DNA methylation landscapes before and after Pacific Oyster Mortality Syndrome are different within and between resistant and susceptible *Magallana gigas*

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Keywords
OsHV-1 µVar, DNA methylation, immune response, epibiomarkers, oyster, POMS

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Abstract
The Pacific oyster faces significant threats from recurring outbreaks of Pacific Oyster Mortality Syndrome (POMS), a polymicrobial and multifactorial disease. Recent researches have underscored the crucial role of epigenetics in shaping oyster resistance and susceptibility through microevolutionary pressures. We conducted a comprehensive characterization of the basal (no infection) and the POMS-induced changes of the methylome in resistant and susceptible oysters focusing on the gills and mantle. Our analysis identified differentially methylated regions (DMRs), which revealed distinct methylation patterns uniquely associated with either susceptible or resistant phenotypes in each tissue. Enrichment analysis of the methylated genes highlighted that these epigenetic changes were specifically linked to immunity, signaling, metabolism, and transport. Notably, among the methylated genes, and regardless of the tissue, 31 genes with well-known immune responses were differently methylated after POMS with contrasted methylation between phenotypes. This suggests that epigenetic changes can also drive rapid adaption, enabling oyster populations to develop enhanced resistance to POMS diseases through heritable, yet environmentally influenced induced changes. We hypothesized that these epigenetic changes during POMS infection may result from a negative feedback loop between transcription and methylation, viral manipulation of host cellular machinery, or interactions between these mechanisms. Additionally, and beyond its biological aspect, this study provided insights into potential epigenetic biomarkers for POMS disease management and targets for enhancing oyster health and productivity. Based on the substantial methylome differences between phenotypes, we identified a set of candidate epibiomarkers that could characterize whether an oyster is resistant or susceptible (1,998 candidates) and whether a site has been exposed to POMS or not (164 candidates). Overall, the findings provide a deeper understanding of the molecular interactions between oysters and POMS infection opening new questions about the broader implications of epigenetic mechanisms in host-pathogen dynamics and offering promising strategies for mitigating the impacts of this devastating disease.

Introduction

The Pacific oyster, *Magallana gigas* (Salvi & Mariottini, 2016, 2021), is one of the most widely exploited species in aquaculture. Nevertheless, the recurrent outbreaks of Pacific Oyster Mortality Syndrome (POMS) have jeopardized the sustainability of the oyster farming industry (EFSA Panel on Animal Health and Welfare (AHAW), 2010; Richard et al., 2021).

POMS is a polymicrobial disease that affects spat and juvenile oysters (Petton et al., 2021). The primary causative pathogen is the herpesvirus OsHV-1 µVar, infecting cells and ultimately the haemocytes leading to an immune suppression state of the oyster (de Lorgeril et al., 2018). This state alters the control of the associated microbial community, leading to the proliferation of a consortium of opportunistic and pathogenic bacteria (Rubio et al., 2019; Oyanedel et al., 2023) resulting in lethal bacteremia (Clerissi et al., 2023; de Lorgeril et al., 2018; Petton et al., 2021). In addition to the polymicrobial aspect, POMS is a multifactorial disease that involves a series of biotic and abiotic factors that influence the outcome of the POMS/oyster interaction (Petton et al., 2021). The genetic component plays a pivotal role in encoding oyster resistance/susceptibility (Dégremont et al., 2015; de Lorgeril et al., 2020). Additionally, factors such as temperature (Petton et al., 2013; Pernet et al., 2015; De Kantzow et al., 2016; Delisle et al., 2018, 2020), ageing (Hick et al., 2018; Peeler et al., 2012; Pernet et al., 2012), food availability (Pernet et al., 2019; Petton et al., 2023), and interactions with associated microbiota (Pathirana et al., 2019; Clerissi et al., 2020; Delisle et al., 2022; Fallet et al., 2022) can also influence the permissivity to POMS (*i.e.,* the development of the disease in a susceptible oyster).

If the adaptive potential of the oyster population toward POMS was previously shown to rely on genomic variations (Azéma et al., 2017; Gutierrez et al., 2017), a recent study revealed that non-genetic variations are also essential (Gawra et al., 2023). Concretely and independently of the DNA sequence, distinct methylation signatures in the CG context (*i.e.,* CpGs), mostly harbored in immune genes, were found to be significantly associated with resistance to POMS in wild *M. gigas* populations (Gawra et al., 2023). Additionally, interactions of oyster larvae (D- to veliger larval stages) with a rich microbial environment were shown to result in epigenetic reconfigurations contributing to immune shaping and increased resistance to POMS even transgenerationally (Fallet et al., 2022). The impact of POMS infection itself on DNA methylation in oysters is however unexplored, but strongly hypothesized since pathogenic interactions are well known to induce DNA methylation changes (Fischer, 2020; Netea et al., 2020).

Interest in epigenetic changes induced by the environment has been steadily increasing in shellfish aquaculture since most of these activities take place in natural open environments, subjecting individuals to continuous exposure to abiotic and biotic environmental fluctuations. Research on *M. gigas* epigenetics with interest in aquaculture has been conducted to better understand the influence of epigenetics changes in sex determination (Jiang et al., 2016; X. Zhang et al., 2018; Sun et al., 2022, 2024), in response to thermal stress and thermotolerance (Fellous et al., 2015; C. Wang et al., 2024), intertidal effects (X. Wang et al., 2023), larvae development (Le Franc et al., 2021) and in the acquisition of resistance or robustness to POMS (Fallet et al., 2022; Gawra et al., 2023). Beyond its interest in fundamental biology, these epigenetic changes (Law & Holland, 2019) can also be used as epibiomarkers for applied research (Bock, 2009; Chan & Baylin, 2010). Indeed epibiomarkers hold potential for various applications, especially in aquaculture (Piferrer, 2023). Epibiomarkers could help farmers to make decisions regarding breeding programs (Anastasiadi et al., 2018), disease control (Moraleda-Prados et al., 2020), identify thermal history events (Valdivieso, Anastasiadi, et al., 2023), environmental contamination events (Rondon et al., 2017), and for a sustainable productivity enhancement (Valdivieso, Sánchez-Baizán, et al., 2023). The protocols for developing epibiomarkers have already been established in several marine organisms (Anastasiadi & Beemelmanns, 2023) and would assist in managing and controlling the POMS. Among the putative solutions raised by epibiomarker, the identification of resistance would improve oyster breeding programs through marker-assisted selection. Furthermore, the ability to identify potential new farming areas free of POMS outbreaks would enable safer growth for the oyster industry.

In this context, our study pursued two objectives. Firstly, we aimed to characterize the methylome of resistant and susceptible oysters to POMS before and after infection on a whole-genome scale on two tissues highly exposed to the environment: gills and mantle. Based on this first approach we secondly aimed to identify potential epibiomarkers that could be used to characterize resistance traits and the presence/absence of POMS at a given geographic location. To achieve these objectives, five oyster F1 populations were produced from wild progenitors collected in the main oyster

production basins in France. To ensure accurate observations of the methylation changes, we employed a non-lethal sampling design to track methylation changes occurring in the same individual.

Material and methods

Oyster sampling and production

First-generation (F_1) oyster populations produced in the hatchery were used in our study (Populations #1 to #5). Four of these F_1 populations were produced from progenitors collected in 2022 from four of the main oyster production basins in France: Thau Lagoon (#1), Arcachon Bay (#2), La Floride (#3), Logonna Daoulas (#4) (**Figure S1)**. These natural populations experienced annual POMS events. The fifth F_1 population SC18 (#5) (**Figure S1)** remained unexposed to POMS events since the year 2007 thanks to a biosecured maintenance in controlled condition at the Institut Français de Recherche pour l'Exploitation de la Mer (Ifremer), La Tremblade facility (France). Each F_1 populations were produced from gametes of 20 females and 10 males according to a protocol previously described (Azéma et al., 2017). The F_1 populations #1–5 were then grown in 150 L tanks for one year, fed with seawater-enriched phytoplankton (*Skeletonema costatum*, *Isochrysis galbana*, and *Tetraselmis suecica*; 550,000 cells/mL at 75 L/h) and maintained under biosecured condition to avoid exposure to POMS. These F_1 populations are referred to as the recipient oysters in the experimental infection procedure (see below).

To induce POMS infection, donor oysters from the F14 family (de Lorgeril et al., 2018) and the NSI population were used (Petton et al., 2013). The F14 consisted of a bi-parental oyster family displaying a high susceptibility to POMS (expected susceptibility > 90%). The NSI population is a genetically diversified standardized oyster spats with an expected 50–60% susceptibility to POMS. We used these two different donor oysters to maximize the production of OsHV-1 µVar without compromising the genetic diversity of the viral populations produced. All oysters were produced and kept in the Ifremerbiosecured facilities at Argenton and Bouin (France), and never experienced POMS events. Before the POMS experiment, donor and recipient oysters were acclimatized for two weeks in a dedicated chamber and fed *ad libitum* with *Skeletonema costatum* (700,000 cells/mL). Every two days, the water temperature was gradually increased by 2 °C until it reached 21 °C, and water was renewed at the rate of 30%/hour (BIO-UV ultraviolet-filtration).

OsHV-1 µVar viral suspension for donor oysters

The viral suspension used for experimental infection was an equimolar mix of suspensions obtained from infected oysters collected from three different locations in France: the Rade de Brest, La Tremblade, and Thau Lagoon. Viral suspension was produced as previously described (Schikorski et al., 2011). Donor oysters were injected with 20 µL of viral suspension (6.0 E^7 genomic units) using a 26-gauge needle attached to a multi-dispensing hand pipette into the adductor muscle to facilitate spreading into the circulatory system.

POMS infection by cohabitation between donor and recipient oysters

After acclimatization, 20 recipient oysters from each of the five F_1 populations (#1–5, N_{size} = 100) were individually tagged, anaesthetized (Suquet et al., 2009), and part of their gills and mantle was excised (5–6 mm^2). The extracted samples were labelled as "Pre-infection-T_0" and was the control group. Finally, the 100 biopsied recipient oysters were monitored for a recovery period of 30 days (**Figure 1A**).

To distinguish between POMS-susceptible from POMS-resistant among the recipient oysters a cohabitation protocol using a randomized complete block design was carried out to mimic "natural" POMS infection (Schikorski et al., 2011; Gawra et al., 2023). The infection began with the inoculation of OsHV-1 µVar viral suspension in 100 donor oysters (F14 = 60 and NSI = 40, **Figure 1B**). After injection, five sets of 20 donor oysters (F14 = 12 and NSI = 8) were randomly distributed into five tanks (volume = 10 L, technical replicates). Each tank was equipped with an air-bubbling system to ensure oxygen saturation and water flow. The infected donor oysters were stand-alone for 24 hours. Then, five sets of 20 biopsied and tagged recipient oysters (four from each of the five F_1 populations #1–5) were introduced into each of the five tanks containing the donors (**Figure 1B**). At 24 hours post-

cohabitation (hpc), the donors were removed and their mortality was assessed daily over seven days in an isolated tank. Simultaneously, one mL of seawater from each of the five tanks was sampled daily for quantifying the OsHV-1 µVar viral load until the end of the experiment.

The POMS progression in the recipient oysters was monitored every two hours to assess their status as either "*susceptible*" or "*resistant*" as described (Gawra et al., 2023). Briefly, an oyster was considered susceptible if it could not close its valves after 30 seconds of emersion. Susceptible oysters were immediately removed from the tanks, and another sampling of its gills and mantle was performed (5–6 mm^2). The experiment ended when no mortality was recorded for 48 hours in all five tanks. All the surviving recipient oysters were categorized as resistant, and their gills and mantle were also sampled (**Figure 1B**). This second sampling, for both susceptible and resistant oysters, was labelled as "Post-infection-T_1" and was the treatment group.

All dissected gills and mantle were promptly flash-frozen in liquid nitrogen and stored at −80 °C. For further analysis, ten oysters were selected, with one resistant and one susceptible oyster for each F_1 population #1–5 among the five tanks.

Survival analysis in donor and recipient oysters

Kaplan-Meier model was used with the *'survfit'* and *'ggsurvplot'* functions of *'survival'* (Therneau & Lumley, 2015) (v3.2-11) and *'survminer'* (Kassambara et al., 2017) (v0.4.9) packages, respectively. Then, the Cox proportional hazard model was performed using the *'coxph'* function from the *'survival'* and results were plotted using the *'ggforest'* function from the *'survminer'* package. Survival was considered significant when below the 5% error level.

DNA extraction of gills and mantle from susceptible and resistant oysters

The genomic DNA (gDNA) was extracted from gills and mantle using a NucleoSpin® Tissue kit (MACHEREY-NAGEL GmbH & Co. KG) with 15 min RNase A digestion to remove co-purified RNA, then stored at −20 °C. The gDNA purity was assessed using a nanodrop spectrophotometer (ND1000; Thermo Fisher Scientific) and concentration was verified using the Qubit 2.0 Fluorometric (Thermo Fisher Scientific) with the dsDNA HS assay kit (Q32851; Thermo Fisher Scientific). The presence of a consistent band of high molecular weight gDNA was evaluated using a 1% agarose gel electrophoresis.

Quantification of OsHV-1 µVar viral load

Quantification of the OsHV-1 µVar viral load was performed using quantitative PCR (qPCR). The 20 µL qPCR reaction consisted: 5 µL of gDNA (5 ng μL^{-1}), 2µL of each primer at the final concentration of 550 nM (Eurogenetec), 1 µL of distilled water and 10 µL of Brilliant III Ultra-Fast SYBR®Green PCR Master Mix (Agilent). The virus-specific primer pairs targeted a region of the OsHV-1 µVar genome predicted to encode a DNA polymerase catalytic subunit (ORF100, AY509253): Forward-TTGATGATGTGGATAATCTGTG and Reverse-GTAAATACCATTGGTCTTGTTCC (Webb et al., 2007; Pepin, 2013). The amplification reactions were carried out using the Mx3005P Real-Time thermocycler (Stratagene) with a program: 3 min at 95 °C followed by 40 cycles of amplification at 95 °C for 5 s and 60 °C for 20 s. A melting temperature curve of the amplicon was generated to verify the specificity of the amplification and absolute quantification of the virus was estimated by comparing the observed cycle threshold (Ct) values to a standard curve of the DP amplification product cloned into the pCR4-TOPO vector for the OsHV-1 µVar.

Enzymatic methyl-seq library preparation and sequencing

NEBNext Enzymatic Methyl-seq (EM-seq™) library preparations and sequencing were carried out by IntegraGen (Evry, France). Briefly, 100 ng of gDNA were end-repaired, A-tailed, and ligated to methylated universal adapters. The libraries were then purified and converted using the NEBNext Enzymatic Methyl-seq Conversion Module according to the manufacturer's recommendations. After PCR amplification and indexing, samples were sequenced in paired-end (PE) reads with a length of 150 base pairs (bp) using an Illumina NovaSeq sequencer.

EM-seq reads the bioinformatics pipeline

The raw PE reads quality for each sample was analyzed using FastQC (v0.53) (Andrews, 2010), and the adapters were trimmed using TrimGalore! (v0.6.7) (Krueger, 2015) with parameters: -q 30 --paired --clip_R1 5 and --clip_R2 5 --Illumina. Any remaining adapters were removed in a second trimming round with default parameters. We utilized Bismark (v0.23.1) (Krueger & Andrews, 2011), employing the *'bismark_genome_preparation'* to perform bisulfite conversion *in silico* of the *M. gigas* genome (cgigas_uk_roslin_v1, Assembly: GCA902806645v1) (Peñaloza et al., 2021). Then, the trimmed PE reads were aligned to the bisulfited converted genome using *'bismark'* with parameters: -q -N 1 --score_min L,0,-0.4. The duplicated aligned reads were removed using the *'deduplicate_bismark'* and the methylation calling was accomplished using *'bismark_methylation_extractor'* with parameters: --no_overlap --cutoff 10 only in the CpG context. To assess the enzymatic conversion efficiency, each sample included unmethylated sequences of the bacteriophage lambda (48,502 bp from cI857ind 1 Sam 7 strain) as a spike-in control. The efficiency was calculated for each sample by aligning the trimmed PE reads to the bacteriophage lambda genome (same procedure as above), and retained samples showing conversion efficiency ≤ 99.0% for the DNA methylation level analysis.

DNA methylation level analysis

The *'MethylKit'* package (v1.24.0) (Akalin et al., 2012) was employed along with the *'bismark_cpg_report2mycpg.pl'* script (available at github.com/avilella/methylKit) to process the input data and identify common CpG sites among the selected samples. After normalization and filtering, Principal Component Analysis (PCA) was conducted to assess sample clustering for each tissue and phenotype before and after POMS infection. For differential methylation analysis, we processed the output from methylation calling using the *'DSS'* package (v2.50.1) (Feng & Wu, 2019) to identify the differentially methylated region(s) (DMR) using a smoothing strategy on 500 bp windows. A DMR was considered significant when containing at least four CpGs in a sequence of 50 bp, a minimum methylation level difference of 10% with a False Discovery rate (FDR) ≤ 0.05. Significant DMRs were automatically merged if they were within 50 bp of each other. In bivalves, as described in *M. gigas* and *Crassostrea virginica,* the methylated fraction primarily occurs within the gene body regions (Gavery & Roberts, 2014; Männer et al., 2021; Venkataraman et al., 2020, 2022). To identify DMRs within gene body regions, we obtained the genic coordinates (*i.e.,* chromosome, start and end positions) of all coding genes of *M. gigas* from the *'biomaRt'* package (v2.54.1) (Durinck et al., 2009) and we overlapped with the significant DMRs using the *'foverlaps'* function from the *'data.table'* package (v1.14.8) (Dowle et al., 2019) obtaining a list of genes displaying DMRs. Finally, the average methylation value was assessed when a gene showed more than one DMR in its boundaries.

Gene Ontology enrichment (GO-terms) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis of methylated genes affected by POMS

We performed a Gene Ontology (GO-terms) enrichment analysis to extract the Biological processes (BP) GO-terms associated with the genes with DMRs affected by POMS infection. The gene list of *M. gigas* containing the complete set of annotated genes was used for functional annotation using binary analysis: a score of 1 or 0 was attributed to genes with DMRs or not, respectively to identify enriched GO-terms (based on Fisher's exact test). We employed the GO_MWU package with adaptive clustering (github.com/z0on/GO_MWU) (Wright et al., 2015) with the parameters: largest = 0.1, smallest = 3, cluster CutHeight = 0.25.. A BP GO-term was considered significant with an FDR correction ≤ 0.05. To visualize the significant BP GO-terms we used the ReViGO (v1.8.1) (Supek et al., 2011) with parameters: large = 0.9, the Whole UniProt database as background and SimRel as the semantic similarity measures of the relationship of the GO-terms. We used the Database for Annotation Visualization and Integrated Discovery platform (DAVID, v2023q4) to obtain the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Huang et al., 2009; Sherman et al., 2022). A KEGG pathway was significant when a minimum of five genes and an EASE Score (modified Fisher exact test) ≤ 0.05.

Statistical analysis and software

Statistical analysis was conducted using R environment (v4.3.2) (Team, 2013) through Rstudio (v2023.06.1). The heatmaps were performed using the '*ComplexHeatmap*' package (v2.14.0) (Gu, 2022; Gu et al., 2016), while plot visualizations were created using the *'ggplot2'* package (v3.4.4) (Wickham, 2009). Data processing was carried out using the *'dplyr'* package (v1.1.4) (Wickham et al., 2020). To plot the map of the sites where the oyster progenitors were collected we used the *'ggspatia'l'* (v1.1.9), *'geodata'* (v0.5-9)*, 'terra'* (v1.7-71), and *'raster'* (v3.6-26) and to illustrate the exons of the genes we used the *'ggtranscript'* (v0.99.9) packages.

Results

Experimental POMS infection and mortality

After the "Pre-infection-T_0" sampling no mortalities were recorded for the recipient oysters indicating the low impact induced by the biopsy (**Figure 1A**). After three weeks of recovery, we phenotyped the five F_1 populations for POMS resistance through a cohabitation process with the infected donor oysters. The first mortalities among recipient oysters were observed at 52 hpc. At the end of the infection experiment, mortality rates among the five F_1 populations displayed significant differences (Log-rank test, *P*-value ≤ 0.001) ranging from 15% of survival for the most susceptible (*i.e.,* SC18, #5) to 85% for the most resistant (*i.e.,* Thau Lagoon, #1) (**Figure 1C**, **Table S1** and **S2**). We did not observe significant differences among the five replicates (Log-rank test, *P*-value = 0.96) (**Figure S2A** and **Table S3**). Mortalities of donor oysters (*i.e.*, F14 and NSI) began at 24 hpc, and the survival rate dropped to 70% for NSI and 0% for F14, as expected from previous experiments (**Figure S2B**). Quantification of the OsHV-1 µVar viral load sampled from the water of the five tanks showed that viral shedding reached 520 ± 340 genome copies/µL at 24 hpc, and peaked at 48 hpc with 5,781 ± 2,360 genome copies/µL (**Figure S2C**). In gills and mantle sampled from the "Post-infection-T_1" point, the OsHV-1 µVar viral load was significantly higher (Mann-Whitney, W = 23, *P*-value = 0.032 for both tissues) in the susceptible oysters compared to their resistant counterparts (**Figure S2D**). All these results confirmed that the experimental POMS infection was efficient with an active viral replication and shedding starting in donors and then in the recipient oysters.

Global methylation levels in susceptible and resistant oysters in gills and mantle

For each phenotype (*i.e.,* 5 susceptible and 5 resistant oysters), we analyzed their DNA methylation profile in two tissues (gills and mantle) and for two-time points: before *(i.e.,* "Pre-infection-T_0") and after (*i.e.,* "Post-infection-T_1") POMS infection, making a total of 40 samples. After demultiplexing from sequencing, the number of raw PE reads per sample was 111,915,407 ± 19,799,732 (mean ± SD), with a mapping efficiency of 43.15 ± 1.85%. The removed duplicated reads represented 8.99 ± 0.71%, and the enzymatic conversion efficiency was 99.90 ± 0.09%. Detailed information for each sample is provided in **Table S4**.

At the genome-wide level, we observed a consistent increase in the global methylation level in both tissues of the "Post-infection-T_1" samples (**Figure S3**). When comparing phenotypes, the DNA methylation levels were substantially higher in the gills (One-way ANOVA; F = 4.62, *P*-value = 0.005) and in the mantle (One-way ANOVA; F = 7.49, *P*-value = 0.002) of the susceptible oysters (**Figure S3A** and **S3B**). In the PCA analysis conducted with CpGs common to all gills (1,886,331 CpGs, **Figure S4A**) and mantle (1,806,224 CpGs, **Figure S4B**) samples we observed that samples at both "Pre-infection-T_0' and "Post-infection-T_1" predominantly clustered accordingly to the populations of origin.

All these results showed that POMS infection increases the whole genome methylation level, especially in susceptible individuals, but that the infection had only a subtle impact on the *M. gigas* cytosine methylation landscape.

Block 1: Resistant and susceptible oysters exhibited distinct methylation patterns in both their gills and mantle when compared before and after POMS infection

We then conducted differential methylation analysis comparing the "Post-infection-T_1" (treated) *vs*. "Pre-infection-T_0" (control) samples for each phenotype and tissue separately. This analysis aimed to identify methylation changes associated with POMS infection and to understand how these changes differ in resistant and susceptible oysters (**Figure S5**).

Methylation changes due to POMS infection

DNA methylation profile in susceptible oysters' gills

In the susceptible oysters' gills, our analysis revealed 3,069 DMRs (**Table S5** and **S6**) located in 2,014 genes (**Table S7**). Among these genes, 1,014 displayed hypermethylation and 1,000 hypomethylation in response to POMS (**Figure 2A** and **Table S7**). The 1,014 genes exhibiting hypermethylation were mostly associated with biological processes involved in immunity and stress response (*e.g.,* DNA damage response, GO:0006281, and Response to virus, GO:0009615), and transport (*e.g.,* Nuclear transport, GO:0046907, and Cellular localization, GO:0051641). With the 1,000 hypomethylated genes, a reduced number of GO enrichments was obtained and corresponded to more generalist biological processes (*e.g.,* RNA metabolic process, GO:0016070) (**Figure 2A**). The KEGG pathways analysis revealed that hypermethylated genes were involved in Base excision repair (crg03410) and Nucleocytoplasmic transport (crg03013). While hypomethylated genes were predominantly associated with metabolic processes (*e.g.,* Biosynthesis of cofactors (crg01240), Purine metabolism (crg00230) and Ubiquinone biosynthesis (crg00130)(**Figure 2A)**.

DNA methylation profile in resistant oysters' gills

In the resistant oysters' gills, our analysis revealed 2,594 DMRs (**Table S5** and **S8**) located in 1,702 genes (**Table S9**). Among these genes, 923 and 779 were hypermethylated and hypomethylated, respectively in response to POMS (**Figure 2B** and **Table S9**). Genes with hypermethylation were associated with biological processes involved in signaling (*e.g.,* Negative regulation of the Wnt signaling pathway, GO:003178, Positive regulation of GTPase activity, GO:0043547; and Protein phosphorylation, GO:0006468), and transport (*e.g.,* Cellular transport, GO:0006810). Hypomethylated genes were associated with transport only (*e.g.,* Nucleobase-containing compound transport, GO:0015931) (**Figure 2B**). The KEGG pathways analysis showed that hypermethylated genes were predominantly involved in the Mitophagy-animal (crg04137). In contrast, hypomethylation was located in genes participating in cofactor biosynthesis such as Pantothenate CoA biosynthesis (crg00770) and Biosynthesis of cofactors (crg01240) (**Figure 2B**).

Similarities in gills between the susceptible and resistant oysters

Based on the above results obtained on gills, we performed a delta rank correlation analysis on GO-terms enriched in response to POMS infection of both phenotypes. Results showed a significant, although moderate, positive correlation (Spearman; *P*-value = 0.0089; $R^2 = 0.31$; **Figure S6A**). Among the 2,014 and 1,702 genes with DMRs associated with the susceptible and resistant phenotypes in gills, respectively, 527 genes overlapped (**Figure S6C**). From these shared genes, GO-terms enrichment in biological processes known to be key in the POMS response were identified; specifically in stress response (*i.e.,* Regulation of apoptotic process, GO:0042981; DNA damage response, GO: 0006974 and Double-strand break repair, GO:0006302) (**Figure S6E**). Additionally, among these 527 shared genes, 277 displayed consistencies in the tendency to the methylation changes after POMS infection, irrespective of the phenotype (*i.e.,* 120 genes hypomethylation and 157 hypermethylation, **Figure S6G).**

However, and even if these changes are common to and in the same way in susceptible and resistant, the methylation level at T_0 and T_1 between phenotypes were strongly different probably thanks to a different genetic background or progenitors history. For example, rapamycin complex 2 subunit MAPKAP1 (G25230) gene, which plays a critical role in the TOR signaling pathway by regulating cell growth, proliferation, and survival, experienced hypomethylation changes in both phenotypes. Methylation values of G25230 ranged from 36.92% in resistant oysters and 35.10% in susceptible oysters at Pre-infection-T_0", to 5.04% and 31.73% at "Post-infection-T_1", respectively.

Methylation profile in susceptible oysters' mantle

In the susceptible oysters' mantle, 2,836 DMRs were identified (**Table S5** and **S10**), encompassing 1,866 genes in which 690 were hypermethylated and 1,176 hypomethylated in response to POMS (**Figure 3A** and **Table S11**). In the 690 hypermethylated genes, we identified by GO term enrichment analysis the involvement of apoptosis (*e.g.,* Regulation of programmed cell, GO:0043067;

and Programmed cell death GO:0012501) and cellular integrity maintenance (e.g., Riboflavin biosynthetic, GO:0009231), while the 1,176 hypomethylated genes were involved in biological processes linked to intracellular transport (*e.g.,* Calcium ion transport, GO:0048193, Nuclear transport, GO:0051169), RNA and ncRNA metabolism (*e.g.* RNA metabolic process GO:0016070; and Regulatory ncRNA processing, GO:0070918) and protein metabolic processes (*e.g.,* Positive regulation of protein metabolic process, GO:0051247) (**Figure 3A)**. The KEGG pathway analysis showed significant enrichment for metabolic pathways with Riboflavin metabolism (crg00740), and β-alanine metabolism (crg00410) in hypermethylated genes. While in the hypomethylated genes pathways such as protein turnover and associated regulation with Proteasome (crg03050) as well as translation (Nucleocytoplasmic transport, crg03013) were found (**Figure 3A**).

Methylation profile in resistant oysters' mantle

In the mantle of resistant oysters, our analysis revealed 5,469 DMRs between infected and non-infected samples (**Table S5** and **S12**). Among DMRs located within gene body regions, 1,683 exhibited hypermethylation and 1,294 hypomethylation in response to POMS (**Figure 3B** and **Table S13**). The 1,683 genes undergoing hypermethylation were associated with biological processes of immunity (*e.g.,* Response to virus, GO:0009615; Response to stress GO:0006950), protein modification (Protein modification, GO:0036211; Protein modification by small protein removal GO:0070646), transport (*e.g.,* Organic substance transport GO:0071702; and Cellular localization GO:0051641), and metabolism (*e.g.,* Amide metabolic process, GO:0043603; and Glycolipid metabolic process, GO:0006664). The 1,294 hypomethylated genes were associated with stress response (*e.g.* Cellular stress response, GO:0033554; Recombinational repair, GO:0000725, and Double-strand break repair GO:0006302), GTPase activity (*e.g.,* Regulation of GTPase activity GO:0043087; Positive regulation of hydrolase activity, GO:0051345), and transport (*e.g.,* Cellular localization, GO:0051641) (**Figure 3B)**. The KEGG pathways analysis performed with the hypermethylated genes showed the involvement of Other glycan degradation (crg00511) and Nucleocytoplasmic transport (crg03013). Finally, hypomethylation was associated with Basal transcription factors (crg03022) and Aminoacyl-tRNA biosynthesis (crg00970) (**Figure 3B)**.

Similarities in mantle between the susceptible and resistant oysters

Based on the above results obtained on mantle, delta rank correlation analysis on GO-terms identified in the mantle of both phenotypes did not show a significant correlation (Spearman; *P*-value = 0.38; $R^2 = 0.0089$; **Figure S6B**). When focusing on the genes with DMRS, we found that 788 genes overlapped between phenotypes (**Figure S6D)**. Within these 788 common genes, several were implicated in key cellular processes, including immunity (*e.g.,* Cellular response to virus, GO:0009615 and Response to virus, GO:0009615) intracellular transport (*e.g.,* Nucleocytoplasmic transport, GO:0000063), and positive regulation of enzymatic activity (*e.g.*, Positive regulation of catalytic activity, GO:0048554) (**Figure S6F**). The analysis of DNA methylation patterns in the mantle of oysters demonstrates distinct responses between susceptible and resistant phenotypes following POMS infection. These changes are primarily associated with processes related to intracellular transport, RNA metabolism, and protein turnover. In contrast, resistant oysters exhibit a broader and more pronounced response, with a larger number of DMRs identified encompassing genes involved in immunity, stress response, protein modification, and metabolism. As it was observed in gills, among the 788 common genes in the mantle, 384 displayed the same directional changes (213 hypermethylation and 171 hypomethylated) irrespective of the phenotype (**Figure S6D** and **S6H**). For example, the interferon-induced protein 44 (G31185) gene displayed a methylation level of 47.52% and 43.64% in resistant and susceptible oysters at T0 and 27.14% in resistant and 12.91% in susceptible oysters at T1.

Genes with DMRs associated with immune response

Our analysis of the genes with DMRs in gills and mantle revealed distinct overlaps and highlighted specific functional categories in two different tissues after POMS infection. Interestingly, some of the genes displaying methylation changes due to the infection process were associated with

immunity. From the lists of genes found in gills, we identified 222 (**Table S7**) and 185 (**Table S9**) immune-related genes in susceptible and resistant oysters, respectively. In the mantle, they were 181 in susceptible (**Table S11**) and 284 in resistant oysters (**Table S13**). When comparing the four lists, 31 genes were common to all tested conditions and displayed differential methylation patterns in response to POMS, regardless of the tissue and phenotype (**Figure S7** and **Table S14**). Among them, we identified the bcl-2 homologous antagonist/killer-like (*bak1,* G17360), the baculoviral IAP repeat-containing protein 7-like (*birc3,* G19919), few genes encoding for E3 ubiquitin-protein ligases (*trim3,* G18939; *trim36*, G18954; and *trim71,* G19802), the ubiquitin-like modifier-activating enzyme 1 (*uba1,* G29533), and the integrin alpha-2-like gene (*itga2*, G31928).

Overall and for both tissues, the methylation level before and after the response to POMS revealed stark distinctions between resistant and susceptible phenotypes at the specific but also common genes level. This dynamic response suggests a nuanced interplay between genetic predisposition or history of progenitors and environmental triggers, where epigenetic modifications might serve as key mediators in orchestrating the host's defense mechanisms. Consequently, while genetic predisposition or progenitors history may shape the epigenetic profile of initial vulnerability, the shared alterations in DNA methylation on same genes may reflect the existence of a core response. This situation also has been observed in those 31 genes related to immune response. This unified mechanism offers valuable insights into potential targets for enhancing oyster resilience against POMS infection.

Block 2: DNA methylation as epibiomarkers for POMS disease management

In our investigation, we uncovered distinct DNA methylation profiles within the gills and mantle of resistant and susceptible oysters before and after POMS infection. Beyond the biological interest of understanding the epigenetic patterns associated with each phenotype, we aimed to identify potential CpG sites and DMR that could serve as predictive epibiomarkers for POMS infection presence (exposed *vs.* unexposed site to POMS) and outcome (resistant *vs.* susceptible to POMS). POMS is a highly virulent syndrome, that, when occurring in the wild decimates all susceptible individuals. In the case of a non-exposed site to POMS, the oyster populations will be composed of a mix of resistant and susceptible individuals displaying the "Pre-infection-T_0" methylation profile. On the contrary, already exposed sites will host only those resistant individuals with a resistant "Post-infection-T_1" methylation profile. It is based on this rationale that we then focused on the identification of the most promising epibiomarker candidates.

Epibiomarkers for phenotype selection

To identify epibiomarker(s) of resistance we made two comparisons where the reference group was the Susceptible-T_0 group (**Figure S8**). We established the following criteria that candidate epibiomarkers must meet: 1) an absolute methylation difference ≥ 20% between |Resistant-T_0 *vs.* Susceptible-T_0|, 2) an absolute methylation difference ≥ 20% between |Resistant-T_1 *vs.* Susceptible-T_0|, and finally 3) an absolute methylation difference ≤ 5% between |Resistant-T_0 *vs.* Resistant-T_1| (**Figure S8**). We thus identified 1,204 DMRs in gills of which 1,030 were located within the gene body region (**Table 1** and **Table S15)**, and 794 DMRs in the mantle of which 718 were located within the gene body region (**Table 1** and **Table S16**). Among these candidate epibiomarkers we have selected one example for description as shown in **Figure 4A**. Specifically, this DMR span over 151 bp with a total of 10 CpGs within the Hedgehog-interacting protein-like gene body region (G9133) (**Figure 4A**). When comparing the mean methylation difference of the DMR between the RT_0 *vs.* ST_0 and the RT_1 *vs.* ST_0 groups, we observed 20.0% and 22.6% hypomethylation, respectively, with only a 2.6% methylation difference between the RT_0 *vs*. RT_1 group. At the single CpG level, the third CpG exhibited significant (One-way ANOVA, F = 5.02; *P*-value = 0.039) differential methylation levels between the susceptible (ST_0, n = 4 samples) and the resistant (RT_0 n = 4, and RT_1 n = 3) oysters (**Figure 4A**). The results using this approach showed that several CpG candidates' epibiomarkers located in gene body regions could be used as the foundation to develop a panel of putative informative DMRs or CpGs suitable for the phenotyping of oyster resistance in all epidemiological contexts.

Epibiomarkers for site selection

To identify epibiomarkers to diagnose the presence/absence of POMS on an unknown site, we made two comparisons where the reference group was the Resistant-T_1 group (**Figure S9**). We established the following criteria that candidate epibiomarkers must meet: 1) an absolute methylation difference ≤ 5% between |Susceptible-T_0 *vs.* Resistant-T_0|, 2) an absolute methylation difference ≥ 20% between |Susceptible-T_0 *vs.* Resistant-T_1|, and finally 3) an absolute methylation difference ≥ 20% between |Resistant-T_0 *vs.* Resistant-T_1|. We thus identified 68 candidates epibiomarkers in gills and 66 of them were located within the gene body regions (**Table 2** and **Table S17**). In mantle 128 DMRs were found which 98 were located within the gene body region (**Table 2** and **Table S18**). Among these candidate epibiomarkers, one from the gills is further developed in **Figure 4B**. This DMR span over 194 bp encompassing five CpG in the gene body region of the THO complex subunit 3-like gene (G14367) (**Figure 4B**). When comparing the methylation differences between non-infected and infected samples their mean methylation was 2.4 (RT_0) and 2.8 (ST_0) times higher than what was quantified for RT_1. Among the 5 CpGs within the DMR, CpG #3 appeared as an example of a putative epibiomarker for site selection.

Discussion

Epigenetic changes during POMS reveal the dynamic interplay of host-pathogen interactions

In the present study, we showed that some epigenetic differences in oysters are associated with their resistant and susceptible phenotype before POMS exposure regardless of their geographical origin. Additionally, the exposure of these oysters to POMS disease-induced DNA methylation changes in both phenotypes. These epigenetic differences between phenotypes and those induced by the disease open new questions about the fundamentals of the interaction between oysters and POMS and about putative applications in POMS management.

Host-pathogen interactions are dynamic and involve constant co-evolutionary processes where each partner constantly tries to circumvent molecular innovation enhancing host resistance or pathogen infectivity. Among these mechanisms, the host epigenome can be a target of choice given its ability to rapidly shape new molecular configurations, with potentially improved fitness both for the host (Netea et al., 2020) and the pathogen (Fischer, 2020). The DNA methylation changes identified in our study in response to POMS infection can therefore rely on the host response or the hijack of the cellular machinery by the OsHV1-µVar.

In hosts, non-lethal biotic interaction inducing an immune response was shown to drive immune priming leading to long-lasting protection against a later pathogen encounter (Lanz-Mendoza & Contreras-Garduño, 2022). This mechanism called “trained immunity” is presented as a phenomenon of immune memory relying on the innate immune system *sensus lato* and metabolic shift (Lanz-Mendoza & Contreras-Garduño, 2022). Metabolic intermediates can serve as substrates and cofactors of chromatin modifiers, and the activity of the related enzymes that fluctuate during infection can regulate innate immune responses via epigenetic mechanisms (Nieborak & Schneider, 2018). While numerous empirical studies show clear evidence of this phenomenon in mollusks, the mechanisms governing it remain misunderstood but may rely, at least partly, on metabolic shift and epigenetic changes (M. Zhao et al., 2023), as in plants and vertebrates (Netea et al., 2020). In the case of oyster/POMS interaction, immune priming was already evidenced (Lafont et al., 2017), and associated with specific regulation of genes of the innate immune response and the metabolism (Lafont et al., 2020). Two studies have linked specific DNA methylation changes or patterns with oyster resistance/susceptibility to POMS. One study showed, in a mesocosm context, that exposure to a microbial-rich environment during the larval stage induced an intergenerational increase of resistance against POMS (Fallet et al., 2022), while the second showed a significant association between CpG methylation level and oyster resistance/susceptibility to POMS, in wild populations (Gawra et al., 2023). These findings underscored that the phenomenon of trained immunity in the interaction between oysters and POMS may be influenced, in part, by alterations in DNA methylation.

Several studies have pointed out that DNA methylation may be an important epigenetic mechanism controlling inducible gene expression in *M. gigas* (Gavery & Roberts, 2010; Riviere et al., 2013). In our study, several genes belonging to biological functions linked to innate immunity and metabolism displayed such changes in response to POMS infection, either in susceptible or resistant

oysters (**Figures 2** and **3**, **Table S7**, **S9**, **S11** and **S13**). This result can be interpreted as a first step for the induction of trained immunity. From a roughly equal number of identified DMRs induced by POMS infection, the affected genes displayed some divergent functions between susceptible and resistant oysters. Mechanistically, these DNA methylation changes targeted to specific genes and functions would be driven by the transcriptional activation/repression of these functions (de Lorgeril et al., 2018; Lafont et al., 2020; Fallet et al., 2022) during POMS challenge. Indeed, as hypothesized in *Acropora millepora*, the gene body methylation levels and transcription could influence each other: a higher transcription rate induces hypermethylation to a certain threshold, whereas a too-strong methylation level would decrease the gene expression in a negative feedback loop (Dixon et al., 2018). The consistent methylation changes observed in specific shared genes in response to POMS infection (**Figures 6C** and **6D** suggest that these genes may play a crucial role in the host's response to POMS. This observation points to the importance of epigenetic mechanism where certain gene regions undergo similar methylation alterations in both resistant and susceptible oysters, reflecting a common response. The initial differences (T0) in methylation level of these genes can however explain, at least partly the phenotypic differences between resistant and susceptible individuals. Thus, these regions appear to be under environmental influence due to OsHv1-µVar pressure, indicating potential epigenetic selection on those genes. From our results, a set of 31 genes showed substantial methylation level changes after POMS infection in two tissues independent of their biological functions. Those genes are involved in the immune response. Among them, the *bak1-like* gene (G9050), the interferon alpha-inducible protein 27 (G9050), and the macrophage mannose receptor 1 gene (G28068) are genes with a well-conserved function in immunity in *M. gigas* (Lafont et al., 2020; Namwong et al., 2023; Qiao et al., 2022).

On the other side of the interaction, pathogens are also known to induce epimutation in the host epigenome. This is especially true in diseases involving viruses where the host cellular machinery is hijacked to optimize the multiplication and transmission of shedding viral particles (Balakrishnan & Milavetz, 2017). Among viruses identified to use epigenetic manipulation, several belong to the Herpesviridae family. OsHV-1 µVar, the primary agent of POMS, also belongs to this viral family (Pei et al., 2020). In general, these viral-induced epigenetic reprogramming are mainly associated with the upregulation of the host DNA methyltransferase (DNMTs) inducing hypermethylation of targeted gene and/or genome-wide hypermethylation (Locatelli & Faure-Dupuy, 2023). Our study mirrors this trend (especially in susceptible oysters) with methylation levels consistently increasing by ≈ 0.54% in gills and ≈ 0.48% in mantle post-POMS infection (**Figure S4**). The case of the Epstein-Barr virus (EBV) has been extensively studied in this context. It has been shown that the major EBV oncoprotein, the latent membrane protein 1 (LMP1), is a pleiotropic factor that reprograms, balances, and perturbs a wide spectrum of cellular mechanisms, including epigenetics (L. Wang & Ning, 2021). Tsai and colleagues showed that LMP1 downregulates the expression of critical host genes using the cellular DNA methylation machinery (C.-N. Tsai et al., 2002), more specifically the LMP1 transcriptionally upregulates the DNA methyltransferase 1 (DNMT1) thanks to its COOH-terminal activation region-2 YYD domain (C.-L. Tsai et al., 2006). We searched for the presence of the YYD domain in the OsHV-1 µVar proteins and we found that 6 of the 128 ORFs encode proteins containing this domain, and only one (ORF088) is predicted to encode a membrane protein (Burioli et al., 2017). Further investigations are required to determine the potential role of the protein encoded by this ORF in the hijacking of the oyster methylome and the epigenetic landscape of DNMTs genes in *M. gigas.*

During Herpesviridae interaction with vertebrate host, the manipulations to hijack cellular machinery have three major objectives: to delay or to impair the host immune response, to induce a metabolic shift (both retrieved in biological processes displaying DNA methylation changes as observed in **Figure 2** and **3**, **Table S7**, **S9**, **S11** and **S13**), and to regulate the latent/lytic cycle (Pei & Robertson, 2020; Locatelli & Faure-Dupuy, 2023). Concerning the former, several viruses of the Herpesviridae family were shown to immuno-modulate through epigenetic changes the expression of different functions of the main antiviral pathways, such as the pathogen recognition, the complement activation, the interferons (IFN) response, the JAK-STAT pathway, and the TGF-β signaling (Locatelli & Faure-Dupuy, 2023). Interestingly, we and others have previously shown that all these pathways are transcriptionally activated in response to POMS and that differences between resistant and susceptible oyster families are mostly characterized by a slight delay in the activation of these

antiviral pathways in susceptible oysters infected by the OsHV-1 µVar (de Lorgeril et al., 2018, 2020; Leprêtre et al., 2021).

Development of putative epibiomarkers for improvement of the Pacific oyster aquaculture

With the constant increase of food demand worldwide, aquaculture displays a permanent expansion thanks to increased production and the setup of new farms in uncultured areas (Gentry et al., 2017). One of the main limitations to this increase in the oyster industry relies on diseases that strongly affect cultured stock but also bring uncertainties for geographical expansion (Pernet et al., 2016). In this context, providing epibiomarkers for genomic selection and site exposition to diseases are two essential aspects that could improve current zootechnical practices and the sustainable growth of the Pacific oyster industry given the outcome of climate change (Reid et al., 2019). For this purpose, we have identified a set of candidate epibiomarkers that could be used to characterize whether an oyster is resistant or susceptible (1,998 candidate epibiomarkers) and whether a site has been exposed to POMS or not (164 candidate epibiomarkers).

Genetic selection for resistance to POMS was shown to be a good approach for oyster farming enhancement since it displays a high heritability (Azéma et al., 2017; Gutierrez et al., 2018, 2020). However, despite the identification of several QTLs (Sauvage et al., 2010; Gutierrez et al., 2018, 2020; Gawra et al., 2023) unique determinant bearing oyster resistance was not yet identified, probably because of the polygenic nature of this trait (de Lorgeril et al., 2020). Recent studies performed by our group showed the major role that epigenetic variation can play in oyster resistance to POMS (Fallet et al., 2022; Gawra et al., 2023). In addition, we have identified by an (epi)genomic population study in wild oyster populations that resistance trait to POMS is indeed polygenic but controlled at the genetic and epigenetic level, with the latter explaining better the phenotypic variation than the former (Gawra et al., 2023). In another study, resistance can be environmentally induced resulting in the appearance in the epigenome of new epialleles (Fallet et al., 2022). The meiotic inheritance in the absence of the environmental inducer of some of these epialleles also demonstrates the suitability of such epimutation for epigenomic selection (Fallet et al., 2022). The difficulty to find genetic markers associated with resistance due to its polygenic nature, coupled with the significant influence of epigenetic variation on oyster resistance to POMS, presents a novel opportunity for POMS management. Utilizing epibiomarker-assisted selection, offers therefore a promising avenue for optimization. These identified epibiomarkers would serve as a potent toolkit for further refinement in POMS management strategies.

Currently, the characterization of the POMS exposure history of a natural site is costly and time-consuming. Indeed, it is still impossible to detect directly the initial agent causing the POMS (*i.e.,* OsHV-1 µVar) in seawater (Richard et al., 2021). The unique applicable method currently available is based on the deployment of dedicated pathogen-free oyster cohorts on the site of interest; their weekly monitoring during 4–5 months of the POMS season and the detection by qPCR of OsHV-1 µVar in moribund individuals. The induction of specific epimutation in response to POMS events is therefore a powerful alternative solution to diagnose the presence or the absence of POMS in a putative oyster farming site hosting a wild oyster population. A solution that would use the set of epibiomarker candidates we have identified in the present study.

To identify the candidate epibiomarkers for phenotyping or site selection, we employed a whole-genome sequencing technique using a limited number of samples. The main interest of a genome-wide approach relies on the identification of candidate epibiomarkers without *a priori* knowledge but it suffers limitations due to the lower number of individuals used, and the conservative threshold of 10X coverage applied to calculate the methylation level of each CpG. For these reasons, subsequent experiments will be necessary to validate the candidate epibiomarkers identified and to translate them into a targeted locus-specific method such as the Multiplex Bisulfite Sequencing (Anastasiadi et al., 2018; Moraleda-Prados et al., 2020; Valdivieso, Anastasiadi, et al., 2023; Valdivieso, Caballero-Huertas, et al., 2023). A method that will be better suited for high throughput characterization routinely. As a first step to validate the candidate epibiomarkers identified in our study, we propose to increase the sample size and to use individuals from independent origins to "transform" these putative candidate loci into biologically strong candidate features using more

stringent statistical thresholds as stated in (Anastasiadi & Beemelmanns, 2023). This first step will enable confirmation of the signal provided by the epibiomarkers by removing false positive candidates and putative biases. In a second step, we propose to apply machine learning procedures to ultimately select a panel of epibiomarkers providing a reproducible diagnostic with high accuracy and sensitivity. All these steps will assess the generalizability and the robustness of the predictive model and will open the use of the validated panel of epibiomarkers for its use in oyster farming development but also other types of diseases in marine species with aquaculture interest.

Conclusion

This study brings on new piece of knowledge about the epigenetic response of oysters against the Pacific Oyster Mortality Syndrome (POMS), highlighting the dynamic host-pathogen interactions. We showed that oysters exhibit distinct DNA methylation patterns associated with resistance or susceptibility to POMS before exposure. We also demonstrated that POMS infection induces further methylation changes in both phenotypes. These findings underscore the putative role of DNA methylation in imprinting the host response and potentially enhancing immune priming and trained immunity. The study reveals that specific genes related to innate immunity and metabolism undergo methylation changes post-POMS infection, indicating their critical role in the host's response. The study also raises the possibility that epigenetic modifications could be leveraged for POMS management, offering new avenues for enhancing oyster industry resilience through targeted breeding or site selection. Overall, the findings provide a deeper understanding of the molecular interactions between oysters and POMS, opening new questions about the broader implications of epigenetic mechanisms in host-pathogen dynamics and offering promising strategies for mitigating the impacts of this devastating disease.

Data availability statement

Raw reads are available at ENA under the project accession number: XXXX

Author contributions

JVD granted the study.
AV and JVD designed the study and performed the data.
LD, BM, and JVD produced the oyster populations
AV, BM, and MM performed the experiment and sampled.
AV and JVD wrote the manuscript.
All the authors revised and approved the manuscript submission.

Conflict of interest

The authors declare no competing or financial interests.

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Tables

Table 1. List of epibiomarkers for phenotype selection in gills and mantle tissue with different levels of DNA methylation (from lower to very stringency conditions)

Tissue	Δ |ST_0 *vs.* RT_0|	Δ |ST_0 *vs.* RT_1| & |RT_0 *vs.* RT_1|	No. DMRs	No. genes with DMRs	Immune response
Gills	≤ 5	≥ 20	1,204	1,030	115
		≥ 30	420	392	45
		≥ 40	133	126	15
		≥ 50	33	32	4
		≥ 60	7	7	0
	≤ 2.5	≥ 20	816	717	79
		≥ 30	272	257	29
		≥ 40	78	76	8
		≥ 50	22	21	2
		≥ 60	5	5	0
	≤ 1	≥ 20	429	391	49
		≥ 30	126	120	14
		≥ 40	34	34	3
		≥ 50	7	7	1
		≥ 60	1	1	0
Mantle	≤ 5	≥ 20	794	718	85
		≥ 30	281	268	32
		≥ 40	77	74	9
		≥ 50	23	23	4
		≥ 60	9	9	1
	≤ 2.5	≥ 20	531	489	61
		≥ 30	199	191	28
		≥ 40	52	50	8
		≥ 50	16	16	4
		≥ 60	7	7	1
	≤ 1	≥ 20	292	278	35
		≥ 30	106	102	17
		≥ 40	28	28	7
		≥ 50	11	11	2
		≥ 60	5	5	0

Table 2. List of epibiomarkers for site selection in gills and mantle tissue with different levels of DNA methylation (from lower to very stringency conditions)

Tissue	Δ |ST_0 *vs.* RT_0|	Δ |ST_0 *vs.* RT_1| & |RT_0 *vs.* RT_1|	No. DMRs	No. genes with DMRs	Immune response
Gills	≤ 5	≥ 20	66	65	2
		≥ 30	22	22	1
		≥ 40	7	7	0
		≥ 50	1	1	0
		≥ 60	0	0	0
	≤ 2.5	≥ 20	35	35	1
		≥ 30	14	14	1
		≥ 40	2	2	0
		≥ 50	0	0	0
		≥ 60	0	0	0
	≤ 1	≥ 20	15	15	0
		≥ 30	4	4	0
		≥ 40	1	1	0
		≥ 50	0	0	0
		≥ 60	0	0	0
Mantle	≤ 5	≥ 20	98	94	9
		≥ 30	34	34	4
		≥ 40	17	17	2
		≥ 50	9	9	1
		≥ 60	3	3	0
	≤ 2.5	≥ 20	66	64	6
		≥ 30	25	25	2
		≥ 40	14	14	1
		≥ 50	9	9	1`
		≥ 60	3	3	0
	≤ 1	≥ 20	41	39	6
		≥ 30	13	13	2
		≥ 40	7	7	1
		≥ 50	5	5	1
		≥ 60	1	1	0

Figures legends

Figure 1. Schematic representation of the experimental design and survival analysis

A) First generation (F_1) offspring from five wild oyster populations (#1–5) were acclimatized in controlled conditions for two weeks and tagged. After they were anaesthetized and a piece of gills and mantle were excised and labelled as "Pre-infection-T_0" (control group). This sampling was followed by 30 days of recovery. **B)** Then, a cohabitation protocol between recipients and donor oysters previously infected by injection with 20 µL OsHV-1 µVar (6.0 E^7 genomic units) was used to mimic an event of Pacific Oyster Mortality Syndrome (POMS). Recipient oysters were phenotyped for susceptibility (moribund, red) and resistance to POMS (those who survived POMS infection, blue). The second sampling of gills and mantle was carried out labelled "Post-infection-T_1" (treatment group). **C)** Kaplan–Meier survival analysis for the five recipient oyster F_1 populations (#1–5) during POMS infection.

Figure 2. DNA methylation analysis in gills comparing "Pre-infection-T_0" *vs.* "Post-infection-T_1" samples during Pacific Oyster Mortality Syndrome (POMS) infection

A) Analysis of 2,014 genes with DMRs in gills of susceptible oysters and **B)** 1,027 genes with DMRs in gills of resistant oysters, depicting their hypo- and hypermethylation changes after POMS infection, respectively. Gene Ontology enrichment (GO-terms) of the Biological Processes (BP) category and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed to elucidate the functional implications and molecular pathways associated with the differential genes with DMRs for each phenotype.

Figure 3. DNA methylation analysis in mantle comparing "Pre-infection-T_0" *vs.* "Post-infection-T_1" samples during Pacific Oyster Mortality Syndrome (POMS) infection

A) Analysis of 1,866 genes with DMRs in the mantle of susceptible oysters and **B)** 2,997 genes with DMRs in the mantle of resistant oysters, depicting their hypo- and hypermethylation changes after the POMS infection, respectively. Additionally, the Gene Ontology enrichment (GO-terms) of the Biological Processes (BP) category and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed to elucidate the functional implications and molecular pathways associated with the differential genes with DMRs for each phenotype.

Figure 4. Example of epibiomarkers for phenotype and site selection in gills tissue

A) Example of a candidate epibiomarker for phenotype selection. Methylation levels of the CpGs located in the 151 base pairs (pb) long DMR located in the gene body of the Hedgehog interacting protein-like gene (G9133) for the "Susceptible T_0", "Resistant T_0", and "Resistant T_1" oysters. Among these CpGs, #CpG3 was differentially methylated (DMC) between phenotypes. **B)** Example of a candidate epibiomarker for site selection. Methylation levels of the CpGs located in the 194 pb long DMR located in the gene body of the THO complex subunit 3-like gene (G14367) for the "Susceptible-T_0", "Resistant-T_0", and "Resistant-T_1".

Supplementary figures legends

Figure S1. Selection of progenitors

Origin of the progenitors used to generate the F1 populations used in the present study. These regions include populations from the Mediterranean Sea: Thau Lagoon (#1) and the Atlantic Ocean: Arcachon Bay (#2), La Floride (#3), Logonna Daoulas (#4), and SC18 (#5).

Figure S2. Survival rates and OsHV-1 µVar load quantification

A) Kaplan–Meier survival curves for the five experimental tanks (n = 20 recipient oyster tanks) during the Pacific Oyster Mortality Syndrome (POMS) infection experiment. Notice that the vertical dashed line at 240 hours post cohabitation (hpc) indicates that no mortality was recorded after this time **B)** Kaplan–Meier survival curves of the donor oysters during POMS experiments. **C)** The OsHV-1 µVar viral load (genome copies, GC/µL) quantified in water from the five experimental tanks along the POMS infection. The letters D and R represent period of time where the oysters where in the tank during the experiment: D = donor only; D+R = cohabitation of donors and recipient oysters for 24 hours and R = recipient only oysters remained in the experimental tanks. **D)** The OsHV-1 µVar viral

load (GC/µL) quantified in the gills and mantle of susceptible and resistant oysters "Post-infection (T_1)". * = $P \leq 0.05$; Kruskal-Wallis test.

Figure S3. Global methylation analysis by tissue
Boxplots illustrating global methylation levels (methylation level in the CpG context) of susceptible and resistant samples before and after POMS infection in **A)** Gills and **B)** Mantle tissues. One-way analysis of variance (ANOVA) showed significant differences in script letters between groups. Significant differences were observed when *P*-value ≤ 0.05.

Figure S4. Principal Components Analysis (PCA) of methylation profiles between the five susceptible (red) and the five resistant oysters (blue) from the "Pre-infection-T_0" (black) and "Post-infection-T_1" (grey border) sampling points.
Methylation profiles **A)** in gills based on the 1,886,331 CpG characterized in all samples , and **B)** in the mantle tissue based on the 1,806,224 CpG characterized in all samples. The code associated to each oyster corresponds to the label assigned (**Table S1**).

Figure S5. Methylation analysis to understand epigenetic changes during the Pacific Oyster Mortality Syndrome (POMS) infection.
Schematic representation to analyze the methylation changes that occurred during POMS infection in susceptible (red) and resistant (blue) oysters by comparing "Pre-infection (T_0)" *vs.* "Post-infection (T_1)" sampling points in gills and mantel tissue separately. The reference group was the T_0. The code inside each oyster corresponds to the label assigned (**Table S1**).

Figure S6. Similitudes between susceptible and resistant oysters in gills and mantle tissues.
A) and **B)** Correlation analysis of the Gene Ontology (GO)-terms. The green and blue dots represent significant GO-terms from genes that were hypermethylated and hypomethylated during the Pacific Oyster Mortality Syndrome (POMS) infection **C)** and **D)** The Venn diagram of illustrating specific and common methylated genes. **E)** and **F)** The Biological Processes GO-terms identified from the common methylated genes. **G)** and **H)** Heatmaps of the methylation changes of the common genes consistently affected during POMS in gills and mantle, respectively.

Figure S7. Venn diagram of methylated genes within the immune response functions.
Venn diagram of the methylated genes of the immune functions extracted from (de Lorgeril et al., 2018). Susceptible (red) and resistant (blue). The 31 common methylated genes regardless of tissue and phenotype are listed in **Table S14.**

Figure S8. Criteria for epibiomarker identification for phenotype selection.
A) Schematic representation to identify putative epibiomarkers for phenotype selection. The analysis was carried out in three steps: 1) comparison between "Resistant-T_0" (RT_0) *v*s. "Susceptible-T_0" (ST_0); 2) comparison between "Resistant- T_1" (RT_1) *vs.* "Susceptible-T_0" (ST_0); and 3) filtering accordingly to the difference in methylation value between RT_0 and RT_1. The reference was the ST_0 in both comparisons. The code inside each oyster corresponds to the label assigned (**Table S1**).

Figure S9. Criteria for biomarkers for site selection.
A) Schematic representation to identify putative epibiomarkers for site selection. The analysis was carried out in three steps: 1) comparison between "Susceptible-T_0" (ST_0) *v*s. "Resistant-T_0" (RT_0); 2) comparison between "Resistant-T_0" (RT_0) *vs.* "Resistant-T_1"; and 3) filtering accordingly to the difference in methylation value between ST_0 and RT_0. The reference was the RT_1 group in both comparisons. The code inside each oyster corresponds to the label assigned (**Table S1**).

Declaration of generative AI and AI-assisted technologies in the writing process
During the preparation of this work the author(s) used ChatGPT in order to manage properly the data tables, improving the codes for plotting figures and improve English for clarity. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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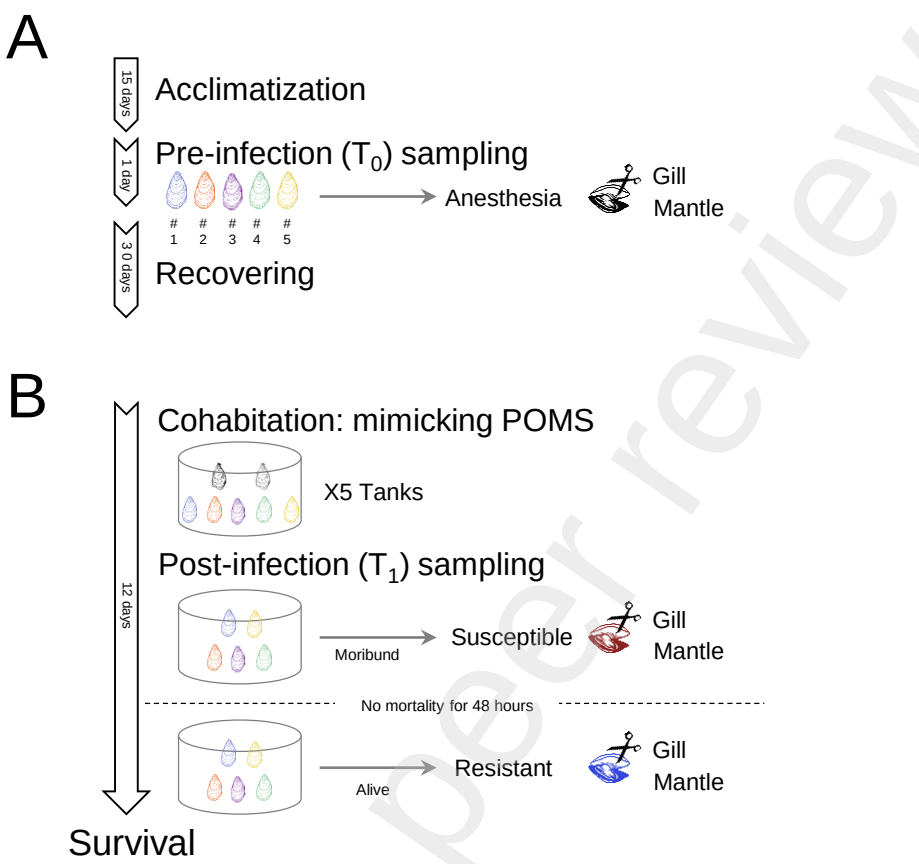
Figure 1



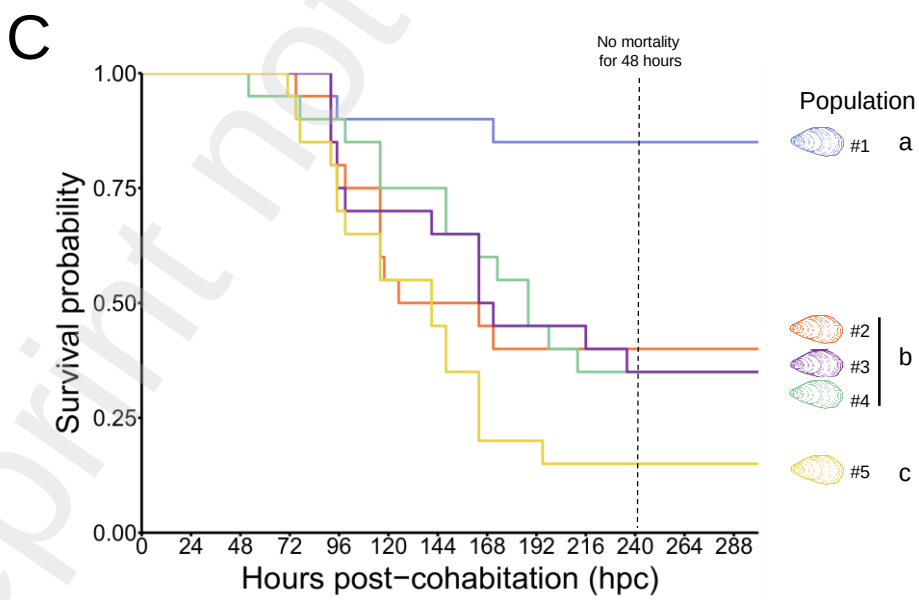
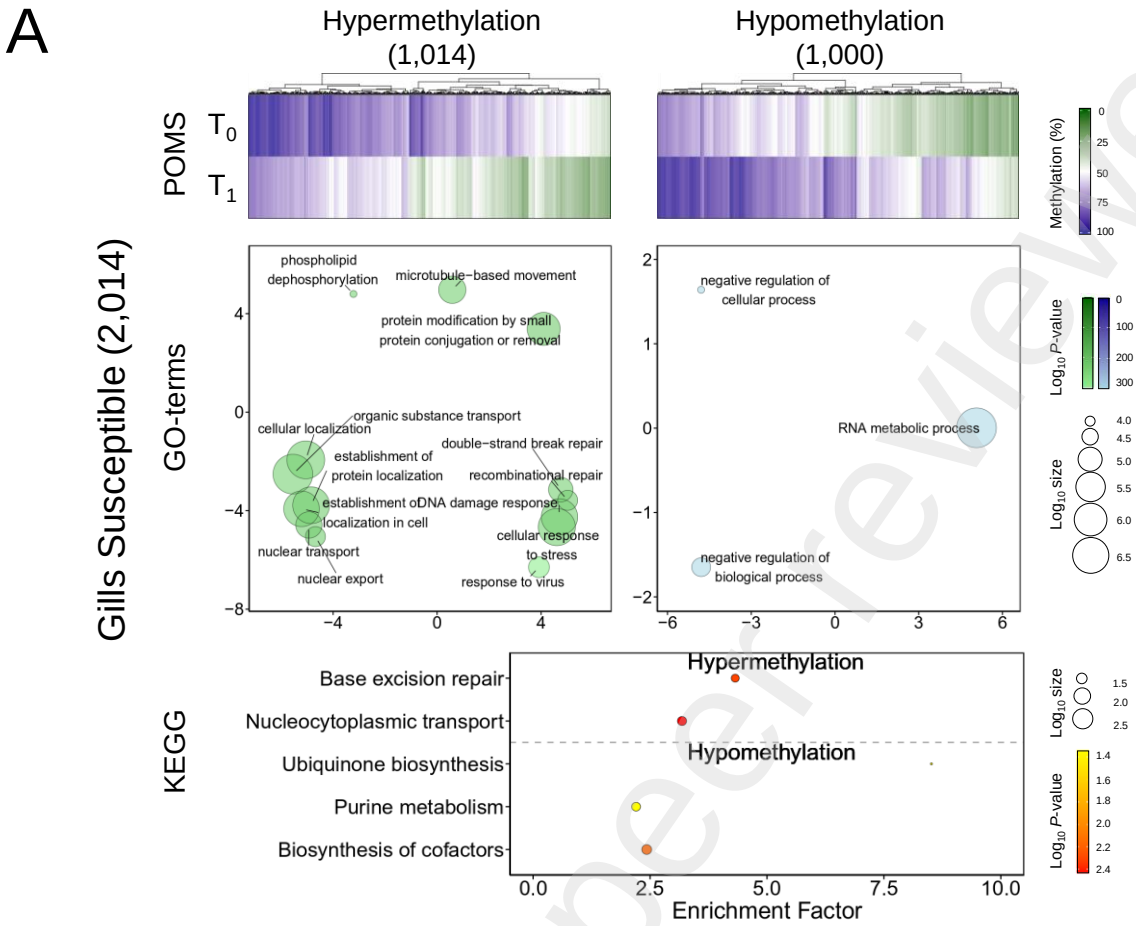


Figure 2



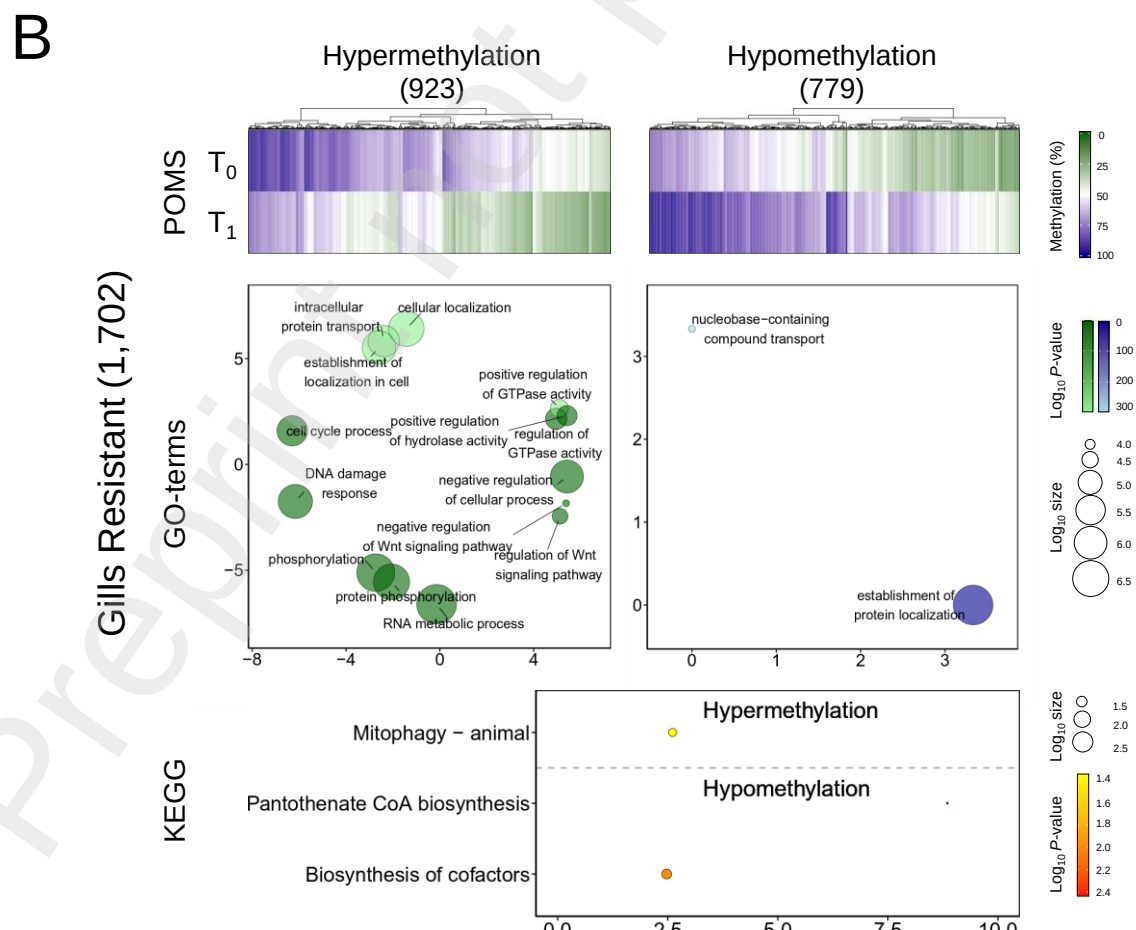
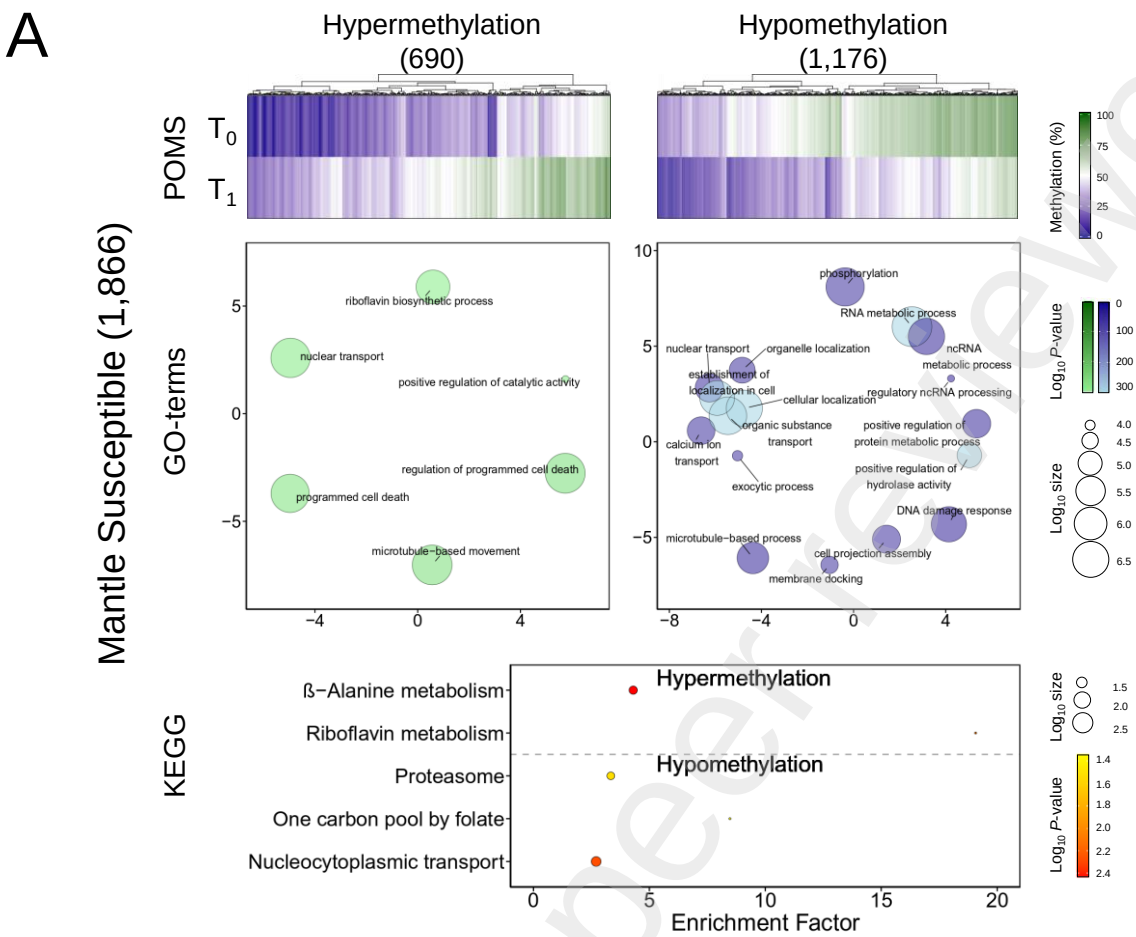


Figure 3



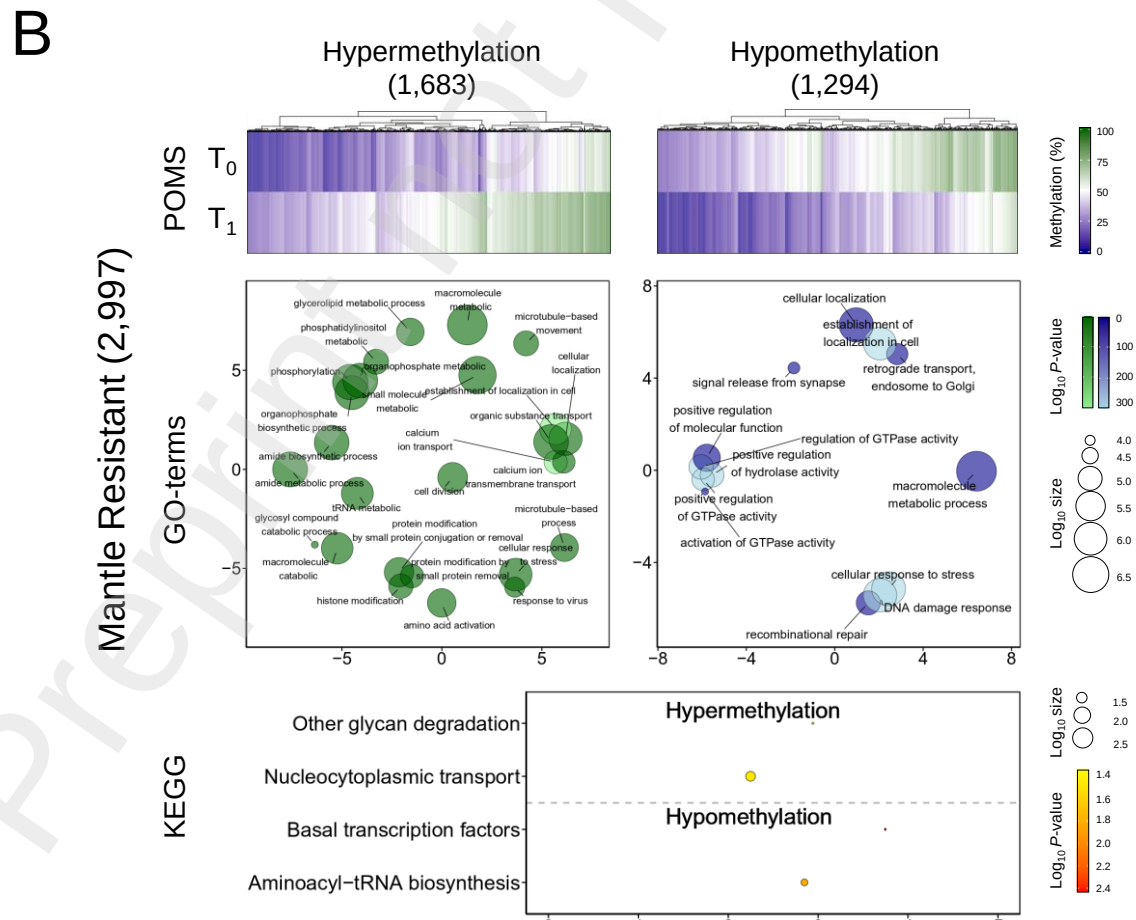


Figure 4