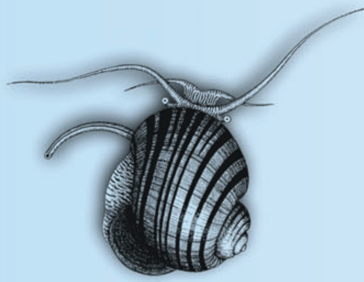

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TRANSCRIPTIONAL RESPONSES OF *MYTILUS CHILENSIS* EXPOSED *IN VIVO* TO SAXITOXIN (STX)

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ABSTRACT

Saxitoxin (STX) is one of the main phycotoxins that contribute to paralytic shellfish poisoning (PSP). This toxin is mainly produced by marine microalgae of the genus *Alexandrium*. There is evidence of changes at the physiological level in bivalves exposed to STX, although transcriptional responses to STX in bivalves have not yet been studied. The present work evaluates the expression of 13 candidate genes associated with cellular stress and immune response in *Mytilus chilensis* exposed to STX. Analysis by qPCR showed higher gene transcription levels in *M. chilensis* injected with STX than in control mussels. High levels of differential gene expression were observed for superoxide dismutase, catalase, ferritin and heat-shock protein genes. To a lesser extent, ependymin, fibrinogen-like, galectin and mytilin B genes were also significantly more expressed in haemocytes of mussels injected with STX that in control mussels. Our results provide insights into how marine toxins could modulate the innate immune system of marine invertebrates.

INTRODUCTION

Saxitoxin (STX) is a marine toxin produced by diverse species of dinoflagellates that may have detrimental effects in marine species and in organisms found at higher levels of the food chain, such as humans, causing paralytic shellfish poisoning (PSP). This group of toxins is mainly produced by dinoflagellates of the genera *Alexandrium* and *Gymnodinium* (Lagos, 1998; Rodrigues *et al.*, 2012) and corresponds to tetrahydropurins, of which 26 structural analogues are known (Wang, 2008). Saxitoxin is the most representative cause of PSP, its lethality being conferred by a mechanism mediated by carbamate (Vale, 2010). Harmful algal blooms have become more frequent in recent decades (Klemas, 2012) and the number of reports of PSP in natural populations has likewise increased.

Suspension-feeding organisms such as bivalve molluscs can concentrate and bioaccumulate toxins such as STX by ingesting dinoflagellates (Estrada *et al.*, 2010). The capacity to bioaccumulate and respond to toxins differs in a species-specific manner among bivalves and the harmful microalgae on which they may feed (Cembella, Shumway & Lewis, 1993; Nagai, Yasumoto & Hokama, 1996; Smolowitz & Shumway, 1997; Shumway, 2006; Hégaret *et al.*, 2007a, b). These relationships suggest that bivalve molluscs are more than simple transference vectors of phycotoxins to humans or other feeders at higher trophic levels. In fact, it has been reported that some types of marine toxins can reduce growth and reproduction rates of bivalves, and could be lethal for some species (Shumway, Burkholder & Springer, 2006; Samson, Shumway & Weis, 2008). In addition, sublethal physiological responses have been documented among bivalve molluscs exposed to toxic microalgae or to their toxins, such as lower filtration rates and clearance rates, as well as changes in feeding and respiration (Morono *et al.*, 2001; Blanco *et al.*, 2006). STX has been described as a neurotoxin that specifically targets the voltage-dependant sodium channel, causing a blockage of sodium conductance in nerve and muscular fibres (Narahashi, 1988) by preventing depolarization and thus transmission of nerve impulses, resulting in neuromuscular paralysis and metabolic stress. On the other hand, more recent studies have shown that this toxin can also block voltage-dependent calcium and potassium channels (Llewellyn, 2006). At the physiological level, for bivalve molluscs this implies changes in valve closure, paralysis of the adductor muscle, mantle retraction, mucus production and changes in cardiac activity (Perovic *et al.*, 2000; Estrada *et al.*, 2010).

There are various hypotheses on how bivalves cope with toxic damage. These include the use of lipid peroxidation as a means

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of detoxification (Galimany *et al*., 2008), as well as interspecific genetic variations that interfere with the capacity for accumulation and sensitivity of toxins (Bricelj *et al*., 2005). Changes in immunological parameters suggest that bivalves have the capacity to control the effects of ingesting toxic dinoflagellates, including changes in haemocyte populations, the number of haemocytes in the haemolymph and their infiltration into target organs such as gonadal follicles and the adductor muscle (Hégaret *et al*., 2007a, b; Haberkorn *et al*., 2010a, b). Additionally, the induction of inflammatory responses has been observed, with evident haemocyte degranulation and diapedesis, with subsequent formation of insoluble granules of lipofuchsin in the alimentary canal, which allows the elimination of microalgae and toxins through faeces (Estrada *et al*., 2007; Galimany *et al*., 2008). These background studies suggest that haemocytes could be involved in the immune defence against marine toxins. Nevertheless, knowledge regarding the mechanisms of such defence by haemocytes at the molecular level is scarce.

Mussels of the genus *Mytilus* have been used as the main sentinel bivalve species for monitoring marine environments in relation to the presence of natural and industrially produced toxins. Previous studies have been conducted to characterize candidate genes obtained from sequenced clone libraries that are involved in physiological responses of interest, and in the evaluation of gene expression levels (Venier, 2003). Biomarkers have been used to assess the physiological response of mussels to high concentrations of metals (Dondero *et al*., 2006a, b) and microarrays developed to assess the expression of hundreds of genes in mussels in response to mercury and crude oil contamination in sea water (Dondero *et al*., 2006a, b). From these studies it has been possible to identify a complex repertoire of genes associated with innate immune responses in bivalves. However, important questions remain unsolved about the effects of STX on bivalve transcriptomes. For instance, are there specific patterns of immune-related and/or stress-related genes involved against STX exposure? The objective of this study was to assess the expression of 13 candidate genes in the haemocytes of the mussel *Mytilus chilensis* exposed to STX at different times, as a first approximation to the study of transcriptional changes in response to this toxin.

MATERIAL AND METHODS

Samples and experimental design

Thirty-six individuals of *Mytilus chilensis* were collected from Laraquete (37°9′S, 73°11′W), Chile. The specimens ($n = 16$ per experimental group) were acclimatized for 7 d at 10°C with constant aeration. The average weight of the mussel tissue content was 3 g and their average shell length was 6 cm. The challenged groups were injected with 150 μl of an extract of *Alexandrium catenella* at a concentration of 15.9 μg equivalents of STX ml^{-1} in the adductor muscle. The concentration used was similar to the international regulatory limit of 80 μg of STX 100 g^{-1} of wet meat. Control groups were injected simultaneously with only the buffer vehicle (3 mM hydrochloric acid). Samples were collected after: (1) a short time period, sampling mussels at 4 and 6 h post-injection (hpi), and (2) after a longer exposure duration, sampling at 48 hpi. At each sampling time, four replicates were included and each replicate was treated separately. Four non-injected individuals were also sampled after the acclimatization period (time = 0 h).

RNA extraction and cDNA synthesis

At the corresponding sampling times, approximately 1 ml of haemolymph was collected from the adductor muscle of each mussel using a sterile 1-ml syringe. The haemolymph of each individual was centrifuged at 1200 rpm for 20 min at 4°C to separate the haemocytes from the aqueous material. Supernatants were then discarded and 1 ml of Trizol® reagent (Invitrogen, Life Technologies, San Diego, USA) was added. Samples were then stored at −80°C until all sampling times were completed. Stored samples were lysed in a Retsch MM 200 Mixer Mill (Retsch Inc., Düsseldorf, Germany) at 20 Hz for 5 min and the standard protocol for RNA extraction using Trizol® reagent was carried out following the manufacturer's instructions. The phases were separated with 100% chloroform and nucleic acid was precipitated with 100% isopropanol after cooling at −20°C. The extracted total RNA was washed repeatedly with 75% ethanol cooled to −20°C and then treated with DNAses (DNAse I, Thermo Scientific, Wilmington, USA) to eliminate genomic DNA contamination. The RNA concentration and purity was measured in a ND-1000 spectrophotometer (Nanodrop® Technologies, Thermo Scientific), and its integrity visualized by electrophoresis in a 1.2% MOPS-agarose gel under denaturing conditions. Finally, the purified RNA was stored at −80°C for later use. The first cDNA strain was retro-transcribed from 200 ng of purified RNA using the RevertAid H Minus First Strand cDNA Synthesis kit (Thermo Scientific) in accordance with the manufacturer's instructions. This process included the addition of ribonuclease inhibitors to avoid RNA degradation used in the synthesis.

Selection and standardization of candidate genes

Thirteen candidate genes were selected, some of which had previously been reported to present differential responses to thermal stress (*HSP90*, *HSP70*), metal contamination (*ferritin*, *metallothionein*) oxidative stress (*SOD*, *CAT*) and pathogens (*galectin*, *PRGP*, *Myt A*, *MytB*, *LITAF*, *fibrinogen-like*, *ependymin*) (Núñez-Acuña *et al*., 2012). *Elongation factor 1*, *28S ribosomal unit*, *18S ribosomal unit* and *α-tubulin* genes were assessed as endogenous controls. Primers were designed with the commercial software Geneious v. 5.1.7 (Biomatters Ltd.®, Auckland, New Zealand) in cases where no primers had been previously described for selected genes. PCR reactions were carried out using 1.5 U of the Taq polymerase enzyme (Fermentas, Thermo Scientific) in a reaction of 25 μl that included 1× enzyme buffer, BSA (0.2 μg μl^{-1}), $MgCl_2$ (1.5 mM) and the respective sense and antisense primers at 500 nM. PCR cycles consisted of an initial denaturing run of 5 min at 95°C, followed by 35 denaturing cycles at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s and a final extension run at 72°C for 7 min. Amplification products were visualized on agarose gels stained with ethidium bromide. For all the primers used, either designed in this study or cross-amplified from other species, we confirmed successful amplifications by size and existence of a sole band through the electrophoresis. Table 1 presents candidate genes including their molecular functions and the primers used in this study with their respective sequences.

Analysis of gene expression with qPCR

Dynamic ranges analyses were conducted to obtain qPCR reactions efficiencies. The calibration curve for these runs consisted of five serial dilutions to a 200 ng of cDNA stock with a serial factor of 1:5, which were amplified with primers for each target gene (see obtained qPCR efficiencies in Supplementary Material 1). Then, to determine the best reference gene, the four candidate housekeeping genes were amplified in six samples to assess their stability index. ΔCt values of these genes were introduced into the bioinformatic tool Normfinder (Andersen *et al*., 2004) and stability analysis was conducted.

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Table 1. Candidate transcripts used in this study. Genbank/Mytibase accession numbers, biological functions and primers used.

Gene	Genbank/ Mytibase Accession number	Molecular function	Primers (5′ to 3′)	Source species	Reference
HSP70	HQ693569	Molecular chaperone	TCAAGCACACAAGCAAGTGTTG GATCTGCATTCAATTCCTCAAACC	*M. chilensis*	Núñez-Acuña *et al.*, 2012
HSP90	HQ693565	Molecular chaperone	AGCCCCATTTGACATGTTTGA TGGGATGAGGTCTTCACAATTGT	*M. chilensis*	Núñez-Acuña *et al.*, 2012
Catalase	HQ693566	Hydrogen peroxide catalysis	CCAGGTGTCCTTCCTGTTTTCT TGTCCATCCTTGTTGACTGTCTTAA	*M. chilensis*	Núñez-Acuña *et al.*, 2012
Ec-SOD Cu/Zn	HQ693564	Dismutation of superoxide catalysis	TCGCTTTCAGTCAACAGAATGG CCAAACTCGTGAACGTGGAA	*M. chilensis*	Núñez-Acuña *et al.*, 2012
Ferritin	HQ693568	Iron storage and metabolism	CAAGTGAACGCCATCAAGGA CGTTGATGCTCTCTTTGTCATACA	*M. chilensis*	Núñez-Acuña *et al.*, 2012
Metallothionein	AJ007506	Heavy-metal binding	CTGTGGTGACGCCTGCAAGT ACACGTTGAAGGTCCTGTACATCCT	*M. edulis*	Primers designed in this study
Mytilin B	HQ693567	Antimicrobial peptide	TGAGGCAGAGGCAAGTTGTG GCAGTAGCAACGCCCTCTGTA	*M. chilensis*	Núñez-Acuña *et al.*, 2012
Myticin A	AF162334	Antimicrobial peptide	CGGAAGCTCATTCGCACGCT TGCGCACATTTTACCGGGATGG	*M. galloprovincialis*	Primers designed in this study
Fibrinogen-like	MGC04809 (Mytibase)	Fibrin precursor	TGGAGGAAATGCGGGTGATGGT CCCTTGTTTCTGGCAGCGCA	*M. galloprovincialis*	Primers designed in this study
LPS-induced TNF alpha factor	MGC06663 (Mytibase)	Cytokine	GTTGGACAGCCGCAGACGTT CCGCCACTATCCAAGCCATCG	*M. galloprovincialis*	Primers designed in this study
Peptidoglycan recognition protein	MGC04209 (Mytibase)	Recognition of pepidoglycan	TGGACAATCGAACATGGGACGA CCGTACGTATGTGCCCCGAC	*M. galloprovincialis*	Primers designed in this study
Galectin	MGC08887 (Mytibase)	Lectin pathway	GCAAGCATGGCACAACCTGC AGGCGGGTTCATCATTGGTGC	*M. galloprovincialis*	Primers designed in this study
Ependymin	MGC03478 (Mytibase)	Cellular regeneration	ACTGGAGGTGCAGTCGAGGG GCGGGTTCCCATGTTTTGTCCT	*M. galloprovincialis*	Primers designed in this study
Elongation factor 1	AY580270	Translation factor	CCAGAAGGAAGTCAGCAGTTAC TGTTGTCTCCATGCCATCC	*M. edulis*	Núñez-Acuña *et al.*, 2012
28S ribosomal unit	Z29550	Ribosomal unit	TCCCGAGACCGAGGATTTGC GTTGCTTCCCGACAGATCGC	*M. edulis*	Núñez-Acuña *et al.*, 2012
18S ribosomal unit	L33448	Ribosomal unit	TCGATGGTACGTGATATGCC CGTTTCTCATGCTCCCTCTC	*M. edulis*	Núñez-Acuña *et al.*, 2012
α-tubulin	HM537081	Microtubule formation	GAGCCGTCTGCATGTTGAGC TGGACGAAAGCACGTTTGGC	*M. galloprovincialis*	Núñez-Acuña *et al.*, 2012

Subsequently, gene expression levels were assessed by the comparative ΔΔCt method with three replicates per sample and the target genes were normalized with the selected endogenous control, *α-tubulin* (see Supplementary Material 2). The runs were made with the Maxima SYBR-Green/Rox qPCR Master Mix 2X kit (Fermentas, Thermo Scientific) according to the manufacturer's instructions in a StepOne Plus Mastercycler (Applied Biosystems®, Life Technologies). A concentration of 500 nM was used for both primers in all the runs, except for the *galectin* gene, which was successfully amplified with 250 nM of each primer. The initial quantity of cDNA used for each reaction was 80 ng based on the results for the dynamic ranges. The PCR programs were conducted in the same way as the endpoint PCR analysis described above, except that the annealing and extension steps were combined into one single step of 60 s at 60°C, following a holding step at 95°C for 10 min to activate the enzyme. After PCR reactions a melting curve was performed in the same instrument to verify the presence of a single amplification product and the absence of contaminations in the negative controls.

Statistical analysis

Data were exported from Microsoft Excel (v. 2011, Microsoft®, USA), in which data per sample and biological groups (experimental and control) were reordered and the RQ value was manually calculated using the ΔΔCt formula (Livak & Schmittgen, 2001). Subsequently, the data were exported to Statistica software (v. 8.0, Statsoft®, Tulsa, USA), in which the distribution of the data and significant differences were evaluated by one-way ANOVA tests for parametrically distributed data and Kruskal-Wallis tests for data that did show nonparametric distribution. In both cases a value of $P < 0.05$ was established for significant differences among groups. To determine the distribution of the data, a Shapiro-Wilk test was conducted for each analysed gene.

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RESULTS AND DISCUSSION

One of the main questions concerning marine bivalves is how they respond successfully to different sources of stress, which in some cases could be lethal for other species, such as toxins produced in harmful algal blooms. Among these toxins, STX is perhaps the most lethal, but little is known about the effect that STX produces at the molecular level in species that accumulate toxins, such as edible marine bivalves. Therefore, to determine if exposure to STX produces changes at the transcriptional level in *M. chilensis*, we evaluated 13 genes associated with thermal stress (*HSP90*, *HSP70*), response to metal contamination (*ferritin, metallothionein*), response to oxidative stress (*SOD*, *CAT)* and response to pathogens (*galectin*, *PRGP*, *MytA*, *LITAF*, *fibrinogen*, *MytB*, *ependymin*). Study of the gene expression of these transcripts could at least suggest potential relationships based on the timing when mRNA abundance increases. Then, approximations to physiological responses could be generated knowing the functions of the genes triggered by exposure to STX. Herein, the transcription levels of these 13 candidate genes in response to STX were evaluated through qPCR assays. We calculated the reaction efficiencies of each target gene (Supplementary Material 1) and the best of four endogenous reference genes to normalize the Ct values, based on their stability values (Supplementary Material 2). The transcriptional response of the 13 target genes was thus normalized using the endogenous control α-tubulin.

In order to assess the transcriptional responses of these genes to STX exposure, we performed an experiment sampling at different durations to assess short-term effects and effects after 2 d. For almost all genes tested, the experimental group exposed to STX had a higher level of expression than the control groups. Levels of mRNA expression after short-term exposure (4 h and 6 h) were 2.8 to 48 times higher in the groups treated with STX than in the control groups. This difference decreased to 2.1 times over the longer exposure at 48 h, although significant differences ($P < 0.05$) could still be observed between the control and challenged groups (Fig. 1). A few studies have already assessed the global transcriptome and/or punctual transcriptional response of bivalves to marine toxins, such as the number of genes expressed and over-expressed at different exposure times (Manfrin *et al.*, 2010) or each gene individually at each exposure time (Brown *et al.*, 2006). In this work, we approached the sum of the changes in the relative expression of the 13 candidate genes. This approach highlights a generalized higher transcriptional response in STX-challenged mussels. However, to assess the relationships among the genes being expressed and their biological functions in response to STX exposure, it is also necessary to identify each of their individual levels of expression.

When individuals were exposed to STX, molecular chaperone genes involved in the response to thermal stress (*HSP90* and *HSP70*) showed the highest average gene expression levels of genes among the 13 candidate genes (Fig. 2); they were also the most over-expressed after injection of STX. These genes have been widely studied as biomarkers in response to the marine environment and, for some toxins (Gibney, Gault & Williams, 2001; Wang *et al.*, 2008; El Golli-Bennour & Bacha, 2011; Jiang *et al.*, 2012), show an increase in their transcription. These proteins perform molecular functions associated with the correct folding of denatured peptide chains, which has several implications at the biological level (Macario & de Macario, 2007). In this context, our results are consistent with results on the effect of brevetoxin on oysters (Mello *et al.*, 2012), showing a similar pattern in *HSP70* gene in response to STX. A recent study also showed a two-fold increase of the level of expression of *HSP70* in *Crassostrea gigas* oysters exposed *in vitro* to STX (Mello *et al.*, 2013). However, not all these differences are similar, due to either different toxin isoforms or modes of exposure, as well as different candidate genes.

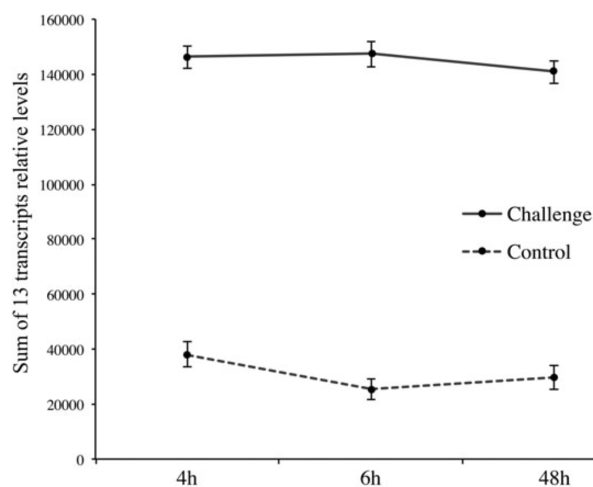

Figure 1. Sum of relative transcript levels for 13 genes in individuals of *Mytilus chilensis* exposed to short-term (4h, 6h) and longer treatments (48h) with STX. Error bars are standard errors.

The second group of genes highly expressed in mussels exposed to STX was of genes involved in the metal response, such as *ferritin* and *metallothionein*. The *ferritin* gene encodes for the main iron-storage enzyme (Orino & Watanabe, 2008). Increases in the levels of expression of this gene were expected, given the findings of studies that demonstrate the response to toxic compounds such as pesticides (Collin *et al.*, 2010) or brevetoxins (Walsh *et al.*, 2010). Nevertheless, it should be considered that this gene responds to other stress factors, such as thermal stress (Salinas-Clarot *et al.*, 2011). The expression level of the *metallothionein* gene did not differ significantly between the control and the exposed group, regardless of the time after injection. The *metallothionein* gene protects the cell from oxidative stress that can be caused by the presence of heavy metals (Sato & Kondoh, 2002) and has previously been used as a gene marker for marine pollution in the northern Adriatic Sea (Mićović *et al.*, 2009). These results suggest the potential specificity of the *metallothionein* gene to metal response (or other stressful factors) in this species, and are not related to the immune response against STX. In contrast, *ferritin* overexpression was maintained after long STX exposure. This could be explained by the multiple biological functions of the *ferritin* gene, which is also involved in iron accumulation, conferring a role in oxidative stress response (Watt, 2011), as shown in the scallop *Argopecten purpuratus*, in which the *ferritin* gene was over-expressed under experimental exposure of individuals to copper that could increase levels of reactive oxygen species (ROS) (Zapata *et al.*, 2009).

Catalase (*CAT*) and *SOD*, two widely studied genes in response to oxidative stress, also showed a high increase of gene transcription when mussels were injected with STX. *SOD* and *CAT* are two genes equally associated with metal stress in marine environments (Lee *et al.*, 2008) and also potentially related to the innate immune system in mussels (Canesi *et al.*, 2010). These genes encode for two enzymes, SOD and CAT, capable of decreasing the damaging effects of ROS, and thus considered as the primary antioxidant enzymes. A recent study showed an increase of the production of ROS in *C. gigas* exposed to *A. minutum* (Haberkorn *et al.*, 2010a, b). Nonetheless, this is the first time that an increase in the expression levels of antioxidant genes has been reported in response to STX injection in marine bivalves. Our results thus highlight the physiological disruption caused by an injection of STX in mussels and suggest the importance of SOD and CAT in the elimination of ROS, as well as

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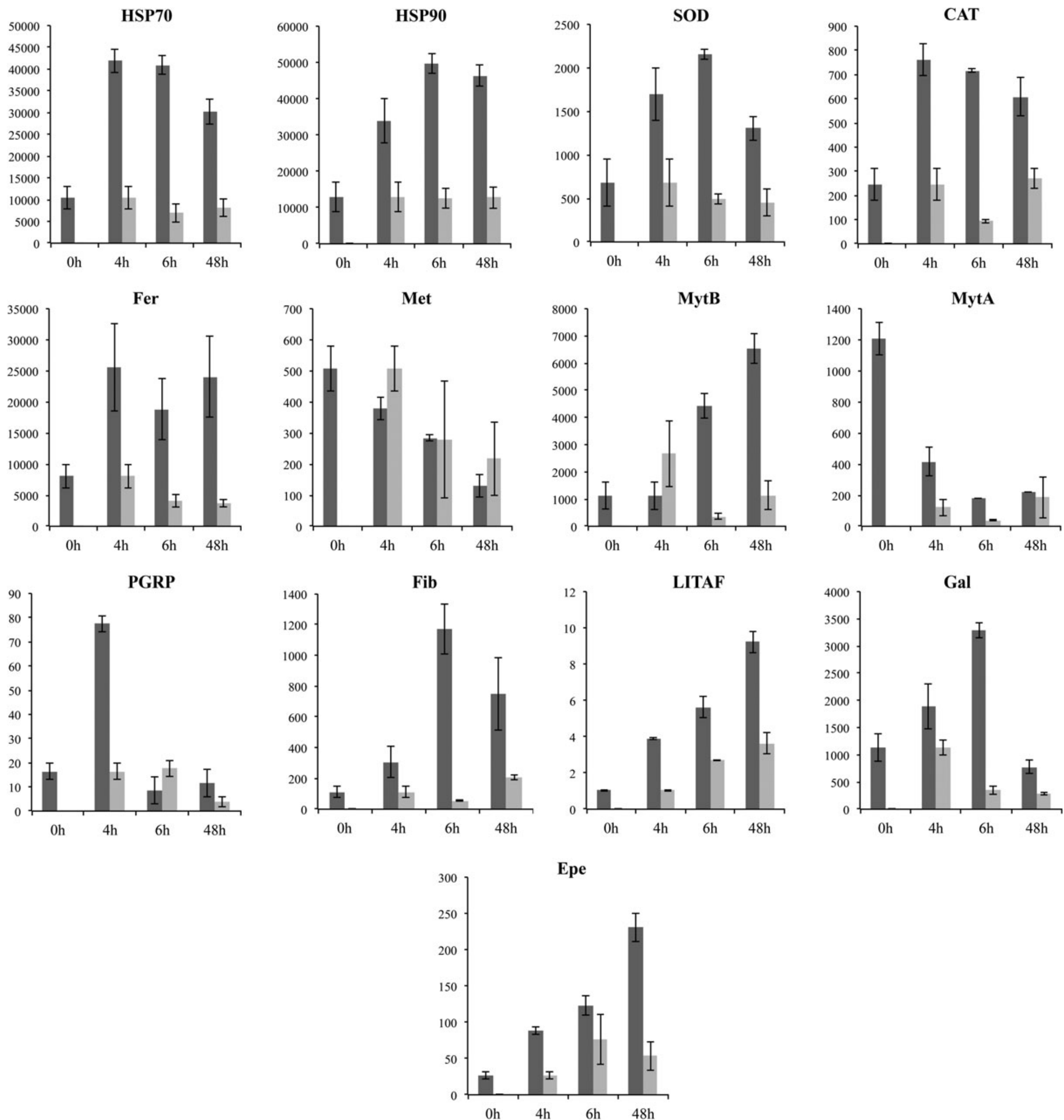

Figure 2. Relative transcript levels for each studied candidate gene of *Mytilus chilensis* after injection with STX or control solution. Dark grey bars = challenged mussels (STX-injected); light grey bars = control mussels (mean ± SE). Grey bar at time = 0 corresponds to non-injected individuals. $N = 4$ per exposure time for each treatment.

their important role as antioxidant molecules following toxin exposure.

The last group of studied genes comprises those involved in response to pathogens (*MytA*, *MytB*, *ependymin*, *galectin*, *PRGP*, *fibrinogen-like* and *LITAF*). Among them the antimicrobial peptides *MytA* and *MytB*, which code for proteins that neutralize pathogenic bacteria and have a direct role in the innate immune system of marine bivalves (Mercado *et al*., 2005). Further, there have been extensive studies to understand their ecological implications in mussel populations (Li *et al*., 2010) and the relationship between single-nucleotide polymorphisms and gene expression levels (Rosani *et al*., 2011). These two antimicrobial peptides, *MytA* and *MytB*, showed an unexpected opposite trend in response to STX exposure. *MytB* displayed over-expression in relation to the exposure time to STX, whereas the expression levels of *MytA* were lower after injection of STX. A previous study in *Mytilus galloprovincialis* exposed to live *Vibrio splendidus* showed an increase of *MytB* transcription 3 h after injection, whereas the expression levels of *MytA* decreased at 3 and 48 h post-exposure (Venier *et al*., 2011). Regarding the *MytA* gene,

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we noted significant differences between the non-injected controls (time = 0) and the controls injected with the buffer at different exposition times. This could suggest a potential relationship between the environment stressor H_2O_2 and this gene, even at very low concentrations of the toxic agent (3 mM). Currently, *MytA* is a poorly studied gene at the transcriptional level; in fact there is no record of studies that measure *MytA* transcript levels in the presence of excess ROS. Further work should be done to study how easily this gene could be triggered in relation to this type of environmental stress. On the other hand, the *ependymin* gene was also involved in response to pathogens, showing over-expression in mussels injected with STX. The *ependymin* gene codes for a protein that may be involved in tissue regeneration in marine invertebrates (Suarez-Castillo & Garcia-Arraras, 2007), although its biological function in *Mytilus* remains unknown. Moreover, several studies have indicated that bivalves exposed to PSP-producers, such as *Alexandrium* sp., showed increased mucus production in gills, melanization of several tissues, degranulation and diapedesis of haemocytes into the alimentary canal as well as evidence of lipid peroxidation, similar to the detoxification pathway described for various xenobiotics, and insoluble lipofuchsin granules (Estrada *et al.*, 2010; Hégaret *et al.*, 2012). These observations clearly suggest that STX could indeed be considered as a non-self molecule, being eliminated by haemocytes via diapedesis, but also responsible for some tissue damage or regeneration, thus causing over-expression of antimicrobial peptides by *MytA* and *MytB* and *ependymin* genes.

In addition to *PGRP*, *galectin* and *fibrinogen-like* genes that are involved in the response to pathogens, several studies have focused on their association with primary signalling pathways of invertebrates (Wang & Zhao, 2004). These three genes in mussels were also over-expressed in response to exposure to *V. splendidus*, which was assessed through microarray technology (Venier *et al.*, 2011). In this study, *galectin* and *fibrinogen-like* were over-expressed in mussels following the STX injection (Fig. 2). Galectins are able to bind glycans present at the surface of microorganisms and therefore allow the entrance of some pathogens inside host cells (Tasumi & Vasta, 2007), but they are also involved in a diverse range of cell activities related to adhesion, immunity, proliferation and death (Perillo, Marcus & Baum, 1998). Galectin proteins were also over-expressed in the oyster *Saccostrea cucullata* exposed to cadmium (Liu, Wang & Wang, 2012). *Fibrinogen-like* genes also code for lectins that bind to carbohydrate residues of foreign and apoptotic cells, or associate with specific serine proteases, initializing the proteolytic complement cascade and pathogen lysis (Venier *et al.*, 2011). The over-expression of both genes following STX injection could suggest that STX is probably considered as a toxic molecule by the mussel's immune system. *PGRP* was, however, not affected by the injection of STX. PGRPs are pattern-recognition receptors that specifically bind to peptidoglycans, a major component of bacterial cell walls, being involved in detection and clarification of invading bacteria.

The last gene studied, *LITAF,* is a transcription factor involved in the activation of the *TNF-α* gene, which has been molecularly characterized in pectinoid bivalves. So far, the expression of *LITAF* has only been observed to be induced by pathogens, such as bacterial endotoxin, bacteria and parasites (Zhu & Wu, 2012). The level of *LITAF* mRNA expression also increases at 3 h of exposure to lipopolysaccharides in *Chlamys farreri* (Yu *et al.*, 2007). In our study, the expression of *LITAF* was not affected by injection of STX, thus suggesting its potential specificity for pathogens or pathogenic molecules.

Expression patterns of the 13 candidate genes were analysed by clustering. Thus, we observed that the genes involved in thermal stress response had similar expression patterns to genes involved in response to oxidative stress such as *SOD*, *catalase* and *ferritin* (Fig. 2). For these five genes, no significant differences could be observed over time (two-way ANOVA with hpi and treatments as the independent variables; data not shown). However, we observed a pronounced over-expression for mussels injected with STX, especially after 48 h (Fig. 2). Indeed, the plot in Figure 3 allows us to correlate the expression of genes according to the first component, which accounts for more than 52% of the variance. To a lesser extent, *ependymin*, *fibrinogen-like*, *galectin* and *mytilin B* genes were also significantly more expressed in mussels injected with STX that in control mussels (Fig. 3). This was particularly observed after 6 h post-injection for the *galectin* and *mytilin B* genes (Fig. 3). These results clearly show that an injection of STX into mussel tissues causes specific effects, which do not cause a whole differential expression. Genes specifically involved in response to pathogens or metals (*methallothionein*, *myticin A*, *LITAF* and *PGRP*) were not affected, whereas genes involved in potentially less-specific processes,

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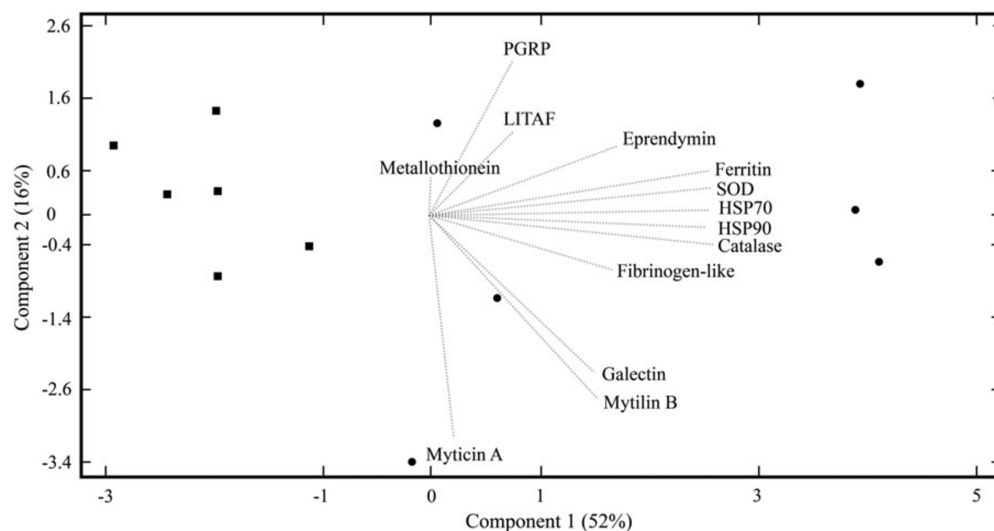

Figure 3. Principal component analysis (PCA). Projection of the individuals (squares: control; circles: STX-injected) in a factorial plane of 13 genes associated with thermal stress (*HSP90*, *HSP70*), response to metal contamination (*ferritin*, *metallothionein*), response to oxidative stress (*SOD*, *CAT)* and response to pathogens (*galectin*, *PRGP*, *MytA*, *LITAF*, *fibrinogen-like*, *MytB*, *ependymin*) in *Mytilus chilensis*.

involving response to stress (*HSP70, HSP90*), the immune system (*mytilin B, ependymin, galectin, fibrinogen-like*), anti-oxidant response (*SOD, catalase, ferritin*) were affected. Until now, few studies have considered the impact of PSP-producers on gene expression in marine invertebrates. However, several authors have shown that exposure to *Alexandrium* sp. and *Gymnodinium catenatum,* both producers of PSP-toxins, can modify feeding behavior and valve movement, but also cause tissue damages, affect immunity, haemocyte cellular responses or excretion (Bricelj & Shumway, 1998; Estrada *et al*., 2010). These findings confirm disruption and stress in bivalves exposed to a PSP-producing dinoflagellate.

The present study demonstrates that STX generates changes at transcriptional levels in *M. chilensis*, mainly related to biological processes associated with the *SOD*, *catalase*, *ferritin* and *HSP* genes. The transcriptional patterns of candidate genes reported in this study provide useful information that could be used for early detection of the effects of toxins on marine bivalves. In this context, the concentration of STX used in the present study is the same as the internationally permitted level in shellfish for human food. However, it remains necessary to determine how a combination of environmental factors can influence the gene expression of bivalves exposed to toxic algae.

CONCLUSIONS

Through the assessment of candidate transcripts, it has been possible to measure the effect of the marine toxin STX, produced by the harmful alga *A. catenella* among others, on the commercially important marine bivalve *M. chilensis*. The overexpression of these transcripts identified significant differences between STX-injected and control specimens, the latter showing no physiological changes. Future studies should focus on understanding the interactions between the transcriptome of this edible bivalve and natural toxins of harmful algae, with the aim of identifying and validating markers at the transcriptomic level, with a sufficient level of resolution to detect the impact of STX and other toxins from the environment on marine bivalves.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Molluscan Studies*.

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