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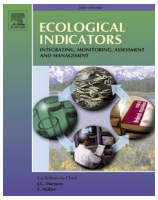

Epigenetic responses in juvenile Lemon sharks (*Negaprion brevirostris*) during a coastal dredging episode in Bimini, Bahamas

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A B S T R A C T

Sharks serve as key predators in coastal areas, with several species using near shore habitats as nursery refuges. With many shark species threatened or endangered, it is now critical to increase the knowledge of how anthropogenic activities impact these populations. This study is the first attempt at characterizing epigenetic modifications in elasmobranchs, and their potential as biomarkers of stress and disease using a dredging event episode in the early 2000′s in Bimini, Bahamas, as a model system. Lemon shark fin samples were selected from a historic archive (1995–2012) of Bimini's North (impacted) and South (control) shark nurseries, and classified as before (1995–2000), during (2001–2006), and after (2007–2012) the dredging event. Genome-wide DNA methylation was analyzed in these samples, along with the quantification of trace metals to investigate temporal and spatial patterns of metal concentrations. The obtained results revealed differential DNA methylation patterns in sharks from the North nursery (closest to the dredging site). While low concentrations of metals were found for all nursery sites and time frames, one trace metal (Manganese) was significantly correlated with the observed differences in DNA methylation. Overall, the findings of this study support DNA methylation as a potential stress biomarker for sharks, even in the absence of a reference genome. Furthermore, the identification of genomic regions differentially methylated in response to stress provides a basis for future analyses aimed at identifying gene networks and subsequent physiological responses to these events. Collectively, these analyses will help improve shark population monitoring, management, and conservation in impacted areas.

1. Introduction

Coastlines around the world experience high amounts of anthropogenic activity and pollution. From contaminated run-off, to coastal development, to high boating activity, there are several stressors to wildlife. These nearshore environments are utilized by a wide variety of organisms, including several shark species playing key predator roles in these ecosystems (Knip et al., 2010). Dredging is a common coastal disturbance that is often conducted in shallow waters such as bays and estuaries, which serve as nursery refuges for different types of sharks (Castro, 1993; Gaitán-Espitia et al., 2017; Roskar et al., 2020). However, there is limited knowledge of the impact that dredging has on these species (Jennings et al., 2008; Landos, 2012; McCook et al., 2015; Wheeler et al., 2020). This information has become increasingly important to obtain given the growing number of sharks being listed on the International Union for Conservation of Nature's (IUCN) Red List as either endangered or threatened (Dulvy et al., 2014). Although fishing remains the top threat to sharks, it remains unclear what other anthropogenic and environmental stressors, like dredging, may be impacting populations and most importantly, how. This gap in knowledge is broadened by the difficulty in studying vagile marine species, therefore requiring new technology and tools to overcome these challenges in shark research.

Over the last decade, the field of epigenetics (the study of potentially heritable phenotypic changes not related to changes in the DNA sequence) has provided new exciting avenues for developing biomarkers across many fields of study ranging from human health to coral reef conservation (Eirin-Lopez and Putnam, 2018; Jeyapala et al., 2020; Ladd-Acosta, 2015; Nikas and Nikas, 2019; Rodriguez-Casariego et al., 2018; Wang et al., 2020). The biomarker potential of these modifications is mainly based on their responsiveness to environmental change, constituting the earliest and main regulators of subsequent changes in genome function leading to phenotypic plasticity. Furthermore, many of these epigenetic marks are reversible (Furtado et al., 2019; Stein, 2012)

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and can be potentially inherited inter- and *trans*-generationally, expanding the current impact of stressors (and the phenotypic responses triggered in organisms) into the future of populations (Atta, 2015). Epigenetic modifications include DNA methylation, histone variants, histone modifications, and various small RNAs, among several others. Genomic DNA methylation (the addition of a methyl group to a Cytosine often next to a Guanine, known as a CpG dinucleotide site) has been found to change in response to environmental variation, modulating gene expression programs in order to produce acclimatized phenotypic responses (Hearn et al., 2019; Putnam et al., 2016; Vandegehuchte et al., 2010). An example of how DNA methylation plays a key role in acclimatization and phenotypic plasticity is the ability for three spined sticklebacks to adjust rapidly to drastically different osmotic pressures (freshwater vs. saltwater) due to variation in DNA methylation, even when genetic diversity is lacking (Artemov et al., 2017).

Changes in DNA methylation have not only been documented in response to immediate environmental change and stress exposure, but also during the onset of altered genomic and epigenomic states leading to disease in humans and other organisms (Kim et al., 2018; Martin and Fry, 2018; Morgan et al., 2018; Wang et al., 2020). Furthermore, it appears that disease risk is a complex interaction between genomes, epigenomes, and the environment (Wang et al., 2018). Collectively, these studies suggest that the characterization of DNA methylation represents a powerful addition to marine biomonitoring toolkits. Accordingly, changes in DNA methylation would assist in identifying exposure and effects of stress, providing information about how organisms are physically responding to such stress, and if they are more susceptible to developing certain diseases as a consequence. In spite of the promise of epigenetic monitoring, its applicability for conservation of vulnerable populations and species remains to be fully assessed. More specifically, little is known about epigenetic responses in fish, especially apex predators such as sharks. The present study takes advantage of a historic sample archive from Lemon sharks in the Bahamas to address the gaps in knowledge surrounding the suitability of DNA methylation analysis for the ecological monitoring of disturbances on environmentally and ecologically relevant species.

In the early 2000s, a major dredging event took place in Bimini, Bahamas, one of the most well studied Lemon shark (*Negaprion brevirostris*) nurseries in the Atlantic (Gruber et al., 2001). The purpose of the dredging was to create a large marina capable of housing several mega yachts for Resorts World Bimini visitors (Fig. 1). During the time of the dredging, a 20–25% decline in survivorship was documented in the North Bimini nursery populations for mangroves, sea grass, sharks and their potential food sources (Jennings et al. 2008). It was proposed that trace metals introduced into the water during dredging could have played a role in the observed decline (Gruber et al., 2001; Jennings et al., 2008). Here, DNA methylation changes in response to this major environmental disturbance are characterized using the technique Methyl-Sensitive Amplification Polymorphism (MSAP). Archived juvenile Lemon shark fin clips from before, during, and after the dredging event for both nurseries (North and South) were used to investigate if 1) DNA methylation significantly changed during the dredging event, 2) if trace metals in the tissues were significantly different between the time frames and 3) if there is a change in DNA methylation, do any of the metals show a correlation with this change. By addressing these questions, this study has found evidence that DNA methylation constitutes a useful monitoring tool for environment quality and population health.

2. Methods

2.1. Study site and sampling

The district of Bimini, Bahamas, consists of two small islands, North Bimini and South Bimini (Fig. 1). Juvenile Lemon sharks maintain small home ranges in these islands during the first three years of life (Morrissey and Gruber, 1993). As such, nursery sites may be considered as

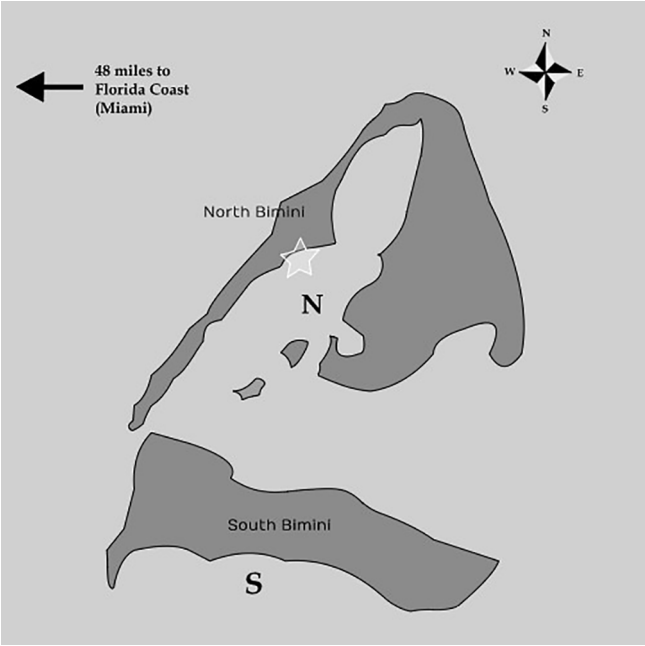

Fig. 1. Study location in Bimini, Bahamas. The white star represents the site where the major dredging was conducted. General location of the nurseries denoted as "N" for the north nursery and "S" for the south nursery.

isolated patches within a Bimini metapopulation in which philopatric behavior of females for parturition specific to their nursery site as juveniles (North or South) has been documented (Feldheim et al., 2014; Fig. 1). The Bimini Shark Lab, located on South Bimini, has been conducting annual sampling of the juvenile Lemon sharks at these nurseries since 1995, generating a large archive of samples held at The Field Museum in Chicago. This archive, along with the small home ranges kept by these sharks, opens a unique window into the past to study how DNA methylation has changed over time. Furthermore, the coastal development that took place in the early 2000s on North Bimini represents the best "natural experiment" to assess the impact of a large disturbance on shark populations and its links with changes in the shark's epigenome. Samples for this study were collected under Bahamas Research Permits (MA&MR/FIS/17B) during the years 1995–2012 using gill nets (Manire and Gruber, 1991). A majority of samples were collected between May and June just after parturition by females, other individuals were sampled opportunistically throughout the rest of the year. Most samples were between 0/young of year (YOY) to 3-years of age with the exception of one 4-year old. There was roughly an equal amount males and females sampled (supplemental table 2; see "extras'' tab). All fin clips were stored in DMSO and held at room temperature for sometime (up to a few years at most) before being stored at -20°C. DNA methylation has been found to be stable even at room temperature and any loss of DNA methylation is reliant upon degradation of the DNA. The samples were archived at the Field Museum in Chicago then shipped frozen overnight to the Environmental Epigenetics Lab at Florida International University where they were further processed for this project. A total of 71 samples were used in this study. Samples were categorized into three time frames as follows: Before Dredging (1995–2000), During Dredging (2001–2006), and After Dredging (2007–2012) and two locations: North and South. Unfortunately, during the years 1995 to 2000, sharks were only sampled in the North nursery, therefore, the following groups discussed in the remainder of this paper are: Before.North (n = 20), During.North (n = 18), During.South (n = 12), After.North (n = 8), and After.South (n = 13).

2.2. DNA extraction and purification

Shark DNA was extracted from fin clip samples following a salt out protocol (Sunnucks and Hales, 1996). Accordingly, a small subsample of fin clip was minced and placed in a mixture of 500 μL of TNES and 10 μL of proteinase K (10 mg/μL) and incubated overnight at 56 °C with shaking. After incubation, 150 μL of 5 M NaCl solution was added and each sample was vortexed for 15 sec and centrifuged at 14,000 × g to pellet the proteins. The supernatant was transferred to a new tube and 1 vol (~650 μL) of 95% ethanol was added to each tube, vortexed, and then centrifuged at 14,000 × g for 15 min to pellet the DNA. The ethanol was then discarded and the pellet was washed by adding 300 μL of 70% ethanol and centrifuged at 14,000 × g for 10 min. The ethanol was again discarded and tubes were centrifuged in a vacuum-centrifuge for 20 min to ensure excess ethanol was fully evaporated off. The DNA pellet was then resuspended in 50 μL of Tris-EDTA buffer and stored at −20 °C until further use.

2.3. MSAP and fragment analysis

Methyl-Sensitive Amplification Polymorphism (MSAP) is an adapted protocol of Amplified Fragment Length Polymorphism (AFLP) that targets the sequence 5′-CCGG-3′ with methylation sensitive enzymes (MSPI and HPAII) that cut depending on the type of methylation present (unmethylated, hemi-methylation, internal C methylation, and hyper-methylation) producing a series of fragments whose presence/absence between the two enzyme treatments represents the DNA methylation pattern present in a sample (Supplementary Table 1). The MSAP protocol used for this study was adapted from (Reyna-López et al., 1997) and consists of an initial parallel restriction ligation (RL) step using either MspI/EcoRI or HpaII/EcoRI, followed by a pre-selective PCR with an additional base pair added to the end of a primer that targets the ligated sequence, then followed by a selective PCR that adds two more base pairs and a fluorescent label (FAM or HEX) for fragment analysis (Supplementary Table 1). Ultimately, this process produced two tubes from each individual sample for each PCR combination, so that MspI/HpaII enzyme treatments could be compared.

The RL protocol used was as follows: 200 ng of DNA was treated with either MSPI/EcoRI or HPAII/EcoRI in the following recipe: 4.5 μL sterile water, 1.1 μL of 10X Ligase Buffer, 1.1 μL of 10X Digestion Buffer, 0.55 μL BSA (1 mg/mL), 0.25 μL EcoR1 Enzyme (4U), either 1U MSPI or 1U HPAII enzyme, 1 μL EcoRI Adapter mix (5 μM), 1 μL MSPI/HPAII Adapter mix (50 μM), and 0.01 μL of T4 Ligase (1U/μL) in a final volume of 11.56 μL for MSPI treated samples or 11.61 μL for HPAII treated samples. Samples were incubated at 37 °C for 2 h. Each RL reaction was diluted with 60 μL of sterile water and then 4 μL of each sample was used in a pre-selective PCR as follows: 10.5 μL sterile water, 2 μL 10X Taq polymerase buffer, 1.5 μL dNTPs mix (10 μM), 1 μL EcoR1 + X (20 μM), 1 μL MSPI/HPAII + X (20 μM), 1 μL $MgCl_2$ (50 μM), and 0.2 μL Taq Polymerase enzyme in a final volume of 21.2 μL. Preselective PCRs were subjected to the following thermocycler protocol, with an initial step of 72 °C for 2 min followed by 20 cycles of 95 °C for 20 sec, 56 °C for 30 sec, and 72 °C for 2 min, ending with a final extension at 60 °C for 30 min, and a final hold temperature of 4 °C. Each pre-selective PCR was diluted with 60 μL of sterile water and then 6 μL was used in a subsequent selective PCR reaction as follows: 14 μL sterile water, 2 μL 10X Taq polymerase buffer, 1.5 μL dNTPs mix (10 μM), 1 μL Selective Primer 1 (20 μM), 1 μL Selective Primer 2 (20 μM), 1 μL $MgCl_2$ (50 μM), and 0.2 μL Taq Polymerase enzyme in a final volume of 26.7 μL. The thermocycler protocol began with an initial denaturation step at 95 °C for 2 min followed by 10 cycles of 95 °C for 20 sec, 66 °C for 30 sec, and 72 °C for 2 min, followed by 20 cycles of 95 °C for 20 sec, 56 °C for 30 sec, and 72 °C for 2 min, ending with a final extension of 60 °C for 30 min and a final hold at 4 °C. Selective PCRs were checked for quality using 2% agarose gel electrophoresis before preparation for fragment analysis with ROX-1000 used as the internal size standard on a 3130XL Genetic Analyzer conducted by the FIU DNA Core Facilities. Raw data was viewed using Peak Scanner 2 software, which consisted of a list of fragments by length along with a plot view of length (x axis) and height (y axis) for fragments which were then recorded as present/absent for each individual for each enzyme treatment (Supplementary Table 2).

2.4. Trace metal analysis

A subset of samples were used for metal analysis (Supplementary Table 3). Seven samples from each group (Before.North, During.North, During.South, After.North, After.South) were selected based on availability of fin sample and chosen to represent a range of ages (0–3 years). Fin samples were weighed and submitted to the FIU CAChE Nutrient Analysis Core Facility for ICP-MS to quantify the concentrations of a panel of metals (Be, V, Cr, Mn, Co, Ni, Cu, Zn, As, Se, Mo, Ag, Cd, Ba, Hg, and Pb). After an initial analysis of metal amounts (Supplementary Table 3) in the different groups, a subset of potentially pertinent metals (Mn, Zn, Se, Cd, and Pb) (Newman et al., 2010); (Pettitt-Wade et al., 2011) present in the samples were selected for further analysis as related to DNA methylation.

2.5. Data analysis

The *msap* package (version 1.1.8) in R was used to classify each locus as one of the four potential methylation states (unmethylated, hemi-methylated, methylated at an internal Cytosine, and fully methylated; “u”, “h”, “i”, or “f”, respectively) based on the presence/absence of fragments from the parallel restriction digest (MspI and HpaII) followed by parallel PCR reactions (Pérez-Figueroa, 2013). While the scenario of no bands may indicate either full methylation or a lack of a target due to genetic variation, this case was considered full methylation due to the documented low genetic structure found in Lemon sharks across the Atlantic and this choice is further supported by previous studies focused on identifying changes due to gradual environmental change (Feldheim et al., 2001; Rodríguez-Casariego et al., 2020). Additionally, by choosing this designation of no bands, we optimize our ability to capture strong physiological responses. For example, it is plausible that changes in gene function (i.e., activation/deactivation) would be mirrored by changes in DNA methylation through hyper- or hypo-methylation of CpG sites especially if a CpG site was located in a CpG island (several CpG sites close together) in a promoter region involved in gene regulation (i.e, transcription). Loci with a methylated state in at least 5% of the samples were defined as Methylation-Susceptible Loci (MSL) (Herrera and Bazaga, 2010). Polymorphic loci were defined as loci with at least two occurrences of both a methylated and unmethylated state (Pérez-Figueroa 2013). All subsequent analyses of DNA methylation patterns focused on the polymorphic MSL.

All distance-based analyses of DNA methylation patterns were performed on Gower distance matrices based on methylation states (u, h, i, or f) of polymorphic MSL (*daisy* function, *cluster* package version 2.1.0) (Gower, 1971). Patterns of DNA methylation were visualized with a Non-metric Multidimensional Scaling Analysis (NMDS) (*metaMDS* function, *vegan* package version 2.5–6; (Faith et al., 1987). A permutational analysis of variance (PERMANOVA) (Anderson, 2001) was performed (*adonis* function) to determine the variation in DNA methylation patterns between locations (North or South) and time periods (Before, During, or After dredging). The *betadisper* function was used to assess the homogeneity of dispersions between spatial and temporal groups (Anderson, 2006).

To further explore differences in DNA methylation patterns between spatial and temporal groups, a Discriminant Analysis of Principal Components (DAPC) was utilized in *adegenet* package version 2.1.3 (Jombart and Ahmed, 2011). Spatial and temporal combinations (e.g., Before Dredging in the North, During Dredging in the North, etc.) were used as *a priori* groups. Two rounds of cross validation using the *xval-Dapc* function were performed to determine the optimal number of

principal components (PCs) to be retained for the analysis, which was further confirmed through an assessment of a-scores (*optim.a.score* function) (Jombart et al., 2010). All four discriminant functions were retained. The probability of membership of samples to each spatial and temporal group was evaluated to describe the level of separation between groups. Patterns of methylation states of the loci with the highest contributions to the first two discriminant functions were also assessed.

A distance-based redundancy analysis (dbDRA) (Legendre and Anderson, 1999) (*vegan* package version 2.5–6; (Oksanen et al., 2016) was performed on the subset of samples (n = 34 out of 71 total samples) for which metal concentrations were measured. To characterize relationships between metal concentrations and patterns of DNA methylation, the formula for the dbRDA was as follows:

Gower Distance (Methylation Patterns) ~ Mn + Zn + Se + Cd + Pb

A permutation test was performed on the dbRDA model (*anova.cca* function) to assess the significance of the full model, axes, and individual metals (Legendre et al., 2011).

3. Results

3.1. Spatial and temporal patterns of DNA methylation in the Lemon shark genome

A total of 935 loci were identified using the three primer pairs (Selective Combos 1–3) defined in the present study (446, 276, and 213 from Primers 1–3, respectively). All but two of these loci were determined to be Methylation-Susceptible Loci (MSL), of which 486 (52%) were polymorphic. Over 80% of MSL were fully methylated, regardless of the location or time period of the sample collection (Fig. 2). Approximately 1–5% were hemimethylated or methylated at an internal cytosine and ~ 2–7% were unmethylated.

The observed patterns of DNA methylation varied across time periods (PERMANOVA, p-value = 0.001), with marginal differences between North and South locations (p-value = 0.095), and no significant interaction between time and location (p-value = 0.128) (Table 1). Differences between time periods may have been driven by both differences in variation as well as differences in dispersion (p-value: $3.287x10^{-9}$) between groups (Supplementary Figure 1). However, the dispersion did not differ between locations (p-value = 0.634).

Cross validation determined that the optimal number of PC's to be retained for the DAPC analysis was 35. Assessment of a-scores confirmed that the retention of 35 PC's allowed for both a relatively higher a-score and cumulative variance. The DAPC analysis further revealed temporal and spatial differences in DNA methylation patterns (Fig. 3). Samples

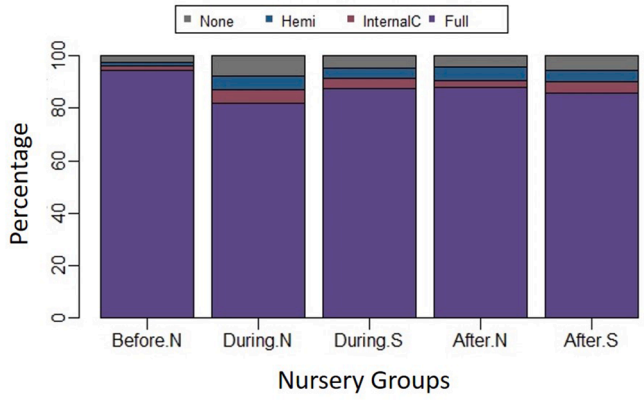

Fig. 2. % DNA methylation states for MSL in Lemon sharks at North (N) and South (S) nurseries before, during, and after the dredging event. None: unmethylated; Hemi: hemimethylated; InternalC: internal Cytosine methylation; Full: fully methylated.

Table 1
PERMANOVA analysis of DNA methylation patterns at MSL in Lemon sharks from Bimini. p-values correspond to MSL (Gower Distance) ~ Time + Location + Time:Location.

Variables	p-value
Time	0.001***
Location	0.095
Time:Location	0.128
Pairwise Comparison	
Before vs During	0.003**
Before vs After	0.003**
During vs After	0.03*

*p ≤ 0.05; **p ≤ 0.005; ***p ≤ 0.001;

collected before dredging (North only) separated from other groups along the first discriminant function (DF1), while samples collected during dredging in the North separated from other groups along the second discriminant function (DF2). DNA methylation patterns from samples collected during dredging in the South, and after dredging in both locations were more similar. The overall probability of assigning samples to their respective spatial and temporal group was 94.37%. Samples from before dredging in the North and during dredging in the South were all correctly assigned to their respective group. Four samples were less distinguished, with less than 90% probability of assignment to one specific group (Fig. 4). Loading plots examining DF1 and DF2 were created to identify fragments that were driving the differentiation in DNA methylation patterns between groups (Fig. 5). Along DF1, the Before.North group differentiates from the rest of the groups (Fig. 3B). The top five loci contributing to differentiation of groups were looked at for each DF as an example of the depth of analysis that can be accomplished in a study such as this. More loci were not investigated due to the time it takes to investigate each individual locus and the limitation of not having a reference genome to discover if each locus belonged to a gene or pathway. Of the top five loci contributing to this variation, four loci were mostly unmethylated before dredging for the North but became methylated to some degree (hemi, internal C, or fully) in the dredging time frame (Supplementary Figure 4). In contrast, the top five loci contributing to the variation of DF2, along which During.North samples differentiated (Fig. 3C), were all highly fully methylated before dredging in the North. During dredging, the frequency of full methylation decreased in the North, but remained high-moderate in the South (Supplementary Figure 4). Of the four loci that experienced a relative "switch" from predominantly fully-methylated to less methylated during dredging in the North, three of those loci demonstrated the reversed pattern after dredging. Although, with hypermethylated loci we are unable to rule out null alleles at this time without sequencing, we find the change in DNA methylation pattern (ex. unmethylated before, hypermethylated during, unmethylated after) to be more likely a change in DNA methylation at these sites rather than a change in the genetic code in these time frames. Being that this is a novel approach for the use of MSAP, future studies incorporating sequencing will be necessary to determine the frequency of null alleles in this type of data.

3.2. Metal levels in Bimini Lemon sharks and relationship with DNA methylation

Metals typically linked to anthropogenic activities in coastal areas (Newman et al., 2010; Pettitt-Wade et al., 2011) were detected (and in small quantities) in fin clip samples regardless of time and location (Table 2, Supplementary Table 3). Patterns of DNA methylation within the subset of samples for which metal concentrations were quantified were similar to those of the full dataset (Supplementary Figure 2). The distance based redundancy analysis (dbRDA) model (Fig. 6), including concentrations of Mn, Zn, Se, Cd, and Pb, significantly explained the variation in DNA methylation patterns (p-value = 0.049). The first axis

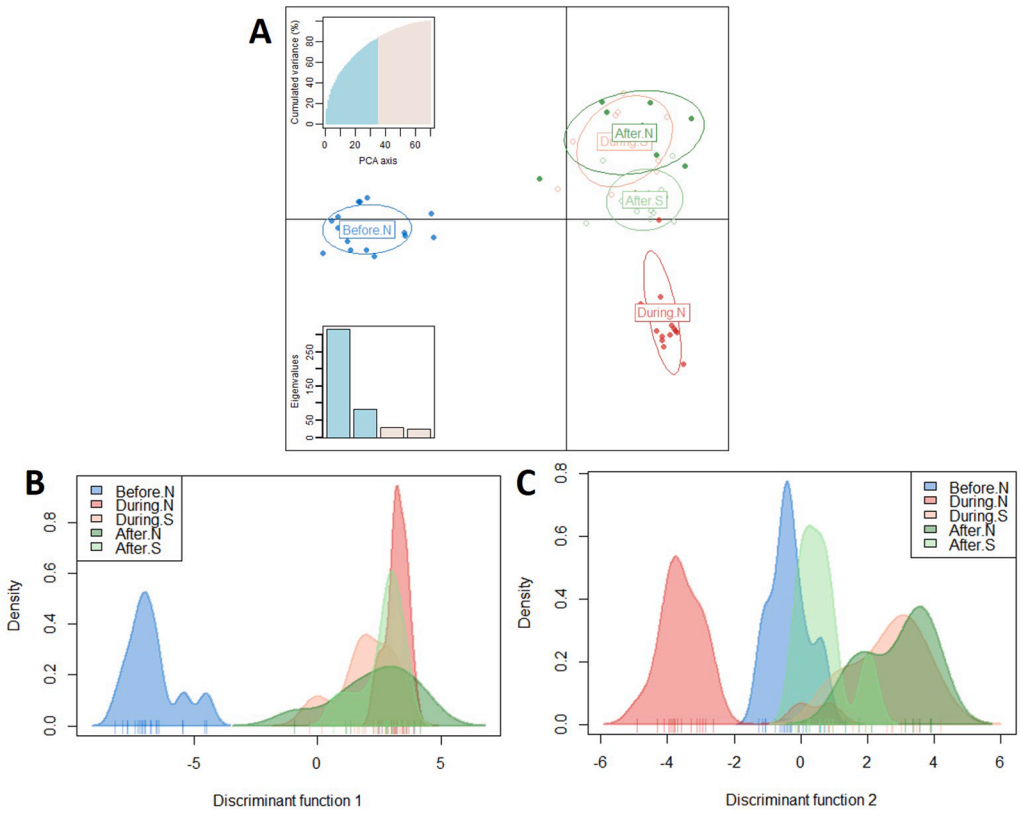

Fig. 3. DAPC showing spatial and temporal differentiation of DNA Methylation patterns in Lemon sharks from North and South nurseries. A, Variation visualized across the first two discriminant functions (x and y axes, respectively). Upper left inset depicts the cumulative variance explained by the retained PCs (35) and the lower left inset displays the amount of variance explained by the 4 retained discriminant variables. B, Density plot of variation across discriminant function 1 (x axis from A), showing the differentiation of Before.North from other groups. C. Density plot of variation across discriminant function 2 (y axis from A), showing the differentiation of During. North from other groups.

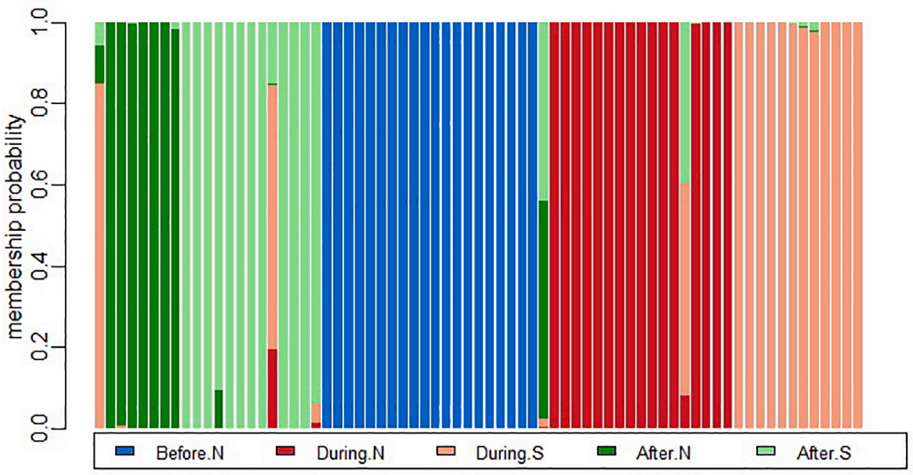

Fig. 4. Probability of membership to assigned sampling group based on the observed DNA methylation patterns. All but four individuals had a high probability (>80%) of being correctly assigned to their respective group.

explained 47.33% of the variation (p-value = 0.077) and the second axis explained 25.28% (p-value = 0.454). Of the five metals included in the model, only manganese (Mn) was significantly correlated with patterns of DNA methylation (p-value = 0.004).

4. Discussion

4.1. DNA methylation patterns in the Lemon shark genome

The current understanding of the role of epigenetic modifications mediating phenotypic plasticity during environmental responses is growing quite rapidly in marine organisms (Eirin-Lopez and Putnam, 2018). However, it still remains little investigated for charismatic groups such as sharks, skates, and rays (Lighten et al., 2016; Peat et al., 2017), where the only DNA methylation data currently available corresponds to the elephant shark (*Callorhinchus milii*) methylome. In that study, it was found that the general genome-wide DNA methylation pattern observed in this chimaera was quite similar to the typical vertebrate methylome structure (Peat et al., 2017). Accordingly, vertebrate genomes display a high amount of DNA methylation, predominantly found at CpG sites (except at CpG islands). These observations are in agreement with the results obtained in the present work, describing high amounts of hypermethylation also in the case of the Lemon shark genome (Fig. 2).

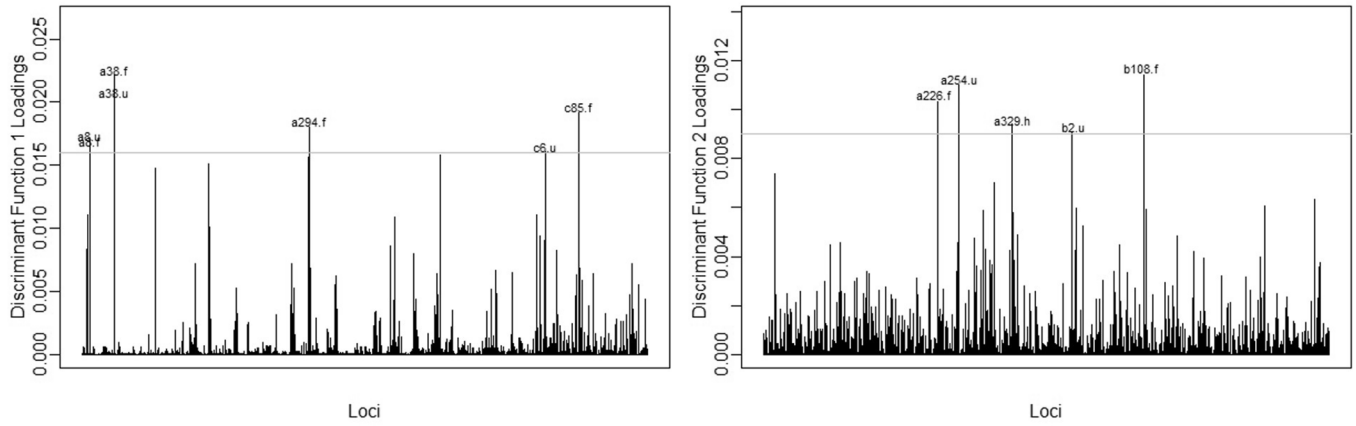

Fig. 5. Loading Plots of fragments driving differentiation along discriminant function 1 (DF1) and discriminant function 2 (DF2) from DAPC. A, Individual loci driving differentiation of the Before.North group from other groups along DF1. The five most influential loci are identified. B, Individual loci driving differentiation of the During.North group from other groups along DF2. The five most influential loci are identified.

Table 2
Metal concentrations (mg/kg) determined in fin clip samples from Lemon sharks at Bimini nurseries before, during, and after the dredging event. Average concentrations and standard errors for each of the focus metals analyzed with DNA methylation data.

Time. Location	Mn	Zn	Se	Cd	Pb
Before.N	0.297 ± 0.144	2.076 ± 0.844	0.153 ± 0.024	0.002 ± 0.001	0.078 ± 0.034
During.N	0.578 ± 0.241	0.044 ± 0.044	0.285 ± 0.135	0.006 ± 0.005	0.132 ± 0.075
During.S	0.293 ± 0.102	0.148 ± 0.077	0.464 ± 0.154	0.001 ± 0.001	0.202 ± 0.129
After.N	0.108 ± 0.017	4.295 ± 3.193	0.609 ± 0.167	0.004 ± 0.002	0.062 ± 0.043
After.S	0.123 ± 0.033	0.904 ± 0.461	0.342 ± 0.055	0.013 ± 0.010	0.065 ± 0.031

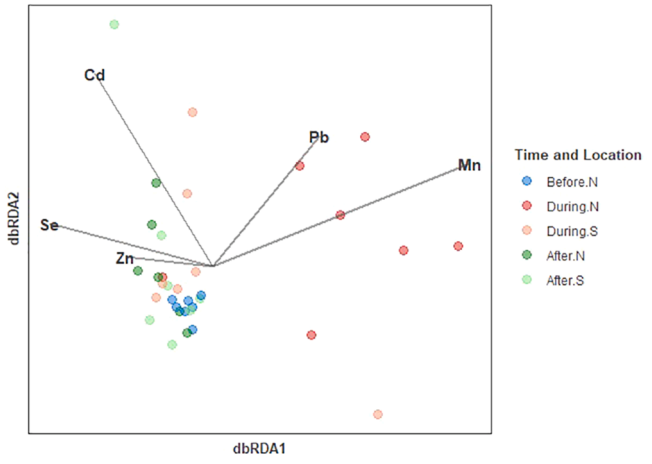

Fig. 6. Correlation between metal concentrations and DNA methylation variation (dbRDA). Vectors show the correlation of metal concentrations to DNA Methylation patterns. Manganese (Mn) was the only metal found to be significantly correlated (p-value = 0.004) to DNA methylation.

One of the main objectives of this study was to determine if DNA methylation patterns are altered as a consequence of the dredging event affecting Lemon shark nurseries in Bimini. To our knowledge, the closest effort to examine the role of epigenetics during stress response in elasmobranchs is represented by a gene expression study in the winter skate (*Leucoraja ocellata*). There, expression differences in genes connected with epigenetic mechanisms, and in particular DNA methylation, were found to be linked to phenotypic plasticity triggered by thermal stress (Lighten et al., 2016). Yet, no DNA methylation levels were estimated as part of this study. The present work begins to fill this gap, using Lemon sharks as a model system, identifying differences in DNA methylation patterns as a consequence of the dredging disturbance in Bimini (Figs. 2 and 3). A possible explanation for the observed DNA methylation patterns found in this study could involve a stress response being initially triggered by the dredging event, as evidenced by the drastic differentiation in DNA methylation observed in the During.North group in respect to the Before.North group (Fig. 3A,C). The During.South and After.South groups exhibited similar patterns and therefore appear to not reflect a change in DNA methylation during the time of dredging. The similarity of the After.North samples to the during and after South samples could reflect a move back towards a "basal" (pre-stress) DNA methylation pattern in the North nursery. These results underscore the importance of developing site and species-specific "baseline" DNA methylation profiles, providing a reference for comparative studies during disturbances (Johnstone and Baylin, 2010; Smallwood et al., 1999). Furthermore, the development of baseline data should be comprehensive, as other studies have found DNA methylation patterns to show seasonality (Rodríguez-Casariego et al., 2020; Scheufen et al., 2017) which could reflect several seasonal changes that may occur within environments including progressive change from year to year, as in global climate change.

4.2. Other contributing factors to DNA methylation differences

Bimini's North and South nurseries display differences and similarities regarding environmental and biological parameters. For example, it has been reported that the North nursery experiences more variation across several environmental abiotic parameters such as temperature and salinity, due to its shallow nature (only 2 to 3 m deep) and its secludedness from tidal changes (Fig. 1; (Voss and Voss, 1960). On the other hand, studies on biotic parameters such as prey abundance and diet composition found that the dominating prey in the diet of Lemon sharks at both nurseries is the yellowfin mojarra, with crustaceans being the second most abundant prey in the North nursery versus parrotfish in the South (Newman et al., 2010). In the present study, both differences and similarities between groups across two different axes of spatial analysis are identified (Fig. 3). Despite the overlap of some of the groups, all had very high (≥85%) assignment probabilities (Fig. 4), further confirming the existence of sharp differences between groups (location X time). It is likely that the collective differences in environmental factors and diet are contributing to the unique patterns found at each

nursery, providing enough information to identify a particular nursery group at a particular time period. The obtained results show time as an explanatory variable producing significantly different patterns (PERMANOVA, $p < 0.05$, Table 1). One possibility is that these results reflect environmental differences at different time periods (i.e., the dredging event). However, this analysis did not find location (North vs South) to be significant, which might be due to the similarity found amongst After.North, During.South, and After.South groups. Nonetheless, major differences between During.North and During.South groups are observed, in agreement with their differential exposure to the dredging event: During.North exposed versus During.South sheltered (Fig. 1, Fig. 3A,C).

4.3. Dredging and metal pollution impact

The types and amount of metal contamination found at coastlines reflect the anthropogenic activities that occur on nearby shores (Nellemann et al., 2008; Wang et al., 2013). Through run-off and direct input, metals make their way to the coast and have a tendency to accumulate in the sediments at shorelines and when coastal development occurs, such as dredging, these metals have the potential to be reintroduced into the environment and made bioavailable for organisms to take up depending on the environmental conditions (Eggleton and Thomas, 2004). Accordingly, a decrease in juvenile Lemon shark survival for the Northern Bimini nursery (closest to the dredging site) was observed at the time of the dredging. Thus, it was hypothesized that trace metals released from the sediments into the water column from the dredging could be a possible explanation for such mortality although there were no water samples taken during this time to confirm this (Jennings et al., 2008). In Bimini there is limited coastal development activity and the most notable coastal development is circumscribed to Resorts World Bimini at North Bimini and the location of our focus disturbance for this study. Consequently, the chance of finding toxic metals would be highest near this site and although we expect to see more essential metals in Bimini, even essential metals at high concentrations can be detrimental to organisms which lends support to the above hypothesis (Wood, 2011).

The archive of samples available for this study consisted entirely of fin tissue (i.e., primarily skin and cartilage with a small amount of muscle tissue). Little is known about the turnover and incorporation rates of trace metals into shark tissues. A previous report assessing metal accumulation in muscle and fin tissue from four shark species collected at a market in China found that, for all metals measured (Cu, Zn, Cd, Hg, and Pb), fin consistently displayed higher amounts of metals (Ong and Gan, 2017). However, this was not an acute exposure study but rather a look at general accumulation of metals over time. In acute exposures, spikes in metal concentrations have been detected mainly in organs involved in uptake and detoxification of the metal concentrations such as intestines, gills, liver, and kidneys for fish (Souid et al., 2013; Bashir et al., 2012; Wood, 2011). In the present work, low concentrations of all metals were found in Lemon shark samples, in agreement with a previous study conducted on prey sources of the Bimini Lemon sharks (Pettitt-Wade et al., 2011). In that study, metals were quantified on both North and South nursery prey items, revealing low amounts of metals. Based on those results, it was hypothesized that Bimini Lemon sharks were possibly deficient in several trace metals (Fe, Cu, and Mn). However, Mn (Manganese) was the only metal that had elevated concentrations for the During.North group in this study versus the rest of the groups (Supplementary Figure 3).

There are two major (although there may be more reasons for this result) factors that should be noted that could be playing a role in the low amounts of metals found in fin tissues for both nurseries across all time frames. One, as mentioned already above, is that fin tissue is not the ideal choice for studying acute exposures. Two, for particularly YOY samples, the fin tissue of these individuals may reflect the mothers exposure state. In regards to the second point, both nursery sites found at Bimini are extremely shallow. This shallowness is what keeps the juvenile sharks safe from predators including bigger sharks. In these nursery areas, mainly juvenile sharks are found except for when adult females come for parturition and especially in the north sound this often must be done at high tide because the water becomes extremely shallow. This would mean that prior to parturition, pregnant adult females were exposed to conditions outside of the nursery area and even outside of the Bimini area as full adults are known to travel far distances from the island at which they were born. This may contribute to the fact that low concentrations of metals were found in the fin tissues due to the mother not being exposed continuously to the dredging activity occurring within the nursery. On the other hand, although metals levels may not change quickly and take time to set in to tissues, DNA methylation changes have been found to occur rapidly as little as within an hour to environmental exposures (Gonzalez-Romero et al., 2017a; Huang et al., 2017).

Although the concentration of Mn was not significantly different among groups, this metal was found to be significantly correlated ($p = 0.004$) with DNA methylation differentiation in the exposed (During.North) group of Lemon sharks (Fig. 6). Manganese is an essential metal to organisms, contributing to proper brain function, glutamine synthetase, and catalyzing redox reactions (Yokel, 2009). At high exposures, it is known to cause neurological effects that resemble Parkinson's Disease in humans (Jiang et al., 2006; O'Neal and Zheng, 2015). Detrimental neurological effects have also been recorded for mice, cows, and fish exposed to high levels of Mn (Arndt et al., 2014; Kikuchihara et al., 2015; Purdey, 2000). Although additional data are required to understand the effect that altered levels of Mn may have on Lemon sharks, it is plausible that its increase in the During.North group is caused by the dredging activity, and is potentially playing a role in some of the DNA methylation changes observed. This hypothesis is further supported by reports describing higher levels of metals are typically found in prey species at the South nursery when compared to the North nursery (Pettitt-Wade et al., 2011). Since environmental records as well as samples of different shark tissues are unavailable for analysis for these time frames, it is not possible to definitely say whether or not metals were increased in the water column with the evidence at hand, however, a possible way forward to understand the impact of metals could involve sequencing the identified driving loci in this study (Fig. 5) and analyze their participation in functional pathways that might use or deal with an increase of Mn in the body. It is pertinent to note, that although only five top loci were chosen to demonstrate the potential of using MSAP to discover genes and pathways, if actually pursuing to characterize these aspects associated with changes, a threshold of contribution could be set which would undoubtedly include several more loci. Furthermore, with the ability to sequence, potential underlying genetic differences could be removed from the data and increase confidence in the loci discovered to contribute to differentiation due to the potential confounding result for one of the methylation types (i.e. hypermethylation) by the MSAP procedure. In a controlled experiment investigating DNA methylation changes in European Eel livers to Cadmium (Pierron et al., 2014), a total of 24 loci were identified from gel electrophoresis of MSAP treated DNA for sequencing analysis. For the current case, along with sequencing of fragments, DNA methylation analysis will need to be gathered alongside more controlled toxicological data to characterize the connection between the metals and DNA methylation changes.

4.4. Identifying and developing epigenetic biomarkers in marine organisms

DNA methylation represents a very powerful candidate for the development of various types of biomarkers and bioindicators for environmental and conservation studies. DNA methylation plays a key role in regulating gene expression, therefore contributing to phenotypic plasticity, acclimatization and adaptation of organisms to their environment (Artemov et al., 2017; Dubin et al., 2015; Razin and Cedar, 1991). Thus, by studying environmentally-induced changes in this

epigenetic modification, it is possible to gain additional insight into the links with phenotypic responses of marine organisms, which can be used as indicators of the quality of their surrounding environment (Arıkan et al., 2018; Baccarelli et al., 2009; Head, 2014; Martin and Fry, 2018; Watson et al., 2017). Compared to other popular molecular analyses such as transcriptomics, DNA methylation studies simply require DNA, which is easier to obtain and stabilize in the field compared to RNA. Thus, by linking DNA methylation to gene function, it is possible to identify altered gene function based on epigenetic analyses. The power of such an approach is best illustrated by the development of medical biomarkers in humans, including the identification of specific types of tumors (Baylin, 2006) and lifestyle-associated hazards [e.g., smokers vs non-smokers (Maas et al., 2019)].

The present study constitutes the first approach at developing epigenetic biomarkers of stress in sharks, including the development of "baseline" patterns necessary to compare exposed and non exposed individuals. This work facilitates the identification of specific patterns in the DNA methylation unique to the group directly exposed to the dredging. Although the identification of unique DNA methylation patterns can be effectively performed using microarrays (able to scan thousands of CpG sites and quantifying the amount of DNA methylation present at each site), this technique requires a detailed reference genome and is expensive. Alternatively, the study of DNA methylation differences can be conducted using the MSAP technique. While the resolution of MSAP is much less than that of microarrays, the former can be implemented without the requirement of a reference genome. Therefore, MSAP provides more information than analyzing global DNA methylation amounts, which can be confounded by the number of different genomic areas simultaneously gaining and losing methylation (some genes turn on, some genes turn off). In addition, this technique has been successfully used on environmental studies on marine invertebrates (Gonzalez-Romero et al., 2017b; Rodríguez-Casariego et al., 2020). In this study, unique DNA methylation patterns for each nursery and for each time frame were identified using the MSAP technique (Fig. 3). Even without a reference genome, the driving loci and key changes in methylation that make a nursery group unique from other groups were identified (Fig. 5, Supplementary Figure 4). Interestingly, 4 out of 5 and 5 out of 5 top loci for DF1 and DF2 respectively showed the same directional change in DNA methylation status (i.e., unmethylated towards more methylation for DF1 and fully methylated towards less methylated for DF2). A plausible hypothesis is that the observed changes in DNA methylation correspond to genes from the same and/or linked pathways inducing a certain physiological response. Further analysis through targeted sequencing of these loci will further clarify the phenotypic consequences of such changes, further elucidating the effect of the dredging episode on Lemon shark nurseries. Overall, this approach constitutes a cost effective way for environmental studies to identify epigenetic biomarkers of exposure and stress.

5. Conclusions and future research

In this study, the epigenetic effects of a major dredging event on juvenile Lemon sharks in North Bimini were investigated, using South Bimini individuals as a control. The obtained results show that 1, the nursery group closest to the dredging area displayed significant changes in DNA methylation patterns; 2, sharks in this impacted group also displayed significantly higher levels of the metal Mn; and 3, the concentrations of this metal were significantly correlated to the observed DNA methylation patterns. Importantly, some of these differentially methylated loci displayed unique DNA methylation patterns correlated with the time of the dredging event for the nursery closest to the disturbance. Overall, this study helps lay the foundation for the development of epigenetic biomarkers of stress in large vagile marine predators (and is a model for other organisms), using a budget-friendly approach that does not require (at least initially) a reference genome. This work opens new avenues for future research, notably supporting the development of a reference genome for Lemon sharks to establish links between environmental triggers, epigenetic modifications, and the regulation of specific genes linked to particular physiological functions involved in phenotypic responses. These driving loci could therefore be potential candidates for biomarkers of exposure and effect. Lastly, the importance of incorporating tissue collection for DNA methylation into routine analysis for studies should be stressed, as that is particularly important to develop a baseline pattern to be able to identify unique changes in DNA methylation in response to subsequent stress and environmental quality changes.

CRediT authorship contribution statement

Andria Paige Beal: Conceptualization, Investigation, Writing - original draft, Writing - review & editing, Data curation, Funding acquisition. **Serena Hackerott:** Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing. **Bryan Franks:** Writing - review & editing. **Samuel H Gruber:** Resources. **Kevin Feldheim:** Resources, Writing - review & editing. **Jose M Eirin-Lopez:** Conceptualization, Resources, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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