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Environmental pharmaceuticals and climate change: The case study of carbamazepine in *M. galloprovincialis* under ocean acidification scenario

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ABSTRACT

Contaminants of emerging concern and ocean changes are key environmental stressors for marine species with possibly synergistic, but still unexplored, deleterious effects. In the present study the influence of a simulated ocean acidification scenario (pH = 7.6) was investigated on metabolism and sub-lethal effects of carbamazepine, CBZ (1 µg/L), chosen as one of the most widely diffused pharmaceuticals in marine organisms. A multidisciplinary approach was applied on mussels, *M. galloprovincialis*, integrating measurement of drug bioaccumulation with changes in the whole transcriptome, responsiveness of various biochemical and cellular biomarkers including immunological parameters, lipid and oxidative metabolism, onset of genotoxic effects. Chemical analyses revealed a limited influence of hypercapnia on accumulation and excretion of CBZ, while a complex network of biological responses was observed in gene expression profile and functional changes at cellular level. The modulation of gamma-aminobutyric acid (GABA) pathway suggested similarities with the Mechanism of Action known for vertebrates: immune responses, cellular homeostasis and oxidative system represented the processes targeted by combined stressors. The overall elaboration of results through a quantitative Weight of Evidence model, revealed clearly increased cellular hazard due to interactions of CBZ with acidification compared to single stressors.

1. Introduction

Challenges for the marine ecosystems have markedly evolved in the last decades in terms of typology of hazards, magnitude and frequency of disturbances. Beside traditional chemicals, contaminants of emerging concern like pharmaceuticals, endocrine disruptors, and microplastics are well recognized environmental stressors (Almeida et al., 2018; Avio et al., 2015; Heye et al., 2019; Mezzelani et al., 2018b). The ubiquitous presence of pharmaceuticals in freshwater and coastal ecosystems is caused by the massive use and often limited removal of those compounds by wastewater treatment plants (WWTPs). Potential bioaccumulation has been shown in aquatic species, with deleterious outcomes highlighted from molecular up to physiological levels in non-target marine organisms, and predictable adverse effects on

development and reproductive success (Álvarez-Muñoz et al., 2015; Klosterhaus et al., 2013; Martínez Bueno et al., 2013; Mezzelani et al., 2016a, 2016b; Miller et al., 2019; Wille et al., 2011). Among human pharmaceuticals, the antiepileptic carbamazepine (CBZ) has been frequently measured in tissues of aquatic invertebrates: in Mediterranean mussels, *Mytilus galloprovincialis*, collected along the Italian coasts, more than 90% of the analyzed samples contained detectable levels of this active pharmaceutical ingredient with concentrations up to 300 ng/ g dry weight (Mezzelani et al., 2020). The onset of oxidative stress, lipid peroxidation, impairment of immune system and genotoxic damage were documented in marine invertebrates exposed to a wide range of environmentally realistic concentrations of CBZ (0.3–3.0 and 6.0–9.0 μ g/L) (Almeida et al., 2014, 2015, 2017; Freitas et al., 2016).

Scientific effort has been dedicated to investigate the biological

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impact of individual pharmaceuticals and, in a few cases, of mixtures (Franzellitti et al., 2019; Mezzelani et al., 2018a; Trombini et al., 2019). Considering the multitude of environmental stressors to which organisms are simultaneously exposed, further research is urgently needed to elucidate also the role that future projections of temperature and CO2driven ocean changes will have on environmental fate, bioavailability and toxicity of such emerging compounds (Delorenzo, 2015). The reduction of seawater pH has both direct effects on several metabolic pathways in marine organisms, as immunocompetence, oxidative status, growth and reproduction (Gazeau et al., 2013; Kroeker et al., 2010), and it also indirectly modulate bioaccumulation and biological responsiveness to other contaminants (Cao et al., 2019, 2018; Giuliani et al., 2020; Götze et al., 2014; Hawkins and Sokolova, 2017; Munari et al., 2019; Nardi et al., 2018a, 2018b). Recent studies on the interaction between ocean acidification (OA) and pharmaceuticals revealed a synergistic increase of CBZ toxicity (3 µg/L) in Scrobicularia plana at pH 7.1, with reduced specimens survival and enhanced oxidative stress (Freitas et al., 2016). At a lower CBZ dose (1 µg/L) and pH of 7.5, Ruditapes philippinarum exhibited changes in mRNA transcription of several genes related to neurotransmission, immunity and biomineralization (Almeida et al., 2018).

However, mechanistic pathways of interactions between different typologies of multiple stressors have been scarcely investigated. The urgency to explore the ecotoxicological potential of compounds of emerging concern (like CBZ) under expected climate changes scenarios is emphasized by the European Commission (EC) within the "Strategic Approach to Pharmaceuticals in the Environment" (COM/2019/128 final). Understanding long term consequences is an important prerequisite also promoted by the 14th of the 17 Sustainable Development Goals (SDGs) of the 2030 Agenda for Sustainable Development.

In this respect, the present study was aimed to investigate the influence of reduced pH on bioaccumulation, excretion and sub-lethal effects of CBZ in M. galloprovincialis. A multidisciplinary approach was applied combining measurement of drug bioaccumulation, transcriptomic responses through RNA-sequencing and a large number of biomarkers reflecting the perturbation of different cellular districts and biochemical pathways: such responses included alteration of immunological parameters, lipid and oxidative metabolism, onset of genotoxic effects. The integration of transcriptomic analyses with functional effects at cellular level was expected to clarify the activation mechanisms of molecular responses and potentially elucidate the intricate Mechanism of Action (MoA) of CBZ in marine mussels. The overall results were elaborated to summarize a cellular hazard index based on toxicological relevance, magnitude of variations and responsiveness of analyzed endpoints. The present study was expected to provide novel insights on the interactive effects of pollutants of worldwide emerging concern with ocean acidification, thus representing a further step to clarify the environmental risk of multiple stressors.

2. Materials and methods

2.1. Animal collection and experimental design

Mussels, *M. galloprovincialis* (6.0 ± 0.5 cm shell length), were obtained in March 2019 from a shellfish farm in an unpolluted area of Central Adriatic Sea (Regoli et al., 2014) and acclimatized for 10 days in eight 20 L aquaria (57 organisms per tank) with aerated artificial seawater (ASW) at local seasonal environmental conditions of salinity (35 practical salinity units), temperature ($13 \, ^\circ$ C) and pH (8.10). Collection and experimental use of these organisms is not subjected to ethical review permissions according to both European and Italian normative (Directive 2010/63/EU, 2010; Italian Legislative Decree n. 26, 4/03/2014), while monitoring guidelines recommend this organism as appropriate bioindicator organism for assessing bioavailability of ecotoxicological effects of environmental pollutants in marine environments (Bocchetti and Regoli, 2006).

At the end of acclimation, mussels were exposed to clean or carbamazepine-contaminated seawater under environmental or reduced-pH conditions, according to the following experimental treatments, each performed in duplicate tanks: control (CTL): pH = 8.10/ $pCO_2 = ~400 \mu atm$; exposure to carbamazepine (CBZ): 1 $\mu g/L$ CBZ, pH = 8.10/ $pCO_2 = ~400 \mu atm$; reduced pH/hypercapnia (ACD): pH = 7.6/ $pCO_2 = ~1700 \mu atm$; ACD + CBZ: pH = 7.6/ $pCO_2 = ~1700 \mu atm$, CBZ 1 $\mu g/L$. After 28 days of exposure, organisms were maintained for additional 10 days in carbamazepine-free ASW at respective pH used during exposure to investigate depuration kinetics.

The exposure dose of CBZ can be found in coastal areas (Birch et al., 2015; Freitas et al., 2016; Gaw et al., 2014; Mezzelani et al., 2018b), while target pH was selected following predicted condition for the end of the century based on CO2 emissions scenario RCP 8.5 (Hoegh-Guldberg et al., 2015). A stock solution of carbamazepine (Sigma Aldrich) was prepared in methanol and stored at room temperature for the duration of the experiment, while working solutions were prepared daily by diluting the stock solution in ASW: during exposures, water was changed every other day and carbamazepine re-dosed. Reduced pH/hypercapnia conditions were obtained by bubbling pure CO₂ in ASW until the desired pH was reached; this condition was monitored and maintained in exposure tanks through pH-stat system (Aquatronica®). For each experimental condition pH was measured continuously, temperature and salinity daily, while total alkalinity (A_T) twice per week according to Dickson et al., 2007. Seawater carbonate parameters (pCO₂ and saturation state (Ω) for calcite and aragonite) were calculated in CO2SYS (Pierrot et al., 2006) using barometric pressure values, as well as A_T, pH, temperature and salinity values of the respective samples; full seawater chemistry is provided in Supplementary Materials (SM1 and Table SMT1), while details of constants used for calculations can be found in Nardi et al., (2017). Mussels were fed 12 h prior the water change with a commercial mixture of zooplankton (50-300 µm) for filter-feeding organisms: zooplankton was preferred to live phytoplankton to avoid any possible interference on the inorganic carbon system. Samples for chemical analyses of accumulated carbamazepine were collected at day 0, 3, 7, 14, 21, 28, 31 and 38, while those for molecular, biochemical and cellular alterations were collected at the end of CBZ-exposure (day 28). For chemical analyses, 3 pools each constituted by the whole tissues of 2 organisms were collected at each time and stored at -20 °C. For transcriptomic, biochemical and histochemical analyses, gills, digestive glands and hemolymph were collected from 24 individuals, pooled in 8 separate samples, each constituted by tissues of 3 individuals, rapidly frozen in liquid nitrogen and maintained at -80 °C. At the same time, aliquots of hemolymph were immediately processed for in vivo analysis of hemocytes lysosomal membrane stability, granulocytes-hyalinocytes ratio, phagocytosis rate and DNA damage; additional aliquots of hemolymph were fixed in Carnoy's solution (3:1 methanol, acetic acid) for the evaluation of micronuclei frequency.

2.2. Chemical analyses

Concentrations of CBZ in mussel tissues were determined by High Performance Liquid Chromatography with fluorimetric and diode array detectors. Information on reagents, as well as detailed extraction and analytical protocols, including QA/QC procedures, are given in SM1.

2.3. Biomarkers analyses

Validated protocols were used to analyze the following classes and typologies of biomarkers (SM1): immunological responses (lysosomal membrane stability LMS, phagocytosis rate, granulocytes/hyalinocytes ratio G/H), genotoxic damage (loss of DNA integrity and micronuclei frequency, MN) and neurotoxic effects (acetylcholinesterase AChE in hemocytes); peroxisomal proliferation (Acyl-CoA oxidase ACOX), single antioxidant defenses (catalase CAT, glutathione S-transferases GST, Sedependent glutathione peroxidases Se-dep. GPx, total GPx, glutathione reductase GR, total glutathione TGSH), total antioxidant capacity (total oxyradical scavenging capacity TOSC toward peroxyl radical ROO•, hydroxyl radical HO• and peroxynitrite ONOO[–]) and lipid peroxidation products (malondialdehyde MDA) in digestive gland. In addition, cryostat sections of digestive glands were analyzed for lipofuscin and neutral lipids content.

2.4. Transcriptomic analysis: RNA extraction, RNAseq library preparation and sequencing

Total RNA was extracted from 5 pools of mussel digestive glands (tissues of 5 specimens for pool) per each experimental group (CTL, CBZ, ACD, ACD + CBZ):RNeasy Mini Kit (Qiagen, Hilden, Germany) was used following the manufacturer's protocol with additional DNAse treatment (Qiagen). RNA purity, concentration and integrity of each pool were checked using Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA Integrity Number (RIN) index was calculated using Agilent 2100 Expert software. Sure Select Strand-Specific mRNA Library (AgilentTechnologies, Santa Clara, CA, USA) was used for the libraries construction according to the manufacturer's protocol (see Milan et al., 2018a). Briefly, the first step of RNA library preparation method was the purification of poly(A) RNA from total RNA using two serial rounds of binding to oligo(dT) magnetic particles. After the chemical fragmentation of poly(A) RNA, single-stranding cDNA, the double-stranding cDNA was obtained through the Adenylate cDNA'3-ends and the ligate adaptors adding. The final step consisted in the adaptor-ligation cDNA library using the PCR amplification. The libraries were quantified with a Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA) and the quality was assessed by Agilent 2100 Bioanalyzer. The library pools were sequenced on 2 lanes of a HighSeq 4000 (Illumina, Davis, CA, USA) with a single 1*100 bp setup, obtaining a total of 673, 705, 767 reads (sequences available in NCBI SRA; https://www.ncbi.nlm.nih.gov/sra; BioProject PRJNA611904).

2.5. Statistical analyses

Statistical analyses for CBZ bioaccumulation and biomarkers data were performed using RStudio (version 1.2.5033). Data were checked for normal distribution (Shapiro-Wilk test) and homogeneity of variances (Levene's test); when assumptions were not fulfilled, data were transformed using Box-Cox transformation (power transformation, R package "MASS"). Effect of the factors CBZ-exposure, pH, time of exposure and their interactions on CBZ bioaccumulation in mussels tissues were assessed using a generalized linear model (three-way ANOVA, SM1-Table SMT2) followed by Tukey HSD (HSD) *post-hoc* test for comparing the means of interest. Similarly, also for biomarkers (measured only at day 28), the effect of CBZ-exposure, pH and of their interaction were assessed using a generalized linear model (two-way ANOVA, SM1-Table SMT4), followed by Tukey HSD *post-hoc* tests. Multivariate principal component analysis (PCA) was applied to visualize the relationships among the different treatments.

For each treatment, the whole dataset of biomarkers results was summarized in a hazard index elaborated through weighted criteria which assign to each biomarker a "weight" based on its toxicological relevance and a "threshold" for the minimum change considered the biological significance (Sediqualsoft, Piva et al., 2011). Variations measured for each biomarker are thus normalized toward their specific thresholds and corrected for the weight and the statistical significance of the difference compared to controls (for full details see Regoli et al., 2019): the calculated Hazard Quotient (HQ) does not include biomarkers with variations lower or equal to their threshold, while it averages or adds the summation (Σ) respectively for those biomarkers with variations up to 2-fold or more than 2-fold greater than the specific threshold (Avio et al., 2015; Benedetti et al., 2012; Mezzelani et al., 2018a; Piva et al., 2011; Regoli et al., 2014). The model finally assigns the elaborated HQ to one of five classes of hazard, from Absent to Severe. Whole calculations and assumptions have been fully given elsewhere (Regoli et al., 2019).

For gene expression statistical analyses, raw reads were quality checked with FastQC/v0.11.5 (https://www.bioinformatics.babraham. ac.uk/projects/fastqc/) and subsequently adaptors and low-quality reads were trimmed using Trimmomatic/v0.36 (Bolger et al., 2014) "Single End (SE)" with default parameters. High-quality reads were subsequently mapped against the reference transcriptome from Moreira et al., 2015, by using Kallisto/v0.46.1 (Bray et al., 2016) with options "-single -fragment-length 100 -sd 40". The count table was generated with the script "abundance_estimates_to_matrix.pl" of the Trinity/v2.9.0 (Haas et al., 2013) suite and imported in R/v3.6.0. The count matrix was filtered to remove contigs with less than 5 reads in at least 4 biological samples to remove contigs with low coverage which would contribute to background noise (Peruzza et al., 2019). A total of 40,552 contigs were maintained for gene expression analyses. To normalize the count table and remove unwanted variation, the functions "betweenLaneNormalization" (with upper quartile method) and "RUVs" (with k = 9 factors) from the R package RUVSeq/v1.14.0 (Risso et al., 2014) were used and the Principal Component Analysis (PCA) was generated with the function "plotPCA". Differential expression (DE) analysis was performed by using a likelihood ratio test with the functions "glmFit" and "glmLRT" of the package edgeR/v3.22.5 (Robinson et al., 2010) on selected pair-wise comparisons: "CBZ vs CTL"; "ACD vs CTL"; "CBZ + ACD vs CTL" and genes were deemed as DE with a cut-off FDR < 0.05 and Fold change (FC) > 2. Subsequently, functional interpretation of differentially expressed genes was obtained by enrichment analysis using Database for Annotation, Visualization, and Integrated Discovery (david ver. 6.8) software (Dennis et al., 2003; Huang, Sherman, & Lempicki, 2009), considering GO Biological Process (BP), Cellular Component (CC) and Molecular Function (MF) Database and KEGG pathways. DAVID retrieves the functional annotation of differentially expressed genes through enrichment analyses based on an integrated biological knowledge-base containing over 40 annotation categories. Since DAVID databases contain functional annotation data for a limited number of species, M. galloprovincialis transcripts were associated with Swissprot sequence identifiers. IDs corresponding to differentially expressed genes revealed in each treatment and to all genes represented in the M. galloprovincialis transcriptome (40,552) were obtained and were then used in DAVID to define a "gene list" and a "background," respectively. A functional annotation was obtained for significant genes identified by each pairwise comparison (CTL vs. CBZ; CTL vs. ACD; CTL vs. ACD + CBZ). DAVID settings: gene count = 2 and ease = 0.1. The same analyses have been performed considering separated up- and down-regulated genes within each treatment.

3. Results

Concentrations of CBZ in tissues of control and exposed mussels are reported in Fig. 1 and in SM1-Table SMT3, while three-way ANOVA results are given in SM1-Table SMT2. Values measured at the beginning of the experiment (day 0) were 37.7 ± 7.5 ng/g dry weight (d.w.) which generally decreased below the detection limit in non-CBZ exposed organisms during the experiment (SMT2). Mussels exposed to CBZ revealed a significant increase in tissue levels of this drug with a peak after 3 days and values always greater than in control groups for the whole duration of experiment. Concentrations markedly increased also in mussels exposed to CBZ at reduced pH with average values comparable to those observed in organisms exposed at control pH. After 10 days of depuration (day 38), levels of CBZ were similar to those observed at the beginning of the experiment. The overall results did not highlight a significant effect of reduced pH on CBZ bioaccumulation and depuration in exposed mussels.

Among immunological biomarkers, the lysosomal membrane stability, the phagocytosis capacity and granulocytes/hyalinocytes ratio



Fig. 1. Concentration at different sampling times of Carbamazepine in the whole tissues of M. galloprovincialis exposed to experimental conditions. Data are referred to both exposure and depuration phase, given in ng/g dry weight (mean values \pm standard deviations, n = 3). Lower case letters are used to highlight significant differences between treatments within the same sampling time, asterisks (*) represent significant difference compared to T0 organisms, "<" indicates concentration below limit of detection, LOD (1.03 ng/g d.w).

were significantly reduced by CBZ, with no additional effect of pH (Fig. 2A-C, SM1-Table SMT4).

A significant induction was caused by CBZ and acidification on the acetylcholinesterase activity, with a greater effect of the drug when

dosed alone (Fig. 2D, SM1-Table SMT4). Considering biomarkers of genotoxic damage, micronuclei frequency increased in all treatments, with a statistically significant effect of hypercapnic condition, either alone or in combination with CBZ (Fig. 2E, SM1-Table SMT4); no effect



Fig. 2. Lysosomal membrane stability (A), phagocytosis rate (B), granulocytes/hyalinocytes ratio (C), Acetylcholinesterase activity (D), frequency of micronuclei (E) and DNA damage (F) in haemocytes of mussels exposed to various treatments. Data are given as mean values \pm Standard Error of the Mean (n = 5). Different letters indicate significant differences between group of means, CTL Control; CBZ Carbamazepine; ACD acidification; ACD + CBZ acidification + CBZ.

was observed in terms of DNA fragmentation (Fig. 2F).

Both CBZ and hypercapnia condition significantly increased lipofuscin content in tertiary lysosomes, with cumulative effects observed in co-exposed organisms (Fig. 3A, SM1-Table SMT4). Neutral lipids were also enhanced by CBZ and hypercapnia, with significant interactions revealed between these two factors (Fig. 3B, SM1-Table SMT4). No variations were measured for MDA content and ACOX activity (Fig. 3C-D).

The antioxidant defenses showed few variations (Fig. 4A-I, SM1-Table SMT4). Among these, glutathione S-transferase was significantly increased by CBZ exposure and lowered by acidification, but these effects were not elicited by interaction of these factors; a significant interaction between CBZ and low pH was revealed by the different effects caused on Se-dependent glutathione peroxidases. A common trend, not statistically significant but worthy to note, was observed for mussels co-exposed to CBZ and low pH for catalase, total glutathione peroxidases and total oxyradical scavenging capacity (TOSC) toward hydroxyl radicals.

The principal component analysis on the whole dataset of biomarkers provided a two-dimensional pattern (Fig. 5), explaining almost 46% of the total variance. A clear separation was observed between control and treated organisms, while a further discrimination occurred between those treated with carbamazepine (alone and at low pH) and mussels exposed only to acidification. The parameters determining the separation along PC1 axis, were phagocytosis rate, lysosomal membrane stability, granulocytes vs hyalinocytes ratio, and lipofuscin content; along the PC2 axis, the separation was mostly determined by MN frequency, TOSC ROO•, TOSC HO• and TOSC ONOO–.

The overall biological significance of cellular responses observed in each experimental condition was summarized in a single hazard index through the application of weighted criteria which consider the toxicological relevance and magnitude of observed variations for all the biomarkers (Fig. 6). The elaborated hazard quotient (HQ) was "Slight" for organisms exposed to individual stressors (CBZ, ACD), while raised to "Moderate" after the co-exposure treatment, further supporting interactive effects of these factors on measured cellular responses.

Results on gene expression profiles were analyzed via Principal Component Analyses (PCA) considering a total of 40,552 contigs. The first axis explaining the 15.32% of the variation of gene expression profiles confirmed a clear separation of the control groups from all

treatments, while the second component (13.53%) discriminated the three tested treatments (ACD, CBZ and ACD + CBZ) (Fig. 7). Pairwise comparisons between the CTL group and ACD, CBZ and ACD + CBZ revealed a total of 972, 697 and 666 DEGs (p-value \leq 0.05, FC > 2), respectively. In all pairwise comparisons, exposed mussels showed a higher number of over-expressed genes compared to CTL group (802, 542, and 512 up-regulated genes in ACD, CBZ and ACD + CBZ, respectively). The whole lists of DEGs and their putative annotation are reported in Supplementary Material 2, SM2. Of all the DEGs, nearly 6% (106 genes) was shared between the three exposed groups compared to control (Fig. 8), while the highest percentage of common DEGs among two treatments was found between ACD and ACD + CBZ exposed mussels (253 DEGs; 14.1%). Enrichment analyses through DAVID software were also carried out to obtain a functional annotation at each treatment, considering the full list of differentially expressed genes as well as up- and down-regulated genes separately (Supplementary Material 3, SM3).

CBZ exposed mussels showed the up-regulation of genes involved in drug metabolism such as Cytochrome P450 4F8 (CYP4F8), Cytochrome P450 3A2 (CYP3A2), Cytochrome P450 3A29 (CYP3A29)) and Sulfotransferase family cytosolic 1B member 1 (ST1B1), as well as the stressinduced proteins Sestrin-3 (SEST3) that has a role in protection against oxidative and genotoxic stresses. Several genes of cell cycle (18 DEGs) and DNA repair (7 DEGs) were also differentially expressed (Table 1). Among the DEGs, Sodium-dependent serotonin transporter (SC6A4) was more than 60 times down-regulated in CBZ exposed mussels compared to CTL. An opposite trend has been revealed for the up-regulation of two genes belonging to GIMAP family (GIMAP4, GIMAP7) with a role in defense, cell differentiation and apoptosis, as well as for three genes coding for Baculoviral IAP repeat-containing protein (BIRC1, BIRC7), which modulate apoptotic processes. It should be also highlighted the significant up-regulation of several genes of immune response and inflammation. Enrichment analyses of DEGs in CBZ exposed mussels confirmed significant changes in xenobiotic metabolism by the enriched terms "response to chemical" (45 DEGs), "cellular response to chemical stimulus" (30 DEGs) and "response to organic cyclic compound" (SM3). Several terms related to synapses and neurotransmission were significantly enriched, such as the CC_terms "synapse" (17 DEGs) and "postsynapse (12 DEGs), and the BP terms "regulation of synapse organization" (6DEGs) and "synapse assembly" (5DEGs), indicating



Fig. 3. Oxidative stress biomarkers in digestive gland of mussels exposed to various treatments. Lipofuscin (A), Neutral Lipids (B), MDA: levels of malondialdehyde (C), ACOX Acyl-CoA oxidase activity (D). Data are given as mean values \pm Standard Error of the Mean (n = 5). Different letters indicate significant differences between group of means, CTL Control; CBZ Carbamazepine; ACD acidification; ACD + CBZ acidification + CBZ.

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Fig. 4. Antioxidant defenses biomarkers in digestive glands of mussels exposed to various treatments. CAT: catalase (A), GST: glutathione S-transferase (B), GR: glutathione reductase (C), TGSH: total glutathione (D), Se-Dep. GPx: Se-dependent glutathione peroxidases (E) total GPx: sum of Se-dependent and Se-independent glutathione peroxidases (F), TOSC ROO•: total oxyradical scavenging capacity toward peroxyl radical (g), TOSC HO•: total oxyradical scavenging capacity toward peroxyl radical (g), TOSC HO•: total oxyradical scavenging capacity toward peroxynitrite. Data are given as mean values \pm Standard Error of the Mean (n = 5). Different letters indicate significant differences between group of means, CTL Control; CBZ Carbamazepine; ACD acidification; ACD + CBZ acidification + CBZ.



Fig. 5. Graphical representation of principal component analysis conducted on biological parameters analyzed in mussels tissues. CTL = Control; CBZ = Carba-mazepine (1 µg/L) exposed; ACD = acidification (pH = 7.6); ACD + CBZ = acidification (pH = 7.6) + Carbamazepine (1 µg/L) d. Arrows represent all variables contribute to the separation.

Treatment	HQ	Class of Hazard	Level
CBZ	8.2	SLIGHT	
ACD	5.9	SLIGHT	
ACD + CBZ	14	MODERATE	

Fig. 6. Weight of Evidence (WOE) classification of biomarkers data for the whole dataset of analyzed parameters for each different laboratory condition. The quantitative hazard quotients (HQ) and the assigned class of hazard are given.

direct effects of carbamazepine in mussels synaptic transmission. In addition, putative *4-aminobutyrate aminotransferase* (GABT) belonging to "GABAergic synapse" KEGG pathway was 10 times down-regulated by CBZ (SM2). Possible tissue damage and inflammation were confirmed by the enriched terms "response to wounding" (10 DEGs), "wound healing" (8 DEGs), "cell death" (28 DEGs), "programmed cell death" (21 DEGs), "inflammatory response" (6 up-regulated genes) and "prostaglandin metabolic process" (3 up-regulated genes).

ACD exposed group revealed significant changes in genes involved in response to stress (41 DEGs), cell death (27 DEGs), apoptotic process (24 DEGs), and cellular response to DNA damage stimulus (6 DEGs) (Table 1 and SM2). A total of 6 transcripts coding for putative *heat shock protein* 70 (*HSP70*), *heat shock protein* 12A (*HSP12A*) and *heat shock protein* 12B (*HSP12B*), as well as several genes involved in immune response were found up-regulated in response to acidification (Table 1 and SM2). Among the enriched GO terms identified by the functional analysis, it should be highlighted the BP_GO terms "cation transport" (16 DEGs), "ion homeostasis" (14 DEGs), the MF_GO terms "ion binding" (83 DEgs), "cation transmembrane transporter activity" (13 DEGs), "calmodulin binding" (7 DEgs) and the KEGG pathway "Calcium signaling pathway" (3 over-expressed genes). While functional analyses of down-regulated genes in ACD exposed mussels revealed the enriched GO_BP term "ion transport" represented by 8 DEGs, the vast majority of genes involved in ion homeostasis were up-regulated by ACD, as demonstrated by the enriched GO terms, "Calcium ion homeostasis" (9 up-regulated genes), "cellular ion homeostasis" (11 up-regulated genes), "inorganic ion homeostasis" (12 up-regulated genes) and "metal ion binding" (67 up-regulated genes) (Table 1). Among the significantly enriched terms, several biological processes were related to energy metabolism including "ATP metabolic process" (9 up-regulated genes), "energy derivation by oxidation of organic compounds" (12 up-regulated genes), "generation of precursor metabolites and energy" (12 up-regulated genes), and "energy reserve metabolic process" (6 up-regulated genes) (Table 1 and SM3).

Half of DEGs detected in mussels exposed to the combination of carbamazepine and acidification were differentially expressed only in response to this co-exposure (344 DEGs; 52%), while a total of 175 and 253 DEGs were common to those detected in response to the individual exposure to CBZ and ACD, respectively (Fig. 8). Among the genes differentially expressed only in response to ACD + CBZ, a total of 10 genes are involved in "calcium ion binding" and Voltage-gated potassium channel subunit beta-3 (KCAB3). The ACD + CBZ exposures revealed significant changes also in transcriptional regulation of Multidrug resistance-associated proteins (MRP5) and Multidrug resistance protein 1 (MDR1) and two DEGs coding for neuropeptide Y (NYP). Additional upregulated genes of immune response such as Lysozyme 3 (LYS3) and Complement C1q-like protein 4 (C1QL4) were also found differentially expressed only in ACD + CBZ exposed mussels. CBZ and ACD downregulated Heavy metal-binding protein (HIP), while DNA damage and changes in apoptosis regulation are suggested by several genes involved in the cellular response to DNA damage stimulus, and by the upregulation of Caspase 2 and Caspase 3, Protein shisa-5 (SHISA5), inhibitor of apoptosis proteins (BIRC3 and BIRC7) and DNA damage-regulated autophagy modulator protein 2 (DRAM2). Possible tissue damage and changes in apoptosis regulation and energy metabolism were supported by the enriched terms "Response to wounding" (9 DEGs), "Intrinsic apoptotic signaling pathway in response to DNA damage" (4 up-



Fig. 7. Principal component analyses (PCA) of the entire set of genes considered for gene expression analyses.



Fig. 8. Venn diagrams reporting for each pairwise comparison (CBZvsCTL; ACDvsCTL; ACD + CBZvsCTL) the number and percentage of DEGs.

regulated genes), "Energy derivation by oxidation of organic compounds" (7 DEGs) and "ATP generation from ADP" (3 up-regulated genes). Enrichment analyses identified also several biological processes and molecular pathways in common with the individual exposure to CBZ and ACD, such as ions transport, synapse, inflammation and immune response. The enriched biological processes "Reproduction" (22 DEGs) and "Sodium ion transport" (5DEGs), including putative *Transient receptor potential cation channel subfamily M member 2 (TRPM2)*, were found enriched only in ACD + CBZ (Table 1; SM3).

4. Discussion

4.1. Carbamazepine bioaccumulation and excretion under ocean acidification scenario

The need to understand the effects of multiple stressors is a major research issue and clear interactions between carbamazepine exposure and future projection of ocean acidification were provided by the present investigation. Uptake, bioaccumulation and excretion are key processes for risk assessment of pharmaceuticals to non-target species but limited pharmacokinetics and pharmacodynamics studies are actually available. CBZ is one of the most persistent pharmaceuticals in aquatic environment (Almeida et al., 2014, 2015, 2017; Miller et al., 2019), as confirmed by concentrations measured in control mussels tissues (37.7 ng/g d.w) which are well within the range of baseline values measured in mussels from unpolluted areas of the Adriatic Sea (Mezzelani et al., 2020).

Our study also revealed a significant uptake of CBZ in mussels exposed to environmentally realistic levels of this drug either when dosed alone or in combination with lower pH, without any influence of the latter on bioaccumulation. The limited time-dependent variations in carbamazepine accumulation during CBZ-exposure phase, suggests a rapid steady-state condition, as reported in previous studies with this drug (Boillot et al., 2015; Serra-Compte et al. 2018) and other pharmaceuticals such as the Non-steroidal Anti-Inflammatory Drugs

(NSAIDs) Diclofenac and Nimesulide (Mezzelani et al., 2018a). Mussels do not typically possess the capability to regulate intracellular concentration of traditional chemical pollutants, but a certain metabolism of pharmaceuticals has been hypothesized (Boillot et al., 2015; Bonnefille et al., 2017; Serra-Compte et al. 2018; Swiacka et al., 2019). The principal metabolic pathway of CBZ in mammals is mediated by the cytochrome P-450 monooxygenases leading to formation of the carbamazepine-10,11-epoxide, followed by hydration to trans-10,11dihydroxy-10,11-dihydro-carbamazepine and conjugation with glucuronic acid. The activity of Cytochrome P450 enzymes is generally very low in invertebrates, and the present study revealed a moderate effect of CBZ on biotransformation enzymes such as glutathione S-transferases. In addition, gene expression profile of CBZ-exposed mussels highlighted the up-regulation of genes involved in drug metabolism, i.e. CYP3A2, CYP3A29, CYP4F8 and ST1B1, suggesting the induction of a rudimental CBZ metabolism in marine mussels. Nevertheless, the recalcitrant behavior of this drug to be fully metabolized and excreted was supported by the limited decrease of CBZ in exposed mussels after 10 days of depuration. Further, detectable levels of CBZ were still present in control mussels after 10 days of acclimation and additional 14 days of experiment, confirming that more than 20 days of depuration are needed to eliminate CBZ levels from mussels tissues (Serra-Compte et al., 2018).

Although ocean acidification can influence both the speciation and bioavailability of several environmental pollutants (Götze et al., 2014; Nardi et al., 2017), our study did not reveal significant differences of CBZ uptake in mussels exposed at normal or reduced pH, as also reported for *Scrobicularia plana* and *Ruditapes philippinarum* (Almeida et al. 2018; Freitas et al. 2016; Serra-Compte et al. 2018;). The high pKa of CBZ (13.9) would prevent a marked ionizable tendency at tested environmentally realistic pH projections, explaining the similar bioavailability; this could allow to hypothesize that ocean acidification would not represent a factor affecting CBZ bioavailability in future oceans, although interaction between these factors was evident in terms of biological effects.

4.2. Immune system alterations

Similar to contaminants mixtures, multiple environmental stressors can elicit synergistic, additive or antagonistic effects, depending on stressors combinations, investigated endpoint and level of biological organization (Heye et al., 2019). One of the main outcomes of this work revealed hemocytes as a target of CBZ, pH and of their interactions. Circulating hemocytes of mussels are involved in important functions, such as cell physiology, intracellular turnover, immune defense, wound healing, metabolite transport, shell deposition, degradation and elimination of pathogens (Gorbi et al., 2013; Munari et al., 2019). In the present study, a significant reduction of lysosomal membrane stability was observed in mussels exposed to CBZ, both at control pH and hypercapnic condition, with the lowest values in organisms co-exposed to both stressors. A similar effect was observed on the inhibition of phagocytosis capacity, which represents one of the most important cellmediate immune responses in mussels, enabling specific hemocytes subpopulations to recognize and eliminate non-self-components. In M. galloprovincialis granulocytes are responsible of phagocytosis, and the observed inhibition of this activity can thus be explained by a decrement of circulating granulocytes, as supported by the lowered granulocytes vs hyalinocytes ratio due to CBZ-exposure. Although reduced LMS due to CBZ exposure was also reported in R. philippinarum (Aguirre-Martínez et al., 2016), the combined modulation of mussels immune responsiveness toward such multiple stressors, was so far never highlighted. In addition, the significant induction of acetylcholinesterase activity in all exposed mussels would support the proposed role of this enzymatic activity in modulation of the immune response of bivalves hemocytes during disturbance (Gerdol et al., 2018; Shi et al., 2014). Worthy to note, the weaker effect of CBZ on AChE induction under hypercapnic condition compared to control pH, allows to hypothesize antagonistic

Table 1

Lists of differentially expressed genes involved in different biological processes and molecular pathways. Enriched GO terms and pathway are reported in bold (↑ and ↓ indicated enriched terms considering up- and down-regulated genes separately). Number of differentially expressed genes for each terms are also reported, as well as the gene name of differentially expressed genes (green: down-regulated genes in exposed mussels; red: up.regulated genes in exposed mussels). Full list of DEGs are reported in Supplementary Material 2 SM2 including the gene description for each Gene ID. Full lists of enriched terms are reported in Supplementary Material SM3.

PATHWAY / BIOLOGICAL PROCESSES	N° DEGs	GENE NAME		
ACD vs CTL				
Response to stress	41	CNO11; KSYK; RAD54; PDE1; FA8; FAK1; PPIP1; M4K4; CO3A1; VI16; CAP1; PLMN; MKC1; COIA1; MMGL; FCN2; NPL4; DCAM; IF4A; OAS1; SRSF6; STML2; SRSF6; DTX3L; COL12; CYAD; TRI25; JAK2; BPI; TFPI2; LIG1; LRC33; HRH4; ZN622; MYSN; C1QT3; CXXC1; XIAP; DMBT1; CREBRF; FMAR		
Cell death	27	BIR7B; MBL; FAK1; M4K4; COIA1; IP3KB; PDE1A; T22D1; PDE1B; SRSF6; MEF2A; SRSF6; DNAIB6 CASP3; JAK2; TIF1A; MEG10; GRAM4; DRAM2; LITFL; ANK2; PEN2; CASP3; ZN622; PIM3:XIAP; FUTSC		
Apoptotic process	24	BIRC7B; MBL; FAK1; M4K4; COIA1; IP3KB; PDE1A; T22D1; PDE1B; SRSF6; MEF2A; DNAJB6; CASP3; JAK2; MEG10; GRAM4; DRAM2; LITFL; PEN2; CASP3; ZN622; PIM3; XIAP; FUTSC		
Cellular response to DNA damage stimulus	6	CNOT1; <mark>LIG1; IF4A;</mark> RAD54; <mark>XIAP;</mark> DTX3L		
Immune response	22	KSYK; FAK1; PPIP1; CO3A1; PLCG1; MRC1; FCN2; OAS1; STML2; COL12; CL004; TRI25; JAK2; BPI: LRC33: DMBT1: MYTB: MYNA: BPI: C1QL3. PLA2GE		
Ion binding	83	ARFG2; FBCD1; ZN474; ZN891; BIR7B; P4HA2; MBL; MAB1; MAB3; PDE1; FA8; CALU; TSSK2; ZC3H1; SC5D; SLIT1; CSTN1; ATS2; PYGM; CO3A1; PLCG1; ATC1; ITA8; COIA1; COX1; ARSB; MATN1; PDE1A; NCAN; FOR2; PN41; SPAN; PGBM; VCB; PDE1B; DCH5; MIG17; UNC22; RO60; CAZ; DTX3L; COL12; LIMA; FNKE; CYAD; CYGB1; T230; TRI25; JAK2; DYH7; TIF1A; PC11X; PPIG; ZN544; ODD1; RNF44; ZSCA2; PPN; LIG1; EFHA2; I17R0; FRR51; VWDE; HMCN2; ZCH10; TITN); DYST; CLIP1; FFCD1; T230; ZN622; KCTD7; HMCN1; CAD23; TSSK4; DZAN1; DSPP; MARK1; CXXC1; ZNFX1; XIAP; NR2E3; IDH3A		
Ion homeostasis	13	FAK1; PLCG1; ATC1; ANK2; TRPM1; STML2; JAK2; TIF1A; HRH4; CAD23; COPT2; COPT1; FMAR		
Ion transport ↓ Metal ion binding / Inorganic ion homeostasis/ Cellular ion homeostasis ↑	74	SZ7AZ; ANK2; SZZAD; SCGA7; ASILA; CAD23; AMIL2; CUF12 ARFG2; FBCD1; ZN474; ZN891; BIR7B; P4HA2; MBL; MAB3; PDE1; FA8; FAK1; CALU; TSSK2; ZC3H1; SC5D; SLIT1; CSTN1; ATS2; PLCG1; ATC1; ITA8; COIA1; COX1; ARSB; PDE1A; NCAN; FCN2; NPL4; PGBM; CYB; PDE1B; OAS1; MIG17; CA2; TRPM1; STML2; LIMA; FNKE; CYAD; CYGB1; T23O; JAK2; DYH7; TIF1A; PC11X; PPIG; ZN544; ODD1; RNF44; ZSCA2; PPN; LIG1; EFHA2; ANK2; FRR51; VWDE; HMCN2; DYST; HR44; CLIP1; FBCD1; T23O; KCTD7; COPT2; TSSK4; COPT1; DZAN1; DSPP; MARK1; CXXC1; ZNFX1; XIAP; IDH3A; FMAR		
Calcium ion homeostasis ↑	9	PLCG1; ATC1; TRPM1; STML2; JAK2; TIF1A; ANK2; HRH4; FMAR;		
Calcium signaling pathway ↑	3	PDE1A; PDE1B; PHKG1		
Generation of precursor metabolites and energy ↑	12	PYGM; INSR; COX1; CYB; NU4M; NU5M; PHKG1; PPIG; ODO1; AAKG2; SRBS1; IDH3A		
ATP metabolic process↑	9	INSR; COX1; ATP6; CYB; NU4M; NU5M; STML2; ODO1; AAKG2		
Cell Cycle 18 BIRC7: CSPP1: CHM24: LIG1: CHER: EPS8: HMCN1: MIE: SIR1: LARK: SEPT7: SRSE2: SC6A4:				
	0	SPG20; ERCC2; TITIN; VPS4B		
Immune Response DNA repair	9	MYNA; LY21; C1QL3, DEFI, MRC; IRG1, IF278, LITAF, PLA2 LIG1; RAD54; MMS22; SIR1; ERCC2; KAT5; HERC2		
Drug metabolism/oxidative stress	4	SESN3; CYP4F8; ST1B1; CYP3A2, CYP3A29; CYP2E1		
Apoptotic process	23	BIRC1; BIRC2; BIRC7; CHIA; DNAJB6; ERCC2; FAK1; FGD1; G2E3; GIMAP4; GIMAP7; HIP1; IP3KB; LITFL; MAX; MBL; MEG10; MIF; ORCT; RBM25; RHBD1; SFRP5; SIR1		
Cell death	28	ANK2; BIRC1; BIRC2; BIRC7; CHIA; DNAIB6; ERCC2; FAK1; FGD1; G2E3; HIP1; IP3KB; UTFL; MAX; MBL; MEG10; MIF; MMP3; ORCT; RBM25; RBM25; RHBD1; SFRP5; SIR1; TCPA; TIF1A; VP548		
Synapses	17	SC6A4; CE112; SPG20; SYNG1; FUS; SYTL4; ESPN; BAI3; ANK2; HIP1; DYST; STX7; EPS8; HRH4; SEPT7; PDZD2; DLG1		
Regulation of synapse organization	6	SYNG1; BAI3; C1QL3; CAZ; FAK1; MDGA1		
Response to wounding	10	ILK; MAX; GABT; MMGL; BIRC1; MYL9; FA8; TFPI2; XCT; IFRD1		
Inflammatory response [†] 6 MMGL; BIRC1; FA8; IRG1; C163A; MIF; HRH4				
Apoptotic process	10	ACD+CBZ vs CTL F4113: ARHGC: NHRE1: BIRC3: ATRV: FRCC3: SRDK3: MIE: SUSAS: LDDG: TIAD: CASD3: TDI30:		
Cellular response to DNA damage	9	CASP3; T22D1; DRAM2; BIRTA; HNRPK; SRSF6 DOTLL: CENPX; SETD2; ATRX; ERCC2; MIF; SHSA5; CASP2: HNRPK		
stimulus	10			
Immune system process↑	18	EPS8; CS066; LYAM3; ERCL2; SKPK2; MIF; OAS1; PLMN; PLCG1; DCTN4; GSLG1; TRPM2; TITIN; FCN2; CASP3; MDR1; MRC1; PLMN CART: S22AD: AMT2: SZ7A2; SCSA1: CCSA9: S22AE: AT1R1: MRD5: NHRE1: CNG1: MIE-		
	18	GRB1, 322AD, AM12, 327A2, 303A1, 303A6, 333AC, A11B1, MAES, MARET, CNG1, MIE, S17A5; PLCG1; TRPM2; S23A1; ANK2; UNC9		
Calcium ion binding	19	ANXA7; HMCN1; TTTN; TRHY; RFP4A; LRP4; PC11X; GLU2B; LRP; PLCG1; PCDH7; SLIT1; NCS2; MMP3; TRPM2; HMCN2; DSPP LPD6: PLCG1: ANK7; TRPM3; EAAP		
concentration	5	EN 0, LEGI, ANKZ, INFINZ, FINAK		
Calcium-mediated signaling↑	4	PLCG1; LYAM3; ANK2; TRPM2		
Soutium ion transport Synapse organization↑	5	INTIGET, 30348; INTIVIZ; ATTB1; 323A1 LRP4: BAI3: SLIT1: MDGA1: SPTCB: KY		
Response to wounding	9	GABT; MMGL; LYAM3; NINJ2; PLMN; IFRD1; TFPI2; SRSF6; PLMN		
Intrinsic apoptotic signaling pathway in response to DNA damage↑	4	MIF; SHSA5; CASP2; HNRPK		
Energy derivation by oxidation of organic compounds	7	INSR; GLYG; IDH3A; GLYG2; PYGM; PPIG; ODO1		
ATP generation from ADP↑	3	ODO1; INSR; AKD1		
Reproduction	25	UNC22; VIT6; VEZP9; VEZP12, VERL, GABT CENPX; CHDM; AT1B1; SETD2; DOT1L; INSR; LRP6; TIAR; PLMN; WBP2L; SPTCB; SP17; ATRX; TBD; CCD63; CO4A1; CYAD; CBR1; CASP2		

interactions between tested stressors.

In analogy with cellular biomarkers, gene expression analyses in digestive glands highlighted the significant up-regulation of several genes with a key role in immune response and inflammation (e.g. MYNA, LYZ1, DEFI, MRC1, IRG1, LITAF and PLA2 among others), suggesting a coordinated activation of mussels immune defenses in response to CBZ exposure. Although immune system has not been characterized as direct target of CBZ in mammals, an increasing body of evidence indicates immunosuppression as side effect due to alteration of membrane phospholipids, inhibition of protein synthesis in lymphocytes, modulation of plasma levels of different interleukins, and a significant increment in cytotoxic activity of NK cells (Beghi and Shorvon, 2011). Results obtained in the present study corroborate such evidence also for nontarget marine invertebrates and highlight an exacerbation of these effects under ocean acidification condition. A large number of genes involved in immunological pathways, appeared to be affected by hypercapnia: the significant up-regulation of MRC1, FCN2, MYTB, MYNA and C1QL3 in ACD-exposed mussels, highlighted the earlier responsiveness of transcriptional level compared to cellular biomarkers. The combination of CBZ and low pH caused the most important activation of immune response among treatments, as shown by the enriched term "Immune system process" represented by 18 up-regulated genes (Table 1), and thus confirming that interactive effects on immune system progressed from molecular up to cellular level.

Among the DEGs only in response to combination of stressors, a significant up-regulation was observed for *Lysozyme 3* (*LYS3*) and *Complement C1q-like protein 4* (*C1QL4*), which act as a critical first-line responder of innate immunity (Leprêtre et al., 2019). An increased number of studies on bivalve species demonstrated that environmental stressors may have a direct effect on microbial composition or/and lead to the decreased host ability to control the symbiotic microbial communities (Milan et al., 2018b; Milan et al., 2019). Overall, from our findings, biochemical and molecular modifications in hemocytes and immune response could partially reflect the attempt to control changes in host-microbiota compositions, suggesting that future studies are needed to understand possible links between host-immune system activation and possible changes related to CBZ and hypercapnia exposures.

4.3. Carbamazepine mechanism of action in M. galloprovincialis

Carbamazepine has three main MoA in mammals species: *i*) it inhibits the depolarization of neurons through the enhancement of inhibitory neurotransmission mediated by the gamma-aminobutyric acid (GABA); *ii*) it increases the concentration of the inhibitory neurotransmitter serotonin at neuronal synapses; *iii*) it binds to the voltagegated sodium ion channels proteins, blocks the channel and reduces the frequency at which impulses are fired during epileptic crisis (Ambrosio et al., 2002; Harkin & Hopkinson, 2010; Ofoegbu et al., 2016).

Analyses on transcriptional profiles provided insights on possible similarities between CBZ MoA in invertebrates and vertebrates, including the activation of a large number of molecular mechanisms related to synapses and neurotransmission in CBZ-exposed mussels. First, the direct involvement of gamma-aminobutyric acid (GABA) pathway is suggested by the down-regulation in CBZ-exposed mussels of GABA transaminase (GABT), responsible for GABA catabolism. Second, the significant modulation of Sodium-dependent serotonin transporter (SC6A4), representing an integral membrane protein that transports the neurotransmitter serotonin from synaptic spaces into presynaptic neurons, suggests additional MoA similarities among species. In particular, the SC6A4 down-regulation in CBZ exposed mussels may lead to a significant increase of extracellular serotonin concentration, which represents a typical effect of CBZ exposure in mammals (Dailey et al., 1998). Third, the combined effects of CBZ and hypercapnia led to significant changes in genes involved in "Sodium ion transport" that can be related

to the blockade of inactivated neuronal sodium channels, one of the main CBZ MoA in vertebrate species (Suwalsky et al. 2006). Among genes involved in this pathway particular attention should be payed to the up-regulation of *TRPM2* coding for a protein forming a tetrameric cation channel permeable to calcium, sodium, and potassium. *TRPM2*, already described to be up-regulated in bivalves in response to chemicals exposures (Ertl et al., 2016), is activated by oxidative stress and mediates mitochondria-dependent apoptosis by increasing calcium influx (Kang et al., 2018; Nazıroğlu and Demirdaş, 2015; Takahashi et al. 2011).

Also the induction of acetylcholinesterase activity observed in CBZexposed mussels reveals a certain parallelism between carbamazepine MoA in vertebrate and invertebrates species. Indeed, CBZ is a mood stabilizer, used in human medicine to treat epilepsy, neuropathic pain and maniac disorders. As mentioned above, voltage-gated sodium ion channels represent the main target of CBZ, but it has also been demonstrated its capability to interact with voltage-gated calcium and potassium ion channels, cholinergic, purinergic systems, signaling pathways (serotonergic, dopaminergic, glutamatergic) and receptors (Ambrosio et al., 2002, Siebel et al., 2010). In this respect, the AChE is necessary for the hydrolysis of the neurotransmitter acetylcholine in cholinergic synapses and plays a critical role in the nervous system. Mizuno et al. (2000), observed that therapeutic doses of CBZ enhanced both acetylcholine release and synthesis in rat striatal and hippocampal brain tissues, demonstrating the modulation of cholinergic system. In non-target species a certain responsiveness of this pathway was observed, but without a unique pattern of variation: it was inhibited in R. philippinarum treated with 1 µg/L of CBZ, while induced in the amphipod Ampelisca brevicornis exposed to sediments spiked with carbamazepine at 0.5 ng/g (Aguirre-Martínez et al., 2016; Maranho et al., 2015). Overall, results obtained in this study confirmed that some pathways of human pharmaceuticals are preserved among species, being modulated also in non-target organisms (Almeida et al., 2018; Aguirre-Martínez et al., 2016; Ofoegbu et al., 2016).

4.4. Oxidative and genotoxic effects

Contrasting results were observed on typical markers of genotoxicity measured in terms of DNA strand breaks or MN frequency. The lack of DNA damage in hemocytes through the Comet assay could be explained by efficient DNA repair mechanisms, as also supported by modulation of genes belonging to these pathways in mussels from all experimental treatments. On the other hand micronuclei frequency increased under reduced pH conditions, allowing to hypothesize that hypercapnia can affect hemocytes cell cycle, as already highlighted in *M. galloprovincialis* and in the scallop *Felxopecten glaber* (Nardi et al., 2017, 2018a, 2018b): the observed micronuclei increment, marker of damage at chromosome level, is supported by the significant upregulation of genes involved in apoptosis in digestive gland of organisms exposed to hypercapnia.

One of the main mechanisms causing the onset of DNA damage is mediated through increased intracellular production of reactive oxygen species (ROS) or impaired efficiency of the antioxidant system (Regoli and Giuliani, 2014). Our study, through the integrated measurement of individual antioxidants and the capability to neutralize specific oxyradicals, evidenced a rather weak involvement of this pathway: limited differences of catalase, total glutathione and glutathione-dependent enzymes, were paralleled by the lack of consistent variations of the total oxyradical scavenging capacity toward peroxynitrite, hydroxyl and peroxyl radicals. These findings indicate that prooxidant mechanisms do not represent the primary mode of action of these environmental stressors.

However, histochemical analyses revealed cumulative and interactive effects of CBZ and hypercapnic condition, on respectively lipofuscin and neutral lipids content. Accumulation of lipofuscin, a typical end product of membrane peroxidation is generally accompanied by lysosomal alterations due to its capability to bind lysosomal hydrolases, with consequent inhibition of protein degradation and autophagic accumulation of undegradable damaged organelles, proteins, phospholipids and lipids, contributing to further lipofuscin formation (Moore et al., 2006). The accumulation of neutral lipids, that represent one of the major energy reserves in bivalves, is considered an indicator of disturbed metabolism (Bocchetti and Regoli 2006). An increment of lipofuscin under ocean acidification scenario was observed in *M. galloprovincialis, Euplotes crassus* and *Sterechinus neumayeri*, while the simultaneous increase of lipofuscin and neutral lipids was reported in *M. galloprovincialis* treated with CBZ (Dell'Acqua et al., 2019; Gomiero et al., 2018; Martin-Diaz et al., 2009; Nardi et al., 2017).

Acidification conditions can alter the intracellular milieu (Ivanina and Sokolova, 2015; Stillman and Paganini, 2015), and our RNAseq results on ACD and ACD + CBZ exposed mussels confirmed changes in gene expression of ion binding, transport and homeostasis processes. The subsequent impairment of cellular homeostasis could be the cause of a complex cascade of events involving gene alterations of calcium signaling pathway, mitochondrial energy metabolism (COX, ATP6, IDH3A, ODO1 among others) and the up-regulation of apoptotic pathways (CASP2, CASP3 among others). This cascade, when combined with CBZ-exposure, could explain the peroxidation processes observed herein in terms of accumulation of lipofuscin and neutral lipids in lysosomes, along with the induction of proteins repair mechanisms (HSP70). In this context, previous studies highlighted the involvement of calciumsignaling pathway in the accumulation of lipofuscin in human retinal pigment epithelial cells (Zhang et al., 2011) and in both lysosomal alterations and oxidative insult in M. galloprovincialis (Dailianis et al., 2009; Raftopoulou et al., 2006).

4.5. Additional interactive effects of carbamazepine and hypercapnia

Several changes detected in response to ACD + CBZ, such as apoptosis, inflammation, immune response, ions transport, synapse and DNA damage showed enhanced but similar trends to those revealed in the individual exposures, suggesting cumulative effects of combined stressors. On the other hand, the 52% of DEGs detected in mussels exposed to the combination of carbamazepine and acidification were differentially expressed only in response to this treatment. Among them, genes involved in "calcium ion binding" "Sodium ion transport" and "Potassium channel" were found differentially expressed. While the capability of CBZ to interact with voltage-gated sodium ion channels and voltage-gated calcium and potassium ion channels have already been discussed above, the effects of OA in the homeostasis of carbonate and calcium ions have been reported in several studies (Beniash et al. 2010; Richards et al. 2018), suggesting possible synergistic effects of the two stressors on ion homeostasis. Nonetheless, the multidrug resistance proteins MRP5, having a role in cell detoxification through their ability to excrete xenobiotic conjugates and metabolites, were down-regulated in organisms co-exposed to CBZ and reduced pH, potentially explaining the slightly delayed elimination of CBZ in organisms exposed to this molecule under reduced pH scenario. In addition, the biological process "Reproduction" was enriched only in mussels exposed to ACD + CBZ, and six down-regulated genes coding for vitellogenin like transcripts (Vitellogenin 6, VIT6; vitelline envelope zona pellucida domain protein 12, VEZP12; vitelline envelope zona pellucida domain protein 9, VEZP9; putative vitelline envelop receptor for lysin, VERL) were also found. Although the controversial role of vitellogenin in marine mussels, to our knowledge, just few studies identified potential effects of carbamazepine and ocean acidification on vitellogenin like transcripts (Harms et al., 2014).

Overall, gene expression profiling indicated several pathways where combined stressors induced additive or even synergistic effects, thus representing a potential risk for bivalve populations exposed to pharmaceuticals under future scenarios of reduced pH.

Single and combined effects of CBZ and acidification in *M. galloprovincialis* were further supported when all the cellular responses were elaborated through the weighted criteria of the

quantitative Sediqualsoft model, which summarizes a hazard index based on the number of changed biomarkers, the toxicological relevance of measured end points and magnitude of variations compared to threshold specific for each response (Benedetti et al., 2012; Piva et al., 2011). With this approach, the calculated HQ for biomarkers raised from "Slight" in mussels exposed to individual stressors, to "Moderate" in organisms co-exposed to CBZ and hypercapnia.

5. Conclusions

This study provided clear evidences of interactive effects of CBZ and acidification in M. galloprovincialis. Despite hypercapnia had a limited influence on CBZ accumulation, modulation of ecotoxicological effects was highlighted by changes in gene expression profile and functional biological responses at cellular level. Transcriptional responses showed the early activation of immune response, inflammation processes, apoptosis, DNA damage, ions transport and changes in energy metabolism. Such alterations progressed from molecular up to functional cellular level, with a frequent synergistic, additive and rather antagonistic, action of CBZ and reduced pH. The integration of chemical, molecular and cellular findings allowed to hypothesize intricate pathways behind the biological effects of acidification and carbamazepine, suggesting potential threats for the health status of M. galloprovincialis in coastal areas characterized by noticeable carbamazepine levels. Given the importance of this species in ecosystem functioning and its economical relevance in seafood market additional long-term studies are recommended to unravel the ecological consequences of contaminants of emerging concern under predicted scenarios of ocean changes.

CRediT authorship contribution statement

Marica Mezzelani: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Project administration. Alessandro Nardi: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Project administration. Ilaria Bernardini: Investigation, Formal analysis, Data curation. Massimo Milan: Investigation, Formal analysis, Data curation, Resources, Writing - review & editing. Luca Peruzza: Investigation, Formal analysis, Data curation. Giuseppe d'Errico: Software, Formal analysis, Data curation. Giuseppe d'Errico: Software, Formal analysis, Data curation. Daniele Fattorini: Data curation, Supervision. Stefania Gorbi: Resources, Supervision. Tomaso Patarnello: Resources, Funding acquisition. Francesco Regoli: Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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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Appendix A. Supplementary material

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