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# The Pacific oyster reproduction is affected by early-life exposure to environmental pesticide mixture: A multigenerational study

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**Abstract :**

Pesticides threat marine organisms worldwide. Among them, the Pacific oyster is a bivalve mollusc model in marine ecotoxicology. A large body of literature already stated on the multiple-scale effects pesticides can trigger in the Pacific oyster, throughout its life cycle and in a delayed manner. In particular, reproductive toxicity is of major concern because of its influence on population dynamics. However, past studies mostly investigated pesticide reprotoxicity as a direct effect of exposure during gametogenesis or directly on gametes and little is known about the influence of an early embryo exposure on the breed capacity. Therefore, we studied delayed and multigenerational consequences through gametogenesis features (i.e. sex ratio, glycogen content, gene expression) and reproductive success in two consecutive oyster generations (F0 and F1) exposed to an environmentally-relevant pesticide mixture (sum nominal concentration: 2.85 μg.L−1) during embryo-larval development (0–48 h post fertilization, hpf). In the first generation, glycogen content increased in exposed individuals and the expression of some gametogenesis target genes was modified. The reproductive success measured 48 hpf was higher in exposed individuals. A multigenerational influence was observed in the second generation, with feminisation, acceleration of gametogenesis processes and the sex-specific modification of glycogen metabolism in individuals from exposed parents. This study is the first to highlight the delayed effects on reproduction induced by an early exposure to pesticides, and its multigenerational implications in the Pacific oyster. It suggests that environmental pesticide contamination can have impacts on the recruitment and the dynamics of natural oyster populations exposed during their embryo-larval phase.

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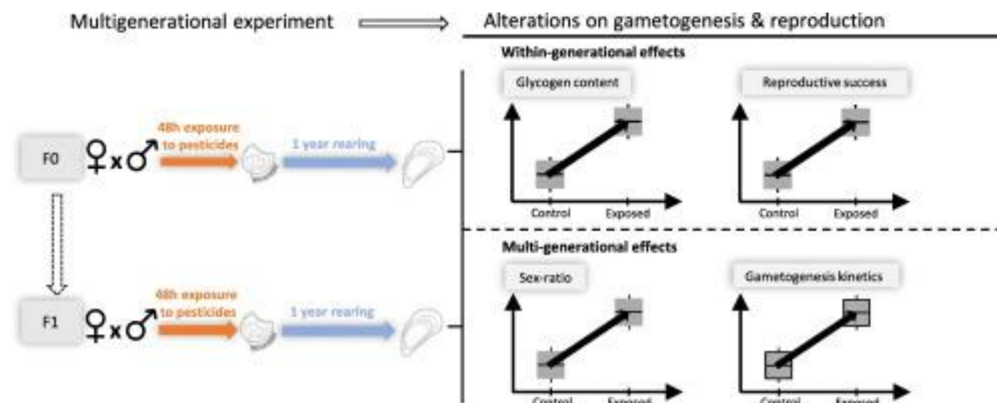
## Graphical abstract




## Highlights

► Embryo-larval pesticide exposure at environmental concentration affects gametogenesis and reproduction in F0 individuals ► A multigenerational influence was observed on sex-ratio and gametogenesis kinetics in F1 individuals ► Contamination of coastal waters is likely to threat the Pacific oyster reproduction

**Keywords** : CSW, contaminated seawater, dpf, Days post-fertilization, GOI, Gonad occupation index, hpf, Hours post-fertilization, NCSW, non-contaminated seawater

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**Abbreviations**

CSW: contaminated seawater

dpf: Days post-fertilization

GOI: Gonad occupation index

hpf: Hours post-fertilization

NCSW: non-contaminated seawater

## 1. Introduction

Despite stringent regulations, the increasing use of pesticides leads to the contamination of continental (Cruzeiro et al., 2017) and coastal waters (Munaron et al., 2020; Tapie and Budzinski, 2018) and constitutes a threat for marine organisms as assessed by a wide range of effects from molecular modifications (Caballero et al., 2023) to population-level disruptions (Moe et al., 2019; Stark and Banks, 2003).from mammals (Tsygankov et al., 2018) to invertebrate species (Brain et al., 2021; Matozzo et al., 2020).

Most of the marine invertebrates exhibit broadcast spawning (Lotterhos and Levitan, 2010) and are thus exposed to environmental stressors like pesticides from the very beginning of their life. By affecting developmental critical windows (Burggren and Mueller, 2015), such early exposures can trigger carry-over effects that may disturb life-history traits (Dupont et al., 2013; Olguín-Jacobson et al., 2021), and sometimes lead to multigenerational alterations (c.a. parental exposure affecting offspring) (Major et al., 2020; Sol Dourdin et al., 2024).

Notably, pesticides can trigger reproductive toxicity (Cao et al., 2016; Mitra & Maitra, 2018; Vidal & Pereira, 2016; C. Yang et al., 2021b), which affects the reproductive success and therefore be of critical impact at the population level (Beckerman et al., 2002; Köhler and Triebskorn, 2013). Reproductive toxicity can emerge as a direct effect of a pesticide

exposure during gametogenesis, or as a delayed effect of an earlier exposure. Direct effects were broadly described in aquatic organisms from diverse taxa (Yang et al., 2021). For instance, pesticides can induce a reduced egg production in *Danio rerio* (i.e. 21d glyphosate-exposure (T. Webster et al., 2014)), a lower proportion of gravid individuals in *Caenorhabditis elegans* (i.e. atrazine or 2,4-dichlorophenoxyacetic acid 24h exposure, (Moya et al., 2022)), an increase in vitellogenin concentration resulting in feminization in the freshwater male mussel *Elliptio complanata* (i.e. 1.5 $\mu g.L^{-1}$ atrazine exposure (Flynn et al., 2013)), or DNA damages in *Crassostrea* (i.e. *Magallana*) *gigas* spermatozoa (Akcha et al., 2012; Mai et al., 2020) as well as a lower D-larval recruitment (Barranger et al., 2014) after broodstock exposure to diuron (2 one week spikes at 0.4 $g.L^{-1}$ and 0.6 $g.L^{-1}$, respectively).

The Pacific oyster, *Crassostrea gigas*, is a widely farmed species (Hough, 2022) with appropriate biological characteristics (benthic, filter feeder, sessile) (Harris, 2008; Miossec et al., 2009) and has been widely studied as a model species in marine ecotoxicology (Gamain et al., 2020; His et al., 1999; Mottier et al., 2013).

The direct impacts of numerous pesticides on the Pacific oyster were extensively described in the context of embryo-larval exposure, highlighting a large variety of effects (*i.e.* DNA damages (Akcha et al., 2020), developmental alterations (Bringer et al., 2021), modification of developmental gene expression and modification of DNA methylation (Sussarellu et al., 2018). Studies also investigated the impacts of environmentally-relevant mixtures of pesticides, revealing their direct impacts on the oyster larval normality and mobility (24 h exposure to a 5 compounds mixture at 0.32 $\mu g.L^{-1}$) (Kuchovská et al., 2021), and on the DNA integrity (24 h exposure to a 14 compounds mixture at 0.16 $\mu g.L^{-1}$) (Mai et al., 2020). Recently, we demonstrated  in the oyster the transcriptomic, epigenetic and physiological carry over effects of an exposure to an environmental mixture of 18 pesticides (sum nominal

concentration: 2.85 µg.$L^{-1}$) during the first 48 hours of development (Sol Dourdin et al., 2023), as well as the multigenerational implication of this exposure (Sol Dourdin et al., 2024). Besides, an exposure to nonylphenol (1µg.L-1) during larval stages increased the proportion of hermaphrodite individuals in 1-year-old oysters (Nice et al., 2003), demonstrating the latent consequences of early exposures to environmental pesticide contamination on the reproductive capacity of the Pacific oyster. However, despite being of prevalent interest, such effects remain poorly investigated to date.

To address this question, we analyzed gene expression, glycogen content, sex ratio, biometric data during gametogenesis and offspring survival at 48 hours post-fertilization in 1-year-old oysters of two consecutive generations (F0 and F1), exposed to an environmentally-relevant cocktail of 18 pesticides (nominal sum concentration: 2.85 µg.$L^{-1}$) during their embryo-larval development (0 hpf – 48 hpf) (these oyster generations were produced in Sol Dourdin et al. (2024, 2023)).

## 2. Materials and Methods

### 2.1. Experimental design

#### 2.1.1. Chemical mixture and seawater chemical analysis

The chemical mixture was elaborated based on a literature review pointing out the main pesticides present in the French oyster farming areas during the reproduction season of the Pacific oyster (i.e. June – August). Overall, 18 pesticides were chosen based on their presence, their concentration, the matrix in which they were measured and their n-octanol/water partition coefficient, as presented in Sol Dourdin et al. (2023). The individual stock solutions of each compound were prepared from powders of pesticides diluted in their respective solvent (ultra-pure water or methanol). The mixture stock solution was established at x100,000 concentration.

The mixture was then inoculated in experimental tanks at an environmentally-relevant total nominal concentration of 2.85 $\mu g.L^{-1}$ (Table 1). Seawater chemical analyses were systematically performed for organic and metallic compounds at the beginning (T0 hpf) and at the end (T48 hpf) of the experimental exposure in order to validate the working concentrations *versus* the nominal concentrations. The protocols and the results were detailed in Sol Dourdin et al. [(2023)] for the F0 generation and in Sol Dourdin et al. (2024) for the F1 generation. Given the complexity of the mixture, it was considered as a whole, based on the top-down approach described in Hernández et al. (2017) for the rest of the study.

*Table 1: Compounds of the mixture and their nominal concentrations.*

| Compound | Nominal concentration ($ng.L^{-1}$) |
|---|---|
| Atrazine | 8.0 |
| Diuron | 15.0 |
| Glyphosate | 590.0 |
| AMPA | 122.0 |
| Terbuthylazine | 0.4 |
| Carbendazime | 1.4 |
| DE-Atrazine | 55.0 |
| Isoproturon | 355.0 |
| Acetochlor ESA | 13.7 |
| Acetochlor | 73.0 |
| Chlortoluron | 57.0 |
| Irgarol | 70.0 |
| Imidacloprid | 30.0 |
| Azoxystrobine | 0.4 |
| Metolachlor | 770.0 |
| Simazine | 80.0 |
| Copper | 600.0 |
| Cadmium | 13.3 |

### 2.1.2. Genealogy, broodstock conditioning, fertilization and embryo-larval exposure

In February 2021, a first oyster generation (F0) was produced from three-years-old Ifremer standardized individuals (males = 4; females = 6) and underwent (i) embryo-larval

development from 0 hpf to 48 hpf in non-contaminated seawater (NCSW: environmental seawater pumped into the bay of Bourgneuf (Bouin, France), 1 μm-filtered, UV-treated and activated carbon-filtered, with solvents methanol and milliQ water representing $5 \times 10^{-5}$ % and $9.5 \times 10^{-4}$ % of the total volume, 25°C, 32 psu, air bubbling) or (ii) embryo-larval development in contaminated seawater (CSW, 1/100,000 v/v dilution of the mixture stock solution in NCSW), at a concentration of 100 embryos.mL$^{-1}$ (50L tanks, n = 3 tanks per condition), resulting in two experimental condition : Control (C) or Exposed (E). The time of exposure (0 hpf – 48 hpf) was selected in order to focus on a critical developmental window and to benefit from the fact that larvae do not feed until 48 hpf to accurately control the level and the route of exposure. After exposure, they were all reared in NCSW from 2 dpf to 1.5 mpf, and then in UV-treated, 10 μm-filtered seawater at environmental temperature (Sol Dourdin et al., 2023) (Fig. 1A). One-year old F0 individuals from each condition were randomly selected (n=150 per condition) and underwent an 8 weeks broodstock conditioning (T0 to T8) in a flow-through system supplied with NCSW at 17°C (fed *ad libitum Skeletonema costatum* and *Isochrysis lutea*) (Fig. 1A). In March 2022, mature F0 individuals were induced to spawn by thermal shock at 30°C. Gametes from each individual (Control: males = 13; females = 6; Exposed: males = 14; females = 8) were collected separately and then pooled by sex and condition in NCSW (4L beakers). Oocytes concentration was estimated under Olympus CK40 inverted microscope (×20 magnification; 3 replicates of 10μL per condition). Spermatozoa were then inoculated into oocytes (5-10 spermatozoa per oocytes, checked under Olympus CK40 inverted microscope) to perform a per-condition fertilization in NCSW. After polar body emission (~30 min after fertilization), embryos were transferred into NCSW or CSW for 48 h (100 embryos.mL$^{-1}$, 50L tanks, n = 3 tanks per condition), resulting in four F1 conditions: Control-Control ($C_C$) and Control-Exposed ($C_E$) descending from the F0 Control condition, and Exposed-Control ($E_C$) and Exposed-Exposed ($E_E$) descending from the F0 Exposed condition (Fig. 1B) (Sol Dourdin

et al., 2024). The F1 individuals underwent the same exposure and rearing protocols than F0 individuals, until producing a F2 generation (March 2023) with three experimental conditions ($CC_C$, $EC_C$, $EE_C$) reared until 48 hpf in NCSW (100 embryos.mL$^{-1}$, 30L tanks, n = 3 tanks per condition).

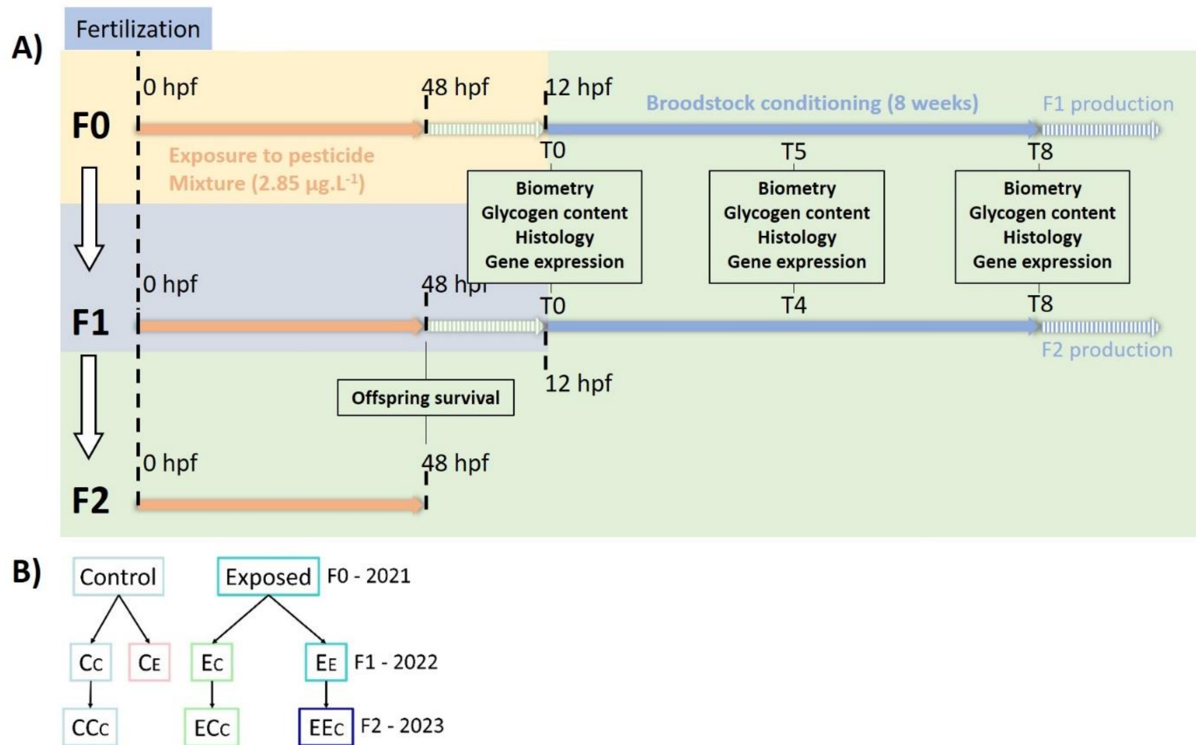


*Figure 1: A) Experimental design exposure. Orange lines represent contaminated seawater; green dotted lines represent NCSW and UV-treated, 10 µm-filtered seawater; blue line represent NCSW; T0: beginning of broodstock conditioning; T4/T5: midcourse of broodstock conditioning; T8: end of broodstock conditioning; hpf: hour post-fertilization; mpf: month post-fertilisation. Yellow background refers to the window investigated in the F0 published paper (Sol Dourdin et al., 2023). Blue background refers to the window investigated in the F1 published paper (Sol Dourdin et al., 2024). Green background refers to the window investigated in the current article. B) Genealogy of the experimental groups, from 2021 to 2023. Each group was represented by three distinct experimental tanks.*

## 2.2. Sampling protocol and analyses

### 2.2.1. Morphometric analysis and tissue samples

In the F0 and F1 generations, individuals were randomly sampled (n=30 per condition and time) at the beginning of the broodstock conditioning (T0), five weeks of conditioning for F0 (T5), four weeks of conditioning for F1 (T4), and the end of conditioning (T8). The earlier mid-term sampling in the F1 generation (4 weeks instead of 5 weeks) was chosen to anticipate a potential fast maturation as observed in the F0 generation. Shell and soft weights were measured for each individual, and the condition index was calculated using wet weights as: (soft weight/total weight) x 100. For each individual, labial palps and gonad tissue were dissected and immediately frozen in liquid nitrogen for subsequent biochemical and molecular analyses, respectively. One transverse section was cut in the gonadal mass of each individual and fixed in Davidson buffer (glycerin 10%, formaldehyde 20%, ethanol 95° 30%, sterile seawater 30%, acetic acid 10%) for 48 h, and then in 70% ethanol until sample processing for subsequent histological analysis.

### 2.2.2. Histology

Fixed transverse sections were dehydrated in a Leica TP 1020 automaton by successive baths of ethanol and butanol and embedded in paraffin wax. Several 3 µm sections were stained following the trichrome method of Prenant-Gabe (Gabe, 1968). Sex and gametogenesis stages were identified in the F0 and F1 generations as previously described by Berthelin et al. (2001): quiescent stage (0), start of gonial mitosis (I), meiosis (II) and ripe (III). The histological sections were digitized with an Olympus VS120 scanner (Olympus France, Rungis, France) equipped with a 20x objective (numerical aperture 0.80). In the F0 only, the whole slide images of oyster sections were observed and processed using the open source software Qupath (Bankhead et al., 2017). For each slide, the adjunct tissues attached to the cross-section (gills, tissue debris) were removed to harmonise the analysis between animals. In order to detect the gonadal tubules, the pixel classification method embedded in Qupath was used and applied on all images to compute the gonadal occupation index (Delgado and Pérez Camacho, 2003). This

index was used as a proxy for reproduction effort and is expressed as the percentage of the gonadal tubule occupation within the total gonadal area of the histological section.

### 2.2.3. Biochemical analysis

For each generation, glycogen content was determined in ~50 mg of freeze-dried powdered samples of labial palps (n=6 per sampling time, condition and sex). Glycogen extraction was based on the trichloroacetic acid (TCA) extraction described by Timmins-Schiffman et al. (2014). The protocol from Laurentin & Edwards (2003) was used to quantify glycogen. Briefly, 3 mL of 15% TCA were added to the powder and samples were stored 1 h at 4°C before centrifugation at 3000 g for 10 min at 4°C. Then 12 mL of absolute ethanol were added to the supernatant to precipitate glycogen through overnight incubation and gentle shaking at 4°C. Pellets were retrieved by centrifugation at 4000 g for 30 min at 4°C. Thereafter, the glycogen pellet was dissolved in 600 µl ultra-pure water in order to obtain purified glycogen in solution. Then, 40 µL of this purified glycogen 1/10 diluted were added in triplicate in a 96-well microplate, mixed gently and incubated for 15 min at 4°C. Then, 100 µL of anthrone (2 $g.L^{-1}$ in sulphuric acid) was added to each well and the plate was incubated at 92°C for 3 min. Reaction was stopped by a 5 min incubation at room temperature. The absorbance (630 nm) was then measured on a microplate reader (Safire TECAN), and purified oyster glycogen (Sigma-Aldrich, ref: G8751) was used as a standard range (from 40 to 1.25 µg). Glycogen content is expressed in mg of glycogen per g of wet weight.

### 2.2.4. Offspring survival

At the end of the 48h exposure, the offspring from the F0 and F1 generations (D larvae) reared in control conditions (offspring from F0: CC and EC; offspring from F1: CCC, ECC, EEC) were sampled in each flow-through tank (3 tanks per condition, 3 samples of 10 mL per tank), stored in 0.1% seawater-formaldehyde solution and counted under an Olympus CK40 inverted

microscope (x20 magnification) in order to determine the survival rate considered as a proxy for reproductive success.

### 2.2.5. Gene expression analysis

For the F0 and F1 generations, total RNA was extracted from freeze-dried gonad tissues (n=6 per sex, condition and sampling time). First, 40-50 mg of samples were ground in 2 mL of TRIzol (Invitrogen) using an Ultra-Turrax homogenizer (IKA©). Two consecutive chloroform-based phase separation steps were carried out. Extracted RNA was precipitated with 70% ethanol and isolated using RNeasy mini kit (Qiagen). RNA purity and concentration were assayed by UV spectrometry (ND-1000 spectrophotometer ThermoScientific, Waltham MA, USA). Samples were treated with DNase I (Ambion, Life Technologies, 0.2 U *per* µg of RNA) following manufacturer's instructions, and precipitated with isopropanol (v/v) and 0.3 M sodium acetate. RNA integrity was assessed using RNA 6000 Nano kits (Agilent Technologies) on an Agilent bioanalyzer for a mean RNA Integrity Number (RIN) of 8.7 ± 1.2. Samples were reverse transcribed using 1 µg of total RNA with iScript Advanced cDNA Kit for RT-qPCR (BIO-RAD) following the manufacturer's instructions.

The level of 10 transcripts, chosen based on a transcriptomic study in oyster gonads (Dheilly et al. 2012) were measured by RT-qPCR in the F0 and F1 generations. *Vitellogenin* (*Vitl*) and *regulator of chromosome condensation* (*Rcc1*) were used as proxies of the gametogenesis course in females, *SH3 domain-containing kinase-binding protein 1* (*SH3KBP1*) and *Binding precursor 1 repeat* (*Bp1r*) were used as proxies of the gametogenesis course in males. *Protein regulator of cytokinesis 1-like* (*Prc1*), *Bloom syndrome protein homolog* (*Blm*), *mitotic-specific cyclin-B-like* (*cyclB*), *Centromere protein F* (*CentrF*) were used as proxies of the gametogenesis course in both sexes. *Glycogen phosphorylase* (*Gly-Phos*) and *Glycogen Synthase* (*Gly-Synt*), were used to assess glycogen metabolism. Specific primers were designed using Primer3 v4.1.0 (Untergasser et al., 2012) (tab.B1 in the Appendix B file). Among 4

putative reference genes, the two displaying the lowest variation coefficient were chosen for each sex. For F0 females, two primers corresponding to *elongation factor 1 alpha* (*EF1* and *EFU*) were chosen as a reference, for F0 males, *elongation factor 1 alpha* (*EFU*) and *ADP-ribosylation factor* (*Adprf2*) were chosen as a reference. For males and females from the F1 generation, on *Adprf2* was used as reference gene. The PCR efficiency (E) was assessed for each primer pair on a sample containing cDNA from both sexes and the three sampling times, on 8 serial dilutions. Efficiencies were between 93.8% < E < 106.4%. Real-time qPCR reactions (20 µl final volume containing SsoAdvanced Universal SYBR Green Supermix (BIORAD), 500 nM of each primer and cDNA diluted to 1/40) were performed in triplicate in 96-wells microplates in a CFX Opus 96 Real-Time PCR System (BIO-RAD). Runs begun with 30 s at 95°C followed by 40 cycles of 15 s at 95°C and 20 s at 60°C. A final melting curve was performed to check for accurate amplification of the target amplicon. A no-template control was performed for each gene. Relative gene expression was normalized on a per-sex basis using the geometric mean of the reference genes with the Pfaffl formula (Pfaffl, 2001) and the specific amplification efficiencies.

### 2.3.Statistical analysis

Results are presented as the mean ± 95% confidence interval (CI) of independent replicates. Data processing and analysis was performed with R/Bioconductor (R Development Core Tem, V4.0.5) (R Core Team, 2022). Pairwise comparisons were carried out on the F0 data using Student's *t* test (X ~ Condition) after verification of data normality and homoscedasticity by Shapiro-Wilk test and Levene's test, respectively. Multiple comparisons were carried out on the F1 data using 2-way ANOVA (X ~ TreatmentF0 : TreatmentF1), followed by Tukey's post-hoc test. An ANOVA was used on the F1 offspring survival data (X ~ Condition). The performance *R* package (performance_0.10.1) (Lüdecke et al., 2021) was used to check for

residue normality and homoscedasticity. When normality was not verified, a permutation test was used as a non-parametric alternative to the 2-way ANOVA (*aovp* function from the lmPerm (V2.1.0) *R* package (Wheeler and Torchiano, 2016). Every significant 2-way ANOVA has outputs presented in the table B2 (in the Appendix B file). The $\chi^2$ test was used to compare distribution frequencies. All tests were performed considering α-threshold = 0.05.

## 3. Results

### 3.1. Histological analyses

Histological analyses were carried out in order to determine sex ratios and the progress of gametogenesis. There was no sex ratio difference between conditions in F0 and F1 ($\chi^2$, $p > 0.05$, Fig. 2 A,B). Considering F1's parental origin, there was a trend toward more females in the F1-exposed offspring ($E_C$+$E_E$) compared to the F0-control offspring ($C_C$+$C_E$) (ratio female/male: 0.5 vs 0.3, respectively; $\chi^2$: $p = 0.07$, Fig. 2B). In the F0 generation, there were overall no differences between control and exposed oysters for gonadal occupation throughout the gametogenesis (fig. A1 in the Appendix A file) (Student's test). Considering both conditions together, the gonadal occupation index increased from T0 to T5 until reaching ~ 66 %, thereafter no increase was observed (fig. A1 in the appendix A file) (ANOVA, $p < 0.0001$). In the F0 generation, two male oysters with remarkable anomalies of the gonad structure were observed. These two oysters were part of the exposed group (one at T5, Fig. A2A in the appendix A file and the other at T8, Fig. A2B in the appendix A file). One of these oysters (T8) had the smallest cutting area of the 160 oysters sampled in F0. This gonad anomaly was characterized by an anarchic development of the germline outside the gonadal tubules, within the connective tissue. These islets of germ cells were not structured in a centripetal manner as usually observed and were not surrounded by a gonadal tubule structure. An abnormal structure of the outer edge of the tubules was also noted (Fig. A2C in the appendix A file). Such

anomalies of the gonad structure were not observed in the F1 generation. In the F0 generation, maximum maturity was reached at T5 and there was no difference in the gametogenesis course between the two conditions (Fig. 2C). In the F1 generation, individuals from exposed parents were more advanced in their gametogenesis process at T4 than those from control parents ($\chi^2$, $p = 0.007$, Fig. 2D).

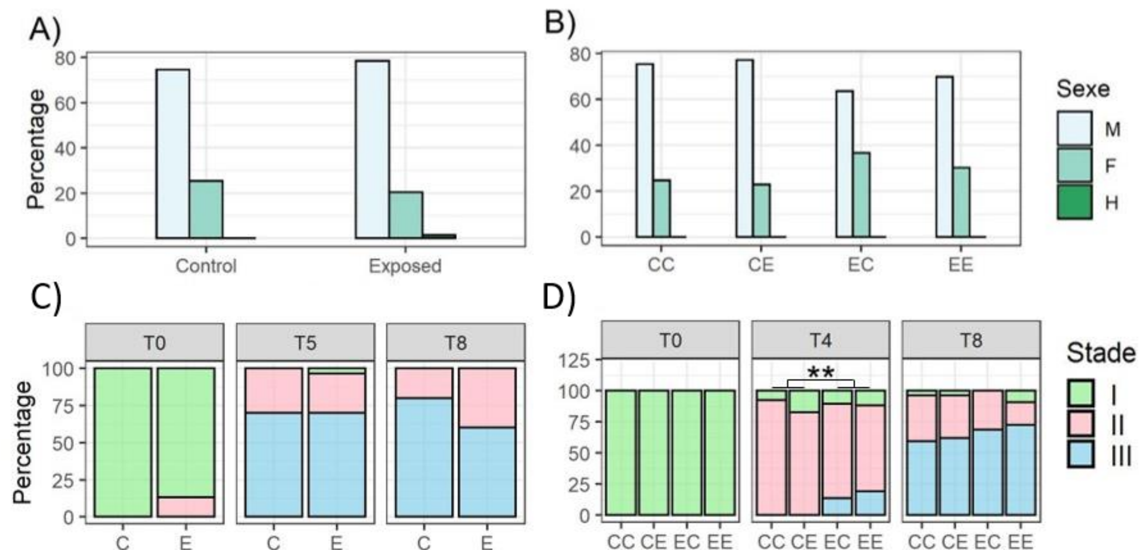


*Figure 2: Histological analyses. A) Percentage of males (M), females (F) and hermaphrodites (H) in the F0 population (N=80 per condition). B) Percentage of males (M), females (F) and hermaphrodites (H) in the F1 population. (N=90 per condition). C) F0 gametogenesis stages (N=30 per condition at T0 and T5, N=20 per condition at T8). D) F1 gametogenesis stages (N = 30 per condition and time), $\chi^2$ test, ** :* p-*value < 0.01. C: Control ; E: Exposed.*

### 3.2. Morphometric data

In the F0, control individuals were heavier than exposed individuals at T0 for soft weight (student's test: $p < 0.01$, Fig. 3A) and shell weight (student's test : $p < 0.01$, Fig. A3 in the Appendix A file), but the difference did not last at T5 and T8. At the end of the broodstock conditioning, the condition index was significantly lower in exposed than in control individuals (student's test: $p = 0.004$, Fig. 3B). In the F1 generation, the F1 treatment affected soft (Fig. 3C) and shell (Fig. A4 in the Appendix A file) weights since control individuals were heavier

than exposed ones regardless the parental origin at T4 (2-way permutation ANOVA, $p < 0.01$) and T8 (2-way permutation ANOVA, $p < 0.01$). Considering the parental origin, individuals descending from the control F0 had a lower shell weight at T8 (2-way ANOVA, $p < 0.02$, Fig. A4 in Appendix A file) and they had higher condition index at T8 (2-way ANOVA, $p < 0.001$, Fig. 3D). There is an F0:F1 treatment interaction at T8, since the condition index tended to increase upon pesticide exposure in individuals from control parents and it decreased in individuals from exposed parents (2-way ANOVA, $p < 0.05$, Fig.3D).

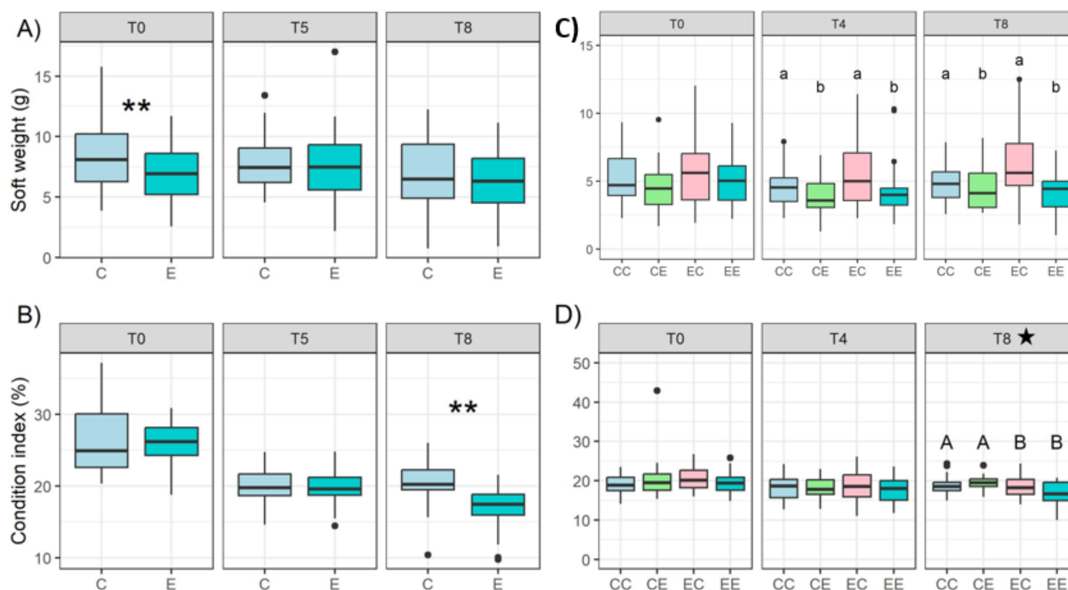


*Figure 3: Biometric monitoring. A) F0 Soft weight (g). B) F0 Condition index (N = 30 per condition at T0 and T5, N = 20 per condition at T8). Student's test.* p-*value < 0.01**. C) F1 Soft weight. 2-way permutation ANOVA. D) F1 Condition index (N = 30 per condition and time). 2-way ANOVA, upper-case letters refer to significant difference between F0 treatments, lower-case letters refer to significant difference between F1 treatments. The black star symbolises the significant interaction effect between F1 treatment and F0 treatment. C: Control; E: Exposed.*

### 3.3. Glycogen content

Glycogen content was measured in labial palps of F0 and F1 individuals during the broodstock conditioning. Regarding the F0 generation, glycogen content was significantly higher in

exposed females at T0 (142.4 ± 68 $mg.g^{-1}$, student's test, $p < 0.05$) compared to controls (59.3 ± 25.9 $mg.g^{-1}$) (Fig. 4A), and the same trend was observed for males (student's test, $p = 0.07$, Fig. 4B). In the F1 generation, there was an opposite effect of the parental treatment on the glycogen content in males, which displayed significantly less glycogen in the exposed group than in the control group at T0 (2-way permutation ANOVA, $p = 0.001$) (Fig. 4D). There was a significant F0:F1 treatment interaction (2-way permutation ANOVA, $p < 0.01$) (Fig. 4D) since the glycogen content decreased upon treatment in males from F0 control parents and it increases in males from F0 exposed parents. An opposite trend was observed for females at T0, as F0 Exposed offspring is likely to have more glycogen than the F0 Control offspring (2-way ANOVA, $p$=0.08, Fig. 4C). Whether F0 and F1 generations, no modifications in glycogen content were observed after T0.

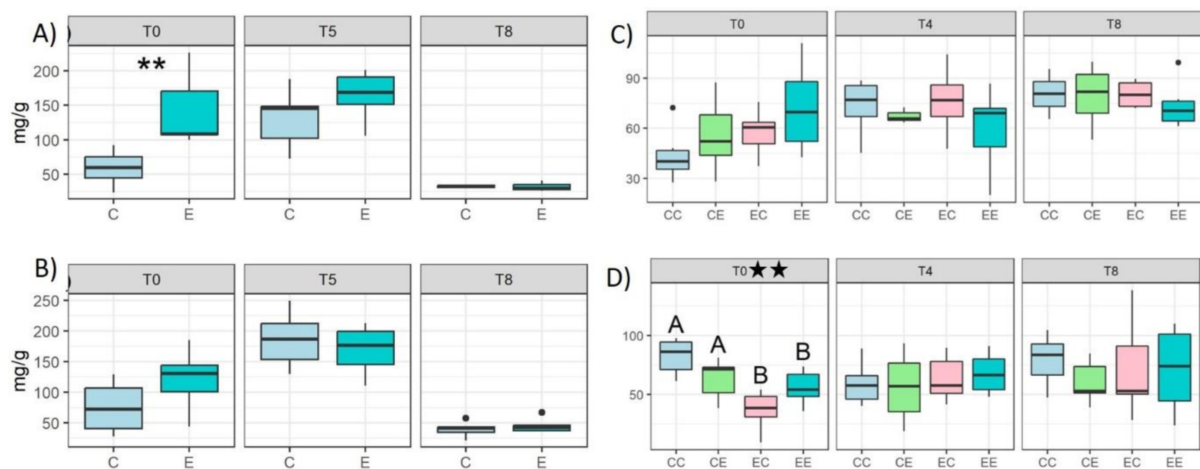


*Figure 4: Glycogen content in labial palps of A) F0 females (N = 6, 7 and 2 for Control condition at T0, T5 and T8 respectively; N = 5, 8, 3 for Exposed condition at T0, T5 and T8, respectively). B) F0 males (N = 6, 6 and 5 for Control condition at T0, T5 and T8 respectively; N = 6, 6, 6 for Exposed condition at T0, T5 and T8, respectively) Student's test. p-value < 0.01** and C) F1 females (N=6 per condition and per time). D) F1 males (N = 6 per condition and per time). 2-way permutation ANOVA (X ~ F0 treatment:F1 treatment), upper-case letters refer to significant differences between F1 treatment, black stars represent significant F0:F1 treatments interaction (p < 0.01). C: Control ; E: Exposed.*

### 3.4. Offspring survival at 48 hours post-fertilization

The reproductive success of the F0 and F1 generations was estimated by the survival rate of their respective offspring in control conditions at 48 hpf. Considering the F0 progeny, the offspring from exposed parents exhibited a higher survival rate (EC: 65.1 ± 8.1%) compared to the offspring from control parents (CC: 51.8 ± 2%, student's test: $p < 0.01$, Fig. 5A). Regarding the progeny of the F1 generation, the offspring from the EC condition had a lower survival rate compared to other conditions (ANOVA $p = 0.04$, post-hoc: Tukey's test, Fig. 5B).

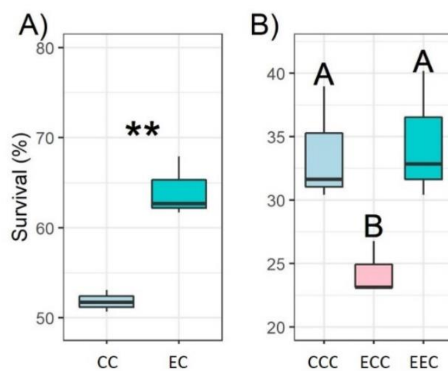


*Figure 5: Offspring survival at 48 hpf. A) F0 offspring. Student's test, p-value < 0.01**. B) F1 offspring. ANOVA, p-value = 0.04. Post-hoc test : Tukey's test. Upper-case letters represent significantly different groups. C: Control; E: Exposed. N = 3 per condition.*

### 3.5. Expression of target genes

The expression of 10 gametogenesis-related genes was measured during the broodstock conditioning in the F0 generation. They were analysed on a per-sex basis. In males, 4 genes exhibited variable expression between conditions (Fig. 6). At T0, *CentrF* and *Blm* were more expressed in non-exposed individuals while *Gly-Phos* was more expressed in exposed individuals. At T8, the expression of *CyclB* was higher in exposed males (Student's test, $p < 0.05$, Fig. 6A). In females, *Gly-Synt* was more expressed at T0 in exposed individuals (Student's test, $p < 0.05$, Fig. 6B). The expression levels of genes without significant expression level

variations are presented in Fig. A5 (in the Appendix A file). In the F1 generation, three genes were differentially expressed. In males, only *SH3KBP1* exhibited a differential expression level at T0, and *Gly-Synt* at T8, both being more expressed in the individuals from the exposed pedigree than from the control pedigree (Fig. 7A). In females, *RCC1* was more expressed at T0 in the exposed pedigree than in controls (Fig. 7B). The expression levels of genes without significant expression level variations are presented in Fig. A6 (in the Appendix A file).

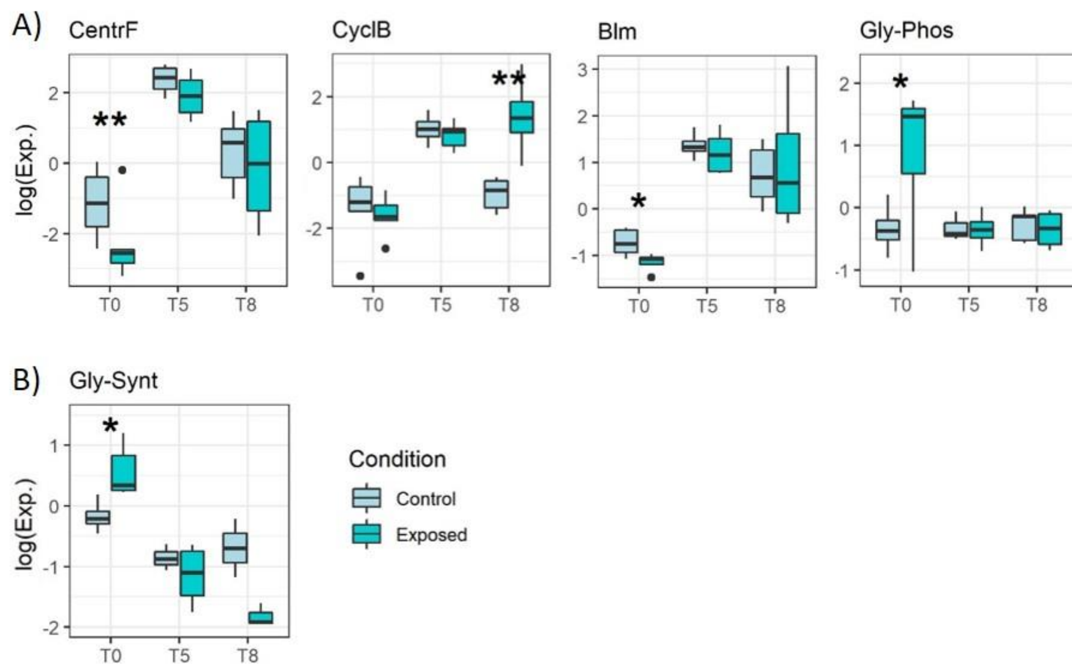


*Figure 6: Relative gene expression level (log) of target genes with significant expression level variations in A) F0 males and B) F0 females. Student's test between condition at each time.* p-*value < 0.05*;* p-*value < 0.01***.

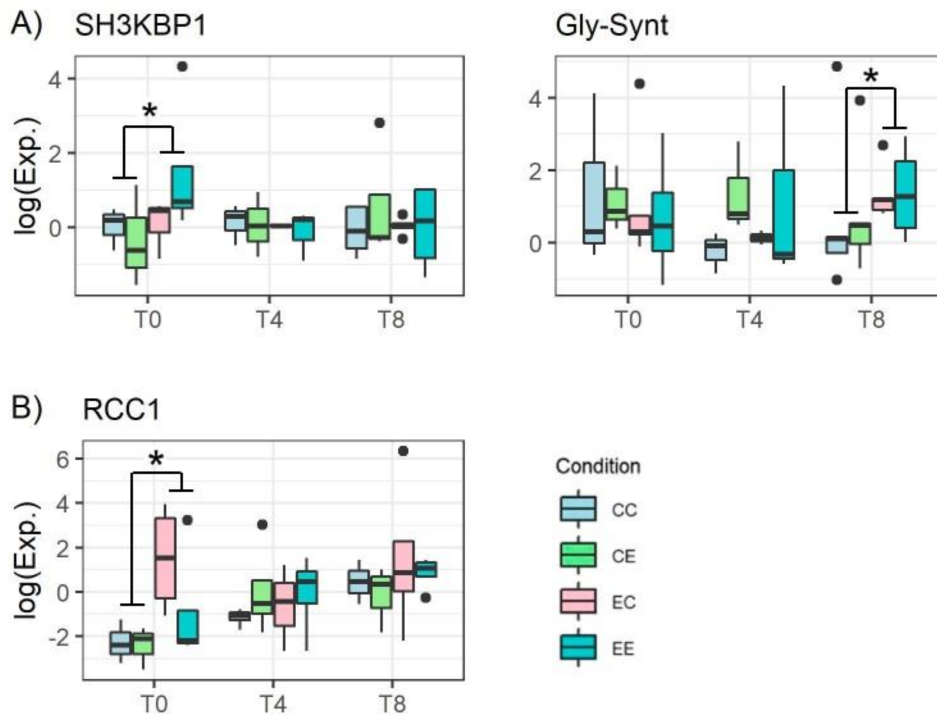


*Figure 7: Relative gene expression level (log) of target genes with significant expression level variations in A) F1 males and B) F1 females. Student's test between pedigrees. p-value < 0.05*. C: Control; E: Exposed.*

## 4. Discussion

This study aimed to investigate carry-over and multigenerational reprotoxic effects of an early exposure to an environmentally-relevant pesticide mixture in the Pacific oyster, *Crassostrea gigas*. Overall, the results showed the substantial influence of the early exposure on the reproductive process of *C. gigas* over F0 and F1 generations through diverse biological endpoints: sex-ratio, gametogenesis time-course, glycogen content in labial palps.

Although the pesticide mixture used in this study already exhibited several delayed effects (Sol Dourdin et al., 2024, 2023), the early exposure appeared to have no significant influence on the sex ratio, which was globally male-biased in both generations in 1-year-old spat, which is consistent with the protandrous hermaphroditism of the Pacific oyster (Park et al., 2012). Hermaphrodite individuals were observed in the F0 exposed condition and a trend toward

feminisation was observed in the F0-exposed offspring (Fig. 2B). Although not significant, this trend is consistent with the increased proportion of hermaphrodite specimen at the expense of males in 1-year-old oysters exposed at larval stage to nonylphenol (1 $\mu g.L^{-1}$) (Nice et al., 2003). Taken together, those results corroborate the influence of the early chemical environment on sex determination in the Pacific oyster. Besides, the modification of sex ratio, especially in offspring, has long been considered as an indicator of pesticide reproductive hazards (De Cock et al., 1995) and it is frequently observed after exposure to endocrine disruptors-like chemicals (EDs). For instance, a 3-day exposure to atrazine (22 $\mu g.L^{-1}$) increased the proportion of females in a 17-day-old zebrafish population (Suzawa and Ingraham, 2008), and an early exposure of medaka fish to an environmental concentration (0.06 $\mu g.L^{-1}$) of chlorothalonil (fungicide) or endosulfuron (herbicide) induced a delayed female-biased sex ratio in 5-year-old fishes (Teather et al., 2004). Recently, the feminization of *Daphnia magna* neonates from parents exposed to pyriproxyfen (insecticide) has been observed (Salesa et al., 2023; Watanabe et al., 2018). The environmentally relevant mixture used in our study contains several EDs pesticides like carbendazim (Jiang et al., 2015), diuron (Kamarudin et al., 2020) or acetochlor (Lu et al., 2023), at environmental concentrations, which might be responsible for the observed sex-ratio modification. However, given the lack of identified endocrine-mediated mode of action in invertebrates (Langston, 2020) and the complexity of the mixture, it is tricky to decipher the role of each compound. Therefore, while these results highlight the risk of endocrine disruption currently faced by marine organisms living in pesticide-contaminated coastal waters, further investigations are needed to precisely draw the underlying mechanisms. Nevertheless, those results indicate that investigating the sex-ratio bias in spat of the year may constitute a potential ecologically-relevant tool for assessing the endocrine hazard of coastal waters.

Reproductive investment and gametogenesis time-course were assessed *via* histological and molecular analyses. Overall, in the F0 generation, the gonadal occupation index (GOI) was

similar between the two conditions and shows a reproductive investment (GOI = 66%) consistent with the literature (Royer et al., 2008) (Fig. A1 in the Appendix A file). The GOI measurements confirmed gonadal development with a maximum GOI reached at T5. Besides, the qualitative analyses did not reveal major pesticide-induced differences in the time-course of gametogenesis in the F0 generation. Almost all individuals from both conditions reached the stage III at the middle of broodstock conditioning (T5, Fig. 4A). Moreover, the expression of gametogenesis-specific genes increased in the F0 generation from T0 to T5 (c.a. stage I to stage III) in accordance with previous studies (Dheilly et al., 2012). Even though *Blm* and *CentrF* were less expressed in exposed males and *CyclB* more expressed in exposed males at the beginning and the end of broodstock conditioning, respectively, such differences did not seem to affect the course of gametogenesis. Nevertheless, given the differences observed at T4 in histological analyses from the F1 generation we cannot exclude that similar differences exist in the F0 generation although they were missed by a late mid-term sampling. Indeed, in the F1 generation, individuals from exposed parents were ahead in the course of gametogenesis than those from control parents at T4 (Fig. 2D). This result is supported at the molecular scale by the increased expression of *Rcc1* (females) and *SH3KBP1* (males) from F0 exposed parents, both genes being increasingly expressed as gametogenesis progresses (Dheilly et al., 2012). The modification of gametogenesis time-course was frequently observed as direct effect of pesticide exposure in fishes (Lal, 2007) or bivalves like zebra mussels (Binelli et al., 2004) and clams (Greco et al., 2011). Recently, oysters exposed to glyphosate (0.1, 1 or 100 $\mu g.L^{-1}$) for 56 days during gametogenesis were slightly more advanced in gametogenesis than controls at the end of the experiment (no statistical test, Mottier et al., 2015). An acceleration of gametogenesis was also observed in oysters after a 7 day exposure to low concentrations of diuron (0.1 $\mu g.L^{-1}$) (Buisson et al., 2008). Therefore, our F1 results are consistent with previous

studies and highlight for the first time a possible multigenerational influence of an early pesticide exposure on the gametogenesis time-course in the oyster.

The glycogen concentration was measured in the labial palps, a storage tissue exhibiting similar glycogen content annual variations to the gonad (Berthelin et al., 2000) of individuals from the F0 and the F1 generation. In the Pacific oyster, energetic metabolism and gametogenesis processes importantly rely on glycogen storage and consumption, which supply maturing gonads in nutritive resources (Berthelin et al., 2000; Mathieu and Lubet, 1993). Glycogen metabolism was frequently described as being modified by environmental stressors (Ansaldo et al., 2006; David et al., 2005; Lee et al., 2023). For instance, a sublethal 7- day exposure to several organophosphorus pesticides (i.e. chlorpyrifos, diazinon) induced a decrease in glycogen content in the shrimp *Litopenaeus vannamei* (Osuna-Flores et al., 2019). The same effect was observed in the foot, mantle and hepatopancreas of the mussel *Lamellidens marginalis* after a 48 h sublethal exposure to malathion (5 ppm) (Ahamad et al., 1978). Interestingly, our results tend to show an opposite delayed effect. Indeed, higher glycogen contents were measured in exposed F0 females at the beginning of gametogenesis (Fig. 4A). This observation was further corroborated by the higher expression level of the Glycogen Synthase gene in F0 exposed females (Fig. 6B). An increased glycogen availability at the beginning of gametogenesis may allow the production of oocytes containing more energetic stocks, thereby likely to exhibit a higher reproductive success, as suggested by Boudry et al. ( 2002). Although further investigations are needed to better characterize the link between glycogen content and reproductive success in our context, this result is consistent with the better survival rate of exposed F0 offspring at 48 hours post-fertilization (Fig. 5A), suggesting a better reproductive success in early exposed oysters. In the F1 generation, the difference in glycogen content between the F0-exposed offspring and the F0-control offspring followed the same trend than in F0 (regarding females) but it was no longer significant and neither correlated to

glycogen-related genes, nor offspring survival. This suggest that the multigenerational effect of the early exposure to low pesticide concentration may not drive the glycogen metabolism in a prevalent fashion.

Interestingly, we observed tissue abnormalities in two exposed male oysters in the F0 generation at T5 and T8 suggestive of neoplasia. Their occurrence is known to be favoured by stressful conditions such as high population densities or high temperatures (Hesselman et al., 1988). In our study, neoplasia could be associated with the dysregulation of *CentrF*, *Blm* and *CyclB*, whose altered expression is known to be associated with the development of cancers (Ababou, 2021; Göbel et al., 2018; Ye et al., 2017). In contrast to other bivlaves such as *Mya arenaria* (Barber and Davis, 1994) or *Mercenaria spp* (Barry and Yevich, 1972), only few cases of gonadal neoplasia were reported in oysters (Balouet et al., 1986; Barber, 2004). Thus, this observation may be an interesting prospect regarding the development of molluscan cell lines (Balakrishnan et al., 2022).

The multigenerational implications of the early exposure to the pesticide mixture (sex-ratio, gametogenesis time-course and glycogen content) suggested by our results question their underlying mechanisms. We previously demonstrated the absence of significant genetic divergence between conditions in F0 and F1 generations, with results favouring the multigenerational influence of the early exposure implying epigenetic cues such as DNA methylation (Sol Dourdin et al., 2024). Similar mechanisms could drive the delayed parental influence highlighted in this study in the F1, however, further investigations are required to gain more insight into this issue.

## 5. Conclusion

This study is the first multi-marker investigation on potential delayed reproductive effects of an early exposure to an environmentally-relevant pesticide mixture in the Pacific oyster. Our work highlighted a possible trend toward feminisation, an accelerated gametogenesis time-course, a modified glycogen metabolism and gene expression disruptions as a result of early pesticide exposure. Taken together, our results underlay that the success of reproduction can be influenced by low pesticide concentrations. The environmental pesticide contamination mimicked in this study has delayed reproductive effects, hence represents a sublethal stress potentially able to trigger population-level damages in an ecologically-relevant model species. Future studies envisioning the whole life cycle and focusing on ecologically-relevant endpoints would be required to better understand the potential impacts of environmental xenobiotics on organism fitness and would therefore be an asset towards an individual-to-population unified knowledge.

## Data availability

Data are available upon request.


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