F, with black arrow).

DISCUSSION

In spite of extensive studies of PST-mediated adverse effects, little information on its effect on gene expression is available, which could contribute to a better understanding of the mechanisms of PST-induced toxicity. From the four studied genes, we found that *Crassostrea gigas* exhibited an increase in the expression level of those genes involved in cell-cycle regulation (*p21*, *cafp55* and *p53*) and initial inflammatory process (*casp1*) in response to *Gymnodinium catenatum* exposure. These genes were selected for their physiological roles and because they could be used as a sensitive tool to detect changes when oysters are exposed to this toxic dinoflagellate. The responses were time- and dose-dependent; the changes in expression levels suggested a direct relationship with ingestion of toxic algae and diet type. Thus, including a toxic control (only *G. catenatum*) in the experimental design confirmed the impact of the dinoflagellate cells or their toxins in the activation of cell-cycle regulation genes.

In addition, the magnitude of change and particular effects were likely based on the susceptibility of each gene to the toxins or other components of the toxic dinoflagellate. In the mixed diet 30×10^3 cell ml^{-1} of *G. catenatum*, oysters could not offset the damage caused by such exposure, so the damage to tissues was major. The genes involved in cell-cycle regulation showed different expression patterns compared with other genes, principally with the two mixed diets (3×10^3 and 30×10^3 cell ml^{-1}). The highest expression levels for *p21* and cafp55 genes were mainly at 7 d.

The gene *p21* or *waf1* codes for a protein that functions as a potent cyclin-dependent kinase (CDK) inhibitor, binding to and suppressing the activity of CDK2 and CDK4 cyclin complexes and thus acting as a cell-cycle progression regulator in G1. The *p53* tumour-suppressor protein tightly controls *p21* expression, by which it mediates the G1 phase-arrest *p53*-dependent cell cycle in response to a variety of stress stimuli (Böttger *et al.*, 2008; Farcy *et al.*, 2008; Mirzayans *et al.*, 2012). The *p53* gene expression was upregulated principally after 7 d of dinoflagellate exposure. Moreover, we observed an increase in *p21* transcript level, probably due to cell-cycle arrest in the

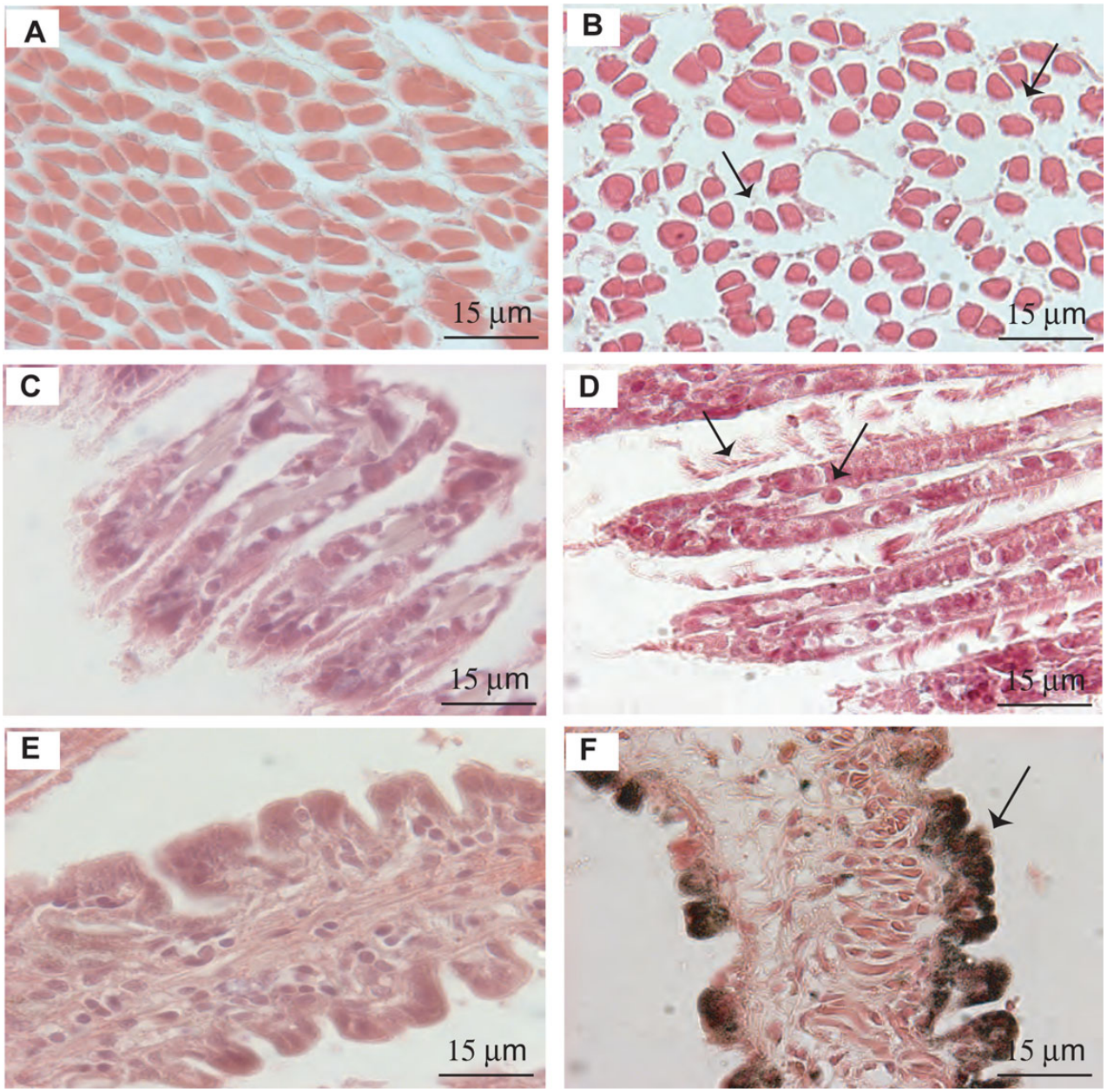

Figure 3. Sections of tissues of *Crassostrea gigas* juveniles exposed to *Gymnodinium catenatum* (haematoxylin-eosin stain). The arrows indicate tissue damage. **A, B.** Adductor muscle fibres. **A.** Nontoxic control oyster fed *Isochrysis galbana* (0.75×10^6 cells ml^{-1}). **B.** Fed mixed diet *I. galbana* + *G. catenatum* (30×10^3 cells ml^{-1}). **C, D.** Gill. **C.** Nontoxic control oyster fed *I. galbana* (0.75×10^6 cells ml^{-1}). **D.** Fed mixed diet *I. galbana* + *G. catenatum* (30×10^3 cells ml^{-1}). **E, F.** Mantle. **E.** Nontoxic control oyster fed *I. galbana* (0.75×10^6 cells ml^{-1}). **F.** Fed mixed diet *I. galbana* + *G. catenatum* (30×10^3 cells ml^{-1}).

oyster by transcriptional activation of *p21* functions to prevent replication of damaged cells. In consequence, these cells might have been involved in apoptosis; therefore, a long-term exposure to toxic dinoflagellates could cause death of the organisms; however, more studies should be performed to confirm this process.

The *cafp55* gene is a histone chaperone protein that plays a critical role in chromatin acetylation, assembly and maturation coupled to DNA replication and repair (Taylor-Harding *et al.*, 2004). It consists of three different subunits of a molecular size of 55, 60 and 150 kDa, of which the 55 kDa subunit is a component of several protein complexes involved in chromatin remodelling (Taylor-Harding *et al.*, 2004). We found that the transcript level reached a maximum value in oysters fed with the mixed diet of 30×10^3 cell ml^{-1} of dinoflagellate after 7 d of exposure. The expression level increase of the *cafp55* gene was closely related to an increased expression of *p21* and *p53* genes, since all three are indispensable for cellular replication and DNA repair mechanisms. This increase suggests an activation of the DNA repair mechanism, which could be related to injury to the tissues that were affected. The regulatory role of the assembly factors (such as *cafp55*) needs to be explored further in the establishment of these chromatin states and their inheritance through the cell cycle and development. The *casp1* gene, which plays a critical role in the execution of the inflammation process leading to apoptosis (Sokolova, 2009), was also upregulated in all treatments at 7 d. Therefore, tissue damage and haemocytic infiltration observed in oysters fed with *G. catenatum* could be involved in inflammatory response regulation, which suggests

the development of a stress syndrome in oysters due to algal toxin accumulation.

Histological examinations revealed that oysters exposed to the dinoflagellate were affected with tissue injuries, principally in the digestive gland. The alterations most commonly reported in this organ have been cellular flattening, swelling, rupture, exfoliation and metaplasia (Howard & Smith 1983; Landsberg, 2002; Galimany *et al*., 2008; Hégaret *et al*., 2012). Therefore, lesions and structural changes of the gastrointestinal epithelium are important indicators of oyster health (Howard & Smith 1983; Galimany *et al*., 2008).

Additionally, in our study changes were observed in the adductor muscle (opaque or striated) structure: oysters exposed to *G. catenatum* showed substantial reduction in muscle fibre volume (Fig. 3A). Oysters fed *I. galbana* did not show tissue injuries. Degeneration of the muscle and changes in the structure and activity has been previously reported in bivalves exposed to PST-producing dinoflagellates (Bougrier, 2003; Hégaret *et al*., 2007, 2009; Pérez-Linares *et al*., 2009; Navarro & Contreras, 2010; Haberkorn *et al*., 2014). The abnormal cell growth observed in some muscle fibres suggests an incidence of oedema, characterized as an increase in cell size.

Gill damage observed in oysters such as epithelial disruption, cilia fusion and loss, could be explained considering that gills are the first organ of direct contact with the toxic cells during the filtration processes. The greatest degree of damage in oysters exposed to *G. catenatum* was with the mixed diet of 30×10^3 cells ml^{-1} and toxic control. Another study has shown that *C. gigas* gills were affected by exposure to *Alexandrium minutum*, finding a significant increase in mucus production (Haberkorn *et al*., 2014), as we observed in oysters exposed to *G. catenatum*.

Melanization in the mantle of bivalves exposed to toxic dinoflagellates has been also been described previously (Haberkorn *et al*., 2014); evidence in this study suggested that melanin production in the different tissues probably resulted from proteolytic cascades involving humoral factors related to serine proteases, such as trypsin and chemotrypsin, which are ubiquitous enzymes involved in many physiological functions. In addition, melanin synthesis is part of the defence against pathogens (Haberkorn *et al*., 2014). Although we did not observe mortality in our experimental setup, the histopathological examination has shown that *G. catenatum* does have a detrimental effect on juvenile oysters. The toxic effects of *G. catenatum* on *C. gigas* assessed here allow us to begin to understand the cellular mechanisms involved in the response of the oysters. The response to *G. catenatum* ingestion was dependent on exposure time, cell concentrations and diet type (mixed or single dinoflagellate). The magnitude of change and the particular effects were likely based on the susceptibility of each gene to the toxins and/or other compounds present in *G. catenatum*.

In conclusion, our results show clear changes in gene expression patterns and histological evidence of tissue injuries due to dinoflagellate exposure, suggesting DNA damage through expression modulation of the analysed key genes, particularly on cell-cycle regulation. The histopathological observations show that *G. catenatum* has a detrimental effect on oysters and reveal the development of an inflammatory process probably mediated by activation of the *Caspase 1* gene. Therefore, it is important to continue research on the effects of HABs on oysters.

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