Effects of Florida Red Tides on histone variant expression and DNA methylation in the Eastern oyster *Crassostrea virginica*

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ABSTRACT

Massive algal proliferations known as Harmful Algal Blooms (HABs) represent one of the most important threats to coastal areas. Among them, the so-called Florida Red Tides (FRTs, caused by blooms of the dinoflagellate *Karenia brevis* and associated brevetoxins) are particularly detrimental in the southeastern U.S., causing high mortality rates and annual losses in excess of $40 million. The ability of marine organisms to cope with environmental stressors (including those produced during HABs) is influenced by genetic and epigenetic mechanisms, the latter resulting in phenotypic changes caused by heritable modifications in gene expression, without involving changes in the genetic (DNA) sequence. Yet, studies examining cause-effect relationships between environmental stressors, specific epigenetic mechanisms and subsequent responses are still lacking. The present work contributes to increase this knowledge by investigating the effects of Florida Red Tides on two types of mechanisms participating in the epigenetic memory of Eastern oysters: histone variants and DNA methylation. For that purpose, a HAB simulation was conducted in laboratory conditions, exposing oysters to increasing concentrations of *K. brevis*. The obtained results revealed, for the first time, the existence of H2A.X, H2A.Z and macroH2A genes in this organism, encoding histone variants potentially involved in the maintenance of genome integrity during responses to the genotoxic effect of brevetoxins. Additionally, an increase in H2A.X phosphorylation (γH2A.X, a marker of DNA damage) and a decrease in global DNA methylation were observed as the HAB simulation progressed. Overall, the present work provides a basis to better understand how epigenetic mechanisms participate in responses to environmental stress in marine invertebrates, opening new avenues to incorporate environmental epigenetics approaches into management and conservation programs.

1. Introduction

The impacts of global change on marine ecosystems are dramatic, as evidenced by growing pollution and acidification, sea level rise, and changes in ocean temperature and currents (Curry and Mauritzen, 2005). Among them, the increase in the frequency and toxicity of episodes of massive algal proliferations known as Harmful Algal Blooms (HABs) represents one of the most important threats to fisheries, aquaculture-based industries, and human populations in coastal areas (Mat et al., 2013; Cook et al., 2015). During HABs, large amounts of potentially harmful biotoxins are produced and recruited into the human food chain through their accumulation by edible marine organisms (Cardozo et al., 2007; Plakas et al., 2008). These biotoxins, among other relevant effects, have the ability to cause DNA damage, both in marine organisms and human consumers of contaminated shellfish (Valdiglesias et al., 2013; Prego-Faraldo et al., 2015; Prego-Faraldo et al., 2016).
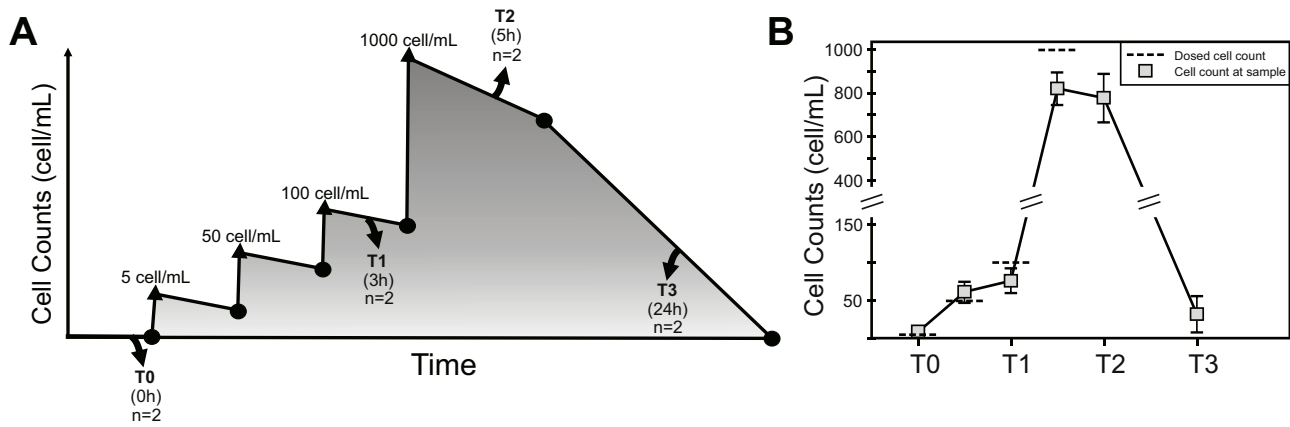
Fig. 1. Florida Red Tide HAB simulation. A, Schematic representation of HAB simulation experiment, including cell concentration (cell/mL) of the brevetoxin-producing dinoflagellate *K. brevis* over time. Samples (n = 2 oysters per biological replicate) were collected at different intervals (T0, 0 h; T1, 3 h; T2, 5 h; T3, 24 h) during the simulation. B, Monitoring of *K. brevis* cell concentrations during HAB simulation. Final cell concentrations used in the present study (5, 50, 100 and 1000 cell/mL) are represented by dotted lines. Each data point represents the average *K. brevis* cell count across biological replicates along with the corresponding standard error in water samples (grey squares), measured after application of *K. brevis* cell cultures to experimental tanks.

The southeastern U.S. is particularly affected by HABs (Flewelling et al., 2005), notably by the so-called Florida Red Tides [FRTs, blooms of the dinoflagellate *Karenia brevis* (Davis, 1948; Brand and Compton, 2007)] and associated brevetoxins (PbTx). FRTs are responsible for high mortality rates of marine invertebrates, fishes and marine mammals (Brand et al., 2012), causing annual losses in excess of $40 million (Twiner et al., 2007). The consumption of brevetoxin-laden shellfish causes Neurotoxic Shellfish Poisoning (NSP) syndrome (McFarren et al., 1965), and exposure to aerosolized brevetoxins is responsible for respiratory distress (Abraham et al., 2005) and severe allergic reactions in humans (Fleming et al., 2007). Furthermore, brevetoxins convey critical disruptive effects at the most fundamental level, as they can induce DNA damage and apoptosis (Radwan and Ramsdell, 2008; Murrell and Gibson, 2009, 2011). Still, while the harmful effects of brevetoxins are well documented, the role these biotoxins play in *K. brevis* is still uncertain (Sunda et al., 2013; Errera et al., 2014).

Coastal areas bear the brunt of HABs, impacting marine communities and commercial resources, especially the aquaculture industry. Consequently, bivalve molluscs are generally used as sentinel organisms to study HAB pollution (Collin et al., 2010; Campos et al., 2012; Fernandez-Tajes et al., 2012; Luchmann et al., 2012; Milan et al., 2013; Suarez-Ulloa et al., 2013; Prego-Faraldo et al., 2015, 2016). Eastern oysters (*Crassostrea virginica)* are particularly relevant due to their abundance, economic importance and major role in the function of estuary ecosystems in areas affected by FRTs (Dame, 1972). During these episodes Eastern oysters rapidly accumulate brevetoxins (Plakas et al., 2002), causing mortality and interfering with larval development (Leverone et al., 2006; Rolton et al., 2014). At the subcellular level, the exposure to high brevetoxin concentrations was previously linked to the activation of specific molecular mechanisms involved in defense, detoxification and stress response in oysters (Mello et al., 2012), including advanced apoptosis and apoptosis-regulating systems (Zhang et al., 2014). Similarly, the presence of oxidative stress was described in other marine organisms including corals, turtles and manatees, as a consequence of exposure to *K. brevis* during FRTs (Ross et al., 2010; Perrault et al., 2014; Walsh et al., 2015).

Traditionally, the ability of organisms to cope with adverse environmental conditions such as those imposed by HABs was attributed to specifically adapted genotypes (Hoffmann and Willi, 2008). However, we now know that responses to environmental changes are largely dependent on epigenetic regulatory mechanisms, in other words, heritable changes in gene expression resulting from modifications in chromatin structure, without involving changes in the genetic information stored in DNA (Allis et al., 2007; Feil and Fraga, 2012). The study of the epigenetic mechanisms mediating exposure-response relationships constitutes the basis for environmental epigenetic analyses (Baccarelli and Bollati, 2009; Bollati and Baccarelli, 2010), providing information about how different environmental factors influence phenotypic variation (Cortessis et al., 2012; Suarez-Ulloa et al., 2015; Etchegaray and Mostoslavsky, 2016). Chromatin, the association between DNA and chromosomal proteins, provides a framework for the study of epigenetic modifications, including DNA methylation, histone variants [a set of minority histone proteins encompassing specialized functions in chromatin metabolism (Henikoff and Smith, 2015)] and their post-translational modifications (PTMs), as well as non-coding RNAs, among others (Kouzarides, 2007; Ptashne, 2007; Arya et al., 2010; Talbert and Henikoff, 2010; Mercer and Mattick, 2013). These modifications define specific epigenomic states throughout the genome that are susceptible of being transmitted trans-generationally (both mitotically and meiotically), setting the basis for short-term acclimatization and long-term adaptation (Migicovsky and Kovalchuk, 2011; Fernandez et al., 2014).

Among marine invertebrates, bivalve molluscs constitute emerging models in environmental epigenetics, as illustrated by recent studies examining the role of DNA methylation in the Pacific oyster (Gavery and Roberts, 2010, 2013, 2014; Diaz-Freije et al., 2014; Olson and Roberts, 2014) and the characterization of chromatin structure and histone variants in mussels (González-Romero et al., 2012; Rivera-Casas et al., 2016a,b). Yet, studies examining cause-effect relationships between environmental stressors, specific epigenetic mechanisms and subsequent responses in marine invertebrates are still lacking. That is primarily due to the absence of genomic information for many environmentally and ecologically relevant organisms, along with a scarce knowledge about their chromatin structure and dynamics (Arenas-Mena et al., 2007; Schulmeister et al., 2007; Suarez-Ulloa et al., 2015). The present work fills that gap by studying the role of two different epigenetic mechanisms during Eastern oyster responses to FRTs. First, given the genotoxic effect of brevetoxins produced during FRTs (Radwan and Ramsdell, 2008; Murrell and Gibson, 2009, 2011), this study targets a group of three histone variants known to be involved in the maintenance of genome integrity, namely H2A.X, H2A.Z, and macroH2A (Li et al., 2005; Ivashkevich et al., 2012; Shi and Oberdoerffer, 2012; Papamichos-Chronakis and Peterson,

2013; Khurana et al., 2014). Although the presence of these variants has not been yet described in oysters, our previous results on other mollusc species support that notion (González-Romero et al., 2012; Rivera-Casas et al., 2016a,b). Second, the study of environmental epigenetic responses is completed by the analysis of genome-wide DNA methylation patterns during FRTs. The obtained results are consistent with a role for histone H2A.X phosphorylation (γH2A.X) and DNA methylation during exposure to the brevetoxin-producing dinoflagellate *K. brevis*.

2. Materials and methods

2.1. Specimen collection and laboratory acclimatization

Eastern oyster specimens were collected at Rookery Bay National Estuarine Research Reserve, Naples FL (coordinates 26.050026, −81.700774). Collected organisms were transported to the laboratory and acclimatized to controlled conditions of temperature ($25 \pm 2\,°C$), aeration (dissolved oxygen >6 mg/L), salinity (30 ± 2 ppt), light cycle and substrate in a recirculating seawater system for 5 weeks. During this period, oyster specimens were fed twice a day using a commercial mix of marine microalgae (1 mL per 30L tank) including *Isochrysis* sp., *Pavlova* sp., *Tetraselmis* sp., *Chaetoceros calcitrans*, *Thalassiosira weissflogii* and *T. pseudonana* (Shellfish Diet 1800, REED MARICULTURE). The concentrated diet was diluted with filtered seawater (1:10) to ensure separation of microalgae cells.

2.2. Experimental HAB simulation

The present work simulated the toxic effect resulting from the exposure of Eastern oysters to an incoming patch of *K. brevis* HAB that disappears within 24 h (Stumpf et al., 2003). HAB simulations were carried out in the laboratory using an exponential culture of the brevetoxin-producing dinoflagellate *K. brevis* (approx. 45,000 cells/mL, courtesy of Dr. K. Rein, Florida International University). Toxin content in microalgae cultures was estimated using LC–MS as 5.0–6.8 pg/cell (Sun et al., 2016). Given that the present work aimed to simulate a natural FRT episode, *K. brevis* exposures were chosen over exposures to purified brevetoxins. The latter have major limitations for that purpose, including the existence of several brevetoxins subtypes (not all can be purified) and their high hydrophobicity, hampering homogeneous solution in seawater. Two groups of oysters (control and treated) were defined in the simulation, each consisting of 3 experimental units (biological replicates) with 20 individuals/unit (80 individuals per group). Each experimental unit consisted of a static 20 L tank where oysters were fed and acclimatized to controlled conditions for 24 h prior to exposure to *K. brevis*. Treated experimental units were subsequently exposed to increasing concentrations of *K. brevis*, starting at 5 cell/mL and reaching 1000 cell/mL after 5 h exposure (Fig. 1A). These conditions mirror *K. brevis* levels during a typical medium-high intensity FRT episode (Stumpf et al., 2003).

Specimens were initially fed with lower microalgae volumes (0.1 mL per 20 L tank) in order to promote feeding behavior. Effects of high algal densities were controlled in tanks exposed to *K. brevis* by monitoring water quality (salinity, pH, dissolved oxygen, and conductivity) and oyster feeding behavior (production of feces). These parameters remained constant within optimal levels throughout the experiment, discarding significant effects of increased algal levels in exposed individuals. Cell concentrations of *K. brevis* were quantified hourly using a Multisizer 4 Coulter Counter instrument (BECKMAN-COULTER). Dosage was adjusted to maintain homogeneous microalgae concentrations across replicates (Fig. 1B). Specimens (n = 2 oysters per biological replicate)

Table 1
qPCR primers used in gene expression analyses, specifically designed to amplify histone variant genes and reference genes in Eastern oyster.

Gene	Primer Name	Sequence (5′ → 3′)
H2A.X	Cv-H2A.X-Fw	AGTTACCATTGCCCAAGGAGG
	Cv-H2A.X-Rv	AAAATTCCTGGGACTGTGACGA
H2A.Z	Cv-H2A.Z-Fw	CGCCATCAGAGGAGACGAAG
	Cv-H2A.Z-Rv	AGCTGTTTTCTGTGTGCCCT
MacroH2A	Cv-mH2A-Fw	TCATTTCCGTATCGGAGCGG
	Cv-mH2A-Rv	CTCTTGCAGCATTTCCAGCC
GAPDH	Cv-GAPDH-Fw	GACAACAGTCCACGCCTACA
	Cv-GAPDH-Rv	GGATGACCTTACCCACTGCC
RPL13	Cv-RPL13-Fw	CCGGGCTCCCAGTAAAATGT
	Cv-RPL13-Rv	TGTCGTATGGGGGAGGGATT

were collected at 4 different time points: T0, before exposure begins; T1, after 3 h exposure; T2, after 5 h exposure; T3, after 24 h exposure. Upon sampling, oysters were shucked, and gills were dissected and immediately flash-frozen in liquid nitrogen. The choice of gill tissue as model system in the present work was motivated by previous reports indicating that gills are the part of the organism first contacted by biotoxins during HABs, experiencing substantial DNA damage (Prego-Faraldo et al., 2015, 2016).

2.3. Gene expression analysis

Total RNA was extracted from gill cells using Ribozol Reagent (AMRESCO) and digested with PerfeCTa DNase I (QUANTA BIOSCIENCES) to eliminate residual genomic DNA. cDNA was synthesized using qScript cDNA Supermix (QUANTA BIOSCIENCES). Expression analyses were performed by means of quantitative PCR (qPCR) experiments. Accordingly, specific primers for Eastern oyster H2A.X, H2A.Z, and macroH2A histone genes were designed based on sequences retrieved from GenBank (Table 1) using Primer-BLAST software (Ye et al., 2012). Primers were also generated for GAPDH and RPL13, used as reference genes for normalization purposes. Primer efficiency was calculated based on the slope of calibration curves constructed using ten-fold dilution steps, according to the formula $E = 10^{-1/slope}$.

Gene expression profiles were examined in gills from Eastern oyster by measuring SYBR green incorporation, using FastStart Essential DNA Green Master (ROCHE) in a LightCycler 96 System (ROCHE). A pre-incubation step of 10 min at 95 °C was included. cDNA amplification was carried out in 45 cycles under the following conditions: denaturation for 10 s at 95 °C, annealing for 10 s at 60 °C, and elongation for 10 s at 72 °C, including a final melting gradient up to 97 °C using a ramp of 4.4 °C/s to check primer specificity. Each individual reaction was carried out in triplicate including negative controls, No Template Control (NTC) and Non-Reverse Transcription Control (NRTC). Results were recorded as normalized ratio values by the LightCycler 96 Software version 1.1 following the Pfaffl method (Pfaffl, 2001). Statistical analyses were carried out using ANOVA after observing an approximately normal distribution with QQ-plots, including a post-hoc Tukey HSD test for multiple comparisons. All analyses were carried out using R-Bioconductor (Gentleman et al., 2004).

2.4. Histone protein extraction, separation and western blot analysis

Histone protein isolation was performed as described elsewhere (Ausio and Moore, 1998), adapting the protocol to oyster gill tissues in the present work. Accordingly, gills were homogenized with a Dounce Tissue Grinder in 100 mM KCl, 50 mM Tris-HCl (pH 7.5), 1 Mm MgCl$_2$, and 0.5% Triton X-100 buffer containing a protease inhibitor mixture. After homogenization and incubation on ice for 5 min, samples were centrifuged for 10 min at 12,000g (4 °C). The

resulting pellets were resuspended in 0.6 N HCl, homogenized and centrifuged again. The HCl supernatant extracts were precipitated overnight with six volumes of acetone at −20 °C and centrifuged for 10 min at 12,000g (4 °C). Acetone pellets were dried using a speedvac concentrator and stored at −80 °C.

The extracted histone proteins were separated using Sodium Dodecyl-Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) in ClearPAGE SDS gels 4–20% (C.B.S. SCIENTIFIC). Gels were stained with 0.2% (w/v) Coomassie blue in 25% (v/v) 2-propanol, 10% (v/v) acetic acid, and destained in 10% (v/v) 2-propanol, 10% (v/v) acetic acid. Western-blot analyses were performed using commercial human-specific antibodies, including anti-H2A.X (ABCAM), anti-γH2A.X (ROCKLAND), anti-H2A.Z (THERMOFISHER SCIENTIFIC); as well as antibodies raised in-house against mollusc macroH2A and H4 histones. Gels were electro-transferred to a nitrocellulose membrane (C.B.S. SCIENTIFIC) and processed as described elsewhere (Finn et al., 2008). Membranes were incubated with a secondary goat anti-rabbit antibody (ROCKLAND) whose signal was subsequently detected using enhanced chemiluminescence (Amershan ECL Prime Western Blotting Detection Reagent, GE HEALTHCARE LIFE SCIENCES) and a CCD based imager (ChemiDoc-It TS2 Imager, UVP).

2.5. Genome-wide DNA methylation analysis

Genomic DNA was purified from gill tissue as described elsewhere (Fernandez-Tajes et al., 2007), adapting the protocol to Eastern oysters in the present work. DNA methylation was analyzed using a Methylation Sensitive Amplified Polymorphism (MSAP) protocol, adapted from (Reyna-Lopez et al., 1997). This method is based on the differential cleavage reactivity to Cytosine methylation (CCGG sites) between the isoesquizomeric endonucleases HpaII and MspI. Accordingly, while HpaII is sensitive to internal Cytosine methylation (i.e., 5′-C^{m}CGG-3′/3′-GG^{m}CC-5′) or hypermethylation states (i.e., 5′-^{m}C^{m}CGG-3′/3′-GG^{m}C^{m}C-5′), MspI is sensitive to external Cytosine methylation, including hemimethylation states where methylation is located on the external Cytosine of the CCGG pattern but on a single DNA strand (i.e., 5′-^{m}CCGG-3′/3′-GGCC-5′) and hypermethylation states. Thus, by comparing both restriction profiles, MSAP analyses provide a basis for establishing global Cytosine methylation patterns and to perform comparisons among different samples (Diaz-Freije et al., 2014). Briefly, gill genomic DNA samples were separately digested with EcoRI/HpaII and EcoRI/MspI in parallel reactions. The resulting fragments were ligated to EcoRI adapters (5′-CTCGTAGACTGCGTACC-3′, 3′-AATTGGTACGCAGTCTAC-5′) and HpaII/MspI adapters (5′-GACGATGAGTCTAGAA-3′, 3′-CGTTCTAGACTCATC-5′). Digestion-ligation reactions were performed simultaneously for 2 h at 37 °C in a solution consisting of 10 nM DNA, 4 U of EcoRI (NEB), 1 U of either HpaII (NEB) or MspI (NEB), 1 U T4 DNA ligase (NEB), 1X ligase buffer (NEB), 1X CutSmart Buffer (NEB). Restriction fragments were selectively amplified through two consecutive PCR reactions as follows: a first reaction containing 1 μL of 1/10 diluted restriction-ligation product, 20 pM of each HpaII/MspI + T (5′-GATGAGTCTAGAACGGT-3′) and EcoRI + A (5′-GACTGCGTACCAATTCA-3′) primers, 1X PCR buffer, 0.5 mM dNTPs (THERMO FISHER SCIENTIFIC), 2.5 mM MgCl$_2$, and 1 U BIOTAQ DNA polymerase (BIOLINE). The PCR conditions included an initial denaturation step of 2 min at 95 °C followed by 20 amplification cycles (20 s at 95 °C; 30 s at 56 °C; 2 min at 72 °C) and a final extension step of 30 min at 72 °C. The second reaction used 0.5 μL of 1/10 of the pre-selective PCR product, 0.83 pM of each 6-FAM labelled selective primer, HpaII/MspI + TAG (5′-GATGAGTCTAGAACGGTCC-3′) and HpaII/MspI + TCC (5′-GATGAGTCTAGAACGGTAG-3′), 1X PCR buffer, 0.5 mM dNTPs, 2.5 mM MgCl$_2$ and 1 U DNA polymerase (BIO-TAQ). Conditions included an initial denaturation step of 2 min at 95 °C followed by 10 amplification cycles (20 s at 95 °C; 30 s at 66 °C; 2 min at 72 °C) and a final extension step of 30 min at 72 °C. Amplified products were run in a ABI Prism 310 Genetic Analyzer (APPLIED BIOSYSTEMS) with a GeneScan 500 ROX in the DNA Core facility at Florida International University.

MSAP restriction profiles were scored using the GeneMapper v.3.7 software (APPLIED BIOSYSTEMS) and the resulting absence-presence matrix was analyzed using the R package MSAP (Perez-Figueroa, 2013). For that purpose, the presence of both EcoRI-HpaII and EcoRI-MspI fragments (1/1) is used as an indicator of unmethylated states; Hemimethylated and internal Cytosine methylated loci are represented by EcoRI–HpaII (1/0) and EcoRI–MspI (0/1) fragments, respectively. Lastly, the absence of both fragments (0/0) mirrors either a target mutation (i.e., recognition site CCGG is not present any more) or hypermethylation, being therefore considered uninformative. Differences among methylation profiles were studied throughout the experiment using Analysis of MOlecular VAriance (AMOVA) and Principal Component Analysis (PCA). Statistical significance was assessed using Fisher's exact tests

(Benjamini & Hochberg multi-test corrections, adjusted $p < 0.05$) based on counts for the four pattern categories (unmethylated, hemimethylated, hypermethylated, and internal Cytosine methylation), identifying loci with non-random distribution of methylation states. Relationship estimates were computed using Gower's Coefficient of Similarity, and the resulting distance matrix was clustered using UPGMA and visualized as a heatmap with the "ComplexHeatmap" R package (Gu et al., 2016).

3. Results and discussion

3.1. Florida Red Tide simulation and feeding response in Eastern oysters

FRTs are usually patchy and highly mobile (i.e., driven by currents and wind), displaying heterogeneous dinoflagellate concentrations as these episodes develop (Carvalho et al., 2011). In order to improve HAB simulation, the present work focused on the first 24 h of a FRT episode, where dinoflagellate concentrations can be predicted more accurately. Accordingly, Eastern oyster individuals were exposed to an exponential increase in the concentration of the brevetoxin-producing dinoflagellate K. brevis (Fig. 1A), simulating a typical medium-high intensity FRT episode (Stumpf et al., 2003). The presence of active feeding in exposed oysters was corroborated by the reduction in the number of K. brevis cells in experimental tanks after each hourly dosage application (Fig. 1B), as well as by additional indicators including oyster valve opening and production of feces. These results, together with the absence of oyster mortality and the stability in water quality parameters throughout the experiment and across treatment groups, support the effectiveness of HAB simulation in ensuring exposure of Eastern oysters to brevetoxins through K. brevis ingestion.

3.2. Eastern oyster exposure to K. brevis does not trigger specific modifications in the expression of H2A.X, H2A.Z and macroH2A

Different epigenetic mechanisms participate in environmental responses, including histone variants encompassing highly specialized functions (Talbert and Henikoff, 2014). Among the different histone families constituting the nucleosome, H2A and H3 display the highest diversity of variants, including H2A.X, H2A.Z, macroH2A, and H2A.Bbd (H2A family); as well as H3.3 and cenH3 (H3 family), among several others [see (Cheema and Ausio, 2015; Henikoff and Smith, 2015) for additional details]. Among H2A variants, H2A.X, H2A.Z and macroH2A stand out due
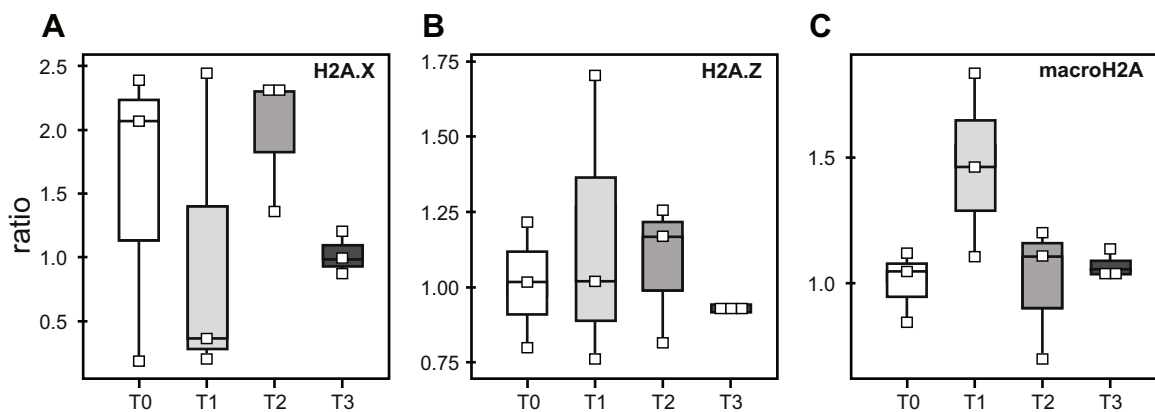
Fig. 2. Histone variant gene expression during exposure to *K. brevis*. Boxplots represent the quartile distribution of the expression levels of Eastern oyster genes encoding histone variants H2A.X (A), H2A.Z (B) and macroH2A (C). Results are categorized by exposure time (T0-T3) and represented as normalized ratios respect to the study calibrator (i.e., gene expression at T0). Boxes and whiskers show a large dispersion in the data for each group of samples, and no significant differences between groups.

Table 2
Frequency (%) of different methylation states at target sequences across different time points.

Band pattern	Methylation state	Time points			
		T0	T1	T2	T3
HPA+/MSP+	Unmethylated	0.1017	0.1085	0.2017	0.1856
HPA+/MSP−	Hemimethylated	0.0958	0.0991	0.1576	0.1763
HPA-/MSP+	Internal Cytosine methylation	0.1059	0.1525	0.0898	0.1398
HPA-/MSP−	Full methylation or absence of target	0.6966	0.6398	0.5508	0.4983

to their involvement (along with PTMs) in the maintenance of genome integrity (Li et al., 2005; Ivashkevich et al., 2012; Shi and Oberdoerffer, 2012; Papamichos-Chronakis and Peterson, 2013; Khurana et al., 2014). Given the ability of brevetoxins to induce DNA damage (Radwan and Ramsdell, 2008; Murrell and Gibson, 2009, 2011), it is hypothesized here that genes encoding H2A.X, H2A.Z and macroH2A will be differentially regulated in response to these stressors. Consequently, the expression of these variants was analyzed during HAB simulation, constituting the first report describing their presence in oysters. The obtained results revealed an absence of significant changes in gene expression across different time points (adjusted *p*-value >0.05) and a substantial amount of inter-individual variation (Fig. 2). That is best exemplified by the expression of H2A.X at time point T1 (Fig. 2A), where individuals under similar experimental conditions display a two-fold factor difference in gene expression. These results suggest that oyster responses to *K. brevis* exposure do not involve specific modifications in H2A.X, H2A.Z and macroH2A transcription. Yet, it still might be possible that specific cause-effect relationships are masked by the high levels of variation observed.

The role of histone variants was further investigated by studying H2A.X, H2A.Z and macroH2A protein expression levels (Fig. 3). This work is the first report describing the presence of these proteins in oysters, as well as the validity of commercial antibodies to detect histone variants in this group. Two of these antibodies constitute commercial products specifically raised against human H2A.X and H2A.Z histones. Their suitability for the study of oyster variants is supported by the high level of evolutionary conservation displayed by these proteins in eukaryotes (Malik and Henikoff, 2003; Eirín-López et al., 2009), as well as by previous reports supporting the experimental application of these antibodies in molluscs (González-Romero et al., 2012; Rivera-Casas et al., 2016b). In the case of macroH2A, an antibody specifically raised against mussel macroH2A (able to specifically detect this variant in invertebrates) was used, further supporting its suitability for oysters (Rivera-Casas et al., 2016a). Similarly to gene expression analyses, western blot

hybridizations did not show substantial modifications in H2A.X, H2A.Z or macroH2A protein levels throughout HAB simulation (Fig. 3B). Altogether, these results suggest that oyster exposure to FRTs does not result in specific transcriptional or translational modifications in the studied histone variants. Nonetheless, an active role for histone variants during environmental responses, without invoking modifications in their expression levels, could still be possible. Accordingly, the dynamic mobilization of pre-existing histone variants to specific chromatin regions was described during environmental responses (Morrison and Shen, 2005; Weber and Henikoff, 2014), including responses to DNA damage (Lopez et al., 2012). Long-term exposure simulations combined with biopsy sampling of specific individuals (Acosta-Salmón and Southgate, 2004) will complement the present results, helping ascertain the nature of the role played by histone variants during environmental responses (Romero-Geraldo et al., 2014, 2016).

3.3. γH2A.X increases in oysters exposed to growing concentrations of K. brevis

The phosphorylation of a Serine residue at the C-terminal domain of the H2A.X protein (γH2A.X) and its subsequent accumulation at damaged chromatin regions (γH2A.X foci) rapidly marks double-stranded DNA breaks for repair, constituting a biomarker of DNA damage widely used in standardized assays (Kuo and Yang, 2008; Turinetto and Giachino, 2015). Since DNA breaks stand out as the most severe effects caused by brevetoxin exposure (Altaf et al., 2007; Radwan and Ramsdell, 2008; Murrell and Gibson, 2009, 2011), protein expression analyses were complemented with the study of γH2A.X dynamics during HAB simulation. The obtained results revealed an increase in γH2A.X, concomitantly with the exposure of Eastern oysters to increasing concentrations of *K. brevis* (T1, T2), and followed by a slight decrease during the recovery phase (T3, Fig. 3C). Based on these results, it seems plausible that γH2A.X formation is triggered by the genotoxic effect of brevetoxins on Eastern oysters (Li et al., 2005; Ivashkevich et al., 2012). This find-
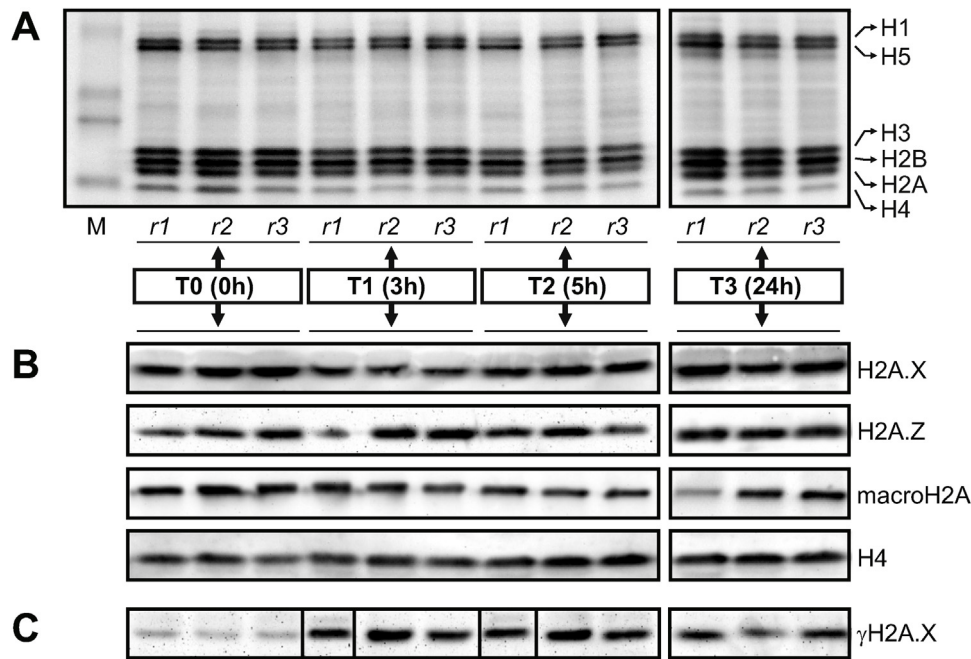
Fig. 3. Histone variant protein expression and H2A.X phosphorylation (γH2A.X) during exposure to *K. brevis*. A, SDS gel showing normalized histones extracted from Eastern oyster individuals at different biological replicates (*r1, r2, r3*) and at different time points (T0, T1, T2, T3). B, Western blot hybridization revealing homogeneous levels of H2A.X, H2A.Z and macroH2A proteins throughout HAB simulation. C, Western blot hybridization showing an increase in γH2A.X formation concomitantly with exposure to increasing *K. brevis* concentrations (T1, T2), followed by a slight decrease during the recovery phase (T3). An H4 antibody was used for normalization purposes. Gels in different boxes denote independent hybridization experiments. M, ClearPAGE Two-Color Marker (C.B.S. SCIENTIFIC).

ing has a dual relevance: first, it corroborates the specialization of histone variants and PTMs in the chromatin of molluscs (González-Romero et al., 2012; Rivera-Casas et al., 2016a,b), supporting a role for γH2A.X during environmental responses in invertebrates (Suarez-Ulloa et al., 2015) and the evolutionary conservation of this mechanism (Kinner et al., 2008; Lee et al., 2014). Second, it validates the application of commercial antibodies to detect γH2A.X in bivalves, opening new avenues for monitoring DNA damage and health in populations of marine invertebrates.

3.4. Global DNA methylation decreases in oysters during HAB simulation

The regulatory role of DNA methylation during environmental responses was previously investigated in marine invertebrates, linking modifications in methylation of stress-responsive genes with phenotypic plasticity and adaptation (Gavery and Roberts, 2010). The present work builds on those results, monitoring DNA methylation in Eastern oysters during HAB simulation. Methylation Sensitive Amplified Polymorphism (MSAP) analyses revealed a total of 428 bands, including 295 methylation-susceptible loci. Among those, 204 (69%) were considered polymorphic (i.e., they have at least two occurrences of both states). The analysis of different methylation states over time revealed a slight increase in unmethylated and hemimethylated states and a loss of fully methylated bands as HAB simulation progressed (T2 and T3, Table 2). More specifically, comparisons among different time points revealed significant differences in DNA methylation (ΦST = 0.1296, $p < 0.05$, Table 3), notably between earlier (T0, T1) and later (T2, T3) exposure, as well as between T2 and T3. Such differences were further evidenced by Principal Component Analysis (PCA), comparing samples in a space of reduced dimensionality (Fig. 4A). Accordingly, the first coordinate (C1) accounts for 16% of the observed variation in DNA methylation, allowing to discriminate between two major groups: one of them corresponding to the early time points (T0 and T1), and the other one to the overexposed oyster samples from T2

Table 3
Pairwise AMOVAs between exposure time points based on complete MSAP methylation profiles. ΦST values and p-values are indicated in the upper and lower diagonal, respectively.

	T0	T1	T2	T3
T0		−0.0087	0.3543	0.4131
T1	0.6489		0.4188	0.4167
T2	0.0311[*]	0.0255[*]		0.3333
T3	0.0291[*]	0.0284[*]	0.0290[*]	

[*] $p < 0.05$.

and T3. The observed differences in DNA methylation were further supported by Fisher exact tests, identifying 10 MSAP loci showing significant differences ($p < 0.05$) between early (T0 and T1) and late (T2 and T3) exposure times (Fig. 4B). These loci were characterized based on methylation profiles, defining two groups: a first one predominantly showing internal Cytosine methylation at T0 and T1, but mostly unmethylated at T2 and T3 (left cluster); and a second group consisting of fully methylated fragments undergoing demethylation towards later exposure times (right cluster). Overall, MSAP results were consistent with a decrease in genome-wide DNA methylation levels as *K. brevis* concentration increased.

Specific DNA methylation profiles contribute to environmentally induced phenotypes, displaying different levels of heritability in invertebrates (Vandegehuchte et al., 2009). In the case of molluscs, DNA methylation participates in the epigenetic regulation of gene expression during development (Riviere et al., 2013; Diaz-Freije et al., 2014), as well as during responses to environmental stress (Gavery and Roberts, 2014). Furthermore, it has been recently suggested that a reduction in global DNA methylation during early invasive episodes could compensate for low genetic variation caused by founder effects in the pygmy mussel (Ardura et al., 2017). Particularly, DNA hypomethylation was described in different organisms during responses to environmental pollution (Vandegehuchte et al., 2009; Chen et al., 2012; Fang et al., 2013; Dimond and Roberts, 2016), including exposure to agents inducing
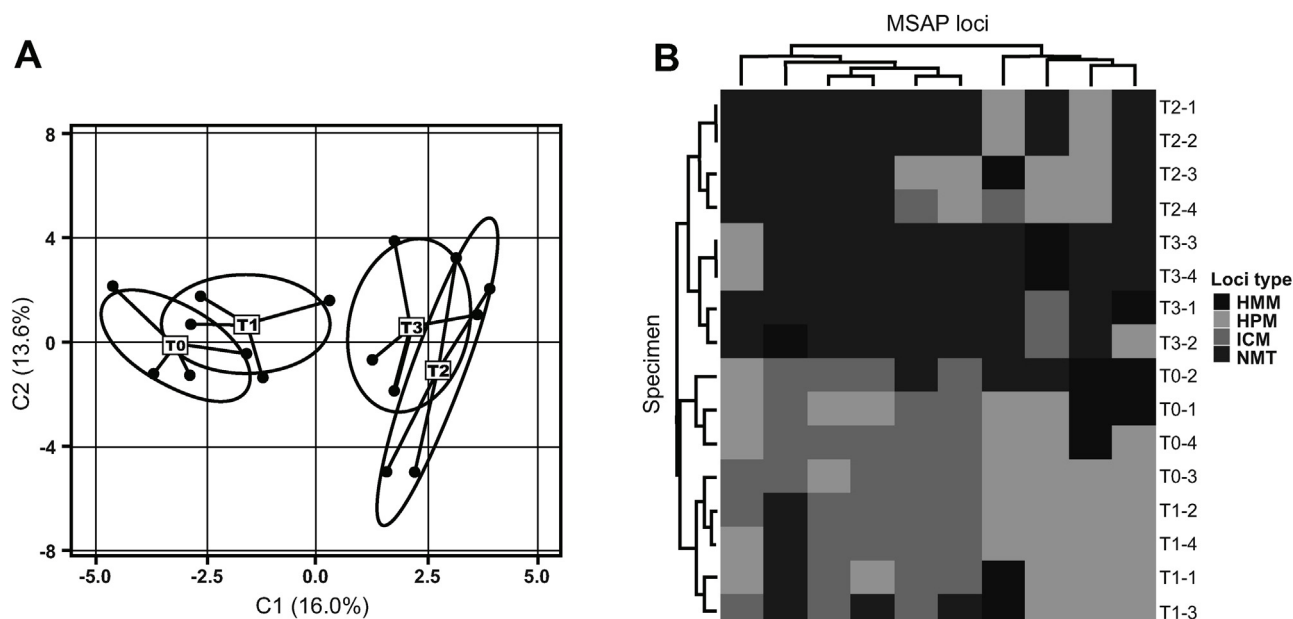
A

B

Fig. 4. Gobal DNA methylation changes during HAB simulation. A, Principal Component Analysis of complete MSAP profiles representing the different treatment groups (i.e., time points), labeled in the centroid of each cluster (T0-T4). The first two principal components are shown, indicating the percentage of the global variance explained on the corresponding axis. Individual samples are represented as points and the variance within each group is represented with an ellipse. Results show that earlier (T0, T1) and later time points (T2, T3) segregate through the C1 = 0 axis, corresponding to differentiated genome-wide methylation patterns. B, Heatmap representing changes in DNA methylation in a group of 10 loci showing a non-random distribution of DNA methylation patterns ($p < 0.05$) throughout the HAB simulation. Methylation profiles observed at T0 and T1 display significant differences from patterns observed at T2 and T3. Rows (specimens) and columns (MSAP loci) were clustered using Gower's Coefficient of Similarity. Loci methylation status is indicated in the right margin of the figure: HMM, hemimethylated, HPM, hypermethylated; ICM, internal cytosine methylation; NMT, non-methylated (unmethylated).

oxidative stress (Mirbahai and Chipman, 2014). Given the ability of brevetoxins to induce oxidative damage, it seems plausible that the observed reduction in global DNA methylation in Eastern oysters is a direct consequence of their exposure to the brevetoxin-producing *K. brevis* during HAB simulation.

4. Conclusions

The present work investigated the effects of FRTs on two types of mechanisms potentially participating in the epigenetic memory of Eastern oysters: histone variants and DNA methylation. This research revealed, for the first time, the existence of H2A.X, H2A.Z and macroH2A genes in oysters along with their active transcription and translation, validating the application of specific qPCR primers, as well as commercial and invertebrate-specific antibodies for their study *in vivo*. While the expression of these variants does not seem to be specifically altered during FRTs, the observed increase in γH2A.X would be consistent with the presence of oxidative DNA damage in Eastern oysters, as a consequence of their exposure to brevetoxins produced by *K. brevis*. Based on these results, the application of γH2A.X as a biomarker of oxidative stress seems plausible, with obvious implications for the conservation and management of Eastern oysters as well as other marine invertebrates. Still, further analyses clarifying the relationship between the magnitude of γH2A.X and the levels of DNA damage will be required to fully elucidate the applicability of this histone variant modification in this group of organisms. Additionally, the study of DNA methylation patterns revealed significant differences between early (T0, T1) and late (T2, T3) stages of the HAB simulation. More precisely, a decrease in genome-wide DNA methylation levels was observed as the simulation progressed. These results are consistent with the documented connection between DNA hypomethylation and environmental responses (Vandegehuchte et al., 2010; Chen et al., 2012; Fang et al., 2013; Dimond and Roberts 2016), thus

supporting an active role for this epigenetic mechanism during oyster responses to *K. brevis* exposure (i.e., modulation of genes specifically involved in responses to brevetoxin stress). Overall, the present work provides a basis to better understand how epigenetic mechanisms participate in responses to environmental stress in marine invertebrates. By doing so, it opens new avenues to incorporate environmental epigenetics approaches into management and conservation programs.

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