Environmental Science & Technology
January 2024, Volume 58, Issue 4, Pages 1865-1876
https://doi.org/10.1021/acs.est.3c08201 https://archimer.ifremer.fr/doc/00872/98346/

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Ancestors' Gift: Parental Early Exposure to the Environmentally Realistic Pesticide Mixture Drives Offspring Phenotype in a Larger Extent Than Direct Exposure in the Pacific Oyster, Crassostrea gigas

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Abstract :

Marine organisms are threatened by the presence of pesticides in coastal waters. Among them, the Pacific oyster is one of the most studied invertebrates in marine ecotoxicology where numerous studies highlighted the multiscale impacts of pesticides. In the past few years, a growing body of literature has reported the epigenetic outcomes of xenobiotics. Because DNA methylation is an epigenetic mark implicated in organism development and is meiotically heritable, it raises the question of the multigenerational implications of xenobiotic-induced epigenetic alterations. Therefore, we performed a multigenerational exposure to an environmentally relevant mixture of 18 pesticides (nominal sum concentration: 2.85 μg·L–1) during embryo–larval stages (0–48 hpf) of a second generation (F1) for which parents where already exposed or not in F0. Gene expression, DNA methylation, and physiological end points were assessed throughout the life cycle of individuals. Overall, the multigenerational effect has a greater influence on the phenotype than the exposure itself. Thus, multigenerational phenotypic effects were observed: individuals descending from exposed parents exhibited lower epinephrine-induced metamorphosis and field survival rates. At the molecular level, RNA-seq and Methyl-seq data analyses performed in gastrula embryos and metamorphosis-competent pediveliger (MCP) larvae revealed a clear F0 treatment-dependent discrimination. Some genes implicated into shell secretion and immunity exhibited F1:F0 treatment interaction patterns (e.g., Calm and Myd88). Those results suggest that low chronic environmental pesticide contamination can alter organisms beyond the individual scale level and have long-term adaptive implications.

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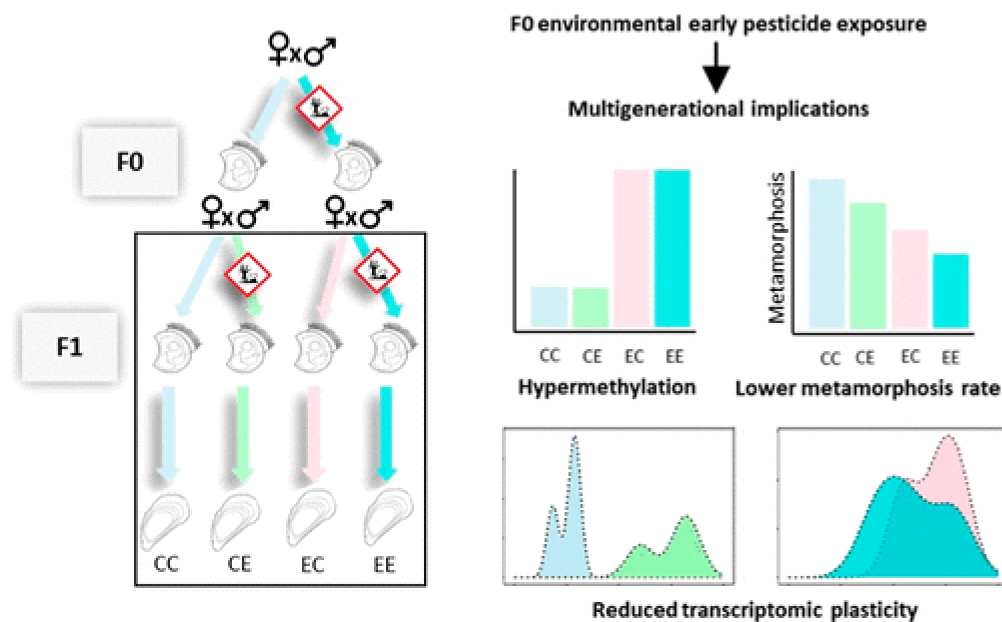
Graphical abstract



Keywords : epigenetics, gene expression, molluscs, DNA methylation, multigenerational, contaminant cocktail

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1. Introduction

The worldwide use of pesticides led to their presence in all of the Earth compartments [1], [2]. As a consequence, although public authorities try and preserve water quality, continental and coastal water bodies are still contaminated by complex mixtures of pesticides [3].

49 Pesticide threat all the integration levels within ecosystems, especially in the marine
50 environment which constitutes their final destination. Indeed, marine organisms are non-target
51 species known to be impacted by pesticides [4]. Among them, many non-vertebrate species like
52 molluscs [5] exhibit external fertilization and their early life stages are exposed to
53 environmental stressors and constitute windows of high susceptibility, potentially leading to
54 long-lasting or delayed effects [6], [7]. The Pacific oyster, *Crassostrea gigas* (Thunberg, 1873;
55 e.g. Magallana gigas), is a historically farmed species of great economic interest worldwide.
56 Moreover, it exhibits specific biological characteristics (filter feeder, sessile, wide spread) that
57 explain its long-standing use as a marine ecotoxicological model [8]–[10].

58 For many years, ecotoxicological studies documented pesticide toxic effects, revealing several
59 outcomes i.e. embryotoxicity [11], [12], genotoxicity [9], [13], [14], reprotoxicity [15] or
60 symbiotoxicity [16]. However, two great challenges are emerging: (i) the study of complex
61 mixtures to better fit environmentally relevant conditions [17] and (ii) the integration of
62 intergenerational and evolutionary perspectives [18]. If a growing body of literature investigates
63 binary and ternary mixtures, the issue of complex cocktails remains less addressed [19], notably
64 in molluscs where the few existing studies do not allow a satisfactory risk assessment [20]–
65 [23]. On the other hand, intergenerational ecotoxicological studies mostly focus on short
66 generation-time species i.e. zebrafish [24] or daphnia [25], which are less ecologically-relevant.
67 Considering longer generation-time species like molluscs, the available studies usually
68 investigated the parental effects of exposure during parental gametogenesis [26], [27], and to
69 our knowledge the multigenerational effects of early exposures in the Pacific oyster has only
70 been addressed once, in the case of a microbiota study [10].

71 Because epigenetic marks can persist through generations, epigenetics became an important
72 tool to investigate the intergenerational issue. In this context, DNA methylation and its link
73 with differential gene expression has gained interest and is increasingly studied in

74 ecotoxicology as a new toxicity endpoint as well as a tool for transgenerational risk assessment
75 [28]. DNA methylation is known to be implicated into gene expression regulation [29] and
76 adaptation to environmental changes within and across generations [30]. In the oyster, DNA
77 methylation is crucial for the development, notably because it is associated to gene expression
78 [31]. Exposure of oyster embryos to contaminants such as copper induces development gene
79 methylation and expression defects together with developmental abnormalities [32]. In
80 addition, we have recently demonstrated that an early exposure to an environmentally-relevant
81 pesticide mixture leads to adverse carry-over effects and the disruption of key developmental
82 stages [6]. Because DNA methylation is affected by pesticides [33], the question of the
83 multigenerational impacts and adaptive consequences of an early exposure in the Pacific oyster
84 is asked. This study investigated the impacts of a multigenerational exposure to an
85 environmentally-relevant cocktail of 18 pesticides (nominal sum concentration: 2.85 $\mu g.L^{-1}$)
86 during the first 48 hours after fertilization. A multi-scale survey was conducted by combining
87 the assessment of larval development, metamorphosis rate, spat growth and on-field survival
88 together with transcriptomic and methylomic approaches.

89

90 **2. Materials and Methods**

91 **2.1 Experimental design**

92 **2.1.1 Chemical mixture**

93 The pesticide mixture used was the same as in the F0 generation [6]. Briefly, the pesticides of
94 the mixture were chosen based on their reported presence in the main French oyster farming
95 areas during the reproduction period of the oyster. The 18 chosen compounds mimic an
96 environmentally relevant pesticide cocktail with a total nominal concentration of 2.85 $\mu g.L^{-1}$
97 (Tab. SI1). Individual stock solutions were prepared from powders in their respective solvents

98 (methanol or ultra-pure water) and combined in a mixture stock solution at x100000
99 concentration.

100 **2.1.2 Broodstock: origin, conditioning and fertilization**

101 The first generation of *Crassostrea gigas* oysters (F0) was reared under two experimental
102 conditions during the first 48 hours after fertilization: (i) control in non-contaminated seawater
103 or (ii) exposed to the 18 pesticides mixture (E, 2.85 $\mu g.L^{-1}$ nominal concentration). Data and
104 analyses of the F0 generation were already published in Sol Dourdin et al. [6]. One-year old
105 individuals from each condition were randomly selected. They underwent broodstock
106 conditioning (NCSW, 8 weeks at 18°C, fed *ad libitum* with *Skeletonema costatum* and
107 *Isochrysis lutea*) and were then induced to spawn by thermal shock (C: 13 males, 6 females; E:
108 14 males, 8 females). Oocytes and spermatozoa were collected as the passing fractions on 80
109 µm and 20 µm sieves, respectively, and then pooled by sex and condition. A per condition
110 fertilization was then performed as previously described [32], resulting in a E progeny and a C
111 progeny.

112

113 **2.1.3 Embryonic development under chemical contamination and larval rearing**

114 Embryos from each fertilization (C and E parental conditions) were divided into 2 treatments
115 : (i) control (C, NCSW with solvents (methanol and milliQ water representing 5×10^{-5} % and
116 9.5×10^{-4} % of the total volume), 25°C, 31.1 psµ, air bubbling, n = 3 tanks, 30 L tanks, 100
117 embryos.mL^{-1}) and (ii) exposed to the environmental pesticide mixture (E: 2.85 $\mu g.L^{-1}$ nominal
118 concentration , 1/100,000 v/v dilution of the mixture stock solution in NCSW, 25°C, 31.1 psµ,
119 air bubbling, n = 3 tanks, 30 L tanks,100 embryos.mL^{-1}). This resulted in 4 experimental
120 conditions: C_C and C_E are embryos from control parents (C parent) that are exposed (CE) or not
121 (Cc) to the mixture in the present generation. E_C and E_E are embryos from exposed parents (E

122 parent) that are exposed (CE) or not (Cc) to the mixture in the present generation., respectively
123 (Figure 1). Embryos were maintained in these conditions for 48 h. Thereafter, they were
124 transferred in a flow-through larval rearing system supplied with NCSW (25°C, 33.4 ± 0.2 psµ,
125 5 L tanks, 5 $L.h^{-1}$, 50 $larvae.mL^{-1}$, n = 3 tanks per condition) with *ad libitum* equal volumes of
126 *Isochrysis lutea* and *Chaetoceros gracils* from 2 dpf to 6 dpf, then *Isochrysis lutea* and
127 *Thalassiosira weissflogii* (homogenous algae outflow = $1.5x10^6$ $mm^3.L^{-1}$ [34]). At 16 dpf (14
128 days post-exposure), about 50% of the larvae from each condition reached the eye-spotted
129 pediveliger stage (MCP larva), which reveals the acquisition of the competence for
130 metamorphosis.

131 **2.1.4 Spat rearing**

132 MCP larvae from each tank were pooled according to their experimental condition to
133 homogenize larval concentrations. They were then transferred in a flow-through raceway for
134 settlement in NCSW (25.0 °C, 32.3 ± 0.6 psµ, 100 L h^{-1}) and reared as previously described
135 [6]. One year old individuals were deployed on-field in La Coupelasse site (47.026571° N /
136 2.030872° W) and checked every two weeks for survival and growth based on the methodology
137 described in Fleury et al. [35].

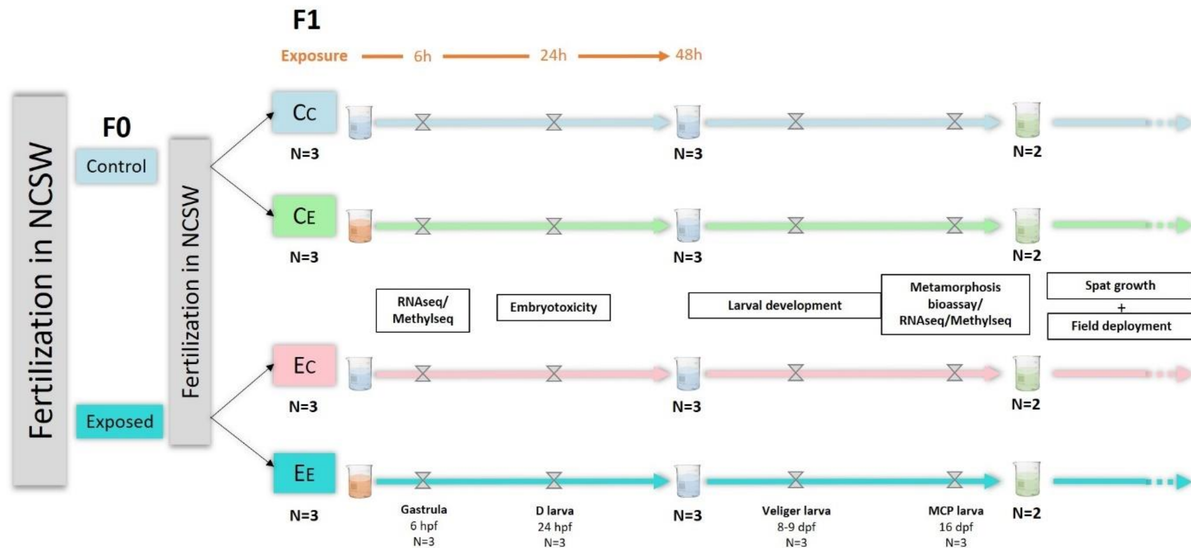

Figure 1 : Experimental design. C_C *= control-control;* C_E *= control-exposed;* E_C *= exposed-control;* E_E *= exposed-exposed; orange beaker= contaminated seawater; blue beaker= non-contaminated seawater (NCSW, 1µm filtered, UV-filtered and active coal-filtered, 25°C), green beaker=pre-treated seawater (UV-treated, 10µm filtered); grey arrows = fertilization in NCSW; Oo= fertilized oocytes; spm= spermatozoa. N = number of samples collected per tank for each analysis and of replicates per conditions.(The F0 generation was analysed and previously published in Sol Dourdin et al.* [6]*. The F1 generation is presented in this paper)*

2.2 Sampling protocols and analyses

2.2.1 Seawater chemical analyses

Seawater chemical analyses were performed for organic and metallic compounds at T0 (before embryo incubation) following the protocols described in [6]. Metallic compounds were measured by ICP-MS (I-CAP-TQ, Thermo), organic compounds were analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS) as described in Sol Dourdin et al. [6].

2.2.2 Ecotoxicology tests and morphometry

The embryotoxicity test was conducted in triplicate according to the previously described protocol [6], following the standardized embryo-larval bioassay ISO 17244:2015, based on the counting of larval abnormalities. During larval rearing, larvae were sampled in each flow-

156 through tank every 2-3 days and stored in 0.1% seawater-formaldehyde solution until image
157 analysis as described in [6]. The metamorphosis bioassay was conducted on competent eye-
158 spotted pediveliger larvae retrieved on a 150 µm sieve (14 dpf, 12 days post-exposure) in 12-
159 well microplates, using epinephrine (Sigma-Aldrich®) at a 10^{-4} M final concentration as
160 described in [6]. Spat growth and on-field survival monitoring were conducted following the
161 protocols used for the F0 generation [6].

162 **2.2.3 Statistical analyses for ecotoxicology and morphometry data**

163 Results are presented as the mean ± 95% confidence interval (CI) of independent replicates. All
164 the data were processed and analysed using R/Bioconductor [36]. Multiple comparisons were
165 carried out using two-way ANOVA (~F0_Treatment×F1_Treatment) to test for interactions,
166 followed by Newman-Keuls' post-hoc test. The normality (Shapiro's test) and
167 homoscedasticity (Bartlett's test) of residues were systematically checked using the
168 performance *R* package (performance_0.10.1) [37]. The Kruskall-Wallis's test (KW test) was
169 performed as non-parametric alternative to compare between the four F1 conditions and the
170 Wilcoxon's test was used as non-parametric alternative to compare between the two lineages.
171 Survival rates were represented as Kaplan-Meier curves and tested for significant differences
172 using a log-rank test with the survival *R* package (survival_3.3-1) [38].

173 **2.2.4 Molecular analyses**

174 **2.2.4.1 Sampling protocol, RNA and DNA extraction and sequencing**

175 Individuals were sampled at gastrula (6 hpf, 6h of exposure, 50,000 embryos per sample, 1
176 replicate per 30 L tanks) and MCP larva stages (16 dpf, 14 days post-exposure, 1,500 animals
177 per 5 L tank). RNA and DNA extraction were performed using Trizol (Invitrogen) and E.Z.N.A
178 Mollusc DNA Kit (Omega Biotek, Norcross, USA), respectively, as previously described [6].
179 RNA sequencing (RNAseq, paired-end 2×150 bp, Illumina NovaSeq6000) and DNA

180 methylation sequencing (Methylseq, paired-end 2×150 bp, Illumina NovaSeq 6000) were
181 performed at Genome Quebec, Montreal, Canada. RNA sequencing led to ca. 1.8 billion of
182 paired-end reads i.e. 77 ± 0.12 million reads per sample, with an average quality score of 36 ± 0
183 and a presumptive ca. 100x transcriptome coverage. Genomic DNA sequencing led to ca. 1.9
184 billion of paired-end reads i.e. 79 ± 0.15 million reads per sample, with an average quality score
185 of 35 ± 0 and a presumptive ca. 35x coverage. Bioinformatic data processing was carried out
186 on the Ifremer's high performance computing cluster (Datarmor).

187 **2.2.4.2 RNAseq analyses**

188 Raw reads were checked using FastQC (fastqc_v0.11.9) [39]: all bases were above 30 Q_c score.
189 Reads were mapped to the indexed reference genome GCA902806645v1 [40] using STAR
190 (star_v2.7.9a) [41] with the following parameters: --alignIntronMin 20 --alignIntronMax
191 1,000,000. The resulting mapping rate was about 48.9 ± 0.1%. Gene expression was quantified
192 by counting the number of reads mapped on mRNA sequences and only genes exhibiting 10
193 reads or more were kept.

194 Principal component analyses (PCA) were performed with the 'plotPCA' function from
195 DESeq2 considering the 1000 more variable genes. The differential expression analyses were
196 performed with the DESeq2 *R* package [42] after *vst* normalization. The Wald's test was used
197 to check for parental effect (|log-2fold change| > 0.5, α threshold = 0.05) and the likelihood-
198 ratio test (LRT) to check interaction between F0 and F1 treatments (α threshold = 0.1).
199 Heatmaps of the differentially expressed genes (DEG) were built from the normalized counts
200 matrix with correlation distance using the pheatmap *R* package (pheatmap_1.0.12) [43]. Rank-
201 based gene ontology (GO) terms enrichment analysis were performed with the GO_MWU
202 method , using log-2 fold change (L2FC) values [44].

203 The global transcriptomic shift upon pesticide exposure was analysed using a discriminant
204 analysis of principal components (DAPC) on normalized counts [45]. The DAPC was
205 performed on a per-lineage (F0 treatment: control *vs* exposed) basis using the adegenet *R*
206 package (adegenet_2.1.5) [46]. This analysis allows the description of a population using
207 variables maximizing inter-group divergence and minimizing intra-group divergence (namely
208 the discriminant function). The same function was used to discriminate samples within each
209 lineage in the two F1 treatment groups: control and exposed. Coordinates distribution of each
210 F0-F1 treatment groups along the first DAPC component were fitted and their difference was
211 tested against a generalised linear mixed model using the Markov chain Monte Carlo
212 ($MCMC_{glmm}$) method (LD1 ~F0Treatment + F0Treatment:F1Treatment) implemented in the
213 MCMCglmm *R* package (MCMCglmm_2.33) [47].

214 **2.2.4.3 Methylseq analyses**

215 Raw read quality was checked with FastQC (fastqc_v0.11.9) [39]. Fastp (fastp_v0.20.1) [48]
216 was used to trim Illumina adapters, the last 20 poor quality bases of reads 1 and 2, and filter
217 reads by length (125 ≥ bp). Trimmed reads were mapped to the indexed GCA902806645v1
218 reference genome [40] with BWA-Meth (bwa-meth_v0.6.1) [49], leading to a 52.9 ± 0.1 %
219 mapping rate. Methylation bias and methylation calling were performed using MethylDackel
220 (methyldackel_V0.6.1) (https://github.com/dpryan79/MethylDackel) with the following
221 parameters: --minDepth 10 –OT 5,125,5,125 –OB 5,125,5,125.

222 Methylation data were summarised on a per-tile basis (tile = 500 bp) using the methylKit *R*
223 package (methylKit_1.16.1) [50]. PCA were performed with the 'PCA()' function from the
224 FactoMineR *R* package (FactoMineR_2.4) (1000 more variable regions) [51], Wald's test was
225 used to check parental effect (|Δ| = 10%, α threshold = 0.05) and the likelihood-ratio test (LRT)
226 to check interaction between F0 and F1 treatments (α threshold = 0.1). Differentially methylated
227 regions (DMR) coordinates were intersected with gene coordinates from the GCA902806645v1

genome assembly, using Bedtools intersect (bedtools_v2.30.0) [52], to identify DMR-related genes (DMG). The differential methylation of DMGs was estimated as the mean of the differential methylation of its related DMRs. Rank-based GO terms enrichment analysis were performed with the GO_MWU method, using Fisher's exact test [44].

The relationship between gene expression and DNA methylation was explored based on the 5000 more variable genes and regions using the Projection to Latent Structures (PLS) method, from the mixOmics *R* package (mixOmics_6.14.1) [53].

2.2.4.4. Genetic divergence

Biallelic single nucleotide polymorphisms (SNP) were identified from the RNAseq data of the F0 and the F1 generations using the nf-core rnavar pipeline 1.0.0 [54]. Variant callings were merged together and then filtered using BCFtools v1.17 [55] with the following parameters "-g 10 -i 'TYPE="snp" & N_ALT=1 & min(FORMAT/DP)>=4 & max(FORMAT/DP)<= 2000 & min(QUAL)>=100 & min(MQ)>=30 & min(AC)>=3 & F_MISSING<0.1'". The allele frequencies were computed for each SNP and each sample, and plotted on a PCA. In order to estimate the genetic divergence between both lineages, the absolute allele frequency difference (AFD) was used as alternative to the F_{ST} metrics [56], using a script developed in Andres et al. [57]. Whether and to what extent SNPs may account for methylation differences (i.e., the physical link between SNPs and methylation variations) was assessed by overlapping SNPs coordinates with between-lineage DMRs coordinates using Bedtools Intersect (bedtools_v2.30.0) [52].

3 Results

3.1 Chemical contamination

251 The measured concentrations (MC) in the control tanks were under the detection or
252 quantification limits for all of the organic contaminants (Tab. SI2). In the exposed batches, the
253 MC of metallic contaminants were consistent with the nominal concentrations. Regarding
254 organic contaminants, the MC are consistent with the nominal concentrations excepted for
255 atrazine, diuron, carbendazime, acetochlor and simazine that exhibit concentrations
256 approximately 2-fold lower than expected.

257 **3.2 Embryotoxicity test, larval development and metamorphosis bioassay**

258 The embryotoxicity test conducted at 24 hpf did not reveal major morphological alterations and
259 there was no difference between the four conditions (C_C: 92.9 ± 1.9%; C_E: 90.7 ± 2.7%; E_C:
260 90.1 ± 2.6%; E_E: 89.8 ± 3.6%; 2-way ANOVA, $p > 0.05$). The larval sizes were different at day
261 2 and 13 (Fig. SI1). At day 2, the C_C larvae were larger than the C_E, E_E and E_C conditions.
262 (KW test, $p < 0.05$), at day 13 C_E was greater than E_E (KW test, $p < 0.05$). At 16 days post-
263 fertilization, the larvae from all conditions reached the competence for metamorphosis, with no
264 statistic difference in eye-spotted pediveliger larvae percentage (C_C: 38.7 ± 29.8; C_E:
265 64.0 ± 30.1; E_C: 41.7 ± 19.3; E_E: 37.7 ± 34.8; 2-way ANOVA, $p > 0.05$).

266 The metamorphosis rate was higher in the control lineage than in the exposed lineage, and there
267 was a significant effect of the F1 exposure which induced a lower metamorphosis rate (Figure
268 2.A). No interaction was observed between F0_Treatment and F1_Treatment (2-way Anova,
269 $p > 0.05$).

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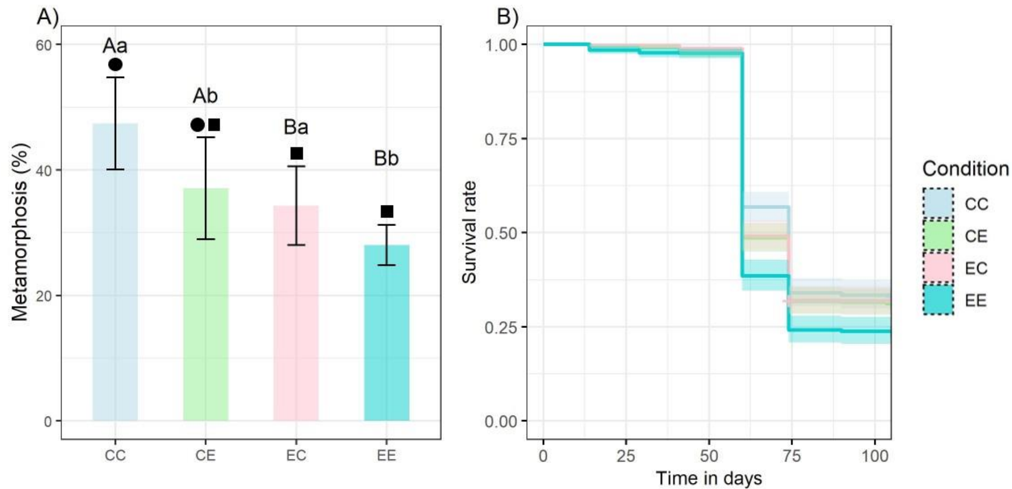


272 *Figure 2: A) Epinephrine induced metamorphosis rate (%). Letters refer to statistically significant differences between*
273 *parental treatment (upper-case) and between F1 treatment (lower-case). 2- way anova, p < 0.001. Black shapes refer to*
274 *statistically differences between de four F1 conditions. 1-way anova, p < 0.05. Error interval represent the 95% CI. B)*
275 *Survival rate of spats in the field. Error interval represents the 95% CI (log-rank test, p < 0.01).*

276

277 **3.3 Spat growth and on-field survival**

278 Spat total weight was statistically different between conditions from month 1 to 3, from month
279 5 to 8 and at month 11 post-fertilization, however no clear trend was observed (Fig. SI2). E_E
280 individuals placed in the field had a lower survival rate than C_C, C_E and E_C groups (24%, 33%,
281 32% and 32% respectively; log-Rank test, $p < 0.005$, Figure 2.B).

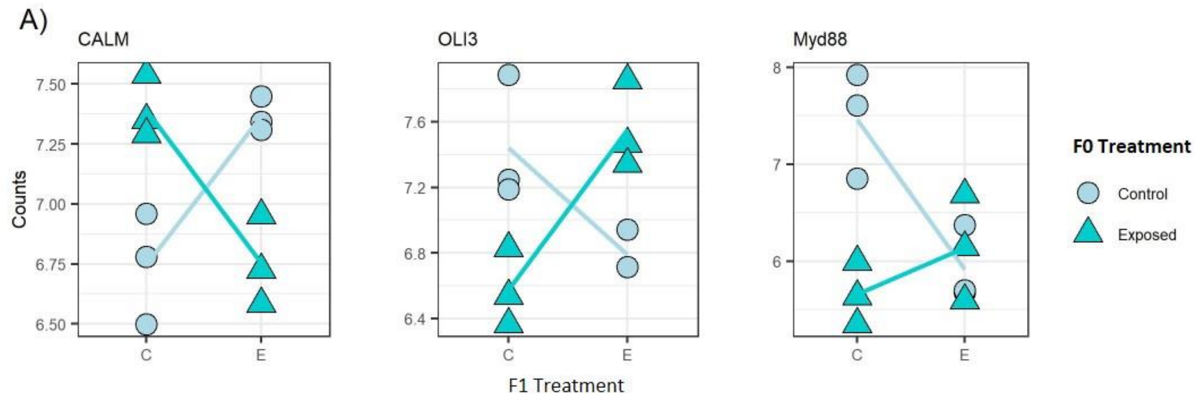
282 **3.4 Gene expression**

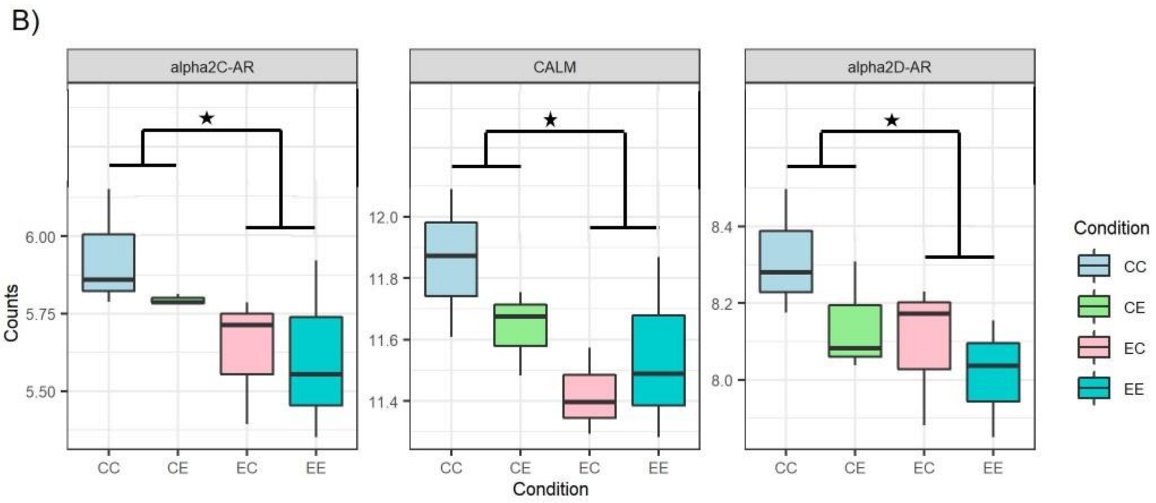
283 In gastrula embryos (6h pesticide exposure), samples were discriminated by the F0 treatment
284 along the second PCA component that explained 14% of the variance (Fig. SI3.A). There were
285 451 genes differentially expressed (DEGs) between parental treatments (Tab. SI3, Wald's test,
286 $p < 0.05$, |L2FC| threshold = 0.5). Of those genes, 203 were induced and 248 were repressed in
287 the exposed lineage compared to the control (Fig. SI4.A). The GO term enrichment analysis
288 revealed that several biological processes as mitochondrial respiratory or peptide biosynthetic

289 process were repressed (Fig. SI5.A). Instead, biological processes implicated in nervous system
290 development, stress response and signalling pathways were induced in the offspring from
291 exposed parents. There were 20 genes exhibiting F1:F0 treatments interaction pattern of
292 expression (LRT, $p < 0.1$) (Fig. SI6.A, Tab. SI4). Three patterns of interaction were observed
293 (Fig. SI6.C): (i) the control lineage expression increases in response to pesticide exposure while
294 the exposed lineage expression decreases, e.g. Calmodulin (CALM, Figure 3.A); (ii) the control
295 lineage expression decreases while the exposed lineage expression remains constant, e.g.
296 Myeloid differentiation primary response protein 88 (MyD88, Figure 3.A), and (iii) the control
297 lineage expression decreases while the exposed lineage expression increases, e.g.
298 Oligodendrocyte transcription factor 3 (OLI3, Figure 3.A).

299 In MCP larvae, transcriptome-wide signatures related to the parental exposure were no longer
300 detected with global PCA analysis (Fig. SI3.B). Anyway, the differential analysis based on the
301 F0 treatment revealed 241 DEGs (Tab. SI3, Wald's test, $p < 0.05$, |L2FC| threshold = 0.5),
302 among which 130 were induced and 111 were repressed in the exposed lineage compared to the
303 control (Fig. SI4.B). GO term enrichment analysis showed the repression of biological
304 processes linked to nervous system development and regulation, and signal transduction, while
305 lipid metabolism, translation or cell motility were induced in exposed F0 offspring (Fig. SI5.B).
306 Five genes exhibited F1:F0 treatment interaction pattern, according to the pattern (i) only (Fig.
307 SI6.B, Tab. SI4). Among those genes, 3 are related to stress response: HSP68, HSP70 and Stress
308 induced protein (Tab. SI4).

309 Three genes putatively implicated in the response of MCP larvae to epinephrine (alpha2C-AR,
310 alpha2D-AR and CALM) (Figure 3.B) were individually tested for differential expression
311 between lineages. All of them exhibited differential expression (one-tailed wilcoxon's test,
312 $p \leq 0.05$), being more expressed in the control than in the exposed lineage.

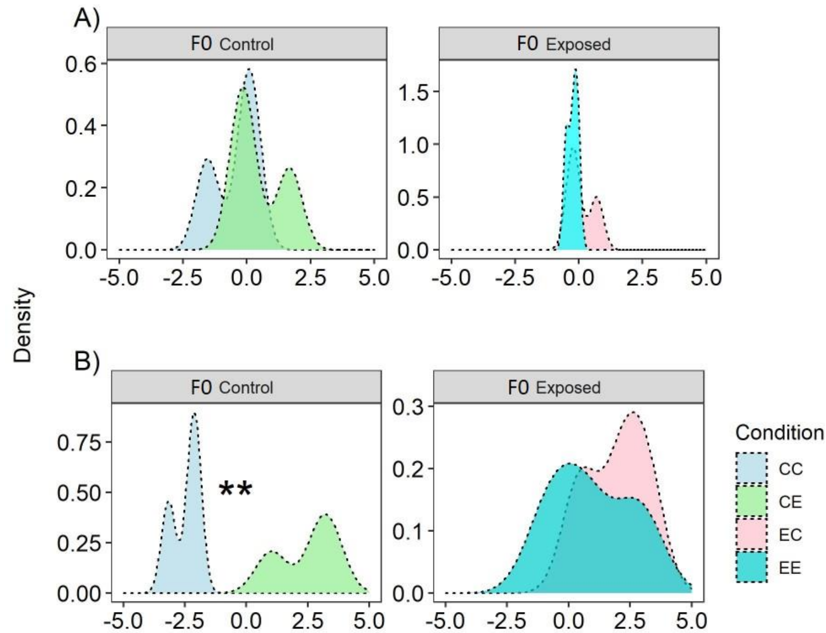




313

314 *Figure 3: A) RNAseq normalized counts plot of three genes exhibiting significant F1:F0 interaction pattern of expression in*
315 *gastrula embryos, LRT, p ≤ 0.1. B) Expression level of metamorphosis-related genes based on RNAseq normalized counts.*
316 *One-tailed Wilcoxon's test between lineages, *: p ≤ 0.05. C = control treatment; E = exposed treatment.*

317 The pesticide-induced transcriptomic plasticity was estimated from the shift in sample
318 distribution on the first DPCA component built on normalized counts matrix (Figure 4). The
319 expression shift was not significant for the parental groups in gastrula embryos (MCMCglmm,
320 $p > 0.05$). In MCP larvae, the expression shift was significant in the control lineage but not in
321 the exposed lineage (MCMCglmm, $p = 0.006$ and $p > 0.05$, respectively) (Figure 4), indicating
322 that transcriptomic response to pesticide exposure is attenuated in MCP larvae from parents that
323 experienced the same stress.



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325 *Figure 4: Transcriptomic plasticity: coordinates distribution of each condition along the first DAPC component for gastrula*
326 *(A) and metamorphosis larvae (B). Samples were discriminated against the same function. (**: MCMCglmm* p-*value < 0.01).*

327

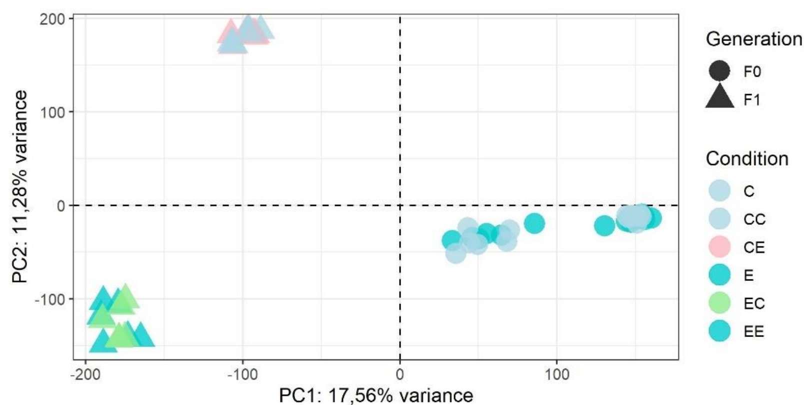
328 **3.5 Differential methylation analysis**

329 In gastrula embryos, overall methylation was strongly influenced by the F0 treatment. Samples
330 discriminated by lineage along the first PCA component, counting for 89.75% of the variance
331 (Fig. SI7.A). Differences in methylation preferentially affected intergenic regions, which
332 exhibit 15% of the constitutive methylation but 27% of the DMRs observed in gastrulae (χ^2 test,
333 $p < 0.05$, Fig. SI8). There were 4349 DMRs (hypo- methylated: 435, hyper-methylated: 3914;
334 Wald's test, $p < 0.05$, |Difference| threshold = 10%) between exposed and control lineage,
335 intersecting 2345 genes (Tab. SI5). Of those genes, 89.5% were hypermethylated. Enriched
336 biological processes are mainly related to cell fate, cell junction and muscle development (Fig.
337 SI9.A). Overall, 84 DMR-related genes exhibited F1:F0 treatment interaction patterns. (Fig.
338 SI10A, Tab. SI6). In MCP larvae, overall methylation did not discriminate on PCA for neither
339 F0 nor F1 treatment (Fig. SI7.B). In MCP larvae, there were 550 DMRs (hypo-methylated: 178,

340 hyper-methylated: 372; Wald's test, $p < 0.05$, |Difference| threshold = 10%) between both
341 lineages, intersecting 337 genes of which 68.8% were hypermethylated. Regarding GO term
342 analyses, enriched biological processes are mainly related to development and organismal-level
343 homeostasis (Fig. SI9.B). There were 69 DMR-related genes exhibiting F1:F0 treatment
344 interaction patterns (Fig. SI10B, Tab. SI6). In gastrula embryos, 51 genes were differentially
345 expressed and methylated (Tab. SI7), but there was no correlation between the level of
346 differential methylation and differential expression for those genes (Pearson's correlation test,
347 $p > 0.05$). None of the differentially methylated genes was differentially expressed in MCP
348 larvae. The PLS analysis revealed that DNA methylation was the main driver discriminating
349 samples at the gastrula stage (Fig. SI11). For methylation, the first latent component accounted
350 for 66.1% of the variance, and discriminated C_C and E_E conditions. For gene expression, the
351 second axis explained 56% of the variance without discriminating samples. In MCP larva stage,
352 PLS analysis did not allow to discriminate conditions (Fig. SI12).

353 **3.6 Genetic divergence**

354 The projection of allele frequencies on a PCA (Figure 5) did not reveal genetic divergence in
355 the F0 generation between exposed and control individuals. A clear differentiation was
356 observed between parents (F0) and offspring (F1) which discriminate along the first component
357 (17.56% variance). In the F1 generation, individuals from each pedigree also discriminate along
358 the second axis of the PCA (11.28% variance). The AFD calculation resulted in a similar
359 differentiation between both F1 lineages (AFD = 0.079) and between F0 and offspring from
360 control pedigree (AFD = 0.077) or exposed pedigree (AFD = 0.083). The intersect of DMRs
361 and SNPs coordinates revealed that the overlapping concerns 0.8% of SNPs and 6% of DMRs
362 in gastrula embryos, and less than 0.0001% of SNPs and 0% of DMRs in MCP larva.



363

364 *Figure 5 : Principal component analysis of the allele frequencies related to the SNPs existing between individuals from the*
365 *F0 and the F1 generation.*

366

367 **4. Discussion**

368 This study investigated two of the main challenges in ecotoxicology: the complex chemical
369 mixtures [19] and the intergenerational effects [58]. Three questions were addressed: (i) does
370 an early exposure to environmentally relevant pesticide mixture induce multigenerational
371 effects in *C. gigas* ?; if so, (ii) does multigenerational inheritance trigger adaptive mechanisms
372 ?; and, (iii) does DNA methylation contributes to the transmission of environmentally-induced
373 effects across generations ? To answer, we assessed molecular and phenotypic endpoints in a
374 multigenerational exposure experiment.

375 Organic contaminants were under detection or quantification limits in all of the unexposed
376 batches (C_C and E_C). Besides, the mean copper and cadmium concentrations in control
377 conditions were representative of the recently measured concentrations in the Loire estuary
378 [59]. Those results confirmed the efficiency of the active carbon treatment and the absence of
379 metallic contamination in the experimental system. Moreover, the measured concentrations
380 (MC) in seawater from all exposed batches (C_E and E_E) were mostly consistent with expected
381 nominal concentrations, except for atrazine, diuron, carbendazime, acetochlor and simazine that

382 were about 2-fold lower than expected (Tab. SI2). However the total MC in contaminated
383 batches (2.75 ± 0.063 $\mu g.L^{-1}$) was lower but within the same range than for the F0 generation
384 (3.75 ± 0.014 $\mu g.L^{-1}$) [6].

385 We chose to investigate gene expression and DNA methylation in the gastrula and MCP larva
386 stages because they correspond to important time windows of direct (gastrula) or delayed (MCP
387 larva) sensitivity to pesticide exposure, where impairments could lead to measurable functional
388 consequences. However, gene expression and DNA methylation are highly dynamic during
389 development [31] and it is not excluded that investigating those life stages fail to thoroughly
390 encompass all the possible consequences of pesticide exposure. Therefore, our results should
391 be considered with the appropriate caution regarding this point.

392 Parental effects were observed at both the DNA methylation and gene expression levels. A clear
393 hyper-methylation trend was observed in gastrula embryos as well as MCP larvae from exposed
394 parents (Tab. SI5). Regarding gene expression, gastrulas were clearly discriminated along the
395 second axis (14% variance) based on their origin rather than on the ongoing pollutant stress
396 (Fig. SI3.A), while the first axis (68% variance) is likely to be driven by the sampling process.
397 Overall, 451 genes were differentially expressed at that stage. In gastrulas, offspring from
398 exposed parents exhibited a repression of genes involved in cellular metabolism while genes
399 related to nervous system development and locomotion were induced (Fig. SI5.A). In MCP
400 larvae, the parental effect still drove the expression of 241 genes, with the repression of
401 biological processes linked to nervous system structure and development in MCP larvae from
402 exposed parents (Fig. SI5.B). Interestingly, a recent study revealed an up-regulation of genes
403 involved in nervous system remodelling just before the initiation of metamorphosis in *C. gigas*
404 [60]. The results of the epinephrine-induced metamorphosis test indicate a disruption of the
405 metamorphosis process in exposed F0 offspring. Indeed, the exposed F1 larvae display lower
406 metamorphosis rates compared to their control counterparts, a trend that was already observed

407 in the parental (F0) generation [6]. Such phenotypic impairment is consistent with the
408 repression of calmodulin (CAM) and two adrenergic-type receptors (AR) genes in larvae from
409 exposed parents, that are key factors of the epinephrine-response pathway in oysters [60]. These
410 delayed effects on metamorphosis, and thereby recruitment, highlight the multigenerational
411 consequences of an early exposure of *C. gigas* embryos to pesticides.

412 However, the assessment of parental effects being the result of non-genetic inheritance of
413 chemical-induced effects requires to detangle non-genetic from genetic effects, which is
414 recognized as a highly tricky task [61]. Indeed, epigenetic mechanisms could mediate non-
415 genetic effects [62] and epigenetic divergence could be faster and predominant over genetic
416 divergence in rapid adaptation of oysters [63]. Besides, the data herein does not allow to
417 quantify the relative prevalence of genetic and epigenetic effects. In addition, the Pacific oyster
418 has a highly variable genome and laboratory experiments are likely to increase genetic drift
419 [64] or selection [65], which may explain the genetic divergence observed here between control
420 and exposed lineages. The inter-lineage divergence in the F1 generation is also very similar to
421 the divergence observed between parents and offspring, and may therefore be considered as a
422 consequence of the reproduction experiment design. Nevertheless, in the present work, the
423 hypothesis of multigenerational effects *per se* may be supported by results at the phenotypic
424 and molecular scales. First, the similar metamorphosis rates between the C_E, E_C and E_E groups
425 (i.e. exposed larvae from control parents and both control and exposed larvae from exposed
426 parents) reinforces the idea that this endpoint is not the consequence of a particular genotype
427 resulting from the protocol of reproduction (Figure 2.A). The same conclusion can be drawn
428 from the transcriptomic plasticity analysis of MCP larvae. The gene expression profiles of the
429 C_E, E_C and E_E conditions were similar (Figure 4), suggesting that expression patterns are likely
430 to be induced by the early exposure rather than the result of a genetic divergence. Moreover,
431 the limited relationship between SNPs and methylation differences at both gastrula and MCP

432 larva stages reinforces the idea that methylation differences are independent of the (limited)
433 genetic variation between pedigrees. We therefore conclude that an early exposure to an
434 environmentally-relevant pesticide mixture can have multigenerational effects at multiple
435 biological levels in the Pacific oyster, including the alteration of critical developmental steps
436 like metamorphosis. This result supports a better consideration of the inheritance of toxicity in
437 future studies [58].

438 Repeating the chemical stress in F1 generation allowed us to investigate whether
439 multigenerational inheritance can trigger adaptive mechanisms. F1:F0 interaction patterns were
440 observed in gastrula and MCP larvae at the molecular level, suggesting an adaptive
441 phenomenon. Some interactions are likely to benefit individuals from exposed parents. For
442 instance, in gastrula embryos, the Myd88 protein, implicated in the innate immune response via
443 the Tool/interleukin-1 receptor (TIR) family signalling pathway [66], [67], was repressed in the
444 control lineage under pesticide exposure and remained constant in the exposed one (Figure 3.B).
445 Later on, the induction of stress-related proteins observed in MCP larvae from control parents
446 after pesticide exposure was mitigated in those from exposed parents. However, non-benefit
447 interactions were also observed. For example, Calmodulin (Calm) is involved in the cellular
448 calcium homeostasis and biomineralization process in marine invertebrates [68], and it is
449 commonly up-regulated in oysters facing ocean acidification [69], [70]. Here, in the case of a
450 multigenerational exposure to pesticides, the expression of Calm is repressed in MCP larvae
451 from the exposed progeny, while it is induced in the control progeny. Therefore, this experiment
452 revealed the possibility for multigenerational exposure to increase the potential negative impact
453 of chemicals on genes involved in shell formation in molluscs larvae, that has already been
454 highlighted in several studies [6], [32], [71]. Nonetheless, because gene expression is highly
455 dynamic during oyster development, the interaction patterns could also be interpreted as a
456 consequence of the subtle effect of the pesticides on the developmental dynamics, which was

457 suggested by the significant differences in the larval size observed at days 2 and 13 post-
458 fertilization. In addition, our observation that the influence of the chemical exposure on the
459 epinephrine-induced metamorphosis rate in MCP larvae was the same in both progenies does
460 not support the presence of strong interaction mechanisms at higher biological levels. However,
461 the field survival rate presented a negative interaction pattern, because a higher mortality was
462 associated with the early exposure in spat descending from exposed parents while no significant
463 effect was measured in the control progeny being exposed. Overall, those results are in line
464 with several studies in marine invertebrates documenting the multigenerational effects of
465 environmental modifications [72], which do not report a clear orientation of the influence of
466 the parental exposure toward profitable or detrimental modifications in the offspring.
467 Investigation including more generations could help deciphering the adaptive value of these
468 modifications.

469 Another hypothesis of this study was the role of DNA methylation in mediating
470 environmentally-induced effects, which is now commonly admitted [73], [74]. First, in gastrula
471 embryos, DMRs were significantly enriched in intergenic regions (Fig. SI9), as already
472 observed in the F0 [6], although dedicated studies are needed to clarify functional consequences
473 of the methylation of non-coding regions and transposons. Second, it is remarkable that
474 pesticide-induced differential methylation followed opposite trends in the F0
475 (hypomethylation) and the F1 (hypermethylation) generations. Such 'rebound' has already been
476 observed in oysters after early microbial exposure [10], but it remains unexplained. Differential
477 DNA methylation revealed F1:F0 treatment interaction patterns, confirming its susceptibility
478 to environmental stressors [75], and the PLS analysis indicated that the overall DNA
479 methylation was the main factor discriminating the F1 conditions (*i.e.* gastrula, first latent
480 component: 66.1% variance). However, there was no direct correlation between differential
481 methylation and gene expression (only 51 genes DMG and DEGs in gastrula among 2345

482 DMGs). Such limited or absent correlations have also been observed in fish models [76], [77],
483 in Anthozoa and Hexapoda [78], and in oysters [10], [26], [79], [80] and can be interpreted in
484 different ways. First, DNA methylation is part of a wide and complex epigenetic network of
485 gene expression regulation mechanisms [29], [81]. Besides, the modification of genome-wide
486 gene expression under stress condition (Figure 4) was already observed in corals in response to
487 changing habitat [45] or in bivalve species in response to temperature stress [82], [83]. In our
488 study, the reduction of transcriptomic plasticity after pesticide exposure in MCP larvae from
489 exposed parents was associated with a global hyper-methylation trend. In this context, the
490 implications of DNA methylation changes could be considered at a higher-order organization
491 level. If in our study specific gene body differential methylation does not trigger differential
492 expression, a genome-wide hyper- or hypo-methylation could substantially modify chromatin
493 structure [84] and global transcriptional potentialities [85], [86]. This could be mediated by
494 potentially DNA methylation-dependent features like transcriptional bursts [87], [88] or
495 alternative splicing [89], [90]. However, alternative splicing was found to be a relatively scarce
496 mechanism potentially occurring in approximately 16% of oyster genes [91] (against 60% in
497 *Drosophilia melanogaster* [92]). Besides, a brief analysis of the alternative splicing events
498 existing between control and exposed lineages failed to highlight evident relation with the
499 observed differential methylation (the analysis is presented in the
500 Supplementary_Material_Variant_Splicing file). Understanding the mechanisms responsible
501 for the functional relationships between methylation changes and the complex process of gene
502 expression therefore requires further studies.

503 To our knowledge, this work is the first investigation of the multi-generational implications of
504 early exposure to an environmentally-relevant pesticide mixture in the Pacific oyster. Our
505 results revealed that an early chemical exposure can substantially affect individuals at critical
506 developmental stages and have multi-generational consequences. On the one hand, we observed

507 the F0 to F1 transmission of environmentally-induced defects (i.e. lower epinephrine-induced
508 metamorphosis rate) and, on the other hand, transcriptomic and methylomics data at gastrula
509 and MCP larva stages suggested potential adaptive or maladaptive phenomenon. This suggest
510 that low, chronic environmental contamination can have substantial adaptive consequences on
511 non-target species. These considerations are of great interest for ecotoxicology. They highlight
512 the need for long lasting and multi-generational studies on environmentally-relevant
513 concentrations of contaminants and the need to improve our understanding of how epigenetic
514 mechanisms act within gene regulation networks to efficiently integrate those new 'epimarkers'
515 in ecotoxicology frameworks.

516 **Abbreviations**

517 DEG: differentially expressed gene

518 DMG: differentially methylated gene

519 DMR: differentially methylated region

520 MC: measured concentration

521 MCP larva: metamorphosis competent pediveliger larva

522 NCSW: non-contaminated seawater

523 **Data availability**

524 RNAseq and Methylseq data are available at the European Nucleotide Archive under the
525 accession numbers: PRJEB58194 and PRJEB58545 respectively, and metadata are presented
526 in the Supplementary_Material_data_1. All non-molecular data are available in the
527 Supplementary_Material_data_2. Codes used for bioinformatic and statistical analyses are
528 available at https://gitlab.ifremer.fr/ccem-public/PESTO-project.

Supporting information

Supplementary_Material_data_1. Accesion ID of the RNAseq and Methylseq data at the European Nucleotide Archive

Supplementary_Material_data_2. Raw non-molecular data used in the study

Supplementary_Material_Figures. All the figures that were referred to as "Figure SM" in the study

Supplementary_Material_Tables. All the tables that were referred to as "Table SM" in the study

Supplementary_Material_Variant_Splicing. Analysis of the alternative splicing and its relation with the differential methylation.

Acknowledgements

We are grateful to the French National Research Agency (ANR) for funding the PESTO project (Projet-ANR-19-CE34-0004). We also thank the EPOC-LPTC platform for the analyses of organic contaminants in the experimental water, the Ifremer Marine Shellfish Platform of Bouin (PMMB) who hosted the experimentations, and Céline Reisser from the MARBEC UMR for the advices she gave abouts genetic analyses.

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Supporting Information

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Summary: 10 pages, 1 tables, 12 figures

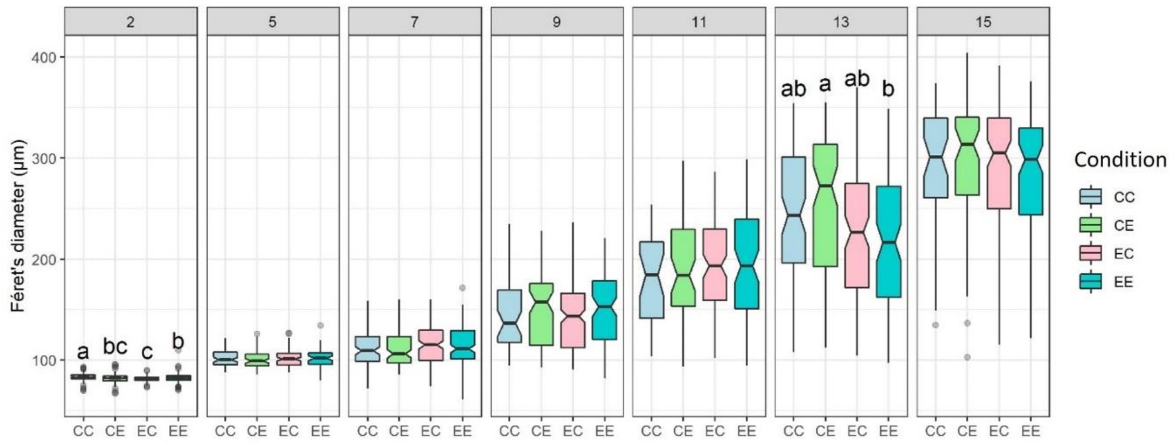

Figure SI1: Larval length (Feret's diameter, µm) from day 2 to 15 post-fertilisation. Notches represent the 95% CI. Letters refer to statistically different conditions (Kruskal's test followed by pairwise wilcoxon's test, $p < 0.05$).

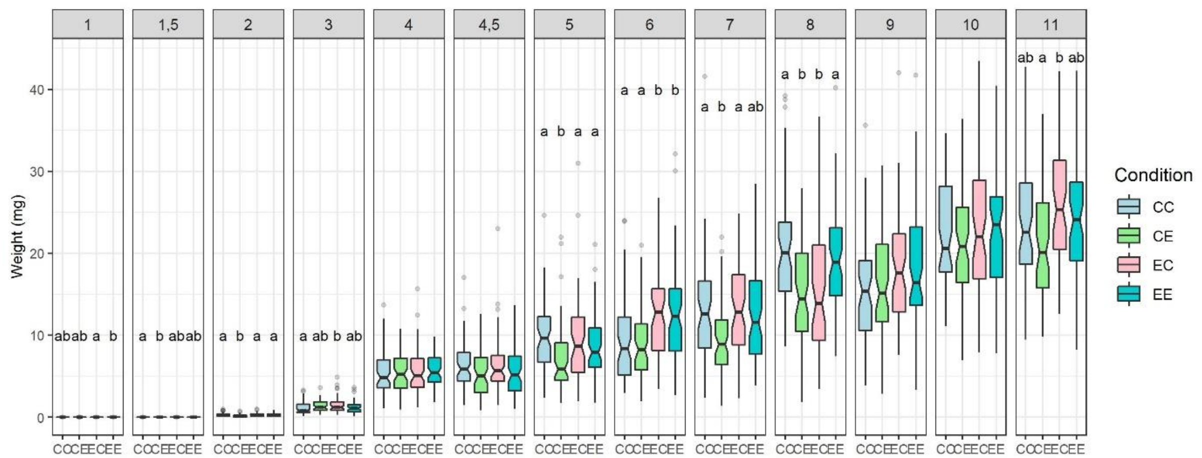

Figure SI2: Spat total weight (g) from month 1 to 11 post-fertilisation. Letters refer to statistically different conditions (Kruskal's test followed by pairwise wilcoxon's test, $p < 0.05$).

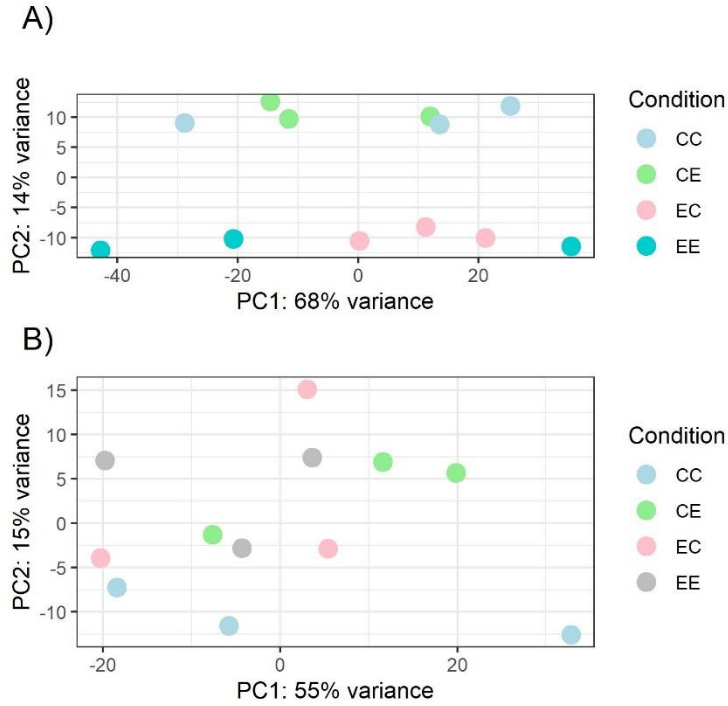

Figure SI3: Samples PCA projection on the two first components based on the gene expression counts normalized by vst *method (1000 more variables genes). A) Gastrula; B) MCP larva.*

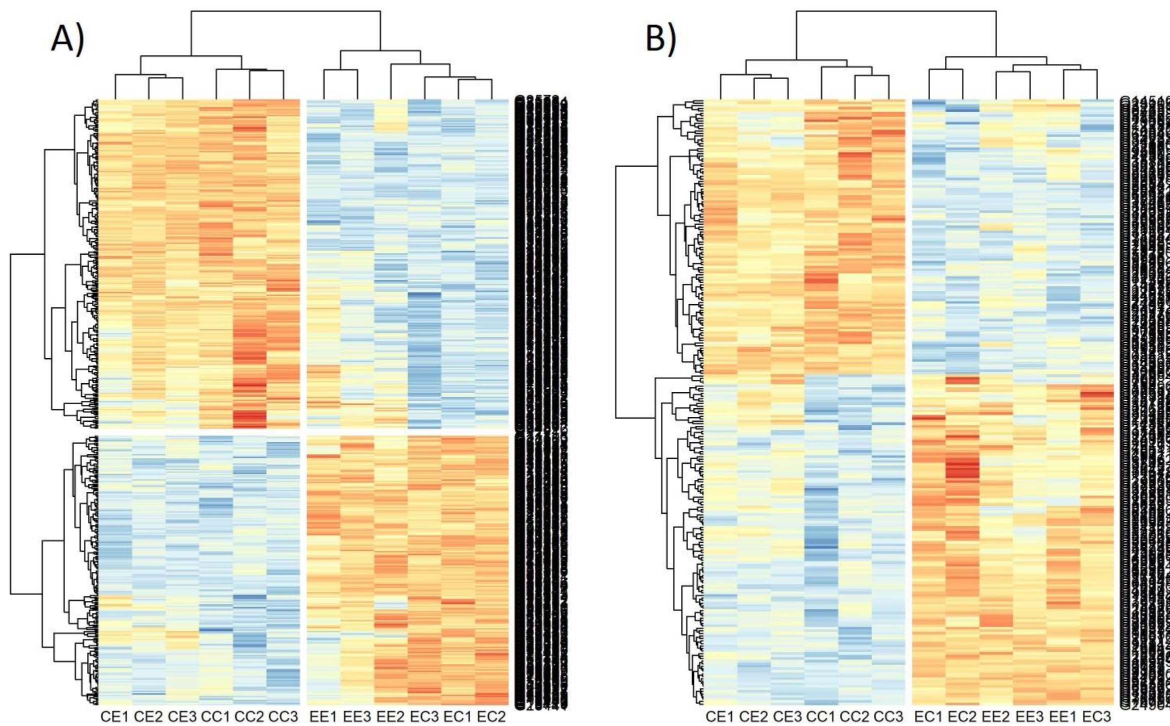

*Figure SI4: Normalized counts (*vst *method) of A) the 451 DEGs between the two experimental lineages in gastrula, B) the 241 DEGs between the two experimental lineages in MCP larva. Cols and rows are clustered based on correlation. Numbers from 1 to 3 refer to the experimental replicates for each condition.*

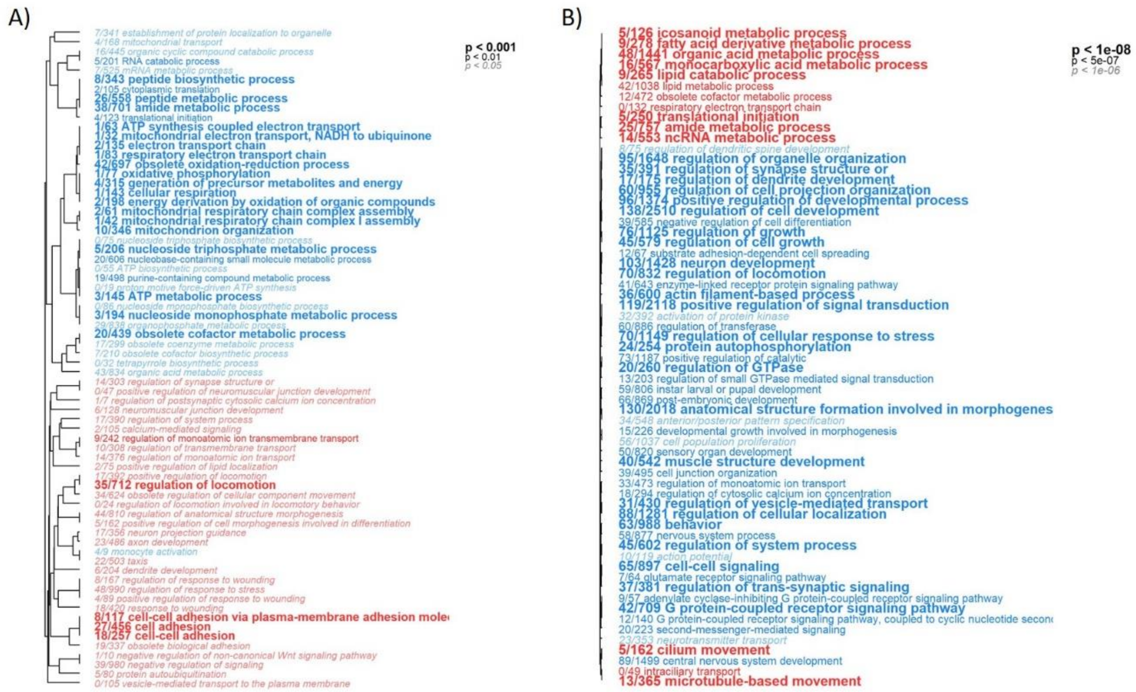

Figure SI5: Enriched biological processes (BP) in A) gastrula and B) MCP larva stages
Red refers to induced BP and blue refers to repressed BP.

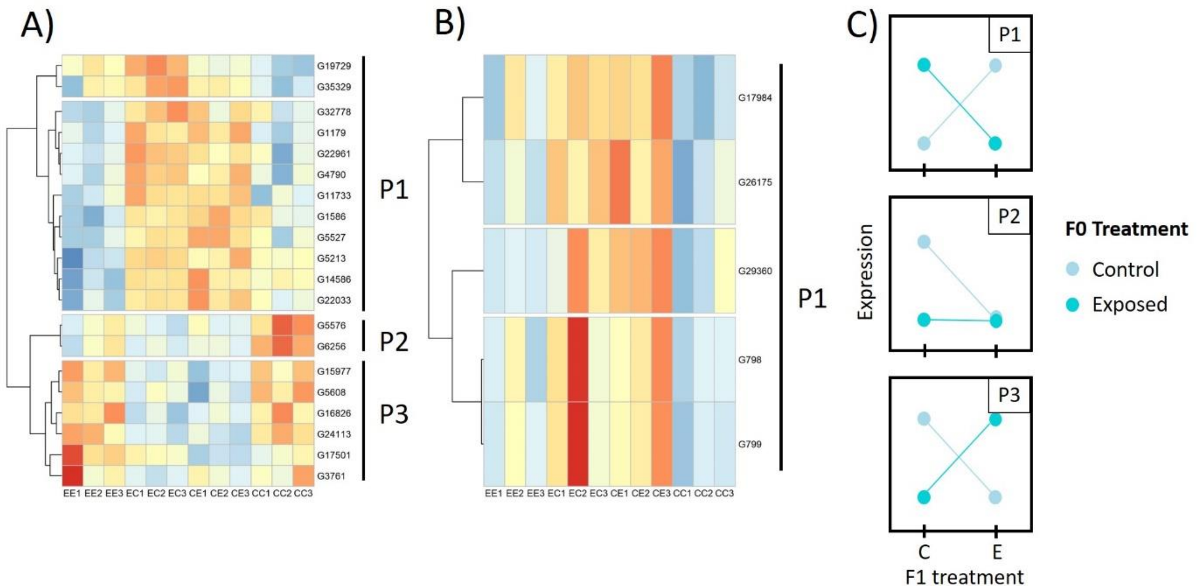

Figure SI6: Normalized counts (vst method) of the F1:F0 treatment interacting genes in A) gastrula and B) MCP larva. Rows are clustered based on correlation. Numbers from 1 to 3 refer to the experimental replicates for each condition. C) Interaction patterns. P1: Cluster 1, P2: Cluster 2, P3: Cluster 3, C: Control, E: Exposed.

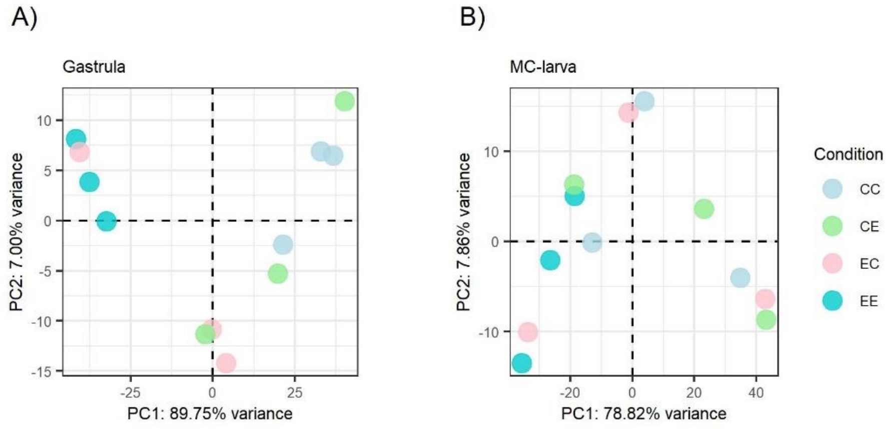

Figure SI7: Samples PCA projection on the two first components based on the tile methylation level (1000 more variable regions). A) Gastrula; B) MCP larva.

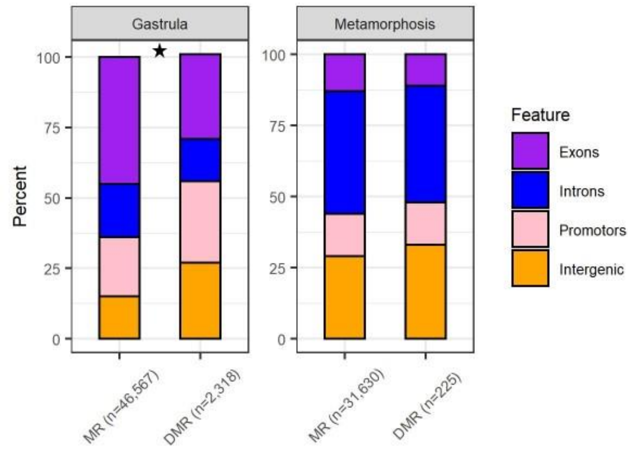

Figure SI8: Genomic features annotation of control methylation regions and between progenies DMRs at gastrula and MCP larva stages

Figure SI9: Enriched biological processes in DMGs from A) gastrula and B) MCP larva.

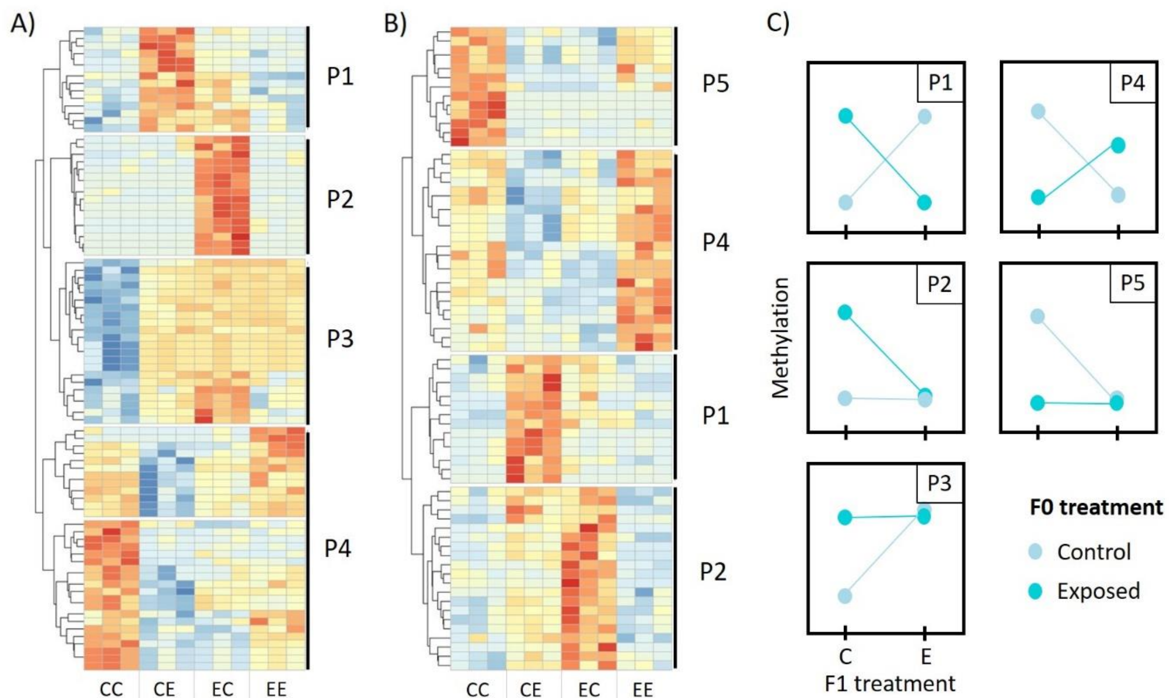

Figure SI10: Methylation level of DMR-related genes exhibiting F1:F0 treatment interaction in A) gastrula and B) MCP larva. 378 Rows are clustered based on correlation. C) Interaction patterns. P1: Pattern 1, P2: Pattern 2, P3: Pattern 3, P4: Pattern 4, 379 P5: Pattern 5 C: Control, E: Exposed.

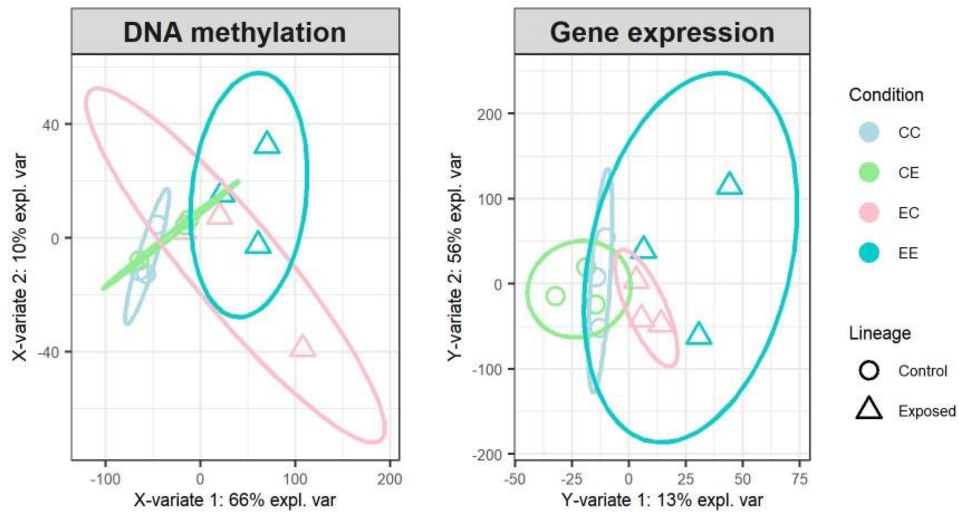

Figure SI11: Projection to latent structures analysis aggregating DNA methylation and gene expression data in gastrula embryos.

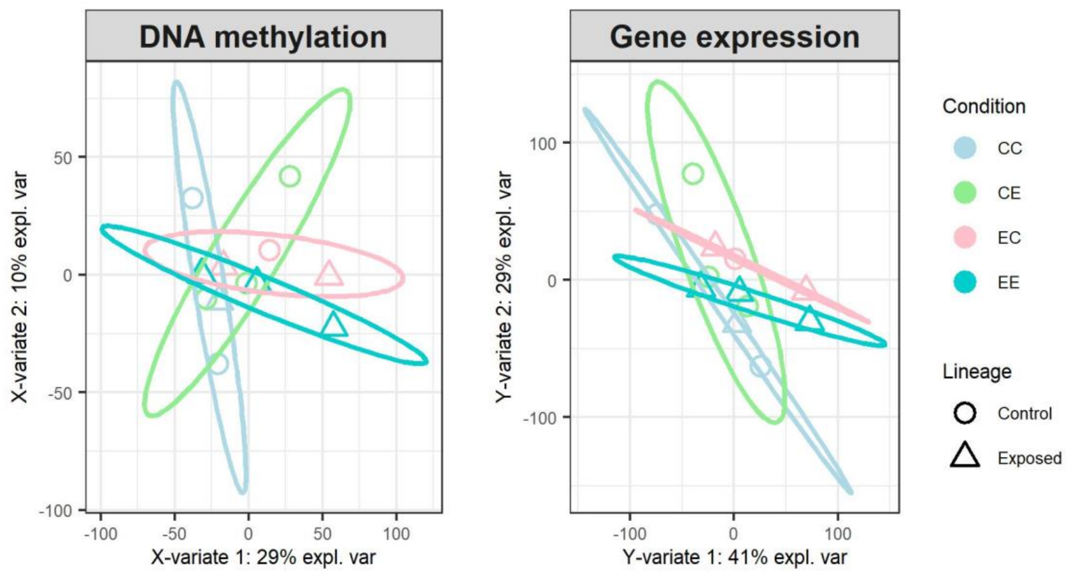

Figure SI12: Projection to latent structures analysis (PLS) aggregating DNA methylation and gene expression data in MCP larva.

Supplementary variant splicing analysis

1. Material and Methods

1.1. Prediction of alternative splicing events

The prediction of alternative splicing events between de control (CC and CE) and the exposed lineages (EC and EE) at the gastrula and MCP larva stages was performed using rMATs (rmats_v4.2.0) [1]. The analysis started from the output BAM files from STAR aligner (star_v2.7.9a) [2]. The first 'prep' and the second 'post' steps were run separately with the default parameters. The output files were split into 5 splicing event types (SE: skipped exon, RI: retained intron, MXE: mutually exclusive exon, A5SS: alternative 5' splice sites and A3SS: alternative 3' splice sites) and 2 counting methods (JC: junction reads only and JCEC: junction and exon reads). The JCEC output files were retrieved and only the significant ($p < 0.05$) splicing events were retained for the subsequent analysis. The files from each splicing event type were merged together and the number of genes implicated was counted. The gene IDs were intersected with the differentially methylated genes in gastrula and MCP larva stages.

2. Results

Overall, there was a limited number of alternative splicing events. In gastrula embryos, there were 535 significant alternative splicing events between control and exposed lineages (230 SE, 153 RI, 51 MXE, 53 A3SS, 48 A5SS) accounting for 428 genes (TabA. & Tab.SM8). In MCP larvae, 729 (334 SE, 194 RI, 78 MXE, 59 A3SS, 64 A5SS) alternative splicing events targeted 545 genes were predicted (Tab.A & Tab.SI8). Representing less than 1% of the *C. gigas* total genes (30,418 [3]). The intersect of the alternatively spliced genes and the differentially methylated genes revealed a weak physical correlation between the variation in DNA methylation and the variation in splicing. In gastrula, 42 genes were differentially methylated and exhibited alternative splicing between control and exposed lineages, accounting for 1.8%

of DMGs and 9.8% of alternatively spliced genes (ASG). In MCP larva, 10 genes were both differentially methylated and alternatively spliced, representing 3% of DMGs and 1.8% of ASG (Tab.A).

Table A: Summary of the predicted splicing events in gastrula embryos and in MCP larva, between control and exposed lineages. Only statistically significant events ($p < 0.05$) are reported. DM-AS genes = differentially methylated and alternatively spliced genes

	Gastrula	**MCP larva**
Skipped exon	230	334
Retained intron	153	194
Mutually exclusive exon	51	78
Alternative 5’ splice site	53	59
Alternative 3’ splice site	48	64
Total events	535	729
Alternatively spliced genes	428	545
DM-AS genes	42	10

Bibliography

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[3] C. Peñaloza *et al.*, “A chromosome-level genome assembly for the Pacific oyster Crassostrea gigas,” *Gigascience*, vol. 10, no. 3, pp. 1–9, 2021, doi: 10.1093/gigascience/giab020.