Divergent selection for cytogenetic quality in mussel species

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Highlights

- *Mytilus edulis* and *M. galloprovincialis* had similar cytogenetic quality in controlled condition
- Low realized heritability for cytogenetic quality for both species

Abstract

France is one of the major mussel producers in Europe with two commercially important species *Mytilus edulis*, and *M. galloprovincialis*. Since 2014, French mussel farms have been vulnerable to Abnormal Mussel Mortality (AMM) with mortality rates ranging from 30 to 100%, and varying spatially and temporally. One of the proposed factors influencing mortality is the haemocyte cytogenetic quality. The purpose of this study was to estimate the response to selection of cytogenetic quality trait using a divergent selection (DS) approach for the two important mussel species cultivated in France, and explore the relationship between the cytogenetic quality and survival using a cohabitation protocol with mussels sampled in a AMM site. In January 2022, wild mussels were sampled in Agnas and in Biarritz representing *M. edulis* and *M. galloprovincialis*, respectively. Their cytogenetic quality was measured using haemolymph by flow cytometry, and a DS was applied with 1.40 intensity of selection to produce contrasted groups (high/low) as well as control group in May/June 2022. The offspring cytogenetic quality was measured in June 2023. The response to selection after one generation of mass selection on the liability scale was 1.44% for *M. edulis* and null for *M. galloprovincialis*. Mortality of high, low and control groups for each species was recorded using a cohabitation experiment from March-October 2023. In October 2023, high mortality was recorded for hatchery-produced mussels (77%), with no significant difference between species, as well as within species between the high, low and control groups. Furthermore, a non-significant from zero phenotypic correlation was observed between cytogenetic quality and survival of offspring. Nevertheless, further investigations are required to validate the genetic basis of the cytogenetic quality of the mussel species cultivated in France.

Keywords: *Mytilus* spp, cytogenetic quality, Realized heritability, Mortality.

1. Introduction

Global mussel production has reached 2.1 million tons in 2020, valued at approximately 4.5 billion USD (FAO, 2022). Aquaculture is by far the primary source of mussels and is responsible for over 90% of total landings (FAO, 2022). France is one of the major mussel producers in Europe with a production value of 61,375 tons for 160 million USD in 2020 (FAO, 2022) with mainly two commercially important species *Mytilus edulis* and *M. galloprovincialis* as well as their hybrids. Most importantly, the French mussel production entirely depends on the wild spat collection (Prou and Goulletquer, 2002).

Recurrent mass mortality outbreaks of bivalves reduce production, cause economic losses, and negatively impact the ecosystem of natural bivalve populations as well as terrestrial food web (Bódis et al., 2014; Soon and Ransangan, 2019). Mass mortality of various cultured mussels have been reported worldwide such as in blue mussels (Avdelas et al., 2021; Lupo et al., 2021), green-lipped mussels (Ericson et al., 2023), and pheasant shell (Putnam et al., 2023), and their occurrence seems to increase in the context of global warming. Since 2014, French mussels farms have been vulnerable to Abnormal Mussel Mortality (AMM) and based on sites/seasons/years the mortality rate fluctuate among years from 30 to 100% (Lupo and Prou, 2016; Normand et al., 2022; Polsenaere et al., 2017) but the peak of mortality outbreaks occurs during spring (Charles et al., 2020a; Degremont et al., 2019). Various investigations are going on to find out the cause(s) of the AMM outbreaks in France and propose solutions to reduce the mass mortalities in mussel

farms/wild stocks. Until now, the etiology of AMM outbreaks remains unclear, but it could be linked to environmental pollutions, seawater characteristics, mussel characteristics, culture practices, and climate change (Lupo et al., 2021; Polsenaere et al., 2017). Pathogens could also be involved in mortality outbreaks as the bacteria *V. splendidus* (Ben Cheikh et al., 2016; Lupo and Prou, 2016; Oden et al., 2016; Polsenaere et al., 2017) and *Francisella halioticida* (Bouras et al., 2023; Charles et al., 2020b), but their role in AMM are still unclear (Benabdelmouna et al., 2018a; Charles et al., 2020b). However, horizontal transmission of one or several putative causal agents were observed from wild stocks that survived to AMM to wild naive stocks, as well as to hatchery-produced lines (Benabdelmouna et al., 2018a). Interestingly, mussel stocks showed significantly different levels of resistance such as *M. edulis* was found more susceptible than *M. galloprovincialis* at the spat stage under laboratory condition (Benabdelmouna et al., 2018a) and at the adult stage in AMM site (Ajithkumar et al., 2024).

Furthermore, in the larger context of the recurring mortality crises decimating shellfish populations in France since 2008, previous studies using ploidy analysis through flow cytometry (FCM) in different bivalve populations (Pacific oysters and blue mussels) have shown that cytogenetic quality trait could be considered as a significant factor of morbidity (and even mortality), as it reveals a significant positive association between the initial cytogenetic quality and the final mortality levels. Thus, it could be considered as an interesting indicator of the health status of an animal as its responds to various biotic and abiotic stresses (Benabdelmouna and Ledu, 2016; Fleury et al., 2023; Normand et al., 2022). In the context of AMM affecting mussel stocks, mussel mortalities were linked to a possible physiological weakening of mussels in relation to a reduction of their cytogenetic quality (Benabdelmouna and Ledu, 2016). The FCM is

a powerful tool to differentiate a good quality individual from poor quality based on their cytogenetic quality (Benabdelmouna et al., 2018b).

The potential for genetic improvement through mass selection is well documented in many bivalve species during the past decades, particularly due to their short generation intervals and their high reproductive capacity allowing the possibility of applying high selection pressures (Gjedrem and Rye, 2018; Tan et al., 2020) and limiting the loss of genetic diversity in breeding populations (Chen et al., 2022). This method is commonly used with positive results in particular for growth, survival and disease resistance traits across various mollusc species, including oysters, mussels and abalone (Degremont et al., 2019; Degremont et al., 2015; Hu et al., 2022; Liu et al., 2015). Divergent mass selection (DS) is defined as selection that acts on two contrasting directions (Hill, 1972). DS scheme is simpler and less expensive than family-based selection, because only two selected groups are produced to assess the breeding potential of desired traits.

The purpose of our study was to estimate the response to selection of cytogenetic quality trait using a divergent selection approach in the two commercially important mussel species (*M. edulis* and *M. galloprovincialis*) cultivated in France. In addition, we explored the relationship between the cytogenetic quality and the resistance to mortality using a cohabitation protocol with donor mussels sampled in site regularly impacted by AMM.

2. Materials and Methods

2.1 Base population and broodstock conditioning

In January 2022, 600 wild mussels were sampled from Agnas (45°87'07'' N and -1°17'67'' W), and another 600 wild mussels were sampled from Biarritz (43°28'20'' N and 1°34'35'' W) (Figure 1). As per Simon et al. (2020), and more lately in Ajithkumar et al. (2024) who sampled at the same area in both sites, the mussel stock from Agnas was identified as pure *M. edulis*, while it was pure *M. galloprovincialis* for the mussel stock from Biarritz. Both stocks were transferred to the experimental hatchery at IFREMER, La Tremblade (45°79'81'' N, 1°15'01'' W) (Figure 1). The length of the mussels ranged from 4 to 6 cm. Upon arrival, the mussels underwent a month-long acclimatization period in separate tanks (one tank per species), using a flow-through system with unheated and UV-filtered seawater at a rate of 250 L per hour. During this period, a consistent food supply (*Isochrysis galbana*, *Tetraselmis suecica*, and *Skeletonema costatum*) was provided to favour the gametogenesis of the mussels and the temperature was deliberately maintained below 10°C to prevent any unintended spawning events.

2.2 Parental selection and Flow cytometry analysis

For each species, the cytogenetic quality of parental population was determined from the haemolymph of 420 mussels to determine their frequency distribution before selection in March/April 2022.

To collect haemolymph, the mussels were anesthetized by using magnesium chloride ($MgCl_2$) at a concentration of 50 g L^{-1}. Once the mussels opened their valves, a sterile 1 ml syringe equipped with a 26-gauge needle was used to gently puncture the adductor muscle. Approximately 0.05 to 0.1 ml of haemolymph was collected from each mussel and preserved in a 1.5 ml Eppendorf microcentrifuge tube containing 1

ml of nuclei extraction buffer solution (5 mM $MgCl_2$, 85 mM NaCl, 10 mM Tris, 0.1% Triton X100, pH 7), while being kept on ice to prevent cell clumping. The nuclei were extracted using the action of detergent (Triton X-100) and facilitated by successive pipetting. To eliminate membranes and larger clumps, the extracted nuclei underwent purification via filtration using a 30 µm nylon sieve (Celltrics, Sysmex). Samples were then simultaneously treated with DNase-free RNase A (Sigma R4875) and propidium iodide (PI, Sigma, P4170) at a concentration of 50 µg ml^{-1} each in a 2 ml final solution. Then, the sample tubes were left at room temperature in a dark environment for a duration ranging from 30 minutes to 2 hours for the staining process before initiating the analysis using FCM.

Flow cytometry analysis was conducted using a Partec PA II flow cytometer equipped with a 590 nm, 30 mW green laser (Sysmex, Sainte Geneviève des Bois, France) to determine the ploidy level of cells. Laser light was utilized to assess nuclei quality. PI fluorescence, which correlates with the DNA content of each nucleus, was detected using the FL3 detector (orange-red fluorescence detector at 550–600 nm). A total of approximately 5000 nuclei were counted per sample under low flow rate conditions (15 µl min^{-1}). Cell-cycle estimations were carried out using the method described by Benabdelmouna and Ledu (2016) for the removal of doublets and debris. To differentiate nuclei in the G2/M phase from doublets of G0/G1 nuclei that share the same DNA content, FL3-area vs. FL3-width dot-plots were employed to isolate single nuclei. A specific region (R1) was defined on these dot-plot representations to distinguish single nuclei from doublets. Once isolated within R1, single nuclei were plotted on a FL3-area histogram with a linear scale of 2048 channels. The use of Triton X-100 is known to remove cell membranes and cytoplasm, leaving only bare nuclei and resulting in distinct DNA peaks. Furthermore, filtration of nuclei through a 30 µm

nylon sieve effectively eliminated clumps and doublets. This was evident when applying FL3-width vs. FL3-area dot plots, which indicated a low occurrence of doublets and debris. This data was used to calculate the percentage of nuclei populations based on their DNA content. Manual peak determination, as described in Delaporte et al. (2008), involved placing specific markers to estimate the percentages of normal diploid G0/G1 nuclei (RN1 gate) and non-diploid nuclei in both the S, tetraploid G2/M and beyond stages (RN2 gate).

2.3 Divergent selection

After analysing the cytogenetic quality of parental population from the two species, a divergent selection was applied to produce low and high selected lines. The selection intensity applied was ±1.40 standard deviation units from the population mean (Falconer and Mackay, 1996). This involved selecting 20% of the population (80 individuals) from each extreme direction to produce two distinct groups of genitors: a first group of low cytogenetic quality genitors (LCQ) from the lowest cytogenetic quality mussels, and a second group of high cytogenetic quality genitors (HCQ) from the highest cytogenetic quality mussels. In addition, a control group was produced from 50 first spawned individuals (25 males and 25 females) from each species before applying selection (Supplementary Table 1).

2.4 Spawning, larval rearing, and grow-out culture

Mussels were induced to spawn in May/June 2022. For each species and each group (HCQ, Control, LCQ), 404/388 mussels (Control) and 80 mussels (HCQ and LCQ) were individually placed in a 400 ml beaker, and spawning was triggered

alternating cold (10°C) and warm seawater (20°C) (Table 1). Depending on the ripeness of the mussels, five to eight replicate spawns per group were produced for each species (Table 2), involving two to six females and three to twelve males (Supplementary Table 1). In total, 36 replicate spawns were produced.

For each replicate spawn, embryos were transferred to a cylindrical tank of 30 L filled with filtered and UV-treated seawater at 20°C. Seawater was changed three times per week, and larvae were fed daily with a mixture of algae (*I. galbana, Chaetoceros gracilis, S. costatum*). Larval density was established at 10 larvae ml^{-1} on day 1 post-fertilization and then progressively reduced to 5 and 3 larvae ml^{-1} on days 7 and 14 post-fertilization, respectively. At the pediveliger stage, the larvae were then transferred into 150-µm sieve-bottomed trays in 120 L tank to accomplish their metamorphosis using flow-through UV-treated seawater enriched with a mixture of four algal species routinely produced at the hatchery (*I. galbana, C. gracilis, T. suecica, and S. costatum*). Each 120 L tank contained seven replicate spawns. The sieves were washed daily and changed regularly depending on spat growth. When spat reached 1 cm, 250 spat from each replicate spawn were transferred into 1000 µm sieve-bottomed tray in a vertical nursery system. Each tray with a 12 L capacity ensured optimal growth conditions by regulating temperature and salinity. During this step, flow-through UV-treated seawater was enriched with *S. costatum, I. galbana,* and *C. gracilis*.

2.5 Progeny cytogenetic quality analysis

Once the progenies reached an appropriate size (minimum = 2.5 g) for haemolymph collection, thirty to forty mussels of each replicate spawn, were randomly sampled (Table 2) and underwent screening for subsequent FCM analysis as

described in section 2.3 (Supplementary Table 2). However, some of the replicate spawn, which were smaller in size (< 2.5 g) were found to be contaminated with gametes in June 2023, and were re-phenotyped with different individuals in August 2023.The individual length (mm) and total weight (g) of each mussel were recorded in addition to assessing cytogenetic quality.

2.6 Estimation of genetic parameters of cytogenetic quality trait

The cytogenetic quality trait is defined as the ratio of normal diploid cells to the total cells (diploid + aneu-polyploid), measured in percentage. The response to selection is the difference in cytogenetic quality between the HCQ and LCQ groups (Hill, 1972). The selection differential is the difference in cytogenetic quality between the selected parents from HCQ and LCQ groups (Hill, 1972). The realized heritability (h^2) of each mussel species after one generation of mass selection for the cytogenetic quality trait was estimated using the regression of individual responses on individual selection differentials (Hill, 1972). The control replicates produced for each species could clarify whether or not selection responses from contrasted lines were symmetric in both lines (Roff, 1997). The selection response for unidirectional selection was measured by comparing selected group (HCQ/LCQ) versus control group, and selection differential is the mean liability of the selected parents in the previous generation as it deviates from their population mean liability, given the intensity of selection (i) as reported in Falconer and Mackay (1996).

2.7 Cohabitation experiment

In March 2023, a cohabitation protocol was set up involving experimental groups (HCQ, LCQ and control) produced in hatchery and donor mussels. The donor mussels were collected from Maison Blanche (46°99'72'' N, -2°20'18'' W) where AMM frequently occurs (Figure 1) (Ajithkumar et al., 2024). Two conditions were tested: treatment versus control. For the treatment condition, three 150 L flow-through tanks were used with a renewal rate of 250 L per hour of unheated and UV-filtered seawater. In each tank, 30 mussels from each replicate spawn and 30 donors were tested for the mortality estimation using small boxes in a tray to keep them separated (Figure 2). The initial total weight of all 30 mussels was recorded for each replicate spawn. In addition, 600 donors (mean weight = 4.2 g, total weight approximately 2.5 kg) were placed at the water intake (Figure 2). Two types of control conditions were followed, both using unheated and UV-filtered seawater. For the first control condition, 90 mussels per replicate spawn were reared without donors in the bottomed tray of a vertical nursery system. For the second condition, after measuring the cytogenetic quality of offspring in June, each replicate spawn was placed in a 150 L flow-through tank. Each week, the tanks were cleaned with freshwater, and mortality counts were performed until the end of the experiment in October 2023. Seawater temperature was recorded hourly using two probes (Progesplus, 59,780, Willems, France).

To identify parasitic pathogens potentially associated with mortality event, mussels from both control and treatment conditions were examined for histopathological studies. 37 moribund mussels (21 *M. galloprovincialis* and 16 *M. edulis*) from the treatment condition and 20 healthy mussels (10 *M. galloprovincialis* and 10 *M. edulis*) from the control condition were sampled during the peak mortality months of July and August. For mussel dissection, shells were opened by cutting the adductor muscle. An approximately 5 mm thick transverse section of mussel tissue

containing digestive tract, gonads, mantle, gills, adductor muscle and foot was excised and fixed into Davidson's fixative for 48h before preserved in 70% ethanol until dehydration. Subsequently, they were embedded in paraffin using standard protocols for histology. Paraffin blocks were sectioned into 2-3 µm sections, followed by staining with haematoxylin and eosin. Histological examination was performed under light microscopy.

2.8 Statistical Analysis

2.8.1 Cytogenetic quality

Parental cytogenetic quality (diploid = 1 and non-diploid = 0) was analysed using a binomial logistic regression with the Glimmix procedure in SAS® 9.4 software according to the following model:

$$\text{Logit}(Y_i) = \mu + \text{species}_i + \text{edv}$$

where Y_i is the probability of cytogenetic quality of mussel in the i^{th} species (*M. edulis* and *M. galloprovincialis*), μ is the intercept and edv is the residual term.

For the progenies, the model was:

$$\text{Logit}(Y_{ijk}) = \mu + \text{species}_i + \text{group}_j + \text{dos}_k + \text{bw} + \text{species}_i \times \text{group}_j + \text{replicate spawn(species)} + \text{edv}$$

where Y_{ijk} is the probability of cytogenetic quality in the i^{th} species (*M. edulis* and *M. galloprovincialis*) from the j^{th} group (HCQ, Control and HCQ) spawned in k^{th} month (May and June), bw is the individual body weight, replicate spawn nested within species used as a random effect.

All factors were fixed except replicate spawn. When a significant interaction was observed, the SLICE option was used allowing a more powerful analysis than rerun the model for each effect as the degrees of freedom are not reduced (Littell et al., 2002).

2.8.2 Mortality analysis

The final cumulative mortality in the treatment condition of the cohabitation experiment was assessed at endpoint of the study using a binomial logistic regression model with the Glimmix procedure in SAS® 9.4 software. The same model as above for the progenies was used, except that the total weight of each replicate spawn as fixed effect was added instead of individual body weight.

Notably, the model was subsequently refined for species-specific analysis. A simple model was performed to test the effect of origin in the mortality outbreak (donors versus hatchery produced mussels).

2.8.3 Correlation analysis

Phenotypic correlations (r) between traits were estimated based on the Pearson correlation between traits using the proc corr in SAS. Significance level was set to $p < 0.001$. The phenotypic correlation between the two traits was calculated as:

$$r\,(x, y) = \frac{\mathrm{Cov}(x,y)}{\sqrt{\mathrm{Var}\ x}\sqrt{\mathrm{Var}\ y}}$$

where Cov(x,y) was the covariance of two traits, Var x and Var y was the variance of trait x and trait y, respectively.

2.8.4 Histological analysis

Prevalence of each pathological condition in two groups were estimated as number of affected mussels * 100/total number of mussels in the sample (Fuentes et al., 2002).

3. Results

3.1 Cytogenetic quality of mussels

3.1.1 Cytogenetic quality of the parents

A total of 420 mussels per species were examined using FCM techniques. Among them, 404 individuals of *M. edulis* and 388 of *M. galloprovincialis* yielded data with clearly distinguishable sample peaks, meeting rigorous standards for acceptability, i.e. with no gametes nor cellular debris (Table 1). The percentage of diploid cells in individuals from parental population before applying selection was significantly higher in *M. galloprovincialis* (91%) than in *M. edulis* (88%) ($p < 0.01$) (Figure 3). The mean proportions of diploid cells for the parents selected to produce the LCQ and HCQ groups were 73%, and 95% for *M. edulis*, and 78%, and 98% for *M. galloprovincialis* (Table 1). Further details about the selected parents of each replicate spawn are provided in Supplementary Table 1.

3.1.2 Cytogenetic quality of the offspring

Approximately 30-40 mussels per replicate spawns were examined per group utilizing FCM techniques. Among them, individuals yielded data with clearly distinguishable sample peaks, meeting rigorous standards for acceptability were used for further analysis. The mean percentage of diploid cells (%) for LCQ, Control, and HCQ were 83%, 80%, and 85% for *M. edulis*, and 86%, 80%, and 86% for *M.*

galloprovincialis, respectively (Figure 4). The mean percentage of diploid cells (%) for each replicate spawn per group per species is shown in Supplementary Table 2. A non-significant interaction was found between group and species (p = 0.98). No significant difference for cytogenetic quality was observed between offspring groups derived from HCQ and LCQ progenitors in both mussel species (p = 0.81; p = 0.99) and both were not significantly different from control (Supplementary Table 3). The mean diploid cells (%) of *M. galloprovincialis* (84%) were slightly higher than *M. edulis* (83%), but no significant difference was found between species for offspring cytogenetic quality (p = 0.85).

3.2 Genetic parameters

The response to selection after one generation of selection on the liability scale was 1.44% for *M. edulis* and null for *M. galloprovincialis* (Table 3). The realized heritability using a divergent selection approach to improve or decrease the cytogenetic quality was 0.10 ± 0.32 for *M. edulis* and -0.14 ± 0.18 for *M. galloprovincialis* (Table 3). Due to similar cytogenetic quality for the HCQ and LCQ groups, and a lower value for the control group, realized heritability were much higher for both species in upward directions (0.45; 0.79), while negative values were obtained in downwards (-0.08; -0.45) (Supplementary Tables 4 and 5). The response to selection after one generation selection, comparing control versus selected groups for cytogenetic quality trait was 5.19%, 3.75% for *M. edulis* and 5.97%, 6.05% for *M. galloprovincialis* (Supplementary Tables 4 and 5).

3.3 Cohabitation experiment/ Mortality analysis

In the cohabitation experiment, temperature was recorded throughout the experimental period, and it exhibited a range from 13.1 to 25.9°C (Figure 5). Notably, the mean seawater temperature exhibited a gradual rise from 14.4 to 21°C between the months of March and June 2023. Following this warming trend, seawater temperature fluctuated around 22°C until October.

In the first control condition, all groups (HCQ, LCQ, and control) for both species exhibited low mortality throughout the trial, reaching 6% mortality at endpoint. For the second control condition, after collecting the haemolymph, the mortality was less than 10% (Supplementary Table 6). In contrast, much higher mortality was observed for the cohabitation condition with donors reaching 77% at the endpoint (Figure 5). Mortality began during the first week of April and occurred until the end of the trial in October. The origin of the mussel had significant effect on mortality and higher mortality for the wild donors (95%) in comparison with hatchery produced mussels (77%) ($p < 0.01$). Cumulative mortality for *M. galloprovincialis* was low in April (1%), and regularly increased from May (11%), to July (34%), to rise up to 63% by August and 76% at endpoint. Likewise, for *M. edulis*, no mortality was observed until April, and then cumulative mortality started low in May (3%), and June (11%), but showed an upward trend during the summer in July (30%), doubling by August to 65% to reach 77% at endpoint. Notably, *M. galloprovincialis* (22%) exhibited significantly higher mortality than *M. edulis* (11%) in the treatment condition in June, period when mussels are normally harvested by farmers in most of the sites ($p < 0.01$). In October, no significant difference was found between species for mortality, with 77% and 76% mortality for *M. edulis* and *M. galloprovincialis*, respectively ($p = 0.42$) (Figure 5). When focus on contrasted groups selected for their cytogenetic quality, the highest percentage of mean mortality was observed in offspring produced from LCQ genitors (81%), followed

by offspring produced from HCQ genitors (78%) and lowest in control (71%) in *M. edulis* (Figure 6) and similar trend followed in *M. galloprovincialis* at the endpoint (Figure 6). No significant different in mortality was found among groups in both species (p = 0.38 - 0.99; p = 0.87 - 0.95). There was no significant interaction observed between species and group concerning mortality (p = 0.70).

A non-significant from zero negative correlation was found between the cytogenetic quality of offspring and their survival at endpoint for *M. edulis* (r = -0.38 and p = 0.12) and *M. galloprovincialis* (r = -0.34 and p = 0.23) (Figure 7). A low significant positive correlation was observed between cytogenetic quality and weight for *M. edulis* (r = 0.2; p < 0.01) and *M. galloprovincialis* (r = 0.11; p < 0.01). Similar pattern was observed between cytogenetic quality and length. As expected, strong significant positive correlations were observed between length and weight for both mussel species (r > 0.92; p < 0.01).

Histological analyses conducted during the cohabitation experiment revealed the absence of known protozoan and metazoan pathogens that induce mortality in mussels, such as trematodes or the protozoan *Marteilia pararefringens.* Similarly, no regulated parasites (*Marteilia sp., Perkinsus sp*., *Bonamia sp*., and *Mikrocytos sp* parasites) were noted. Undetermined ciliates were observed along the gills and mantle in treatment condition for both mussel species but they were absent in mussels from control condition (Table 4). Various tissue lesions were observed in mussels from the treatment condition, including haemocyte infiltration and necrosis of the connective tissue in different organs (Table 4). The main lesions observed were severe necrosis of muscular fibres, connective tissues of the mantle and gills and the epithelia of digestive diverticula essentially in animals from treatment tanks (100% of detection). The epithelia of digestive diverticula appeared vacuolised with cell desquamation and

lysis evident; in comparison, the digestive duct presents few lesions (Figure 8A and B). Additionally, severe necrosis of muscular fibres was observed often associated with bacteria proliferation (Figure 8C). Other bacterial foci were located in connective tissues of the mantle (Figure 8D). Haemocyte infiltration was also noted and mainly concerning the connective tissues of mantle and digestive gland. In some cases, granuloma was also observed but only in mussels from the treatment condition; they were found in the connective tissue of digestive gland and mantle (Figure 8E); their detection was limited with 10% of individuals infected.

4. Discussion

It is importance to improve the survival of commercially important mussels when they are facing a massive mortality outbreak for sustainable development of the industry as well as ecological niche. One approach to overcome mortality issue is to adopt a selective breeding program to improve the survival of the mussels. In many aquaculture species, challenge experiment under controlled environment has been proven to be an effective method to improve the disease resistance against a known pathogen (Odegard et al., 2011). However, the cause(s) of abnormal mussel mortality in France is/are more complex and still unknown. Consequently, applying the selection of resistant populations through challenge-based methods is problematic. In the context of mass mortality outbreaks affecting mussels in France, cytogenetic quality has been identified as one of the potential factors as it showed highly significant positive correlation with the survival of the mussels in wild environmental conditions (Benabdelmouna and Ledu, 2016).

Pure crosses of the two commercially important mussel species farmed in France, *M. edulis* and *M. galloprovincialis* were used to investigate the cytogenetic quality. The overall objective of the present study was to evaluate the response to selection/realised heritability to increase or decrease the cytogenetic quality in mussels for each species. Additionally, a cohabitation experiment using donors (wild cultivated mussels sampled in a AMM site) and recipient mussels (hatchery-produced mussels) was conducted to identify the relationship between cytogenetic quality and mortality.

4.1 Cytogenetic quality of mussels

In our study, we analysed the cytogenetic quality using haemolymph as the target tissue. It's worth noting that the cytogenetic quality of mussels in haemocytes may vary in different tissues, such as gills or gonads. Indeed, it was demonstrated in Pacific oysters that genomic abnormalities were detected in haemocytes and also in spermatozoa and hence could be transferred to offspring, leading to negative effects on development. For example, a high percentage of aneuploid cells in embryos could lead to higher mortality in the larval stage and reduced the larval growth in the Pacific oyster (Barranger et al., 2014).

The percentage of diploid nuclei in each individual mussel provides a nuanced perspective on the variation in cytogenetic quality within the parental population, offering insights into their underlying genetic makeup. In parental population, the higher mean proportion of diploid cells in haemolymph was observed in *M. galloprovincialis* (91%) compared to *M. edulis* (88%), which may be due to various factors, such as sampling sites, age groups, previous occurrence of mortality outbreaks, or better environmental conditions (replicate their good health status) for mussel culture. In a

previous study, Benabdelmouna and Ledu (2016) reported that diploid cell percentages in French mussel stocks (*Mytilus spp*) showed extended variation ranging from 79% to 98% prior to mortality events. Burioli et al. (2019) also reported a wide range of variation in diploid cell percentages in the haemolymph of mussel stocks (*Mytilus spp*) from five sites along the French coast between September 2017 and June 2018. These findings imply that mussel populations in France exhibited diverse cytogenetic profiles before experiencing mortality outbreaks.

For the progenies of each mussel species, our analysis revealed that there were no statistically significant differences for cytogenetic quality between the selected groups (HCQ/LCQ). Additionally, no significant difference was observed between the selected groups and the control group, with consistent results as the both selected groups showed a better cytogenetic quality than controls for both species. Regardless of the cytogenetic quality of the parents (HCQ/Control/LCQ group), all the corresponding offspring from replicate spawns could be considered to have LCQ status as defined by Benabdelmouna et al. (2018b) that suggested that the threshold value of non-diploid nuclei in the haemolymph should be lowered to 5% to delimit HCQ mussels from LCQ mussels. In our study, most of the replicate spawns exhibited genomic abnormalities (GA%) more than 10% at the time of FCM analysis (Supplementary Table S2). It could be assumed that these genomic abnormalities caused by several stress-related factors could significantly contribute to the morbidity and mortality of mussels cultured in France (Benabdelmouna et al., 2018b; Fleury et al., 2023). Therefore, all these mussels can also be considered as highly vulnerable to the mortality outbreaks as they have lower diploid cells (Elston et al., 1990).

Our study revealed significant differences in the cytogenetic quality between the two mussel species collected in different areas. However, there were no significant

differences among their offspring, which were produced in the same location under identical hatchery conditions. This suggests that the cultivation in analogous environmental conditions may lead to an identical cytogenetic status in mussels, regardless of the geographical origin, age, or life-history trait of the parental population. This finding also suggest that cytogenetic quality trait is highly influenced by environmental factors and is an integrative trait that results from the interaction between the molluscs and their varying exposome. These results were consistent with previous reports on other bivalves indicating that environmental factors play a major role influencing the cytogenetic quality of the species (Barranger et al., 2014; da Silva et al., 2018; Delaporte et al., 2008; Morgan et al., 2012).

4.2 Response to selection

The realized heritability for cytogenetic quality is a pivotal metric in understanding the genetic underpinnings of this trait and assessing the potential for selective breeding to improve overall health status and consequently the survival potential. This is crucial for both cultured and wild stocks of *M. edulis* and *M. galloprovincialis*, which are suffering from heavy mortality outbreaks in France. To the best of our knowledge, our study is the first to report findings on the response to selection for cytogenetic quality in mussels or in any bivalves. In the case of *M. edulis*, the realized heritability for cytogenetic quality was low (Table 3). In contrast, the realized heritability for *M. galloprovincialis* was negative. This result indicates that the response to selection for cytogenetic quality was not consistent for the two mussel species, despite variations found in the parental population. The low or null response to selection could be attributed to the low intensity of selection applied for the parents (20% for each selected group) or especially in short term selection experiments (one

generation) where stochasticity of response can be highly inconsistent due to genetic variation, environmental variation, population size, complex traits or other factors (Roff, 1997). Although multiple replicate spawns were used in our study to enhance the precision of estimating the response to selection (Aggrey et al., 2003; Roff, 1997), the heritability estimates gave strong reason to doubt the adequacy of the liability model (Falconer and Mackay, 1996). Consequently, several generations of mass selection could be necessary to monitor the genetic variation in the successive generations to ensure the accuracy of the estimation.

To date, there is no available report that allows for a direct comparison of our results with those of other bivalve species. Indeed, most of the heritabilities estimated in bivalve species have been reported for growth parameters (Guo et al., 2018; He et al., 2008; Li et al., 2011; Nguyen et al., 2014), shell nacre colour (Bai et al., 2017), mantle colour (Pino-Querido et al., 2015), toxin accumulation (Pino-Querido et al., 2015), calcification (Kingston et al., 2018), and survival (Degremont et al., 2007; Degremont et al., 2019; Mallet et al., 1986).

Overall, the limited genetic influence on cytogenetic quality trait was observed in both mussel species underscores the complexity of this trait. Consequently, selective breeding programs aimed to enhancing cytogenetic quality in mussel populations may face considerable challenges and necessitate a deeper understanding of the underlying genetic factors. Further exploration and research are essential to unravel the genetic architecture of this trait in mussel species and to develop effective strategies for selective breeding aimed at improving overall health status.

4.3 Mortality analysis

A lower mortality was observed for the control condition (<8%), while higher mortality was observed for all groups in the cohabitation experiment (77%). This suggests that horizontal transmission occurred from donors originated from AMM infected sites to hatchery-produced mussels, as previously observed by Benabdelmouna et al. (2018a). In France, there have been recent occurrences of different mussel mortality outbreaks, notably in spring (AMM outbreaks) (Charles et al., 2020a; Degremont et al., 2019) and in summer (Seuront et al., 2019). Our study revealed a distinct mortality patterns between species from March to June with higher mortality for *M. galloprovincialis*, while it was similar between July and September. Regarding the first period of mortality occurring between March and June, similar pattern and mortality rates were observed when testing different mussel species in the intertidal area in Charente Maritime (Ajithkumar et al., 2024). It differed from AMM outbreaks in the field where *M. edulis* exhibited higher susceptibility compared to *M. galloprovincialis* (Ajithkumar et al., 2024). We hypothesized that the cause of mortality observed in our study might differ from AMM outbreaks in the field emphasizing species-specific responses to the experimental conditions. Additionally, the peak mortality occurred during July-September, it suggests the possibility of summer mortality.

Weak species by group interaction (based on cytogenetic quality) at endpoint in October 2023 suggesting that group had no impact on mortality for both species, irrespective of the cytogenetic quality of genitors (Figure 6). At the endpoint of our experiment, no species-specific response was observed on mortality rate, both exhibiting similar mortality (76-77%) (Figure 5). As experiment progressed especially during long time, both species were susceptible to this unknown mortality outbreaks

observed in the cohabitation experiment. These results indicate that the progenies of mussels sampled from Agnas and Biarritz had absence of local adaptation and no prior exposure with this particular mortality outbreak. Overall, this result suggests that multiple factors involved in the mortality outbreaks as noted for mass mortality episodes of the blue mussels (*Mytilus spp*) reported in France (Lupo et al., 2021; Polsenaere et al., 2017). Furthermore, in a controlled condition the Agnas stock in our study and in a previous study in 2015 (Benabdelmouna et al., 2018a) observed less than 10% mortality, it suggests that the Agnas stock has not encountered any AMM outbreaks since 2014 and will probably be highly susceptible to such mortality outbreaks in the future.

A non-significant from zero phenotypic correlation was observed between cytogenetic quality and survival for *M. edulis* and *M. galloprovincialis*, respectively (Figure 7). Correlation results from both species implying that there was no substantial association between cytogenetic quality and mortality, while previous studies emphasized strong significant positive association between genetic abnormalities and mussel mortality (Benabdelmouna and Ledu, 2016; Benabdelmouna et al., 2018b). This result is not surprising, as our study correlated cytogenetic quality and mortality from siblings, while previous studies used the cytogenetic quality and mortality from the individual mussel. It's important to note that the cytogenetic quality of siblings may not accurately represent the true value of the mussels tested in the cohabitation experiment, particularly when there is low genetic variation. Most importantly, cytogenetic quality of the offspring might yield different results if the replicate spawns would have been tested in sites experiencing AMM outbreaks. The diploid cells (%) of all replicate spawns were below 90% and according to Benabdelmouna and Ledu (2016), all replicate spawns were in LCQ status and vulnerable to the mortality

outbreaks. In general, low cytogenetic quality mussels significantly contribute to morbidity, with influence from the other factors like temperature, pathogens lead to the mortality (Benabdelmouna et al., 2018a). Further experimentation would be necessary to unravel the precise mechanisms behind the observed correlations between cytogenetic quality and survival in mussel species.

In our study, no parasites were found in the moribund mussels from the treatment condition. Histopathological analysis revealed similar lesions in all mussels in the treatment condition, suggesting that the cause of mortality was likely identical. However, the exact cause of mortality remains unknown, as observed in previous studies (Charles et al., 2020a; Villalba et al., 2001). Since 2015, several studies highlighted the presence of numerous inflammatory granulomas in French mussels affected by mortalities in the Atlantic coasts without identifying any cause (Charles et al., 2020a; Pépin et al., 2018; Pépin et al., 2017; Travers et al., 2016), and recent mass mortality of mussels in Netherlands (Capelle et al., 2021). In our study, some mussels presented granulomas but their detection frequency was low. The main observed lesion was characterized by degeneration and necrosis of the digestive diverticula epithelia, which may be indicative of digestive epithelial virosis (DEV). DEV is suggestive of a viral etiology, disrupting digestive processes and contributing to high mortality rates (Rolton et al., 2023). However, it's also plausible that DEV represents a natural cycle in mollusk digestive diverticula, coinciding incidentally with observed morbidity and mass mortalities due to physiological stress (Rolton et al., 2023). Our study suggests that this mortality outbreak may be caused by an infectious agent potentially a virus or bacteria. Furthermore, the use of metagenomic analyses could provide a valuable insight into the potential existence of viruses in the mortality outbreak. In the previous investigations, the gram-negative bacteria *Vibrio splendidus*

and *Francisella halioticida* were detected on mussels in sites experiencing AMM outbreaks in France but their role in the mortality outbreaks still unclear (Ben Cheikh et al., 2016; Bouras et al., 2023; Charles et al., 2020b). Additionally, further testing should be conducted on selected lines in challenge tests and at field test sites concurrently for identifying potential pathogens in order to establish the role of a specific pathogen in AMM outbreaks.

5. Conclusion

To the best of our knowledge, our study is the first to report findings on the response to selection for cytogenetic quality trait in mussel as well as in bivalves. The low response to selection was observed (1.44%) for cytogenetic quality trait in *M. edulis*. A non-significant from zero phenotypic correlation was observed between cytogenetic quality and survival for each mussel species, indicating that selection for cytogenetic quality would not result in any responses in survival. Understanding the interplay between cytogenetic quality and survival can have broader implications for the study of shellfish diseases and conservation efforts. Our study revealed that cytogenetic quality is influenced by multiple factors and limited availability of genetic variation. Further studies needed to validate the genetic variation of cytogenetic quality in mussel species.

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Author contributions

Munusamy Ajithkumar: Methodology, Data curation, Formal analysis, Writing - original draft, Writing - review & editing. **Lionel Dégremont:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing. **Céline Garcia**: Methodology, Writing - review & editing. **Christophe Ledu:** Methodology. **Abdellah Benabdelmouna:** Conceptualization, Methodology, Supervision, Writing - review & editing.

Data availability

Data will be made available upon reasonable request.

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