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SEASONAL DNA METHYLATION VARIATION IN THE FLAT TREE OYSTER *ISOGNOMON ALATUS* FROM A MANGROVE ECOSYSTEM IN NORTH BISCAYNE BAY, FLORIDA

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ABSTRACT Epigenetic analyses constitute an emerging approach for better understanding of the mechanisms underlying environmental responses and their role during acclimatization and adaptation across diverse ecosystems. The expansion of environmental epigenetic studies to a broader range of ecologically and environmentally relevant organisms will enhance the capability to forecast ecological and evolutionary processes, as well as to facilitate a retrospective assessment of stress exposures in biomonitor organisms through "epigenetic footprinting" analyses. With such purpose, the present study monitored spatial and temporal variation in abiotic parameters (temperature, salinity, pH, and horizontal visibility) over a 2-y period in a mangrove ecosystem located in North Biscayne Bay (North Miami, FL). The obtained data were subsequently compared with epigenetic modifications (global genome-wide DNA methylation levels) in the flat tree oyster *Isognomon alatus*, used as a sentinel model organism across experimental sites. The obtained results revealed a certain level of seasonality in temporal DNA methylation patterns, which seem to be primarily associated with changes in temperature and horizontal visibility. These results constitute the first long-term study combining spatial and temporal epigenetic analyses in a marine organism in its natural environment, laying the initial groundwork to assess the biomonitoring potential of environmental epigenetic analyses.

KEY WORDS: flat tree oyster, *Isognomon* alatus, biomonitoring, coastal oceans, DNA methylation, global climate change, pH, salinity, stress, temperature

INTRODUCTION

The intensification of extreme events is emerging as one of the most important aspects of global climate change, imposing strong selective pressures on marine organisms at different levels, including individual, physiological, and genetic responses (Jentsch et al. 2007, Poloczanska et al. 2016). Epigenetics, defined as the study of phenomena and mechanisms that cause chromosome-bound, heritable (both mitotically and/or meiotically) changes to gene expression that are not dependent on changes to DNA sequence (Deans & Maggert 2015), constitutes a promising frontier to understand how molecular mechanisms regulate these responses (Bollati & Baccarelli 2010, Cortessis et al. 2012, Costantini 2014). Given the contribution of epigenetic mechanisms to phenotypic plasticity and acclimatization (Baccarelli & Bollati 2009, Bollati & Baccarelli 2010, Etchegaray & Mostoslavsky 2016, Torda et al. 2017), their study in a broader range of organisms [notably ecologically and environmentally relevant species (Suarez-Ulloa et al. 2015, Rivera-Casas et al. 2017)] will enhance the capability to forecast ecological and evolutionary processes (Beal et al. 2018), as well as to facilitate a retrospective assessment of stress exposures in biomonitor organisms through "epigenetic footprinting" analyses (Eirin-Lopez & Putnam 2018).

Oceans bear the brunt of global climate change, a fact which is best exemplified by the negative impact experienced by charismatic marine ecosystems such as coral reefs and mangrove forests (Lotze et al. 2006). These oligotrophic environments play critical roles in coastal oceans because of the high diversity of species they harbor and for the services they provide (Hoegh-Guldberg & Bruno 2010). Among them, mangrove habitats have been particularly affected during the last two decades, including the loss of at least 35% of their area, exceeding those for coral reefs and tropical rainforests (Valiela et al. 2001). Unfortunately, and even though mangroves provide critical ecosystem services (Lee et al. 2014) and protect coasts from erosion and extreme weather episodes (Alongi 2008), their alarming degradation has received much less publicity in mainstream media (Valiela et al. 2001). Precisely, a side effect of their protective properties is that mangroves are constantly affected by natural stressors [e.g., changes in salinity and temperature (Feller et al. 2010)] and by anthropogenic disturbance, especially in the proximity of densely populated areas (Delabie et al. 2006, Bala Krishna Prasad 2011, Zhang et al. 2014).

Among the high diversity of organisms hosted by mangrove forests, the flat tree oyster *Isognomon alatus* (Gmelin, 1791) stands out because of its association with mangrove roots forming dense patches in the red mangrove *Rhizophora mangle* (Fig. 1A) and for its ubiquitous distribution throughout the Caribbean, from Colombia to the Gulf Coast of the United States (Felder 2009, Tëmkin 2010). Both characteristics, along with a filter-feeding and sessile lifestyle, make *I. alatus* a great sentinel species for health-biomonitoring studies in mangrove ecosystems (Saed et al. 2002, Yap et al. 2010, Graniero et al. 2016). Its choice is further supported by the general value of bivalve molluscs as sentinel organisms widely used in biomonitoring studies (Suarez-Ulloa et al. 2015), by the availability of multiple bivalve "-omic" resources (Suárez-Ulloa et al. 2013), and by the emergence of this group as a model system for epigenetic analyses (Rivera-Casas et al. 2017). Accordingly, recent studies have described a role for DNA methylation in epigenetic effects during development and intragenerational responses to stress in oysters (Gavery & Roberts 2014, Olson &

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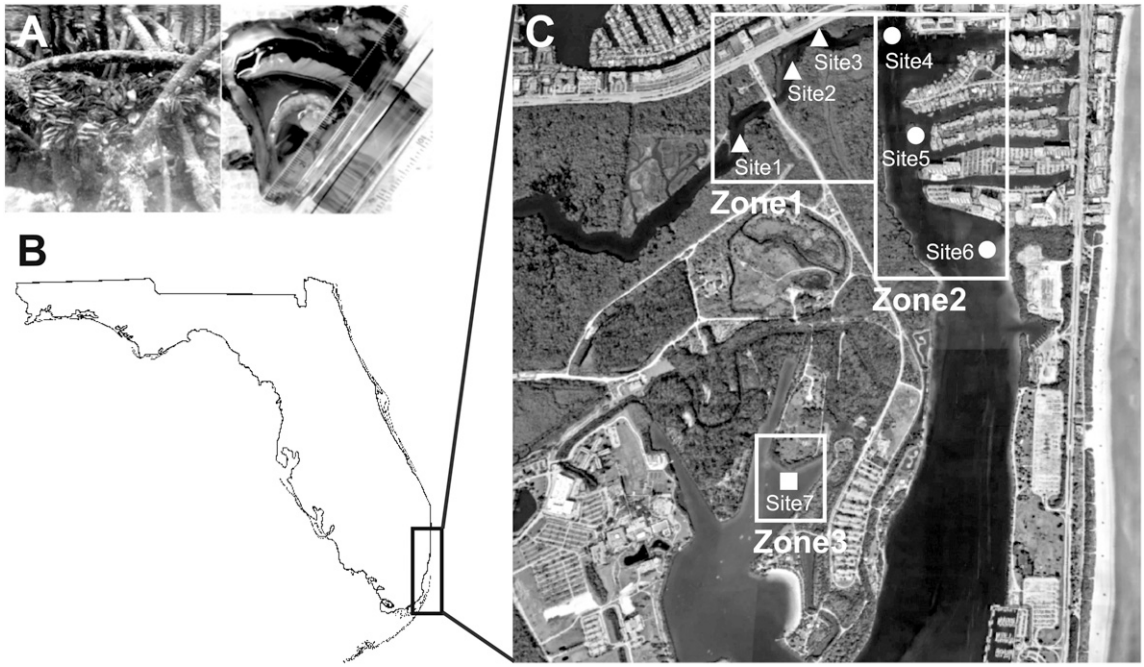

Figure 1. The flat tree oyster *Isognomon alatus* (A) forms tightly packed clusters attached to prop mangrove roots in tropical areas of the American continent (credit: Caroline Rogers, U.S. Geological Survey, Public domain). Individuals with a shell length (hinge to edge as shown) ranging from 5 to 10 cm were collected, and their gills were dissected to analyze genome-wide levels of DNA methylation. The present study was conducted in a mangrove ecosystem located in NBB, FL, surrounded by highly urbanized areas of the Miami metropolis (B and C; credit: Google Maps). Sampling sites were organized into three main zones, including (C) Zone 1, upstream Oleta River; Zone 2, lagoon area; and Zone 3, ocean inlet area. By following such distribution, this study covers areas differentially exposed to freshwater (i.e., Zone 1) or oceanic seawater inputs (i.e., Zone 3) and with higher and lower direct contact with anthropogenic activity (e.g., intensive boat traffic in Zone 2).

Roberts 2014, Gonzalez-Romero et al. 2017), as well as in transgenerational plasticity [i.e., epigenetic inheritance of DNA methylome (Jiang et al. 2016, Rondon et al. 2017)]. In addition, studies showing fitness increase in offspring from parents or grandparents previously exposed to stress could potentially be explained by stable epigenetic modifications (Parker et al. 2015, 2017, Zhao et al. 2017a).

Research efforts investigating the cause–effect relationships between marine environmental stressors and subsequent epigenetic mechanisms modulating phenotypic responses (i.e., environmental epigenetic analyses) have flourished during the last 5 y (Eirin-Lopez & Putnam 2018). Yet, their implementation in biomonitoring across diverse marine ecosystems and species remains hampered by the intrinsic experimental complexity of most epigenetic techniques, by the difficulty to accommodate epigenetic population analyses consisting of large sample sizes, and by the lack of seasonal baseline data. The present work aims to strengthen those aspects and explore the potential of epigenetic-based biomonitoring tools by developing the first seasonal epigenetic monitoring study in a marine ecosystem. For that purpose, genome-wide DNA methylation levels were analyzed in flat tree oysters from a mangrove ecosystem during a 2-y period, providing very valuable baseline information about the impact of natural seasonal variation on the oyster epigenome. These results will facilitate the development of long-term studies and impact assessment of potential future events that may threaten mangrove ecosystems.

MATERIALS AND METHODS

Specimen Collection and Study Area Description

Flat tree oyster (*Isognomon alatus*) specimens were collected from seven different sites across North Biscayne Bay (NBB), FL, during a 2-y period (July 2015 to June 2017). Biscayne Bay is a marine lagoon categorized as an aquatic preserve, located between the U.S. mainland and a barrier of islands, as part of the Miami metropolitan area in southeastern Florida (Fig. 1B, C). This body of water stretches from the Oleta River in the north to the Card Sound in the south, comprising the Biscayne National Park in its central core (Caccia & Boyer 2005). The NBB area overlaps with the Oleta River State Park, harboring a critically endangered mangrove ecosystem subject to multiple water environmental gradients caused primarily by freshwater input from the Oleta River and coastal urban development in the cities of North Miami and North Miami Beach. The Oleta River flows southward close to a major road and densely populated areas, with mangrove populations lining both sides of the river. Over its course, the influence of oceanic input and anthropogenic impact (e.g., boat traffic) has increased progressively on these mangroves. The integrity of this ecosystem is further jeopardized by the Munisport Landfill adjacent to the Oleta River State Park, categorized as a superfund site (see https://cumulis.epa.gov/for additional details).

Given the observed gradients, the present study defined three major geographic zones expected to differ in different types of abiotic parameters, including an area upstream Oleta River

(Sites 1–3, Zone 1) and another in the adjacent lagoon (Sites 4–6, Zone 2). In addition, a third area (Site 7, Zone 3) farther from the river and close to the inlet of the ocean at Haulover Park was added as an out-group in the analyses (Fig. 1C). Oyster specimens for epigenetic analyses were collected at the described sampling sites every 2 mo during a 2-y period (July 2015 to June 2017). The oyster patches were visually inspected at mangrove roots or at clusters on the seabed, identifying adult individuals based on their size range (5–10 cm, longest distance from the hinge to the opposite edge of the shell, Fig. 1A). Five adult oysters were sampled at each site and immediately flash-frozen in liquid nitrogen on collection (transported in the boat using a portable dewar). The samples were subsequently placed on dry ice and transported to the laboratory, where they were stored at –80°C until further processing.

Quantification of Abiotic Parameters

The abiotic baseline data characterization of study sites consisted of monthly measurements of those environmental parameters potentially subject to sharpest seasonal changes, including seawater temperature, salinity, horizontal visibility, pH, and precipitation. Seawater temperature and salinity levels were monitored using a YSI Handheld Multiparameter Instrument (Xylem Analytics, OH) with corresponding calibrated probes. Horizontal visibility was quantified using a Secchi disk, determining the maximum distance at which the disk could still be discerned through a horizontal line close to the surface (Steel et al. 2002). In addition, water samples were collected at different sites and transported to the laboratory to immediately quantify pH levels using a VWR SympHony benchtop pH meter (VWR International, PA) at the same temperatures as those recorded in the field sites. Last, precipitation data for experimental sites during sampling days were obtained from the DBHYDRO database (S29_R station, Arch Creek, www.sfwmd.gov/science-data/dbhydro).

Genome-Wide Global DNA Methylation Analysis

DNA methylation was characterized using gill tissue from oysters collected at 13 different time points distributed across a 2-y period (2015 to 2017). This work builds on previous studies recommending the use of gill tissue for monitoring studies based on its prime contact with the surrounding aquatic environment (Prego-Faraldo et al. 2016, 2017) and specifically in the case of epigenetic analyses (Gonzalez-Romero et al. 2017). Consequently, gills were dissected from all five individuals collected from each site and independently pooled into CTAB lysis buffer (100 mM Tris-HCl, 20 mM ethylenediaminetetraacetic acid, 1.2 M NaCl, and 2% CTAB), constituting a biological sample (i.e., each sampling site produces a biological sample or pool per time point). Genomic DNA was subsequently extracted from pooled samples following the modified low-salt CTAB protocol (Arseneau et al. 2017) and adapted to flat tree oyster in the present work. Briefly, the samples were incubated at 55°C for 3 h with CTAB lysis buffer and proteinase K, and the DNA was extracted with chloroform: isoamyl alcohol (24:1) and precipitated after incubation with diluted CTAB buffer (100 mM Tris-HCl, 20 mM ethylenediaminetetraacetic acid, and CTAB 2% w/v). The concentration of the obtained DNA was measured using a Qubit 2.0 Fluorometer (Thermo Fisher, Waltham, MA) following the instructions provided by the manufacturer.

Genome-wide DNA methylation levels were quantified in genomic DNA samples by measuring the amount of 5-methyl cytosines (5-mC), using the MethylFlash Global DNA Methylation (5-mC) ELISA Kit (Epigentek, Farmingdale, NY). Accordingly, the DNA samples were assayed in duplicate using 100 ng as the starting material. The methylated fraction of the DNA was detected by using a 5-mC antibody and quantified colorimetrically by reading the optical density at 450 nm in an ELx808IU microplate reader (BioTek, Winooski, VT). Percentage of methylated DNA was calculated based on the slope of the standard curve generated from samples of known methylation levels, following the calculations suggested by the manufacturer. The intra-assay coefficient of variation (CV) was determined as 0.08.

Data Analysis

The abiotic parameters and DNA methylation levels measured in the present work were organized into a database and analyzed using R. The simultaneous analysis of all the parameters was implemented through a dimensionality reduction using principal component analysis (PCA). This method allows the visualization of multivariate data in a simple bidimensional space, emphasizing those variables that seem to have a stronger influence in the observed variation. Subsequently, sampling sites were additionally clustered in the principal component space using k-means, setting a predefined number of groups ($k = 3$) motivated on their geographical distribution: Zone 1, upstream Oleta River; Zone 2, lagoon area; and Zone 3, ocean inlet area (Fig. 1). The purpose of the clustering step is to classify the different sites into groups based on the similarity of their temporal profiles. Unsupervised hierarchical clustering (agglomeration method = average and distance measure = Euclidean) was used to group sampling sites based on the similarities of their temporal patterns. A multiscale bootstrap resampling was performed to generate P values for each cluster (Suzuki & Shimodaira 2006), representing the uncertainty associated with the obtained cluster distribution. Approximately unbiased (au) P values represent the results of the multiscale bootstrap resampling, which is considered more accurate than the bootstrap probability (bp). The clustering distribution was considered significant if au >95 (Suzuki & Shimodaira 2006).

The analysis of seasonal modifications in the measured parameters (across sampling sites and time) was carried out by plotting a time series for each variable individually (Fig. 2). Yearly modifications between wet and dry seasons were studied using a t-test (two-tailed, unless specified otherwise). Such comparison was chosen based on the dominance of a tropical climate of the study area, with marked differences between wet (May to October) and dry (November to April) seasons, potentially affecting all abiotic parameters studied in the present work. The pairwise correlation between patterns of abiotic variation and genome-wide DNA methylation levels were individually tested using nonparametric Spearman pairwise rho coefficients. Last, generalized linear models (GLMs) were constructed from the complete multivariate dataset, choosing the one with the lowest Akaike information criterion as the best among the different models tested. The obtained GLM model was tested with Hosmer and Lemeshow goodness-of-fit test. Data and code are available on Github (https://github.com/eelabfiu/FTO).

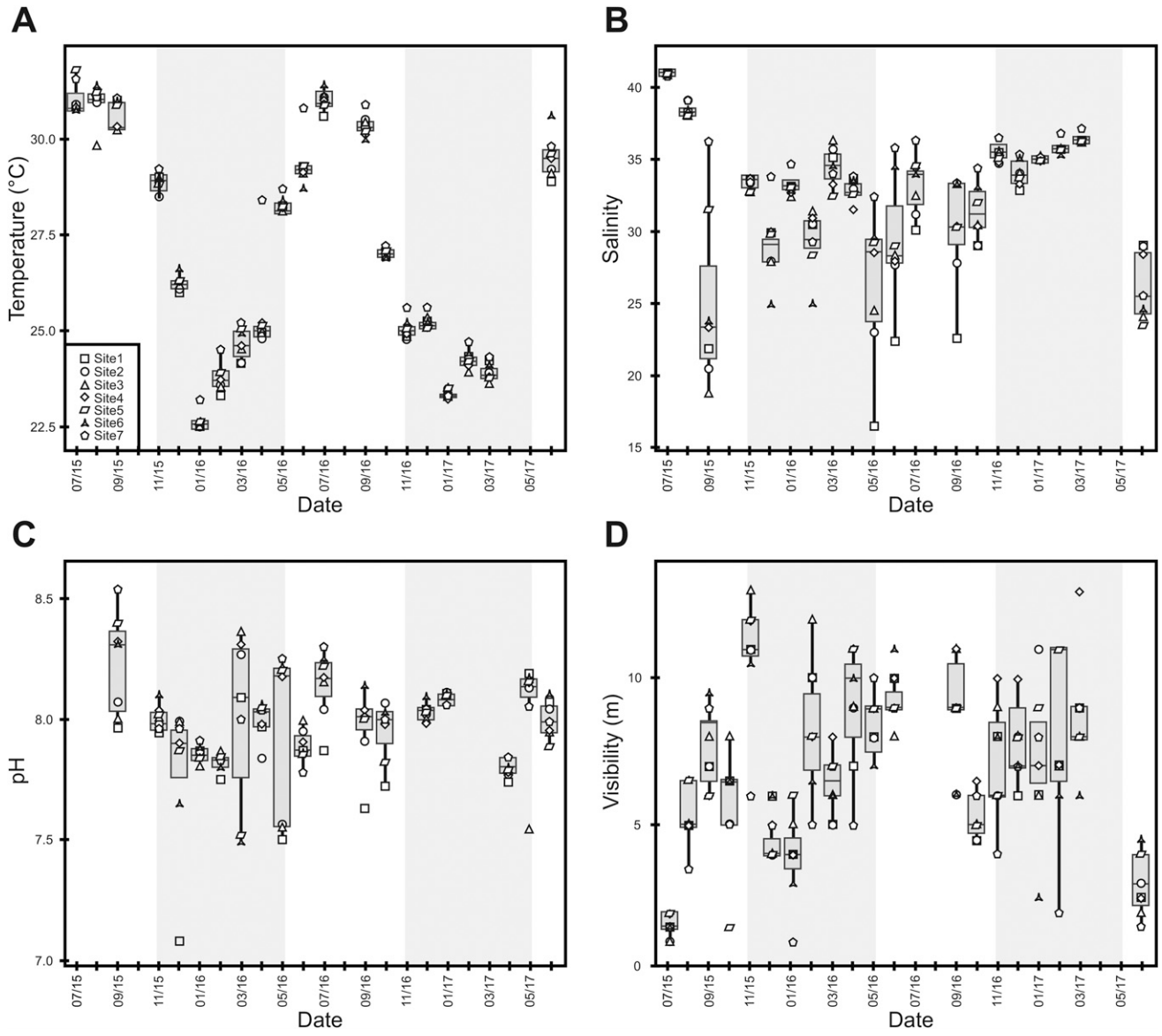

Figure 2. Temporal variation observed in different environmental parameters, including temperature (A), salinity (B), pH (C), and horizontal visibility (D), across different experimental sites in NBB. Boxplots represent the data distribution in quartiles. Temperature constitutes the only parameter displaying a conspicuous seasonal variation, with salinity, pH, and horizontal visibility being less influenced by seasonal changes. In this latter case, the effect of singular extreme weather events (e.g., hurricanes) and anthropogenic pressures (e.g., pollution) are also possible. Seasonal differences between wet and dry seasons are statistically significant (P value < 0.05, t-test) in all cases.

RESULTS

Abiotic Characterization of NBB

The present study provides a unique opportunity to profile the environmental conditions shaping the mangrove ecosystem of NBB across different gradient areas. Accordingly, information about precipitation, seawater temperature, salinity, horizontal visibility, and pH was collected every month at each sampling site across NBB (Fig. 2). The precipitation data show a significant seasonal variation throughout the duration of the present study, with averages of 0.18 inches/day and 0.12 inches/day in wet (May to October) and dry (November to April) seasons, respectively (P value = 0.01, t-test). The effects of the marked tropical seasonality were also corroborated for the case of water temperature, a well-known stressor of marine organisms (Bruno et al. 2015, Clements et al. 2018), with a maximum of 31.8°C in July 2015 and a minimum of 22.5°C in January 2016 (Table 1), producing average and median temperatures of 27.1°C and 26.9°C, respectively, across all monitored sites. Although no obvious thermal spatial patterns were found across the sampling sites (Fig. 2A), significant differences were observed when comparing wet and dry seasons (P value = 1E-35, t-test).

The salinity levels determined at the experimental sites ranged from 16.5 to 41.1, with an average of 32.5 (Table 1). Although seasonal trends were not obvious in this case (Fig. 2B), the largest variation in this parameter was observed during the wet season, displaying an inverse correspondence

TABLE 1.

Summary statistics for abiotic parameters combining data from all sites over the course of the present study.

	Temperature (°C)	Salinity	pH	Horizontal visibility (m)
Maximum	31.8	41.1	8.5	13.0
Minimum	22.5	16.5	7.1	1.0
Average	27.1	32.5	8.0	6.9
Median	26.9	33.5	8.0	7.0
SD	2.9	10.3	0.2	2.9

with precipitation (Fig. 3), which is even more evident when analyzing seasonal data independently (i.e., salinity decreases significantly during the wet season, P value = 0.008, t-test). A significant difference was also observed in the analysis of horizontal visibility data (Fig. 2D) between wet and dry seasons (P value = 0.006, t-test), likely determined by the increased flow of freshwater contributed by the Oleta River during the wet season. The analysis of pH, on the other hand, suggests that this parameter does not follow the fluctuation patterns that are conspicuous for other abiotic parameters such as temperature (Fig. 2C). Nonetheless, significant differences were again observed when comparing wet (high pH) and dry (low pH) seasons (P value = 0.02, t-test, Table 1).

Spatial clustering analyses (based on all abiotic factors simultaneously) grouped Sites 1, 2, and 3 (upstream Oleta River) independently from sites in the adjacent lagoon (Sites 4, 5, and 6). Site 7 was clustered independently, representing the most divergent site closer to the ocean inlet. On the other hand, the intracluster comparisons among individual sites revealed a small level of variation in all cases, as shown by the nonsignificant probability values obtained in hierarchical clustering analyses (au <95, Fig. 4C). Particularly, Site 4 seemed to be more similar to sites within Zone 1 than to other geographically related sites within Zone 2 when using a hierarchical method instead of k-means. Such a result could be attributed to the transitional nature of this site (i.e., outside the mouth of the Oleta River, Fig. 1C). Consistently, a comparison among different zones using analysis of variance revealed nonsignificant results (P value > 0.05) for all tested abiotic parameters.

Abiotic parameters were further analyzed, revealing the presence of variation patterns. Accordingly, a low thermal variation was found among sites, with a maximum CV for measurements in a single time point of 5.1%, and an average CV across different months of 1.4%. On the other hand, salinity was less homogeneous, as revealed by the larger fluctuations observed in comparisons between sites in Zones 1 and 2. In that case, a maximum CV of 49% was observed, with an average CV value of 8.4% across different time points. Furthermore, individual comparisons revealed a gradient between sites closer to the Oleta River and sites closer to the ocean inlet during the months with high precipitation levels (Fig. 3). This observation is consistent with the expected effect of tidal regimes and heavy precipitation episodes in river outlets, constituting the main source of freshwater into the bay. Last, the study of pH and horizontal visibility did not show any type of geographical trends within the studied areas. Nonetheless, these parameters could be affected by other potential sources of variation, notably the high levels of nutrient runoff pollution reported in NBB (Caccia & Boyer 2005, 2007).

DNA Methylation Levels in the Flat Tree Oyster Isognomon alatus

Global genome-wide DNA methylation levels were monitored in flat tree oysters across the different experimental sites for a period of 2 y. The DNA methylation levels ranged from 0.02% to 1.28%, revealing a high level of variation across samples (average = 0.55% ± 0.03%). These results are consistent with previous estimations reported for other invertebrates (Zemach et al. 2010) and with the DNA methylation levels described in gill tissues from other oyster species using high-throughput sequencing methods (Gavery & Roberts 2013). The spatial analysis of DNA methylation revealed that the specific location of the different experimental sites in NBB (i.e., Zones 1 and 2) does not seem to carry significant changes in this epigenetic modification; however, consistently with the results obtained for abiotic factors, both hierarchical clustering and PCA revealed that DNA methylation levels in oysters from Site 7 (Zone 3) are significantly different from those found in oysters from Zones 1 and 2 (Fig. 4).

Spatial analyses were completed with the temporal study of oyster DNA methylation levels across experimental sites,

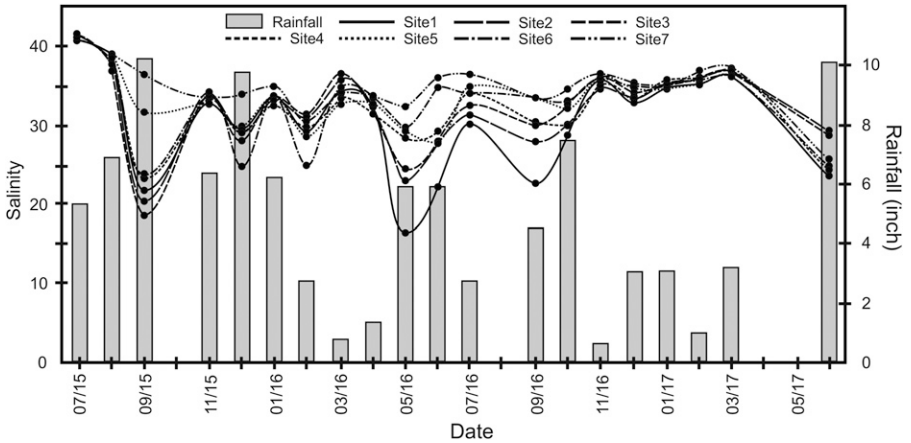

Figure 3. Time series overlapping levels of salinity and precipitation during the sampling months. A greater salinity variability across sites can be observed in periods with greater rain input, more noticeably in the decrease of salinity levels at sites within Zone 1 (upstream Oleta River).

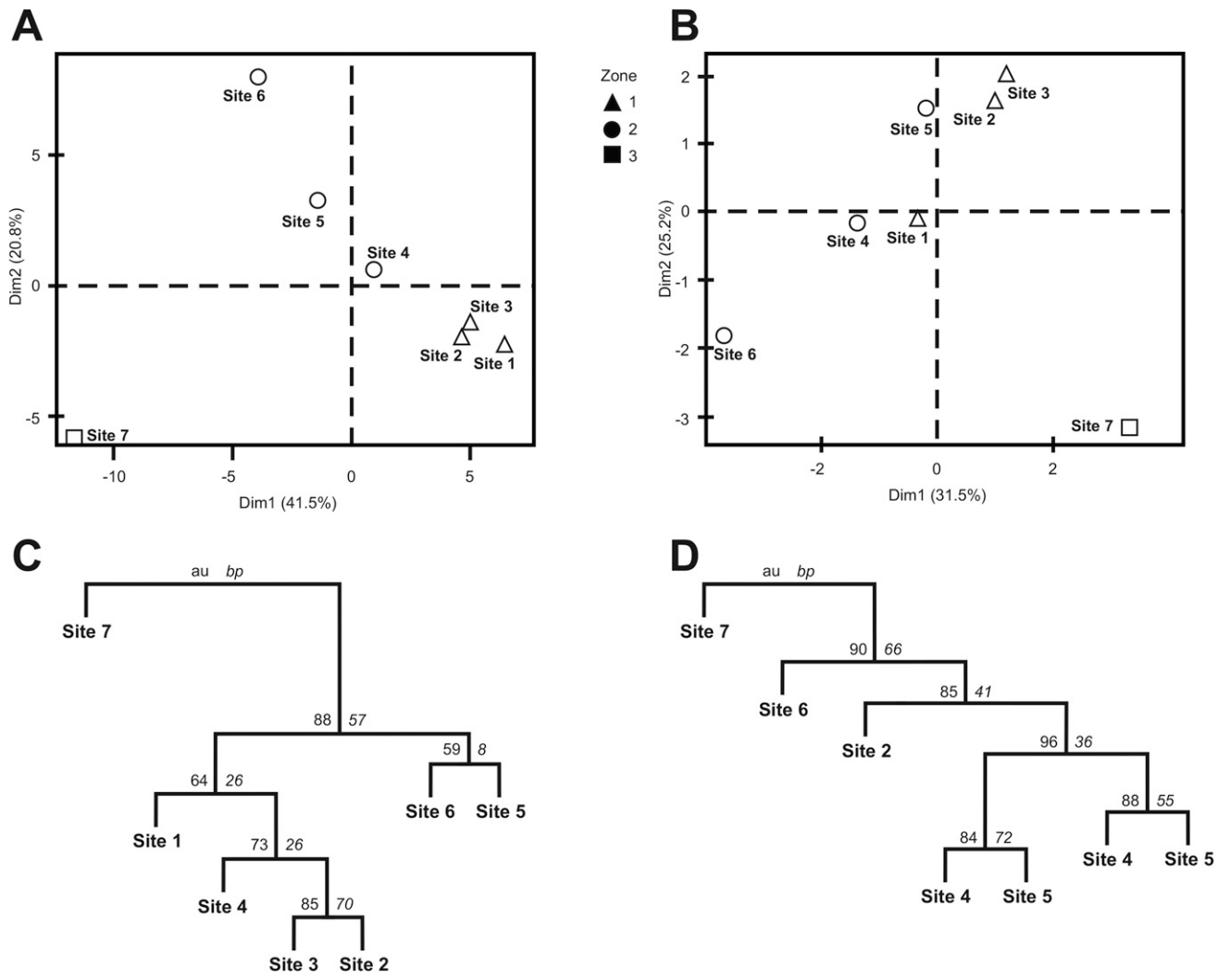

Figure 4. Experimental site analysis based on combined temperature, salinity, pH, and horizontal visibility data, using PCA clustering analysis of abiotic parameters by *k*-means (A) and based on the time series of percentage of DNA methylation observed for the flat tree oysters (B). The corresponding unsupervised hierarchical classification of abiotic factors (C) and DNA methylation data (D) is shown. The PCA plot of abiotic parameters (A) shows the two first principal components or dimensions (Dim 1 and Dim 2) explaining up to 41.5% of the variability for the combined abiotic data (C). In the case of DNA methylation, the PCA plot (B) explains up to 31.5% for the DNA methylation data (D). The site clustering defined by the PCA space is in agreement with the geographical distribution of the experimental sites in NBB, supporting the existence of environmental gradients. On the other hand, differences among sites are too subtle to be unequivocally resolved through hierarchical clustering (probability values au and bp < 95), especially in the case of transitional Site 4. Approximately unbiased (left value on nodes) *P* values represent the results of the multiscale bootstrap resampling, which is considered more accurate than the bp (right value on nodes).

revealing the presence of seasonal patterns in this epigenetic mark. More precisely, higher DNA methylation levels were found during the dry season months as compared with the wet season (P value = 0.002, t-test; Fig. 5A). The seasonal variation in oyster DNA methylation was complemented by studying the extent to which each of the characterized abiotic parameters contributes to this epigenetic mark. Accordingly, significant inverse correlations were found between DNA methylation and temperature, as well as between DNA methylation and horizontal visibility (P value < 0.05, Spearman's rho; Table 2, Fig. 5B). These results were reinforced by multivariate statistical analysis using a GLM approach, where the best model includes only temperature and horizontal visibility as independent variables (Akaike information criteria value = 6.5). Such a model is described by the linear equation: DNA methylation = [0.1472 × (temperature + 0.1313) × (visibility – 3.0375)]. According to the Hosmer–Lemeshow goodness-of-fit test, the predicted global DNA methylation values fit well with the observed data (P value = 0.6087, chi-square).

DISCUSSION

Abiotic Characteristics of NBB are Influenced by Tropical Seasonal Variation

The abiotic characterization of the NBB mangrove ecosystem revealed that this area is subject to multiple environmental gradients. The organization of the different environments is consistent with their spatial distribution defining three different zones, supporting the assumptions of the experimental design.

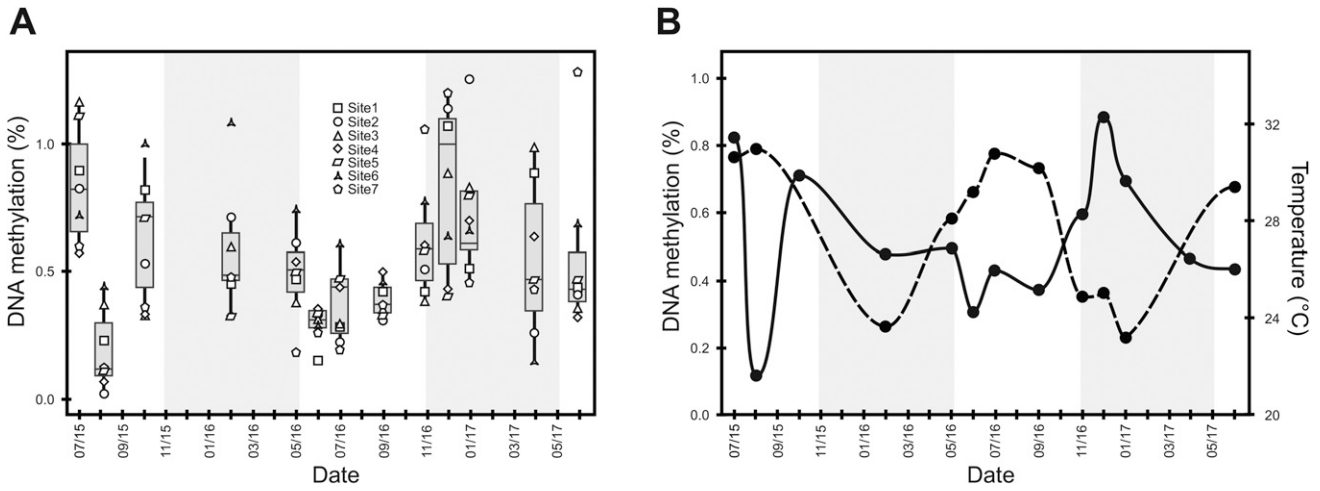

Figure 5. (A) Seasonal variation in global genome-wide DNA methylation in flat tree oysters from NBB over the course of the present experiment, displaying significant differences between averages in dry and wet seasons (P value = 2E-3, t-test). Each site is represented by a biological sample of pooled gill tissue from five oyster individuals. Months corresponding to dry seasons are highlighted by gray boxes, as opposed to wet season months, over the 2-y experiment. (B) DNA methylation data (solid line) are compared with temperature data (dashed line, medians across sites) showing opposite seasonal trends.

Although the contribution of each individual abiotic parameter to the overall definition of these zones is still uncertain, the clustering analysis of their seasonal profiles (i.e., k-means in PCA space with a predefined $k = 3$, Fig. 4A) supports the existence of environmental gradients. These are especially conspicuous when comparing Zone 3 with Zones 1 and 2, as expected by their geographical location. On the other hand, Zones 1 and 2 were not clearly distinguishable from each other based on the measured abiotic factors. In addition, a clear trend toward seasonal variation was observed, driven by differences in precipitation and temperature (Biedinger & Lushine 1993) and defining wet (May to October) and dry (November to April) seasons. It should be noted that the years 2015 and 2016 (when this study was conducted) were subject to strong effects of El Niño (Jacox et al. 2016), which is reported to have increased precipitation levels in South Florida (Schmidt et al. 2001). As a consequence, seasonal climate variation was less pronounced than usual during this period.

The obtained abiotic parameter results are in agreement with the tropical climate characteristics expected from the study location in South Florida. These include high mean temperatures and levels of precipitation from May to October, concomitantly with a decrease in salinity levels to ranges previously recorded for the Biscayne Bay area (Caccia & Boyer 2005, Kelble et al. 2007). Salinity regimes bear critical implications for marine invertebrates, given their role on cellular homeostasis and modulating the effects of acidic seawater conditions (Dickinson et al. 2013). Indeed, this parameter can change drastically in Biscayne Bay because of the effects of frequent extreme weather events such as tropical storms and hurricanes (Caccia & Boyer 2005, Kelble et al. 2007). Similarly, horizontal water visibility can be influenced by meteorological conditions (i.e., precipitation), but it may be also linked to different levels of suspended organic matter, algal presence, and pollution affecting sessile filter-feeder organisms (Davies-Colley & Smith 2001). Although the cause–effect relationships between marine pollutants and horizontal water visibility are beyond the scope of this work, such relationship constitutes an attractive avenue for future research (O'Sullivan 1971, Caccia & Boyer 2005, 2007), especially by considering the proximity of a superfund landfill site to the studied area. Last, seawater pH critically affects marine organisms, especially shellfish with carbonate-based shells, which can be chemically degraded in acidic conditions (Gazeau et al. 2007, Liu et al. 2016, Zhao et al. 2017b, Su et al. 2018). Seasonal variation in pH levels was also observed, with more acidic conditions during the dry season.

TABLE 2.

Pairwise correlation analysis between global genome-wide DNA methylation and each of the abiotic parameters studied in this work (i.e., temperature, salinity, pH, and horizontal visibility), using the nonparametric Spearman coefficient (rho).

	DNA methylation vs.			
	Temperature	**Salinity**	**pH**	**Visibility**
Spearman rho	–0.38	0.17	0.09	–0.24
P value	6E-4***	0.13	0.46	0.039*

* $P < 0.05$, *** $P < 0.001$.

Flat Tree Oysters Display Seasonal Changes in DNA Methylation

The changes in environmental conditions trigger different phenotypic responses in marine organisms, requiring modifications in gene function across different cell types that are regulated by modifications in epigenetic marks. DNA methylation is arguably the most studied epigenetic modification, displaying a well-established correlation with the regulation of gene expression (Klose & Bird 2006). Accordingly, DNA methylation at upstream promoters and enhancers is usually associated with the transcriptional repression of downstream genes (Deaton & Bird 2011), whereas the DNA methylation of gene bodies is correlated with highly active genes, reduction of transcriptional noise, and regulation of alternative splicing

(Shukla et al. 2011, Jones 2012, Huh et al. 2013). Although the present work does not discriminate between these two types of DNA methylation, the identification of changes in global DNA methylation profiles constitutes the first necessary evidence supporting the existence of active epigenetic responses to environmental variation in the flat tree oyster. Importantly, by characterizing the abiotic parameters in NBB, this work allows to study the epigenetic mechanisms (e.g., DNA methylation) linking local and seasonal environmental changes to subsequent phenotypic (e.g., transcriptomic) responses in marine organisms inhabiting that area.

Hierarchical clustering and PCA analysis revealed that seasonal patterns in global DNA methylation are more similar in oysters from zones that are geographically close (i.e., Zones 1 and 2), which in turn display consistently larger differences with respect to oysters from Zone 3. Similarly, and supporting the results obtained in the analyses of abiotic parameters, DNA methylation levels did not display significant differences in comparisons between Zones 1 and 2. The spatial correlation between DNA methylation and changes in abiotic factors is also supported by the temporal analyses developed in this work, revealing higher levels of DNA methylation during the dry season in comparison with the wet season. More precisely, temperature and horizontal visibility (to a lesser extent) represent the most likely drivers of the observed DNA methylation patterns. Such observations agree with previous studies describing temperature as one of the major drivers of epigenetic changes both in vertebrates and invertebrate organisms, with an effect which is largely dependent on the developmental stage of the organism (Marsh & Pasqualone 2014, Anastasiadi et al. 2017). Similarly, horizontal visibility and DNA methylation were significantly correlated (Table 2); however, because this parameter is intimately related with other environmental stressors that have been shown to affect DNA methylation levels or trends in other aquatic organisms [e.g., pollutants (Aluru et al. 2018), harmful algal blooms (Gonzalez-Romero et al. 2017), or nutrient input (Rodriguez-Casariego et al. 2018)], the results obtained in this work evidence the need of studying additional parameters to better characterize environmental epigenetic responses in marine invertebrates.

Oppositely, the observed changes in pH and salinity levels do not seem to drive significant modifications in oyster DNA methylation patterns. Nonetheless, the contribution of these parameters cannot be ruled out, especially by considering recent reports describing significant epigenetic modifications in marine organisms driven by these factors. For instance, a link between pH and global DNA methylation has been reported in the environmentally sensitive coral *Pocillopora damicornis*, but not in the most resistant *Montipora capitata* (Putnam et al. 2016). Similarly, widespread DNA methylation changes were also observed in the coral *Stylophora pistillata* after long-term exposure to low pH levels (Liew et al. 2018), evidencing a role for DNA methylation in regulating the expression of genes involved in responses to pH stress. Salinity, although less studied, has been also linked to DNA methylation changes in fish (Li et al. 2017, Metzger & Schulte 2018) and molluscs such as the Pacific abalone *Haliotis discus hannai* (Kong et al. 2017). The magnitude of the influence of pH and salinity on DNA methylation in flat tree oysters is difficult to ascertain. Although these parameters are likely to modulate the methylation status of specific genes, further analyses will be required to evaluate their effects on the physiological response of these organisms.

Overall, the findings presented in this work are consistent with the regulatory role of environmentally induced epigenetic modifications in marine organisms, potentially affecting phenotypic plasticity. Along with studies describing seasonal gene expression patterns in other marine invertebrates (Banni et al. 2011), this work constitutes one of the first necessary steps toward better understanding the cause–effect relationships between environmental variation and epigenetic responses in marine organisms. Although these observations open new exciting avenues for the application of environmental epigenetic analyses (e.g., biomonitoring of marine ecosystems), several important considerations need to be addressed, notably the contribution of additional environmental parameters. Accordingly, the effect of anthropogenic pollution cannot be discarded, given the presence of a superfund site near the study area and the intense urban development and recreational boating activities in NBB. In addition, the developmental and physiological conditions of the studied oysters will play a critical role in influencing epigenetic responses, potentially contributing to the observed interindividual variability. For instance, the link between gene expression and reproductive cycles in mussels (Banni et al. 2011) raises questions about the contribution of the latter to seasonal DNA methylation patterns in flat tree oysters and whether these cycles are primarily modulated by environmental cues (Siung 1980). Last, family or population effects along with sample pooling effects could result in biased epigenetic estimations, requiring independent genotyping and subsequent analysis of individual samples.

CONCLUSIONS

The present work depicts a complex relationship between environmental abiotic parameters and epigenetic marks in marine organisms, constituting the first long-term study combining spatial and temporal epigenetic analyses in a marine species in its native environment. This is best illustrated by the observed seasonal epigenetic variation in flat tree oysters, potentially affected by changes in temperature and horizontal visibility in a tropical mangrove ecosystem. In doing so, this effort helps to increase the current understanding about marine environmental epigenetics, taking additional steps to assess its potential assisting environmental monitoring analyses. That goal remains contingent to clarifying the contribution of other types of environmental factors, notably anthropogenic effects (e.g., pollution) and developmental effects (e.g., physiological status), that may complement or modulate the epigenetic effect of the abiotic parameters studied. Similarly, further studies will be required to understand the mechanistic relationships between environmentally induced epigenetic modifications and the onset of specific phenotypes, requiring the identification of specific genes responsive to key environmental stressors. Overall, the progressive development of environmental epigenetic studies is opening new avenues to provide answers to long-standing basic scientific questions and the application of this knowledge to solve immediate environmental challenges.

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