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Differential expression of serine protease inhibitors 1 and 2 in *Crassostrea corteziensis* and *C. virginica* infected with *Perkinsus marinus*

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ABSTRACT: Proliferation of *Perkinsus marinus* (Dermo) *in vitro* is inhibited by the action of 2 serine protease inhibitors belonging to the I-84 family. We compared the levels of expression of serine protease inhibitors 1 and 2 (SPI-1 and SPI-2) in 2 oyster species (*Crassostrea virginica* and *C. corteziensis*) inoculated with the parasite *P. marinus*. *C. virginica* is well known to be susceptible to this parasite, whereas *C. corteziensis* is apparently more tolerant. Oysters were inoculated with trophozoites (1×10^6 trophozoites oyster^{-1}) of *P. marinus* while control oysters were injected with saline solution. Oysters were maintained in a closed water system for 2 wk. The oysters were then sacrificed and parasite burden, histological damage, and gene expression were evaluated. The results showed that the challenged oysters presented a significant increase in parasite burden, which generated histological damage in digestive gland and gills. Quantitative PCR detected significant differences in SPI-1 and SPI-2 expression levels in the 2 oyster species, with *C. corteziensis* showing higher expression levels than *C. virginica* as a response to *P. marinus* inoculation. Our results provide valuable information for the understanding of the defense response in *C. corteziensis* and a possible explanation for its tolerance to the parasite.

KEY WORDS: Eastern oyster · Protozoon · Dermo · Serine protease inhibitor

INTRODUCTION

The protozoan parasite *Perkinsus marinus* is the etiological agent of Dermo disease, responsible for severe mortalities of the eastern oyster *Crassostrea virginica* (Gmelin 1791) along the east coast of North America and the Gulf of Mexico (Burreson & Ragone Calvo 1996, Ford & Tripp 1996). In the Chesapeake Bay region, Dermo has decimated oyster populations, whereas in the Gulf of Mexico, the market-size component of oyster populations suffers an estimated 50% yearly mortality from *P. marinus* (La Peyre et al. 2003).

The transmission of the parasite is waterborne and direct from oyster to oyster (Perkins & Menzel 1966). It is characterized by hemocyte infiltration followed by tissue fibrinolysis and blockages of hemolymph vessels, culminating in most cases in the death of the oyster by emaciation (Ford & Tripp 1996). According to Villalba et al. (2004), *Perkinsus* spp. have 3 main life stages: trophozoite, hypnospore, and zoospore. All stages of *P. marinus* are infective and seem to occur when the oyster ingests the parasite, which then crosses the external epithelia of the oyster's mantle, palps, gills, and gut (Dungan et al. 1996, Chintala et al. 2002). The trophozoite stage, which is

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considered to be the primary agent for disease transmission (Villalba et al. 2004), is a single-cell stage (uninucleate stage) that proliferates by palintomy giving rise to 4 to 32 (often 8 to 16) trophozoites (Perkins 1996). When the tomont wall ruptures, a new generation of immature trophozoites is released, which are ready to infect more host cells (Sunila et al. 2001). Dermo has the potential to kill large numbers of *C. virginica* oysters; experimental challenges have shown that exposure to *P. marinus* for 60 d causes mortality of up to 80% of affected individuals (Meyers et al. 1991, Barber & Mann 1994). Other oyster species (e.g. *C. gigas* and *C. ariakensis*) are also vulnerable, but they seem to be more tolerant (Calvo et al. 1999, 2001, Moss et al. 2006). Meyers et al. (1991) performed challenges in which 40% of *C. gigas* became infected with *P. marinus* compared with 100% of *C. virginica* after 83 d of exposure. Calvo et al. (2000) found that triploid oysters of *C. ariakensis* exhibited lower disease prevalence and infection intensity, and superior survival and growth than *C. virginica*. Even though *P. marinus* has been reported in *C. corteziensis* (Cáceres-Martínez et al. 2008), the absence of die-offs may indicate that this species is tolerant to Dermo disease.

The oyster *C. corteziensis* (Hertlein, 1951) is distributed from the Gulf of California to Panama (Cáceres-Martínez et al. 2008). Oyster production in Mexico is around 50 000 t yr^{-1}, of which 1500 t correspond to *C. corteziensis* (CONAPESCA, www.conapesca.sagarpa.gob.mx/wb/cona/consulta_especifica_por_produccion). Farming of *C. corteziensis* began in the 1970s in San Blas, Nayarit, Mexico, and has increased since then. Suitable temperature for culture ranges from 22.4 to 32.2°C. Higher temperatures (32°C) promote a better growth rate (0.528 mm d^{-1}), whereas at lower temperatures (22°C) the growth rate barely reaches 0.235 mm d^{-1}. *C. corteziensis* reaches its market size in less than 13 mo when temperatures are above 18°C (Chávez-Villalba et al. 2007, Mazón-Suástegui et al. 2011).

The pathogenesis mechanism of *P. marinus* and the immunological–physiological resistance of the oysters are still poorly understood. It is known that in a host–pathogen relationship, the pathogen's success of infection depends on the internal defense system of the host and/or the ability of the pathogen to avoid this defense (Chu & La Peyre 1993, Iwanaga & Lee 2005). In invertebrates, hemocytes are the primary defense cells and are involved in inflammation, wound repair, phagocytosis, and oxidative burst activity. Normally, *P. marinus* is recognized and phagocytosed by oyster hemocytes (Roberts et al. 2012). However, hemocytes are not always able to kill the parasite since *P. marinus* cells can survive and proliferate within them, transforming reactive oxygen species to less harmful components like hydrogen peroxide and oxygen through superoxide dismutase. This defense strategy allows the parasite further invasion of the tissues and fluids, causing a systemic infection that eventually kills the host (De Groote et al. 1997, Asojo et al. 2006). The molecular responses involved in the host–parasite interaction between oysters and *P. marinus* include the synthesis of proteases and protease inhibitors (Oliver et al. 1999, La Peyre et al. 2010). The inhibitors of proteases have evolved as important elements in the system of host defenses against pathogens and as regulators of endogenous proteases.

Inactivation of pathogen-secreted proteases has the potential to restrict the steps of invasion and the dispersal in the internal milieu of those parasites that have successfully invaded and inactivated other elements of host immunity (Armstrong 2001). Protease inhibitors are usually classified as aspartic protease inhibitors, cysteine protease inhibitors, metalloprotease inhibitors, or serine protease inhibitors (SPIs; Laskowski & Kato 1980). These inhibitors are involved in a wide variety of immune and physiological processes (Rawlings et al. 2004) and are known to play important roles in the immune system of marine invertebrates (Iwanaga & Lee 2005, Cerenius et al. 2008, Xue et al. 2009). They also regulate protease activity implicated in the coagulation of hemolymph, the activation of prophenoloxidase, and the synthesis of cytokines and antimicrobial peptides (Iwanaga & Lee 2005). The protease inhibitors can also act as effectors of host defenses by directly inactivating proteases of pathogens, since proteases are important virulence factors (Iwanaga & Lee 2005, Ranasinghe & McManus 2013), or possibly through antimicrobial activities independent of protease inhibitory activity (Yeaman & Yount 2007, Baranger et al. 2008). Recently, 2 SPIs, cvSI-1 and cvSI-2, belonging to the same family (I-84) and sharing similar amino acid sequences (54% similarity; Rawlings et al. 2004, Xue et al. 2009) were purified and identified from the plasma of *C. virginica* (Xue et al. 2006, 2009, La Peyre et al. 2010). These SPIs play a potential role in the host defense against *P. marinus* because they strongly suppress perkinsin and subtisilin A, the major extracellular proteases of the parasite (Xue et al. 2009, La Peyre et al. 2010). Moreover, cvSI-1 inhibited the proliferation of *P. marinus in vitro* (La Peyre et al. 2010). The expression levels of cvSI-1 and cvSI-2 are significantly greater in oysters with

increased resistance to *P. marinus* compared to susceptible oysters (Xue et al. 2009, La Peyre et al. 2010).

The objective of our work was to analyze the expression of SPI-1 and -2 in *C. virginica* (a susceptible species) and *C. corteziensis* (a more tolerant species) challenged with *P. marinus* under laboratory conditions. The hypothesis was that *C. corteziensis* presents higher expression levels of SPI-1 and -2 compared to *C. virginica* as a defense mechanism against *P. marinus* infection.

MATERIALS AND METHODS

Oysters

The individuals were collected during June of 2012. *Crassostrea virginica* were obtained from the Gulf of Mexico (Paraiso, Tabasco, Mexico, at 30 ppt; 29.7 ± 2°C), whereas *C. corteziensis* were obtained from the Gulf of California (Guasave, Sinaloa, Mexico, at 28.7 ± 1.8°C). Sixty oysters (shell length, 7.2 ± 1.2 cm) were sampled for each species.

The oysters were shipped on ice to the Laboratory of Immunology at the Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional (CIIDIR), Sinaloa, Mexico. Oysters were depurated and acclimated for 14 d in 2 separate outdoor plastic tanks (1000 l capacity) with 200 l of seawater (filtered at 20 µm; 28 ppt; 28 ± 2°C) and with continuous aeration. The oysters were fed daily through a drip system with 1 l of Shellfish Diet 1800® (1×10^6 cells ml^{-1}; Reed Mariculture) containing *Isochysis* (40%), *Pavlova* (15%), *Tetraselmis* (25%), and *Thalassiosira weissflogii* (20%). Wastewaters were treated with household bleach (10%). Prior to the bioassay, samples of 10 oysters from each species batch were used for *Perkinsus marinus* diagnosis using histology, whole body burden, and PCR.

Histological analysis

Oyster tissues fixed in Davidson's solution were embedded in paraffin. Sections (3 mm each) were obtained and stained with hematoxylin and eosin (Humason 1979). Slides were observed under a light microscope (Leica Olympus® BX41) connected to a video camera (CooISNAP-ProColor™). The images recorded were digitalized using image analysis software (Image Pro® Plus version 4.5). Positive samples for *P. marinus* were scored according to the associated tissue alterations using 3 categories according to Cáceres-Martínez et al. (2008). Prevalence was calculated according to Cáceres-Martínez et al. (2012).

Whole body burden assay (WBBA) and infection intensity index (Mackin scale)

After taking samples for RNA, DNA, and histology, the rest of the oyster was weighed, minced, and incubated in Ray's fluid thioglycollate medium (RFTM) during 7 d (Ray 1966, Fisher & Oliver 1996, Kim et al. 2006). After incubation, samples were centrifuged, and the tissue pellets were resuspended in 20 ml of 2 M NaOH and incubated at 60°C for 3 h. Digested samples were washed twice with distilled water and resuspended in 1 ml of Lugol's iodine diluted 1:10 with dH_2O. Cells were immobilized on a 0.45 µm filter (Whatman™), and dark blue parasites were counted at 100× magnification, multiplied by the dilution factor, and divided by the respective wet weight (WW). The result is the estimation of infection intensity as number of parasites per gram of tissue WW. Additionally, the number of parasites obtained by WBBA was used to establish the intensity of infection according to the Mackin scale, which ranked from 0 (negative) to 5 (heavily infected) and which we modified using absolute average values of the number of stained *P. marinus* hypnospores contained in each experimental group (Mackin 1962, Craig et al. 1989, Choi et al. 2002).

PCR analysis

Pieces (0.1 g) of gills, mantle, and digestive gland were fixed in 1 ml absolute ethanol for DNA isolation. The DNA was extracted by a modified salting-out method and Proteinase K (Promega®) digestion. The samples were incubated in lysis buffer (50 mM Tris pH 8.0, 100 mM NaCl, 100 mM EDTA pH 8.0, 1% sodium dodecyl sulfate, SDS) for 20 min at 65°C in a thermal bath. After incubation, the DNA was separated from nucleoproteins with 6M NaCl and precipitated with absolute ethanol. The ethanol was removed and the pellet was dried in an oven at 30°C for 5 min. Afterwards, the DNA pellet was resuspended in 100 µl of molecular grade water (Sigma-Aldrich). DNA samples were stored at −20°C until used. Diagnosis by PCR was performed using the non-transcribed spacer (NTS) region of *P. marinus* using forward primer NTS-1 (5′-CAC TTG TAT TGT GAA GCA CCC-3′) and reverse primer NTS-2 (5′-

TTG GTG ACA TCT CCA AAT GAC-3′) as reported by Robledo et al. (1999). For genotyping, the internal transcribed spacer (ITS) region was amplified using primers PerkITS1F (5′-GAG ATG GGA TCY CCG CTT TGT TT-3′) and PerkITS1R (5′-GAA TCG CGT GAT CRA GGA ACA CG-3′) as reported by Park et al. (2006). The PCR mix was prepared as follows: 100 ng of DNA template, 1× PCR Buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs (each), 0.4 µM of each primer, and 0.5 U of GoTaq® Flexi DNA Polymerase (Promega®) in a final volume of 12.5 µl. The amplification conditions were: 94°C for 3 min, 35 cycles at 94°C for 1 min, 58°C (NTS oligos) and 55°C (ITS oligos) for 1 min, and 72°C for 1 min. A positive control (plasmid DNA from infected tissue of *C. virginica*) and a negative control (water, molecular grade, Sigma-Aldrich) were included in all the reactions. The amplicons were visualized in a 1.5% agarose gel, stained with ethidium bromide (1 µg ml^{-1}), visualized under UV light, and photographed. The marker PhiX174 DNA *Hae*III (Promega®) was used as a reference. All PCR analyses were done in triplicate, and positive fragments were forward and reverse sequenced at Macrogen Inc., South Korea. All sequences were aligned using the ClustalW option in the MEGA 4 program (Tamura et al. 2007). The similarities of the consensus sequences were searched in GenBank with BLAST®.

P. marinus cultures

P. marinus was isolated from infected oysters collected in Nayarit, Mexico. The cultures were routinely maintained in sterile flasks containing Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with Ham's F-12 Nutrient Mixture (Sigma-Aldrich), and 2% fetal bovine serum at 28°C (Invitrogen™). The subcultures were maintained for 6 wk prior to the experimental infections with 200 U ml^{-1} of antimycotic nystatin (Mycostatin®) and an antibiotic solution containing 400 U ml^{-1} penicillin G, 0.4 mg ml^{-1} streptomycin sulfate, 0.2 mg ml^{-1} gentamicin, 0.4 mg ml^{-1} kanamycin A, 0.4 mg ml^{-1} erythromycin, and 0.2 mg ml^{-1} neomycin in sterilized artificial seawater. *P. marinus* growth was evaluated every 6 or 7 d by measuring the viability, density, and morphology until the bioassay was carried out. Cell morphology was observed with a light microscope using differential interference contrast optics (Zeiss Axiovert 25, Carl Zeiss®). Cell viability was measured every sampling day, adding 10 µl of neutral red to 50 mg l^{-1} (Sigma-Aldrich) to each well of the plate and then incubated at 28°C for 30 min. Live cells (stained) and dead cells (unstained) were counted until the total number of cells exceeded 200 $well^{-1}$, and the percent viability was calculated by dividing the number of live cells by the total number of cells counted. Cell viability was determined from duplicate wells. Cell density was determined after the cells from each well were resuspended and passed 5 times through a 25 gauge needle attached to a 1 ml syringe to break up clumps, ensuring that the culture was well mixed. The cells were stained with neutral red (10 mg l^{-1}) and then quantified using a Neubauer Bright-LineR hemocytometer (Reichert) (La Peyre et al. 2010). The activation of the parasite was carried out on Day 7, prior to the experimental infections. Parasite cultures were centrifuged at 5000 × *g* (15 min), and the cells were washed twice with sterile seawater and resuspended to a final concentration of 1×10^6 cells ml^{-1} in sterile seawater at 28 °C. Parasites from culture were genotyped by PCR.

Experimental infections

The bioassay was performed to evaluate SPI gene expression in *C. corteziensis* and *C. virginica* inoculated with 1×10^6 cells of *P. marinus* per oyster. The bioassay was carried out at CIIDIR. One week before infections, the oysters were separated into 4 groups (n = 15 $group^{-1}$; 72 ± 3 mm height) and transferred to 4 cylindrical plastic tanks (1000 l capacity) with 200 l of seawater at 28 ± 2°C and 28 ppt. Throughout the experiment, oysters were fed daily. The oysters were grouped as follows: (1) *C. corteziensis* challenged (CC+); (2) *C. corteziensis* non-challenged (CC–); (3) *C. virginica* challenged (CV+); (4) *C. virginica* non-challenged (CV–).

Oysters from the challenged groups were injected into the shell cavity, near the gills (without penetrating soft tissues), with 0.2 ml of seawater containing 1×10^6 cells ml^{-1} of *P. marinus*. Individuals from the unchallenged groups were injected with 0.2 ml of sterile seawater.

Five oysters of each group were sampled at 0, 7, and 14 d post-injection (D0, D7, and D14, respectively), in which D0 corresponds to samples injected with 0.2 ml sterile seawater and collected 4 h later in order to evaluate the effect caused by injection. The opened oysters were placed in a Petri dish, and the soft tissues were examined for the presence of abnormalities.

The soft body was removed from the shell, and 100 mg of digestive gland, mantle, and gills were

fixed in 0.5 ml RNA-later® Stabilization Reagent (Qiagen) for gene expression analysis. Pieces of the same tissues (0.1 g) were fixed in 1 ml of absolute ethanol for DNA isolation and PCR diagnostic of *P. marinus*, in Davidson's solution (Humason 1979) for histological analysis, and in 4% paraformaldehyde (Thermo Scientific™, Richard-Allan Scientific) for *in situ* hybridization analysis. The rest of the tissues were weighed and collected in RFTM solution for WBBA. WBBA and PCR were performed as mentioned above.

Gene expression of SPI-1 and SPI-2 by qRT-PCR

Total RNA was extracted from each tissue fixed in RNA-later® using Trizol® Reagent (Invitrogen™) according to the manufacturer's recommendations, and treated with DNase I (Sigma-Aldrich). The RNA quantity was measured using a nanodrop (NanoDrop 2000, Thermo Scientific™). The quality was examined through a 0.7% agarose gel stained with ethidium bromide (1 µg ml^{-1}) and visualized under a UV lamp. Total RNA (3 µg) from each sample was used to synthesize cDNA using ImProm-II™ Reverse Transcriptase supplemented with oligo-dT primer and RNase inhibitor (all 3 reagents from Promega®) according to the manufacturer's instructions. PCR primers were designed with the computer program Primer 3 (Rozen & Skaletsky 1998) using GenBank accession number DQ092546 (SPI cvSPI-1) and AB468967 (SPI cvSPI-2). For SPI-1, the forward primer was SPI-1qF (5′-TAA TGA ACT CCA GTG CGC AAG-3′) and the reverse primer was SPI-1qR (5′-ACT GTC CAC ACA ATG CCA GA-3′). For SPI-2, the forward primer was SPI-2qF (5′-CGA TGG AGA TTG CAA GAA CA-3′) and the reverse primer was SPI-2qR (5′-AAG CAA CGG CAT TTA GCA TC-3′). PCR amplifications of both genes were performed as previously described for *P. marinus* diagnostic and the amplicons were sequenced. Quantitative real time RT-PCR (qRT-PCR) was carried out in an ABI 7000 Sequence Detection System (Applied Biosystems). PCR amplifications were obtained by mixing 1 µl (100 ng) of cDNA, 13.5 µl of 2× Sybr PCR Master Mix (Applied Biosystems), 0.4 µM of each primer, and 10 µl molecular grade water in a total volume of 25 µl. The reaction conditions were 50°C for 2 min, 95°C for 10 min, 40 cycles of 15 s at 95°C, and 1 min at 60°C. The amplification efficiency was calculated with a qPCR efficiency calculator (Thermo Scientific®) from 1/10 serial dilutions of cDNA prepared from digestive glands showing high SPI-1 and SPI-2 gene expression in a preliminary experiment. The 28S rRNA was used as a reference gene to depict the relative expression level, since its expression was stable according to Vandesompele et al. (2002). Primers used for 28S rRNA were: 28sF (5′-GAT GCT GGG AAC TGT GGT G-3′) and 28sR (5′-CTT TGG GCT GCA TTC TCA A-3′). The coefficient of variation (CV) was calculated for each gene according to $CV\% = SD / (1 + E)^{-Ct}$, where E is PCR efficiency and Ct is cycle threshold. The SPI-1 and SPI-2 gene expression was calculated with comparative C_T Method $2^{-\Delta\Delta C_T}$ according to Applied Biosystems (2004). Every challenged sample was calibrated twice, first with its respective unchallenged sample on D7 and D14, and then with the D0 sample of its respective group. Data were analyzed by a 2-way ANOVA followed by Tukey's test when significant differences ($p < 0.05$) were found.

In situ hybridization for SPI-1 gene expression

Tissues fixed in 4% paraformaldehyde were analyzed to detect *P. marinus* DNA using *in situ* hybridization (ISH) according to Le Roux et al. (1999). We used the DIG DNA Labeling and Detection Kit® (Roche Molecular Biochemicals) to label by random priming the NTS PCR product of *P. marinus* with digoxigenin-dUTP (DIG) and to detect the parasite using an anti-DIG antibody coupled to alkaline phosphatase. As a negative control, slides were incubated with hybridization solution without probe. Once the presence of *P. marinus* was confirmed by ISH, the expression of both SPIs was evaluated. PCR products were obtained from cDNA from digestive glands using the SPI-IqF and SPI-IqR primers, and ligated into a pGEM®-T Easy plasmid vector (Promega®). The recombinant plasmid was linearized with *Apa*I and *Pst*I restriction enzymes. DIG-labeled sense and antisense RNA probes were synthesized with T7 or SP6 RNA polymerases *in vitro* using the linearized plasmid and the DIG RNA Labelling Kit® (SP6/T7; Roche Applied Science).

Tissues were embedded in paraffin after dehydration, and 4 µm sections were obtained and mounted onto polyprep slides (Sigma-Aldrich). Several serial sections were prepared from each tissue block and deparaffinized with xylene, rehydrated through a graded series of ethanol concentrations, immersed in phosphate-buffered saline (PBS, pH 7.3) and incubated at 37°C for 15 min in PBS containing 10 µg ml^{-1} of Proteinase K. After washing twice in PBS, the tissue was incubated in

100 µl hybridization solution (DIG DNA Kit locking solution in 0.1 M maleic acid, 0.15 M NaCl, pH 7.5) containing 3 µl of probe at 42°C overnight in a humidity chamber. The tissues were washed twice with 2× saline sodium citrate (SSC)/0.1% SDS, twice with 0.5 SSC/0.1% SDS, and incubated with blocking solution and anti-DIG antibody solution (1:1000). Finally, the alkaline phosphatase substrate nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate was used for detection. The slides were examined under a microscope.

RESULTS

Diagnosis of oysters prior to challenge

Ten oysters of each species were diagnosed prior to challenge, and all were found to be infected. Mean *Perkinsus marinus* infection intensities at the beginning of the study were 116 ± 56 parasites g^{-1} WW in *Crassostrea corteziensis* and 984 ± 380 parasites g^{-1} WW in *C. virginica*. According to the Mackin scale, *C. corteziensis* reached 0.96 (light infection) and *C. virginica* reached 2.04 (light–moderate infection). Diagnosis by PCR confirmed the presence of *P. marinus* in all oysters using both NTS and ITS region primers. Results produced by BLAST searching showed that the NTS fragment (307 bp) obtained from *C. corteziensis* and *C. virginica* had 98 and 99% similarity, respectively, to *P. marinus* TXsc obtained from Texas, USA (GenBank AF497479). The PerkITS1 primer set amplified the 532 bp of the ITS region of *P. marinus* obtained from *C. corteziensis* and *C. virginica*, showing a 100% similarity and coverage with the ITS of *P. marinus* from Texas (AF497479). Parasites from culture were also amplified and sequenced, using NTS and ITS primers as described above, and results were identical to the *P. marinus* genotypes obtained from experimental oysters.

WBBA and infection intensity index

Non-challenged oysters sampled on D7 and D14 showed no statistical differences in parasite burden when compared to D0 except in CV– on D7, when significant differences were found compared to D0 (Fig. 1). Parasite burden expressed as means showed that CC– and CV– had 73 ± 25 (SD) and 1027 ± 173 parasites g^{-1} WW, respectively, on D0; these values were in the light and light–moderate

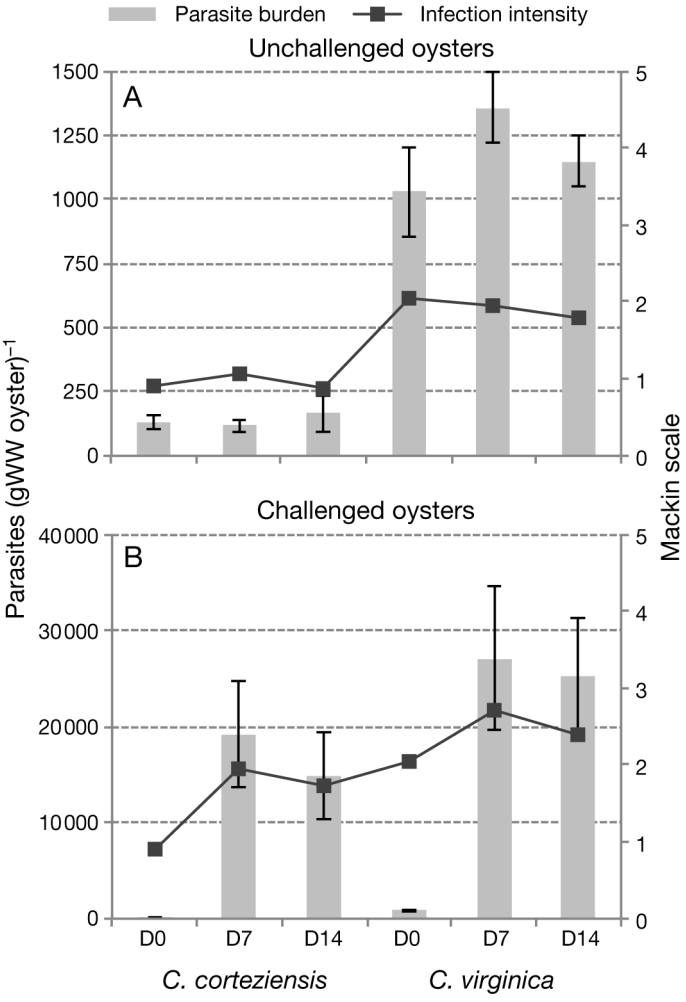

Fig. 1. Relationship between *Perkinsus marinus* infection and parasite burden in oysters *Crassostrea virginica* and *C. corteziensis* from Day 0 (D0) to Day 14 (D14) of the experiment. Infection intensity was determined by the Mackin rating system with Ray's fluid thioglycollate medium (RFTM) assay, where parasite burden count was used to estimate the Mackin scale. (A) Oysters in the unchallenged group were injected with 0.2 ml of sterile seawater. (B) Oysters in the challenged group were injected 0.2 ml containing 1×10^6 cells ml^{-1} of *P. marinus*. WW: wet weight. Error bars are SD

categories, respectively, according to the Mackin scale. Challenged oysters showed important differences in the number of parasites observed compared to D0. CC+ reached 19 230 ± 5546 parasites g^{-1} WW on D7 and 14 811 ± 4461 parasites g^{-1} WW on D14. On the other hand, CV+ had 27 073 ± 7493 and 25 172 ± 6219 parasites g^{-1} WW on D7 and D14, respectively. According to the Mackin scale, CC+ reached values corresponding to a light–moderate infection level (1.95 ± 0.1), while CV+ reached a moderate level (2.72 ± 0.2); significant differences were found between both species using

1-way ANOVA ($p < 0.05$). Mortality was not observed in any group.

PCR-based detection of *P. marinus*, SPI-1, and SPI-2

PCR-based analyses confirmed the presence of *P. marinus* in the tissues of each experimental oyster which generated amplicons of the expected size: 307 bp (NTS primers; Fig. 2) and 532 bp (ITS primers). All sequenced fragments were identical. The BLAST search revealed 98 and 100% identity for NTS and ITS, respectively, to *P. marinus* strain TXsc (AF497499). SPI-1 and SPI-2 were detected from digestive glands and gills from both species. The PCR products (180 and 200 bp, respectively) were sequenced and aligned with previously published records of cvSI-1 and cvSI-2. No differences in nucleotide sequences were found.

Histological analyses and ISH

Histological analysis (Fig. 3) confirmed the presence of *P. marinus* in the tissues of all oysters. The examination of tissue slides revealed the presence of cellular stages resembling *P. marinus*. Unchallenged *C. corteziensis* oysters were classified as having light infections (scored as Category 1), because some parasites were observed in the epithelia of the gut with no hemocyte infiltrations. *C. virginica* were also scored as Category 1, although some oysters were scored as Category 2 since they appeared to have parasites in the epithelia of the digestive gland and the gut, with some hemocyte infiltration. Challenged oysters of CC+ sampled on D7 and D14 were scored as Category 2, but CV+ from the same sampling days were scored between Categories 2 and 3. All challenged oysters from both species presented invasive infiltration of hemocytes and phagocytosis, and trophozoites were disseminated in the connective tissue surrounding the epithelia of the digestive gland, gills, mantle, and some brown cells. Selected challenged oysters were used for ISH, which revealed the presence of *P. marinus* (Fig. 4). SPI-1 mRNA was expressed in the digestive epithelia of oysters from groups CC+ and CV+ (Fig. 5). Unchallenged oysters showed a poor hybridization signal.

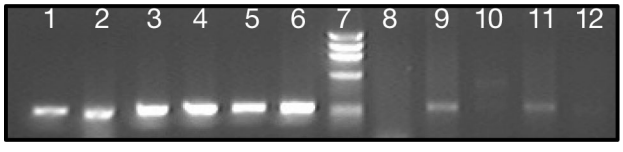

Fig. 2. Agarose gel showing *Perkinsus marinus* non-transcribed spacer PCR products (307 bp) from infected and uninfected oysters. Lanes 1, 2: *Crassostrea corteziensis* at 7 and 14 d post-inoculation, respectively; Lanes 3, 4: *C. virginica* at 7 and 14 d post-infection respectively; Lane 5, Positive control (*C. virginica* infected); Lane 6: Positive plasmid control; Lane 7: Ladder PhiX 174/*Hae*III; Lane 8: Negative control (molecular biology grade water); Lane 9: *C. virginica* on Day 0; Lane 10: *C. corteziensis* on Day 0; Lane 11: *C. virginica* on Day 0; Lane 12: *C. corteziensis* on Day 0

SPI-1 and SPI-2 gene expression

Quantitative RT-PCR was performed to evaluate the expression of SPI-1 and SPI-2 genes. Amplification efficiency for all genes was about 96%, and the CV was less than 6% for the reference gene, whereas for the SPI-1 and SPI-2 genes it ranged from 6.7 to 8.9%. Fig. 6 shows the fold differences related to D0 detected in SPI-1 and SPI-2 gene expression in different tissues from the 2 species. Parasite inoculation had a strong effect on the expression of both genes, since a considerable increase was observed between D0 and D7 samples for both species and tissues. Also, differences were found between D7 and D14 of challenged oysters in both species and tissues, except mantle. Comparison between species showed significant differences in gene expression in challenged oysters, where *C. corteziensis* presented higher expression levels than *C. virginica*, except in mantle for SPI-1 on D7 and gills on D14 for SPI-2. Analysis of unchallenged oysters on D7 and D14 compared with D0 presented a general decrease in gene expression in both species and genes. Significant differences were evident in the digestive gland, where *C. corteziensis* presented a −4.66-fold decrease and *C. virginica* a −7.5-fold decrease for SPI-2 compared to D0.

DISCUSSION

In this study, we evaluated SPI-1 and SPI-2 gene expression in 2 oyster species experimentally infected with *Perkinsus marinus.* Significant differences in SPI gene expression were found between species and between days after parasite inoculation. Our results showed that parasite prevalence was 100% at both sampled sites (Gulf of California and Gulf of Mexico). Although *P. marinus* has been reported since 2008 in northwestern Mexico, its prevalence has varied from 1 to 100% (Cáceres-

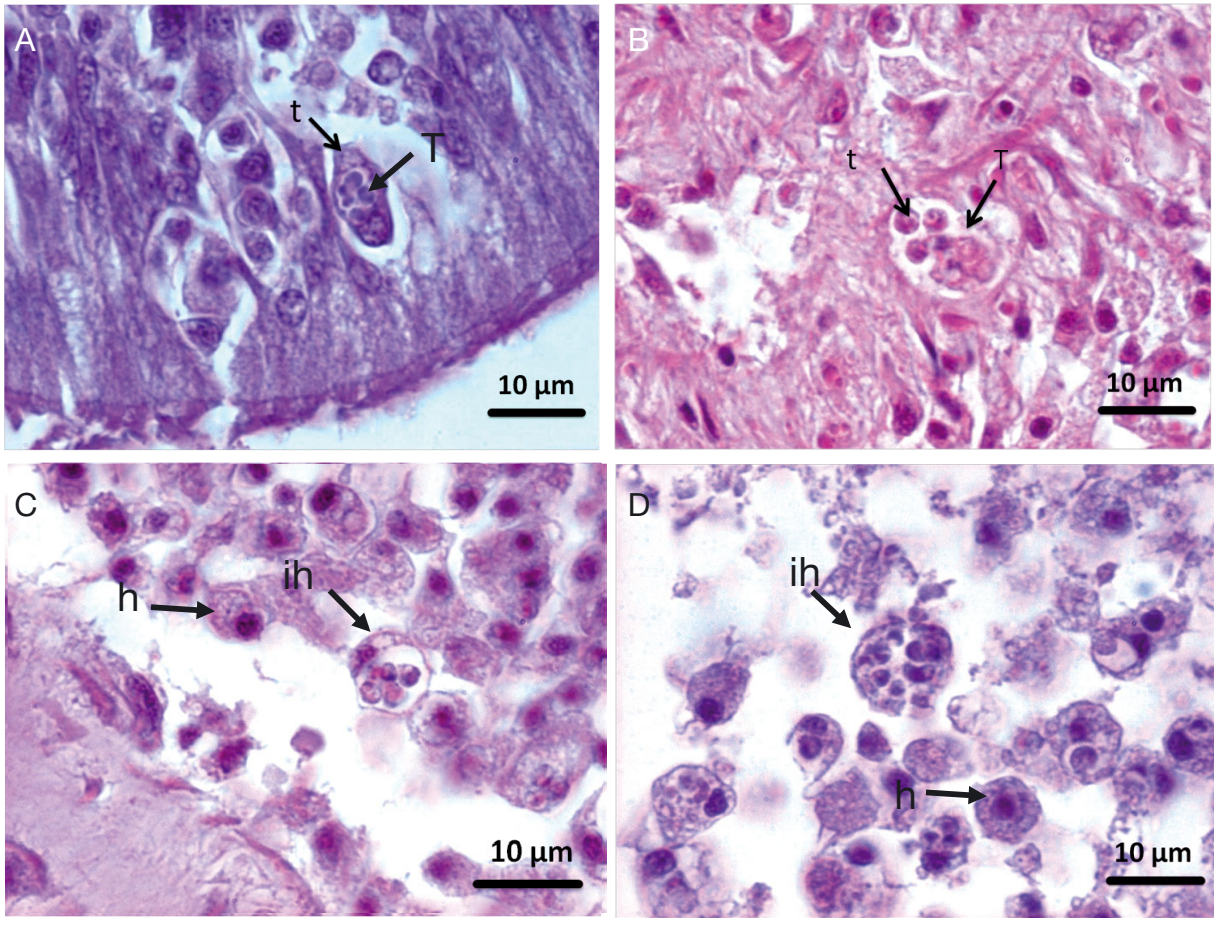

Fig. 3. Cross sections of digestive glands from (A,C) *Crassostrea corteziensis* and (B,D) *C. virginica* infected with *Perkinsus marinus*. (A,C) Epithelium showing different developing stages of the parasite: tomont (T), different stages of trophozoites (t), hemocytes (h), and infected hemocytes (ih). (B,D) Epithelium showing different developing stages of the parasite: tomont (T) containing about 4 developing trophozoites (t). Scale bars = 10 µm. Hematoxylin-eosin staining

Martínez et al. 2008, 2010, Enríquez-Espinoza et al. 2010, Escobedo-Fregoso et al. 2013), whereas in the Gulf of Mexico, it has varied from 73 to 100% (Curiel-Ramírez-Gutíerrez et al. 2012). In our study, although we were unable to collect *Perkinsus*-free oysters, we have shown that direct inoculation of *P. marinus* into the shell cavity induces the activation of protease inhibitors (He et al. 2012). It has been demonstrated that parasite inoculation is an effective method to achieve desired levels of infection and to alter the immune response (Romestand et al. 2002), and it has been shown to be a more efficient method for *Crassostrea virginica* in comparison to feeding oysters with trophozoites (Bushek et al. 1997) or by immersion in seawater containing *P. marinus* (Meyers et al. 1991, Barber & Mann 1994).

In this study, the parasite-burden assay, histological analysis, PCR-based assay, and ISH confirmed that the inoculation method used was efficient. The parasite-burden assay showed important differences in the number of parasites observed between the challenged oysters on each sampling day. The highest values were obtained 7 d post-inoculation in both species, whereas on D14, the infection intensity slowly decreased. This pattern of infection was similar to that reported by Romestand et al. (2002), Meyers et al. (1991), and Barber & Mann (1994), in which *P. marinus*-challenged oysters showed similar trends. Such a trend of infection consists of a short period with a light infection, increasing in intensity, followed by a gradual decrease, until parasites are no longer detectable. Faisal et al. (1998) suggested a possible role of protease inhibitors in parasite elimination. Xue et al. (2006) reported that cvSI-1 is able to inhibit perkinsin, the major protease of *P. marinus*, and subsequently inhibit its propagation.

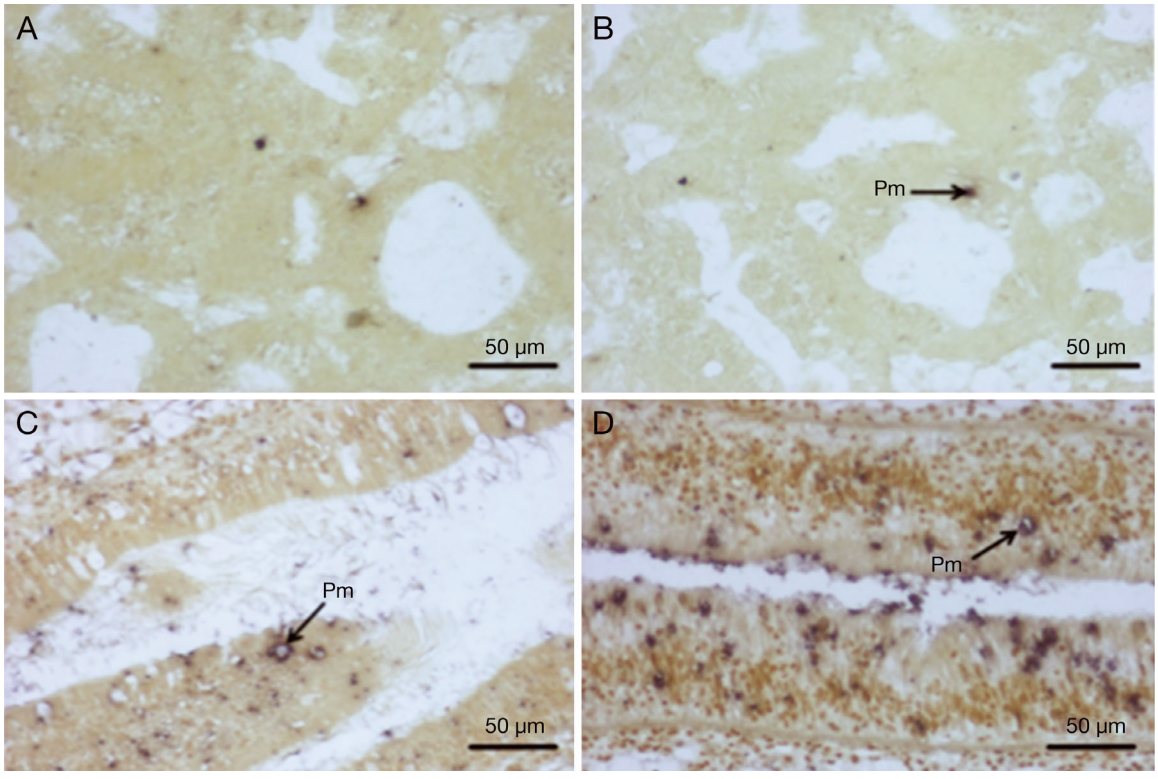

Fig. 4. Cross sections of digestive tissues from *Crassostrea corteziensis* and *C. virginica* to localize *Perkinsus marinus* (Pm) non-transcribed spacer region of the rRNA gene cluster by *in situ* hybridization. (A) Digestive gland of uninfected *C. corteziensis*; (B) digestive gland of uninfected *C. virginica*; (C) digestive epithelium of infected *C. corteziensis*; (D) digestive epithelium of infected *C. virginica*. Scale bars = 50 µm

The histological analysis of infected *C. corteziensis* was consistent with previous reports concerning parasite development and lesions (Cáceres-Martínez et al. 2008). ISH using an SPI-1 riboprobe revealed that SPI-1 mRNA was present in the epithelia of digestive tubules. Similar findings were reported by Xue et al. (2009) for cvSI-2 and by La Peyre et al. (2010) for cvSI-1. No hybridization was observed in gills and mantle, probably due to low gene expression levels, which were also observed in qPCR analysis. Xue et al. (2009) and La Peyre et al. (2010) reported similar results; they reported digestive tubules as target tissue and no signal of detection in other tissues.

Sequence analysis of NTS and ITS regions of the parasite showed that *Perkinsus* from culture, from oysters collected prior to challenge, and from challenged oysters had the same nucleotide sequences and close relationship to the variety from Texas. Analysis of our sequences was consistent with the results reported by Escobedo-Fregoso et al. (2013) and Cáceres-Martínez et al. (2008, 2012). Escobedo-Fregoso et al. (2013) found a high similarity of ITS sequences between samples collected along the Atlantic shoreline, suggesting the possible introduction of *P. marinus* by transport of mollusks from the Atlantic to the Pacific. This was also suggested by Cáceres-Martínez et al. (2008) and is supported by our results.

C. corteziensis (unlike *C. virginica*), appear to be tolerant to *P. marinus* as well as to diverse pathogens and adverse physicochemical conditions (Mazón-Suástegui et al. 2011). Resistance to *Perkinsus* has been mentioned in previous studies (Meyers et al. 1991, Barber & Mann 1994, Faisal et al. 1998, Romestand et al. 2002). The exact mechanisms mediating the resistance are not known, but preliminary studies revealed that the presence of protease inhibitors in the plasma can disable the proteases produced by parasites (Faisal et al. 1998, Tall et al. 1999). Meyers et al. (1991) and Barber & Mann (1994) found significantly higher resistance to *P. marinus* in *C. gigas* compared to *C. virginica*, with greater protease inhibitory activity (Faisal et al. 1998).

Despite the differential levels of initial infections between species, gene expression was greater in *C. corteziensis*; the main effects were evaluated and

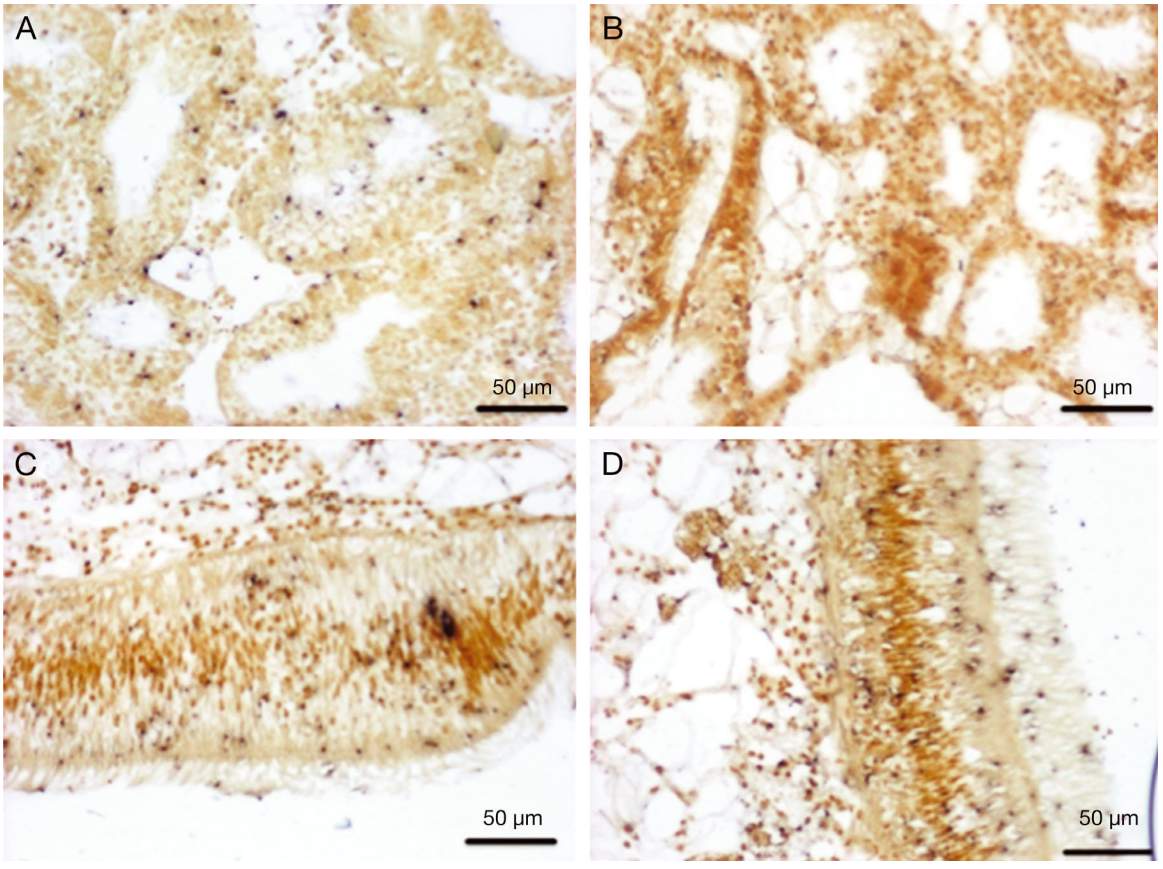

Fig. 5. Cross sections of digestive tissues from *Crassostrea corteziensis* and *C. virginica* to localize serine protease inhibitor (SPI-I) gene expression by *in situ* hybridization. (A) Digestive gland of *C. corteziensis* on Day 0; (B) digestive gland of *C. virginica* on Day 0; (C) digestive epithelium of *C. corteziensis* on Day 7; (D) digestive epithelium of *C. virginica* on Day 7. Scale bars = 50 µm

were consistent with those reported previously by La Peyre et al. (2010). In general terms, our results showed that SPI-1 and SPI-2 expression levels increase gradually after infection, which may be the result of the oysters' protease inhibitors counteracting the parasite proteases, consistent with a previous report by Faisal et al. (1998), who suggested a role of protease inhibitors in parasite elimination or neutralizing pathogenic proteases. According to our results, gene expression of SPIs in *C. corteziensis* was greater than in *C. virginica*, as was parasite burden. Romestand et al. (2002) observed that the highest protease inhibitory activities (3–10 d post-inoculation) preceded parasite elimination (initiated at 7 d post-challenge), which is consistent with our results.

The immune response was equally altered by parasite injection; however, the digestive gland showed higher gene expression of SPI-1 and -2 than the rest of the tissues. Although SPIs in mollusks have been identified in hemolymph in the Pacific oyster *C. gigas*, eastern oyster *C. virginica*, softshell clam *Mya arenaria*, and surf clam *Spisula solidissima* (Kanost 1999, Xue et al. 2009, Nikapitiya et al. 2010), the digestive gland seems to be a target organ since maximum values of gene expression were detected there (La Peyre et al. 2010). La Peyre et al. (2010) found that the cvSI-1 gene was mainly expressed in basophilic epithelial cells of digestive tubules and that its expression level in wild oysters was significantly higher than in gills, style-sac-midgut, mantle, and other tissues. Xue et al. (2009) found by ISH that cvSI-2 mRNA was present in the epithelia of digestive tubules. Comparison between serial sections indicated that the specific presence of cvSI-2 mRNA was limited to basophilic cells. cvSI-2 was not found in other tissues, although we found a low expression level in gills and mantle compared to the digestive gland.

The digestive gland of unchallenged oysters showed a significant decrease in gene expression on D7 com-

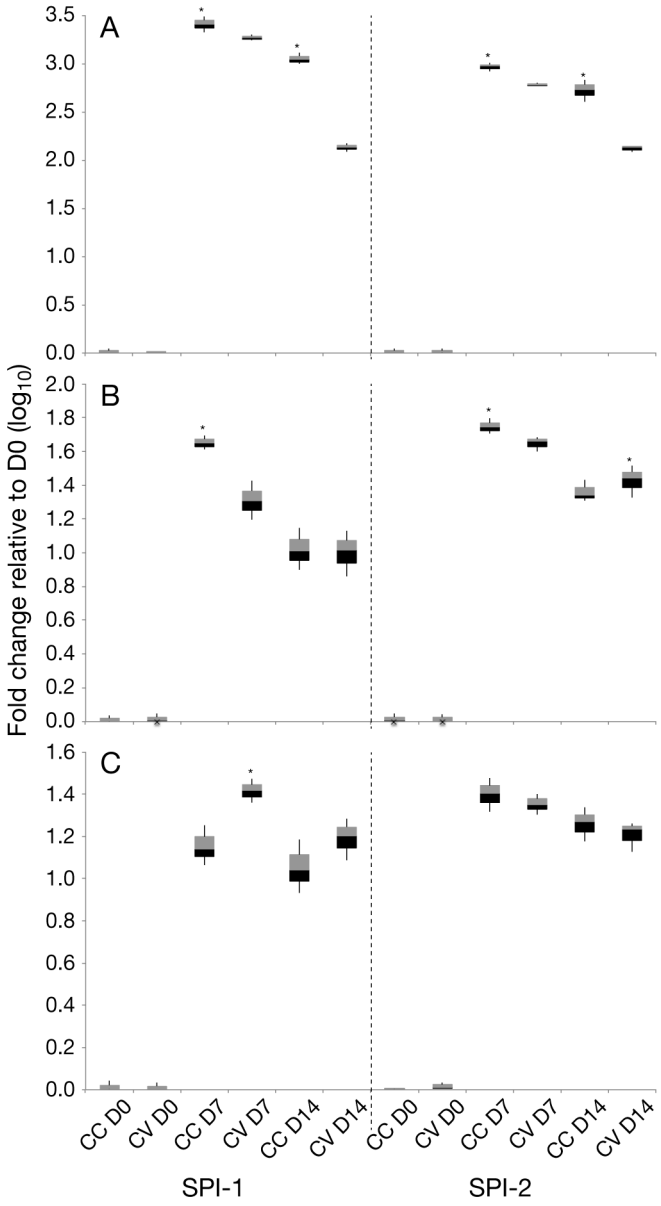

Fig. 6. Relative mRNA expression of serine protease inhibitors (SPI-1/SPI-2) genes between non-challenged and challenged oysters on Days 0, 7, and 14 (D0, D7, D14). (A) Digestive gland; (B) gills; (C) mantle. The grey and black hinges of the box indicate 75% and 25% of the variation, respectively; the thick horizontal line within the box indicates the median. The whiskers indicate minimum/maximum range. Asterisks indicate significant mean differences according to Tukey's test ($p < 0.05$). CC: *Crassostrea corteziensis*; CV: *C. virginica*

pared to D0. This may be explained by the trauma effect imposed on oysters when the saline solution was injected. A similar response was observed by Lacoste et al. (2001), who argued that mechanical stress induced by injection may increase the susceptibility of the organism. In our study, the parasite load did not provoke any mortalities, probably because the exposure time was too short or the parasite burden too low. However, the presence of *P. marinus* may provoke negative effects in both *C. corteziensis* and *C. virginica* such as reduced growth rate, unsuccessful gametogenesis, and increased susceptibility to other pathogens (Villalba et al. 2004).

Since the first detection of *Perkinsus* in *C. corteziensis* in 2007, there have been no mass mortalities in this species. The implications of host–parasite coevolution in aquatic models, particularly in the context of epidemiology, are weakly recognized in spite of the ever growing reports on emergent pathogens around the world, which open many questions on how both resistance and infectivity traits evolve in such new associations. As stated by Thompson & Burdon (1992), coevolution may be particularly important as a means of maintaining resistance and infectivity and/or virulence polymorphisms.

The results obtained in this study emphasize the role of SPIs in the response of *C. corteziensis* against *P. marinus*, and open the door for further studies on perkinsosis in this and other oyster species. Our work suggests that *C. corteziensis* possesses a more efficient defense mechanism against the parasite compared to *C. virginica*.

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