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Short Communication

Differential expression of immune response genes in Pacific oyster, *Crassostrea gigas* spat, fed with dinoflagellates *Gymnodinium catenatum* and *Prorocentrum lima*

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ABSTRACT. The effects of *Gymnodynium catenatum* and *Prorocentrum lima*, dinoflagellate species present in the Gulf of California, on marine bivalves, have demonstrated physiological alterations and changes in gene expression patterns; however, modification effects of the genes involved in immune defense are still poorly understood. This study analyzed the mRNA levels of five genes encoding for the immune defense of oyster *Crassostrea gigas* spat fed with a control diet (*Isochrysis galbana* alone) and a combination of the two toxic dinoflagellates. Expression levels of lipopolysaccharide (LPS)-binding protein 1,3-glucan and cavorting genes were higher in oysters exposed to the combined diets in all treatments compared to the non-toxic diet at day three, which was probably related with an activation of the oysters' immediate immune response during the first 24 h. Protein 44 interferon-induced gene expression level was repressed in treatments with the highest dinoflagellate concentration and overexpressed in the diet with equal dinoflagellate concentration. Interaction protein-Toll and immunoglobulin gene transcript levels reached the highest values at day seven in oysters exposed to all combined diets. Then, the immune defense appeared to be activated in oyster spat as a response of toxins and/or extra-cellular compounds produced by the dinoflagellates.

Keywords: *Crassostrea gigas*; *Prorocentrum lima*; *Gymnodinium catenatum*; dinoflagellates; gene expression; immune response

Crassostrea gigas is one of the most cultured marine species in the world (Shumway, 1990). The Pacific oyster is cultured mainly in the northwestern region of Mexico. The occurrence of harmful algal blooms (HABs) have been identified previous to mortality episodes, but a clear correlation has not been established yet (Wikfors, 2005). During blooms, the bivalve can accumulate large amounts of marine toxins, causing behavioral and physiological symptoms (Galimany *et al.*, 2008; Haberkorn *et al.*, 2010); the accumulation of diarrheic and paralytic toxins produced by dinoflagellates *Prorocentrum lima* and *Gymnodinium catenatum*, respectively, has been reported for *C. gigas* (Shumway, 1990). *P. lima* is a toxic benthic, epiphytic dinoflagellate, and known producer of diarrheic shellfish poisoning (DSP). Diarrheic shellfish toxins (DSTs) are lipophilic polyether compounds, including okadaic acid (OA), dinophysistoxin-1 (DTX1), DTX2 and other derivative forms (Blanco *et al.*, 2007). *G. catenatum* is an athecate species, chain-forming dinoflagellate, and responsible for paralytic shellfish poisoning (PSP). Paralytic shellfish toxins (PSTs) are a group of neurotoxic alkaloids, grouped into three structural families: carbamate (saxitoxin-STX, neosaxitoxin-NEO and gonyautoxins-GTX1 to GTX4), decarbamoyl and N-sulfocarbamoyl (Oshima, 1995).

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These dinoflagellate species have been associated with DSP and PSP episodes in different parts of the world, and in the Gulf of California, they have also been extensively used in aquatic toxicological studies (Pinto-Silva *et al.*, 2005; Wikfors, 2005; Flórez-Barrós *et al.*, 2011; García-Lagunas *et al.*, 2013, 2015; Romero-Geraldo *et al.*, 2014, 2016).

Molecular studies with both toxins cause differential expression level on the genes involved in immune (García-Lagunas *et al.*, 2013; Mello *et al.*, 2013; Lassundrie *et al.*, 2014, 2015a,b). In other hand, proteins of immune system (as carvotin, Interaction protein-Toll, immunoglobulin and Protein 44 interferon-induced) on oyster spats had a detectable and differentiated concentration in response to the low (3×10^2 cell mL^{-1}) and high concentrations (3×10^3 cell mL^{-1}) of *P. lima* (M.A. Matus, *pers. comm*.). In this context, genes with functions in early immune response were selected, such as toll-interacting protein (*tlp*), a ubiquitin-binding protein that interacts with TLR signaling components and is highly conserved in evolution from invertebrate to vertebrate (Zhang *et al.*, 2015); cavortin (*cvt*), which is the major hemolymph protein of the Pacific oyster, characterized as the hemocyte Cu/Zn superoxide dismutase (Lambert *et al.*, 2007; Scotti *et al.*, 2007); lipopolysaccharide and β-1,3-glucan-binding protein (*lgbp*), which is a pattern recognition protein (PRP) that recognizes lipopolysaccharide and β-1,3-glucan (βG) and subsequently triggers innate immunity (Romero-Geraldo *et al.*, 2014). Additionally, interferon-induced protein 44 gene (*if44*) that may induce a cellular GTP depletion involved in cell cycle arrest (Renault *et al.*, 2011) and immunoglobin domain (Ig) [and Leucine-rich repeat (LRR)] gene (*imm*) was selected because it is also a competent immune recognition module domain present in some proteins, which could function as immune effector or pro-inflammatory factor in oyster (Wang *et al.*, 2017). Therefore, the aim of this study was to determine the effects of the simultaneous presence of two toxic dinoflagellates, *P. lima* and *G. catenatum*, on oyster C. *gigas* spat in combined diets. The specific objectives were to 1) assess their effects related to time and dinoflagellate cell density, 2) determine oysters' feeding preference in the combined diets, and 3) examine whether or not the combined diets induced differential expression of genes involved in oysters' immune response.

Gymnodinium catenatum was reared as described in García-Lagunas *et al.* (2013). *P. lima* and *Isochrysis galbana* were grown as described in Romero-Geraldo *et al.* (2014). Diploid individuals (n = 180) of Pacific oyster spats (3-4 mm of length) were obtained from the hatchery and acclimated under controlled conditions of temperature, salinity, and oxygen for 10 days. The cell concentration of treatments was chosen by previous research by we work group (García-Lagunas *et al.*, 2013, 2015; Romero-Geraldo *et al.*, 2014). Oysters were exposed to four diets: (T1) combined diet of *G. catenatum* (3×10^3 cell mL^{-1}) and *P. lima* (3×10^2 cell mL^{-1}) and a fixed amount of *Isochrysis galbana* (7.5×10^5 cell mL^{-1}); (T2) combined diet of *G. catenatum* (1.5×10^3 cell mL^{-1}), *P. lima* (1.5×10^3 cell mL^{-1}) and *I. galbana* (7.5×10^5 cell mL^{-1}); (T3) combined diet of *G. catenatum* (3×10^2 cell mL^{-1}) and *P. lima* (3×10^3 cell mL^{-1}), *I. galbana* (7.5×10^5 cell mL^{-1}); and (T4) a non-toxic control diet of *I. galbana* alone (7.5×10^5 cell mL^{-1}). Groups of 50 oysters (in triplicate) were maintained in 100 mL transparent polypropylene containers with a 1:1 microalgal mixture in a final volume of 50 mL. The microalgal ration was provided each day as a single dose; aeration was used during feeding experiments to avoid cell sedimentation. Five organisms of each experimental unit were randomly sampled after one, three, five and seven days. The samples were placed on Eppendorf® tubes, washed with sterile seawater and frozen at -80ºC until use. The number of filtered cells (FC) by the oysters was calculated using the Sedgwick-Rafter and hemocytometer counting chambers and optic microscope (Olympus BX41, Tokyo, Japan); the number of filtered cells was expressed as cell mL^{-1} (Fig. 1). Samples were thawed on ice, and total RNA was extracted with TRI Reagent® (Life Technologies, Carlsbad, California). Samples (n = 5) were homogenized using a glass pestle. RNA quality and cDNA synthesis were verified and performed as described in García-Lagunas *et al.* (2013). Partial sequences of *C. gigas* were used to design a set of five primer pairs. The efficiency of each primer pair was determined using the standard curve method (Table 1). All qPCR reactions were conducted as described in García-Lagunas *et al.* (2013). The comparative CT method (Livak & Schmittgen, 2001) was used to analyze gene expression levels. Data were analyzed using one-way ANOVA; significant differences were obtained with Fisher's multiple test comparison (α = 0.05). All analyses were performed with Statistic 8.0® software (StatSoft, Tulsa, Oklahoma).

The feeding behavior of C. *gigas* fed only with *I. galbana* diet maintained normal and completely consumed their food after three hours; the same behavior was observed during the experiment. However, the oysters did not wholly consume to *I. galbana* in T2 and T3 at 24 h (Fig. 1a), because the oysters fed the combined dinoflagellate diet showed a low feeding activity and production of pseudo-feces containing intact *P. lima* cells when the dinoflagellate was present. The oysters showed a preference for *G. catenatum* cells

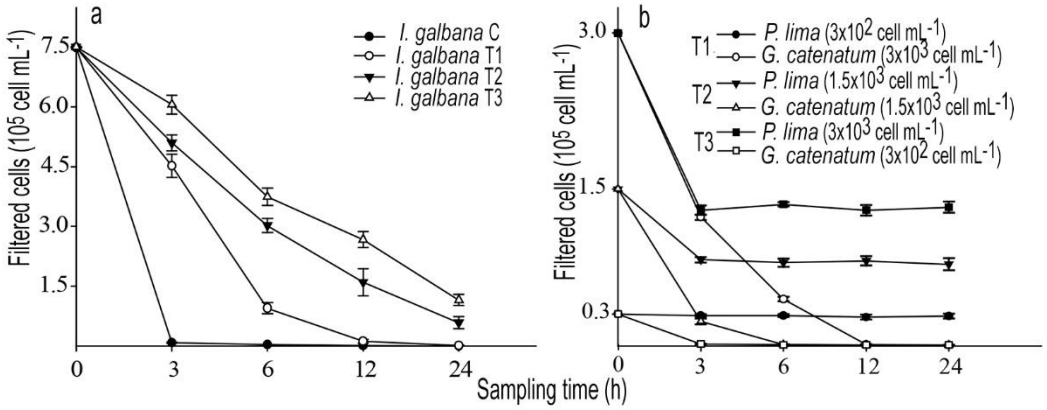

Figure 1. Consumption of control microalga and dinoflagellate cells by *C. gigas* in terms of filtered cells (FC) per mL in all treatments at 24 h of exposure; Bars represent mean ± SD.

Table 1. Primer sequences, expected amplicon size and efficiency corresponding to target (immune response) and reference genes* from *Crassostrea gigas* used for gene expression analysis after challenge with *Prorocentrum lima* and *Gymnodinium catenatum*. Fw: forward, Rv: reverse.

Name	Sequence 5´-3´	Gene	PCR efficiency	Access number GenBank
lgbp-Fw	TTGTCCAGTTCTCCCAGCTTCC	LPS binding protein and β-1,3 glucan	1.95	CB617438
lgbp-Rv	GACACTGGAATGGGATGAAGAAC			
cvt-Fw	ATCTCCATCTCCATGACGACG	Cavorting	1.90	AY256853
cvt-Rv	CGATGACAGCGGTATGAGAGG			
if44-Fw	CATACTGGACGGCAACATACC	Interferon-induced protein 44	1.92	FJ440108
if44-Rv	ATGAATCCTGTCCCCGAGAT			
tlp-Fw	GGCTTTCTCTATCCGTGGTA	Toll-interacting protein	1.85	EKC34473
tlp-Rv	GTTCTTGGCACCGTTGTAAG			
imm-Fw	ATTTACAGCCGCTCCCATTCT	Inmunoglobulin	1.89	EU678312
imm-Rv	GCATCTCATTCGGTAAGGACTG			
ef1-Fw*	ACCATACAGTGAGGCTCGATTC	Elongation factor 1-alpha	1.98	AB122066
ef1-Fw*	GTGGAAGCCTCAATCATGTTATC			
gapdh-Fw*	GTTCAAATATGATTCAACTCACGG	Glyceraldehyde 3 phosphate dehydrogenase	2.0	AJ544886
gapdh-Rv*	TGGATCCCGTTCGCAATATACG			

in T1 treatment (Fig. 1b). The cellular concentration of *G. catenatum* decreased at 12 h while that of *P. lima* was stable until 24 h (T1). Similarly, when the concentration of *G. catenatum* and *P. lima* was equal (T2), *C. gigas* preferred to feed on *G. catenatum* and completely consumed it at six hours (Fig. 1b) while *P. lima* cell concentration was stable until 24 h; the same behavior was observed during the experiment. Similar feeding behavior of *C. gigas* was observed in T3, in which the oysters preferred to feed on *G. catenatum* (Fig. 1b).

The expression level for *if44* gene in the diet with *G. catenatum* > *P. lima* (T1) significantly ($P < 0.05$) down-regulated during the experiment on *C. gigas*. The diet with equal cell concentration of *G. catenatum* and *P. lima* caused a significant ($P < 0.05$) increase from day three (Fig. 2a). The *cvt* gene expression level was constant until day three when it decreased significantly ($P < 0.05$) in all combined diets, below the control diet (Fig. 2b). For *lgbp* gene, the expression level upregulated until day three in all combined diets; after that, the expression level decreased significantly ($P < 0.05$) except in T2. The treatment with *G. catenatum* < *P. lima* (T3) provoked mostly a decrease in the expression of *lgbp* (Fig. 2c).

Notably, the *imm* gene showed a down-regulation below the control diet on the first day, and increased significantly ($P < 0.05$) in expression level (twice more than the control level) in all combined diets at day seven (Fig. 2d). For the *tlp* gene the expression pattern was similar, with a notable decrease in expression level at day one of exposure to combined diets and an increase

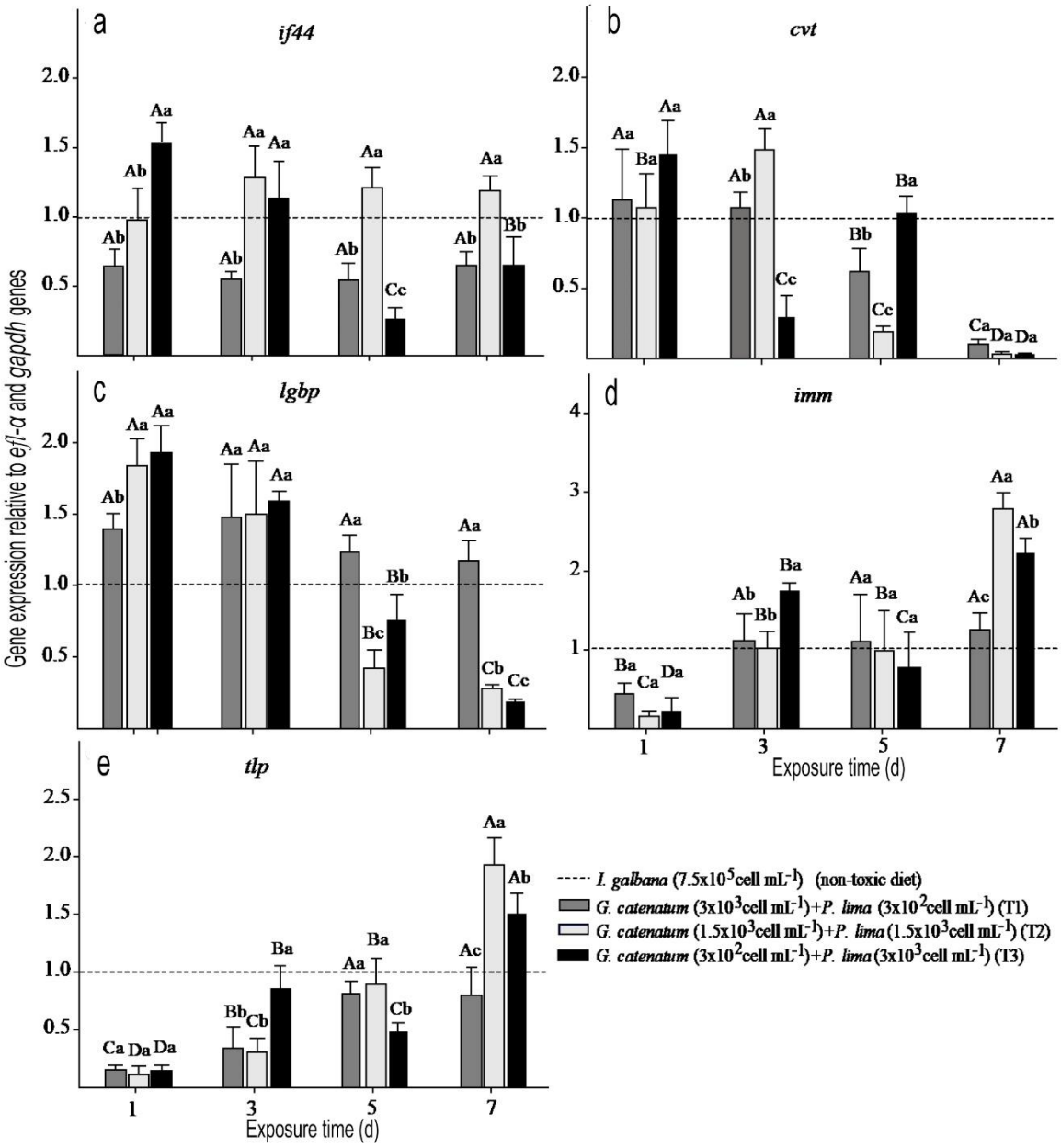

Figure 2. Expression level for *if44*, *cvt*, *lgbp*, *imm*, *tlp* genes respectively, in *Crassostrea gigas* spat fed with *Isochrysis galbana* (7.5×10^5 cell mL^{-1}) non-toxic diet and mixed diets of *Gymnodinium catenatum* and *Prorocentrum lima* cells. *G. catenatum* (3×10^3 cell mL^{-1}) + *P. lima* (3×10^2 cell mL^{-1}) (T1); *G. catenatum* (1.5×10^3 cell mL^{-1}) + *P. lima* (1.5×10^3 cell mL^{-1}) (T2), and *G. catenatum* (3×10^2 cell mL^{-1}) + *P. lima* ($3\times x10^3$ cell mL^{-1}) (T3). Bars represent mean ± SD. Values in column denoted by different lowercase letters indicate significant differences among the treatments, while different capital letters indicate significant differences among each exposure time (one-way ANOVA, Fisher´s HSD test, $P < 0.05$). The values with same letters show non-significant differences at $P < 0.05$.

of expression level at day seven in combined T2 and T3 diets (Fig. 2e).

When *C. gigas* was fed on *P. lima* and *G. catenatum* (T1, T2 and T3 treatments) besides *I. galbana*, oysters changed food preferences. The feeding behavior experiments showed that *C. gigas* preferred to be fed on *G. catenatum* than *P. lima*. In the three treatments, the concentration of *P. lima* cells was stable from three hours to the end of the experiment (Fig. 1b) and also provoked the production of pseudo-feces containing

Table 2. Summary of one-way ANOVA for gene expression levels involved in immune response from *Crassostrea gigas* with diets (treatments) and exposure time as factors.

Source of variation	Treatment and exposure time	
Gene	F-value	*P*-value
if44	80.99	<0.0000
Cvt	15.4	0.02
Lgpb	7.96	0.013
Imm	11.02	0.000
Tlp	4.17	0.033

intact *P. lima* cells. The presence of dinoflagellate cells in feces is due to digestion activity decrease (Bauder *et al.*, 2001), so the decrease of the digestion activity of *C. gigas* on *P. lima* could be related to the thecal valve and toxicity of this dinoflagellate species. Possibly, *C. gigas* was more affected by DSP toxins of *P. lima* related to PSP. Factors range from sensitivity, accumulation and elimination of the toxin by the bivalve, density of toxin-producing algae, exposure time and metabolic and structural consequences caused by the accumulation of toxins in bivalves (Bauder *et al.*, 2001; Blanco *et al.*, 2007).

The analysis of our results showed that the expression level in the studied genes was affected by cell concentration of the toxic dinoflagellates, as well as exposure time on *C. gigas* spat. These observations were consistent with those reported by other authors Romero-Geraldo & Hernández-Saavedra, 2014; García-Lagunas *et al.*, 2013, 2015; Romero-Geraldo *et al.*, 2014, 2016). The expression level of *cvt* and *lgbp* gene showed a similar tendency, up-regulated for the three treatments at day three and down-regulated until the end of the experiment, indicating that DSP and PSP toxins inhibited the expression of these genes after five days of feeding. This observation was consistent with previous findings in the working group (García-Lagunas *et al.*, 2013; Romero-Geraldo *et al.*, 2014). Early activation of the immune response can be seen in the overexpression of these genes at first days. The *lgbp* gene plays a crucial role in the innate immunity of invertebrates (Girón-Perez, 2010), which suggests these genes could play a critical role in *C. gigas*-*G. catenatum*-*P. lima* interaction, allowing oysters to identify dinoflagellates as foreign or invasive elements. Carvotin gene in bivalves has been studied little. This study is the first report that has addressed the expression level of this gene about harmful algae.

For *imm* and *tlp* genes, the tendency of expression was similar in T1 and T3, although the expression level was higher in T3. On the contrary, in T2 (*G. catenatum* = *P. lima*), these genes showed a different tendency with high expression at day seven. The presence of immune response could also be suggested by the overexpression of *imm* and *tlp* genes, as a result of a synergistic effect when both toxic dinoflagellates were present. Studies have suggested that bivalves, and principally bivalve cells of the immune system, can be adversely affected by HAB (Galimary *et al.*, 2008; Lassudrie *et al.*, 2015b). In some cases, a clear modulation of hemato-immunological parameters was observed, especially cellular immune response although most of these studies were performed under controlled laboratory conditions (Ferraz-Mello *et al.*, 2010; Haberkorn *et al.*, 2010; Mello *et al.*, 2013; Lassudrie *et al.*, 2015a). Little is known about the effect of harmful algae and their phycotoxins on responses in the immune system on oyster adults and less in oyster spats, due to the small size of spat, the study of immunity at the cellular level is challenging.

Then probably the co-occurrence of the two dinoflagellates could promote an increased susceptibility to infections in *C. gigas* spats. However, the possible molecular mechanism triggered by dinoflagellate toxins in mollusks is still not understood. Given that HAB may continue to intensify in the future (Hallegraeff, 2010), the co-occurrence of HAB and their toxins may become more common, so more research is imperative, mainly due to the unknown additive effects of multiple marine toxins on bivalve health.

Based on the results of this study, oyster *C. gigas* spats behaved differently in the presence of *P. lima* and *G. catenatum*. The simultaneous presence in combined diets of two dinoflagellates generated change in feed behavior of oysters with higher preference for feeding *G. catenatum*. The consumption of toxic dinoflagellates by oyster caused changes at the expression level of the genes involved in the immune response. The expression level was affected by cell concentration of the dinoflagellates, as well as exposure time when the immune response in oyster spats was probably activated for the defense of PST or DST toxins and/or extra-cellular compounds produced by dinoflagellates.

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