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Can temperature rise change the impacts induced by e-waste on adults and sperm of *Mytilus galloprovincialis*?

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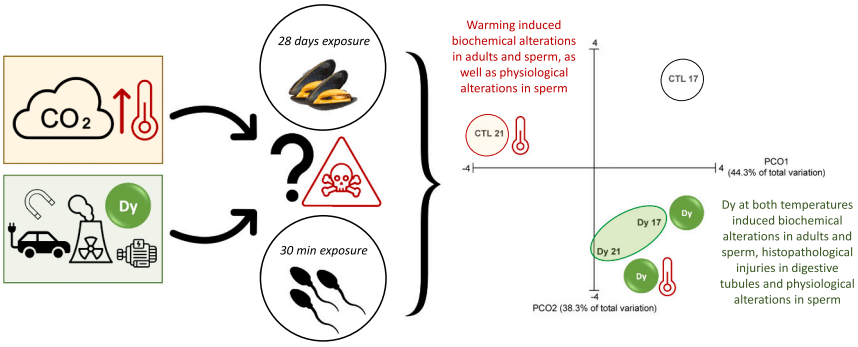
HIGHLIGHTS

- Mussels accumulate dysprosium (Dy) at 17 and 21 °C
- All the stressors tested induced loss of redox balance and cellular damage in adults
- Dy at both temperatures caused histopathological injuries in digestive tubules
- Sperm motility was impaired by all stressors.
- When combined, the impacts of Dy overlap temperature effects

GRAPHICAL ABSTRACT



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ABSTRACT

Nowadays, it is of utmost importance to consider climate change factors, such as ocean warming, since the risk of negative impacts derived from increased surface water temperature is predicted to be high to the biodiversity. The need for renewable energy technologies, to reduce greenhouse gas emissions, has led to the increasing use of rare earth elements (REEs). Dysprosium (Dy) is widely used in magnets, motors, electrical vehicles, and nuclear reactors, being considered a critical REE to technology due to its economic importance and high supply risk. However, the increasing use of this element contributes to the enrichment of anthropogenic REEs in aquatic systems. Nevertheless, the information on the potential toxicity of Dy is limited. Moreover, the effects of pollutants can be amplified when combined with climate change factors. Thus, this study aimed to assess the effects of Dy (10 μg/L) in the species *Mytilus galloprovincialis* under actual (17 °C) and predicted warming conditions (21 °C). The Dy concentration in contaminated mussels was similar between temperatures, probably due to the detoxification capacity in individuals under these treatments. The combined stressors affected the redox balance, but higher impacts were caused by Dy and warming acting alone. In terms of cellular damage, although Dy acting alone was prejudicial to mussels, warming and both stressors acting together induced higher levels of LPO and

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PC. The histopathological effects of Dy in the digestive tubules were independent of the temperature tested. Regarding effects on sperm, only warming induced cellular damage, while both stressors, alone and together, impaired sperm movement. Overall, this study highlights that warming might influence the effects induced by Dy, but greater impacts were caused by the element. Eventually, the tested stressors may have consequences on mussels' reproduction capacity as well as their growth, abundance, and survival.

1. Introduction

Since the beginning of industrialization, human influence has been a major contributor to climate change, constantly damaging the environment (Ghazali and Ali, 2019; IPCC, 2021). It is stated that the release of greenhouse gases (GHG) by human activity into the atmosphere is the major source of climate change (IPCC, 2021; Montzka et al., 2011; Wang et al., 2017). The concentrations of GHG have been increasing since the industrial revolution (Ghazali and Ali, 2019), with the growth in carbon dioxide (CO_2) and methane (CH_4) concentrations far exceeding the natural changes between glacial and interglacial periods (IPCC, 2021). The emissions of CO_2 are believed to be the main source of global warming (Soytas et al., 2007). The latest report of the United Nations Intergovernmental Panel on Climate Change (IPCC) demonstrated that in 2019 the concentration of CO_2 reached 410 parts per million (ppm) and that from the pre-industrial period until 2019 the GHG emissions contributed to a temperature rise between 1 and 2 °C (IPCC, 2021). Atmospheric and land warming, as well as ice loss, accounted for 9 % of the observed heating increase between the pre-industrial period and 2018, while ocean warming corresponded to the remaining 91 % (IPCC, 2021). From the pre-industrial time until 2020 the ocean temperature increased by 0.88 °C. It is expected that the ocean surface temperature will increase between 2.01 and 4.07 °C in the worst case scenario (SSP5–8.5) between the periods 1995–2014 and 2081–2100 (IPCC, 2021). Furthermore, it is predicted that climate change will have negative consequences on biodiversity, leading in the worst scenario to the extinction of species (Bellard et al., 2012; Cahill et al., 2013; Thomas et al., 2004). Several studies, such as the ones conducted by Leite et al. (2023), Maia et al. (2022), Velez et al. (2017) and Suárez et al. (2020) already showed that the rise in water temperature (4 °C increased compared with control temperature in the case of mussels, clams and shrimps, and 16 °C in the case of anemones) can cause negative impacts on aquatic organisms, such as histopathological, physiological and biochemical alterations.

To reduce the emissions of GHG, the so-called "renewable energy technologies" such as solar and wind power as well as hybrid and electric vehicles have gained attention. Some of the main raw materials used in these technologies are rare earth elements (REEs) (Groves et al., 2023). These elements were defined by the International Union of Pure and Applied Chemistry (IUPAC) as a group of seventeen elements including the fifteen lanthanoids plus scandium and yttrium (Balaram, 2019). Because of their unique magnetic, catalytic and phosphorescent properties, in addition to renewable energy technologies, REEs are also used in electronics, medicine and manufacturing (Balaram, 2019). Dysprosium (Dy), discovered by Paul Émile Lecoq de Boisbaudran in 1886 (Szabadvary, 1988), is considered a heavy REE due to its high atomic weight (Schüler et al., 2011) and was identified by the European Commission Report (European Commission, 2020) as a critical element due to its high economic importance and high risk of supply. Among the lanthanides, Dy has one of the highest magnetic properties (Ganjali et al., 2016) and most of the demand comes from the permanent magnet industry (Hoenderdaal et al., 2013). Due to its magnetic properties, Dy is also used in wind power (Groves et al., 2023), magnetic resonance imaging as well as X-ray imaging (Rim et al., 2013), and in Terfenol-D alloy (Hoenderdaal et al., 2013). Furthermore, due to its high capability to sorb thermal neutrons it is used in the nuclear industry (Sinha and Sharma, 2005). Additionally, its phosphorescent properties have led to its utilization in white light-emitting diodes (Ganesh Kumar et al., 2020), while its electrical properties make it suitable for implementation in multilayer ceramic capacitors (Kim et al., 2011). The increased use of REEs in the last decades led to the release of these elements into the environment (Merschel and Bau, 2015). The amount of Dy found in the environment differed between locations, with low values found at the Rhine estuary (between 0.0035 and 0.0162 μg/L, Sneller et al., 2000); Bonnail et al. (2017) detected 4.80 μg/L of Dy in the Odiel river that is affected by acid mine drainage; in the ex-mining pit lake in Malaysia the Dy concentration found was 12.1 μg/L (Adeel et al., 2019); while at the Berkeley Pit Lake in Montana higher concentrations were found as it is an abandoned open pit mine (ranging from 172 to 186 μg/L, Gammons et al., 2003). Due to the increased concentration of Dy in aquatic systems, several authors highlight the importance to study the effects of this element, such as Freitas et al. (2020) showing that Dy induced cellular damage and loss of redox balance in the species *Mytilus galloprovincialis*. Exposure of *Paracentrotus lividus* to Dy resulted in loss of fertilization success, increase of mitotic aberration, higher production of reactive oxygen species (ROS), and caused defects in the development of the larvae (Oral et al., 2017). Hanana et al. (2021) showed that Dy can induce genotoxic and inflammatory effects in rainbow trout.

In addition to the direct consequence of temperature rise in organisms, warming can alter the behaviour and distribution of pollutants (Manciocco et al., 2014), as well as change their bioaccumulation (Bai and Wang, 2020; Leite et al., 2023). Furthermore, warming can also increase the toxicity of the pollutants and change the organisms' responses towards stress (Coppola et al., 2018; Figueiredo et al., 2020; Guo et al., 2018). Bivalves, in particular mussels, are ideal organisms for monitoring environmental stressors and are globally used as bioindicators due to their tolerance to contaminants, high accumulation capacity, abundance, wide geographical distribution, and sedentary lifestyle (Goldberg, 1975; Zhou et al., 2008). Therefore, this study aimed to investigate the effects of Dy (10 μg/L) in the species *Mytilus galloprovincialis* under actual (17 °C) and predicted (21 °C) warming conditions, assessing the influence of temperature rise on the impacts caused by this element. For this, histopathological and biochemical alterations were evaluated in adult mussels, as well as biochemical and physiological alterations in mussels' sperm. Freitas et al. (2020) already showed the effects of Dy on the same species, however to our knowledge no information is available on the influence of temperature rise on the biochemical and histopathological effects of Dy on adults of the species *M. galloprovincialis*, as well as its effects on male gametes of this species.

2. Materials and methods

2.1. Sampling and experimental conditions

The experiment with adult mussels was carried out for twenty-eight days and for this *Mytilus galloprovincialis* specimens were collected in the Ria de Aveiro coastal lagoon (Portugal). The exposure of sperm to the treatments lasted 30 min and mussels were collected in the Gulf of La Spezia (Italy). Although the adult mussels used for both experiments were from two different populations, previous studies demonstrated similar responses in adults of both locations under control conditions (de Marchi et al., 2020; Leite et al., 2023).

The tested treatments were: CTL 17 °C (0 μg/L of Dy at control temperature – similar to the temperature registered in the sampling site at the time of the collection), CTL 21 °C (0 μg/L of Dy at warming conditions – considering an increase of 4 °C that resembles the

maximum value for the end of the century considering the worst-case scenario of the IPCC report (IPCC, 2021)), Dy 17 °C (10 μg/L of Dy at control temperature) and Dy 21 °C (10 μg/L of Dy subjected to warming conditions). The Dy concentration was chosen based on the values found in contaminated environments in Malaysia (12.1 μg/L of Dy, Adeel et al., 2019), Spain (4.80 μg/L of Dy, Bonnail et al., 2017) and Portugal (0–52 μg/L of Dy, with some sites presenting concentrations around 10 μg/L, Gomes et al., 2022). Furthermore, the concentration tested considered a previous study on Dy impacts on mussels (0–40 μg/L of Dy, Freitas et al., 2020).

2.2. Adults' exposure to dysprosium and warming

In the laboratory, adults of *M. galloprovincialis* (shell length: 66 ± 3.0 mm; width: 37 ± 2.6 mm) were placed in aquaria for acclimation to the laboratory conditions (temperature 17.0 ± 1.0 °C; salinity 30 ± 1; pH 8.0 ± 0.1; natural photoperiod) for the first week. In the subsequent week, mussels were divided into two groups in separate climatic rooms: one group remained at 17 °C, while the other one was acclimated to warming conditions, with a gradual increase in temperature in order to achieve 21 °C in the seawater at the beginning of the experiment. The remaining conditions were maintained similar to the first week and mussels were fed with Algamac protein plus (150,000 cells/animal/day). Seawater was prepared artificially by mixing a commercial salt (RED SEA SALT, Éilat, Israel) with deionized water. During this period, the seawater was continuously aerated and renewed every two days.

During the experimental period mussels were maintained in two climate rooms, one at 17 ± 1 °C (control temperature) and the other at 21 ± 1 °C (warming scenario). In each climatic room, mussels were distributed in different aquaria with 3 L of seawater each (five mussels per aquarium and three aquaria per treatment). Mussels were exposed to the previously described test treatments for twenty-eight days and were fed three times a week with the same food used for acclimation. During this period, the water in the aquaria was continuously aerated, and salinity, pH and photoperiod conditions were the same as in the acclimation period. Weekly Dy concentration was re-established, and seawater was totally renewed. Water samples were collected in the first week from the exposure aquaria (the ones with mussels) immediately after spiking to obtain the real exposure concentration of Dy, and from blanks (aquaria with 10 μg/L of Dy at both temperatures without organisms) immediately after spiking (0 h) and after 24, 48 and 168 h (before water renewal) to verify the Dy stability at both temperatures for one week. After the exposure period, one mussel per aquarium (three per treatment) was collected for histopathological analyses and three mussels per aquarium (nine per treatment) were used for biochemical analysis, with the remaining tissue from one mussel (three per treatment) being used for Dy quantification.

2.2.1. Dysprosium quantification

The quantification of Dy in water samples and mussels' tissues was performed by inductively coupled plasma mass spectrometry (ICP-MS) following the procedure explained by Leite et al. (2023). For the Dy quantification in tissues, one mussel per aquarium (three per treatment) was used. The quality control for the acid digestions of tissues was assured by running procedural blanks (without sample), certified reference material (CRM) BCR-668 (Mussel Tissue; 89 ± 0.6 μg/kg of Dy) and duplicates. Blanks were always below the quantification limit (LOQ) for Dy (0.01 μg/L), the percentage of recovery for CRM was 93 %, and the coefficient of variation of duplicates was 5 %.

2.2.2. Biochemical analysis

To perform the biochemical analysis mussels were homogenized individually using a mortar and a pestle under liquid nitrogen. After that the mussels were divided into five aliquots of 0.5 g fresh weight (FW). The biochemical parameters selected for this study are related to energy balance (electron transport system (ETS) activity, total protein (PROT) content); antioxidant and detoxification capacity (superoxide dismutase (SOD), catalase (CAT), carboxylesterases with *p*-nitrophenyl acetate (CbEs-pNPA) , glutathione S-transferases (GSTs), enzymes activities); redox balance and cellular damage (ratio between reduced and oxidized glutathione (GSH/GSSG), lipid peroxidation (LPO) and protein carbonylation (PC) levels). The extraction procedure and methodology for the determination of each biochemical parameter are described in detail in Andrade et al. (2022c) and Almeida et al. (2015).

2.2.3. Histopathological analysis

One mussel per aquarium was fixed in Davidson solution (glycerol, formalin, 95 % ethanol and seawater) for 24 h at room temperature. After that mussels were transferred to 70 % ethanol. Histopathological analyses were conducted in gills and digestive tubules. The inclusion and staining process, as well as the evaluation of the histopathological index (I_h) are described in Leite et al. (2020a, 2020b).

2.3. Sperm' exposure to dysprosium and warming

In the laboratory adult mussels (shell length: 60–70 mm) were kept in 10 L aquaria for acclimation to the laboratory conditions (temperature: 17.0 ± 1.0 °C and natural photoperiod) for two weeks. Natural seawater filtered at 0.45 μm was used and the initial salinity was 38 (similar to the sampling site), which was gradually decreased to a salinity of 30, so that these individuals were exposed to the same salinity as the mussels for the adults' test. During this period, seawater was continuously aerated and renewed every two days. After the seawater renewal mussels were fed with Algamac protein plus (CA, USA, 150,000 cells/animal/day).

The sperm collection was performed according to Mikhailov et al. (1997) protocol. For each parameter, sperm suspensions from three males (previously checked for morphology and activity) were collected by cutting their mantle and the resulting sperm masses were pooled and kept on ice before the analyses. The sperm concentration (cells/mL) of each pool was estimated by using a Bürker counting chamber and an Olympus CH-2 optical microscope (20×). After that, the pools were diluted to the desired final concentrations inherent to each parameter. The sperm suspensions were incubated for 30 min with 0 and 10 μg/L of Dy (contaminant 1:99 sperm suspension) at different temperatures (17 and 21 °C). The incubation period was selected considering sperm longevity as well as fertilization time followed by natural spawning (Sedano et al., 1995; Toro et al., 2012). Then, the suspensions were centrifuged at 3000 *g*, at 4 °C for 5 min and the pellets were resuspended with filtered natural seawater.

2.3.1. Biochemical analysis

To assess the biochemical alterations caused by Dy at different temperature scenarios on sperm cells of *M. galloprovincialis*, a battery of biomarkers was evaluated, such as the production of reactive oxygen species (ROS) (superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2)) and lipid peroxidation (LPO) levels. The methodology used is described in Cuccaro et al. (2021).

2.3.2. Physiological analysis

To evaluate the physiological alterations in sperm the motility, average path velocity (VAP) and respiration rate (RR) were analysed. For motility and VAP, the relative protocols are described in Cuccaro et al. (2022b), while, for RR, the procedure is already explained in Bordalo et al. (2022).

2.4. Statistical analyses

Adults' responses, including biochemical markers (ETS, PROT, SOD, CAT, CbEs-pNPA, GSTs, GSH/GSSG, LPO, PC), histopathological indexes ($I_{h\ DT}$, $I_{h\ G}$), Dy concentrations, as well as sperm responses, namely physiological (motility, VAP, RR) and biochemical alterations (ROS

(O_2^-), ROS (H_2O_2), LPO) were submitted to a non-parametric permutational analysis of variance (PERMANOVA + add-on in PRIMER v6) (Anderson et al., 2008). When significant differences were observed in the main test, pairwise comparisons were performed. Values lower than 0.05 ($p < 0.05$) were considered significantly different. Considering Dy concentrations, as well as adult and sperm responses, the null hypothesis tested was that no significant differences existed between treatments (CTL 17 °C, Dy 17 °C, CTL 21 °C, Dy 21 °C). Significant differences are represented in figures with different letters.

The data (Dy concentration in tissues, markers in adults, histopathological indexes, markers in sperm) was used to perform a Principal Coordinates (PCO) ordination analysis. To calculate the distance between centroids, the Euclidean distance similarity matrix was calculated using the different treatments (CTL 17 °C, Dy 17 °C, CTL 21 °C, Dy 21 °C). The Spearmans' correlation vectors, relating to the data descriptors with a correlation higher than 75 %, were superimposed on the PCO graph.

3. Results

3.1. Dysprosium quantification

The Dy concentration in the water samples collected from CTL at both temperatures was below the quantification limit (0.2 μg/L), while the concentration in the water collected immediately after the spiking with Dy was 11.5 ± 0.1 μg/L in the case of 17 °C and 11.7 ± 0.2 μg/L under 21 °C (Table 1). No significant differences were noticed between temperatures (Table 1).

Regarding the stability of Dy, assessed by the collection of water samples for one week (0, 24, 48, 168 h), variations of 5 % at 17 °C and 4 % at 21 °C were observed, which allow considering Dy a stable element during each week of exposure (Table 2).

Regardless of the temperature, the Dy concentration in mussels' tissue, after twenty-eight days of exposure, was significantly higher in Dy-exposed mussels compared to CTL ones. The concentration of Dy in CTL mussels was 0.02 ± 0.01 μg/g dry weight (DW) (17 °C) and 0.04 ± 0.03 μg/g DW (21 °C), while in Dy-exposed mussels was 0.37 ± 0.14 μg/g DW (17 °C) and 0.46 ± 0.17 μg/g DW (21 °C). No significant differences were noticed between both temperatures (Table 1).

3.2. Adults' exposure to dysprosium and warming

3.2.1. Biochemical alterations

3.2.1.1. Energy balance. The ETS activity was significantly higher in non-contaminated mussels at 21 °C and Dy-exposed mussels at 17 °C compared to non-contaminated mussels at 17 °C (Fig. 1A).

Mussels exposed to 21 °C in the presence or absence of Dy presented significantly higher PROT content than non-contaminated ones at 17 °C (Fig. 1B).

3.2.1.2. Antioxidant and detoxification enzymes. Significantly higher activity of SOD was observed in Dy-exposed mussels at 17 °C compared to the remaining treatments (Fig. 1C).

The activity of CAT showed no significant differences among all treatments (Fig. 1D).

Mussels exposed to Dy at both temperatures presented significantly higher CbE's-*p*NPA activity than non-contaminated ones at 17 °C (Fig. 1E).

The activity of GSTs was significantly higher in non-contaminated mussels at 21 °C compared to non-contaminated mussels at 17 °C and Dy-exposed ones at 17 °C (Fig. 1F).

3.2.1.3. Redox balance and cellular damage. Non-contaminated mussels at 17 °C presented significantly higher reduced/oxidized glutathione ratio values in comparison to the remaining treatments, with the lowest values in non-contaminated mussels at 21 °C and contaminated ones at 17 °C (Fig. 1G).

Stress conditions induced significantly higher LPO and PC levels, with the highest values in mussels at 21 °C, both in the presence and absence of Dy (Fig. 1H and I).

3.2.2. Histopathological alterations

Regardless of the temperature, mussels exposed to Dy showed significantly higher I_h values in their digestives tubules compared to non-contaminated ones (Fig. 2A), with higher lipofuscin aggregates, atrophy and the appearance of necrosis (Fig. 3A).

Regarding the I_h for gills, no significant differences were observed among treatments (Fig. 2B). However, mussels exposed to Dy at 21 °C showed an increase of lipofuscin aggregates and enlargement of the central vessel compared with the ones exposed to the remaining treatments (Fig. 3B).

3.3. Sperm' exposure to dysprosium and warming

3.3.1. Biochemical alterations

In the case of the production of O_2^--derived ROS, significantly lower values were found in non-contaminated mussels at 21 °C compared to mussels exposed to the other treatments. Mussels exposed to Dy at both temperatures showed significantly higher values than the non-contaminated ones (Fig. 4A).

Regarding the production of H_2O_2-derived ROS, significantly lower values were observed in non-contaminated mussels at 21 °C compared to the ones at 17 °C (non-contaminated and Dy-exposed mussels) (Fig. 4B).

In terms of the levels of lipid peroxidation (LPO), significantly higher values were observed in non-contaminated mussels at 21 °C compared to the individuals exposed to the other treatments. Mussels exposed to the combination of warming and Dy presented significantly lower values than the ones under the remaining treatments (Fig. 4C).

3.3.2. Physiological alterations

In the case of the respiration rate (RR), no significant differences were noted among all treatments, although higher RR values were obtained at 21 °C both for contaminated and non-contaminated mussels (Fig. 4D).

The percentage of motility was significantly higher in Dy-exposed mussels at both temperatures compared to non-contaminated mussels (Fig. 4E).

Table 1
Dysprosium (Dy) concentrations in water samples (μg/L) collected after spiking, from CTL and Dy-contaminated exposure aquaria; and in mussels' whole soft dried tissue (μg/g dry weight (DW)) after 28 days exposed to different temperatures (17 and 21 °C) in the absence (CTL) and presence of dysprosium (Dy). Results are the mean ± standard deviation. Significant differences ($p < 0.05$) among treatments are identified by different letters.

		Water (μg/L)	Mussels' tissue (μg/g DW)
CTL	17 °C	$< 0.2^a$	0.02 ± 0.01^a
	21 °C	$< 0.2^a$	0.04 ± 0.03^a
Dy	17 °C	11.5 ± 0.1^b	0.37 ± 0.14^b
	21 °C	11.7 ± 0.2^b	0.46 ± 0.17^b

Table 2
Dysprosium (Dy) concentrations (μg/L) in seawater samples collected from blanks in the first week at 0, 24, 48 and 168 h after spiking at two different temperatures. Results are the mean ± standard deviation.

	Dy concentrations (μg/L)			
	0 h	24 h	48 h	168 h
17 °C	12.0 ± 1.2	11.7 ± 0.4	11.6 ± 0.2	11.4 ± 0.2
21 °C	11.4 ± 0.1	11.5 ± 0.1	11.2 ± 0.2	11.0 ± 0.2

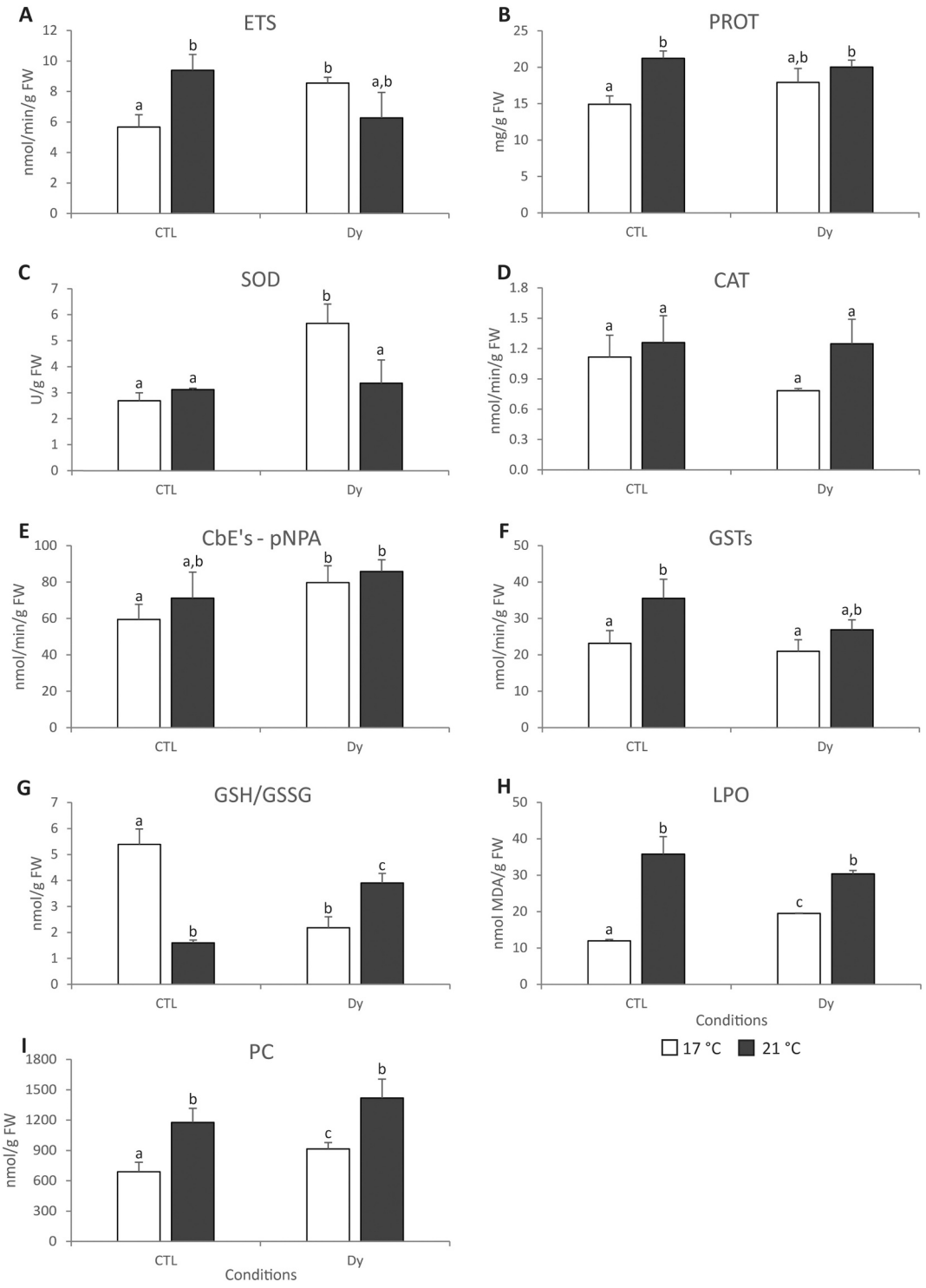

Fig. 1. A: Electron transport system (ETS) activity, expressed in nmol per min per g of fresh weight (FW); B: Protein (PROT) content expressed in mg per g of FW; C: Superoxide dismutase (SOD) activity, expressed in U per g of FW, where U is a reduction of 50 % of nitroblue tetrazolium (NBT); D: Catalase (CAT) activity, expressed in nmol of formaldehyde per min per g of FW; E: Carboxylesterases with *p*-nitrophenyl acetate (CbEs-pNPA) activity, expressed in nmol per min per g of FW; F: Glutathione S-transferases (GSTs) activity, expressed in nmol of dinitrophenyl thioether per min per g of FW; G: Ratio between reduced and oxidized glutathione (GSH/GSSG), expressed in nmol per g of FW; H: Lipid peroxidation (LPO) levels, expressed in nmol of malondialdehyde (MDA) per g of FW; I: Protein carbonylation (PC), expressed in nmol per g FW, in adults of *Mytilus galloprovincialis* exposed to CTL (0 μg/L of Dy) and to Dy (nominal concentration: 10 μg/L; measured concentration: 11.5 ± 0.1 μg/L) at 17 °C, as well as to CTL (0 μg/L of Dy) and to Dy (nominal concentration: 10 μg/L; measured concentration: 11.7 ± 0.2 μg/L) at 21 °C for 28 days. Results are the mean ± standard deviation. Significant differences ($p < 0.05$) among treatments are identified by different letters.

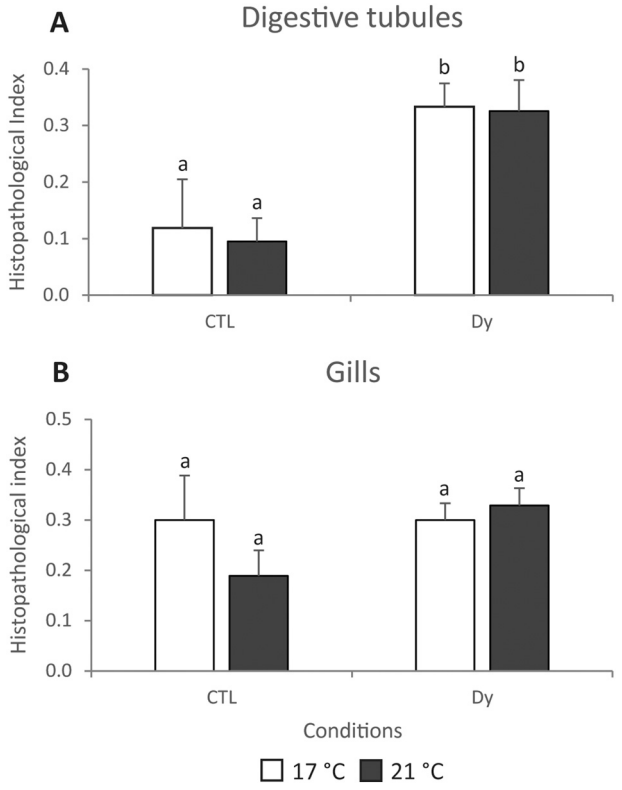

Fig. 2. A: Histopathological index in digestive tubules ($I_{h\ DT}$); B: Histopathological index in gills ($I_{h\ G}$), in adults of *Mytilus galloprovincialis* exposed to CTL (0 μg/L of Dy) and to Dy (nominal concentration: 10 μg/L; measured concentration: 11.5 ± 0.1 μg/L) at 17 °C, as well as to CTL (0 μg/L of Dy) and to Dy (nominal concentration: 10 μg/L; measured concentration: 11.7 ± 0.2 μg/L) at 21 °C for 28 days. Results are the mean ± standard deviation. Significant differences ($p < 0.05$) among treatments are identified by different letters.

The average path velocity (VAP) was significantly lower in mussels under the stress treatments in comparison to non-contaminated ones at 17 °C (Fig. 4F).

3.4. Multivariate analysis

The results of the PCO analysis (Fig. 5) showed that the first principal component (PCO1) accounted 44.3 % of the total variation, distinguishing non-contaminated mussels under increased temperature (on the negative side) from the other treatments (on the positive side). The PCO2 accounted 38.3 % of the total variation, dividing non-contaminated mussels at both temperatures (positive side) from Dy-exposed mussels at both temperatures (negative side). Along with the CTL mussels at 21 °C, GSTs and CAT also showed a strong negative correlation with the PCO1 ($p = -0.8$ for both parameters). On the other hand, together with CTL mussels at 17 °C, H_2O_2 ($p = 1$) and GSH/GSSG ($p = 0.8$) showed a high association with the positive side of PCO1, as well as the VAP with the positive side of PCO2 ($p = 1$). Furthermore, along with Dy-exposed mussels at both temperatures, % Mot, $I_{h\ G}$ and O_2^- showed a strong correlation with the negative side of the PCO2 ($p = -0.8$ for the three parameters).

4. Discussion

A set of histopathological and biochemical biomarkers was used to investigate the responses of adult mussels to the tested stressors. In addition, the effects of the stressors were also assessed in mussels' sperm' quality to predict possible impacts on fertilization and reproduction success. In general, the combined analysis of all parameters, represented by the PCO graph, showed three distinct groups: non-contaminated mussels at 17 °C, non-contaminated mussels at 21 °C and contaminated mussels at both temperatures. The PCO graph showed that Dy treatments are close to each other and separated from the remaining treatments, indicating that the influence of temperature rise on Dy impacts was limited.

4.1. Adults

The results demonstrated that regardless of the temperature mussels were able to accumulate Dy, as shown by the PCO graph, through which the Dy concentration in mussels' tissue is closely associated with the Dy treatments at both temperatures. Freitas et al. (2020) already showed that this species under control temperature was able to accumulate Dy, with increasing concentration along an exposure gradient (0–40 μg/L). Furthermore, the present study demonstrated that temperature did not influence the accumulation of Dy by mussels. Although non-contaminated mussels increased their metabolism at 21 °C compared to their counterpart at 17 °C, when in the presence of Dy at 21 °C the organisms reduce their metabolism indicating an effort to avoid Dy accumulation. This behaviour was also observed in this species when exposed to other contaminants, including another REE (lanthanum, La) (Andrade et al., 2022a), which might be associated with bivalves' physiological mechanism of closing their valves to reduce the filtration rate and avoid the accumulation of contaminants (Gosling, 2003; Ortmann and Grieshaber, 2003).

To assess the energy balance of an organism, the energy consumption, commonly measured by the activity of the mitochondrial respiration capacity, and energy reserves content can be evaluated (Sokolova et al., 2012). When exposed to Dy or warming, mussels increased their metabolic capacity, while when both stressors were combined the ETS activity was lower than values recorded when each stressor was acting individually. This response might be associated with valves' closure as explained before. Such protective behaviour was already reported by Andrade et al. (2022a) in mussels exposed to La and warming. On the other hand, the results here presented demonstrated that the metabolic capacity in mussels exposed individually to Dy and warming was similar. Such findings can be associated with an activation of the metabolism to fuel up defence mechanisms when the stress was below their threshold limit (in this case Dy contamination and increased temperature acting alone). Lopes et al. (2022a) showed an increase in the metabolic capacity in female mussels exposed to sodium lauryl sulfate, while Velez et al. (2017) showed that the clam species *Ruditapes decussatus* increased the metabolism when exposed to a temperature rise. Regarding the energy reserves, mussels exposed to the tested stress factors (Dy, warming and both stressors together) but especially the ones maintained at 21 °C showed higher PROT content than non-contaminated mussels at 17 °C, which might be associated with higher enzyme production. Such findings were also shown by Coppola et al. (2017), in which mussels exposed to the combination of mercury (Hg) and higher temperature for twenty-eight days increased their PROT content compared with the ones at control conditions, but with similar PROT content to mussels exposed to Hg and warming acting alone. Such results may further indicate that mussels under stressful conditions were probably using other energy sources. Previously, Freitas et al. (2020) demonstrated that mussels exposed to 10 μg/L of Dy did not use PROT but instead glycogen.

Under stressful situations, the production of reactive oxygen species (ROS) is enhanced, potentially leading to damage in lipids, proteins, and DNA, as well as loss of redox balance. To eliminate ROS and prevent damage, organisms can activate defence mechanisms, such as antioxidant enzymes including superoxide dismutase (SOD) and catalase (CAT). In addition, to eliminate the contaminants, organisms can activate the detoxification strategies, namely through the activation of carboxylesterases (CbEs) and glutathione S-transferases (GSTs) enzymes (Regoli and Giuliani, 2014; Regoli and Winston, 1999). Nevertheless,

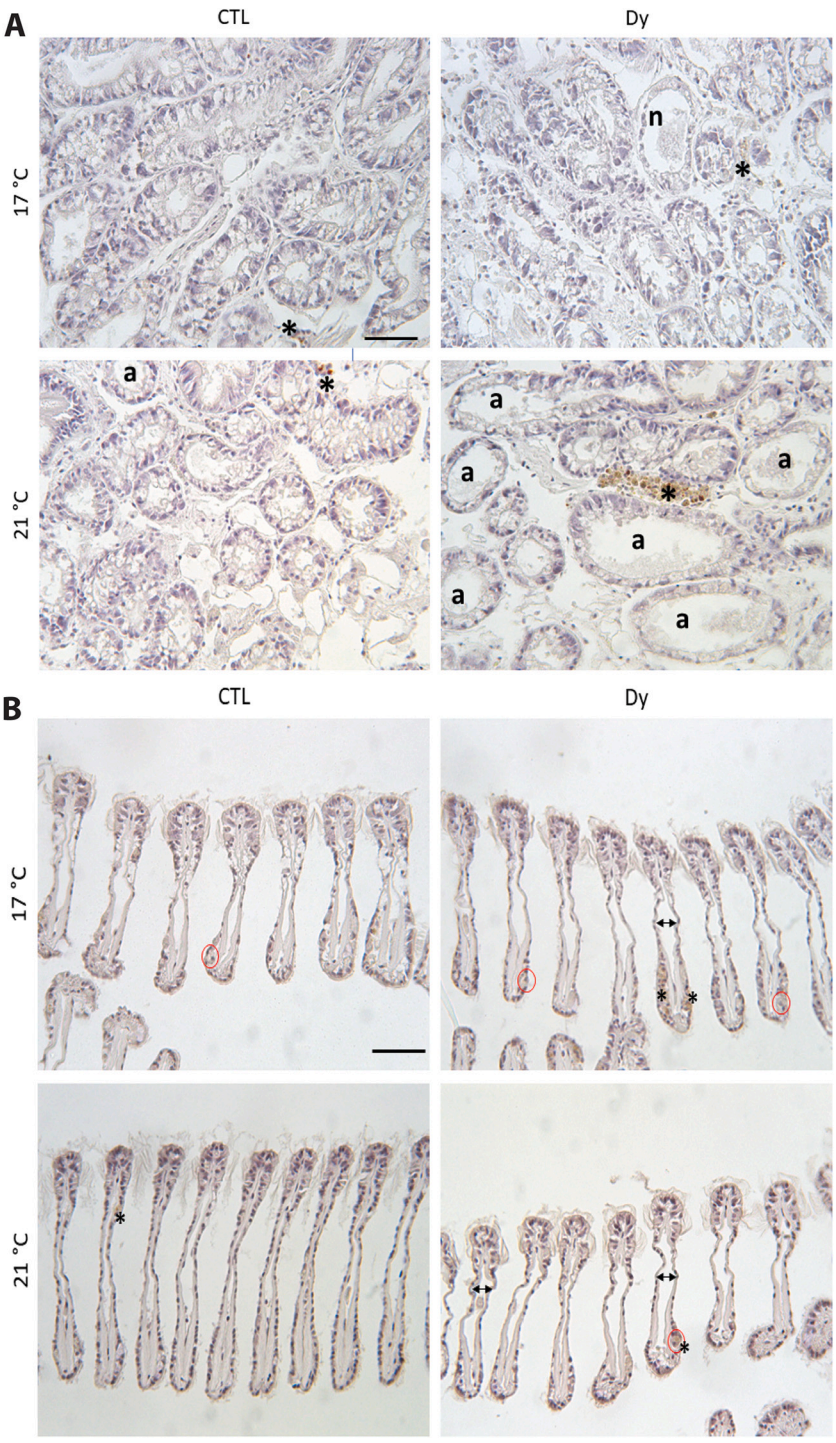

Fig. 3. Micrographs of histopathological alterations observed in A: Digestive tubules and B: Gills of *Mytilus galloprovincialis* exposed to CTL (0 μg/L of Dy) and to Dy (nominal concentration: 10 μg/L; measured concentration: 11.5 ± 0.1 μg/L) at 17 °C, as well as to CTL (0 μg/L of Dy) and to Dy (nominal concentration: 10 μg/L; measured concentration: 11.7 ± 0.2 μg/L) at 21 °C for 28 days stained with hematoxylin. Alterations in digestive tubules: atrophied digestive tubule (a); necrosis (n); lipofuscin aggregates (*). Alterations in gills: lipofuscin aggregates (*); enlargement of the central vessel (double head arrows); hemocytes infiltration (circle). Scale bar 50 μm.

the obtained results highlighted the lack of antioxidant defences in mussels maintained at 21 °C, even if in the presence of Dy, pointing out the effect that temperature might have on mussels' defence mechanisms. A similar response was demonstrated by Freitas et al. (2019) in mussels maintained under increased temperature and lead. On the other hand, Dy-exposed mussels at 17 °C presented the ability to activate SOD to eliminate ROS, accompanying the increase in metabolism and higher protein content observed in mussels of this treatment. The activation of SOD, while CAT activity remains similar to CTL was also revealed when the same species was exposed to Dy (Freitas et al., 2020) and yttrium (Y) (Andrade et al., 2022b).

The present study further demonstrated that regardless of the temperature a similar concentration of Dy was accumulated by mussels which can be explained by the similar detoxification capacity demonstrated at both temperatures. Nevertheless, contaminated mussels tended to present higher CbEs activity than non-contaminated ones while GSTs seem to be not involved in Dy detoxification. Previous studies also showed that the same concentration of other REEs (Gd, Y and samarium) did not induce the increase of GSTs in the freshwater mussel *Dreissena polymorpha* (Hanana et al., 2017, 2018).

Regarding the redox balance, this study highlighted that all stress conditions tested affected mussels' redox balance, which is in accordance with previous studies that demonstrated the impacts of pollutants and warming (acting alone or in combination) on bivalves' redox status

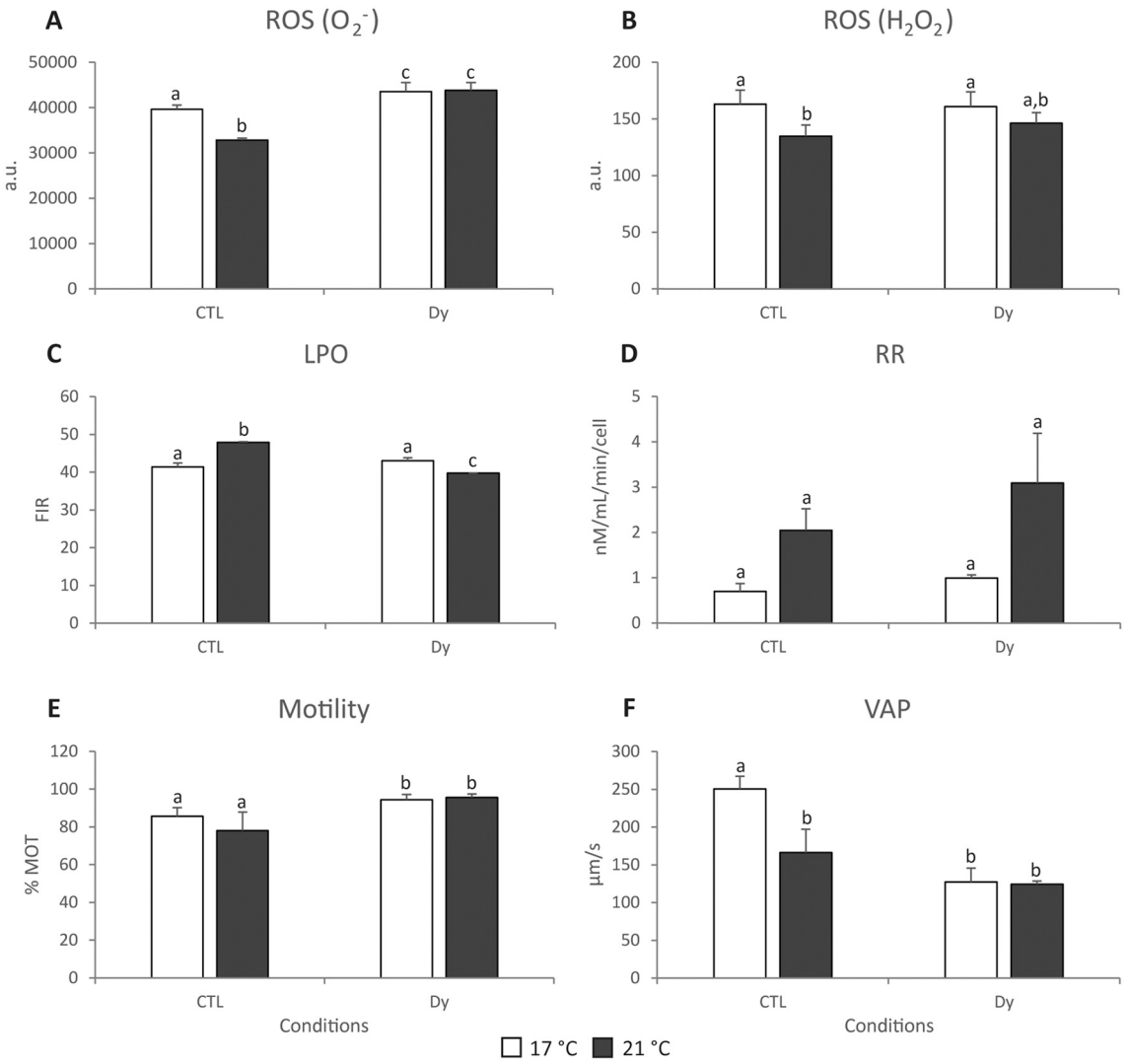

Fig. 4. A: Superoxide anion-derived reactive oxygen species (ROS ($O_2^{\cdot-}$)) production, expressed in arbitrary units of fluorescence intensity (a.u.); B: Hydrogen peroxide-derived reactive oxygen species (ROS (H_2O_2)) production, expressed in arbitrary units of fluorescence intensity (a.u.); C: Lipid peroxidation (LPO) levels, expressed in fluorescence intensity ratio (FIR); D: Respiration rate (RR), expressed in nM of O_2 per mL per min per cell; E: Motility, expressed as a percentage (%); F: Average path velocity (VAP), expressed in µm per s, in sperm of the species *Mytilus galloprovincialis* exposed to different temperatures (17 and 21 °C) in the absence (CTL) and presence of dysprosium (Dy) for 30 min. Results are the mean ± standard deviation. Significant differences ($p < 0.05$) among treatments are identified by different letters.

(Coppola et al., 2021; Freitas et al., 2020; Lopes et al., 2022b). Because of low antioxidant capacity and increased ETS activity, the responses obtained showed that warming, Dy and the combination of both stressors led to lipids and protein damage, especially noticed at 21 °C. This behaviour was already reported by Figueiredo et al. (2022) when exposing the surf clam *Spisula solida* to Gd and a temperature increase of 4 °C.

In terms of histopathological alterations this study showed that Dy induced negative alterations in digestive tubules regardless of the temperature. In this case, compared to Dy-exposed mussels at 17 °C, the contaminated ones at 21 °C presented an increase of lipofuscin aggregates, which was previously associated with oxidative stress (Höhn and Grune, 2013) and higher atrophy. Regarding the I_h of mussels' gills, no differences were noted between all the treatments. However, although the index did not show differences, it was possible to note that Dy-exposed mussels at 21 °C presented a higher number of alterations compared to the remaining treatments, including enlargement of the central vessel and increase of lipofuscin aggregates. Such results clearly pointed out that digestive glands are negatively affected by Dy while the gills are not. Coppola et al. (2020a) also showed that polyethyleneimine affected the digestive tubules while gills were not impacted. Furthermore, Coppola et al. (2020b) revealed that Hg caused impacts on bivalves' digestive tubules independently of the temperature.

4.2. Sperm quality impairments

Mussels are free-spawning organisms, which means that their gametes are released into the environment and are exposed to contaminants present in the water or to climate change-related factors (Lewis and Galloway, 2008; Marçal et al., 2020). The success of reproduction depends on the quality of the gametes (Budhwar et al., 2017; Lewis and Ford, 2012), so it is important to understand if exposure to different stressors may impair sperm quality.

Regarding biochemical changes, it is known that to maintain cell homeostasis and oxidative status it is necessary to balance between the production and elimination of ROS. If this balance is not achieved the overproduction of ROS can affect the sperm membranes negatively affecting sperm functioning (Alahmar, 2019; Balercia et al., 2003). The

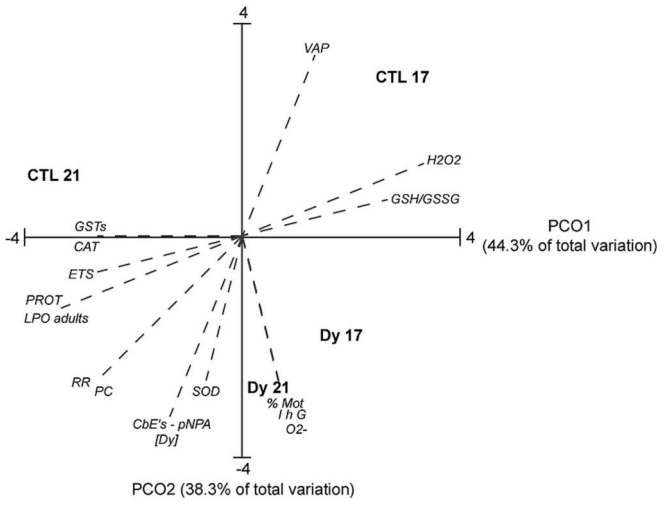

Fig. 5. Centroids ordination diagram (PCO) based on Dy concentration in mussels' tissue, biochemical and histopathological parameters measured in adults of *Mytilus galloprovincialis* exposed to tested treatments (CTL 17 °C, Dy 17 °C, CTL 21 °C, Dy 21 °C); and biochemical and physiological parameters assessed in sperm of *M. galloprovincialis* exposed to tested treatments (CTL 17 °C, Dy 17 °C, CTL 21 °C, Dy 21 °C). Spearman correlation vectors are superimposed as supplementary variables ($r > 0.75$): [Dy], ETS, PROT, SOD, CAT, CbE's – *p*NPA, GSTs, GSH/GSSG, LPO adults, PC, I_{hG}, O_2^-, H_2O_2, RR, % MOT, VAP.

results here presented showed an overproduction of O_2^- in sperm exposed to Dy at both temperatures, however, no changes were observed in the production of H_2O_2. The results also demonstrated that no lipid peroxidation was detectable in Dy-exposed sperm at both temperatures, so we can hypothesize that antioxidant enzymes were activated and efficiently eliminated the excess of ROS avoiding damage to lipids. Bordalo et al. (2022) also observed this response in mussels' sperm exposed to different concentrations of avobenzone. Relatively to the sperm exposed to increased temperature acting alone, it seemed that an increase of 4 °C inhibited the enzymes' activity (O_2^- and H_2O_2 activity) and LPO was observed.

Regarding the physiological parameters, respiration rate and motility are associated with mitochondrial status (Cuccaro et al., 2021; Rolton et al., 2022). The results obtained showed no changes in respiration rate, although higher values were observed at 21 °C (which might be associated with adults' increased metabolic capacity at 21 °C, especially in non-contaminated mussels). The tendency to increase oxygen consumption (not significantly different) was already reported by Andrade et al. (2019) in mussels exposed to warming conditions, as a behavioural mechanism associated with the increase of metabolism to activate defence mechanisms and avoid damage. Motility is an important parameter to assess fertilization success (Lewis and Ford, 2012). This study showed that Dy, regardless of the temperature, had the ability to increase the percentage of sperm motility. A study performed by Cuccaro et al. (2022a) also showed an increase in sperm motility of the same species exposed to UV-filter 4-methylbenzylidenecamphor, and this hyperactivation may be correlated with the increase of ROS (O_2^-) since the relationship between ROS and motility was already reported by other studies (de Lamirande et al., 1997; Thompson et al., 2013). This behaviour can be helpful for fertilization success as described by Thompson et al. (2013). However, stress factors, specially Dy exposure, induced a decrease in the velocity of the sperm (the average velocity path decreased), which may reduce the fertilization ability. A study by Fitzpatrick et al. (2008) tested different concentrations of copper (Cu) (0–100 μg/L) in the sperm of the mussel species *Mytilus trossulus*. A 100 min exposure resulted in a decrease in sperm velocity with the increase of Cu concentrations, along with a reduced fertilization capacity.

5. Conclusion

The present study highlighted the toxicity of Dy, warming and the combination of both stress factors on different biological levels of the species *Mytilus galloprovincialis*, including histopathological and biochemical alterations in adults, as well as biochemical and physiological alterations in sperm cells. Moreover, although temperature might influence Dy impacts, the effects of Dy overlap the ones caused by the temperature, with similar responses given by Dy-exposed mussels regardless of the temperature tested. In the long term, the increasing use of Dy may negatively impact the reproductive capacity of mussels and impair their general health *status*, which may eventually affect population maintenance. It is important to continue to investigate the impacts of REEs on organisms and how climate change-related factors may alter them.

CRediT authorship contribution statement

Carla Leite: Laboratory experiments; Formal analysis; Quantifications; Writing - Original Draft.

Tania Russo, Gianluca Polese, Alessia Cuccaro: Formal analysis.

João Pinto: Quantifications.

Amadeu Soares: Funding.

Rosa Freitas, Eduarda Pereira, Carlo Pretti: Conceptualization; supervision; Writing - Review & Editing; funding.

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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