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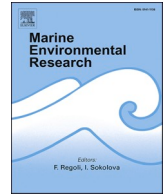
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# Molecular and behavioural responses of the mussel *Mytilus edulis* exposed to a marine heatwave

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## ABSTRACT

Marine heatwaves (MHW) threaten marine organisms and tend to increase in frequency and intensity. We exposed the blue mussel *Mytilus edulis* to a MHW lasting 23 days, including two 10-d periods of thermal intensity increase of +5 °C (20 °C–25 °C) interspersed by 1 day back to 20 °C, followed by a 4-d recovery period. We investigated behaviour responses of mussels and gene expression changes relative to the circadian rhythm (*Per*), oxidative stress (*SOD*), cellular apoptosis (*CASP3*), energy production (*ATPs*), and general stress response (*hsp70*). Results showed that the MHW disturbed the valve activity of mussels. Particularly, mussels increased the number of valve micro-closures, showing a stressful state of organisms. Mussels also decreased *Per*, *CASP3*, *ATPs*, and *Hsp70* gene expression. Some behavioural and molecular effects persisted after the MHW, suggesting a limited recovery capacity of individuals. This work highlighted the vulnerability of *M. edulis* to a realistic MHW.

## 1. Introduction

Climate change is responsible for the increase in frequency and intensity of extreme temperature events such as marine heatwaves (MHW) since the last century (Oliver et al., 2018). According to the largely accepted definition of Hobday et al. (2016), temperature anomalies are considered as MHWs if they exceed a threshold value (90th percentile) determined based on a climatological reference period (30 years) for at least 5 days. MHWs are responsible of major and immediate adverse effects recorded on marine ecosystems and ecosystem services provided (Smale et al., 2019). The most significant ecological impacts have been documented on coastal areas with, for example, mass mortalities (e.g. corals, Hughes et al., 2017; gorgonians and sponges, Garrabou et al., 2009; mussels, Galil et al., 2022, Seuront et al., 2019), changes in species distribution and abundance (e.g. algae, fish, crustaceans, Lonhart et al., 2019), or change in community composition in favor of warm-water adapted species (e.g. sea urchins, Smale et al., 2017). In these coastal habitats, Madeira et al. (2012) notes that species living in thermally cyclic environments (e.g. intertidal areas) would be particularly vulnerable to extreme temperature events as they live near their thermal tolerance limits, any further increase in temperature would likely push them over these limits. Given future predictions of increases in intensity (x10) and frequency (x50) of MHWs by the end of the 21st century

following the current CO<sub>2</sub> emission scenario (IPCC et al., 2021), it becomes critical to assess how these MHWs affect ectothermic species whose physiology is directly temperature-dependent. For decades, research has focused on the influence of incremental temperature changes on the physiology of organisms (Ericson et al., 2023; Lassoued et al., 2021; Matoo et al., 2021; Thyrring et al., 2015). Other studies have considered the physiological effects of abrupt changes (Bakhmet et al., 2022; Wang et al., 2018; Jiang et al., 2016; Yu et al., 2009), but few have simulated the complex nature of temperature changes during a MHW as defined by Hobday et al. (2016) (Grimmelpont et al., 2023; Xu et al., 2023; Minuti et al., 2021). Consequently, the physiological impacts of a MHW on organisms remain poorly understood. Developing a thorough understanding of how organisms respond at sub-individual and individual levels to MHWs is essential for enhancing our comprehension of the ecological impacts observed in the environment. This becomes especially pertinent as it is claimed that predictions of ecological impacts would be more accurate if MHWs were considered in addition to incremental temperature changes due to the current global warming (Cheung and Frölicher 2020).

While mobile species can move to a more thermally favourable environment, sessile species such as the blue mussel *Mytilus edulis* are forced to stay in place and are particularly vulnerable to MHWs (Smale et al., 2019). *M. edulis* is a eurythermal species living in the intertidal

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and subtidal zone, with a wide repartition in cold and temperate regions (Seuront et al., 2019; Tran et al., 2023). It can live at temperatures between 5 °C and 25 °C (Kamermans and Saurel 2022) with a temperature limit of 30 °C that leads to the death of individuals (Kamermans and Saurel 2022). Despite its wide range of thermal tolerance, mass mortalities related to temperature anomalies such as MHW have been recorded in this species (Seuront et al., 2019). Considering its ecological and economic importance, it is therefore important to understand the physiological responses of these organisms to MHWs to assess their level of vulnerability or resistance.

To counteract the effects of thermal stress caused by MHWs, several authors have highlighted that bivalves can make a succession of adjustments at the sub-individual scale including the management of oxidative stress, the production of Heat Shock Protein (HSP) to prevent cellular damage, or changes in the expression levels of metabolic, reproductive, and immune-related genes (Peruzza et al., 2023; Xu et al., 2022; Amorim et al., 2020). Thus, recent studies have investigated the impacts of MHWs on the expression of genes related to diverse functions in bivalves. However, to the best of our knowledge, none of these studies have investigated the molecular responses in *M. edulis* exposed to MHWs and, to date, no studies have been conducted on the impacts of MHWs on genes related to the circadian clock, which regulate biological rhythms essential for the survival of organisms (Payton et al., 2017). The physiological responses of organisms to stress are closely related to their behavioural responses and a change of behaviour may lead to ecological consequences. In bivalves, analysis of valve activity has often been used to establish the impact of xenobiotics such as trace metals (Tran et al., 2004), or toxic algae (Tran et al., 2010) on the behaviour of individuals. However, few have considered the effect of thermal stress on the valve activity of bivalves (May et al., 2021; Gleason et al., 2017; Rodland et al., 2009; Anestis et al., 2007) and, to our knowledge, no study has yet evaluated the effect of a MHW on this behaviour. However, the valve closure for long-term can influence the feeding rate and respiration of mussels and consequently their physiological performance (Ortmann and Grieshaber 2003).

The aim of this study was to assess the response of the blue mussel *M. edulis* during a realistic scenario of MHW of an intensity of 5 °C (Hobday et al., 2016). To do so, mussels were exposed to a MHW lasting 23-days, with two 10-d periods of thermal intensity increase of +5 °C (20 °C–25 °C), interspersed by 1 day back to 20 °C, followed by a 4-d recovery period. Through an integrative level with the measure of the behavioural activity, the first objective was to determine whether experimental MHW modify the valve opening duration (VOD), including at an amplitude ranging between 50 and 75 % (VOD<sub>amp:50-75</sub>), and the number of micro-closures (VMC) in *M. edulis*. At the molecular scale, the second objective was to assess the impact of the experimental MHW on the expression of genes related to cellular response to stress and specifically to energy metabolism and circadian rhythm in the gills of *M. edulis*, as gills are one of the primary tissues involved in the mechanisms of adaptation to environmental change. The results of this study will allow us to better understand the behavioural and molecular mechanisms involved in acute heat stress such as during a MHW.

## 2. Materials and methods

### 2.1. Sampling and acclimation

The study was conducted on 104 blue mussels *M. edulis* (shell length: 43.2 ± 0.4 mm, mean ± standard error ES) collected in a foreshore (location: La Flotte, Ré island, France, 1° 19.85 W 46° 11.83 N) in May 2022 and transferred to the laboratory for acclimation in 145 L holding tanks supplied with renewed seawater. The water temperature in the holding tanks was raised gradually using submersible heaters placed in the tanks, starting from 15 °C (corresponding to the water temperature in the field at the time of mussels collection) and increasing to 20.0 °C at a rate of 1.0 °C per day. A period of 14 days at this temperature of 20 °C

was observed before the beginning of the experiment. The temperature of 20 °C corresponds to the average summer seawater temperature in La Rochelle (<https://www.seatemperature.org/europe/france/la-rochelle-august.htm>). During this period, the temperature was recorded every day using a WTW Cond 3310 multimeter (Xylem Analytics Germany GmbH) and mussels were acclimated to a photoperiod of 14 h light/10 h dark.

### 2.2. Experimental procedure

#### 2.2.1. Experimental system

Two homogeneous groups of mussels were placed in 33 L exposure aquariums isolated from external vibrations by an antivibration bench (n = 52 mussels per condition, of which 16 were equipped with electrodes for behavioural monitoring). Mussels were acclimated for 5 days at 20 °C (20.2 ± 0.1 °C, mean ± ES) before to start the experiment. Both aquaria receive continuous natural and filtered (at 5 µm) seawater from two 145 L buffer tanks with an average flow rate of 47.5 ± 0.4 mL min<sup>-1</sup>. Throughout the acclimation and experimental periods, mussels were fed continuously with a commercial mixture of 5 marine microalgae (Shellfish Diet 1800® containing 4 different genera: *Isochrysis*, *Pavlova*, *Tetraselmis*, *Thalassiosira*) at an average rate of 1.4 ± 0.1 mL min<sup>-1</sup> at a mean concentration of 451.7 ± 42.1 cells.mL<sup>-1</sup>. To ensure good oxygenation of the seawater, bubbling was set up in the aquaria and buffer tanks. Salinity (33.0 ± 0.1 ‰), nitrate (≤1.0 mg.L<sup>-1</sup>), nitrite (≤0.2 mg.L<sup>-1</sup>), and ammonium (<0.1 mg.L<sup>-1</sup>) levels were kept constant. Submersible heaters placed in the aquaria and tanks, associated with the air conditioning system of the experimental room, allow the regulation of the aquaria temperature. Temperature was recorded every minute using a HOBO probe (HOBO Tidbit V2 - UTBI-001 - HOBO Onset, Onset Computer Corporation, USA) placed in the aquaria.

#### 2.2.2. Protocol of MHW chosen

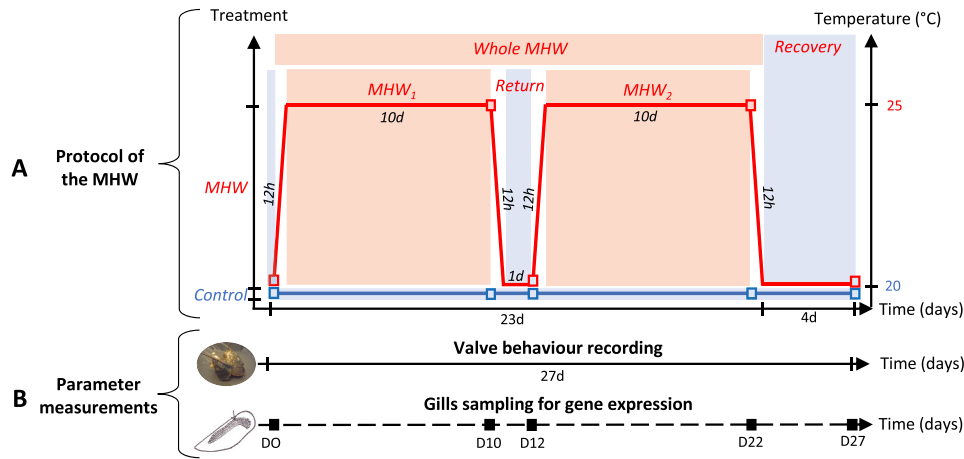
Fig. 1A shows the protocol of MHW exposure undergone by the mussels. The first group of mussels (MHW group) was exposed to an experimental 23-day MHW of +5 °C (Whole MHW) followed by a 4-day Recovery phase at 20 °C (20.0 ± 0.1 °C), while the second group (Control group) was maintained at a stable temperature of 20 °C (20.1 ± 0.1 °C) during the 27 days of experimentation. For the MHW group, the Whole MHW consisted in 3 phases, each separated by rapid temperature changes (average kinetics of 0.4 ± 0.1 °C. h<sup>-1</sup>) as follows:

- First rapid increase in temperature from 20 °C to 25 °C in 12 h
- MHW<sub>1</sub>: first 10-day phase at 25 °C (25.0 ± 0.1 °C)
- First rapid decrease in temperature from 25 °C to 20 °C in 12 h
- Return: 24-h phase at 20 °C (20.1 ± 0.1 °C)
- Second rapid increase in temperature from 20 °C to 25 °C in 12 h
- MHW<sub>2</sub>: second phase of 10 days at 25 °C (25.1 ± 0.1 °C).
- Second rapid decrease in temperature from 25 °C to 20 °C in 12 h

The Whole MHW is considered as a single event as the Return phase lasts less than 48 h, as defined by Hobday et al. (2016).

#### 2.2.3. Measurement of valve activity

Valve activity of *M. edulis* was recorded under both treatments for the whole experimental period (27 days) using two HFNI ("high-frequency non-invasive") valvometers (Tran et al., 2023). Each valvometer can record the behaviour of up to 16 mussels belonging to the MHW and Control groups. Electromagnets (<1 g) were fixed face-to-face on each of the mussel valves and connected to a valvometer by flexible cables. The principle is to measure the voltage variations produced by the electromagnetic current between the two electrodes. The measurement of the electromagnetic force  $\epsilon$  (volts) is an application of Maxwell's law,  $\epsilon = -N \cdot (d\phi B/dt)$ , where N is the number of turns of the electromagnet contained in the electrodes,  $\phi B$  is the magnetic flux (webers), and t is time. The sampling rate for each animal was 0.2 Hz. Data were processed



**Fig. 1. Experimental design. A: Protocol of the MHW.** Two groups were tested: *Control* (blue line) and *MHW* (red line) groups. The *MHW* group was exposed to a 23-day MHW of +5 °C from 20 °C to 25 °C (*Whole MHW*) followed by a 4-day *Recovery* phase at 20 °C. The *Control* group was kept at 20 °C during 27 days. The *Whole MHW* consisted in 3 phases separated by rapid temperature changes: *MHW<sub>1</sub>* (first 10-day phase at 25 °C), *Return* (24-h phase at 20 °C) and *MHW<sub>2</sub>* (second phase of 10 days at 25 °C). **B: Parameter measurements.** The valve behaviour was recorded during 27 days and gills sampling were performed for gene expression at days 0, 10, 12, 22, 27.

using LabView 8.6 software (™National Instruments, Austin, TX, USA).

Different valve activity parameters were analysed for each individual. The hourly VOD is expressed as the percentage of time the mussel spends with its valves open. 100 % and 0 % indicate that the valves are open and closed for the entire hour, respectively. The hourly  $VOD_{amp:50-75}$  is expressed as the percentage of time the mussel spends with its valves open at an amplitude between 50 % and 75 % (100 % being the maximum valve opening amplitude, specific to each individual). The hourly number of VMC is the number of partial and rapid closures of the valves over 1 h.

A mean value of the 3 parameters (VOD,  $VOD_{amp:50-75}$  and VMC) are calculated for each phase studied (*Whole MHW*, *MHW<sub>1</sub>*, *Return*, *MHW<sub>2</sub>* and *Recovery*).

## 2.2.4. Measurement of gene expression

**2.2.4.1. Mussel sampling.** For each of the two groups, six animals were collected from the aquaria at 5 different days (30 mussels per treatment in total) during the 27-day experimental period, i.e. at D0, D10, D12, D22 and D27 (Fig. 1B). For the *MHW* group, these days correspond to:

- D0: the end of the acclimation period, just before the start of the MHW when the water temperature was at 20 °C.
- D10: the end of the *MHW<sub>1</sub>* phase when the water temperature was at 25 °C.
- D12: the end of the *Return* phase when the water temperature was at 20 °C.
- D22: the end of the *MHW<sub>2</sub>* phase when the water temperature was at 25 °C.
- D27: the end of the *Recovery* phase, 4 days after the end of the *Whole MHW*.

For each mussel, gills samples were dissected and then placed in RNAlater (Thermo Fisher Scientific) before being stored at -80 °C for later analysis.

**2.2.4.2. RNA extraction and cDNA synthesis.** Total RNA extraction was performed from 30 to 50 mg of gills by adjusting the protocol of the SV-Total RNA Isolation System kit (Promega). The purity and quantity of the extracted RNAs were controlled by spectrophotometry (Synergy HTX, BioTeK). The cDNA synthesis was performed on 1.5 µg RNA

equivalent using the GoScript™ Reverse Transcription System kit (Promega).

**2.2.4.3. BRYT green qPCR.** Real-time quantitative PCR was performed with the GoTaq® qPCR Master Mix kit (Promega) on 10 ng of cDNA equivalent, with a final concentration of 200 nM for each of the primers, on the Light Cycler® 480 Instrument machine (Roche). Five genes of interest were studied, related to: oxidative stress *Superoxide dismutase* (SOD), cellular apoptosis *Caspase 3* (CASP3), metabolism *ATP synthase* (ATPs), circadian clock *Period* (Per), general stress response *Heat Shock Protein 70* (*hsp70*). Two candidate housekeeping gene expressions were also measured: *Elongation factor 1* (*Ef1*) and *18S ribosomal RNA* (*18S*). All primers sequences used in the study are listed in Table S1 in the Supplementary material section. All samples were run in duplicates. The  $2^{-\Delta Ct}$  method (Livak and Schmittgen 2001) was used to determine the relative expression (RE) of the genes of interest, where  $\Delta Ct_{gene\ of\ interest} = Ct_{gene\ of\ interest} - Ct_{housekeeping\ gene}$  with  $Ct$ , the cycle threshold at which amplification enters the exponential phase. The *Ef1* gene was selected as the housekeeping gene using BestKeeper, Normfinder and GeNorm (Xie et al., 2012).

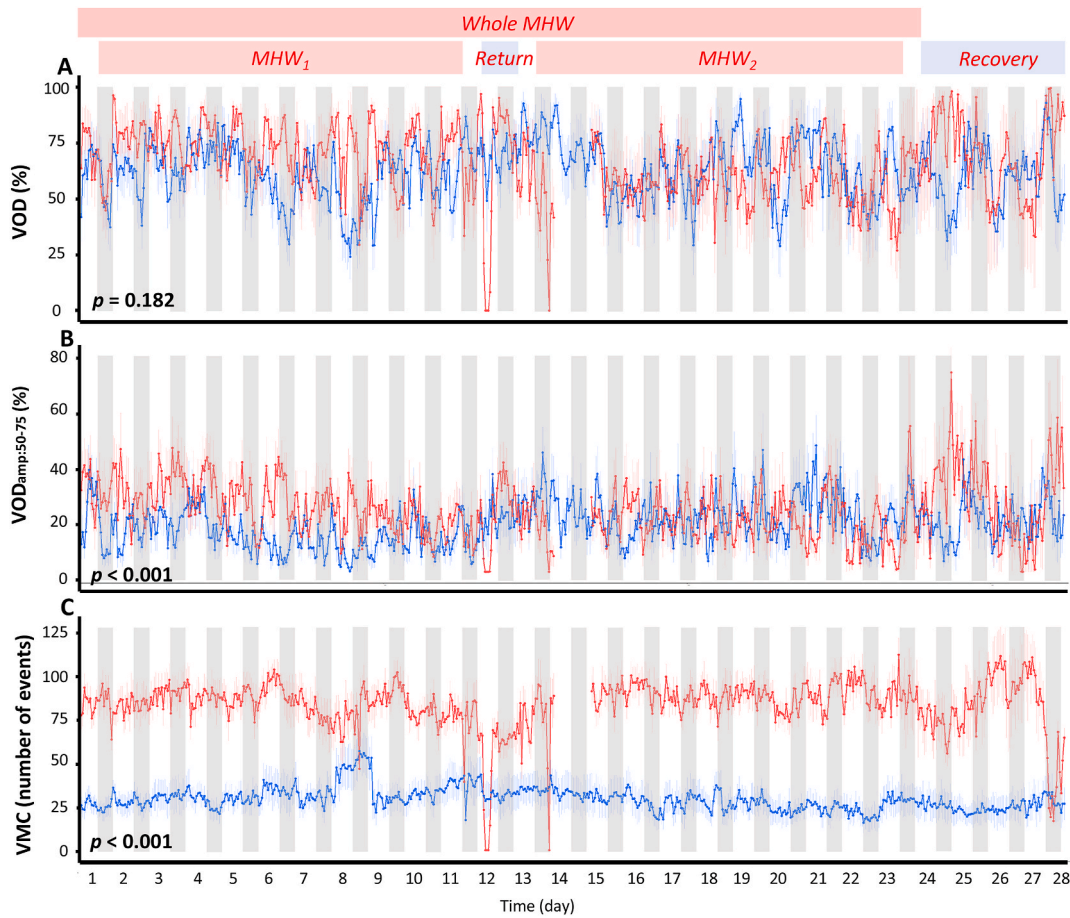
## 2.3. Statistical analyses

Data are expressed as mean ± SE (standard error). Differences between the *Control* and *MHW* groups for each variable studied (RE of gene, VOD,  $VOD_{amp:50-75}$ , VMC) were tested using Student's *t*-test. Prior to analyses, assumptions regarding normality of the data distribution (Shapiro-Wilk test) and homogeneity of variances (Brown Forsythe test) were tested. If one of the assumptions was not met, the nonparametric Mann-Whitney test was used. A significant difference was accepted for a *p*-value = 0.050.

## 3. Results

### 3.1. Valve activity behaviour

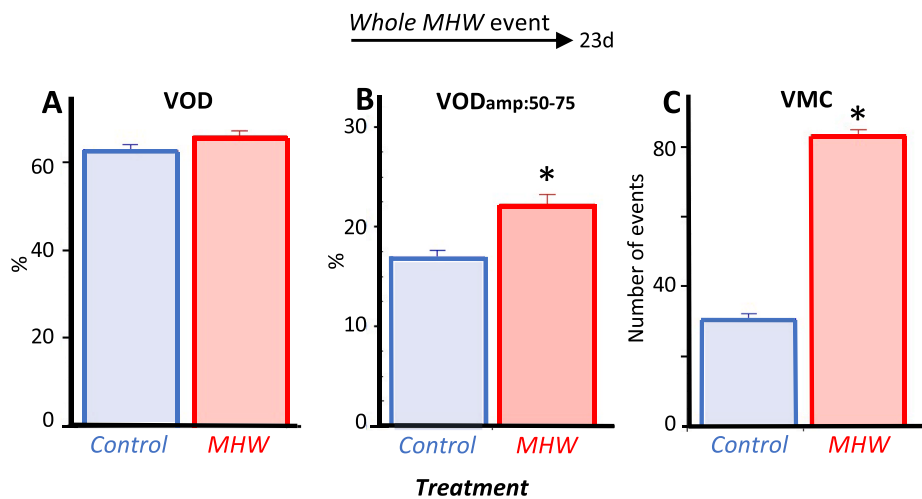
The mean hourly VOD (%),  $VOD_{amp:50-75}$  (%), and number of VMC events over the 27 days of the experiment in the *Control* and *MHW* groups are shown in Fig. 2A, B, 2C respectively. Note that a mortality was observed all along the experiment, including mussels equipped with electrodes, with a 2-times higher mortality for the *MHW* condition (*n* =



**Fig. 2. Hourly valve behaviour of *M. edulis* during the 27-day experiment.** Hourly valve opening duration (VOD, %), hourly valve opening duration at an amplitude ranging from 50 to 75 % (VOD<sub>amp:50-75</sub>, %), and hourly valve micro-closure events (VMC, number of events) in the Control (blue line) and MHW (red line) groups during the 27 days of experiment (mean ± SE). Grey bands indicate the 10 h night.

12) than for the Control condition (n = 6). No statistical difference was found for VOD between the two groups ( $p = 0.182$ ), whereas a significant increase in mean VOD<sub>amp:50-75</sub> ( $p < 0.001$ ) and mean VMC ( $p < 0.001$ ) was measured. To investigate the effect of the experimental

MHW, statistical analyses were performed on the 23 days of MHW (Whole MHW) and presented in Fig. 3. Regarding VOD, no significant statistical difference was observed between the Control and MHW groups (Fig. 3A). On the other hand, significant increases in mean VOD<sub>amp:50-75</sub>



**Fig. 3. Valve behaviour of mussels during the Whole MHW.** Mean VOD (%), VOD<sub>amp:50-75</sub> (%), and VMC (number of events) in the Control (blue) and MHW (red) groups during the Whole MHW (23 days) (mean ± SE). Stars indicate statistical differences between groups ( $p < 0.050$ ).

(+31.7 %,  $p < 0.001$ , Fig. 3B) and mean VMC (+170.7 %,  $p < 0.001$ , Fig. 3C) were measured in the *MHW* group compared to the *Control* group. For further information about the MHW effects, the mean valve activities of mussels during the *MHW*<sub>1</sub>, *Return*, *MHW*<sub>2</sub> and *Recovery* phases were presented in Fig. 4. Regarding VOD (Fig. 4A), the mean value was statistically higher in the *MHW* group compared to the *Control* group during the 10 days of the *MHW*<sub>1</sub> phase (+21.0 %,  $p < 0.001$ ). When the temperature returned to 20 °C for 24 h during the *Return* phase, the mean VOD in both groups was not significantly different. The *MHW*<sub>2</sub> phase then caused a significant decrease in mean VOD in the *MHW* group (-14.2 %,  $p = 0.004$ ). This difference disappeared during the *Recovery* phase ( $p = 0.097$ ). For VOD<sub>amp:50-75</sub> (Fig. 4B), the mean value was statistically higher in the *MHW* group compared to the *Control* group during the 10 days of the *MHW*<sub>1</sub> phase only (+92.7 %,  $p < 0.001$ ), no statistical difference was measured between the two groups during the *Return*, *MHW*<sub>2</sub> and *Recovery* phases. For the mean VMC (Fig. 4C) and compared to the *Control* group, the *MHW* group significantly increased the number of micro-closures during the *MHW*<sub>1</sub> (+163.5 %,  $p < 0.001$ ), *Return* (+66.5 %,  $p = 0.012$ ), *MHW*<sub>2</sub> (+206.6 %,  $p < 0.001$ ) and *Recovery* (+221.0 %,  $p < 0.001$ ) phases.

### 3.2. Relative gene expression

The effect of the MHW on the relative expression of the 5 genes of interest (*Per*, *ATPs*, *hsp70*, *SOD*, *CASP3*) is shown in Fig. 5. No statistical difference in relative expression was measured for *Per*, *ATPs*, *hsp70*, *SOD* genes between the *Control* and *MHW* groups at D0, contrary to the *CASP3* gene where an overexpression was measured in the *MHW* group

( $p = 0.013$ ). Regarding the effect of the MHW on gene expression, similar results were obtained for *Per* and *CASP3* genes. Indeed, the expression of *Per* and *CASP3* genes in mussel gills exposed to the MHW was significantly reduced compared to the *Control* group, either at the end of *MHW*<sub>1</sub> phase (D10), at the end of the *Return* phase (D12), or at the end of *MHW*<sub>2</sub> phase (D22) (for the *Per* gene: at D10  $p = 0.009$ , at D12  $p = 0.006$ , at D22  $p = 0.019$ ; for the *CASP3* gene: at D10  $p = 0.012$ , at D12  $p = 0.017$ , at D22  $p = 0.006$ ). This difference disappeared at the end of the *Recovery* phase (D27), 4 days after the MHW. For the *ATPs* gene, the MHW did not lead to a difference in relative expression at the end of the *MHW*<sub>1</sub> phase (D10) between the two groups. But then, its expression was significantly reduced at D12 and D22 in the *MHW* group ( $p = 0.003$  and  $p = 0.030$ , respectively) and this under-expression persisted at D27 ( $p = 0.008$ ) during the *Recovery* phase. For the *hsp70* gene, no significant difference in relative expression was measured during the MHW between the two groups (at D10, D12 and D22). Only an under-expression of the gene was measured during the *Recovery* phase (D27) for the *MHW* group ( $p = 0.009$ ). Finally, no significant differences in the relative expression of *SOD* were observed over the experiment between the *Control* and *MHW* groups.

### 4. Discussion

The aim of this study was to evaluate the response of *M. edulis* during a realistic 23-day MHW and to assess its ability to recover 4 days after the MHW. Our results show that the MHW altered the behaviour of the mussels and led to a general under-expression of the studied genes.

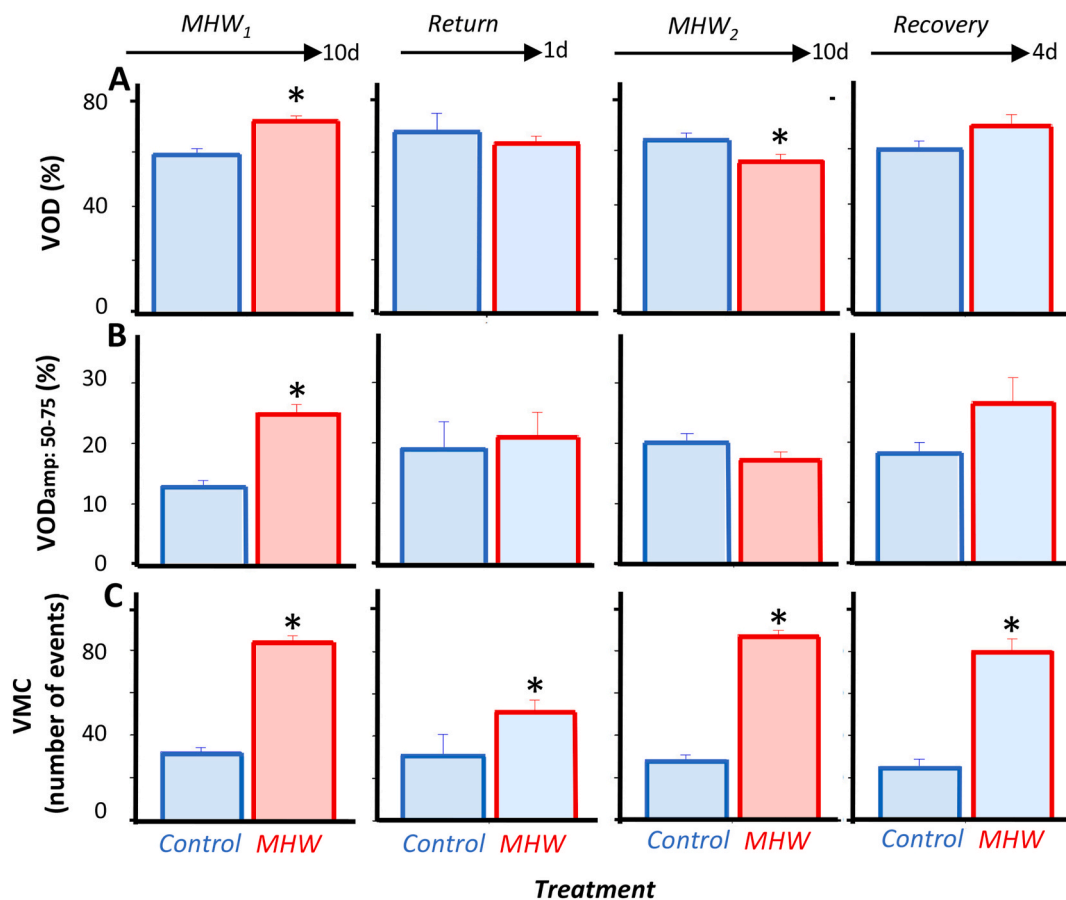


Fig. 4. Valve behaviour of mussels during the different phases of the Whole MHW and the Recovery phase. Mean VOD (%), VOD<sub>amp:50-75</sub> (%), and VMC (number of events) in the *Control* (blue line) and *MHW* (red line) groups during the *MHW*<sub>1</sub> (10 days), *Return* (1 day), *MHW*<sub>2</sub> (10 days), and *Recovery* (4 days) phases (mean ± SE). The interior of the barplots is color-coded to indicate the temperature mussels experienced during the measurement of their valve behaviour: blue is for 20 °C, and red is for 25 °C. Stars indicate statistical differences between groups ( $p < 0.050$ ).

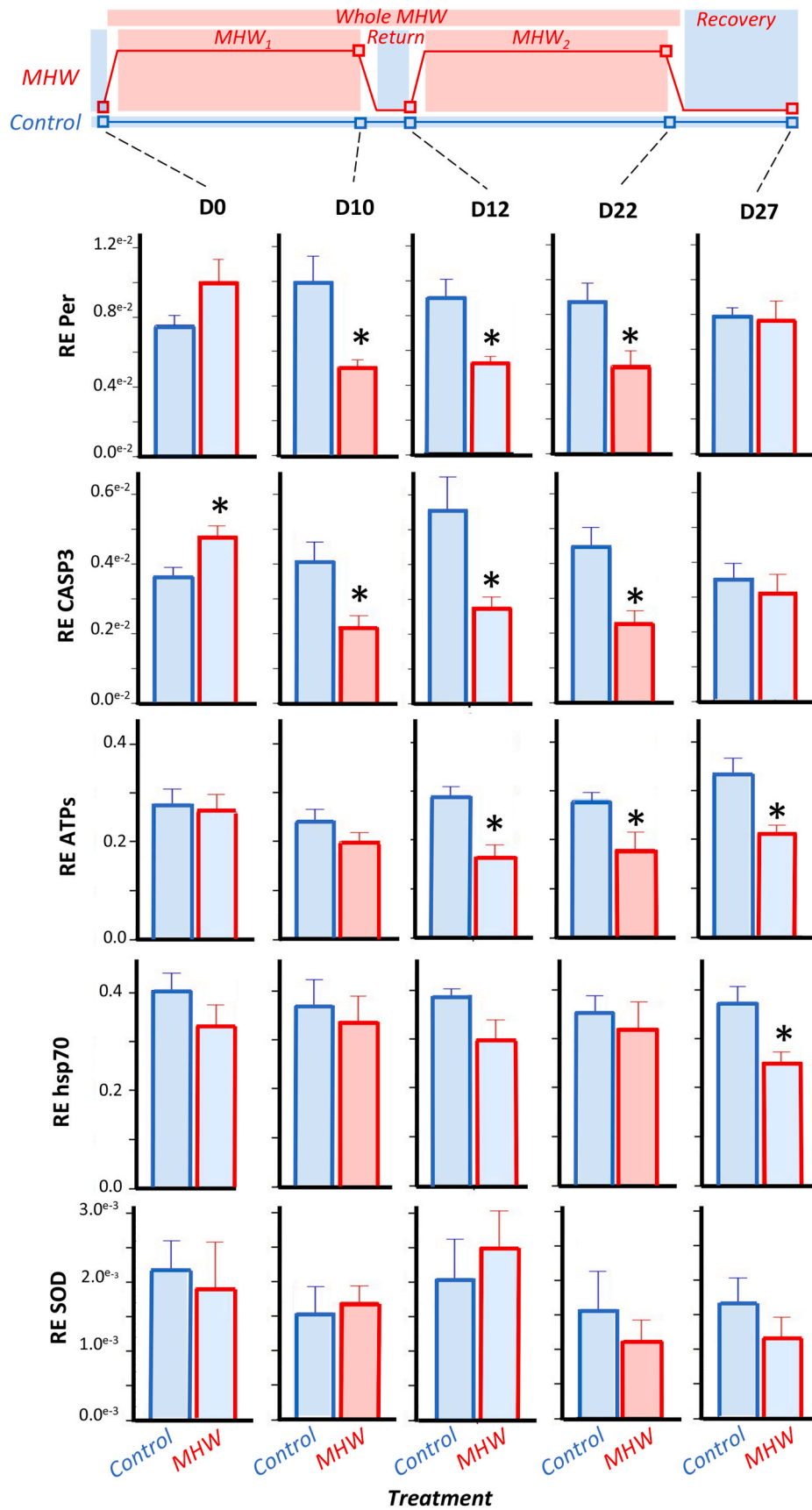


Fig. 5. Relative expression level (RE) of 5 genes of interest in the gills of *M. edulis* during key phases of the MHW event. RE of *Per*, *CASP3*, *ATPs*, *hsp70*, and *SOD* in gills of the Control (blue line) and MHW (red line) groups at D0 (before the Whole MHW), D10 (end of MHW<sub>1</sub>), D12 (end of the Return phase), D22 (end of MHW<sub>2</sub>) and D27 (end of the Recovery phase) (mean ± SE, n = 6 per condition and per sampling day). The interior of the barplots is color-coded to indicate the temperature at which mussels were collected from the aquaria: blue is for 20 °C, and red is for 25 °C. Stars indicate statistical differences between groups (p < 0.050).

#### 4.1. Disruption of mussel valve activity by the MHW

Valve activity in bivalves can be used as an indicator of environmental stress (Payton et al., 2017). However, to our knowledge, no study has yet evaluated the effect of a MHW on this behaviour. First in our study, we observed a higher mortality of mussels exposed to the MHW of +5 °C (from 20 to 25 °C). Conversely, in the study of Kamermans and Saurel (2022) the authors showed that *M. edulis* can survive within a temperature range of 3–25 °C when acclimated progressively. Our findings align with those of Feidantsis et al. (2020), who measured a gradual increase in *M. galloprovincialis* mortality 10 days after an acute temperature change from 18 to 26 °C. Our results, along with those of Feidantsis et al. (2020), suggest that the intensity and duration of thermal stress associated with MHWs reduce the thermal tolerance of mussels. This suggestion is further supported by observations of mortality events among mussels in the field linked to MHWs (Galil et al., 2022; Seuront et al., 2019). Then, the results show that the experimental MHW significantly affected the behaviour of *M. edulis*, in particular by increasing the number of micro-closure events, revealing a stressful condition. Micro-closures are rapid valve movements achieved through contraction of the adductor muscle that allow for the renewal of water in the mantle cavity. Several studies have highlighted an increased number of micro-closures as a marker of stress in bivalves for example during exposure to trace metals (Tran et al., 2004), or toxic algae (Tran et al., 2010). In our study, the number of VMC increased throughout the MHW in individuals exposed to temperature variations. Moreover, while the increase in the number of VMC was reduced during the 24 h-Return phase after  $MHW_1$ , the increase of VMC was maximal during the 4-day Recovery phase at the end of the experiment, which indicates again the cumulative stressful effect of the multiple temperature variations ( $MHW_1$  and  $MHW_2$ ) that persist even after MHW exposure. Regarding the effect of MHW on valve opening duration, and valve opening duration at high amplitude (i.e. ranging from 50 to 75 %) during the Whole MHW (23 days), a stable VOD and an increased  $VOD_{amp:50-75}$  were observed. However, when the effect of the MHW is analysed in finer detail, we find again a cumulative effect of multiple temperature variations. Indeed, while in the first phase of the MHW ( $MHW_1$ ), individuals increased their VOD and  $VOD_{amp:50-75}$ , in the second phase ( $MHW_2$ ), VOD dropped and  $VOD_{amp:50-75}$  was no longer significantly different from Control individuals. This change in behaviour could reflect a change in their metabolism due to heat stress. Indeed, an increase in VOD and  $VOD_{amp:50-75}$  during the  $MHW_1$  suggests an increase in oxygen consumption or higher food intake to meet the increased temperature-related energy demand (Sokolova et al., 2012). The study by Thyrring et al. (2015) is consistent with this hypothesis. The authors measured an increase in Standard Metabolic Rate (SMR, metabolic rate measured via O<sub>2</sub> consumption of individuals and reflecting their maintenance cost) up to the maximum acclimation temperature of 25 °C beyond which SMR dropped in *M. edulis* collected in a temperate area. This study indicates that at 25 °C individuals have an increased energy requirement to sustain life, expressed by the increase in their oxygen consumption. Additional stress could therefore lead them to exceed their tolerance threshold. In this sense, the rapid temperature variations of the second phase of the MHW ( $MHW_2$ ) could represent an additional stress for *M. edulis* that would explain the decrease in VOD compared to the Control treatment. In bivalves, valve-closing behaviours are considered a defensive mechanism in response to stressful conditions (Tran et al., 2004) and can induce metabolic depression in an effort to conserve energy (Ortmann and Grieshaber 2003). Indeed, the closure of the valves interrupts the oxygen and nutrient supply and slows down the metabolism. In this sense, Anestis et al. (2007) noted an increase in the duration of valve closure (x 6) in *M. galloprovincialis* progressively (0.6 °C.h<sup>-1</sup>) exposed to 24 °C compared to individuals acclimated to 18 °C. This closure was associated with a metabolic depression expressed by a decrease in the activity of the enzyme pyruvate kinase PK (indicating low glycolytic rates to produce ATP). Thus, based on the

results of our study, it would have been interesting to measure aerobic metabolism (e.g. O<sub>2</sub> consumption) and/or anaerobic metabolism (e.g. lactate content) to understand whether valve closure is indeed a strategy used by mussels to switch metabolism and conserve energy when exposed to a MHW.

So far, little is known about the behavioral responses of bivalves exposed to MHWs. A recent study highlighted that a 30-day MHW of +10 °C (from 20 to 30 °C) can alter the behaviour of clams *Ruditapes philippinarum* by increasing the duration of burrowing, which could lead to higher vulnerability to predation and decrease species fitness, as suggested by the authors (Peruzza et al., 2023). In our study, a change in valve behavior of individuals exposed to a MHW is of particular concern as it is closely linked to important physiological processes such as respiration and feeding (Ortmann and Grieshaber, 2003) and could impact the fitness of individuals. Alterations in mussel populations due to MHWs could lead to ecosystem-level responses. As habitat-forming species, mussels play a critical role in their environment by creating diverse species assemblages. Their function as filter feeders provides essential ecosystem services by removing toxins and bacteria, and they serve as a food source for various organisms, including humans (Zippay and Helmuth, 2012). Thus, the cascading impacts on other species due to mussel's beds disturbance could have significant ecological and economic implications.

#### 4.2. Changes in gene expression in *M. edulis* during the MHW

Overall, our study showed an under-expression of genes related to cellular response to stress, energy metabolism, and the circadian clock in the gills of *M. edulis* exposed to an experimental MHW.

This transcriptional depression generated by heat stress has also been measured in other studies, including the transcriptomic analysis of He et al. (2023) performed on the pearl oyster *Pinctada maxima* exposed to two consecutive MHWs. Among the processes affected were bio-mineralization, cell proliferation, molecular defence, and energy metabolism, suggesting a strong negative response of individuals to the MHWs.

##### 4.2.1. Effect of the MHW on the expression of a gene related to cellular metabolism: ATPs

The ability of individuals to maintain energy homeostasis is critical to their stress tolerance (Sokolova et al., 2012). Such as in mammals, mitochondrial ATP synthase in bivalves is an enzyme that serves to produce cellular energy in the form of ATP using oxygen as the final electron acceptor. Changes in ATP synthase activity reflect alterations in cellular energy demands, metabolic flux, and mitochondrial function, making it a sensitive indicator of energy metabolism. The first phase of the MHW ( $MHW_1$ ) did not alter *ATP synthase* gene expression in *M. edulis* compared to individuals in the Control group that remained at 20 °C. However, the expression level of the gene encoding the enzyme was reduced at D12 and D22 of the MHW, as well as after 4 days of recovery at D27, which could suggest a decrease of the activity of the ATP synthase and consequently a decrease of ATP production by the aerobic pathway. It would be pertinent to measure the activity of this key enzyme, the ATP synthase, in order to confirm the hypothesis of a reduced aerobic metabolism due to the exposure of the MHW. The down-regulation of *ATP synthase* gene expression measured in our study appears to be synchronous with a decrease in VOD in the MHW group during the  $MHW_2$  phase, and strengthens the hypothesis of a reduced aerobic metabolism. In the study by Torossian et al. (2020), a heat shock from 17.5 °C to 25.5 °C (in 2 h) did not alter the activity of citrate synthase (a mitochondrial respiratory chain enzyme) in *M. edulis* adductor muscle tested 1 h after the thermal shock. However, a heat shock from 17.5 °C to 31.5 °C, a temperature close to the lethal thermal limit of *M. edulis* (Kamermans and Saurel 2022), led to a decreased activity of the citrate synthase as well as a decreased heart rate of the mussels suggesting, according to the authors, a metabolic arrest. The



decreased *ATP synthase* expression level in our study suggests that even far below thermal limit, the duration of MHWs and multiple temperature variations of MHWs could also compromise mussel's metabolism and impair their fitness. Furthermore, the persistence of gene down-regulation 4 days after the MHW highlights the low recovery capacity of mussels and may limit the tolerance of individuals exposed to additional heat stress as in the case of multiple MHWs (Seuront et al., 2019).

#### 4.2.2. Effect of the MHW on the expression of a gene related to response to stress: *hsp70*

HSPs are major players in cellular response to stress by stabilizing or restoring the three-dimensional structure of denatured proteins (Parsell and Lindquist 1993). HSP70 are among the most studied stress proteins in marine invertebrates and may be involved during heat stress in *Mytilus* sp. (Lockwood et al., 2010). In our study, no changes in expression level of the *hsp70* gene were observed during the MHW. Our results are consistent with those of Xu et al. (2022), who observed no change in the regulation of the *hsp70* gene in the gills of the oyster *Pinctada maxima* exposed to two successive MHWs of similar intensity compared to our experiment (+4 °C, from 24 to 28 °C). It is known that the expression levels of *hsp*s may vary with time (Liu et al., 2014; Dimitriadis et al., 2012). For example, Feidantsis et al. (2020) measured the mRNA expression levels of *hsp70* and *hsp90* in the mantle and posterior adductor muscle of *M. galloprovincialis* exposed to a temperature increase from 18 to 26 °C. They measured an initial increase of *hsp*s expression level within the first 5 days of exposure, peaking within 3–5 days, followed by a decrease on the 10th day. Notably, the expression levels of *hsp70* and *hsp90* remained elevated at day 10 for mussels exposed to 26 °C compared to those exposed to 18 °C. In our study, we exposed *M. edulis* to temperature changes of a similar range (from 20 to 25 °C) than the study of Feidantsis et al. (2020), however we measured the *hsp70* expression levels later, i.e. 10 days after the two water temperature increase events. It is possible our sampling times did not allow us to measure any change in *hsp70* expression levels, even if it happened. It can be suggested that *M. edulis* first increased their *hsp70* expression levels, then decreased them substantially to reach the control levels within or before the 10 days after the water temperature changes. To confirm this hypothesis, it would be relevant to measure the kinetics of *hsp70* expression levels during the MHW at different times following the temperature changes. Furthermore, measuring the HSP70 protein levels could enhance our comprehension of the cellular stress response in mussels exposed to a MHW, as their translation may not correlate with mRNA transcription (Feidantsis et al., 2020). In our study, we also measured a decreased expression 4 days after the MHW, indicating a delayed effect of heat stress. In the study by Wang et al. (2018), the authors also showed that *hsp70* gene synthesis could be inhibited in the gills of the mussel *Perna viridis* following heat stress of +17 °C (from 25 to 42 °C, 5.7 °C.h<sup>-1</sup>). The authors suggest that this acute temperature change caused extreme stress in this species. Thus, our results suggest again that the duration and the complexity of the 23-day MHW temperature changes could represent an important stress to *M. edulis*, even if the temperature increase is limited to 5 °C.

#### 4.2.3. Effect of the MHW on the expression of a gene related to cell apoptosis: *CASP3*

When cellular damage exceeds a threshold and cannot be properly repaired, a programmed cell death called apoptosis can be initiated. Caspase-3 plays a key role in the apoptosis signalling pathway as an executor (Earnshaw et al., 1999). Previous studies have shown that caspase-3 is induced during heat stress in bivalves (Wang et al., 2018). In our study, the mRNA level of *CASP3* was decreased in the gills exposed to the MHW during the whole extreme event (D10, D12, D22), while a difference in gene expression between exposed and control groups was no longer observed after 4 days of recovery. In the study by Yao and Somero (2012), a heat shock from 13 °C to 28 °C induced in

*M. galloprovincialis* an increase in *CASP3* mRNA level after 8 h. In contrast, a more intense heat shock (from 13 °C to 32 °C) did not alter gene expression, yet DNA damage was highest at 32 °C. This result suggests that the decrease in *CASP3* expression between individuals exposed to 28 °C and 32 °C is not the result of a reduction in cellular damage to be eliminated by apoptosis but rather the result of greater heat stress at 32 °C in *M. galloprovincialis*, which disrupted the synthesis of *CASP3*, a major component of the cellular stress response. In our study, it would have been interesting to assess if the reduced expression of *CASP3* was associated with an increase in cellular damages by measuring for example the lipid peroxidation products (malondialdehyde) or the lysosomal integrity (accumulation of lipofuscin and neutral lipids).

#### 4.2.4. Effect of the MHW on the expression of a gene related to antioxidant defence: *SOD*

Previous studies have highlighted the pro-oxidant nature of heat stress and the antioxidant response of bivalves (Wang et al., 2018). For example, after an 11-day exposure to a MHW of an intensity of 8 °C (17 °C–25 °C, 1.8 °C.h<sup>-1</sup>), Amorim et al. (2020) measured an elevation of antioxidant defences activity (*SOD* and *CAT*) in the bivalve *Scrobicularia plana*, which would have protected cells from cellular damage, as illustrated by the lack of appearance of malondialdehyde. Wang et al. (2018) measured an increase in ROS (reactive oxygen species) production as well as significant activation of *SOD*, *CAT*, *GSH-Px* enzyme activity in gills of *P. viridis* mussels exposed to a thermal ramp from 25 °C to 35 °C over 3 h. *SOD* is a primary scavenger of ROS in tissues and converts superoxide radicals to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and oxygen molecule (O<sub>2</sub>). In our study, the expression level of *SOD* remained identical between the exposed and control mussels throughout and after the MHW. It is possible that other antioxidant mechanisms were involved to counteract the effects of ROS. In this way, Nardi et al. (2022) measured in the digestive gland of *M. galloprovincialis* exposed to a 11-days MHW of an intensity of 4.5 °C (from 18 °C to 22.5 °C, 0.9 °C.day<sup>-1</sup>) a significant increase in the enzymatic activity of glutathione reductase (*GR*), glutathione peroxidase (*GPx*), while *SOD* and *CAT* had unchanged levels on the sixth day of the MHW as well as immediately at the end of the extreme episode (day 11) and 10 days after it. In our study, measurement of additional genes related to antioxidant defence such as *CAT* or *GR*, as well as measurement of markers of lipid peroxidation, may have revealed an oxidative stress induced by our experimental MHW. Another hypothesis is that the lack of MHW effect on the level of *SOD* expression in our study would be due to a slowdown of aerobic metabolism. Indeed, the major ROS production (90 %) is the result of the mitochondrial respiratory chain activity (Wilhelm Filho 2007). As discussed previously, the reduced expression of *ATP synthase* and the reduced *VOD* observed in our study in organisms exposed to MHW would suggest a decrease in aerobic metabolism in response to MHW. This defence mechanism would thus lead to a reduced production of ROS that could explain the absence of an increase of antioxidant defence.

#### 4.2.5. Effect of the MHW on the expression of a circadian clock gene: *Per*

Circadian clock genes regulate biological rhythms, essential for the synchronisation between physiological functions and cyclic environmental parameters, optimizing the fitness and survival of organisms (Payton et al., 2017; Tran et al., 2020). Recent studies have characterized these genes in mussels (Connor and Gracey 2011) including the *Per* gene (Chapman et al., 2020). The effect of a MHW on clock genes has never been studied before. However, it is known that high temperature during emersion influences the rhythmicity of clock genes (Connor and Gracey 2011). In our study, the 23 days of the MHW induced under-expression of the *Per* gene. If *Per* expression is altered, this may suggest that daily rhythm expression is disrupted upon exposure to MHW. The resulting disruption of the circadian clock mechanism by MHW could have ecological implications in mussels since biological

rhythms are essential for organisms to adapt to their biotope (Tran et al., 2020). However, the expression level of the *Per* gene was not disturbed anymore 4 days after the end of the MHW, suggesting a return to a normal daily rhythm.

## 5. Conclusion

This study provided valuable data on the behaviour of bivalves exposed to a realistic MHW of +5 °C. Our results showed that the thermal stress of the MHW negatively impacted the valve activity of *M. edulis* and this behaviour changed over time suggesting the cumulative effect of temperature variations. The change of behaviour recorded could reflect a change in the metabolism of *M. edulis* due to heat stress, as valve opening influences access to environmental oxygen. This hypothesis was supported by the under-expression of a gene related to energy metabolism: ATPs. Thus, it would be relevant to further analyze the effects of this MHW on the metabolism of mussels, for example by measuring the mitochondrial efficiency (ATP/O, i.e. ratio between ATP synthesis and oxygen consumption). For some behavioural parameters and genes expressions, the effects persisted after the MHW suggesting a limited ability of mussels to recover rapidly from the MHW and indicating the importance of considering this period when attempting to understand the effects of MHWs. Together, these results suggest that the stress generated by the MHW could compromise the fitness of the mussels.

## CRedit authorship contribution statement

**Margot Grimmelpont:** Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Laura Payton:** Writing – review & editing, Methodology, Formal analysis, Conceptualization. **Christel Lefrançois:** Funding acquisition, Conceptualization. **Damien Tran:** Writing – review & editing, Methodology, Formal analysis, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.marenvres.2024.106418>.

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