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Will extreme weather events influence the toxic impacts of caffeine in coastal systems? Comparison between two widely used bioindicator species

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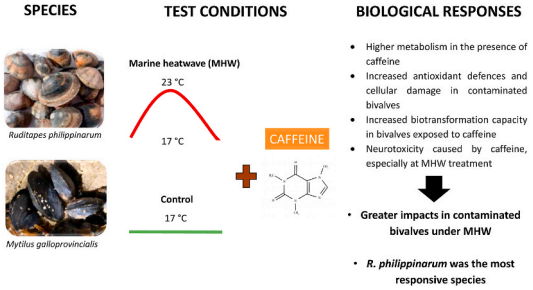
HIGHLIGHTS

- Greater impacts in contaminated bivalves under marine heatwave (MHW) treatment.
- Higher metabolism in the presence of caffeine.
- Increased antioxidant defences and cellular damage in contaminated bivalves.
- Increased biotransformation capacity in mussels and clams exposed to caffeine.
- Neurotoxicity caused by caffeine, especially at MHW treatment.

GRAPHICAL ABSTRACT



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ABSTRACT

In the recent years, marine heatwaves (MHWs) have caused devastating impacts on marine life. The understanding of the combined effects of these extreme events and anthropogenic pollution is a vital challenge. In particular, the combined effect of MHWs on the toxicity of pharmaceuticals to aquatic life remains unclear. To contribute to these issues, the main goal of the present investigation was to evaluate how MHWs may increase caffeine (CAF) toxicity on the clam *Ruditapes philippinarum* and the mussel *Mytilus galloprovincialis*. Bioaccumulation levels and changes on oxidative stress, metabolic capacity and neurotoxic *status* related biomarkers were investigated. The obtained results revealed the absence of CAF accumulation in both species. However, the used contaminant generated in both bivalve species alteration on neurotransmission, detoxification mechanisms induction as well as cellular damage. The increase of antioxidant defence mechanisms was complemented by an increase of metabolic activity and decrease of energy reserves. The obtained results seemed magnified under a simulated MHWs, suggesting to a climate-induced toxicant sensitivities' response. On this perspective, understanding of how toxicological mechanisms interact with climate-induced stressors will provide a solid platform to improve effect assessments for both humans and wildlife.

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1. Introduction

During the last decades, the cumulative impacts resulting from anthropogenic activities have evolved in a complex scenario of multiple stressors, with a general consensus that these activities are responsible for the worldwide climate change effects observed (Stern and Kaufmann, 2014; Gao et al., 2018). Climate change effects include sea level rise, seawater temperatures enhancement, ocean acidification and increasing intensity of extreme events (Ross and Behringer, 2019). Among these changes, extreme events are becoming more frequent and intense (IPCC et al., 2019; Sánchez-Benítez et al., 2020). Marine heatwaves (MHWs) are recognized as extreme weather events, associated with seawater temperature rise over a short period of time (Frölicher and Laufkötter, 2018; Jacox et al., 2020). There are recent evidences that MHWs will not only become more frequent in the near future, but also their duration and intensity are very likely to increase (IPCC et al., 2019; Laufkötter et al., 2020; Sánchez-Benítez et al., 2020). The minimum number of consecutive high temperature days required to be considered as heatwave vary across the Globe. Using Berkeley Earth temperature dataset and key heatwave metrics Perkins-Kirkpatric and Lewis (2020), systematically examined regional and global heatwave trends. Authors observed that the rate of MHWs increased since the mid-twentieth century. Moreover, trend magnitudes are not globally uniform and are higher over regions known to experience disproportionately more adverse impacts of climate change. Recently, Sánchez-Benítez et al. (2020) defined an average of 3.3 days for Iberian MHWs, expecting to increase +3.3 days during the next 10 years. The abrupt nature of these extreme events can have more lethal effects on marine life than an average gradual temperature increases of seawater, severely affecting marine organism's health, populations and ecosystem functions (Smale and Wernberg, 2013; Smale et al., 2019). On this subject, it has been reported shifts on species geographical distribution, changes in community's composition and increasing mortalities of a significant number of organisms (Ummenhofer and Meehl, 2017; Frölicher and Laufkötter, 2018), including economically and ecologically important *taxa*, as bivalves (Leung and ConnellRussell, 2017; Seuront et al., 2019) and fish (Frölicher and Laufkötter, 2018). Few recent works reported adverse effects caused by MHWs on these organisms, including impacts in terms of filtration rate, lysosomal membrane stability and massive mortality (Ferreira-Rodríguez et al., 2018); oxidative stress generation (Amorim et al., 2020); individuals' thermal tolerance limits, resulting in an inability to acclimate and high mortality (Carneiro et al., 2020); decrease in scope for growth (SFG), reduction in burrowing activity and high mortality (Domínguez et al., 2021).

Anthropogenic activities are not only responsible for climate change but are often associated with hazardous chemicals production and release into aquatic systems, causing detrimental effects on environment and human health (Häder et al., 2020). For this reason, it is expectable that combined occurrence of climate change related events and pollution will increase, with inevitable consequences for marine ecosystems and inhabiting organisms. These scenarios are of particularly concern regarding the increasing proliferation of contaminants of emerging concern. Among them, caffeine (1,3,7-Trimethylxanthine) (CAF) is known to be one of the most consumed psychoactive drugs, present in medicines, beverages, foodstuff and several other products (Rogers and Dernoncourt, 1998; Cruz et al., 2016; Quadra et al., 2020). Due to its high use and wide occurrence in the environment, CAF is currently recognized as a marker of anthropogenic activity (Quadra et al., 2020). The main recognized sources of this psychoactive drug in the aquatic environment include wastewater excretory residues, inappropriate deposition of expired or unwanted CAF containing pharmaceutical products, manufacturing plant wastes, hospital wastes and sewage treatment due to the inefficiency of the sewage treatment plants (Cruz et al., 2016; Korekar et al., 2020; Vieira et al., 2022). Li et al. (2020) have carried out a global literature review of studies reporting results of CAF levels in marine and estuary aquatic systems. The authors showed that more than 50% of the sea water samples presented CAF concentrations higher than 18 ng/L, encountering concentrations up to 10 μg/L in some continents (e.g., 10.2 μg/L in Lebanon river waters; 11 μg/L in Australia and 19.3 μg/L in Jundiaí River, Brazil). Once entering into the environment, CAF remains relatively stable and water-dispersible under different environmental conditions because of its chemical-physical properties such as high-water solubility (aprox. 13.0 g/L), low octanol-water coefficient (log kow = −0.07) and low volatility (Lin et al., 2009; Edwards et al., 2015). Due to the reported high residual concentrations in the environment as well as their chemical-physical properties, concerns have been raised on the potential adverse impacts of CAF on the ecological safety and human health (Li et al., 2020). The bioaccumulation in aquatic wildlife has been reported in recent field studies, including macroalgae (Ali et al., 2018), aquatic plants (Zhou et al., 2018), bivalves (Bayen et al., 2013; Burket et al., 2019) and fish (Ali et al., 2018). Moreover, several laboratorial studies have also evidenced significant effects of CAF residues in marine organisms, including mortality, reproduction and development changes in polychaetes (Li et al., 2012; Pires et al., 2016), oxidative stress induction in bivalves and fish (Pires et al., 2016, Aguirre-Martínez et al., 2016), behaviour alterations on fish (Cruz et al., 2016), changes on energy reserves content and metabolic capacity in polychaeta and bivalves (Cruz et al., 2016; Pires et al., 2016), as well as DNA damage in bivalves and decapods (Maranho et al., 2015; Aguirre-Martínez et al., 2016).

The research on the assessment of the effects caused by environmental changes on biota requires the selection of effective bioindicators and test organisms. Bivalves comprise a wide range of species with different tolerances to anthropogenic stressors, including pollutants as pharmaceuticals and factors related with climate changes (Cruz et al., 2016; Aguirre-Martínez et al., 2018; Steeves et al., 2018; Soon and Zheng, 2020; Costa et al., 2020a, b). Considering the importance of bivalves in aquatic systems, as ecosystems engineers, keystone species (Steeves et al., 2018; Ysebaert et al., 2019) and important species for human consumption, it is vital to increase the effort to understand the effects of pollutants on these organisms, especially considering predicted climate change scenarios. The knowledge on the effects of MHWs on the accumulation of pharmaceuticals, as CAF, and the resulting toxicity to aquatic life, is very scarce. This information is necessary to improve climate change models and it is also crucial to understand the potential effects and accumulation in seafood to guarantee food safety and public health.

Overall, the main objective of the present study was to identify possible effects of CAF in the clam *Ruditapes philippinarum* and the mussel *Mytilus galloprovincialis* and if the effects were magnified by the presence of dynamic fluctuation of temperature regime. This will contribute to understand and compare the impacts of most popular and widely used stimulant compound by humans simulating a future heat wave scenario. To achieve this goal, a wide battery of biomarkers on multilevel responses was employed (oxidative stress, energetic and neuro biomarkers), following an experimental design which simulated an increase of 6 °C during 4 days for a total of 28 days of exposure.

2. Material and methods

2.1. Species sampling and acclimation

Ruditapes philippinarum (32.41 ± 0.65 mm) and *Mytilus galloprovincialis* (46.22 ± 0.80 mm) were collected in the Ria de Aveiro (NW Atlantic coast of Portugal), during the beginning of September 2020. After field sampling, bivalves were transported to the laboratory using thermally isolated boxes with water from the collection site. Specimens were acclimated for 14 days using artificial seawater (ASW) (salinity 30), prepared with artificial sea salt (Tropic Marin® Sea Salt) and deionized water in a temperature-controlled room (17 ± 0.4 °C), continuous aeration and natural photoperiod conditions. During this period, animals were fed with AlgaMac Protein Plus (150 000 cells/

animal/day) every 2–3 days after water renewal. Bivalves were observed at least once *per* day and no mortality was recorded during the acclimation period.

2.2. Experimental setup and exposure conditions

The study included two complimentary experimental set-ups: **Group I**: bivalves were exposed simultaneously for 28 days to 2 μg/L of caffeine (CAF) in 17 L glass aquarium filled with ASW under constant control temperature (17 °C) (**CAF 17°C**). Negative controls (**CTL 17 °C**) (organisms not exposed to CAF) and positive controls (**BLANK I**) (4 glass aquaria (17 L each) contaminated with CAF but without organisms) were used to verify the correct functioning of the test. **Group II**: bivalves were exposed simultaneously for 28 days to 2 μg/L of CAF in 17 L glass aquarium filled with ASW under marine heatwaves (MHWs) (7 days at 17 °C followed by 1 °C increase *per* day during 4 days, 4 days at 23 °C followed by a 1 °C decrease *per* day during 4 days, and 7 days at 17 °C) (**CAF MHW**). As for Group I, four positive controls were simultaneously used (**BLANK II**). Within each Group (I and II), 3 aquaria were used *per* treatment and *per* sampling day, with 7 individuals *per* aquarium and *per* species. The increase in temperature in MHW aquaria was achieved with submersible heaters (Jäger 3612, EHEIM, Deizisau, Germany).

Abiotic factors were maintained as for the acclimation period and daily checked. Individuals were measured and weighed at the beginning and at the end of the experiment and additional food was provided every 2–3 days. Test media and CAF concentration were renewed and re-established every week allowing conditions to be constant during the entire experiment.

Selected CAF concentration was based on: i) values reported in different Iberian coastal ecosystems (Dafouz et al., 2018); and ii) concentrations used in previous studies with different bivalve species (Cruz et al., 2016; Aguirre-Martínez et al., 2016). MHW duration was based on Sánchez-Benítez et al. (2020) study, defining an average of 3.3 days for Iberian MHWs. Selection of the highest temperature was based on previous works (e.g., Dasari et al., 2014; Ferreira-Rodríguez et al., 2018).

2.3. Chemical analyses

Caffeine (1,3,7-trimethylxanthine, 99%) (CAF) was obtained from Sigma Aldrich. The stock solution (1000 ppm) was suspended in methanol; then further dilutions were made in mobile phase and stored in vials at 4 °C. Distilled water and acetonitrile (Sigma Aldrich) of HPLC-grade were used. Solid-phase extraction cartridges, packed with 200 mg of Oasis HLB, were purchased from Waters (Milford, MA, USA). The chromatographic analyses were performed using an HPLC system with a PerkinElmer Series 200 variable flow pump, coupled to a PerkinElmer UV-VIS detector (PerkinElmer), which was set at 272 nm. The system was controlled by a PerkinElmer interface module (NCI 900 Network Chromatography Interface) and chromatograms were processed by a PerkinElmer TotalChrom Navigator software. Separation was carried out on a 250 × 4.6 mm chromatographic column X-Bridge C18 5 μm (Waters) at room temperature. The mobile phase consisted of an acetonitrile-water solution (20:80, isocratic) at a flow rate of 0.8 mL/min and an injection volume of 100 μL was used.

2.3.1. Caffeine concentrations in water

Immediately after medium renewal and after 3 and 7 days (only for positive controls (BLANKs) of each group tests (I and II)), 50 mL water samples ($N = 4$) were collected to determine CAF concentration and assess drug stability along the experiment. The solid phase extraction (SPE) procedure was performed by using Oasis HLB Cartridges 6 cc extraction columns as described by use manual. Before extraction, each HLB cartridge was pre-conditioned with 3 mL of methanol followed by rinsing with 3 mL deionized water. The water sample, 150 mL, was passed through the HLB-cartridge at a flow rate of about 1 mL/min. When the extraction was completed, the cartridge was washed with 3 mL of water and subsequently air dried under a vacuum. The CAF was then eluted from the cartridge with 3 mL of methanol. The extract was completely evaporated to dryness with a stream of nitrogen at a temperature of 40 °C. The residue was redissolved with 500 μL of mobile phase and injected into HPLC. The detection limit, calculated as a signal-to-noise ratio of 3:1, was 0.02 μg/L for water samples.

2.3.2. Bioaccumulation of caffeine in R. philippinarum and M. galloprovincialis specimens

Seven organisms *per* species and *per* treatment were randomly sampled at days 1, 7, 12, 16, 21 and 28 and immediately frozen. Samples were then ground and homogenized. About 2 g of each sample was weighed into a centrifugal tube and was added with 10 mL of dichloromethane-ethyl acetate (50:50%, v/v). The sample was shaken on a vortex mixer for 3 min and subjected to ultrasonic extraction for 10 min. The sample was then centrifuged at 4000 rpm for 10 min at 4 °C and the supernatant was collected in a glass tube. The above extraction procedure was repeated two times and the extracts were combined (about 20 mL in total). The extract was completely evaporated to dryness under nitrogen flow at a temperature of 40 °C. The residue was redissolved with 500 μL of mobile phase and injected into HPLC. The recovery was >80% for water samples and >75% for soft tissues. The detection limit, calculated as a signal-to-noise ratio of 3:1, was 0.1 ng/g for soft tissues.

2.4. Biochemical responses

As for the CAF quantification, other seven organisms *per* species and *per* treatment were randomly sampled at days 1, 7, 12, 16, 21 and 28 and immediately frozen. The whole body was pulverized individually with liquid nitrogen and divided into aliquots of 0.5 g fresh weight (FW). About 0.5 g fresh weight (FW) tissues were homogenized in 1:2 w/v with specific buffers (Andrade et al., 2018) and then centrifuged for 20 min at 10,000 *g* (S9 fraction) or 3,000 *g* (mitochondrial fraction) at 4 °C (Costa et al., 2020a;b). Biochemical measurements were carried out either immediately or after storage at −80 °C in order to determine: neurotoxicity (acetylcholinesterase activity, AChE), cellular damage (lipid peroxidation levels, LPO), antioxidant and biotransformation capacity (activities of the enzymes catalase, CAT and glutathione S-transferases, GSTs) and indicators of energy metabolism (electron transport system activity, ETS; glycogen content, GLY). The AChE activity was determined following the method described by Ellman et al. (1961) and modifications by Mennillo et al. (2017). The LPO levels were measured according to Ohkawa et al. (1979) and modifications made by Carregosa et al. (2014). The activity of GSTs was determined according to Habig et al. (1974) and CAT activity was measured according to Johansson and Borg (1988), with modifications performed by Carregosa et al. (2014). The activity of ETS was measured following King and Packard (1975) and De Coen and Janssen (1997) methods. The GLY content was quantified according to the sulphuric acid method from Dubois et al. (1956). Specific protocols' details were already described and reported in Andrade et al. (2018).

All biochemical analyses were performed in triplicate and absorbance was read by microplate reader Synergy HT (BioTek ®Inc.).

2.5. Data analysis

The normality and homogeneity of variances of all data were analysed using Kolmogorov-Smirnov and Levene tests. When necessary, log (x+1) and square root transformations were also applied. Results are expressed by means and standard deviation. A Tukey test ($p < 0.05$) was used to test the effects of temperature and time. All data preliminary analyses, transformations and Tukey tests were performed using SPSS (IBM version 21).

3. Results

3.1. Caffeine concentrations in water

Regardless the sampling time and the temperature, CAF concentration was similar to the used nominal concentration (2 μg/L) in water samples collected from positive controls of both groups (BLANKS) and from aquaria maintained at 17 °C (Group I) (Table 1).

3.2. Bioaccumulation of caffeine in R. philippinarum and M. galloprovincialis specimens

Caffeine was not detected in specimens from both species, regardless the treatment and sampling time (Table 2).

3.3. Biochemical responses

3.3.1. Neurotoxicity

A significant increase of AChE activity was observed in *M. galloprovincialis* and *R. philippinarum* exposed to CAF at constant temperature of 17 °C (Group I) after 7, 16 and 28 days. Considering MHW treatment (Group II) in *M. galloprovincialis*, the AChE activity significantly increased after 12 days and decreased at the end of experimental period (day 28). Regarding *R. philippinarum*, a significant increase of AChE activity was observed after 7 days under MHW treatment. In both species, no significant differences were found along the experimental period in non-contaminated individuals (control treatment) (Fig. 1A and B).

3.3.2. Oxidative stress

In both species, cellular damage occurred in bivalves exposed to CAF, regardless the temperature scenario (Groups I and II), with the highest values in bivalves exposed to the MHW treatment (significant after 12 days of exposure for both species). In non-contaminated organisms (CTL 17 °C) no significant differences were observed in both species along the experimental period (Fig. 2A and B).

The activity of CAT significantly increased in organisms under MHW treatment, especially after 16 days of exposure in mussels and 12 days of exposure for clams. In the case of bivalves exposed to CAF 17 °C, significant CAT enhancement was only detected in *R. philippinarum* after 28 days of exposure. Under control conditions, bivalves showed similar CAT activity along the entire experimental period (Fig. 2C and D).

In bivalves maintained at constant temperature (Group I) the activity of GSTs was only significantly higher in *R. philippinarum* after 12, 16, 21 and 28 days of exposure period. Biotransformation capacity significantly increased during the MHW after 12 days of exposure in mussels and clams, with higher values compared with contaminated bivalves maintained at 17 °C. In both species, the activity of GSTs in CTL 17 °C treatment showed no significant differences among sampling periods (Fig. 2E and F).

Table 1
Concentration of caffeine (2 μg/L) in water samples collected in aquaria with (exposure aquaria) and without (blanks) organisms. Regarding **Blanks conditions**, water collection was performed in two temperature groups and in different times of sampling: G**roup I**, characterized by constant temperature at 17 °C and the water was collected at T0 (immediately after spiking), T72h (after 72 h of exposure), T168h (after 168 h of exposure); G**roup II**, characterized by marine heatwave simulation (MHW) and the water was collected at 17 °C immediately after caffeine spiking (T0), a peak of temperature of 23 °C (T0) and then followed by returning to the initial temperature (17 °C) (T0). For E**xposure aquaria -Group I**, characterized by constant temperature at 17 °C, water was collected immediately after caffeine spiking (T0). **LOD** = 0.02 μg/L.

Condition	Group	Temperature	Sampling time	μg/L
Blanks	I	17 °C	T0	1.90
			T72h	1.92
			T168h	1.95
	II	17 °C	T0	1.88
		23 °C	T0	2.06
		17 °C	T0	1.98
Exposure aquaria	I	17 °C	T0	2.30

Table 2
Bioaccumulation of caffeine in both species soft tissues (*Mytilus galloprovincialis* and *Ruditapes philippinarum*) collected at the beginning (T0) and at the end (T28d) of the experimental period. **LOD** = 0.1 ng/g.

Organisms	Temperature	Sampling time	μg/L
M. galloprovincialis	17 °C	T0	<LOD
		T28d	<LOD
	23 °C	T0	<LOD
		T28d	<LOD
R. philippinarum	17 °C	T0	<LOD
		T28d	<LOD
	23 °C	T0	<LOD
		T28d	<LOD

3.3.3. Energy reserves and metabolic activity

At 17 °C the ETS activity was also enhanced in contaminated bivalves (CAF 17 °C), with a significant increase after 16, 21 and 28 days in *M. galloprovincialis* and after 12, 16, 21 and 28 days in *R. philippinarum*. In both species, the metabolic capacity significantly increased in organisms subjected to the MHW treatment after 12 days of exposure, with higher ETS values compared with organisms maintained at constant temperature. Under control conditions (CAF 17 °C), no significant differences were observed along the experimental period for both species (Fig. 3A and B).

Regardless the species, contaminated organisms showed a significantly decrease in the GLY content after 12 days of experiment, both under 17 °C and MHW treatments. Bivalves maintained under CTL 17 °C showed no significant differences in terms of GLY content (Fig. 3C and D).

4. Discussion

Previous research has shown and highlighted impacts of marine heatwaves (MHWs) and related thermal stress on marine organisms (Roberts et al., 2019; Lugo et al., 2020). However, the number of studies considering the effects of MHWs on the toxicity of contaminants of emerging concern to bivalves, remains scarce. The research on this topic is crucial to understand the potential ecological effects and accumulation in seafood to guarantee food safety and public health.

Caffeine (CAF) represents one of the most widely consumed neuroactive compounds on the planet, described as a common organic waste contaminant in natural waters (Sharma and Paul, 2013). Environmental Exposure Distributions (EEDs) generated from a literature review of CAF levels, reported that 28% and 69% of sea and estuary water samples respectively, resulted in a hazard quotient >1 caused by CAF contents (Dafouz et al., 2018). Due to CAF 'half-life of 100–240 days (Hillebrand et al., 2012), this psychoactive compound is already acknowledged as an emerging contaminant, being toxic to marine organisms and even to humans (Quadra et al., 2020). The present finding for CAF concentrations in water samples collected at different sampling times corroborates its stability in seawater which, in turn, favours its dispersion (Li et al., 2020). However, the 28-day exposure did not lead tissue' accumulation (<LOD) in bivalves maintained under both scenarios (constant temperature and marine heatwave simulation). Similarly, Capolupo et al. (2016) did not observe accumulation after exposing *M. galloprovincialis* to a range of CAF concentrations of 0.005–0.5 μg/L during 7 days. Differently from most pharmaceuticals, CAF is a highly hydrophilic compound, which displays a low octanol-water repartition coefficient (−0.07) (Garvin et al., 2015), a property that provides a high readiness of metabolization and a very low potential of bioaccumulation. This

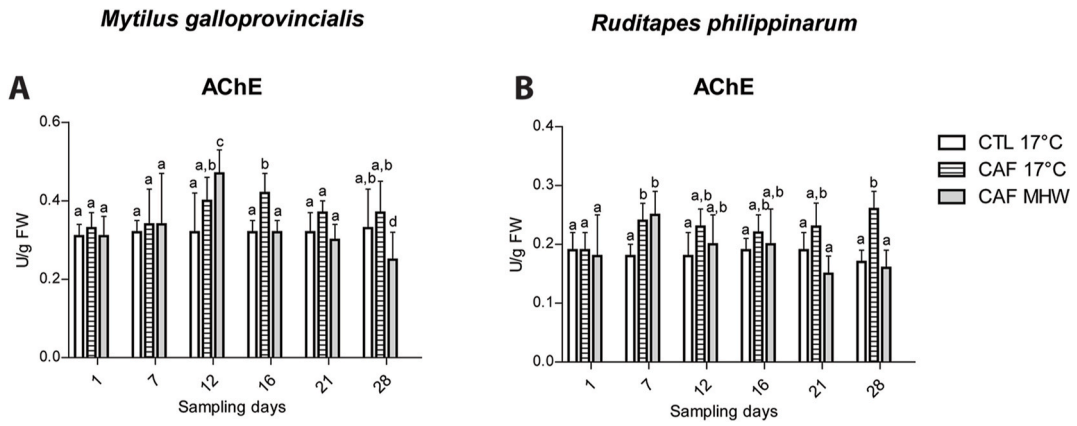

Fig. 1. A & B: Acetylcholinesterase (AChE) activity in *Mytilus galloprovincialis* and *Ruditapes philippinarum* exposed to different treatments: control (CTL 17 °C - 17 °C without caffeine), Group I (CAF 17 °C - 17 °C with caffeine); Group II (CAF MHW - marine heatwave with caffeine) for 28 days. Results are means with standard deviations. Significant differences ($p < 0.05$) among treatments are identified with different lowercase letters.

characteristic may justify the lack of CAF bioaccumulation observed in both bivalve species. Furthermore, low CAF accumulation may be explained by the increased biotransformation capacity observed in both species regardless the temperature scenario. Given CAF concentrations reported in the environment (Vieira et al., 2022) and the role of bivalves as ecosystem keystone species, both from ecological and economic perspectives, this low accumulation can be considered positive in terms of environmental risks and human health scenarios.

Looking on biochemical effects, CAF is an important central nervous system stimulant (Li et al., 2012; Aguirre-Martínez et al., 2016) recognized as a competitive antagonist of adenosine receptors (Stiles, 1992). The activity of AChE enzyme has been used to assess potential neurotoxic effects of several classes of contaminants, including CAF (Maranho et al., 2015). Previous studies demonstrated significant AChE inhibition in marine bivalves, including both species subjected to this study, when exposed to higher CAF concentrations (mg/L) (Aguirre-Martínez et al., 2016). Similarly, this organic compound induced significant neurotoxicity in both species maintained under constant temperature (Group I) comparing with non-contaminated ones. The increase of AChE activity was already identified as a neurotoxic effect in clam species (Liu et al., 2011), due to an inhibition of the enzyme activity which causes an accumulation of the neurotransmitter acetylcholine. Differently, when organisms were subjected to CAF combined with temperature dynamic fluctuation, AChE activity decreased significantly at the end of the exposure period. It has been shown that there are different relevant ways in which variables affected by Global Climate Change could interact with chemicals in terms of producing adverse effects. These responses can be in terms of cellular, metabolic or structural responses (Hooper et al., 2013). This hypothesis may justify the opposite neurotransmitter response obtained in presence/essence of MHWs, suggesting how chemical- and climate-specific variables interact to lead to different outcomes.

Evaluation of oxidative stress *status* has been recommended as early warning indicator of toxicity effects in bivalves exposed to contaminants, under different temperature scenarios (Aguirre-Martínez et al., 2015; Serra-Compte et al., 2018; Almeida et al., 2018, 2021; Costa et al., 2020a; Freitas et al., 2020). Recent evidences demonstrated that CAF may enhance the production of reactive oxygen species (ROS), inducing antioxidant responses (Pires et al., 2016; Li et al., 2020). These responses are usually mediated by antioxidant enzymes, including catalase (CAT), in order to prevent lipid peroxidation (LPO) as a consequence of oxidative stress (Aguirre-Martinez et al., 2013, 2016). Also, glutathione S-transferases (GSTs) can be involved in ROS elimination during the detoxification process. In this context, GSTs quench reactive molecules and catalyse the conjugation of GSH to an array of hydrophobic and electrophilic substrates, thus, protecting the cell from oxidative burst (Park et al., 2020). In the present study, toxic effects were detected in contaminated organisms with an activation of both species antioxidant and biotransformation responses (CAT and GSTs) and a significant membrane peroxidation. Strong concentration-response relationship was also observed in the clam species *Corbicula fluminea* when exposed to 0.1, 1, 5, 10, 15, 50 μg/L of CAF, with an induction of antioxidant and biotransformation enzymes responses and a significant rise in LPO levels (Aguirre-Martínez et al., 2015). Bioavailability of CAF during the experiment and its metabolization by bivalves was also evidenced in other studies (e.g. Cruz et al., 2016; Piscopo et al., 2021a, b) corroborating the potentiality of this compound to generate oxidative stress. Significant cellular damage and antioxidant system activation were also detected in the presence of temperature fluctuation after 12 days of exposure. Considering that a greater sensitivity of bivalves to heat exposure reflected as lethal and sub-lethal responses in bivalves was already reported (Olabarria et al., 2016), it seems that response of each species to contaminant-induced stress could be influenced by dynamic fluctuation of temperature regime. In agreement, Piscopo et al. (2021a) showed inefficient biotransformation capacity and consequently increase of cellular membrane damages when *R. philippinarum* was expose to CAF under warming conditions, suggesting to a climate-induced toxicant sensitivities' response.

The mitochondrial electron transport system (ETS) consists of four enzyme complexes that transfer electrons from donors like NADH to oxygen, the ultimate electron acceptor. This electron flow is coupled with the generation of a proton gradient across the inner membrane and the energy accumulated in the proton gradient is used to produce ATP (Birsoy et al., 2015). The activity of ETS tended to increase to limit oxidative stress and their energy storage expenditures (Cruz et al., 2016). The metabolic activities of marine organisms have been demonstrated to be enhanced after CAF exposure (Li et al., 2020). The adverse effects elicited by increase of temperature and chemical toxicant exposures were also confirmed by the present metabolic results. In fact, both *M. galloprovincialis* and *R. philippinarum* exposed to MHW treatments, magnified their stress reactions when compared with isolated effects, showing a major increase of their metabolism, measured by ETS activity, in an attempt to fight against oxidative stress with expenditure of their energy storages. Alterations in bivalves' metabolic capacity were already recorded by Cruz el al. (2016) when exposing *R. philippinarum* to CAF. Moreover, considering that distribution and survival of marine ectotherms are temperature-dependent and warmer conditions affect indirectly organism susceptibility to other biotic and abiotic stressors (Sokolova and Lannig, 2008), Piscopo et al. (2021b) showed major metabolic pathways in the clam *R. decussatus* under high temperature condition when exposed to CAF. Similarly, Delorenzo (2015) reported in his review paper different examples regarding how warmer conditions

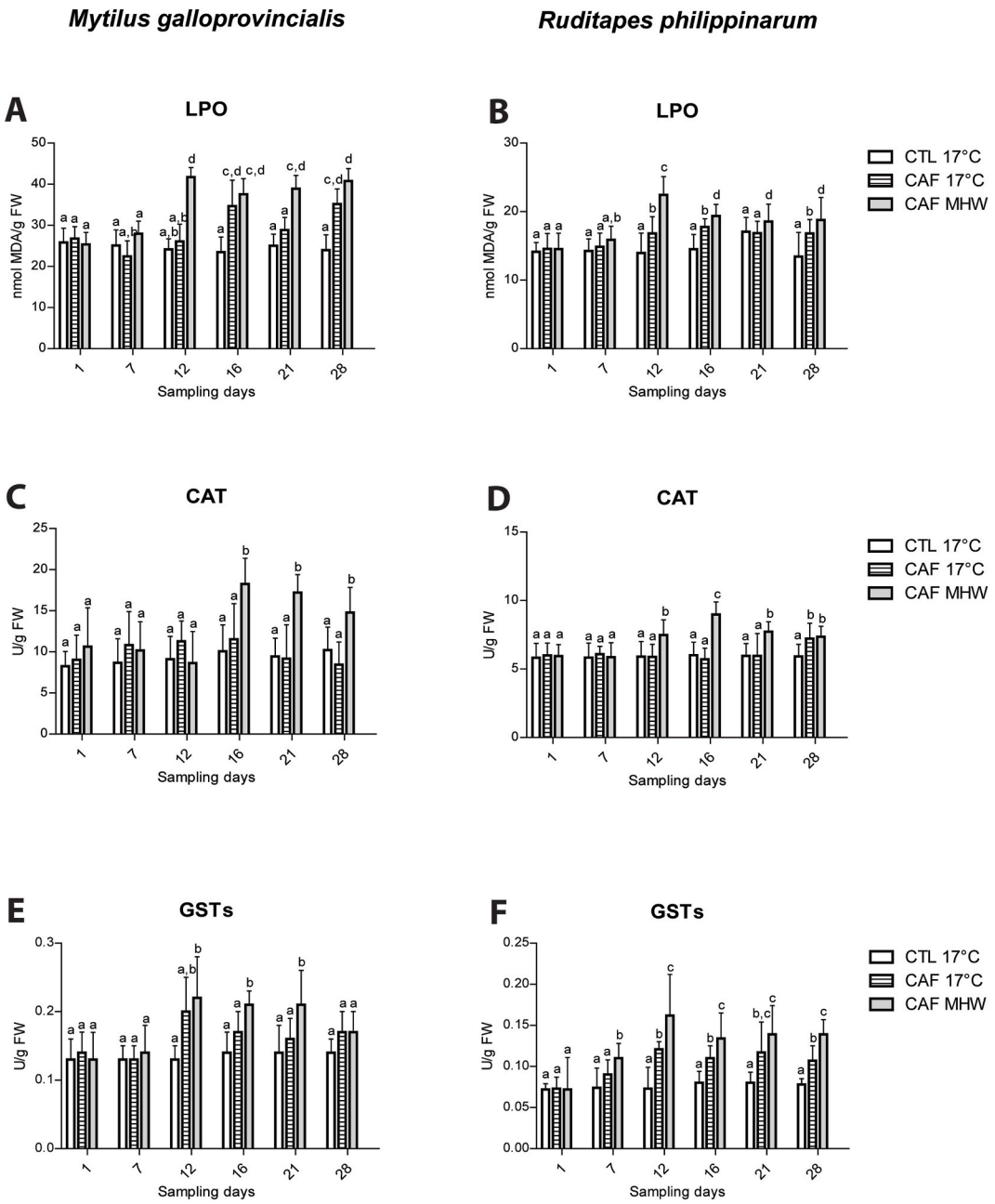

Fig. 2. A & B: Lipid peroxidation (LPO) levels; C & D: Catalase (CAT) activity; E & F: Glutathione S-transferase (GSTs) activity in *Mytilus galloprovincialis* and *Ruditapes philippinarum* exposed to different treatments: control (CTL 17 °C - 17 °C without caffeine), Group I (CAF 17 °C - 17 °C with caffeine); Group II (CAF MHW - marine heatwave with caffeine) for 28 days. Results are means with standard deviations. Significant differences ($p < 0.05$) among treatments are identified with different lowercase letters.

altered physiological and biochemical responses (both in terms of oxidative stress and metabolic pathways) of different invertebrate species when exposed to different contaminants. The authors concluded that the environmental parameters themselves may not cause mortality in estuarine species as the those used in the present research, but they may modify the chemical toxicity. Perhaps, membrane structure and fluidity change in response to temperature, which may increase permeability and chemical uptake.

The results obtained further indicate that *R. philippinarum* was the most responsive species, with early activation of cellular responses than *M. galloprovincialis*. Previous studies showed that mussels may be more resistant than clams to both emerging contaminants exposure and climate change scenarios (Munari et al., 2018), though dependent from seasonal and abiotic variability (Moschino et al., 2011). Despite both selected species are intertidal, they are representative of different habitats with different characteristics: *M. galloprovincialis* lives in the water column attached to hard substrates and the clam species burrows in sediments (Matozzo et al., 2012). These ecological features can result in different biochemical and immune-related responses to cope with multiple stressors, including higher thermal stress resistance in mussels (Munari et al., 2018). Both mussels and clams include species of high ecological and economic importance, being mainly produced in aquaculture. MHWs are predicted to become more frequent and intense due to anthropogenically induced climate change, affecting the bivalve vulnerability and variability in natural ecosystems and also commercial production (Steeves et al., 2018).

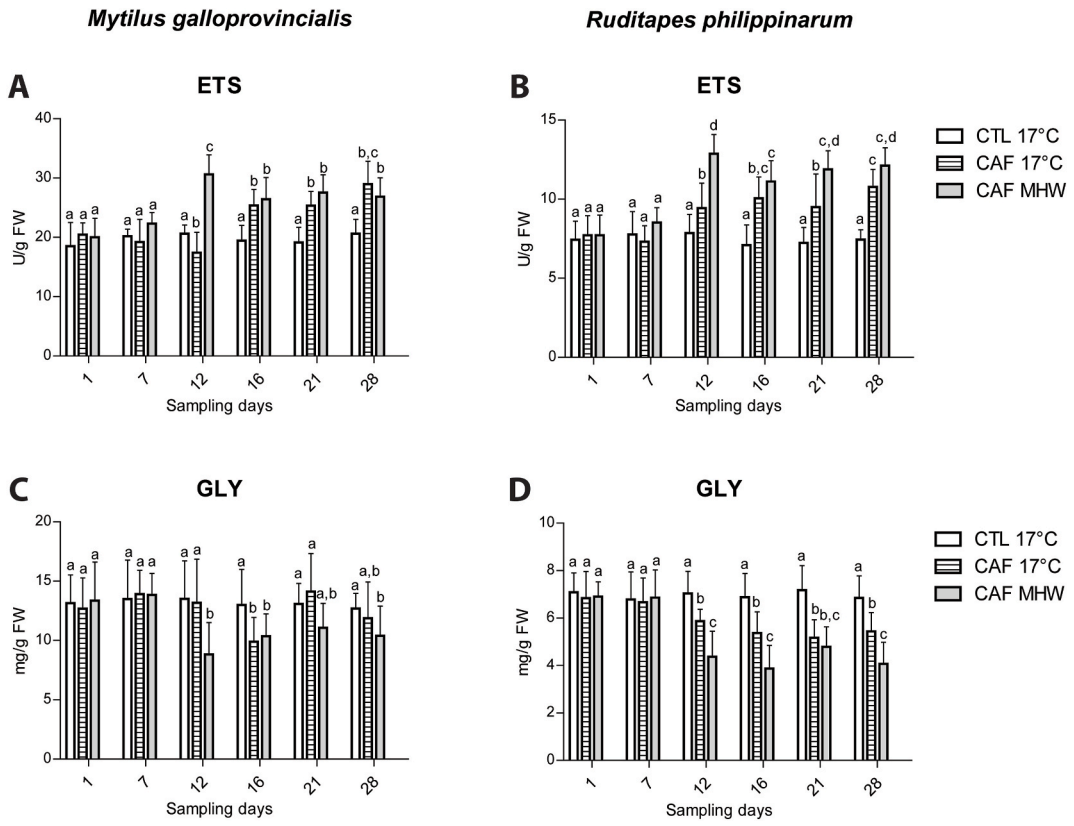

Fig. 3. A & B: Electron transport system (ETS) activity; C & D: Glycogen (GLY) content in *Mytilus galloprovincialis* and *Ruditapes philippinarum* exposed to different treatments: control (CTL 17 °C - 17 °C without caffeine), Group I (CAF 17 °C - 17 °C with caffeine); Group II (CAF MHW - marine heatwave with caffeine) for 28 days. Results are means with standard deviations. Significant differences ($p < 0.05$) among treatments are identified with different lowercase letters.

5. Conclusions

This study confirmed toxic effects of CAF in both bivalve species showing an alteration on neurotransmission, induction of detoxification mechanisms and cellular damage. Moreover, the increase of antioxidant defence mechanisms was associated to an increase of bivalves' metabolic activity and decrease of energy reserves. The obtained results seem magnified in the presence of predicted warming conditions, suggesting to a climate-induced toxicant sensitivity responses. Considering the increasing adverse impacts of CAF pollution in the environment as well as the potential consequences of elevated temperatures and associated heatwaves on marine biodiversity, the present results highlight the urgent need to evaluate potential interactions between toxic chemicals and environmental conditions influenced by Global Change Scenario. On this perspective, understanding of how toxicological mechanisms interact with climate-induced stressors will provide a solid platform for improved effect assessments for both humans and wildlife.

Author contributions

De Marchi, L.: Formal analysis; Methodology; Writing – original draft; **Vieira, L.R.**: Formal analysis; Methodology; Writing – original draft; Intorre, **L., Meucci**, **V., Battaglia**, **F., Pretti**, C.: Resources; Methodology, Writing – review & editing; Funding, Soares, **A.M.V.M.**: Funding, **Freitas, R.**: Conceptualization; Project administration; Resources; Supervision; Writing – review & editing.

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.

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