Marine heatwaves hamper neuro-immune and oxidative tolerance toward carbamazepine in Mytilus galloprovincialis

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Abstract

The increased frequency and intensity of short-term extreme warming phenomena have been associated to harsh biological and ecosystem outcomes (*i.e.,* mass mortalities in marine organisms). Marine heatwaves (MHWs), occurring when seasonal temperature threshold is exceeded for at least 5 consecutive days, may reduce the tolerance of coastal species toward additional pressures, but interactions between such multiple stressors are virtually unexplored. The present study aimed to characterize in *Mytilus galloprovincialis* the influence of a simulated MHW scenario on the toxicological effects of the pharmaceutical carbamazepine (CBZ), ubiquitously detected in the marine environment and chosen as model compound for this relevant class of emerging contaminants. The bioaccumulation of CBZ and responsiveness of various biological parameters, including immune system, antioxidant status, lipid metabolism and cellular integrity, were analyzed in exposed mussels both during and after the end of the heatwave.

MHW appeared to strongly modulate accumulation of CBZ, paralleled by weakened immunocompetence and onset of oxidative disturbance that finally evolved to cellular damages and lipid metabolism disorders. Elaboration of the overall results through a quantitative Weight of Evidence model, revealed the highest hazard in organisms exposed to both the stressors 10 days after the end of the heatwave, suggesting that MHWs could leave a footprint on the capability of mussels to counteract CBZ toxicity, thus affecting their vulnerability and predisposition to adverse effects toward multiple stressors.

Introduction

The global ocean warming trend, with a 0.06°C temperature increase per decade since 1900, is a direct consequence of anthropogenic CO_2 (Garcia-Soto et al., 2021; Bindoff et al., 2013). Ocean adsorbed almost 90% of excess heat from Earth system since the industrial revolution, reaching an unprecedented heat content in 2021, the hottest year ever recorded (Cheng et al., 2022). This long-term and gradual warming is superimposed to an increased frequency of marine heatwaves (MHWs), short-term extreme events with seawater temperature exceeding a seasonally varying threshold (usually the 90th percentile) for at least 5 consecutive days (Collins et al., 2019; Frölicher et al., 2019; Oliver et al., 2019; Hobday et al., 2018; Perkins et al.; 2012).

The number of anomalous warm days per year has increased globally by 50% since the early twentieth century and average intensity of MHWs showed a linear trend of almost +0.1°C per decade since 1982 (Oliver et al., 2019, 2018). These phenomena, attributed to the anthropic impact on climate stability, are projected to worsen on a global scale by the end of the century, with many areas of the ocean experiencing a nearly permanent state of MHW (Collins et al., 2019; Oliver et al., 2019). The Mediterranean Sea represents one of the most endangered areas, where frequency and intensity of MHWs are predicted to increase, causing severe consequences on marine ecosystems structure, functioning and services (Ainsworth et al., 2020; Darmaraki et al., 2019; Genevier et al., 2019; Oliver et al., 2019; Galli et al., 2017). In this respect, MHWs have already been linked to harsh outcomes and mass mortality events of Mediterranean invertebrates (Strydom et al., 2020; Smale et al., 2019; Rubio Portillo et al., 2016; Garrabou et al., 2009).

The consequence of environmental temperature changes can seriously affect organisms health condition since temperature is fundamental in shaping aerobic capacity and physiological homeostasis of marine ectotherms (Sokolova, 2021; Hemraj et al., 2020). Exposure to constantly elevated temperature caused adverse effects ranging from impairment of molecular and cellular

64 pathways (mitochondria functioning, oxidative stress, signalling), up to energy trade-offs and
65 alterations of physiological processes, as immune system efficiency, embryonic development,
66 fertilization and reproduction (Arribas et al., 2022; Crespo et al., 2021; Armstrong et al., 2020; Balogh
67 and Byrne, 2020; Velez et al., 2017; Bartolini et al., 2013; Byrne et al., 2013; Parker et al., 2009;
68 Monari et al., 2007; Abele et al., 2002). Biological responsiveness to stress influences the possibility
69 that organisms resist (maintain performance), recover (restore performance after a decline) or
70 collapse after thermal stress deriving from marine heatwaves (Leung et al., 2019), and possible
71 delayed outcomes of such stress have been reported (Minuti et al., 2021; Amorim et al., 2020).
72 In addition to direct effects, MHWs are of interest also for biological consequences of their
73 interaction with other co-occurring stressors (Arrigo et al., 2020; Przeslawski et al., 2015; Kroeker et
74 al., 2013), including harmful algal blooms and emerging contaminants (ECs) such as perfluorinated
75 compounds, microplastics and pharmaceuticals (Carniero et al., 2021, Lim et al., 2021, Reichert et al.,
76 2021). The widespread occurrence of pharmaceuticals represents a growing risk for coastal areas
77 (COM/2019/128 final), being a direct consequence of the limited removal by wastewater treatment
78 plants, inadequate waste handling and accidental discharges, associated to a global increase of drugs
79 consumption in human medicine and animal husbandry farms (Boxall et al., 2012). Of the 4000
80 pharmaceuticals released in natural ecosystems, the antiepileptic carbamazepine (CBZ) is one of the
81 most environmentally relevant, being ubiquitously detected in water column (up to tens of μg L^{-1})
82 and frequently measured in aquatic invertebrates (Mezzelani et al., 2020; Miller et al., 2019; Álvarez-
83 Muñoz et al., 2018). Among these organisms, previous studies showed that dissolved and particulate-
84 bound carbamazepine can be accumulated in *Mytilus galloprovincialis* through both gills and
85 digestive gland, being rapidly uptaken and metabolized (Serra-Compte et al., 2018; Boillot et al.,
86 2015). Carbamazepine was also reported to affect several physiological and reproductive processes

87 in aquatic non-target species with potential deleterious consequences on wild populations and
88 ecosystem services (Mezzelani and Regoli, 2022; Mezzelani et al., 2021; Almeida et al., 2018).
89 Despite previous studies revealed that ocean and acidification can modulate the bioavailability and
90 effects of several classes of contaminants, *e.g*. trace metals and polycyclic aromatic hydrocarbons
91 (Giuliani et al., 2021; Kibria et al., 2021; Nardi et al., 2021, 2018, 2017; Wu et al., 2020; Moreria et
92 al., 2016; Sokolova and Lannig, 2008), similar interactions were only recently confirmed for
93 pharmaceuticals, including carbamazepine (Mezzelani et al., 2021; Costa et al., 2020; Almeida et al.,
94 2021). In addition, the majority of studies were carried out at constant or linearly increasing
95 temperature values, while the effects of pulsed thermals stress (MHWs) or the possibility of carry-
96 over alterations, have been so far poorly investigated.
97 In the present study, Mediterranean mussel *Mytilus galloprovincialis* were exposed to carbamazepine
98 under a realistic MHW scenario, to test the hypothesis that marine heatwave has detrimental or
99 delayed outcomes on drug accumulation and effects. *M. galloprovincialis* was selected as test species
100 due to its ecological, economic and ecotoxicological value (Musella et al., 2020), being recognized as
101 a suitable bioindicator in the Mediterranean Sea also toward the increasing occurrence of MHWs and
102 pharmaceuticals, as carbamazepine (Almeida et al., 2021; Mezzelani et al., 2020; Darmaraki et al.,
103 2019; Galli et al., 2017). Drug bioaccumulation and a wide panel of biochemical, cellular and
104 histological markers were selected to represent the main pathways of cellular disturbance and stress-
105 response: the neuroendocrine-immune system, antioxidant defenses, lipid metabolism and oxidative
106 damages were measured in mussels tissues during, at the end and ten days after the MHW event.
107 The overall results were finally integrated through a quantitative Weight Of Evidence (WOE) model
108 that elaborates specific hazard indices based on the number, magnitude and toxicological relevance
109 of observed responses (Regoli et al., 2019). This approach provides quantitative insights on multiple

stressors, useful to highlight vulnerability of marine species to interactions between commonly found pharmaceuticals and the increasing intensity, frequency and duration of marine heatwaves.

Materials and Methods

Animal collection and experimental design

Mussels, *Mytilus galloprovincialis* (5.3 ± 0.5 cm shell length), were obtained in October 2019 from a shellfish farm in an unpolluted area of central Adriatic Sea (Mezzelani et al., 2020). As soon as their arrival to laboratory facilities, the whole soft tissues of 15 organisms were dissected for the determination of CBZ levels in wild specimens before the acclimation period. Organisms were maintained for 7 days in aquaria with aerated artificial seawater (ASW; Instant Ocean®) at local seasonal environmental conditions of salinity 35, temperature 18 °C and pH 8.20.

Mussels were then randomly assigned to eight tanks, each containing 60 organisms in 20 L, and exposed for 20 days to clean or carbamazepine contaminated ASW either at constant seasonal environmental temperature or under marine heatwave scenario, according to the four following treatments, performed in duplicate: CTL, control condition at seasonal climatological sea surface temperature (T_{CSST} = 18°C); CBZ, carbamazepine exposure (1 µg L^{-1}) at T_{CSST}=18°C; MHW, marine heatwave scenario (peaking temperature = 22.5 °C, further details are given below and in Supplementary Information Figure SF1); MHW + CBZ, carbamazepine exposure (1 µg L^{-1}) under marine heatwave scenario. The exposure dose of carbamazepine reflects environmental levels in coastal areas (Mezzelani et al., 2018, 2020; Almeida et al., 2021), while marine heatwave scenario was reconstructed based on oceanographic data of events occurred in the Adriatic Sea in late summer – early autumn of 2014 and 2019 (www.marineheatwaves.org/tracker, coordinates: 43.625, 13.625; cumulative intensity of events: 26.8°C and 40.4°C, respectively; duration: 12 and 15 days, respectively). Temperature for marine heatwave scenario was increased daily by 0.9 °C until the peak was reached (22.5 °C, day 5 and 6), then it was daily decreased with the same slope until the climatological mean temperature was restored (18 °C, day 11), and maintained constant for 10 additional days, as “recovery” phase from the marine heatwave. Water was changed every other day,

and carbamazepine re-dosed. Organisms in each tank were fed 24h prior the water change with 500 µL of an unpolluted commercial mixture of zooplankton (Brightwell Zooplanktos-S, size range 50-300 µm) for filter-feeding organisms, according to manufacturer indications.

From each experimental condition, 40 organisms (20 per duplicate tank) were randomly sampled and dissected at three experimental times: T1 (day 6), T2 (day 11) and T3 (day 20) which represented the peak of heatwave (T1), the end of heatwave (T2), and 10-days post heatwave (T3) respectively. The choice of 10 days after the end of the heatwave was considered appropriate to highlight persistence or onset of adverse outcomes after a comparable period of pulsed thermal stress.

The whole soft tissues of 15 individuals were used for analyses of CBZ bioaccumulation (5 pools, each constituted by 3 organisms, stored at -20°C after dissection) while the haemolymph was withdrawn (5 pools, each constituted by 3 organisms), partly immediately processed for in vivo analyses and partly fixed in Carnoy's solution until nuclear alterations analysis. For biomarker analyses, digestive glands and hemolymph were collected from other 20 individuals (5 pools, each constituted by tissues of 4 organism, rapidly frozen in liquid nitrogen and maintained at -80°C). For histological analyses, digestive glands of 5 additional organisms were excised, flash frozen separately and maintained at -80°C.

Chemical analyses

Concentrations of CBZ in mussels whole soft tissues (n=5) were determined by High Performance Liquid Chromatography with fluorometric and diode array detectors. Information on reagents, as well as detailed extraction and analytical protocols, including QA/QC procedures, are given in Supplementary Information.

Biomarkers analyses

160 Validated protocols were used to analyze the following classes and typologies of biomarkers
161 (n=5, details are given in Supplementary Information): immunological responses (haemocytes
162 lysosomal membrane stability, granulocytes vs hyalinocytes ratio and phagocytosis rate), cholinergic
163 effects (acetylcholinesterase activity in haemolymph); levels of antioxidant defenses (activity of
164 catalase, Se-dependent glutathione peroxidases, total glutathione peroxidases, glutathione S-
165 transferases, glutathione reductase, content of total glutathione) and total oxyradical scavenging
166 capacity (TOSC) toward peroxyl radical ROO•, hydroxyl radical HO• and peroxynitrite ONOO-, in the
167 digestive gland; lipid metabolism (acyl-CoA oxidase activity and neutral lipids content) and
168 peroxidation (malondialdehyde concentration and lipofuscin content) in the digestive gland; onset
169 genotoxic damage (DNA fragmentation and micronuclei frequency, in haemocytes).
170
171 *Statistical analyses and weighted elaboration*
172 Data visualization and statistical analyses for CBZ bioaccumulation and biomarkers data were
173 performed using RStudio (version 1.2.5033). Data were checked for normal distribution (Shapiro-Wilk
174 test) and homogeneity of variances (Levene's test); when assumptions were not fulfilled, data were
175 transformed using Box-Cox transformation (power transformation, R package "MASS"). Effect of the
176 factors "CBZ Exposure" (two levels: 0 and 1 µg L^{-1}), "Temperature Scenario" (two levels: constant and
177 marine heatwave), "Experimental Time" (three levels: T1, T2 and T3) and their interactions on CBZ
178 bioaccumulation and biological parameters were assessed using a generalized linear model (three-
179 way ANOVA, Table 1); Tukey HSD (HSD) *post-hoc test* was applied for comparing the means of interest
180 between different treatments at each sampling time and between different sampling times within
181 the same treatment. Multivariate principal component analysis (PCA) of bioaccumulation and
182 biomarkers results was applied to visualize the relationships among the different treatments at each
183 sampling time.

184 The results on bioaccumulation and biomarkers analyses were further elaborated through a
185 quantitative Weight Of Evidence (WOE) model that provides synthetic hazard indices for each
186 typology of data (or Line of Evidence, LOE) before their final integration (Regoli et al., 2019).
187 Independent elaborations for bioaccumulation (LOE-2) and biomarkers (LOE-3) were based on
188 magnitude of observed variations, statistical significance compared to controls and weights assigned
189 to toxicological relevance of measured endpoints. After normalization of indices to a common scale,
190 individual hazard indices were integrated through a classical weight of evidence approach, and level
191 of risk assigned to 1 of 5 classes, from Absent to Severe. Whole calculations, detailed flow-charts,
192 rationale for weights, thresholds and expert judgements have been described in detail in
193 Supplementary Information (Regoli et al., 2019).

194 ## Results

195 *Carbamazepine bioaccumulation*

196 Carbamazepine in tissues of mussels was below the limit of detection of 1.03 ng g^{-1} (d.w.) at the
197 moment of collection (data not shown). Levels of carbamazepine in whole soft tissues of exposed
198 mussels highlighted significant interactions between CBZ exposure, temperature scenario and
199 experimental time (Table 1, Fig. 1). Average CBZ values in MHW+CBZ organisms were almost double
200 at the peak of heatwave (T1) and 10-days post heatwave (T3) compared to those of mussels exposed
201 to CBZ-alone: at control temperature, the peak of accumulation was reached at T2, then remaining
202 almost constant at T3.

203

204 *Immunological and cholinergic alterations*

205 Lysosomal membrane stability in haemocytes was affected by CBZ exposure under the MHW scenario
206 (Table 1, Fig. 2a): the significant reduction of LMS observed at T1 (peak of heatwave), persisted even
207 at the end of heatwave (T2), and 10-days post heatwave (T3). No differences among time were
208 reported for any of the treatments. Significant interactions between CBZ, temperature scenario and
209 exposure time occurred in granulocytes vs. hyalinocytes ratio (Table 1, Fig. 2b): a bell-shaped
210 variation characterized MHW+CBZ treatment, which showed a significant increase at T2 followed by
211 a consistent decrease at T3. Similarly, phagocytosis rate (Table 1, Fig. 2c) was significantly reduced in
212 MHW+CBZ organisms compared to CBZ alone at T1 and T3. Significant differences of AChE activity
213 were measured in organisms exposed to CBZ alone compared both to control condition (CBZ vs. CTL)
214 and to MHW+CBZ organisms, with significantly lower activity in these treatments at T1 and T3 (Table
215 1, Fig. 2d).

Single antioxidant defenses and total oxyradical scavenging capacity

Catalase activity significantly varied along time with a bell-shaped trend (Table 1, Fig. 3a) in organisms exposed to CBZ and MHW+CBZ. Similar effects were observed for Se-dep. glutathione peroxidases (Table 1, Fig. 3b), with enhanced activity measured at T2 compared to T1 and T3; in addition, MHW alone caused a significant induction of Se-dep- GPx activity persisting from the peak up to 10 days after the end of heatwave. In MHW+CBZ organisms, total glutathione peroxidases activity significantly declined at T3 compared to T1 (Table 1; Fig. 3c), while glutathione S-transferases showed a trend of increasing activity reaching a peak of significant induction at T3 in CBZ-exposed organisms, independently of temperature scenario (Table 1; Fig. 3d). Glutathione reductase was enhanced by single and combined stressors, particularly evident at T2 (Table 1; Fig. 3e). Total glutathione increased in all treatments at T1 remaining elevated at T3 in organisms exposed to CBZ, particularly in MHW+CBZ condition (Table 1; Fig. 3f). Significant effects of carbamazepine and MHW were highlighted on TOSC ROO•, TOSC HO• and TOSC ONOO- (Table 1, Fig. 4a, b and c respectively). TOSC ROO• increased at T2 in organisms exposed to CBZ at control temperature, while in marine heatwave scenarios (MHW and MHW + CBZ) this trend was inverted and exacerbated by the presence of the drug. An increased capability to counteract HO• was observed in organisms exposed to marine heatwave (MHW and MHW + CBZ) at T1 and to MHW+CBZ also at T3. Organisms co-exposed to MHW+CBZ showed significantly lowered capability to counteract peroxynitrite (TOSC ONOO-) compared to single stressors both at T1 and T2.

235 *Lipid metabolism and peroxidation*

236 CBZ exposure under MHW scenario resulted in a lowered activity of Acyl CoA oxidase compared to
237 control temperature at T1 (Table 1, Fig. 5a).

238 The content of neutral lipids generally raised in all the treatments with significant interactions
239 between CBZ exposure, MHW and time (Table 1, Fig. 5b). CBZ showed a prolonged and increasing
240 effect for the whole duration of exposure under constant temperature scenario (CBZ treatment),
241 while the effect was not time dependent when CBZ exposure was combined to MHW. Different
242 effects of MHW and CBZ were highlighted by divergent responses when organisms were exposed to
243 single or combined stressors: organisms exposed to MHW also exhibited a marked increase of neutral
244 lipids at the peak of heatwave (T1), followed by a rapid decrement at T2 and T3. CBZ and MHW (Table
245 1, Fig. 5c) caused slight and irregular changes of malondialdehyde (MDA) at T1 and T2, respectively,
246 while no variations were observed in co-exposed organisms, which showed MDA content always
247 comparable to CTL.

248 Lipofuscin content was significantly affected by interactions of CBZ exposure, MHW and experimental
249 times (Table 1, Fig. 5d). The drug, independently of the temperature scenario, led to increased levels
250 of lipofuscin at T1 and T2 in CBZ and MHW+CBZ organisms, while values were comparable to CTL at
251 T3. A biphasic trend was observed also in MHW treated organisms which showed an initial increase
252 of lipofuscin at T1, followed by the decrement at T2 and T3 with values which, however, remained
253 higher compared to CTL even after 10 days of recovery.

Genotoxic damage

Exposure to CBZ and heatwave, alone or in combination determined a significant loss of DNA integrity at T3 (Table 1, Fig. 6a); in addition, a three phases trend was observed in MHW scenario treatments (MHW and MHW + CBZ): haemocytes experienced DNA damage accumulation at T1, which dropped at T2 and furtherly increased at T3. CBZ-exposure also led to an enhancement of micronuclei frequency at T3 (Table 1, Fig. 6b): interestingly, in MHW+CBZ organisms, the highest frequency of MN was observed at T1 when no effects were caused by single stressors.

Principal components analysis and weighted elaboration

Principal components analysis carried out for each experimental time on the whole dataset of results (Fig. 7) provided two-dimensional patterns of separation between treatments, always explaining more than 50% of total variance. At T1 (Fig. 7a), divergence was observed between the two temperature scenarios, at T2 single and combined stressors were separated (Fig. 7b), while at T3 a relevant discrimination was further observed for CBZ exposure along Dimension 1 and for MHW along Dimension 2, producing a split between MHW+CBZ and other treatments (Fig. 7c).

Synthetic hazard indices for each experimental treatment and time of exposure were provided by the weighted elaboration of bioaccumulation (LOE-2) and biomarkers (LOE-3) results (Fig. 8 and details in Supplementary Information). Bioaccumulation hazard (LOE-2) for CBZ treatment was classified as "Slight" at T1, "Major" at T2 and "Moderate" at T3, while for MHW+CBZ treatment was classified as "Moderate" at T2 and "Major" at T1 and T3. The absence of CBZ exposure and bioaccumulation in MHW treatment resulted in an "Absent" hazard classification for this treatment. The elaboration of biomarkers (LOE-3) based on the magnitude of variations compared to CTL organisms and the toxicological relevance of each analyzed parameter produced for CBZ-treated organisms (CBZ and MHW+CBZ) a "Moderate" hazard classification at T1 and T2 and a "Slight" hazard classification at T3. In MHW organisms, hazard was classified as "Moderate" at T1 and "Slight" at T2 and T3. The

278 contribution of each investigated parameter to such hazard classifications, is summarized in
279 Supplementary Information Table ST2. The final weighted integration of LOE-2 and LOE-3, shown in
280 Figure 8, revealed a level of risk constantly classified as “Moderate” or “Slight” for the carbamazepine
281 treatment (CBZ) and marine heatwave scenario (MHW), respectively. When carbamazepine was
282 dosed under marine heatwave scenario (MHW+CBZ), a “Moderate” risk was assigned at the peak and
283 at the end of heatwave (T1-T2), while it increased to “Major” after 10 days of recovery from the event
284 (T3).

Discussion

This study provided clear evidence of the capability of marine heatwaves to modulate the susceptibility to the pharmaceutical CBZ in mussels.

Beside the confirmed capability of *M. galloprovincialis* to accumulate this drug, organisms exposed under MHW scenario exhibited the highest CBZ tissue levels, even after a recovery period from the heatwave: to our knowledge this is the first study observing delayed effects on pharmaceuticals uptake after the exposure to a pulsed thermal stress, which characterizes MHWs. Additional evidence on the modulation of the toxicity of pharmaceuticals in future oceans derives from the onset of immune system alterations caused by combined rather than single stressors. This was particularly evident on haemocytes lysosomal membranes, which lost integrity in co-exposed organisms during all phases of the heatwave and recovery period, consistently with the elevated accumulation of CBZ. We hypothesize an early disturbance triggered by CBZ (Mezzelani et al., 2021; Franzellitti et al., 2019; Aguirre-Martínez et al., 2013; Martin-Diaz et al., 2009) and sustained thereon by thermal stress (Marigómez et al., 2017; Parisi et al., 2017), supported by the reduced capability of lysosomes to recover heat-damaged membranes, as previously observed in *M. galloprovincialis* exposed to a secondary stressor (*e.g.* cadmium, Múgica et al., 2015). Synergistic effects of CBZ and MHW were evident also on haemocytes sub-populations and their functional activity. In co-exposed organisms, granulocytes-hyalinocytes ratio and phagocytosis activity showed a bell-shaped trend of variation over MHW phases: a counteracting phase, characterized by an increase of these immunological parameters at the end of the heatwave, was followed by a significant inhibition observed after 10 days of recovery from the event, confirming that MHWs modulate CBZ toxicity and the immune system of mussels, potentially increasing their long-term vulnerability to stressors. The mechanisms behind these effects may involve CBZ-mediated alterations of the neuroendocrine-immune system (Liu et al., 2018): CBZ was shown to increase ACh synthesis in non-target organisms (Mizuno et al., 2000) and this neurotransmitter has been recently suggested to suppress bivalves phagocytosis through the alteration of NF-kB and Ca^{2+} signaling pathways (Cao et al., 2021; Du et al., 2020). The differences of AChE activity observed in this study between organisms

exposed to CBZ alone or under marine heatwave scenario, support the hypothesis that the impairment of immune system in co-exposed organisms is, at least partially, caused by a reduced ACh hydrolysis. Such alterations might have been exacerbated by a reduced functionality of haemocytes due to an increased oxidative pressure caused by thermal stress of MHW (Benedetti et al., 2022; Rahman et al., 2019), and confirmed in co-exposed organisms by the appreciable responsiveness of antioxidant defenses in the digestive gland.

Catalase and Se-dependent glutathione peroxidases in CBZ-treated mussels (CBZ and MHW+CBZ) showed a temporary increase at the end of the heatwave suggesting a delayed demand of hydrogen peroxide detoxification (Regoli and Giuliani, 2014), possibly reflecting CBZ-metabolism: in non-target species, this drug is biotransformed through phase I and II pathways, that may thus promote an intracellular formation of ROS (Mezzelani et al., 2021; Benedetti et al., 2022). The increased activity of the phase II enzymes glutathione S-transferases observed in the present study, along with variations of glutathione reductase and levels of total glutathione, support the hypothesis of biotransformation as a source of ROS worsened by thermal stress and an increased demand of glutathione metabolism to neutralize derived ROS.

The responsiveness of individual antioxidants was paralleled by alterations of the total oxyradical scavenging capacity: in co-exposed organisms the capability to neutralize hydroxyl and peroxyl radicals (TOSC HO• and TOSC ROO•, respectively), showed trends of variations complementary to CAT and Se-dependent GPx and analogous to TGSH levels. The early increase of defenses against these ROS and the following variations, confirm non-synchronous responsiveness of single antioxidants and oxidative challenge during exposure. On the other hand, the initial depletion of capability to neutralize peroxynitrite (TOSC ONOO-) was followed by its relevant increase after the thermal stress, suggesting that the acute disturbance of this redox pathway was partly compensated by the mutual interplay with other antioxidant mechanisms (Regoli and Giuliani, 2014). Overall, the persistence of antioxidants alterations after the recovery from the heatwave, further corroborates the additional impact of MHWs on oxidative effects of CBZ, with potential energetic costs and metabolic trade-offs to restore redox homeostasis (Sokolova, 2021).

335 In this respect, a disturbance of lipid metabolism was evidenced in co-exposed organisms, which showed a
336 moderate decrease of Acyl-CoA oxidase and accumulation of neutral lipids (NL). Both carbamazepine and
337 thermal stress have been previously suggested to have a role on neutral lipids accumulation (Múgica et al.,
338 2015; Mezzelani et al., 2021; Martin-Diaz et al., 2009; Dimitriadis et al., 2004), but the effects observed in
339 this study for combined stressors were different from those of single ones. In particular, co-exposed
340 organisms accumulated NL in the early phases of the heatwave, and these remained constant throughout
341 the exposure, despite the end of thermal stress; however, after ten days of recovery, levels of NL were lower
342 compared to those of organisms exposed to CBZ alone, allowing to hypothesize that lipid metabolism is
343 affected by reciprocal but still unknown interactions between thermal stress and carbamazepine.
344 Lipofuscin content in tertiary lysosomes was increased by all stressors (alone or in combination) highlighting
345 that the sustained activation of the antioxidant system could not prevent lipid peroxidation processes in the
346 early phases of stress, and confirming the progression of oxidative imbalance from the activation of
347 biochemical defenses to cellular disturbance.
348 Oxidative damages were not limited to lipid peroxidation but also involved DNA integrity and micronuclei
349 formation: exposure to single stressors promoted DNA fragmentation, particularly at the end of recovery
350 period, with similar outcomes but no synergistic effects in co-exposed organisms. Conversely, a subtle
351 enhancement of micronuclei frequency was observed in CBZ-treatments (CBZ and MHW+CBZ), with an
352 earlier and more evident effect in co-exposed organisms, highlighting a hastening of temperature-mediated
353 effect on CBZ genotoxicity (Nardi et al., 2017; Yao et al., 2013; Salazar et al., 2009).
354 Principal components analysis provided a detailed perspective on the prevalence of different stressors in
355 each phase of the experiment. At the peak of the heatwave (T1) a major separation occurred between
356 organisms exposed at control temperature and those under MHW scenario independently of CBZ exposure,
357 indicating the early onset of disturbance due to the extreme thermal stress; conversely, at the end of the
358 heatwave (T2), a major disturbance of CBZ was revealed, suggesting that the need to cope thermal stress
359 was rapidly met but may be disadvantageous in terms of protection toward chemical disturbance. Lastly,

after 10 days from the end of the heatwave (T3), a clear separation occurred between single and combined stressors, supported by the synergic effects in co-exposed organisms.

Differences among treatments and experimental phases were summarized by the weighted elaboration of bioaccumulation and biological responses data through the Weight of Evidence model (Sediqualsoft). The elaboration of bioaccumulation results (LOE-2), based on the levels of CBZ and the magnitude of variation compared to CTL organisms, revealed a consistently higher hazard in co-exposed organisms (MHW+CBZ). For this treatment, the overall integration of the wide panel of analyzed biological traits (LOE-3) revealed a "Moderate" hazard at the heatwave peak, classification supported by the accumulation of lipid peroxidation products, immune parameters alterations and effects on lipid metabolism and redox homoeostasis; this "Moderate" hazard classification persisted until the end of the heatwave, sustained by the effects on lipid peroxidation and metabolism, immune parameters and antioxidant pathways; the hazard decreased to "Slight" after the recovery phase, with still evident effects on certain immune and antioxidant responses. The final integration of chemical and biological results through a WOE approach, provided a synthetic hazard index with the worst classification ("Major") in co-exposed organisms after 10 days of recovery from the heatwave, thus highlighting long-lasting synergic interaction of stressors and corroborating the hypothesis of marine heatwave footprint in organisms coping with CBZ.

Conclusions

This study showed that the occurrence of short-term and pulsed events of temperature extremes may enhance the accumulation of CBZ in mussels, causing effects that persist even 10 days after the end of the heatwave. Interactions among stressors synergistically impaired both the neuroendocrine-immune and oxidative system of organisms, addressing the intensification of extreme events as a key environmental challenge, which can exacerbate the consequences of the increasing release of pharmaceuticals. Due to the ecological relevance of this study referred to Mediterranean conditions, where both MHWs and pharmaceuticals represent emerging challenges, our findings encourage further investigations to better

385 elucidate reciprocal interactions of such multiple stressors, recovery capacity and long-term perspectives

386 for biodiversity conservation, environmental impact and climate change risk assessment.

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Data availability

The data supporting the conclusions of this article are available at the following link: https://figshare.com/s/9c28068c95c8d5661f86.

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References

Abele, D., Heise, K., Pörtner, H.O., Puntarulo, S. Temperature-dependence of mitochondrial function and production of reactive oxygen species in the intertidal mud clam *Mya arenaria*. *Journal of Experimental Biology*, 205 (13), 1831-41, (2002).

Amorim, V.E., Gonçalves, O., Capela, R., Fernández-Boo, S., Oliveira, M., Dolbeth, M., Arenas, F., Cardoso, P.G. Immunological and oxidative stress responses of the bivalve *Scrobicularia plana* to distinct patterns of heatwaves. *Fish and Shellfish Immunology*, 106, pp. 1067-1077. DOI: 10.1016/j.fsi.2020.09.024, (2020).

Aguirre-Martínez, G.V., Buratti, S., Fabbri, E., Del Valls, T.A., Martín-Díaz, M.L. Stability of lysosomal membrane in *Carcinus maenas* acts as a biomarker of exposure to pharmaceuticals. *Environmental Monitoring and Assessment*, 185 (5), DOI: 10.1007/s10661-012-2827-2, (2013).

Ainsworth, T.D., Hurd, C.L., Gates, R.D., Boyd, P.W. How do we overcome abrupt degradation of marine ecosystems and meet the challenge of heat waves and climate extremes? *Global Change Biology*, 26 (2), DOI: 10.1111/gcb.14901, (2020).

Almeida, Â., Calisto, V., Esteves, V.I., Schneider, R.J., Soares, A.M.V.M., Figueira, E., Freitas, R. Effects of single and combined exposure of pharmaceutical drugs (carbamazepine and cetirizine) and a metal (cadmium) on the biochemical responses of *R. philippinarum*. *Aquatic Toxicology*, 198, DOI: 10.1016/j.aquatox.2018.02.011, (2018).

Almeida, Â., Soares, A.M.V.M., Esteves, V.I., Freitas, R. Occurrence of the antiepileptic carbamazepine in water and bivalves from marine environments: A review. *Environmental Toxicology and Pharmacology*, 86, DOI: 10.1016/j.etap.2021.103661, (2021).

Álvarez-Muñoz, D., Rodríguez-Mozaz, S., Jacobs, S., Serra-Compte, A., Cáceres, N., Sioen, I., Verbeke, W., Barbosa, V., Ferrari, F., Fernández-Tejedor, M., Cunha, S., Granby, K., Robbens, J., Kotterman, M., Marques, A., Barceló, D. Pharmaceuticals and endocrine disruptors in raw and cooked seafood from European market: Concentrations and human exposure levels. *Environment International*, 119, DOI: 10.1016/j.envint.2018.07.006, (2018).

Armstrong, E.J., Dubousquet, V., Mills, S.C., Stillman, J.H. Elevated temperature, but not acidification, reduces fertilization success in the small giant clam, Tridacna maxima. Marine Biology, 167 (1), art. no. 8, . DOI: 10.1007/s00227-019-3615-0, (2020).

Arribas, L.P., Alfaya, J.E.F., Palomo, M.G., Giulianelli, S., Nieto Vilela, R.A., Bigatti, G. Ocean warming lead to heat shock protein expression and decrease in the feeding rate of the Patagonian sea star *Anasterias minuta.* Journal of Experimental Marine Biology and Ecology, 546, art. no. 151661, . DOI: 10.1016/j.jembe.2021.151661, (2022).

Arrigo, K.R., van Dijken, G.L., Cameron, M.A., van der Grient, J., Wedding, L.M., Hazen, L., Leape, J., Leonard, G., Merkl, A., Micheli, F., Mills, M.M., Monismith, S., Ouellette, N.T., Zivian, A., Levi, M., Bailey, R.M. Synergistic interactions among growing stressors increase risk to an Arctic ecosystem. *Nature Communications*, 11 (1), DOI: 10.1038/s41467-020-19899-z, (2020).

Balogh, R. & Byrne, M. Developing in a warming intertidal, negative carry over effects of heatwave conditions in development to the pentameral starfish in *Parvulastra exigua*. *Marine Environmental Research,* 162, DOI: 10.1016/j.marenvres.2020.105083. (2020).

Bartolini, F., Barausse, A., Pörtner, H.-O., Giomi, F. Climate change reduces offspring fitness in littoral spawners: A study integrating organismic response and long-term time-series. *Global Change Biology*, 19 (2), DOI: 10.1111/gcb.12050. (2013).

Benedetti, M., Giuliani, M. E., Mezzelani, M., Nardi, A., Pittura, L., Gorbi, S., Regoli, F. Emerging environmental stressors and oxidative pathways in marine organisms: Current knowledge on regulation mechanisms and functional effects. *BIOCELL*, 46(1), DOI: doi:10.32604/biocell.2022.017507, (2022).

Bindoff, N.L. et al. Detection and Attribution of Climate Change: from Global to Regional. In: *Climate Change 2013: The Physical Science Basis.* Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, 867–952. (2013).

Boillot, C., Martinez Bueno, M.J., Munaron, D., Le Dreau, M., Mathieu, O., David, a., Fenet, H., Casellas, C., Gomez, E. In vivo exposure of marine mussels to carbamazepine and 10-hydroxy-10,11-dihydro-carbamazepine: bioconcentration and metabolization. Sci. Total Environ. 532, 564-570; https:// doi.org/10.1016/j.scitotenv.2015.05.067, (2015).

Boxall, A.B., Rudd, M.A., Brooks, B.W., Caldwell, D.J. Pharmaceuticals and personal care products in the environment: what are the big questions? Environ. Health Perspect. 120, 1221-229; https://doi.org/10.1289/ehp.1104477, (2012).

Byrne, M., Ho, M.A., Koleits, L., Price, C., King, C.K., Virtue, P., Tilbrook, B., Lamare, M. Vulnerability of the calcifying larval stage of the Antarctic sea urchin *Sterechinus neumayeri* to near-future ocean acidification and warming. *Global Change Biology*, 19 (7), DOI: 10.1111/gcb.12190, (2013).

Cao, Y., Jiao, Y., Zheng, C., Zhan, S., Gu, Z., Xiong, X., Wang, Q., Du, X. Transcriptome analysis provides insights into the function of the cholinergic system in pearl oyster transplantation immunity. *Aquaculture*, 545, DOI: 10.1016/j.aquaculture.2021.737205, (2021).

Carneiro, A.P., Soares, C.H.L., Pagliosa, P.R. Does the environmental condition affect the tolerance of the bivalve Anomalocardia flexuosa to different intensities and durations of marine heatwaves? Marine Pollution Bulletin, 168,112410, ISSN 0025-326X, https://doi.org/10.1016/j.marpolbul.2021.112410, (2021).

Cheng, L., Abraham, J., Trenberth, K.E., Fasullo, J., Boyer, T., Mann, M.E., Zhu, J., Wang, F., Locarnini, R., Li, Y., Zhang, B., Tan, Z., Yu, F., Wan, L., Chen, X., Song, X., Liu, Y., Reseghetti, F., Simoncelli, S., Gouretski, V., Chen, G., Mishonov, A., Reagan, J. Another record: ocean warming continues through 2021 despite La Niña conditions. Advances in Atmospheric Sciences, DOI: 10.1007/s00376-022-1461-3, (2022).

Collins, M., Sutherland, M., Bouwer, L., Cheong, S.-M., Frölicher, T., Jacot Des Combes, H., Koll Roxy, M., Losada, I., McInnes, K., Ratter, B., Rivera-Arriaga, E., Susanto, R.D., Swingedouw, D., Tibig, L. Extremes, Abrupt Changes and Managing Risk. In: *IPCC Special Report on the Ocean and Cryosphere in a Changing Climate* [H.-O. Pörtner, D.C. Roberts, V. Masson-Delmotte, P. Zhai, M. Tignor, E. Poloczanska, K. Mintenbeck, A. Alegría, M. Nicolai, A. Okem, J. Petzold, B. Rama, N.M. Weyer (eds.)]. In press. (2019).

Communication from the Commission to the European Parliament, the Council and the European Economic and Social Committee. European Union Strategic Approach to Pharmaceuticals in the Environment COM/2019/128 final, 2019.

Costa, S., Coppola, F., Pretti, C., Intorre, L., Meucci, V., Soares, A.M.V.M., Freitas, R., Solé, M. The influence of climate change related factors on the response of two clam species to diclofenac. *Ecotoxicology and Environmental Safety*, 189, DOI: 10.1016/j.ecoenv.2019.109899, (2020).

Crespo, D., Leston, S., Rato, L.D., Martinho, F., Novais, S.C., Pardal, M.A., Lemos, M.F.L. Does an invasive bivalve outperform its native congener in a heat wave scenario? A laboratory study case with *Ruditapes decussatus* and *R. Philippinarum.* Biology, 10 (12), art. no. 1284, DOI: 10.3390/biology10121284, (2021).

Dimitriadis, V.K., Domouhtsidou, G.P., Cajaraville, M.P. Cytochemical and histochemical aspects of the digestive gland cells of the mussel *Mytilus galloprovincialis* (L.) in relation to function. *Journal of Molecular Histology*, 35 (5), DOI: 10.1023/B:HIJO.0000045952.87268.76, (2004).

Du, X., Tang, Y., Han, Y., Ri, S., Kim, T., Ju, K., Shi, W., Sun, S., Zhou, W., Liu, G. Acetylcholine suppresses phagocytosis via binding to muscarinic- and nicotinic-acetylcholine receptors and subsequently interfering Ca^{2+} and NFκB-signaling pathways in blood clam. *Fish and Shellfish Immunology*, 102, 152-160. DOI: 10.1016/j.fsi.2020.04.030, (2020).

Franzellitti, S., Balbi, T., Montagna, M., Fabbri, R., Valbonesi, P., Fabbri, E., Canesi, L. Phenotypical and molecular changes induced by carbamazepine and propranolol on larval stages of *Mytilus galloprovincialis*. *Chemosphere*, 234, DOI: 10.1016/j.chemosphere.2019.06.045, (2019).

Frölicher, T.L., Fischer, E.M., Gruber, N. Marine heatwaves under global warming. *Nature* 560, DOI: 10.1038/s41586-018-0383-9, (2019).

Galli, G., Solidoro, C., Lovato, T. Marine Heat Waves Hazard 3D Maps and the Risk for Low Motility Organisms in a Warming Mediterranean Sea. *Frontiers in Marine Science*, 4, DOI: 10.3389/fmars.2017.00136, (2017).

Garcia-Soto, C., Cheng, L., Caesar, L., Schmidtko, S., Jewett, E.B., Cheripka, A., Rigor, I., Caballero, A., Chiba, S., Báez, J.C., Zielinski, T., Abraham, J.P. An Overview of Ocean Climate Change Indicators: Sea Surface Temperature, Ocean Heat Content, Ocean pH, Dissolved Oxygen Concentration, Arctic Sea Ice Extent, Thickness and Volume, Sea Level and Strength of the AMOC (Atlantic Meridional Overturning Circulation). Front. Mar. Sci. 8:642372. DOI: 10.3389/fmars.2021.642372, (2021).

Garrabou, J., Coma, R., Bensoussan, N., Bally, M., Chevaldonné, P., Cigliano, M., Diaz, D., Harmelin, J.G., Gambi, M.C., Kersting, D.K., Ledoux, J.B., Lejeusne, C., Linares, C., Marschal, C., Pérez, T., Ribes, M., Romano, J.C., Serrano, E., Teixido, N., Torrents, O., Zabala, M., Zuberer, F., Cerrano, C. Mass mortality in Northwestern Mediterranean rocky benthic communities: Effects of the 2003 heat wave. *Global Change Biology*, 15 (5), DOI: 10.1111/j.1365-2486.2008.01823.x, (2009).

Genevier, L.G.C., Jamil, T., Raitsos, D.E., Krokos, G., Hoteit, I. Marine heatwaves reveal coral reef zones susceptible to bleaching in the Red Sea. *Global Change Biology*, 25 (7), DOI: 10.1111/gcb.14652, (2019).

Giuliani, M.E., Nardi, A., Di Carlo, M., Benedetti, M., Regoli, F. Transcriptional and catalytic responsiveness of the antarctic fish *trematomus bernacchii* antioxidant system toward multiple stressors. *Antioxidants*, 10 (3), DOI: 10.3390/antiox10030410, (2021).

Hemraj, D.A., Posnett, N.C., Minuti, J.J., Firth L.B., Russell, B.D. Survived but not safe: Marine heatwave hinders metabolism in two gastropod survivors. *Marine Environmental Research*, 162. DOI: 10.1016/j.marenvres.2020.105117, (2020).

Hobday, A.J., Oliver, E.C.J., Gupta, A.S., Benthuysen, J.A., Burrows, M.T., Donat, M.G., Holbrook, N.J., Moore, P.J., Thomsen, M.S., Wernberg, T., Smale, D.A. Categorizing and naming marine heatwaves. *Oceanography*, 31 (2 Special Issue), DOI: 10.5670/oceanog.2018.205, (2018).

Kibria, G., Nugegoda, D., Rose, G., Haroon, A. Climate change impacts on pollutants mobilization and interactive effects of climate change and pollutants on toxicity and bioaccumulation of pollutants in estuarine and marine biota and linkage to seafood security. *Marine Pollution Bulletin*, *167*, DOI: 10.1016/j.marpolbul.2021.112364, (2021).

Kroeker, K.J., Kordas, R.L., Crim, R., Hendriks, I.E., Ramajo, L., Singh, G.S., Duarte, C.M., Gattuso, J.-P. Impacts of ocean acidification on marine organisms: Quantifying sensitivities and interaction with warming. *Global Change Biology*, 19 (6), DOI: 10.1111/gcb.12179, (2013).

Leung, J.Y.S., Russell, B.D., Connell, S.D. Adaptive Responses of Marine Gastropods to Heatwaves. *One Earth*, 1 (3), pp. 374-381. DOI: 10.1016/j.oneear.2019.10.025, (2019).

Lim, Y. K., Park, B. S., Kim, J. H., Baek, S. S., & Baek, S. H. Effect of marine heatwaves on bloom formation of the harmful dinoflagellate Cochlodinium polykrikoides: Two sides of the same coin?. *Harmful algae,* 104, 102029. https://doi.org/10.1016/j.hal.2021.102029, (2021).

Liu, Z., Li, M., Yi, Q., Wang, L., and Song, L. The neuroendocrine-immune regulation in response to environmental stress in marine bivalves. *Front. Physiol.* 9 DOI: 10.3389/fphys.2018.01456, (2018).

Nardi, A., Benedetti, M., Gorbi, S., Regoli, F. Interactive Immunomodulation in the Mediterranean Mussel Mytilus galloprovincialis Under Thermal Stress and Cadmium Exposure. Frontiers in Marine Science, 8, art. no. 751983, . DOI: 10.3389/fmars.2021.751983, (2021).

Marigómez, I., Múgica, M., Izagirre, U., Sokolova, I.M. Chronic environmental stress enhances tolerance to seasonal gradual warming in marine mussels. *PLoS ONE*, 12 (3), DOI: 10.1371/journal.pone.0174359, (2017).

Marin, M., Feng, M., Phillips, H.E., Bindoff, N.L. A Global, Multiproduct Analysis of Coastal Marine Heatwaves: Distribution, Characteristics, and Long-Term Trends. *Journal of Geophysical Research: Oceans*, 126 (2), DOI: 10.1029/2020JC016708, (2021).

Martin-Diaz, L., Franzellitti, S., Buratti, S., Valbonesi, P., Capuzzo, A., Fabbri, E. Effects of environmental concentrations of the antiepilectic drug carbamazepine on biomarkers and cAMP-mediated cell signaling in the mussel *Mytilus galloprovincialis. Aquatic Toxicology*, 94 (3), DOI: 10.1016/j.aquatox.2009.06.015, (2009).

Mezzelani, M., Fattorini, D., Gorbi, S., Nigro, M., Regoli, F. Human pharmaceuticals in marine mussels: Evidence of sneaky environmental hazard along Italian coasts. *Marine Environmental Research*, 162, DOI: 10.1016/j.marenvres.2020.105137, (2020).

Mezzelani, M., Nardi, A., Bernardini, I., Mila, M., Peruzza, L., d'Errico, G., Fattorini, D., Gorbi, S., Patarnello, T., Regoli, F. Environmental pharmaceuticals and climate change: The case study of carbamazepine in *M. galloprovincialis* under ocean acidification scenario. *Envrionment International,* 146, DOI: 10.1016/j.envint.2020.106269, (2021).

549 Mezzelani, M., Regoli, F. The Biological Effects of Pharmaceuticals in the Marine Environment. *Annual*
550 *Review of Marine Science*, 14. DOI: 10.1146/annurev-marine-040821-075606, (2022).

551 Miller, T.H., Ng, K.T., Bury, S.T., Bury, S.E., Bury, N.R., Barron, L.P. Biomonitoring of pesticides,
552 pharmaceuticals and illicit drugs in a freshwater invertebrate to estimate toxic or effect pressure.
553 *Environment International*, 129, DOI: 10.1016/j.envint.2019.04.038, (2019).

554 Minuti, J.J., Byrne, M., Hemraj, D.A., Russell, B.D. Capacity of an ecologically key urchin to recover from
555 extreme events: Physiological impacts of heatwaves and the road to recovery. *Science of the Total*
556 *Environment*, 785, DOI: 10.1016/j.scitotenv.2021.147281, (2021).

557 Mizuno, K., Okada, M., Murakami, T., Kamata, A., Zhu, G., Kawata, Y., Wada, K., Kaneko, S. Effects of
558 carbamazepine on acetylcholine release and metabolism. *Epilepsy Research*, 40 (2-3), DOI: 10.1016/S0920-
559 1211(00)00129-7, (2000).

560 Monari, M., Matozzo, V., Foschi, J., Cattani, O., Serrazanetti, G.P., Marin, M.G. Effects of high temperatures
561 on functional responses of haemocytes in the clam *Chamelea gallina*. *Fish and Shellfish Immunology*, 22 (1-
562 2), DOI: 10.1016/j.fsi.2006.03.016, (2007).

563 Moreira, A., Figueira, E., Soares, A.M.V.M., Freitas, R. The effects of arsenic and seawater acidification on
564 antioxidant and biomineralization responses in two closely related *Crassostrea* species. *Science of the Total*
565 *Environment*, 545-546, DOI: 10.1016/j.scitotenv.2015.12.029, (2016).

566 Múgica, M., Sokolova, I.M., Izagirre, U., Marigómez, I. Season-dependent effects of elevated temperature
567 on stress biomarkers, energy metabolism and gamete development in mussels. *Marine Environmental*
568 *Research*, 103, DOI: 10.1016/j.marenvres.2014.10.005, (2015).

569 Musella, M., Wathsala, R., Tavella, T., Rampelli, S., Barone, M., Palladino, G., Biagi, E., Brigidi, P., Turroni, S.,
570 Franzellitti, S., Candela, M. Tissue-scale microbiota of the Mediterranean mussel (*Mytilus galloprovincialis*)
571 and its relationship with the environment. *Science of the Total Environment*, 717, art. no. 137209, . DOI:
572 10.1016/j.scitotenv.2020.137209, (2020).

573 Nardi, A., Benedetti, M., d'Errico, G., Fattorini, D., Regoli, F. Effects of ocean warming and acidification on
574 accumulation and cellular responsiveness to cadmium in mussels *Mytilus galloprovincialis*: Importance of
575 the seasonal status. Aquatic Toxicology, 204, DOI: 10.1016/j.aquatox.2018.09.009, (2018).

576 Nardi, A., Mincarelli, L.F., Benedetti, M., Fattorini, D., d'Errico, G., Regoli, F. Indirect effects of climate
577 changes on cadmium bioavailability and biological effects in the Mediterranean mussel *Mytilus*
578 *galloprovincialis. Chemosphere*, 169, DOI: 10.1016/j.chemosphere.2016.11.093, (2017).

579 Oliver, E.C.J., Burrows, M.T., Donat, M.G., Sen Gupta, A., Alexander, L.V., Perkins-Kirkpatrick, S.E.,
580 Benthuysen, J.A., Hobday, A.J., Holbrook, N.J., Moore, P.J., Thomsen, M.S., Wernberg, T., Smale, D.A.
581 Projected Marine Heatwaves in the 21st Century and the Potential for Ecological Impact. *Frontiers in Marine*
582 *Science*, 6, DOI: 10.3389/fmars.2019.00734, (2019).

583 Oliver, E.C.J., Donat, M.G., Burrows, M.T., Moore, P.J., Smale, D.A., Alexander, L.V., Benthuysen, J.A., Feng,
584 M., Sen Gupta, A., Hobday, A.J., Holbrook, N.J., Perkins-Kirkpatrick, S.E., Scannell, H.A., Straub, S.C.,
585 Wernberg, T. Longer and more frequent marine heatwaves over the past century. *Nature Communications*,
586 9 (1), DOI: 10.1038/s41467-018-03732-9, (2018).

587 Parisi, M.G., Mauro, M., Sarà, G., Cammarata, M. Temperature increases, hypoxia, and changes in food
588 availability affect immunological biomarkers in the marine mussel *Mytilus galloprovincialis*. *Journal of*
589 *Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology*, 187 (8), 1117-1126. DOI:
590 10.1007/s00360-017-1089-2, (2017).

591 Parker, L.M., Ross, P.M., O'Connor, W.A. The effect of ocean acidification and temperature on the
592 fertilization and embryonic development of the Sydney rock oyster *Saccostrea glomerata* (Gould 1850).
593 *Global Change Biology,* 15 (9), DOI: 10.1111/j.1365-2486.2009.01895.x, (2009).

594 Perkins, S.E., Alexander, L.V., Nairn, J. Increasing frequency, intensity and duration of global heat waves and
595 warm spells. *Geophysical Research Letters*, 39, 20. DOI: 10.1029/2012GL053361, (2012).

596 Przeslawski, R., Byrne, M., Mellin, C. A review and meta-analysis of the effects of multiple abiotic stressors
597 on marine embryos and larvae. *Global Change Biology*, 21 (6), DOI: 10.1111/gcb.12833, (2015).

598 Rahman, M. A., Henderson, S., Miller-Ezzy, P., Li, X. X., and Qin, J. G. Immune response to temperature stress
599 in three bivalve species: pacific oyster *Crassostrea gigas*, Mediterranean mussel *Mytilus galloprovincialis*
600 and mud cockle *Katelysia rhytiphora*. *Fish Shellfish Immunol.* 86, doi: 10.1016/j.fsi.2018.12.017, (2019).

601 Reichert, J., Tirpitz, V., Anand, R., Bach, K., Knopp, J., Schubert, P., Wilke, T., & Ziegler, M. Interactive effects
602 of microplastic pollution and heat stress on reef-building corals. *Environmental pollution* (Barking, Essex :
603 1987), 290, 118010. https://doi.org/10.1016/j.envpol.2021.118010, (2021).

604 Regoli, F. & Giuliani, M.E. Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine
605 organisms. *Marine Environmental Research*, 93, DOI: 10.1016/j.marenvres.2013.07.006, (2014).

606 Regoli, F., d'Errico, G., Nardi, A., Mezzelani, M., Fattorini, D., Benedetti, M., Di Carlo, M., Pellegrini, D., Gorbi,
607 S. Application of a weight of evidence approach for monitoring complex environmental scenarios: The case-
608 study of off-shore platforms. *Frontiers in Marine Science*, 6, DOI: 10.3389/fmars.2019.00377, (2019).

609 Roth, O., Kurtz, J., Reusch, T.B.H. A summer heat wave decreases the immunocompetence of the
610 mesograzer, *Idotea baltica*. *Marine Biology*, 157, DOI: 10.1007/s00227-010-1433-5, (2010).

611 Rubio-Portillo, E., Izquierdo-Muñoz, A., Gago, J.F., Rosselló-Mora, R., Antón, J., Ramos-Esplá, A.A. Effects of
612 the 2015 heat wave on benthic invertebrates in the Tabarca Marine Protected Area (southeast Spain).
613 *Marine Environmental Research*, 122, DOI: 10.1016/j.marenvres.2016.10.004, (2016).

614 Salazar, A.M., Sordo, M., Ostrosky-Wegman, P. Relationship between micronuclei formation and p53
615 induction. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*, 672 (2), DOI:
616 10.1016/j.mrgentox.2008.10.015, (2009).

617 Serra-Compte, A., Maulvault, A.L., Camacho, C., Alvarez-Muñoz, D., Barceló, D.,Rodríguez-Mozaz, S.,
618 Marques, A. Effects of water warming and acidification on bioconcentration, metabolization and depuration
619 of pharmaceuticals and endocrine disrupting compounds in marine mussels (M*ytilus galloprovincialis*).
620 *Environmental Pollution* 236, 824–834 https://doi.org/10.1016/j.envpol.2018.02.018, (2018).

621 Smale, D.A., Wernberg, T., Oliver, E.C.J., Thomsen, M., Harvey, B.P., Straub, S.C., Burrows, M.T., Alexander,
622 L.V., Benthuysen, J.A., Donat, M.G., Feng, M., Hobday, A.J., Holbrook, N.J., Perkins-Kirkpatrick, S.E., Scannell,

H.A., Sen Gupta, A., Payne, B.L., Moore, P.J. Marine heatwaves threaten global biodiversity and the provision of ecosystem services. *Nature Climate Change,* 9, DOI: 10.1038/s41558-019-0412-1, (2019).

Sokolova, I.M. Bioenergetics in environmental adaptation and stress tolerance of aquatic ectotherms: linking physiology and ecology in a multi-stressor landscape. *Journal of Experimental Biology*, 224, DOI: 10.1242/jeb.236802, (2021).

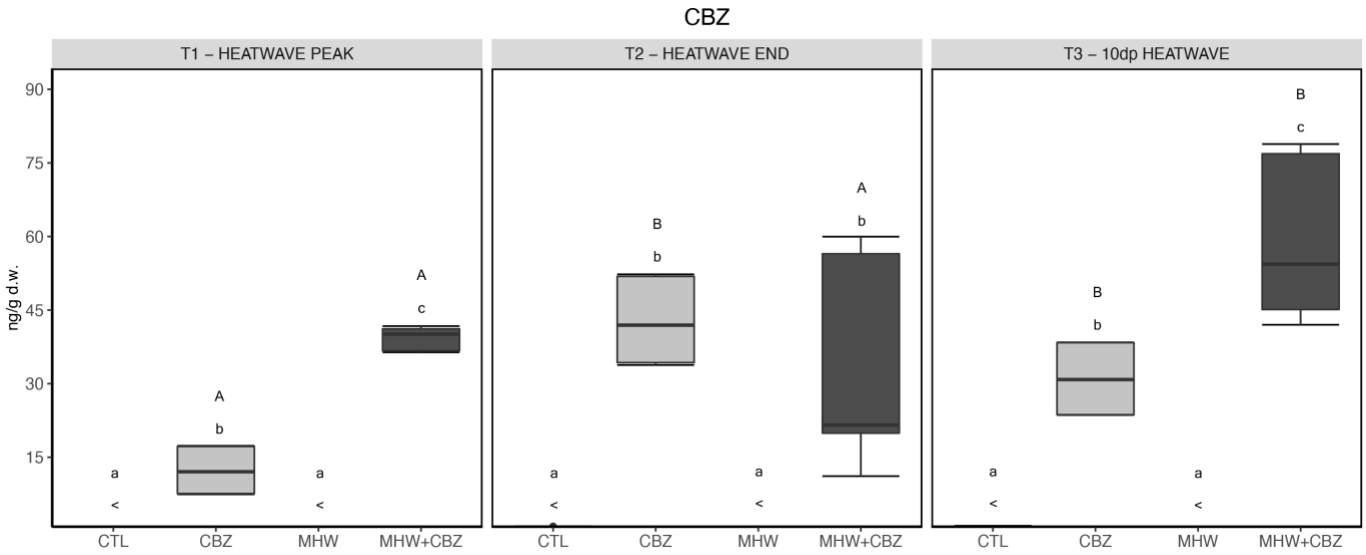
Sokolova, I.M., Lannig, G. Interactive effects of metal pollution and temperature on metabolism in aquatic ectotherms: Implications of global climate change. *Climate Research*, 37 (2-3), DOI: 10.3354/cr00764, (2008).

Strydom, S., Murray, K., Wilson, S., Huntley, B., Rule, M., Heithaus, M., Bessey, C., Kendrick, G.A., Burkholder, D., Fraser, M.W., Zdunic, K. Too hot to handle: Unprecedented seagrass death driven by marine heatwave in a World Heritage Area. *Global Change Biology*, 26 (6), DOI: 10.1111/gcb.15065, (2020).

Velez, C., Figueira, E., Soares, A.M.V.M., Freitas, R. Effects of seawater temperature increase on economically relevant native and introduced clam species. *Marine Environmental Research*, 123, DOI: 10.1016/j.marenvres.2016.11.010. (2017).

Wu, F., Falfushynska, H., Dellwig, O., Piontkivska, H., Sokolova, I.M. Interactive effects of salinity variation and exposure to ZnO nanoparticles on the innate immune system of a sentinel marine bivalve, *Mytilus edulis*. *Science of the Total Environment*, 712, DOI: 10.1016/j.scitotenv.2019.136473, (2020).

Yao, C.-L. & Somero, G.N. Thermal stress and cellular signaling processes in hemocytes of native (*Mytilus californianus*) and invasive (*M. galloprovincialis*) mussels: Cell cycle regulation and DNA repair. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology,* 165 (2), DOI: 10.1016/j.cbpa.2013.02.024, (2013).



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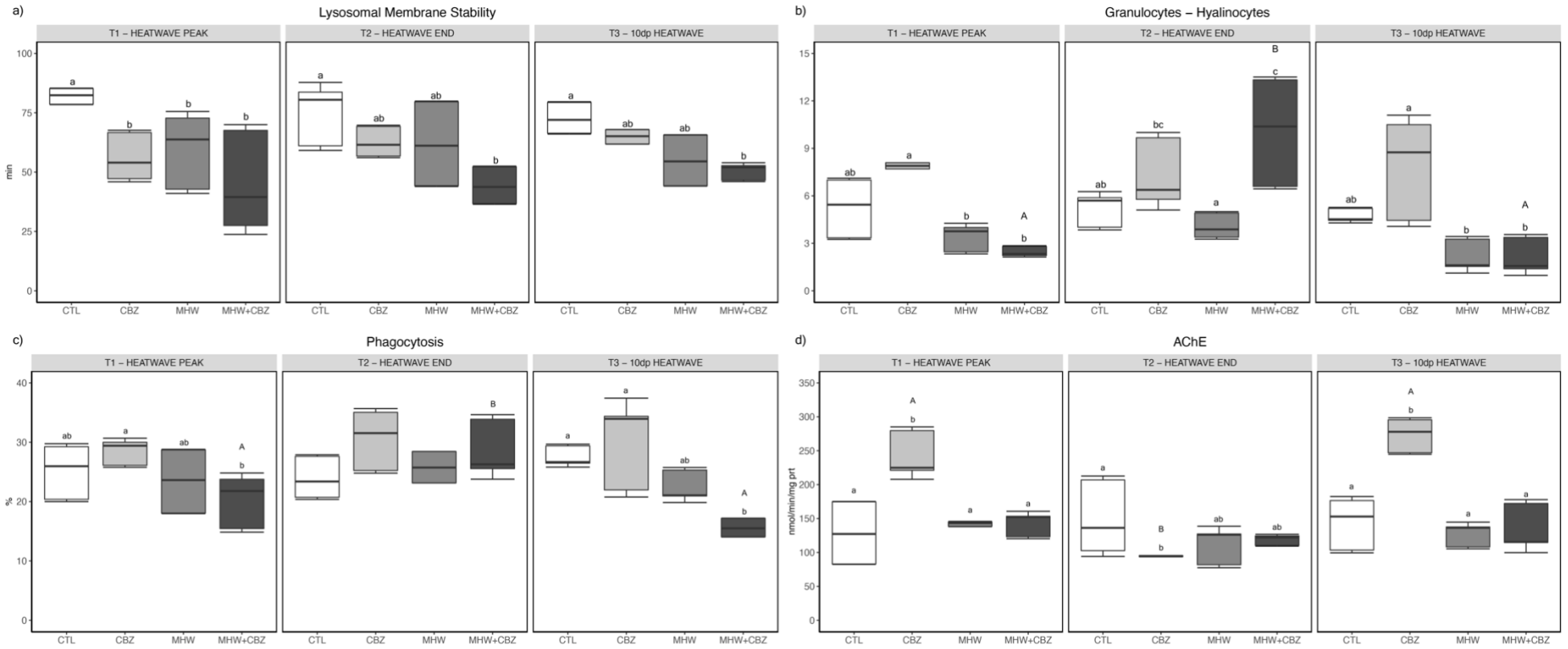
645 **Figure 1 - Carbamazepine concentration in whole soft tissues of exposed organisms**. Data are given in ng g^{-1} dry weight. Lower
646 and upper boundaries of each box represent 25th and 75th percentile, respectively, line within the box indicates the median and
647 whiskers above and below each box mark highest and lowest values, respectively. CTL (white boxes), control condition: constant
648 temperature; CBZ (light grey boxes), CBZ-exposure at constant temperature; MHW (grey boxes), marine heatwave scenario;
649 MHW+CBZ (dark grey boxes), CBZ-exposure under marine heatwave scenario. Letters are used to highlight significant differences
650 between treatments within the same sampling time (lower case) and among the same treatment along time (upper case). "<"
651 indicates concentrations below limit of detection, LOD (1.03 ng g^{-1} d.w.).



652

653 **Figure 2 – Immune and cholinergic alterations.** Lysosomal membrane stability (a), granulocytes/hyalinocytes ratio (b), phagocytosis rate (c), acetylcholinesterase activity (d) in
654 haemocytes of exposed mussels. Lower and upper boundaries of each box represent 25th and 75th percentile, respectively, line within the box indicates the median and whiskers
655 above and below each box mark highest and lowest values, respectively. CTL (white boxes), control condition: constant temperature; CBZ (light grey boxes), CBZ-exposure at constant
656 temperature; MHW (grey boxes), marine heatwave scenario; MHW+CBZ (dark grey boxes), CBZ-exposure under marine heatwave scenario. Letters are used to highlight significant
657 differences between treatments within the same sampling time (lower case) and among the same treatment along time (upper case).

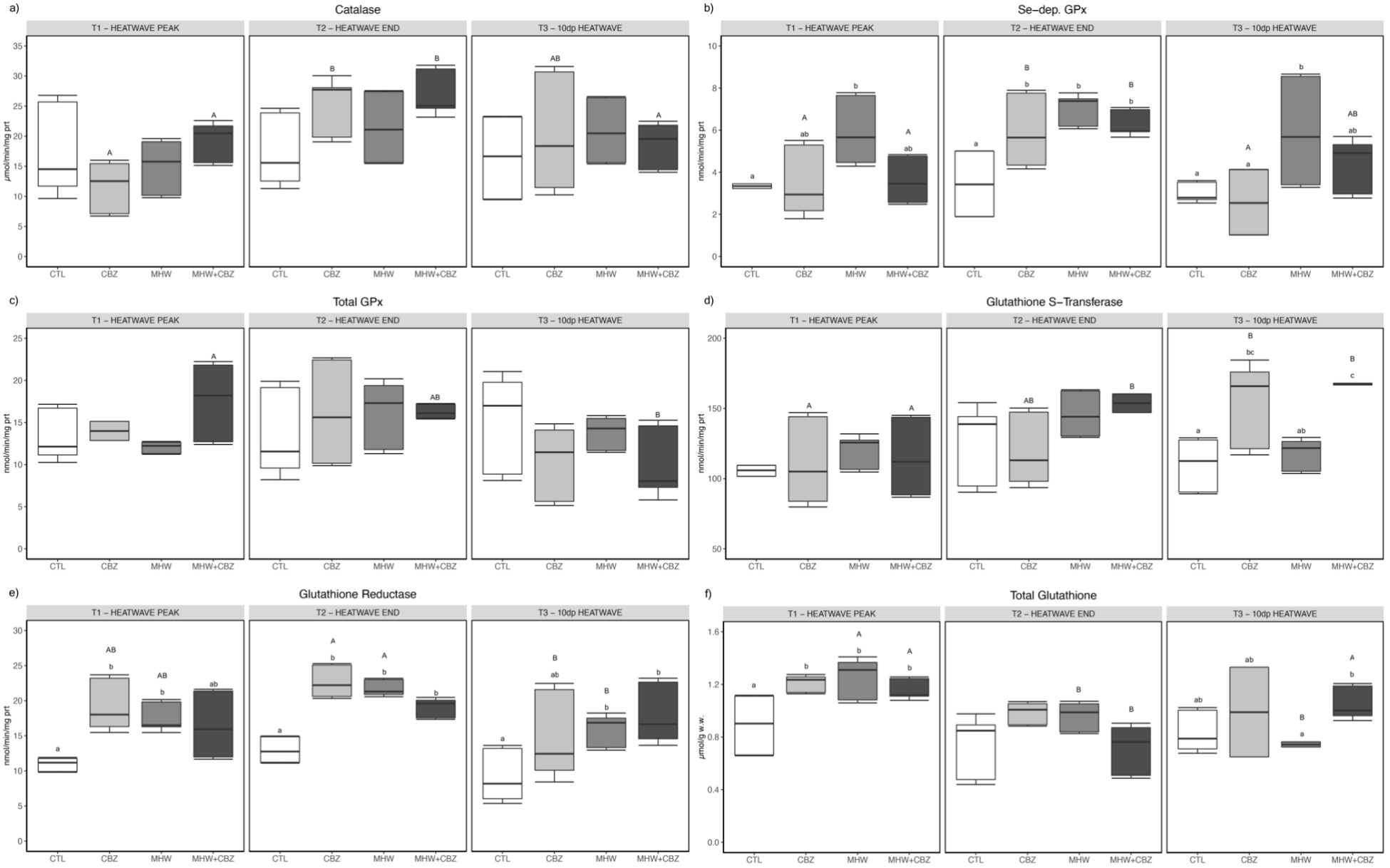


Figure 3 – Single antioxidant defenses. Activity of catalase (a), Se-dependent glutathione peroxidases (b), total glutathione peroxidases (c), glutathione S-transferases (d), glutathione reductase (e) and content of total glutathione (f). Lower and upper boundaries of each box represent 25th and 75th percentile, respectively, line within the box indicates the median and whiskers above and below each box mark highest and lowest values, respectively. CTL (white boxes), control condition: constant temperature; CBZ (light grey boxes), CBZ-exposure at constant temperature; MHW (grey boxes), marine heatwave scenario; MHW+CBZ (dark grey boxes), CBZ-exposure under marine heatwave scenario. Letters are used to highlight significant differences between treatments within the same sampling time (lower case) and among the same treatment along time (upper case).

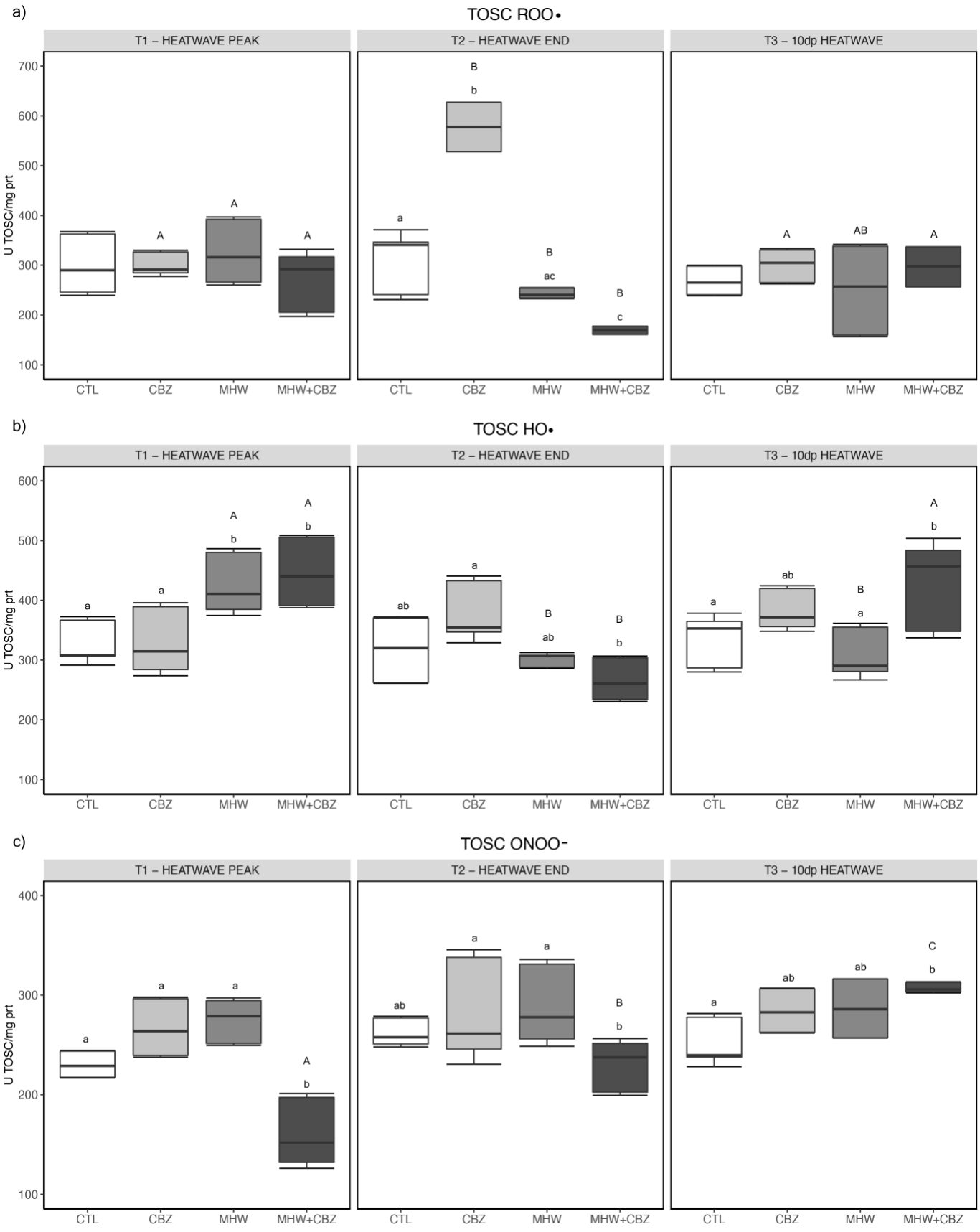
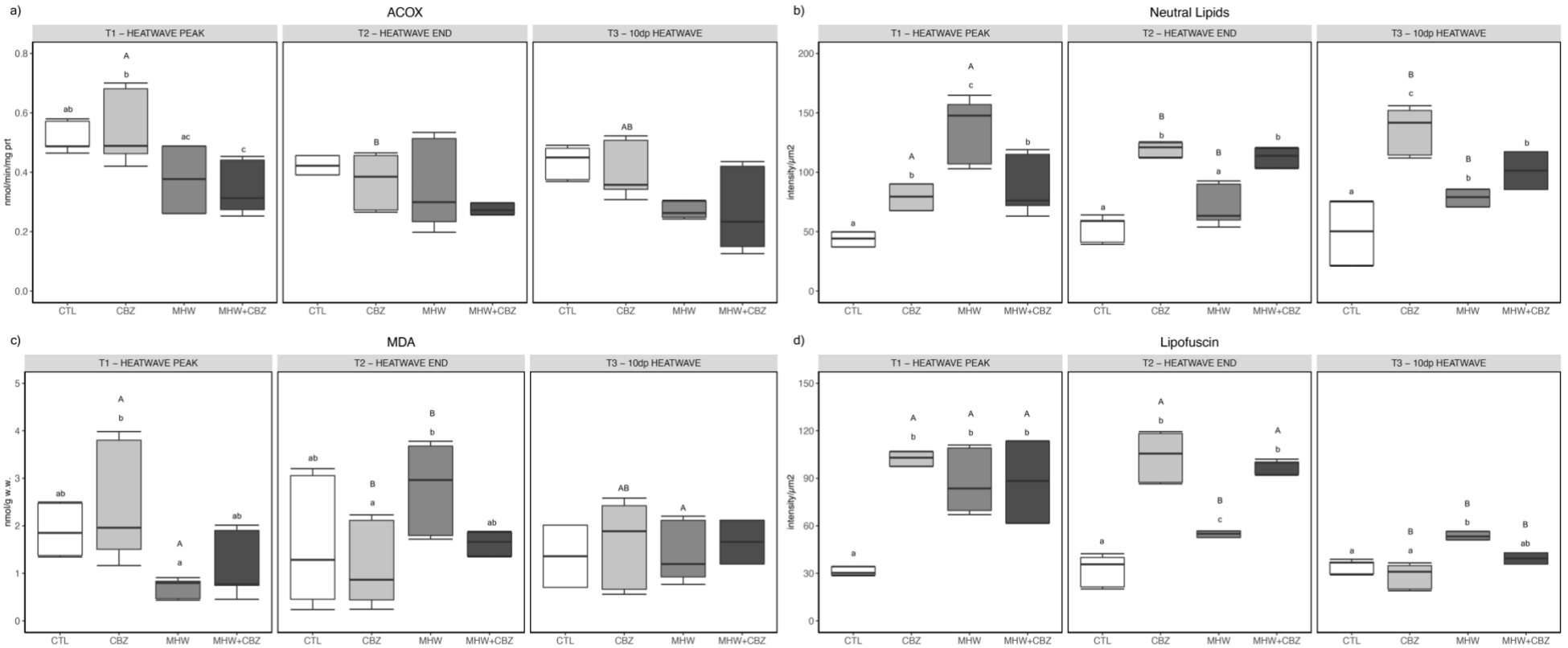


Figure 4 - Total oxyradical scavenging capacity. Overall defenses against peroxyl radical (a), hydroxyl radical (b) and peroxynitrite (c). Lower and upper boundaries of each box represent 25th and 75th percentile, respectively, line within the box indicates the median and whiskers above and below each box mark highest and lowest values, respectively. CTL (white boxes), control condition: constant temperature; CBZ (light grey boxes), CBZ-exposure at constant temperature; MHW (grey boxes), marine heatwave scenario; MHW+CBZ (dark grey boxes), CBZ-exposure under marine heatwave scenario. Letters are used to highlight significant differences between treatments within the same sampling time (lower case) and among the same treatment along time (upper case).



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673 **Figure 5 - Lipid metabolism and peroxidation**. Acyl Co-A oxidase activity (a), neutral lipids content (b), malondialdehyde concentration (c) and lipofuscin content (d). Lower and upper
674 boundaries of each box represent 25th and 75th percentile, respectively, line within the box indicates the median and whiskers above and below each box mark highest and lowest
675 values, respectively. CTL (white boxes), control condition: constant temperature; CBZ (light grey boxes), CBZ-exposure at constant temperature; MHW (grey boxes), marine heatwave
676 scenario; MHW+CBZ (dark grey boxes), CBZ-exposure under marine heatwave scenario. Letters are used to highlight significant differences between treatments within the same
677 sampling time (lower case) and among the same treatment along time (upper case).

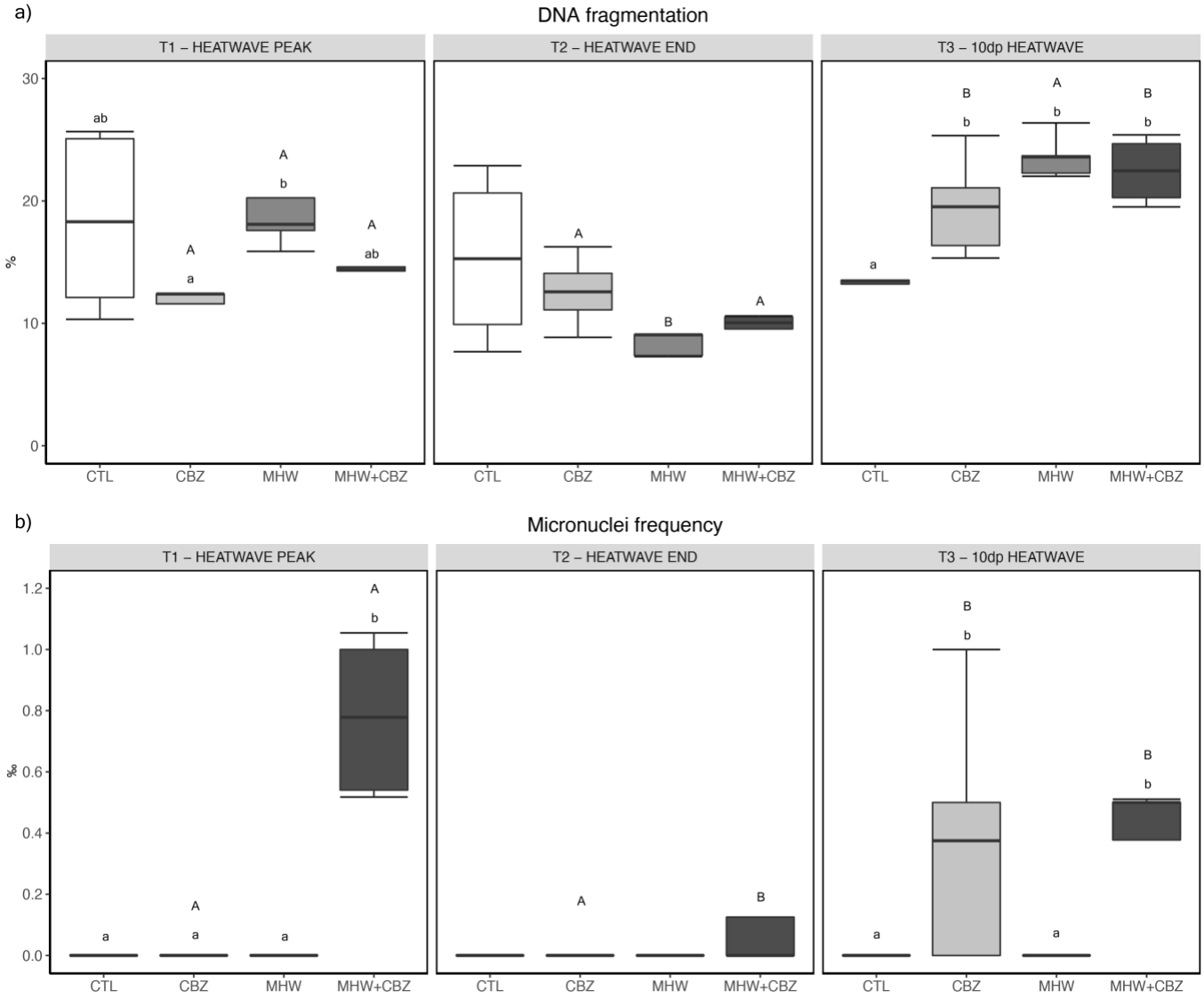
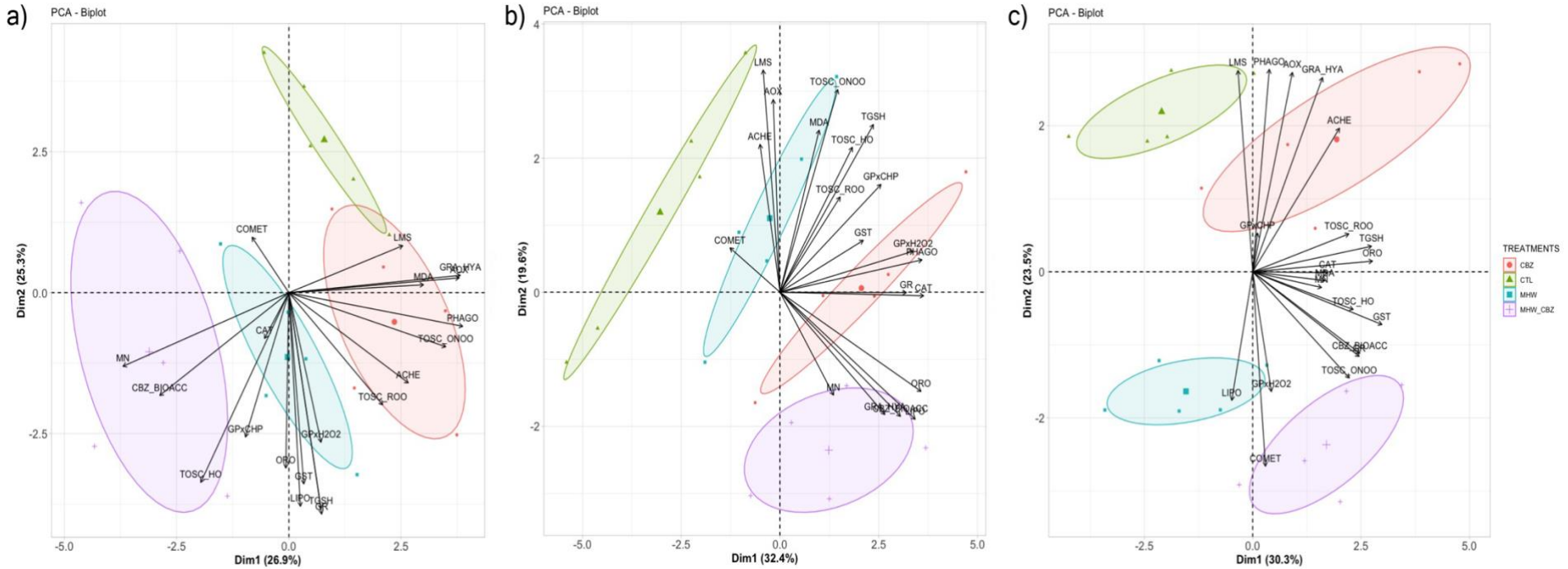


Figure 6 - Genotoxic damage. DNA fragmentation (a) and frequency of micronuclei (b) in haemocytes of exposed mussels. Lower and upper boundaries of each box represent 25th and 75th percentile, respectively, line within the box indicates the median and whiskers above and below each box mark highest and lowest values, respectively. CTL (white boxes), control condition: constant temperature; CBZ (light grey boxes), CBZ-exposure at constant temperature; MHW (grey boxes), marine heatwave scenario; MHW+CBZ (dark grey boxes), CBZ-exposure under marine heatwave scenario. Letters are used to highlight significant differences between treatments within the same sampling time (lower case) and among the same treatment along time (upper case).



685

686 **Figure 7 - Two-dimensional representation of Principal Components Analysis (PCA) of whole dataset for each experimental checkpoint.** T1, day 6, peak of heatwave (a); T2, day 11,
687 end of heatwave (b); T3, day 20, 10-days after heatwave (c).

Treatment	Experimental Checkpoint	LOE-2 (Bioaccumulation)	LOE-3 (Biomarkers)	WOE
CBZ	T1	SLIGHT	MODERATE	MODERATE
	T2	MAJOR	MODERATE	MODERATE
	T3	MODERATE	SLIGHT	MODERATE
MHW	T1 (peak)	ABSENT	MODERATE	SLIGHT
	T2 (end)	ABSENT	SLIGHT	SLIGHT
	T3 (10dp)	ABSENT	SLIGHT	SLIGHT
MHW+CBZ	T1 (peak)	MAJOR	MODERATE	MODERATE
	T2 (end)	MODERATE	MODERATE	MODERATE
	T3 (10dp)	MAJOR	SLIGHT	MAJOR

Figure 8 - Weighted elaboration of whole dataset for each treatment in all experimental checkpoints. Classes of hazard (Absent, white; Slight, light blue; Moderate, yellow; Major, red; Severe, black) are given for each treatment-experimental checkpoint combination for CBZ bioaccumulation (LOE-2), biological effects (LOE-3) and their weighted integration (WOE).

691 **Table 1** – Three-way ANOVA results for single factors and their interactions; F-values with degrees of freedom and p-values are
692 reported for each tested stressors and interactions for each analyzed parameter. Significant effects are highlighted in bold and
693 italic.

Parameter	CBZ Exposure	Temperature Scenario	Experimental Time	CBZ Exp. x Temp. Scen.	CBZ Exp. x Exp. Time	Temp. Scen. x Exp. Time	CBZ Exp. x Temp. Scen. x Exp.Time
CBZ bioaccumulation	***$F_{(1,48)}$ = 236.1*** ***p < 0.001***	***$F_{(1,48)}$ = 10.76*** ***p < 0.01***	***$F_{(2,48)}$ = 5.979*** ***p < 0.01***	***$F_{(1,48)}$ = 11.05*** ***p < 0.01***	***$F_{(2,48)}$ = 5.858*** ***p < 0.01***	***$F_{(2,48)}$ = 6.771*** ***p < 0.01***	***$F_{(2,48)}$ = 6.933*** ***p < 0.01***
Lysosomal membrane stability	***$F_{(1,48)}$ = 19.73*** ***p < 0.001***	***$F_{(1,48)}$ = 28.45*** ***p < 0.001***	$F_{(2,48)}$ = 0.001 *p = 0.999*	$F_{(1,48)}$ = 0.286 *p = 0.596*	$F_{(2,48)}$ = 1.672 *p = 0.1987*	$F_{(2,48)}$ = 0.014 *p = 0.986*	$F_{(2,48)}$ = 0.724 *p = 0.490*
Granulocytes - Hyalinocytes ratio	***$F_{(1,48)}$ = 22.48*** ***p < 0.001***	***$F_{(1,48)}$ = 25.55*** ***p < 0.001***	***$F_{(2,48)}$ = 10.64*** ***p < 0.001***	$F_{(1,48)}$ = 1.114 *p = 0.296*	***$F_{(2,48)}$ = 4.664*** ***p < 0.05***	***$F_{(2,48)}$ = 11.70*** ***p < 0.001***	***$F_{(2,48)}$ = 6.602*** ***p < 0.01***
Phagocytosis rate	$F_{(1,48)}$ = 0.480 *p = 0.492*	***$F_{(1,48)}$ = 18.62*** ***p < 0.001***	***$F_{(2,48)}$ = 3.707*** ***p < 0.05***	***$F_{(1,48)}$ = 8.163*** ***p < 0.01***	***$F_{(2,48)}$ = 3.587*** ***p < 0.05***	***$F_{(2,48)}$ = 6.229*** ***p < 0.01***	$F_{(2,48)}$ = 0.542 *p = 0.585*
AchE activity	***$F_{(1,48)}$ = 18.24*** ***p < 0.001***	***$F_{(1,48)}$ = 28.32*** ***p < 0.001***	***$F_{(2,48)}$ = 16.12*** ***p < 0.001***	***$F_{(1,48)}$ = 12.53*** ***p < 0.001***	***$F_{(2,48)}$ = 13.27*** ***p < 0.001***	***$F_{(2,48)}$ = 6.033*** ***p < 0.01***	***$F_{(2,48)}$ = 14.19*** ***p < 0.001***
Catalase activity	$F_{(1,48)}$ = 1.9024 *p = 0.174*	$F_{(1,48)}$ = 2.025 *p = 0.161*	***$F_{(2,48)}$ = 6.743*** ***p < 0.01***	$F_{(1,48)}$ = 0.057 *p = 0.812*	$F_{(2,48)}$ = 2.122 *p = 0.131*	$F_{(2,48)}$ = 0.115 *p = 0.891*	$F_{(2,48)}$ = 2.584 *p = 0.086*
Se-dep.glutathione peroxidase activity	$F_{(1,48)}$ = 1.071 *p = 0.306*	***$F_{(1,48)}$ = 24.90*** ***p < 0.001***	***$F_{(2,48)}$ = 8.486*** ***p < 0.001***	***$F_{(1,48)}$ = 9.220*** ***p < 0.01***	$F_{(2,48)}$ = 3.076 *p = 0.055*	$F_{(2,48)}$ = 0.566 *p = 0.572*	$F_{(2,48)}$ = 0.656 *p = 0.523*
Total glutathione peroxidase activity	$F_{(1,48)}$ = 0.005 *p = 0.943*	$F_{(1,48)}$ = 0.255 *p = 0.616*	$F_{(2,48)}$ = 3.13 *p = 0.053*	$F_{(1,48)}$ = 0.379 *p = 0.541*	***$F_{(2,48)}$ = 4.086*** ***p < 0.05***	$F_{(2,48)}$ = 0.300 *p = 0.742*	$F_{(2,48)}$ = 0.928 *p = 0.402*
Glutathione S-transferases activity	***$F_{(1,48)}$ = 9.044*** ***p < 0.01***	***$F_{(1,48)}$ = 7.998*** ***p < 0.01***	***$F_{(2,48)}$ = 8.069*** ***p < 0.001***	$F_{(1,48)}$ = 0.063 *p = 0.803*	***$F_{(2,48)}$ = 7.443*** ***p < 0.01***	$F_{(2,48)}$ = 1.163 *p = 0.321*	$F_{(2,48)}$ = 0.37 *p = 0.693*
Glutathione reductase activity	***$F_{(1,48)}$ = 17.56*** ***p < 0.001***	***$F_{(1,48)}$ = 12.27*** ***p < 0.01***	***$F_{(2,48)}$ = 9.137*** ***p < 0.001***	***$F_{(1,48)}$ = 22.75*** ***p < 0.001***	$F_{(2,48)}$ = 0.036 *p = 0.964*	$F_{(2,48)}$ = 0.964 *p = 0.389*	$F_{(2,48)}$ = 2.309 *p = 0.110*
Total glutathione	***$F_{(1,48)}$ = 6.572*** ***p < 0.05***	$F_{(1,48)}$ = 0.740 *p = 0.394*	***$F_{(2,48)}$ = 14.17*** ***p < 0.001***	***$F_{(1,48)}$ = 7.350*** ***p < 0.01***	$F_{(2,48)}$ = 2.090 *p = 0.135*	$F_{(2,48)}$ = 1.618 *p = 0.210*	***$F_{(2,48)}$ = 5.185*** ***p < 0.01***
TOSC ROO•	***$F_{(1,48)}$ = 7.529*** ***p < 0.01***	***$F_{(1,48)}$ = 39.46*** ***p < 0.001***	***$F_{(2,48)}$ = 3.903*** ***p < 0.05***	***$F_{(1,48)}$ = 23.94*** ***p < 0.001***	***$F_{(2,48)}$ =7.692*** ***p < 0.01***	***$F_{(2,48)}$ = 33.07*** ***p < 0.001***	***$F_{(2,48)}$ = 17.27*** ***p < 0.001***
TOSC HO•	***$F_{(1,48)}$ = 8.07*** ***p < 0.01***	$F_{(1,48)}$ = 1.789 *p = 0.187*	***$F_{(2,48)}$ = 9.645*** ***p < 0.001***	$F_{(1,48)}$ = 0.046 *p = 0.831*	***$F_{(2,48)}$ = 3.342*** ***p < 0.05***	***$F_{(2,48)}$ = 15.04*** ***p < 0.001***	***$F_{(2,48)}$ = 3.434*** ***p < 0.05***
TOSC ONOO-	$F_{(1,48)}$ = 1.87 *p = 0.178*	$F_{(1,48)}$ = 0.488 *p = 0.488*	***$F_{(2,48)}$ = 14.72*** ***p < 0.001***	***$F_{(1,48)}$ = 28.20*** ***p < 0.001***	***$F_{(2,48)}$ = 6.288*** ***p < 0.01***	***$F_{(2,48)}$ = 5.349*** ***p < 0.01***	***$F_{(2,48)}$ = 7.001*** ***p < 0.01***
Acyl-CoA oxidase activity	$F_{(1,48)}$ = 1.056 *p = 0.309*	***$F_{(1,48)}$ = 28.49*** ***p < 0.001***	***$F_{(2,48)}$ = 6.621*** ***p < 0.01***	$F_{(1,48)}$ = 0.159 *p = 0.692*	$F_{(2,48)}$ = 0.705 *p = 0.499*	$F_{(2,48)}$ = 1.228 *p = 0.302*	$F_{(2,48)}$ = 0.255 *p = 0.776*
Neutral Lipids	***$F_{(1,48)}$ = 56.51*** ***p < 0.001***	***$F_{(1,48)}$ = 16.45*** ***p < 0.001***	$F_{(2,48)}$ = 0.266 *p = 0.768*	***$F_{(1,48)}$ = 39.79*** ***p < 0.001***	***$F_{(2,48)}$ = 19.24*** ***p < 0.001***	***$F_{(2,48)}$ = 13.24*** ***p < 0.001***	***$F_{(2,48)}$ = 3.277*** ***p < 0.05***
Malondialdehyde	$F_{(1,48)}$ = 0.004 *p = 0.947*	$F_{(1,48)}$ = 0.397 *p = 0.532*	$F_{(2,48)}$ = 0.679 *p = 0.512*	$F_{(1,48)}$ = 0.530 *p = 0.399*	***$F_{(2,48)}$ = 3.521*** ***p < 0.05***	***$F_{(2,48)}$ = 7.601*** ***p < 0.01***	$F_{(2,48)}$ = 0.232 *p = 0.794*
Lipofuscin	***$F_{(1,48)}$ = 82.17*** ***p < 0.001***	***$F_{(1,48)}$ = 24.29*** ***p < 0.001***	***$F_{(2,48)}$ = 62.96*** ***p < 0.001***	***$F_{(1,48)}$ = 35.89*** ***p < 0.001***	***$F_{(2,48)}$ = 42.88*** ***p < 0.001***	$F_{(2,48)}$ = 1.480 *p = 0.238*	***$F_{(2,48)}$= 9.439*** ***p < 0.001***
DNA fragmentation	$F_{(1,48)}$ = 2.322 *p = 0.134*	$F_{(1,48)}$ = 1.576 *p = 0.215*	***$F_{(2,48)}$ = 21.93*** ***p < 0.001***	$F_{(1,48)}$ = 0.440 *p = 0.511*	***$F_{(2,48)}$ = 5.786*** ***p < 0.01***	***$F_{(2,48)}$ = 10.21*** ***p < 0.001***	$F_{(2,48)}$ = 2.270 *p = 0.114*
Micronuclei	***$F_{(1,48)}$ = 41.69*** ***p < 0.001***	***$F_{(1,48)}$= 12.48*** ***p < 0.001***	***$F_{(2,48)}$ = 6.240*** ***p < 0.01***	***$F_{(1,48)}$= 12.48*** ***p < 0.001***	***$F_{(2,48)}$= 6.240*** ***p < 0.01***	***$F_{(2,48)}$= 7.926*** ***p < 0.01***	***$F_{(2,48)}$= 7.926*** ***p < 0.01***

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