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Does the environmental history of mussels have an effect on the physiological response to additional stress under experimental conditions?

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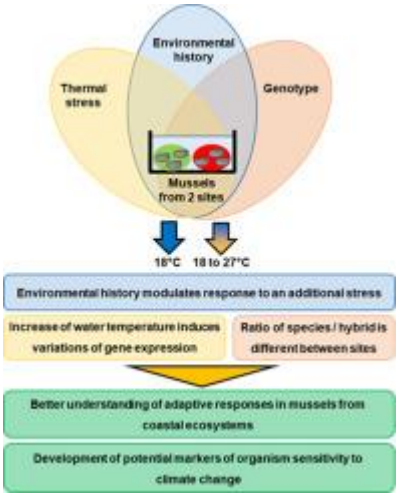
Abstract :

Expected effects on marine biota of the ongoing elevation of water temperature and high latitudes is of major concern when considering the reliability of coastal ecosystem production. To compare the capacity of coastal organisms to cope with a temperature increase depending on their environmental history, responses of adult blue mussels (Mytilus spp.) taken from two sites differentially exposed to chemical pollution were investigated during an experimental exposure to a thermal stress. Immune parameters were notably altered by extreme warming and transcriptional changes for a broad selection of genes were associated to the temperature increase following a two-step response pattern. Site-specific responses suggested an influence of environmental history and support the possibility of a genetic basis in the physiological response. However no meaningful difference was detected between the response of hybrids and M galloprovincialis. This study brings new information about the capacity of to cope with the ongoing elevation of water temperature in these coastal ecosystems.

Keywords : Environmental history, Mussel, Thermal stress, Transcriptomic response, Trade off, Genotype

Graphical abstract



Highlights

► Identifying the role of environmental history in the response to temperature increase ► Environmental history modulated response to an additional stress and genotype proportion. ► Increase of temperature induced variations of gene expression and immunomodulation.

Keywords : Environmental history, Mussel, Thermal stress, Transcriptomic response, Trade off, Genotype

1. Introduction

In the intertidal zone and coastal ecosystems, marine species are often exposed to steep environmental gradients and periods of stressful conditions (Helmuth and Hoffmann, 2001). Among these environmental factors, temperature is one of the most selective because it affects several levels of organization in the ecosystem. Environmental temperature can impact the survival and metabolism of organisms but also their geographical distribution (Monaco and McQuaid, 2019; Collins et al., 2020). Numerous studies showed the effects of a temperature increase due to global warming on the physiology of marine organisms already subjected to anthropogenic effluents (Lannig et al., 2006, Lacroix et al., 2015; Marigomez et al., 2017; Mlouka et al., 2019; Pereira et al., 2020; Clark et al., 2021). Other studies tend to show that exposure to a stressor prior to an experiment leads to a faster stress response and better resilience of organisms (Clegg et al., 1998; Pereira et al., 2020). Indeed, the increase in seawater temperature leads to a greater sensitivity of organisms to pollutants whose toxicity and bioavailability are then modified (Lannig et al., 2006; Sokolova and Lannig, 2008; Izagirre et al., 2014; Marigomez et al., 2017). Conversely, the presence of pollutants in seawater as well as the effect of other environmental biotic and abiotic factors influence the capacity of organisms to respond to heat stress, leading to metabolic adjustments due to the high energy demand of cellular protection mechanisms (Sokolova, 2013; Mlouka et al., 2019). However, organisms living in contaminated environments may present adaptations that allow them to improve their survival in the face of chronic stress by adjusting (1) their energy reserves according to the availability and/or quality of food, particularly in eutrophicated environments (Carmichael et al., 2012; Marigomez et al., 2017), (2) their behaviour in presence of competitors (Olabarria et al., 2017) or (3) by tolerance acquisition towards stressors (Johnston, 2011, Milan et al., 2016). This tolerance can be accompanied or not by a

decrease in fitness, and lead to co-tolerance over some other stressors, and/or a decrease of tolerance towards other stressors.

Along the European coasts, mussels of the genus *Mytilus* are one of the most important marine bivalves at both economic and ecologic point of view. They colonized a wide range of environments from estuary to open coastal ecosystems, and consequently challenged a large variety of fluctuating parameters such as salinity, temperature, tide, acidification or anthropic effluents. Three species of mussels *M. galloprovincialis, M. trossulus* and *M. edulis* are identified from Mediterranean Sea to North Sea, with hybrid zones located in Spain and around Brittany (Bierne et al., 2003, Dias et al., 2008; Kijewski et al., 2011; Hilbish et al., 2012). The two main species were limited in their expansion respectively by cold temperature for *M. galloprovincialis* (<9°C) and warm temperature for *M. edulis* (>20°C) (Fly et al., 2015), *M. trossulus* being absent from the French coasts. Although these organisms are intertidal and therefore potentially exposed to high temperatures during emersion, mussels are highly resistant to extreme air temperature (Clark et al., 2021), but very sensitive to changes in water temperature because they perform most of their metabolic activities while submerged (Jones et al., 2009). They are frequently used as model species in biomonitoring programs (Bodin et al., 2004; Auffret et al., 2006; Lacroix et al., 2014) and laboratory exposure studies (Matozzo et al., 2012; Marigomez et al., 2017; Collins et al., 2020; Clark et al., 2021).

The present study aimed to investigate how wild mussels exposed to various levels of chronic chemical contamination and therefore having distinctive environmental history may respond to an increase of temperature under experimental conditions. For that purpose, mussels of the genus *Mytilus* collected at two sites of the Bay of Brest (Brittany, France) were exposed to graduate warming and cellular and molecular responses relevant for physiological stress assessment were measured. In addition, considering that two mussel species (*M.*

galloprovincialis, *M. edulis*) and their hybrids are present in the study area, we investigated possible differential responses and sensitivity of species and hybrids to environmental factors.

2. Material and methods

2.1. Animal sampling, experimental conditions and species determination

Adult bivalves were randomly collected in the Bay of Brest (Figure 1) at the end of 2012 summer from natural mussel beds inhabiting either rocky shores or harbor wharfs, at a level corresponding to the mid-littoral tidal zone. Collection by sea was achieved at low tide and the animals were transported in cooled containers to the laboratory. The Commercial Port (Cp, 48°22'51.59"N, 4°28'59.50"W; mean shell length ± SD = 65.6 ± 4.8.2 mm) has been historically exposed to anthropogenic inputs due to a combination of maritime activities, urban run-off and occasional release from sediments. Values available for mussel populations there (Lacroix, 2014; Lacroix et al., 2015) reflect a chronic, moderate contamination corresponding to "harbor" conditions (Table 1). A reference site, Pointe de l'Armorique (Ar; 48°19'20.29"N, 4°27'13.51"W; mean shell length ± SD = 58.5 ± 4.6 mm), is located in the central zone of the Bay at a distance of any identified source of contamination (Figure 1). Selected physico-chemical parameters were measured in water upon collection. Temperature at Ar and Cp respectively, was 18.4 and 17.9°C, oxygen was 7.22 and 6.39 mg/L, pH was 8.22 and 8.11 and salinity (PSU) was 34.8 and 34.6.

All mussels were acclimated for 7 days in the laboratory at 18°C, close to the environmental temperature and maximal annual value occurring at these sites (see actual values above). The tanks were connected to a continuous flow of aerated filtered seawater and the mussels were daily fed with microalgae. After then, for each population, 6 groups of 33 mussels were

transferred into the experimental tanks: three maintained at 18°C as a control and 3 submitted to temperature elevation. The thermal stress was applied by step, corresponding to 3 consecutive heat-ramps of +1°C per day for 3 days. Between each temperature ramp, ie 18 to 21°C, 21 to 24°C and 24 to 27°C, a recovery period of 4 days without temperature change was applied (Figure 2A). A light/dark cycle (12h/12h) was applied and daily monitoring of the physico-chemical parameters (temperature, dissolved oxygen, pH and salinity) was carried out in the different tanks during the experiment (Figure 2A and 2B). At the end of each recovery period, samples of 15 mussels were collected in test and control tanks (Figure 2A). Gills (used for RNA extraction and gene expression) and adductor muscle (used for DNA extraction and species identification) were dissected and immediately frozen in liquid nitrogen and stored at -80°C until use. Prior to dissection, hemolymph was collected from adductor muscle to measure immune parameter (see section 2.3).

As collection sites are inside the hybrid zone between *Mytilus edulis* and *M. galloprovincialis*, mussel species was determined using locus *Glu-5'* (Inoue et al., 1995). DNA was extracted individually from the adductor muscle of each mussel used in the experiment using the chloroform phenol extraction method. The Glu-5' locus was amplified by PCR with primers Me-15 and Me-17 and the genotypes were determined from fragment-length variation on 2.5% agarose gels. A χ^2 test was used to compare species distributions between sites. The genotyping indicated that *M. galloprovincialis* (53% and 75% at Ar and Pc; respectively) and hybrid individuals (42% and 24% at Ar and Pc; respectively) largely dominated *M. edulis* (4% et 1% at Ar and Pc; respectively) at both sites (χ^2, p=0.012).

2.2. Condition index of mussels during experimental warming

Mussel condition was assessed in control and exposed mussels by calculating a condition index (CI) relating shells and dry soft body mass. A number of 15 mussels, were cleaned from fouling and opened for dissection. Shells and soft bodies were separately dried at 60°C for 7 days. Walne and Mann CI (‰) was calculated according to the following formula:

CI = 1000 x (tissue dry mass (g) / shell dry mass (g))

2.3. Immunological status of mussels during experimental warming

Approximately 300 µL of hemolymph was withdrawn from the adductor muscle sinus of mussels (n=15 per tank) and maintained on ice until processing. All measurements were made in individual samples using a flow cytometer (FACSCalibur™, Becton Dickinson) as described by Auffret et al. (2002). Briefly, the flow rate was measured to allow absolute, total hemocyte counts (THC). Differential counts (DHC) were performed by labelling cell population with a heterologous lectin. Cell mortality was measured using the propidium iodide-exclusion method (final concentration = 10 µg.L^{-1}). These three parameters describe immunopathological alterations. In addition, to assess hemolymph cell competence, their phagocytic activity was measured *in vitro* by incubating hemolymph cells with 2 µm latex beads at a 1:30 cell-to-bead ratio. A phagocytic index (IP expressed in % positive cells) was calculated as the percent of cells that had engulfed three beads and more, a means to consider only the most active hemocytes. An overall phagocytic capacity of the hemocyte population was calculated from the histogram of relative fluorescence intensity distribution (provided by the CellQuest™ software, as the geometric mean of values provided by positive cells. This raw value was normalized as the ratio to the fluorescence level of a single bead to obtain a new variable (CPN expressed in arbitrary units).

Two sets of statistical analysis were performed. First, each thermal stress condition versus its respective control (student t-test), after a check of homoscedasticity and normality. Then, we compute the mean of each control (18°C at the three exposure time), and calculate the ratio between each individual of the three exposed condition at each site and the mean of its respective control. Ratios were compared with two-way ANOVA with an interaction term, on raw data for IP and CPN, and log-transformed data for THC to meet parametric conditions. Tukey post-hoc were performed when either principal or interaction effects were identified.

2.4. Gene expression

The mRNA expression of 36 genes was followed by real-time PCR in treated and control mussels in gill tissue. Genes were chosen according to their involvement in different metabolic pathways: energetic metabolism, oxidative stress scavenging, stress response, detoxication, immune response and cell cycle regulation. Total RNA was extracted from gill tissue of all individuals (180 mussels) by using TRI Reagent® solution (Ambion) according to the manufacturer's instructions. Three µg of total RNA were reverse transcripted using M-MLV reverse transcriptase (Promega, Madison, WI), random hexamers (Promega) and an anchor-oligo(dT) primer (5'-CGCTCTAGAACTAGTGGATCT$_{(17)}$-3'). The relative mRNA expression of genes selected for their involvement in different metabolisms was estimated by real-time PCR amplification using specific primers designed from available data (Supplementary material Table 1; genbank and Bassim et al, 2014). A volume of 2.1 µl of each diluted reverse transcription product (1:100) was subjected to real-time PCR in a final volume of 5 µl containing 0.4 µM of primers and 1X LightCycler® 480 SYBR green I master (Roche, Germany). The amplification was carried out as follows: initial enzyme activation at 95°C for 6 min, then 40 cycles of 95°C for 10 sec, hybridization at 60°C for 30 sec and

elongation at 72°C for 30 sec. Calibration curves were obtained using serial dilutions of a cDNA pool from 12 individuals. In this study, we used a fragment of 28S was used as a reference gene, because other commonly used reference genes (such as EF1α or actin) are often found regulated in stressed bivalves (Boutet et al, 2004; Tanguy et al, 2004; David et al, 2005). Relative expression of each gene was calculated according to comparative Ct method using the formula: $RQ = 2^{-\Delta Ct}$ where ΔCt is obtained by calculating the difference between Ct of exposed mussels and Ct of control ones. Thus, at each sampling and for each site, gene expression in exposed individuals was normalized by the average obtained for the respective controls maintained at 18°C.

At each sampling point, significant differences between exposed and control individuals were detected using a non-parametric Wilcoxon Mann-Whitney test. Same tests were conducted between *M. galloprovincialis* and hybrids at each temperature. Hierarchical clustering analyses were also carried out between the genes studied and the conditions using the TmeV function with a hierarchical classification method by means of average linkage clustering with Pearson correlation (Saeed et al., 2003).

2.5. Statistical analysis of complete dataset

All analyses were performed with RStudio version 1.1.463, with R version 3.6.3 (R Core team 2020; RStudio team 2016). An analysis of the complete data set was done considering 4 structuring variables: species, site, temperature and time. For this analysis, the dataset should not contain any missing data that were replaced by the mean value in a given condition and the cyp1A1 gene was removed from the analysis because too much data was missing. The results are plotted on a Venn diagram and redundancy analyses (rda function, vegan package

version 2.5-6, Oksanen et al., 2019) are performed to determine the contribution of each variable to explain the whole dataset.

In order to evidence modifications of transcriptomic response' patterns according to time and temperature, all the dataset was analyzed at once, by using a PLS-DA (Partial Least Square Discriminant Analysis, MixOmics package version 6.6.2., Le Cao et al., 2016). This method allows for experimental design with far more variables than individuals within each group, and when the dataset contains missing values. It allows to identify the gene expression levels that are the most suitable to separate exposure conditions, ie whom variations are characteristics of each condition. Important genes were selected based on the VIP (Variable Importance in the Projection) values, with a threshold value of 1. Exposure conditions were then plotted on a factorial plane combining the two first axis of the PLS-DA, with only the 95% confidence ellipses plotted to gain in clarity. Variables were plotted on a correlation circle, giving the correlation of each variable with the axis of the PLS-DA. The significance of the exposure conditions discrimination was assessed by a cross validation test (Westerhuis et al., 2010; Szymańska et al., 2012, RVAideMemoire package version 0.9-75).

3. Results

3.1. Physiological status

Condition index. No significant difference was observed with respect to the controls whatever the site or temperature (ANOVA, $p>0.05$, data not shown).

Immune status. Immune parameters were compared for each thermal stress condition versus its respective control (18°C) at the same date (Figure 3). THC were significantly increased at

the maximum temperature applied (27°C) for both populations. Phagocytosis was altered only for the Ar population, as revealed by a marked decrease of the number of active cells (IP) at 27°C and a reduction of the cell capacity (CPN) at 24°C and 27°C, (see Supplementary Material Table 2 for detailed analysis). Between-temperatures comparisons evidenced only an effect of temperature, and not significant site nor interaction, whatever the endpoint considered (Supplementary material Table 3). For THC and CPN, Tukey post-hoc tests evidenced differences between 21°C and 27°C ($p=0.018$ for THC, $p=0.001$ for CPN) and between 24°C and 27°C ($p<0.001$ for THC, $p<0.001$ for CPN). For IP, only the most extreme temperature was different (21°C vs 27°C, $p=0.03$).

3.2. Determining the relevance of variables

Main variables likely to influence the physiology of these mussels were sampling site, species, imposed temperature and exposure. Redundancy analysis on the data shows that the residual variance is 88% and the remaining 12% is explained by the 4 variables, significantly for the site ($p<0.001$), exposure time ($p<0.001$) and temperature ($p=0.003$), but not significantly for the species ($p=0.477$) (Venn diagram, Figure 4). The individuals carrying the *M. edulis* genotype were therefore removed from the analysis (very low number at each site, see section 2.1), as well as the species variable. Mussels with *M. galloprovincialis* genotype and hybrids will be considered as a homogeneous group in the subsequent global analyses such as in previous study (Lacroix et al, 2014; 2015).

3.3. Effect of mussel conditioning on gene expression

Even if no mortality was observed during experiment, a discriminant analysis (PLS) was performed to compare expression levels in all control groups (tanks maintained at 18°C until the end of the experiment) to seek for any effect of conditioning. After 3 weeks, two groups (ArT3 and CpT3) were separated both from the other points and between each other. Gene expression in Ar mussels varied with conditioning duration (Figure 5B), when Cp mussels were found different only at the last sampling point (Figure 5B). A strong inter-individual variability was noticed within each group with a high variance along axis 2 for the first two sampling points (T1 and T2 for Ar and Cp) and a distribution of the variance on the 2 axes for the third point (ArT3 and CpT3) (Figure 5A). The correlation circle (Figure 5C) focused on genes with VIP>1, the threshold considered significant in PLS analysis (Supplementary material Table 4). Among the genes analyzed, eleven showed significant variations in expression between individuals and were involved in the response to stress and detoxification (*hsp40, hsp22* and *mdr*), energy metabolism (*ldh, idh, odh, pk, mit-mdh* and *cyt-mdh*) and oxidative stress (*perox6* and *cat*).

3.4. Effect of temperature increase on gene expression

Effect of temperature at a global scale. Analysis of the results showed that the increase in temperature had a significant impact on the gene expression profile between mussels at the same site on the one hand and between sites on the other. Mussels exposed to a temperature elevation of 3°C (Ar 21°C and Cp 21°C) compared to the control temperature (18°C) did not show significant variations in gene expression (Figure 6A and 6B). Significant differences with the control appeared when the temperature reaches 24°C then 27°C in the mussels from the 2 sites (Figure 6A and 6B). Expression profiles were also not significantly different between mussels exposed at 21°C and 24°C within each site (Ar 21°C *vs.* Ar 24°C and Cp

21°C *vs.* Cp 24°C), while the pattern of mussels exposed at 27°C was significantly different from the other 2 in each site (Figure 6A and 6B). The factorial plan (Figure 6A) showed that there was inter-individual variability within each group along axis 2, indicating that individuals did not all react in a same way to an increase in temperature. At an inter-site scale, the factorial plan (Figure 6A) and statistical tests (Figure 6B) showed that at 21 and 27°C, the ellipses of the 2 sites did not overlap, whereas they did for individuals exposed to 24°C.

Among the genes analyzed, eleven show significant variations in expression between individuals (Figure 6C and Supplementary material Table 2) and were involved in the response to stress and detoxification (*Hsp40*, *Hsp22* and *Mdr*), energy metabolism (*ldh, idh, odh*, *mit-mdh*, *pepck* and *pk*) and oxidative stress (*perox6* and *cat*).

Effect of temperature in the control population (Ar). The hierarchical clustering showed an effect of temperature (Figure 7A) and indicated an induction of gene expression at 21 and 24°C compared to 27°C that showed a decrease in gene expression. The hierarchical clustering highlights 4 groups of genes differentially regulated by temperature (Figure 7A). The group 1 consisted of genes whose regulation was similar whatever the temperature (Figure 7A). The group 2 was composed of more than half of the genes studied (i.e. 20 genes) and consisted of genes over-expressed at 21 and 24°C but repressed at 27°C (Figure 7A). The group 3 showed an inverse trend with genes induced at high temperatures (Figure 7A). Finally, the group 4 consisted of genes that were over-expressed at 21°C but not at 24°C and appeared repressed at 27°C (Figure 7A).

Effect of temperature in the contaminated population (Cp). An effect of temperature was highlighted by the hierarchical clustering (Figure 7B). However, contrary to what was observed for the mussels collected at Ar, the responses detected at 24°C were closer to those

observed at 27°C than at 21°C. Although the general trend was towards overexpression at 21°C (significant for *hsp90β*, *Phydrox* and *HFI*). At 24°C, only HP was significantly overexpressed. The hierarchical clustering carried out on the genes highlighted two groups that differ mainly by the responses observed at 27°C. The group 1 consisted of genes that were repressed at 27°C and the group 2 consisted of genes that tend to be overexpressed at 27°C (Figure 7B).

Effect of temperature on different species. No significant differences in gene expression were detected between hybrids and *M. galloprovincialis* (data not shown). At Ar site, the hierarchical clustering distinguishing *M. galloprovincialis* from hybrids at each temperature showed that hybrids and *M. galloprovincialis* were not grouped together at 21 and 24°C, but presented the same expression pattern at 27°C (Supplementary material Figure 1A). At Pc site, the hierarchical clustering distinguishing *M. galloprovincialis* from hybrids showed that the expression patterns were the same at each temperature (Supplementary material Figure 1B).

4. Discussion

4.1. *Mytilus* complex as an indicator of site health

The collection sites of mussels were located inside the hybrid zone between *M. edulis* and *M. galloprovincialis* (Bierne et al., 2003, Hilbish et al., 2012). It was previously shown that the mussel populations located in Brittany are dominated by *M. galloprovincialis* alleles (Hilbish et al., 2012) and our results confirmed the higher proportion of *M. galloprovincialis* compared to *M. edulis*, as well as the presence of hybrid individuals in both sites. We observed a

significant difference in the proportions of *M. galloprovincialis*, *M. edulis* and hybrids between the two sites which could indicate an effect of environmental conditions on mussel distribution, the highest proportion of *M. galloprovincialis* being detected in the most polluted site. Although, when considering the species status, our results did not show significant differences in mRNA expression between *M. galloprovincialis* and hybrids in response to temperature elevation under experimental conditions. The question of the physiological performance of hybrids between *M. galloprovincialis* and *M. edulis* has been relatively little addressed. Studies are generally focused on pure species (Fly and Hilbish, 2013; Fly et al., 2015) or mostly showed that hybrids presented a fitness similar to that of *M. galloprovincialis* and superior to that of *M. edulis* in terms of developmental stability and developmental rate (Gardner, 1995), growth rate (Gardner et al., 1993) viability (Gardner and Skibinski, 1991; Willis and Skibinski, 1992), fertility and fecundity (Gardner and Skibinski, 1990) and also resistance to parasites (Coustau et al., 1991). A model of directional selection favoring *M. galloprovincialis* nuclear alleles over those of *M. edulis* (leading to remove hybrid mussels presenting predominantly *M. edulis* nuclear genotypes) has been suggested and such selection naturally occurred in hybrid mussel populations (Skibinski and Roderick, 1991; Rawson and Hilbish, 1998). The reasons why hybrids showed preferential *M. galloprovincialis* performance have not yet been identified. A study suggested that the distribution of species in a hybrid zone was related to their energetic abilities to maintain themselves in that environment (especially cold and warm limits) and the level of introgression of alleles from the cold species (*M. edulis*) into the genetic background of the warm species (*M. galloprovincialis*). Recently, Mlouka et al (2019) showed that mussel larvae exposed to copper and increased temperature showed different morphological and physiological responses depending on the species (*M. galloprovincialis*, *M. edulis* and hybrids). *M. galloprovincialis* larvae and hybrids resulting from *M. galloprovincialis* females appeared to

be more tolerant to these conditions than *M. edulis* mussels and hybrids resulting from *M. edulis* females. *M. galloprovincialis* are known to harbor an unusual mode of mitochondrial DNA inheritance (doubly uniparental inheritance) with females being homoplasmic for the F mitotype which is inherited maternally, whereas males are heteroplasmic for this and the paternally inherited M mitotype (Skibinski et al., 1994; Zouros et al., 1994). The question of the importance of this mode of transmission has not been addressed in the understanding of the physiological response of hybrids. Further experiments are needed to address this issue, taking into account the possibility that physiological responses may also be modulated by gender and indirectly studied the relative contribution of the two mitotypes in the mussel adaptive response.

4.2. Immune status is altered by warming

Immune parameters measured here were moderately affected by experimental conditions. The only conspicuous response was a marked increased number of circulating hemocytes in mussels exposed to the highest temperature, for both populations. These invertebrates have a semi-closed circulatory system and cell migration toward or from tissue compartments may generate total hemocyte count alterations in haemolymph. Many observations of natural (seasonal) or stress-induced variations have been documented in bivalve molluscs (see Auffret, 2005 for review; Matozzo et al., 2012; MacKenzie et al., 2014). Variations of hemocyte counts may then be considered as a systemic response to stress, here a sub-lethal temperature, rather than a consequence of cytopathological alterations in haemolymph. Phagocytosis is an immune function which relies on complex cell mechanisms. Observations during a seasonal survey of blue mussel populations in this bay have revealed a marked influence of natural, water temperature fluctuations on this function (Lacroix et al, 2017). In

the present study, the number of active cells and their capacity to engulf foreign particles was found altered by the thermal stress, but only in the Ar population. Non-toxic immunomodulation in invertebrates results in deleterious consequences for the physiological performance of animals (see Matozzo and Marin, 2011, for review). Based on a long-term, experimental study where mussels were acclimated to increased temperatures, MacKenzie et al. (2014) assumed that the immunological response mounted in stressed mussels may incur energetic costs including altered energy metabolism, but also, altered biochemical composition of hemolymph, as fatty acid composition. More extensively, such signs of immunomodulation could be considered as clinical markers of deep physiological changes or even, general decline in condition of the organism. Indeed, observations discussed in the following sections of a repression of mRNA expression for key-genes involved in metabolic pathways support the hypothesis of an energy limitation. Interestingly, the population collected in the Commercial port did not respond to the acute change applied here. An explanation could be that those mussels inhabiting such confined areas did naturally experience at summertime from their youngest stages episodes of higher temperature compared to populations settled in the bay.

Measuring the mRNA expression of three antimicrobial peptides (AMP) and of lysozyme in gill tissues allowed to explore early non-cellular immune responses of mussels. The level of mRNA of *myticin B*, *mytilin C* and *lysozyme* was significantly more regulated at Ar, when no response for any of the four genes could be detected at Cp. At low stress levels (21 and 24°C), the mRNA level of *myticin B* and *mytilin C* was increased, when *mytilin C* and *lysozyme* mRNA level was decreased at 27°C compared to the control. Interestingly, hemocyte counts were increased in these mussels. A migration of hemocytes from gill tissue to hemolymph could explain this decrease because AMPs are produced in hemocytes (Mitta et al., 2000; Li et al., 2008, 2010). Although the exact role of AMPs in the response to increased temperature

was not yet clear, the observed induction of transcription of genes encoding AMPs in heat shocked *M. galloprovincialis* could be partly explained by the presence of heat shock elements in the promoter region of some genes (Cellura et al., 2007).

4.3. Mild temperature induced an increase in mRNA expression.

Acclimation of organisms in the laboratory aimed to minimize potential effects of stress due to sampling (collection and transport) and changes in environmental conditions (continuous immersion, feeding conditions). For bivalves, periods ranging from 24 hours to 3 weeks have been documented (Boutet et al., 2004, 2005; Tanguy et al., 2005; David et al., 2005, Matozzo et al., 2013). In this study, all mussels were maintained for 7 days before the temperature augmentation and a control group was kept at the initial, field temperature (18°C). When gene expression in control mussels were compared among sites, profiles were significantly altered after 3 weeks (T3), indicating that laboratory conditions themselves might trigger unexpected biological effects after extended conditioning under an artificial environment.

A moderate temperature elevation from 18 to 21 then 24°C induced a significant increase in mRNA expression of genes involved in all the metabolic pathways with the exception of cell cycle regulation in Ar mussels. At 21 and 24°C, the mussels collected at Ar showed a greater response in terms of the number of genes regulated compared to the mussels at Cp. The overexpression of genes involved in antioxidant defenses and stress response suggested higher oxidative pressure and damage in Ar mussels. In these organisms, variations of mRNA level of stress proteins (HSPs), oxidative stress scavenging (CuZnSOD) and detoxification proteins (MT20 and MDR) were widely described in response to heat stress, particularly at the transcriptional level (Eufemia and Epel, 2000; Lockwood et al., 2010; Negri et al., 2013; Tomanek, 2014; Mlouka et al., 2019; Feidantsis et al., 2020). Such an increased expression of

mRNAs encoding stress proteins reflected that animals were still in an optimal range to ensure efficient and adequate physiological performance. Similarly, a regulation of genes encoding for proteins involved in oxidative stress scavenging (CuZnSOD) and in detoxification mechanisms (MT20 and MDR) was detected in mussels exposed to 21 then 24°C.

The activation of different metabolic functions in response to temperature, in particular the activation of stress response, had a significant energy cost and suggested a situation of physiological stress caused by temperature. Previous work conducted on mussels showed that a temperature elevation induced an activation of energy metabolism related genes (Lockwood et al., 2010), an increase in respiration rates and consequently of the energy metabolism (Artigaud et al., 2014). In our study, overexpression of genes involved in glycolysis (*hk* and *pk*), in the Krebs cycle (*cyt-mdh*) and in the anaerobic pathways (*ldh* and *odh*) suggested an activation of energy production. Furthermore, most of the genes induced at 21°C were no longer induced at 24°C (only octopine-dehydrogenase remained over-expressed at 24°C in Ar mussels). It was assumed that the opine pathway, less toxic than lactate, could be the preferred anaerobic pathway during persistent stress in blue mussels (Harcet et al., 2013; Han et al., 2017). The balance between aerobic and anaerobic energy production was a fundamental mechanism when the demand for energy became too high in stress conditions (Sommer et al., 1997; Frederich and Pörtner, 2000; Sokolova et al., 2012; Sokolova, 2013). At the physiological level, when the temperature exceeded a given threshold, the capacity to supply oxygen became limiting in the organism and an imbalance was created between oxygen supply and demand, inducing situations of systemic hypoxia (Pörtner, 2001). In our study, the expression of several genes involved in the detection of oxygen was regulated in response to temperature. For example, the gene coding for the hypoxia up-regulated protein 1 (*hp*) was overexpressed at 21 and 24°C in Ar-originated mussels suggesting systemic oxygen

limitation, which was consistent with the hypothesis of activation of anaerobic metabolism at these temperatures.

4.4. High temperature induced a decrease in mRNA expression.

Contrary to what was observed at 21 and 24°C, a decrease in transcription was observed in mussels from both sites exposed to 27°C, except for a few genes that appeared over-expressed, suggesting a shift towards a situation of metabolic depression. The animals might therefore be under severe physiological stress and had a limited ability to survive at this temperature. It was consistent with the known lethal temperatures of *M. edulis* and *M. galloprovincialis* which were described between 26 and 28°C (Jones et al., 2009). The transition to metabolic depression resulted in a limitation of energy consumption and allowed survival when environmental conditions became extreme (Storey and Storey, 2010, 2012; Sokolova et al., 2012). As this response was to the detriment of activity, growth and reproduction, it was therefore limited in time and reflected a situation of severe stress (Sokolova et al., 2012). This mechanism is also dependant on intrinsic capacities of organisms to manage their energy metabolism (Lannig et al., 2006; Lacroix et al., 2014, 2015, 2017; Marigomez et al., 2017; Collins et al., 2020). Metabolic depression processes mainly concerned energy-consuming mechanisms such as transcription by maintaining only a few genes involved in vital functions such as energy metabolism, antioxidant defences and maintenance of protein integrity (Storey and Storey, 2010, 2012; Sokolova et al., 2012). The activation of antioxidant defences and heat shock proteins, described in many species, could prevent and/or minimized damage to macromolecules while the body was not able to cover the large demand for ATP required for degradation and *de novo* synthesis of molecules (Boutet et al., 2009; Storey and Storey, 2010, 2012). In the present study, overexpression of

genes involved in energy metabolism (*mit-mdh* and *cyt-mdh*), antioxidant defences (*cat*) and maintenance of macromolecule integrity (*hp*, *hsp78* and *hsp60*) was observed in mussels from both sites exposed to 27°C. In parallel, a decrease in mRNA level was observed for other genes involved in energy metabolism (*odh*, *ldh*, *idh* and *pk*), redox regulation (*CuZn-sod*), protein integrity (*hsp22* and *hsp40*) and apoptosis (*caspase 8* and *caspase 3/7_4*). Regarding the regulation of energy metabolism, the repression of mRNA expression of *odh*, *ldh* and *pk* coupled with the induction of *cyt-mdh* suggested an anaerobic production of energy *via* the glucose-succinate and/or aspartate-succinate pathways and supported the hypothesis of an energy limitation (Grieshaber et al., 1994; Sokolova et al , 2012). These similar responses in mussels from both sites suggested that the glucose-succinate and aspartate-succinate pathways could play an important role in energy production when the mussels were exposed to limiting temperature conditions.

5. Conclusions

This experimental study brings new information on the capacity of the blue mussel, a representative coastal, sessile organism, to cope with different levels of temperature increase, as an additional stress to low-level environmental contamination. At an intermediate level of stress (24°C), distinctive population trajectories occurred, with a more pronounced response when mussels experienced daily exposure to a low level of contamination (Ar). At the highest, limit temperature, a similar trade-off was observed in both populations, as expected by the demonstration by Luoma (1996) and Baird and Barata (1999) that extreme levels of stress would canalize biological responses towards mechanisms that allow survival. Comparable effects were induced as on one hand, increased expression of genes coding for stress-response proteins and on the other hand, a depression of genes involved in energy

metabolism. In addition, the step-by-step stress induction applied here allowed to recognize a progressive implementation of the adaptive response to stress. Finally, this study suggests that these mussel populations would respond to additional stress depending not only on their environmental history (exposure to chronic pollution), but also on seasonal and habitat conditions (availability of food, tidal *vs.* subtidal), as suggested by observed patterns of metabolism-related gene expression. In a context of global warming, the environmental history of exposure could modify the ability of species to cope with such an additional pressure.

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Figure legends

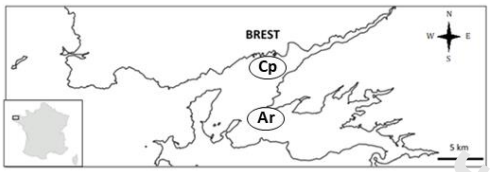
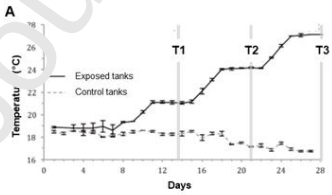


Figure 1. Location of the Bay of Brest within France and collection sites of Ar (Pointe de l'Armorique) and Cp (Commercial Port) populations.



B

	Control tank	Exposed tank
O_2 (mg/L)	7.39 ± 0.34	6.98 ± 0.29
O_2 (% saturation)	95.4 ± 4.3	97.0 ± 3.6
pH	8.21 ± 0.07	8.16 ± 0.03
S (PSU)	35.2 ± 0.0	35.2 ± 0.1

Figure 2. (A) Experimental design and monitoring of the temperature in control (dotted line) and exposed tanks (continuous line). (B) monitoring of physico-chemical parameters in the tanks (given as mean ± SD).

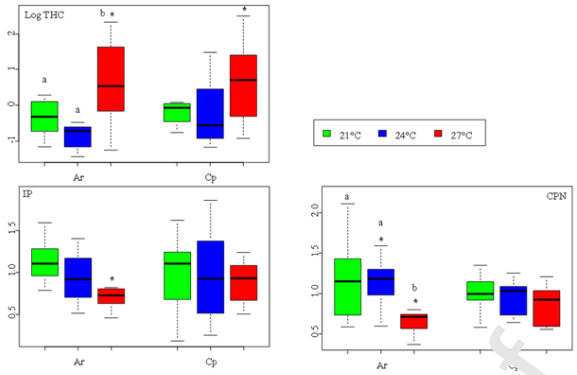


Figure 3. Boxplot of comparison of immune descriptors in experimental trials among mussel populations: total hemocyte count (THC), phagocytic index (IP) and overall phagocytic capacity (CPN). Relative values were calculated as the ratio of each temperature condition to its control (initial temperature). The central line represents the median, the box represents the first and third quartile and whiskers extend to the most extreme value that do not exceed 1.5 times the interquartile range. Outliers are not shown. Values are groups by site. * indicates significant differences between individual exposed at a given temperature and its control (see also Supplementary Material Table 2). Letters indicates differences between temperature within each site, obtained with the Tukey HSD on Anova results.

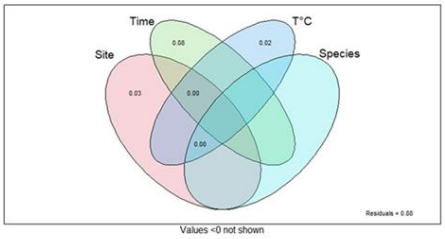


Figure 4. Venn diagram representing the distribution of variance among the 4 main variables in the dataset.

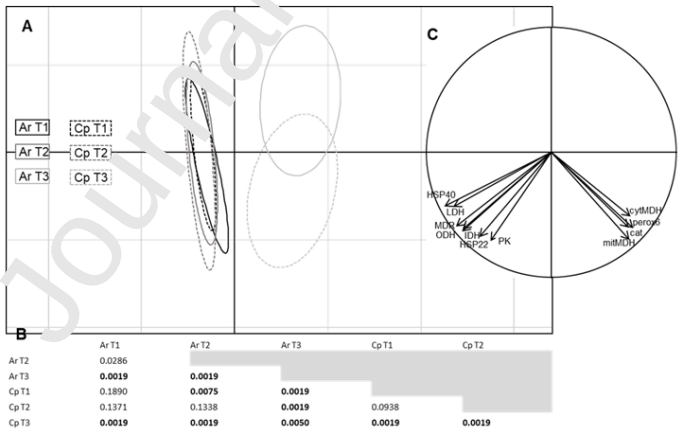


Figure 5. Gene selection based on the VIP (Variable Importance in the Projection) values in mussels maintained at 18°C. (A) For the factorial plane dataset where obtained after PLS-DA (Partial Least Square Discriminant Analysis. Exposure conditions were plotted on a factorial plane combining the two first axis of the PLS-DA. (B) Pairwise comparisons using

permutation tests based on cross model validation (999 permutations; P-value adjustment method: fdr). (C) Variables were plotted on a correlation circle, giving the correlation of each variable with the axis of the PLS-DA.

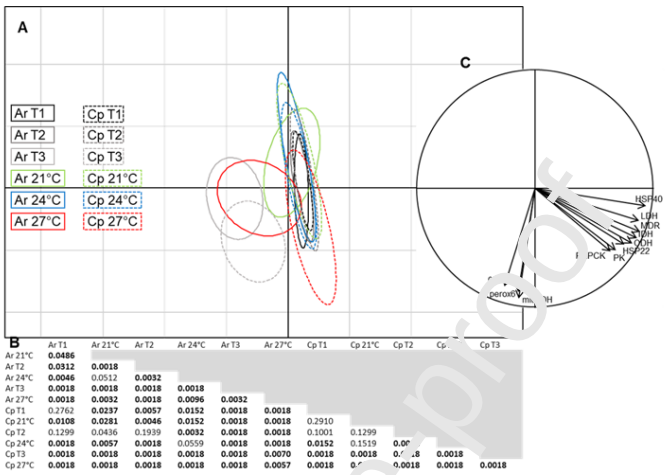


Figure 6. Gene selection based on the VIP (Variable Importance in the Projection) values in mussels of all groups (control and exposed to increase temperature). (A) For the factorial plane dataset where obtained after PLS-DA (Partial Least Square Discriminant Analysis. Exposure conditions were plotted on a factorial plane combining the two first axis of the PLS-DA. (B) Pairwise comparisons using permutation tests based on cross model validation (999 permutations; P-value adjustment method: fdr). (C) Variables were plotted on a correlation circle, giving the correlation of each variable with the axis of the PLS-DA.

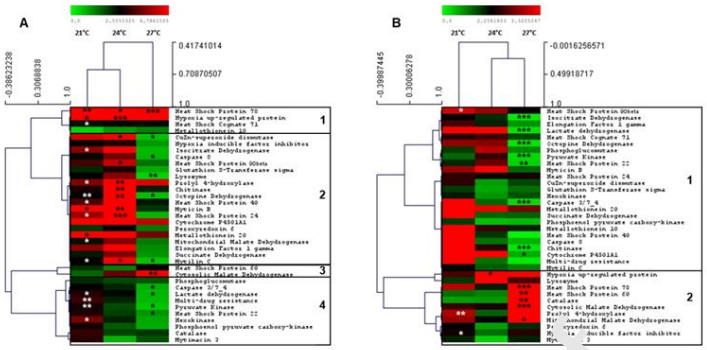


Figure 7. mRNA gene expression followed during temperature increase experiment in mussels collected at Armorique (A) and Commercial Port (B). The genes are grouped in different sets (4 for the Ar site and 2 for the Cp site) according to their expression profile. Relative mRNA expression is expressed as xfold control mussel expression level (green represent gene with less expression than control; red represent gene with more expression than control). Significant differences with control: * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

CRediT roles:

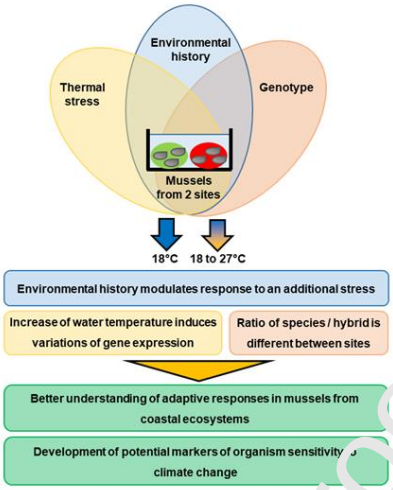
Isabelle Boutet: Formal analysis, Investigation, Writing-Original draft preparation. **Camille Lacroix**: Formal analysis, Investigation, Writing-Original draft preparation. **Simon Devin**: Formal analysis, Writing-Original draft preparation. **Arnaud Tanguy**: Conceptualization, Writing-Original draft preparation. **Dario Moraga**: Conceptualization, Writing-Original draft preparation. **Michel Auffret**: Conceptualization, Supervision, Project administration, Funding acquisition, Investigation, Writing-Original draft preparation.

Competing interests: The authors declare no competing interests.

Table 1. Comparison of concentrations in mussel tissues from both sites (Lacroix, 2014; Lacroix et al., 2015) to the National median as a reference value for coastal waters for the 2003-2007 period provided by the French ROCCH Network (wwz.ifremer.fr/envlit/Surveillance-du-littoral/Contaminants-chimiques/). F/P: Fluorenthene/Pyrene. LOQ: Limit of Quantification. PAH: Sum of 16 compounds according to USEPA recommendations.

	Tissue burden (mg.kg^{-1})		
Contaminant	**Cp**	**Ar**	**National median**
Cd	0.9	0.9	0.7
Pb	3.8	2.9	1.4
Zn	203	134	107
PAH	256	4	
F/P	0.6	< LOQ	

Graphical abstract



Highlights

Identifying the role of environmental history in the response to temperature increase

Environmental history modulated response to an additional stress and genotype proportion

Increase of temperature induced variations of gene expression and immunomodulation