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Single and combined effects of caffeine and salicylic acid on mussel *Mytilus galloprovincialis*: Changes at histomorphological, molecular and biochemical levels

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ABSTRACT

Caffeine (CAF) and salicylic acid (SA) are frequently detected in waterbody, though information on their biological impact is poor. This work assesses the effects of CAF (5 ng/L to 10 μg/L) and SA (0.05 μg/L to 100 μg/L) alone and combined as CAF+SA (5 ng/L+0.05 μg/L to 10 μg/L+100 μg/L) on mussel *Mytilus galloprovincialis* under 12-days exposure by histomorphology of digestive gland and oxidative stress defense at molecular and biochemical levels. Besides evaluating tissue accumulation, absence of histomorphological damage and haemocyte infiltration highlighted activation of defensive mechanisms. Up-regulation of *Cu/Zn-sod*, *Mn-sod*, *cat* and *gst* combined with increased catalase and glutathione *S*-transferase activity were found in CAF-exposed mussels, while SA reduced ROS production and mitochondrial activity. CAF+SA exposure induced differential responses, and the integrated biomarker response (IBR) revealed more pronounced effects of SA than CAF. These results enlarge knowledge on pharmaceuticals impact on non-target organisms, emphasizing the need for proper environmental risk assessment.

1. Introduction

Pharmaceutical residues represent a large group of "emerging" contaminants, being continuously discharged into the aquatic environment via different routes, mainly by wastewater treatment plants (WWTPs) (Daughton, 2004). Pharmaceuticals from different therapeutic classes, including antibiotics, analgesics and non-steroidal anti-inflammatories (NSAIs), beta-blockers, etc., have been detected in the aquatic compartments worldwide at the range of nanograms (ng) to micrograms (μg) per liter (Aus der Beek et al., 2016; Desbiolles et al., 2018; Afsa et al., 2020; Madikizela et al., 2020). These biologically active compounds could negatively affect aquatic flora and fauna, even at very low concentrations, due to their pseudopersistant character and liposolubility thereby favouring bioaccumulation by exposed organisms, which leads to various physiological changes (Küster and Adler, 2014; Afsa et al., 2022a).

Caffeine (CAF; 1,3,7-trimethylxanthine) is a central nervous system psycho-stimulant belonging to the group of methylxanthines. It is naturally present in different plants (e.g., coffee beans, cacao beans, tea leaves, etc.) and mostly found in beverages and caffeinated food. Additionally, CAF is commonly used in the formulations of a plenty of medicines such as painkillers, analgesics, stimulants, etc. (Buerge et al., 2003; Moore et al., 2008). Due to its large consumption worldwide (Quadra et al., 2020), CAF is one of the most frequently detected psychoactive substances in the aquatic compartments being reported at relatively high concentrations in various water matrices (Table 1). Consequently, the presence of CAF in surface water has been recognized as an indicator of anthropogenic contamination, namely by sewage

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(Buerge et al., 2003).

In addition to CAF, salicylic acid (SA; 2-hydroxybenzoic acid) is another commonly detected pharmaceutical compound in the aquatic environment. It represents the main metabolite and the active form of acetylsalicylic acid (ASA, aspirin ®) (Davison, 1971), a drug belonging to the therapeutic class of NSAI drugs. ASA is a widely consumed drug due to its various pharmacological activities (occurring mostly through the inhibition of cyclooxygenases responsible of the synthesis of prostaglandin and prostanoids), effectiveness, and low cost (Brooks and Day, 1991; Cleuvers, 2004; Freitas et al., 2020a). Moreover, SA is used individually to treat dermatological disorders and as an active ingredient in some cosmetic products (Rendon et al., 2010; Madan and Levitt,

Table 1
The detected concentrations (ng/L) of CAF in aquatic environments and effluents worldwide.

Aquatic compartment	Concentration	Frequency of detection	Country/ Location	Reference
HOSPITAL	293,000 *	-	Norway	Weigel et al. (2004)
WASTEWATER	74,800* ± 15,500	-	Saudi Arabia	Al Qarni et al. (2016)
	27,500* ± 2000	-		
	4894.9* (treated)	-	France	Mazzitelli et al. (2018)
	12,300–42,000	-	Greece (Ioannina)	Kosma et al. (2010)
	9272 – 48,583	100%	Greece (Ioannina)	Kosma et al. (2020)
	12,100 – 182,000	92%	Korea	Sim et al. (2011)
	5800*	-	Cameroon	Mayoudom et al. (2018)
	45,740 – 325,000	-	USA	Oliveira et al. (2015)
	200,000 (max)	-	USA	Oates et al. (2017)
	82,500–902,000	100%	Tunisia (Mahdia)	Afsa et al. (2020)
UWW	36,600–59,100	100%	Spain (Gran Canaria)	Afonso-Olivares et al. (2017)
INFLUENT	14,000 – 145,000	100%		
	51,969–82,844	100%	Spain (Cadiz)	Biel-Maeso et al. (2018)
	2444 – 59,408	-	North-eastern Tunisia	Moslah et al. (2018)
	54,700*	-	Norway	Weigel et al. (2004)
	14,300* ± 760	-	South Africa	Matongo et al. (2015)
	66,400* ± 15,447	100%	South Carolina, USA	Hedgespeth et al. (2012)
	82,882* ± 16,359	100%		
	17,100 – 113,200	-	Greece (Ioannina)	Kosma et al. (2010)
	1940 – 75,700	93%	Korea	Sim et al. (2011)
	69,900*	81.2%	Costa Rica	Ramírez-Morales et al. (2020)
	139,900–439,400	100%	Tunisia (Mahdia)	Afsa et al. (2020)
UWW	17 – 1780	16.7%	Spain (Gran Canaria)	Afonso-Olivares et al. (2017)
EFFLUENT	19 – 3260	100%		
	166 – 186	100%	Spain (Cadiz)	Biel-Maeso et al. (2018)
	1056 – 34,578	-	North-eastern Tunisia	Moslah et al. (2018)
	47,700	-	Norway	Weigel et al. (2004)
	6870* ± 1230	-	South Africa	Matongo et al. (2015)
	924* ± 865	100%	South Carolina, USA	Hedgespeth et al. (2012)
	207* ± 498	91.6%		
	1900 – 13,900	-	Greece (Ioannina)	Kosma et al. (2010)
	52 – 3180	35.2%	Korea	Sim et al. (2011)
	6420*	53.3%	Costa Rica	Ramírez-Morales et al. (2020)
	89–19,800	100%	Tunisia (Mahdia)	Afsa et al. (2020)
SEAWATER	5.2 – 78.2	-	Greece	Alygizakis et al. (2016)
	522 (max)		Greece	Nödler et al. (2016)
	3068 (max)	95%	Greece-Turkey	Nödler et al. (2014)
	1110 (max)	100%	Italy (Venice)	Nödler et al. (2014)
	8230 (max)		Japan	Murakami et al. (2011)
	7.2 – 522	-	Spain (Cadiz)	Baena-Nogueras et al. (2016)
	9.8 – 42.9	-		
	6.1 – 327.3	100%	Spain (Cadiz)	Biel-Maeso et al. (2018)
	4.3 – 96.6	100%		
	7 – 87	-	Norway (Tromsø)	Weigel et al. (2004)
	n.d.– 5000	-	New York	
	130 – 1400	-	Canada	Comeau et al. (2008)
	7708 (max)	-	Saudi Arabia	Ali et al. (2017)
	BQL–410	100%	Tunisia (Mahdia)	Afsa et al. (2020)
	84.4 – 648.9	-	Brasil	Pereira et al. (2016)
	350 – 1300	-	Brasil	Roveri et al. (2020)
	8.07– 3060	100%	China	Sun et al. (2016)
	677 (max)		Germany	Nödler et al. (2014)
GROUNDWATER	6 – 25 (GW)	-	North Carolina, USA	McEachran et al. (2016)
SURFACE WATER	5 – 7 (SW)	-		
RIVER WATER	19* ± 11(SW)	98.6%	South Carolina, USA	Hedgespeth et al. (2012)
	n.d – 9250 (RW)	-	South Africa	Matongo et al. (2015)
	913* (RW)	-	France	Mazzitelli et al. (2018)
	902 – 7051 (RW)	-	China	Zhou et al. (2010)
	4–3360 (RW)	-	India	Chakraborty et al. (2021)
	41.7–28,439 (RW)	-	Brazil	Peteffi et al. (2018)
	BQL-1040 (RW)	-	Brazil	Sposito et al. (2018)
	384–426 (RW)	100%	Malaysia	Khalik et al. (2020)
	600–804	-	Portugal	Gonzalez-Rey et al. (2015)

[GW= Groundwater; SW= Surface water; RW= River water; n.d.= not detected; (-) = not available data; (*) = value represents the mean; BDL = below detection limit; BQL = below quantification limit; UWW= urban wastewater]

2014). Consequently, this compound has been frequently found in different aquatic matrices with concentration ranging from several ng in freshwater and seawater to hundreds of μg in wastewaters, as reported in detail elsewhere (Afsa et al., 2022b).

Interestingly, relevant concentrations of CAF were reported in tissues of marine biota including bivalves (up to 68 ng/g, *Mytilus* spp.), fish (up to 73.6 ng/g, *Gerres oyena*) and algae (up to 41.2 ng/g, *Turbinaria conoides*), as recently reviewed by Vieira et al. (2022). Additionally, according to the study of Dafouz et al. (2018), high Hazard Quotient (HQ) has been registered for CAF (using the maximum concentration of CAF detected in seawater). For instance, HQ of CAF reached 117, 164.6 and 220 respectively in USA, Japan and Australia. In addition to CAF, SA was also detected in tissues of few bivalves, namely the marine species *Mytilus edulis* (Wille et al., 2011) and the freshwater species *Dreissena bugensis* (Bai and Acharya, 2019). This data creates an interest in better understanding the impact of both compounds on non-target aquatic organisms.

Previous reports have documented that both compounds were able to cause significant adverse effects under laboratory conditions on several aquatic species including vertebrates (Nunes et al., 2015; Zivna et al., 2015; Santos-Silva et al., 2018; Dos Santos et al., 2021; Muñoz-Peñuela et al., 2021) and invertebrates (Cruz et al., 2016; Pires et al., 2016; Aguirre-Martínez et al., 2018; Freitas et al., 2019; Nunes, 2019; Szabelak and Bownik, 2021). These studies have reported that CAF and SA could impair development and reproduction, behaviour and physiological parameters, metabolic activities and antioxidant enzymatic activities of tested organisms. Nevertheless, when exploring the effects of CAF and SA on bivalves, the available data is still limited and poorly understood particularly for their molecular combined effects (Table 2). The mussel *Mytillus galloprovincialis* is a sentinel bivalve species capable to accumulate a wide spectrum of contaminants from the marine water systems given its filter feeding habits. This model organism represents an excellent tool used in ecotoxicological studies due to a series of features namely its ecological relevance, abundance in the marine coastal areas, the well knowledge on its physiology, ease of handling and maintenance under controlled-laboratory conditions (Caricato et al., 2019; Cappello et al., 2021). Recently, in our previous work (Afsa et al., 2022), we revealed the presence of an unbalance in the antioxidant system and expression of oxidative stress related genes in the digestive gland (DG) of *M. galloprovincialis* after being exposed to SA. Moreover, an hemocytic infiltration confirmed by a flow cytometric analysis was also previously reported (Afsa et al., 2022b). Nevertheless, to date, there is no studies addressing the impact of CAF isolated and in combination with SA on the DG of this species at molecular level. In fact, despite all drugs occur naturally as a complex mixture in the aquatic environment (Kümmerer, 2008), little is known about their combined effects (De Marco et al., 2022a). Therefore, toxicological studies with emphasis on the interactive effects of pharmaceuticals, reflecting realistic scenarios, are needed in order to provide more reliable information about the toxicity of mixtures (Fent et al., 2006). Hence, this study aimed to evaluate the effects of CAF and SA as ~~a~~ single compounds and in combination (CAF+SA) on the DG of the Mediterranean mussel *M. galloprovincialis* under a 12 days exposure period. Transcript levels of oxidative stress related genes, namely *superoxide dismutase* (*Cu/Zn-sod* and *Mn-sod*), *catalase* (*cat*) and *glutathione S-transferase* (*gst*), were assessed over different periods of treatment along with the activities of a battery of biochemical biomarkers (CAT, GST and lipid peroxidation, LPO). Parallelly, DG histomorphology of the challenged mussels was also evaluated through histological tools.

2. Material and methods

2.1. Selection of pharmaceuticals

The concentrations of CAF (Fluka; CAS Number 27600, purity ≥ 99%) and SA (Sigma-Aldrich; CAS Number 84210, purity ≥ 99%) herein tested were selected following the level reported in aquatic environments and effluents worldwide (described here in Table 1 and previously in Afsa et al., 2022b). For CAF, the chosen dosages were as follows: C1 = 0.005 μg/L, C2 = 0.05 μg/L, C3 = 0.5 μg/L, C4 = 5 μg/L and C5 = 10 μg/L. For SA, the selected concentrations, as previously described in Afsa et al., 2022b, were: C1 = 0.05 μg/L; C2 = 0.5 μg/L; C3 = 5 μg/L; C4 = 50 μg/L and C5 = 100 μg/L. The dosages selected for CAF+SA mixture were: C1 = [0.005 μg/L+ 0.05 μg/L], C2 = [0.05 μg/L+ 0.5 μg/L], C3 = [0.5 μg/L+ 5 μg/L], C4 = [5 μg/L+ 50 μg/L] and C5 = [10 μg/L+ 100 μg/L].

2.2. Acclimation and maintenance conditions

Adult mussels (*M. galloprovincialis*) of similar size (5–6 cm shell length) were obtained from a mussel farming (S.A.Co.M) located in Messina (southern Italy). Mussels were immediately transported in seawater tanks to the IRBIM-CNR (equipped with "Mesocosm Facility") of Messina, Italy, where they were subjected to an acclimation period of 15 days in natural filtered seawater (300 μm) under controlled laboratory conditions consisting of a stable temperature of 18 ± 1 °C, pH of 8 ± 0.02, salinity of 35‰ and 12 h:12 h light/dark photo-cycle. During all this period, mussels were supplied with constant aeration. Three times a week, the filtered seawater was completely changed, and mussels were fed with a mixture of commercial algal solution (Liquizell, Hobby).

2.3. Experimental design

Mytilus galloprovincialis individuals were exposed for 12 days to CAF and SA alone and to their combination as CAF+SA. For each exposure, the tested mussels were subject to six experimental conditions, including a negative control group free of drugs. For each group, fourteen specimens were selected randomly and immediately placed in glass aquaria filled with 10 liters of filtered seawater freshly spiked with pharmaceuticals. All experiments were performed in duplicate.

For CAF and SA exposure, the selected nominal concentrations were prepared by adding the corresponding volume of the respective stock solution (CAF: 1 mg/L; SA: 10 mg/L) to each aquarium. Likewise, final concentrations of the mixture were prepared by diluting volumes from the stock solution of CAF (1 mg/L) and SA (10 mg/L) directly in each aquarium. Stock solutions were freshly prepared by dissolving the pure compound (powder) in artificial seawater (ASW) and kept in glass vials at 4 °C. During all the stages of the experiment, mussels were submitted to the same conditions as described for the acclimation period. Thrice per week, exposure medium was completely renewed, mussels were fed using the same algal mixture, and the test conditions re-established. Mortality was monitored regularly during the whole experiment.

2.4. Sampling method and tissues collection

To evaluate the time-dependent effect of the tested pharmaceuticals, three mussels per replicate (n = 6 per treatment condition) were sampled at the beginning of the experiment (T0), after 3 days (T3), 5 days (T5) and at the end of the exposure (T12). At each sampling time, mussels were immediately dissected and a sample of digestive gland tissue (DG), from each test organism, was rapidly stored at − 80 °C prior to processing for molecular and biochemical analyses. For histological analysis, a small piece of each DG tissue was immediately immersed in paraformaldehyde (PFA 4%) fixing solution to preserve the tissue.

2.5. CAF and SA quantification in mussel DG

The samples were extracted using a methanol–formic acid (95:5) micro-liquid-liquid extraction. The sample methanolic extract was then filtered through a 0.45 μm nylon filter directly into an HPLC vial, and sealed. The CAF and SA were determined through the HPLC Agilent 1260 Infinity II. A reversed phase (Column Waters XTerra RP 18, 4.6

Table 2
Individual and combined effects of CAF and SA on marine and freshwater bivalves, under laboratory conditions. Tested concentrations, exposure period and media, biological sample, biomarkers and observed significant effects are indicated.

Test-organism	Tested concentrations (µg/L)	Exposure period	Exposure medium	Biological sample	Biomarkers	Biological effects	References
CAF							
Mytilus galloprovincialis (Seawater)	0.05, 0.5	21 d*	Natural seawater	-Haemolymph	Immune system biomarkers: -Hemocyte volume -Hemocyte diameter -Hemocyte proliferation -THC -LMS	-Increase of haemocyte volume -Increase of haemocyte diameter -THC reduction	Munari et al. (2020)
Mytilus galloprovincialis (Seawater)	0.005, 0.05, 0.5	7 d	Artificial seawater	-Gills -Digestive gland -Haemocytes	-Lysosomal parameters of general stress (LMS, LYS/CYT, NL, LIF) -Oxidative stress biomarkers (MDA, GST, CAT) -Neurotoxicity biomarkers (AChE) -Genetic damages biomarkers	-Reduction of LMS -Increase of NL level -Increase of GST activity	Capolupo et al. (2016)
Mytilus galloprovincialis (Seawater)	0.005, 0.05, 0.5, 5, 10	12 d*	Natural filtered seawater	-Gills -haemolymph	-Histomorphology -THC -Oxidative stress biomarkers (SOD, CAT, GST, MDA) -Neurotoxicity biomarker (AChE)	-Haemocyte infiltration (moderate) -No changes in THC (Slight increase of halinocytes sub-population and decrease of granular haemocytes sub-population) -increase in GST activity -Oxidative stress induction -AChE alterations	De Marco et al. (2022a)
Mytilus californianus (Seawater)	0.05, 0.2, 0.5	30 d*	Natural seawater	-Gills -Mantle	-Hsp70 concentration	-Inhibition/ up-regulation	Del Rey et al. (2011)
Ruditapes philippinarum (Seawater)	0.5, 3, 18	28 d	Artificial seawater	-Soft tissues (Pooled samples)	-Antioxidant system biomarkers -Phase II biotransformation biomarkers -Energy reserves and metabolism biomarkers	-cellular redox status alterations -oxidative stress induction -metabolic activity increase	Cruz et al. (2016)
Ruditapes philippinarum (Seawater)	0.1, 5, 15, 50	14 d	Natural seawater	-Haemolymph -Digestive gland tissues	-General stress biomarker (LMS) -Biochemical biomarkers [Phase I (EROD and DBF) Phase II (GST)] -Oxidative Stress biomarkers (GR, GPX, LPO) -Neurotoxicity (AChE) -Genotoxicity (DNA damage)	-Reduction of LMS -Induction of detoxification metabolism -Induction of oxidative stress -AChE changes (induction/ inhibition)	Aguirre-Martínez et al. (2016)
Corbicula fluminea (Freshwater)	0.1; 5; 15; 50	21d	Water (N.P)	-Gonad tissues -Digestive gland tissues	-DOP levels -MAO, COX, VTG, MET -Total lipids -Energy expenditure -Neurotoxicity (AChE)	-Increase of DOP levels, -COX induction -Alteration of AChE activity -Energy expenditure increase	Aguirre-Martínez et al. (2018)
Corbicula fluminea (freshwater)	0.1, 5, 15, 50	21 d	Water (N.P)	-Haemolymph -Digestive gland tissues	-General stress biomarkers (LMS) -Phase I and II biotransformation biomarkers (EROD, DBF, GST) -Oxidative stress biomarkers -Genotoxicity (DNA damage)	-LMS changes (inhibition) -Induction of biotransformation phases -Induction of oxidative stress (GPX and GR increase) -DNA damage	Aguirre-Martínez et al. (2015)
Elliptio complanata (freshwater)	0.08, 0.04, 2, 10[a]	48 h	Injection (via abductor muscle)	-Gonad -Digestive gland tissues	-Oxidative stress biomarkers -Genotoxicity biomarkers	-Induction of oxidative stress -DNA damage	Martín-Díaz et al. (2009)
Elliptio complanata (freshwater)	2.5, 25, 50, 100[b]	24 h	*In vitro* treatment	-Haemolymph	-Phagocytic activity -Intracellular esterase activity	-Induction of phagocytic activity -Lipid peroxidation -Inhibition of cell adherence	Gagné et al. (2007)

(*continued on next page*)

Table 2 (*continued*)

Test-organism	Tested concentrations (μg/L)	Exposure period	Exposure medium	Biological sample	Biomarkers	Biological effects	References
					-Cell adherence -Lipid peroxidation		
SA							
Mytilus galloprovincialis (Seawater)	5, 50, 500, 5000	28 d	Seawater	-Soft tissues	-RR -Energy reserves and metabolic capacity biomarkers (GLY, PROT, ETS) -Oxidative stress biomarkers (SOD, CAT, GPx, GSH/ GSSG, LPO) -Phase II biotransformation (GST) -Neurotoxicity (AChE)	-RR decrease -Metabolic activation -Energy reserves increase -Antioxidant defences alteration (inhibition/ increase) -Induction of phase II metabolism -Inhibition of AChE	Freitas et al. (2019)
Mytilus galloprovincialis (Seawater)	4000	28 d	Seawater	- Soft tissues	-Oxidative stress biomarkers (SOD, CAT, GPx, GSH/ GSSG, LPO) -Energy reserves and metabolic capacity biomarkers (GLY, PROT, LIP, ETS)	-Decrease of CAT activity -Reduction of GSH/GSSG -No changes in LPO -No changes in SOD -Increase of ETS -Decrease of GLY content	Freitas et al. (2020a)
Mytilus galloprovincialis (Seawater)	4000	28 d	Seawater	-Soft tissues	-Oxidative stress biomarkers (SOD, CAT, GPx, GSH/ GSSG, LPO) -Energy reserves and metabolic capacity biomarkers (GLY, PROT, LIP, ETS)	-Reduction of GSH/GSSG -Decrease of CAT activity -No changes in SOD -No changes in LPO -Increase of ETS -Decrease of GLY content	Freitas et al. (2020b)
Mytilus galloprovincialis (Seawater)	0.05, 0.5, 5, 50, 100	12 d*	Natural filtered seawater	-Digestive gland -Haemolymph	-Histomorphology (H&E) -THC -Oxidative stress related genes expression	-Haemocyte infiltration -THC raise (85%) -changes in the expression levels of oxidative stress related genes (down-regulation/up-regulation)	Afsa et al. (2022b)
Mytilus galloprovincialis (Seawater)	0.05, 0.5, 5, 50, 100	12 d*	Natural filtered seawater	-Gills -Haemolymph	-Histomorphology (H&E) -Oxidative stress biomarkers (SOD, CAT, GST, MDA) -Neurotoxicity biomarker (AChE)	-Haemocyte infiltrations (intense) -Increase in GST activity -Altered activity of CAT and SOD (increase/decrease) -Increase in MDA at only T3 (no relevant alteration at prolonged exposure) -Inhibition of AChE at prolonged exposure.	De Marco et al. (2022b)
Mytilus spp (seawater) exposed to ASA	0.1, 1, 10, 100	96 h	Artificial seawater	-Gills -Digestive gland	-Energy reserves biomarkers (GLY) -Oxidative stress and metabolism biomarkers (GST, CAT, LPO)	-Increase of GLY level	Piedade et al. (2020)
[CAF+SA]							
Mytilus galloprovincialis (Seawater)	CAF+SA [0.005 + 0.05] [0.05 + 0.5] [0.5 + 5] [5 + 50] [10 + 100]	12 d*	Natural filtered seawater	-Gills -Haemolymph	-Histomorphology (H&E) -THC -Oxidative stress biomarkers (SOD, CAT, GST, MDA) -Neurotoxicity biomarker (AChE)	-Haemocyte infiltration (intense) -THC raise (200%) -Changes in GST activity -Induction of oxidative stress -AChE alterations (induction/ inhibition)	De Marco et al. (2022a)

[a]Concentrations expressed in mM, [b] Concentrations expressed in μM, * The exposure period includes different time of sampling, N.P. = Not precised.
[ASA = acetylsalicylic acid; THC = Total Hemocyte Counts; LMS = Lysosomal Membrane Stability; LYS/CYT = Lysosome to cytoplasm volume ratio; NL = Unsaturated Neutral Lipids; LIF = LIpoFuscin; SOD = Superoxide dismutase; CAT = Catalase; GST= Glutathione *S*-Transferase; GSH = Reduced glutathione; GSSG = Oxidized glutathione; EROD = EtoxyResorufin O-Deethylase; DBF = DiBenzylFluorescein dealkylase; GPX = Glutathione Peroxidase; GR = Glutathione Reductase; DBF = Dibenzylfluorescein Dealkylase; DOP = Dopamine; MAO = MonoAmine Oxidase activity; COX = CycloOXygenase activity; VTG = ViTelloGenin-like proteins; MET = Mitochondrial Electron Transport; AChE = Acetyl CholinEsterase; LPO = Lipid Peroxidation; MDA = Malondialdehyde; RR = Respiration Rate; GLY = Glycogen; PROT = Proteins; ETS = electron transport system; LIP = Lipid].

×150 mm; 5 μm) was used with an isocratic elution in a water-acetonitril-acetic acid mobile phase (1 mL/min) at a wavelength of 275 nm. The calculated linearities were R2 = 0.99985 (0.04, 0.4, 2, 20 μg/L) for CAF, and R2 = 0.99941 (0.5, 5, 50, 100 μg/L) for SA, respectively. The overall recoveries were 97% and 83% for CAF and SA, respectively. Both the drugs were adequately resolved in less than 8 min. Also, the limit of detection (LOD) were of 0.02 ng/mL and 0.1 ng/mL for CAF and SA, respectively. No analytes were disclosed in blank samples.

2.6. Histological analysis

After the fixing step for 4 h in PFA 4% (in 0.1 M phosphate-buffered saline solution, pH 7.4), tissue samples were dehydrated by immersion in a series of increasing concentrations of ethanol. Then, samples were

included in paraffin blocks (Bio-Optica, Milano, Italy) after being cleared with Sub-x. Sections of 5 μm thickness were obtained using a rotary automatic microtome (Leica Microsystems, Wetzlar, Germany). Therefore, rehydrated sections were stained with hematoxylin–eosin (H&E) (Bio-Optica, Milano, Italy), washed and again dehydrated with successive baths of ethanol. The histological structures of DG were observed at 100 × and 400 × through a motorized Zeiss Axio Imager Z1 microscope (Carl Zeiss AG, Werk Göttingen, Germany) equipped with an AxioCam digital camera (Zeiss, Jena, Germany) (Maisano et al., 2017).

2.7. Molecular analysis

2.7.1. Total RNA extraction and retro-transcription

Total RNA was extracted from 50 to 100 mg of *M. galloprovincialis* DG tissue (n = 6 per treatment condition) using TRIzol™ Reagent (Invitrogen, Milano – Italy) following the protocol provided by the manufacturer. The quality and quantity of the isolated RNA were checked respectively by gel electrophoresis (agarose 1%) and spectrophotometry (NanoDrop 2000, Thermo Scientific, USA) (Giannetto et al., 2015). For each sample, 1 μg of total RNA was reverse transcribed into complementary DNA (cDNA), as indicated by Giannetto et al. (2014), using the QuantiTect Reverse Transcription Kit (Qiagen) after elimination of genomic DNA contamination according to the manufacturer's instructions. Thereafter, cDNA samples were kept at − 20 °C.

2.7.2. Quantitative real-time PCR (qPCR)

The transcriptional profile of gene associated with oxidative stress response was determined by qPCR using the QuantiTect SYBR® Green PCR Kit (Qiagen). The qPCR reactions containing ten-fold diluted DG cDNA (50 ng), 1X Rotor-Gene SYBR Green PCR Master Mix, and 1 μM of each gene-specific primers (Listed in Afsa et al., 2022b) in a total volume of 25 μL were run in duplicate in a Rotor-Gene Q 2 plex Hrm thermocycler (Qiagen). In each reaction, two controls were included, no template and minus reverse transcriptase. The most stable reference genes, namely *18 S ribosomal RNA (18 S rRNA)* and *actin (act)*, were selected to calculate the normalization factor (http://medgen.ugent.be/~jvdesomp/genorm/) in order to correct raw data and calculate the relative gene expression (Giannetto et al., 2014). The PCR efficiency (E) was determined using a standard curve from a mixture of RNA, and the specificity of the reaction was confirmed from a melting curve analysis (Giannetto et al., 2015).

2.8. Biochemical analyses

2.8.1. Sample preparation

An amount of 50 mg of mussel DG was homogenized in 10 volumes (w/v) (500 μL) of 100 mM Tris-HCl buffer, pH 7.5, for 5 min at 50 vibrations/s using a Qiagen TissueLyser LT bead mill (3.2 mm stainless steel beads). After this first step, a centrifugation of the homogenized samples was made at 9000 g for 15 min at 4 °C. Supernatants were then collected and stored at − 80 °C for the biochemical determinations. All the analyses were conducted at 25 °C using a Shimadzu UV-1240 spectrophotometer (Maisano et al., 2016). The Pierce BCA Protein Assay Kit (Thermo Scientific) (Bradford, 1976) was used for the calculation of the total protein content.

2.8.2. Biotransformation and antioxidant activity

The assessment of GST activity (nmoL/min/mg protein) was performed following the protocol of Habig et al. (1974), based on the measurement of the absorbance at 340 nm raised by the conjugation of the substrate 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH).

The measurement of the dismutation of H_2O_2 at 240 nm for 90 s following a colorimetric technique (Sureda et al., 2011) was used for the assessment of CAT enzymatic activity (μmoL/min/mg protein).

2.8.3. Lipid peroxidation (LPO)

The thio-barbituric acid reactive substances (TBARS) method (Wills, 1987) was used to estimate the LPO. Briefly, 0.5 mL of mussel DG supernatants was combined to 1 mL of TCA-TBA mixture (15% w/v trichloroacetic acid; 0.375% w/v thio-barbituric acid), and heated in boiling water bath at 90 °C for 15 min. The cooled mixture was measured at 532 nm to quantify the malondialdehyde (MDA) formation. To generate a calibration curve of MDA, the 1,1,3,3-tetraethoxypropane (TEP) was employed, as described by Botsoglou et al. (1994). TBARS content in DG samples was expressed as nmoL of MDA/mg of proteins.

2.9. Integrated biomarker response (IBR)

Data from molecular (*Cu/Zn-sod*, *Mn-sod*, *cat*, *gst*) and biochemical (CAT, GST, LPO) assays for CAF, SA and CAF+SA exposure were merged to gauge the Integrated Biomarker Response index (IBR), following the method initially proposed by Beliaeff and Burgeot (2002), and then improved by Devin et al. (2014). The average IBR values, employed to assess the biomarker responses for each experimental condition (CAF, SA, CAF+SA) were calculated for each concentration and sampling time, by the CALculate IBR Interface (CALIBRI https://liec-univ-lorraine.shinyapps.io/calibri/) developed within the Interdisciplinary Laboratory for Continental Environments (LIEC) of Lorraine University, France.

2.10. Statistical analysis

SigmaPlot software package (Systat software), GraphPad (Prism 7.0, San Diego, CA, USA) and Excel were used to test the significance of accumulation, molecular and biochemical data (p value < 0.05). For molecular data, intergroup concentration and intragroup time-point differences were evaluated using the analysis of variance followed by the Student–Newman–Keuls post-hoc tests. Accumulation and biochemical data were established by analysis of variance (two-way ANOVA) followed by the Tukey's multiple comparison test for different experimental concentrations and exposure time-points. All data were expressed as mean ± standard deviation (SD).

3. Results

It is worthy to note that the results about the exposure of mussels to SA alone described herein come from the same experimental design and were previously published in Afsa et al. (2022). These data are also reported in this work for completeness and comparative purposes among the three experimental groups.

3.1. Accumulation of CAF and SA in mussel DG

Data on accumulation of CAF and SA in the DG of mussels are displayed in the Fig. 1. CAF concentrations (Fig. 1A) were statistically significant ($p < 0.05$) and enhanced in specimens exposed to the two highest CAF dosages (C4; C5) at 5 (T5) and 12 (T12) days of exposure, while the CAF levels in samples exposed to C3 were relevant raised at T12. The highest concentrations (3.8 ± 0.160 μg/g; 4.3 ± 0.25 μg/g) were recorded in the DG of mussels exposed to C4 and C5, respectively, at T12.

With regards to mussels exposed to SA (Fig. 1B), a significant enhancement in its level was noted for all the tested concentrations (C1 to C5) at each exposure time (T3,T5, T12). A time-dependent rise in SA level was observed in mussels exposed to C5, that exhibited the highest recorded concentration of SA at T12 (73.98 ± 3.49 μg/g).

In the combined exposure of CAF+SA (Fig. 1C, 1D), the level of CAF (Fig. 1C) was statistically significantly ($p < 0.05$) increased in mussels exposed to the two highest dosages (C4; C5) at T5 and T12, as well as in mussels exposed to C3 at T5. However, the highest CAF concentration (6.7 ± 0.730 μg/g) was recorded in mussel DG exposed to C5 at T5. In mussels from the same combined exposure, the levels of SA (Fig. 1D)

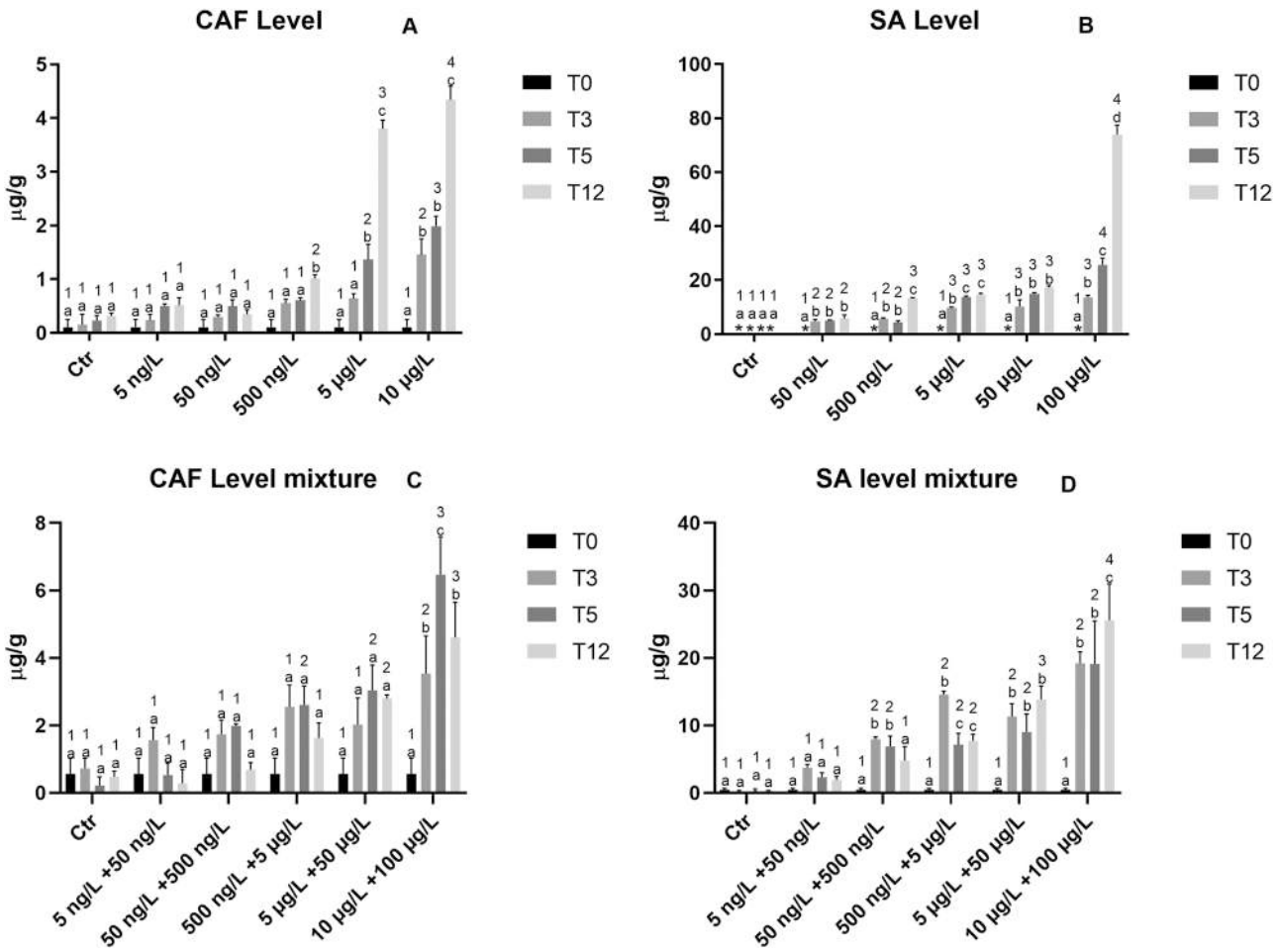

Fig. 1. Caffeine (CAF) and salicylic acid (SA) level (µg/g) in digestive gland of mussel *M. galloprovincialis* from control group (Ctrl) and exposed to different concentration of (A) CAF (C1: 5 ng/L; C2: 50 ng/L; C3: 0.5 µg/L; C4: 5 µg/L; C5: 10 µg/L), (B) SA (C1: 0.05 µg/L; C2: 0.5 µg/L; C3: 5 µg/L; C4: 50 µg/L; C5: 100 µg/L) and (C, D) CAF+SA (C1: 5 ng/L+0.05 µg/L; C2: 50 ng/L+0.5 µg/L; C3: 0.5 µg/L+5 µg/L; C4: 5 µg/L+50 µg/L; C5: 10 µg/L+100 µg/L) at 0 (T0), 3 (T3), 5 (T5) and 12 (T12) days of exposure. Results are shown as means ± SD. Different letters above each column denote significant difference among different exposure times within the same concentration of CAF or SA ($p < 0.05$). Different numbers above each column denote significant difference among different concentrations of CAF or SA ($p < 0.05$). Columns marked with asterisks (*) represent values lower than the detection limit (LOD = 0.02 ng/mL for CAF; 0.1 ng/mL for SA).

were raised in DG exposed to all dosages from C2 to C5 at each time-point (T3, T5, T12), except for C2 at T12. However, the highest concentration of SA was recorded in the DG of mussels exposed to C5 at T12 (27.57 ± 3.98 µg/g).

3.2. Histological observations

Qualitative histological examination of the DG of *M. galloprovincialis* showed the typical organization of the digestive tissue of marine bivalves. Noteworthy, the 12-days exposure to different concentrations of CAF alone (Fig. 2), SA alone (Afsa et al., 2022), and their combination CAF+SA (Fig. 3) did not induce morphological abnormalities in the digestive diverticula. Nevertheless, a haemocyte infiltration was observed among the digestive tubules of all individuals under CAF, SA and CAF+SA treatments.

In regard to CAF exposure, at both T3 (Figs. 2B, 2C) and T5 (Figs. 2D, 2F) the haemocyte distribution in mussel DG appeared to be uniform at all the tested doses. At the end of the exposure (T12), although an important reduction in the haemocyte occurrence was noticed at C1 (Fig. 2G), a remarkable presence of haemocytes was observed at high dosages (Figs. 2H, 2I).

For SA exposure, at T3 and T5 the haemocyte infiltration was more extended at the highest tested concentration C5. Interestingly, at the end of the exposure (T12), a slight reduction of haemocytes was observed in mussel DG, in a dose-dependent manner (Afsa et al., 2022).

Furthermore, three days following the beginning of the exposure to the mixture CAF+SA (T3), a higher inductive effect was observed among mussel DG at the augmented dosage C5, elucidating a dose-dependent trend (Figs. 3B, 3 C). At T5 and T12, a remarkable stronger effect, to some extent, was found at C3 (Figs. 3E, 3H) compared to C1 and C5.

3.3. Effects on gene expression

The effect of CAF and SA, individually and in combination (CAF+SA) on oxidative stress related genes expression was studied using RT-qPCR. The mRNA levels of *Cu/Zn-sod*, *Mn-sod*, *cat* and *gst* were determined at different time-points after exposure to a range of concentrations of the selected active compounds.

3.3.1. Effects on sod relative gene expression

For *Cu/Zn-sod,* at the end of the treatment with CAF (T12), a significant increase in the relative expression level was observed in the DG of mussels at all the tested dosages compared to control. Noteworthy, the obtained results for *Cu/Zn-sod* gene appear to follow a dose-dependent pattern at T12 except for the highest concentration C5, when a slight non-significant decrease in the relative gene expression was noticed compared to C4 treated group. Additionally, a significant time-dependent profile was observed when mussels were exposed to the

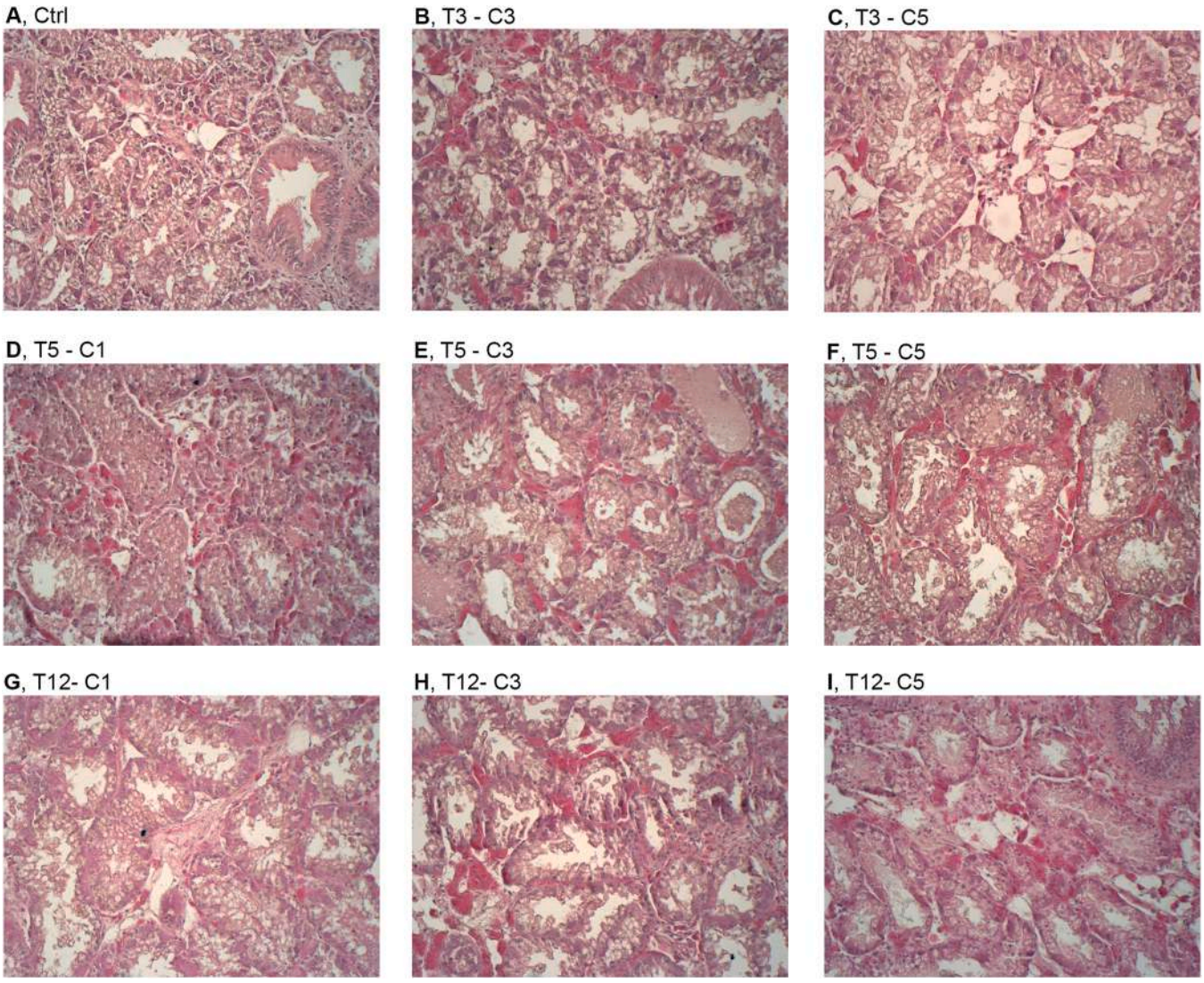

Fig. 2. Hematoxylin and Eosin (H&E) staining of digestive gland (DG) of mussel *Mytilus galloprovincialis* exposed to CAF (0.005 μg/L (C1), 0.05 μg/L (C2), 0.5 μg/L (C3), 5 μg/L (C4), 10 μg/L (C5), or without CAF (Ctrl) as negative control) for 0 (T0), 3 (T3), 5 (T5) and 12 (T12) days of exposure. (A) Representative histological organization of digestive tissue in control mussels. (B-C) Representative histological organization of digestive glands after three days of CAF exposure (T3) showing important haemocytic infiltration at an intermediate CAF dosage (C3) and occurring up to the highest CAF concentrations (C5). (D-F) Representative images of mussel DG exposed to CAF for five days (T5) with an uniform presence of haemocytes among mussel digestive tubules at all the tested doses. (G-I) Representative images of mussels exposed to CAF for 12 days (T12) showing a reduction in the number of haemocytes at the lowest dosage (C1) and a remarkable presence of haemocytes at high dosages. Images were taken at 40x.

three highest concentrations (Fig. 4a). Regarding the gene encoding for the mitochondrial SOD (*Mn-sod*), exposed mussels were significantly responsive only to the highest CAF concentration (C5 = 10 μg/L) at only T12, as clearly depicted in Fig. 4b.

When treated with SA, a different response compared to CAF, was observed for both gene isoforms encoding for SOD. Interestingly, the exposure to SA induced a significant decrease in the transcriptional levels of both targeted genes, in a dose-dependent manner. This profile was observed when mussels were exposed to the three highest SA concentrations (C3, C4 and C5) at T3 and T5. By the end of the exposure (T12), challenged individuals were only responsive to the highest concentration (C5), where a down-regulation of *sod* genes was observed, with a greater extent, in respect to control (Afsa et al., 2022).

Interestingly, mussels exposed to CAF+SA mixture exhibited a different response in the DG tissue compared to those treated with CAF alone. Indeed, the here obtained results clearly indicate a significant decrease in the mRNA levels of *Cu/Zn-sod* gene only at the end of the exposure (T12) when mussels were exposed to the four highest concentrations, namely C2, C3, C4 and C5 (Fig. 5a). For *Mn-sod* gene, a down-regulation of its expression was only observed at C4 and C5 at T12, whereas no significant difference was observed under the other experimental conditions with respect to control group (Fig. 5b).

3.3.2. *Effects on cat relative gene expression*

As shown in Fig. 4c, all tested concentrations of CAF induced a significant increase in *cat* relative gene expression after 5 days and 12 days of the exposure in comparison with control organisms. At T12, a remarkable dose-dependent effect was noticed among all the tested conditions. However, at T5, the gene expression levels appear to follow a dose-dependent trend at only C1, C2 and C3 and there were no significant differences between groups treated with the three highest concentrations. Additionally, a clear and significant time-depending increase of *cat* transcript was found when mussels were exposed to 10 μg/L.

In regard to SA, the obtained results for *cat* gene were similar to those related to SOD encoding genes when exposing mussels to SA alone.

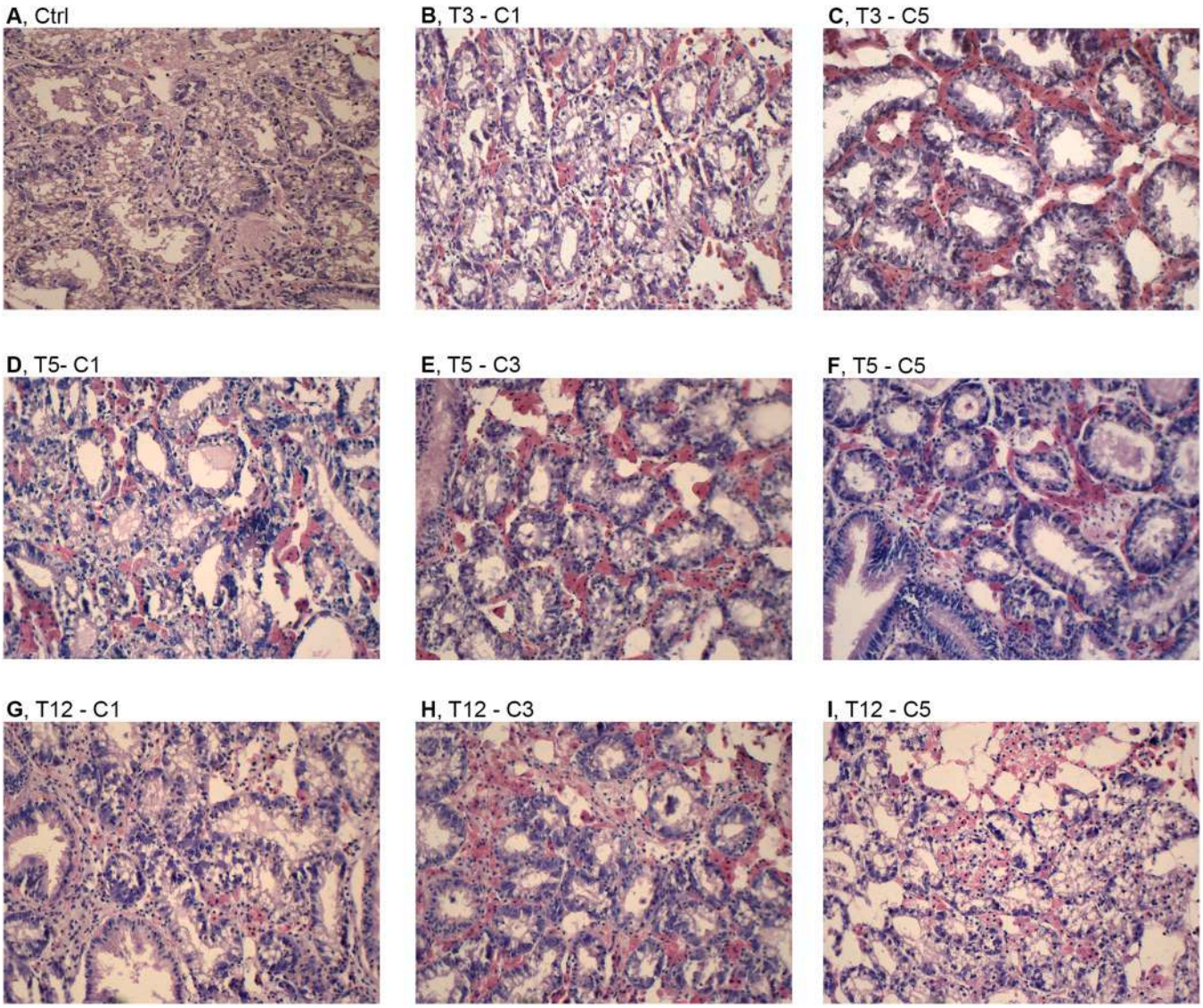

Fig. 3. Hematoxylin and Eosin (H&E) staining of digestive gland (DG) of mussel *Mytilus galloprovincialis* exposed to CAF+SA mixture (0.005 µg/L+0.05 µg/L (C1), 0.05 µg/L+ 0.5 µg/L (C2), 0.5 µg/L+ 5 µg/L (C3), 5 µg/L+ 50 µg/L (C4), 10 µg/L+ 100 µg/L (C5), or without CAF+SA (Ctrl) as negative control) for 0 (T0), 3 (T3), 5 (T5) and 12 (T12) days of exposure. (A) Representative histological organization of digestive tissue in control mussels. (B-C) Representative histological organization of digestive glands after three days of CAF+SA exposure (T3) showing an increasing dose-dependent haemocytic infiltration among mussel digestive tubules. (D-F) Representative images of mussel DG exposed to CAF+SA for five days (T5) with a greater presence of haemocytes at the intermediate dosage (C3) compared to the lower and higher concentrations. (G-I) Representative images of mussels exposed to CAF+SA for 12 days (T12) showing a greater presence of haemocytes at the intermediate dosage (C3) compared to the lower and higher concentrations. Images were taken at 40x.

Likewise, a significant down-regulation was found in DG of mussels exposed to the three highest dosages, namely at T3 and T5. At the end of the experiment, a significant decrease of *cat* transcript levels was observed at C2, C3, C4 and C5 compared to control with a greater extent for C5 treated group (Afsa et al., 2022).

The exposure to the mixture of CAF+SA initially provoked a significant increase of the relative gene expression of *cat* compared to control group, namely when mussels were exposed to the three highest concentrations at T3. However, this profile did not persist for the entire period of exposure. Indeed, after five days of the exposure, the transient up-regulation was followed by a slight significant decrease in mRNA levels of *cat* when test organisms were exposed to C2, C3, C4 and C5 with respect to control. Interestingly, at the end of the experiment, the exposure to the mixture of CAF+SA did not induce any change in the relative gene expression of *cat* relative to control group, except for mussels treated with C2 (Fig. 5c).

3.3.3. *Effects on gst relative gene expression*

The exposure to CAF induced a significant increase in *gst* mRNA levels after 5 and 12 days of the exposure when compared to control group. As indicated in Fig. 4d, the up-regulation of the *gst* gene was observed when tested individuals were exposed to the three highest concentrations of CAF, namely 0.5 µg/L, 5 µg/L and 10 µg/L. The observed effect of CAF appears to follow a time-dependent trend when mussels were exposed to C3, C4 and C5 in respect with those collected at T0. Interestingly, both at T5 and T12, a slight down-regulation was detected at the highest dosage (C5), with a significant difference when compared to only C4 treated group.

Mussels exposed to SA individually showed a significant rise in *gst* mRNA levels when compared to control. This response was observed at the three highest concentrations of SA (C3, C4 and C5) after 3, 5 and 12 days from the beginning of the treatment (Afsa et al., 2022).

Following the exposure to the mixture of CAF+SA, an up-regulation of *gst* gene expression was observed in mussel DG tissues during the first

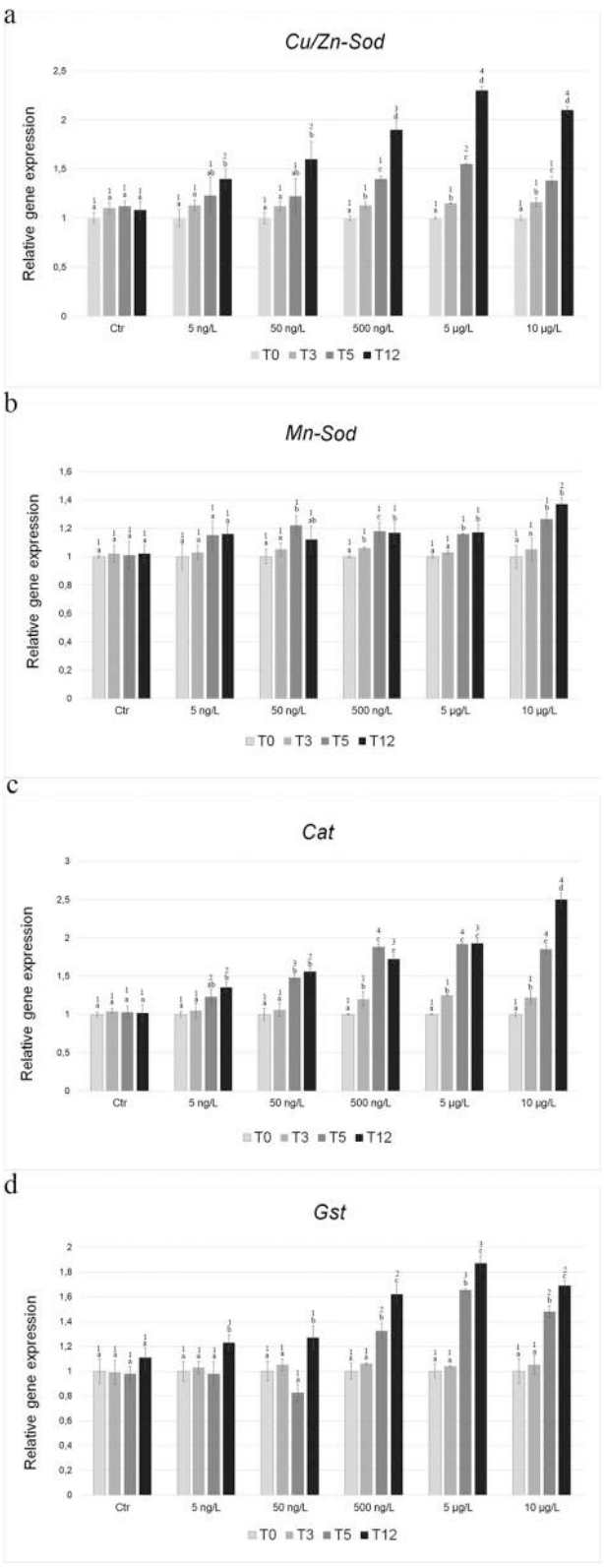

Fig. 4. Gene expression analysis of (a) Cu/Zn-Sod, (b) Mn-Sod, (c) Cat and (d) Gst in digestive gland (DG) of mussel *M. galloprovincialis* exposed to CAF (0.005 µg/L (C1), 0.05 µg/L (C2), 0.5 µg/L (C3), 5 µg/L (C4), 10 µg/L (C5), or without CAF (Ctr) as negative control). Transcript levels at 0 (T0), 3 (T3), 5 (T5) and 12 (T12) days of exposure were evaluated by qPCR. Results are shown as means ± SD. Different letters above each column denoted significant difference among different exposure times within the same concentration of CAF ($p < 0.05$). Different numbers above each column denoted significant difference among different concentrations of CAF within the same time ($p < 0.05$).

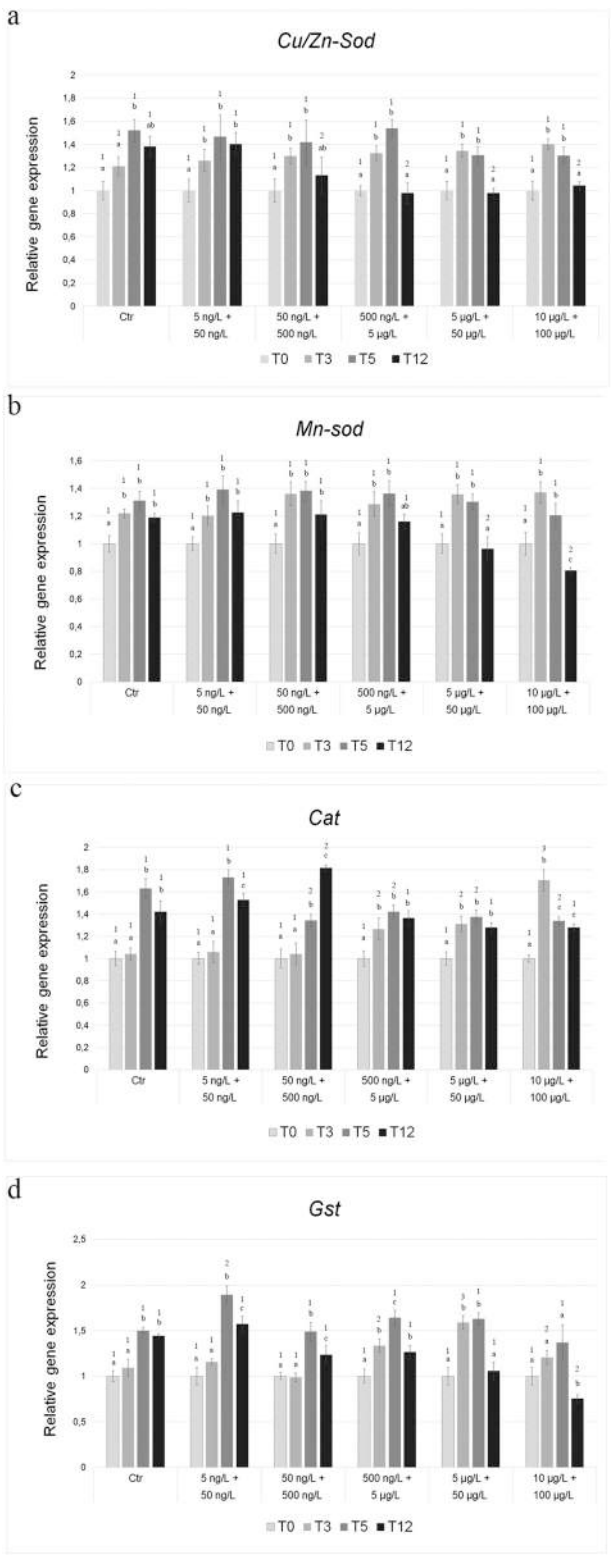

Fig. 5. Gene expression analysis of (a) Cu/Zn-Sod, (b) Mn-Sod, (c) Cat and (d) Gst in digestive gland (DG) of mussel *M. galloprovincialis* exposed to CAF+SA mixture (0.005 µg/L+0.05 µg/L (C1), 0.05 µg/L+ 0.5 µg/L (C2), 0.5 µg/L+ 5 µg/L (C3), 5 µg/L+ 50 µg/L (C4), 10 µg/L+ 100 µg/L (C5), or without CAF+SA (Ctrl) as negative control). Transcript levels at 0 (T0), 3 (T3), 5 (T5) and 12 (T12) days of exposure were evaluated by qPCR. Results are shown as means ± SD. Different letters above each column denoted significant difference among different exposure times within the same concentration of CAF ($p < 0.05$). Different numbers above each column denoted significant difference among different concentrations of CAF within the same time ($p < 0.05$).

days of the exposure (T3) with respect to control group. In details, this behaviour was observed when mussels were treated with the three highest concentrations of the combination C3, C4 and C5 (Fig. 5d). At T5, apart an increase detected at C1, no significant changes were observed for GST encoding gene mRNA transcript under the other treatment conditions. At the end of the exposure (T12), a significant down-regulation was observed, compared to control, when mussels were treated with the highest concentration (C5). Noteworthy, a slight non-significant decrease in the fold induction was also observed at C2, C3 and C4 test conditions.

3.4. Effects on biotransformation and antioxidant activities

3.4.1. Effects on GST activity

As observed in Fig. 6A, CAF exposure induced a significant increase of GST activity in DG tissues of challenged mussels ($p < 0.05$) at the two highest (C4; C5) concentrations after 5 and 12 days (T5; T12).

After SA exposure, a significant increase ($p < 0.05$) in GST activity, as displayed in Fig. 6B, was observed in the DG of mussels exposed to the highest concentrations (C4; C5) during the first exposure phases (T3; T5), whereas after 12 days (T12) no significant change was observed.

The combined SA+CAF exposure (Fig. 6C) displayed a significant enhancement ($p < 0.05$) of GST activity in the DG of specimens exposed to the highest combined concentrations (C4; C5) at T5, followed by a significant drop ($p < 0.05$) of GST activity in mussels exposed to the same concentrations (C4; C5) after 12 days of exposure (T12).

3.4.2. Effects on CAT activity

The exposure to CAF (Fig. 6D) revealed a significant increase ($p < 0.05$) in CAT in the DG of mussels exposed to the four highest concentrations (C2 to C5) at T5 and T12. The highest dosage (C5) provoked significant effects ($p < 0.05$) after 3 days of the exposure (T3).

Contrariwise, the effect of SA exposure, observable in Fig. 6E, revealed a significant decrease ($p < 0.05$) of CAT activity in the DG of individuals exposed to the three highest concentrations (C3, C4, C5) at T5 and T12. Regarding the combined exposure (SA+CAF) (Fig. 6F), at T3 a significant increase ($p < 0.05$) in CAT was observed in mussels exposed to the experimental concentrations C3 and C4. At T5 a significant drop ($p < 0.05$) was observed for the two highest concentrations (C4; C5), combined with a significant increase ($p < 0.05$) for the C2 dosage. At T12, no significant changes were observed for any experimental concentration.

3.4.3. Effects on LPO

CAF exposure (Fig. 7A) elicited a significant augmentation ($p < 0.05$) in LPO levels within the DG of mussels exposed to all experimental concentrations (C1 excluded) after 12 days of exposure (T12). No relevant alteration was observed during SA and SA+CAF exposure (Figs. 7B, 7 C).

3.5. Integrated biomarker response (IBR)

The results of the IBR are shown in Fig. 8 and Table 3. With regard to CAF (Figs. 8A, 8B, 8C), a dose-dependent induction effect is particularly marked at T5 for all the concentrations tested (Fig. 8B, Table 3) and T12 (Fig. 8C) for C2-C5 concentrations.

Concerning SA (Figs. 8D, 8E, 8F), apart from GST and *gst* responses, a slight inhibition trend was detected for the other biomarkers, particularly for the two highest concentrations (C4; C5) at T12, and highlighted by the IBR data (Table 3).

In the combined exposure (CAF+SA) (Figs. 8G, 8H, 8I), a general rise of the chosen biomarkers was recorded at T3 (Fig. 8G), while at T5 (Fig. 8H) and particularly at T12 (Fig. 8I), a reduction at the two highest concentrations (C4; C5) was revealed (Table 3).

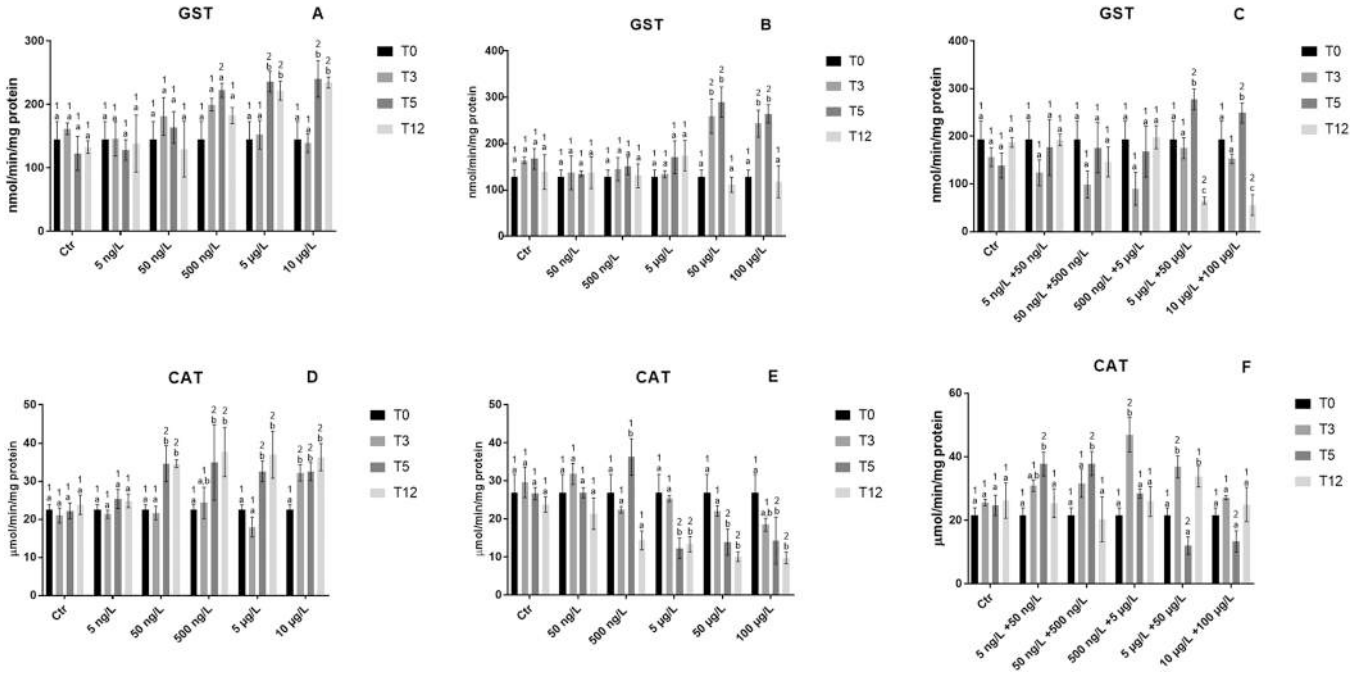

Fig. 6. Activity of enzymes glutathione *S*-transferase (GST) and catalase (CAT) in digestive gland of mussel *M. galloprovincialis* from control group (Ctrl) and exposed to different concentration of caffeine (CAF; C1: 5 ng/L; C2: 50 ng/L; C3: 0.5 µg/L; C4: 5 µg/L; C5: 10 µg/L), salicylic acid (SA; C1: 0.05 µg/L; C2: 0.5 µg/L; C3: 5 µg/L; C4: 50 µg/L; C5: 100 µg/L), and CAF+SA (C1: 5 ng/L+0.05 µg/L; C2: 50 ng/L+0.5 µg/L; C3: 0.5 µg/L+5 µg/L; C4: 5 µg/L+50 µg/L; C5: 10 µg/L+100 µg/L) at 0 (T0), 3 (T3), 5 (T5) and 12 (T12) days of exposure. The graphs refer to the activities of GST (nmol/min/mg protein) in mussels exposed to (A) CAF, (B) SA, and (C) CAF+SA, and to the activities of CAT (µmol/min/mg protein) in mussels exposed to (D) CAF, (E) SA, and (F) CAF+SA. Results are shown as means ± SD. Different letters above each column denote significant difference among different exposure times within the same concentration of CAF, SA or CAF+SA ($p < 0.05$). Different numbers above each column denote significant difference among different concentrations of CAF, SA or CAF+SA within the same time-point ($p < 0.05$).

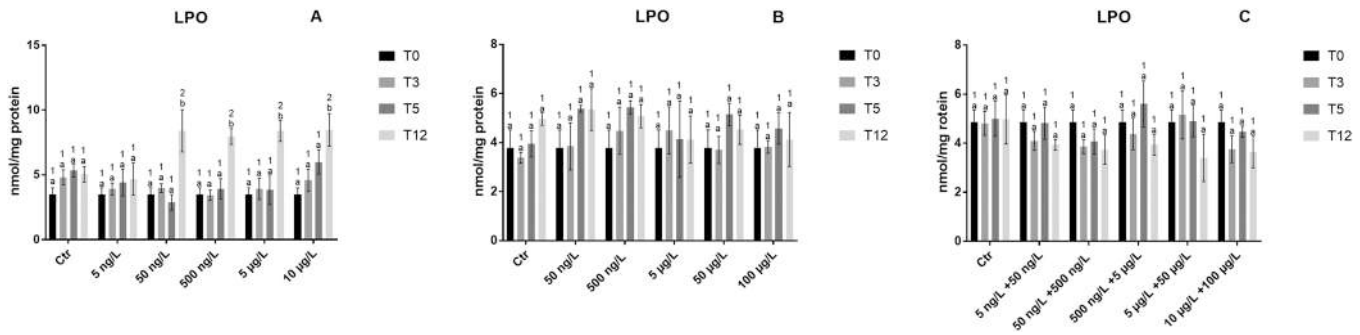

Fig. 7. Assessment of lipid peroxidation (LPO) in digestive gland of mussels from control group (Ctrl) and exposed to different concentration of caffeine (CAF; C1: 5 ng/L; C2: 50 ng/L; C3: 0.5 µg/L; C4: 5 µg/L; C5: 10 µg/L), salicylic acid (SA, C1: 0.05 µg/L; C2: 0.5 µg/L; C3: 5 µg/L; C4: 50 µg/L; C5: 100 µg/L), and CAF+SA (C1: 5 ng/L+0.05 µg/L; C2: 50 ng/L+0.5 µg/L; C3: 0.5 µg/L+5 µg/L; C4: 5 µg/L+50 µg/L; C5: 10 µg/L+100 µg/L) at 0 (T0), 3 (T3), 5 (T5) and 12 (T12) days of exposure. The graphs refer to level of LPO assessed by malondialdehyde (MDA) assay (nmol/mg protein) in mussels exposed to (A) CAF, (B) SA, and (C) CAF+SA. Results are shown as means ± SD. Different letters above each column denote significant difference among different exposure times within the same concentration of CAF, SA, or CAF+SA ($p < 0.05$). Different numbers above each column denote significant difference among different concentrations of CAF, SA, or CAF+SA within the same time-point ($p < 0.05$).

4. Discussion

Aquatic organisms are living under a constantly changing environment. Human-made chemicals such as endocrine-disrupting compounds, pesticides, polycyclic aromatic hydrocarbons, pharmaceuticals, nano and microplastics are ones of the most studied stressors during the last decades (Maisano et al., 2017; Ranjbar Jafarabadi et al., 2018; Pereira et al., 2019; Cappello et al., 2021; Jebara et al., 2021; Missawi et al., 2021; Parrino et al., 2021a; Abouda et al., 2022; Afsa et al., 2022b; Zitouni et al., 2022). Whatever the type of stress factors, adaptive defensive responses are required to survive. Oxidative stress response pathway is one of the most activated pathways under stress conditions. Oxidative stress is defined as an imbalance between reactive oxygen species (ROS) and antioxidant defense system that could lead to either adaptive consequences or to prevailing oxidizing conditions resulting in toxic outcomes, under specific circumstances (Lushchak, 2016). Although several pharmaceuticals are known to induce an enzymatic imbalance mainly through their oxidizing metabolism (Martín-Díaz et al., 2009; Aguirre-Martínez et al., 2018; Trombini et al., 2019; Nogueira and Nunes, 2021), the assessment of gene expression biomarkers in response to pharmaceutical pollutants is, to date, in its infancy, yet. Hence, in the present study molecular biomarkers were combined with traditional biochemical assays in order to better elucidate the time- and dose-dependent biological effects triggered by emerging pollutants like pharmaceuticals (CAF and SA, alone and in mixture) in mussels in the early phase of exposure, thus before the effects became costly to remedy.

4.1. Accumulation of CAF and SA in mussel DG

The current data on CAF and SA levels in the DG of *M. galloprovincialis* during single and combined exposure confirm the ability of mussels to accumulate PhACs in their tissues (Mezzelani et al., 2020). Regarding CAF, it is interesting to observe its occurrence, albeit in moderate amounts, even in control samples collected from a monitored area such as a mussel farm, as also herein reported. This data emphasises the capacity of this substance to be easily absorbed by non-target organisms and to be thus transferred along the food chain (Li et al., 2020). In particular, the data hereby provided reveal how CAF concentrations above 5 µg/L are capable of accumulating significantly in mussel DG even after 5 days of exposure, unlike concentrations in the ng/L range (Capolupo et al., 2016), thus confirming the relevance of monitoring environmental levels of this compound (De Marco et al., 2022a).

Similarly, also SA revealed to be an easily absorbable PhACs in mussel DG within a few days of exposure. The SA levels herein recorded in the range of µg/g appear to be higher than those reported in other works, like Freitas et al. (2019). A possible explanation is likely related to the organ chosen for investigation. Indeed, the DG is a key organ with regards to the biotransformation of xenobiotics (Piedade et al., 2020), able to easily internalize several kinds of pollutants (Cappello et a, 2021; Beyer et al., 2017) at concentrations in the µg/g range (Chandurvelan et al., 2015).

An unexpected absorption trend was observed in the combined (CAF+SA) than in the single exposures. In fact, while CAF levels in the combined exposure were found to be higher than in single exposure, exactly the opposite trend was observed in the case of SA exposure. A comparable scenario was reported in Mezzelani et al. (2023) in mussels treated with a mixture of carbamazepine and valsartan. One possible explication for these modulations in terms of uptake of PhACs could be linked to a possible mutually competitive effect of PhACs among the various major biotransformation (i.e., cytochrome P450) and drug transporter (i.e., ATP binding cassette) systems (Mezzelani and Regoli, 2022). Thus, it is conceivable to consider not only potential competing processes but also interactions among the various PhACs, capable of modulating the observable responses in organisms.

4.2. Individual and combine effects of CAF and SA on DG histomorphology

In bivalves, DG plays an important role not only in digestion functions but also in the metabolism and detoxification of several xenobiotics that preferentially accumulate in this organ (Cappello et al., 2018), sometimes inducing profound histological changes (Zhang et al., 2020). Thus, DG was targeted in this study to better understand the effect of two well-known pharmaceuticals at different levels of biological organization. When examining DG tissue, the exposure to CAF and SA, alone and in combination, revealed an induction of haemocyte infiltration among the digestive tubules of all individuals under the different treatments. In bivalve molluscs, haemocytes are involved in several physiological and defensive functions such as nutrient transport, storage and digestion, immunosurveillance of tissues and detoxification process through their developed lysosomal system (De Vico and Carella, 2012; Carella et al., 2015; Parrino et al., 2021b). Therefore, the high occurrence of haemocytes in DG tissue may play a pivotal role against oxidative stress generated following the exposure, favouring a protection against the prooxidant effects of the tested compounds, as confirmed by the absence of histo-morphological disturbance and tissue damage in this study.

Previous studies have demonstrated that pharmaceuticals can markedly impair the haemostasis of haemocytes and the

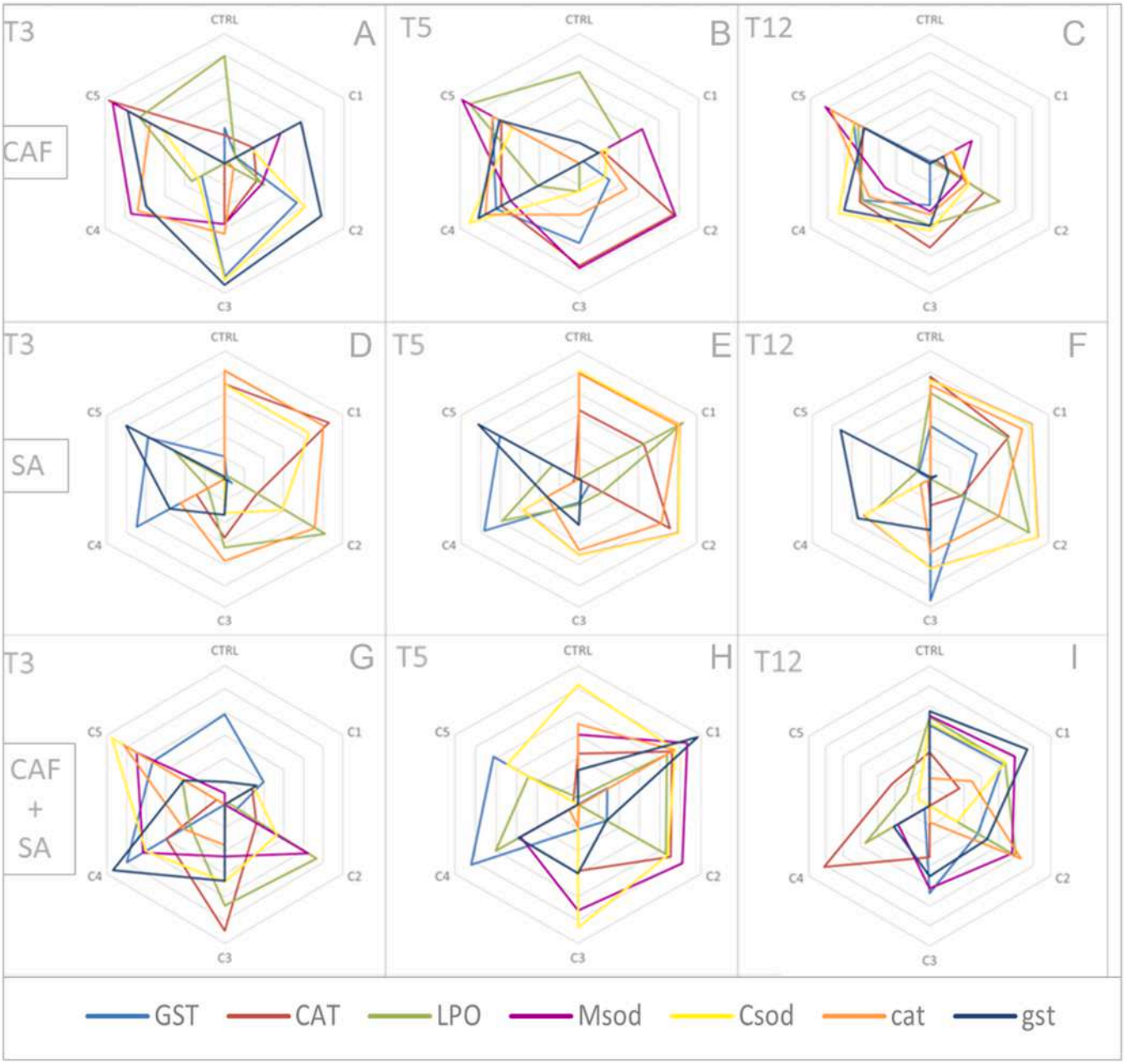

Fig. 8. Star plots for biomarker responses in digestive gland of mussel *M. galloprovincialis* exposed to caffeine (CAF), salicylic acid (SA) and to CAF+SA. The graphs refer to molecular and biochemical biomarker responses in mussels exposed to different concentrations (C1 to C5) of CAF, SA or CAF+SA at 3 (T3; A, D, G), 5 (T5; B, E, H) and 12 (T12; C, F, I) days of exposure.

Table 3
IBR value in the digestive gland of mussel *M. galloprovincialis* exposed to different concentrations of caffeine (CAF), salicylic acid (SA) and caffeine and salicylic acid in mixture (CAF+SA) at 3 (T3), 5 (T5) and 12 (T12) days of exposure.

IBR							
CAF		**Ctr**	**5 ng/L**	**50 ng/L**	**500 ng/L**	**5 µg/L**	**10 µg/L**
	T3	0.55	1.43	4.51	8.37	3.76	10.15
	T5	0.13	1.33	2.65	8.45	11.59	13.64
	T12	0.05	0.58	2.74	6.96	11.35	15.01
SA		**Ctr**	**50 ng/L**	**500 ng/L**	**5 µg/L**	**50 µg/L**	**100 µg/L**
	T3	4.85	4.58	3.98	2.83	1.68	1.37
	T5	4.66	7.34	5.53	2.93	4.37	1.14
	T12	4.77	4.92	3.81	3.3	1.32	0.11
CAF + SA		**Ctr**	**5 ng/L + 50 ng/L**	**50 ng/L + 500 ng/L**	**500 ng/L + 5 µg/L**	**5 µg/L + 50 µg/L**	**10 µg/L + 100 µg/L**
	T3	4.39	4.76	6.44	9.74	13.21	11.72
	T5	4.19	17.85	7.84	5.47	3.87	1.54
	T12	4.18	4.73	3.07	1.33	1.01	0.08

histomorphology of mussels (Munari et al., 2014; Afsa et al., 2022b; Guo et al., 2022; De Marco et al., 2022a; De Marco et al., 2022b). Munari et al. (2020) found that CAF at concentrations of 0.05 and 0.5 μg/L was effective to reduce the total haemocyte count (THC) in the haemolymph of *M. galloprovincialis* exposed for 21 days. The decrease in THC is the result of either the increased movement of cells from haemolymph into tissues or cell lysis (Pipe and Coles, 1995). De Marco et al. (2022a) documented that CAF at 5 ng/L was able to induce a moderate haemocyte infiltration in the gills of *M. galloprovincialis* only after 12 days of exposure. An intense haemocyte infiltration was instead observed in gills of mussels exposed to different dosages of SA (De Marco et al., 2022b), whereas the mixture of CAF+SA provoked a massive haemocyte infiltration in mussel gills and a raise in THC (up to 200% compared to control) in haemolymph exposed to the highest PhAC concentrations (De Marco et al., 2022a).

Overall, the recruitment of haemocytes in the DGs of exposed mussels could be attributed to occurrence of oxidative unbalance and it might be related to the ability of some contaminants including pharmaceuticals to stimulate the mitotic activity of haemocytes (Matozzo et al., 2008), to protecting tissue structure and function.

4.3. Effects of CAF on mussel DG

4.3.1. Effects of CAF on oxidative stress

To better understand the effects of the tested compounds on DG of exposed mussels, the histological observation was followed by molecular and biochemical analyses. The exposure to CAF showed a significant up-regulation of different genes related to oxidative stress defence system, namely *Cu/Zn-sod*, *Mn-sod*, *cat* and *gst*, associated to a significant rise of the antioxidant and biotransformation enzyme activities, GST and CAT, as well as LPO. The selected genes encode for antioxidant enzymes that plays a pivotal role in detoxifying ROS and other pro-oxidants, thereby maintaining the cellular equilibrium between generation and removal of ROS, which preserves the cellular integrity (Lushchak, 2011; Regoli and Giuliani, 2014; Lushchak, 2016). Thus, the observed molecular and biochemical up-regulation, mainly after 5 and 12 days of the exposure, may be correlated to an elevated requirement for antioxidants in cells to rapidly detoxify the oxidizing species that may be formed following the exposure to CAF.

The ability of CAF to induce oxidative stress has been well documented in both saltwater and freshwater bivalve species like *M. galloprovincialis* (De Marco et al., 2022a), *Ruditapes philippinarum* (Aguirre-Martínez et al., 2016; Cruz et al., 2016), *Corbicula fluminea* (Aguirre-Martínez et al., 2015) and *Elliptio complanata* (Martín-Díaz et al., 2009). In these organisms, the induction of oxidative stress has been justified parallelly by the increment of antioxidant activities and the raise of lipid peroxide levels after short (from 48 h) and long termed exposure (up to 28 days). In addition, it has been reported that CAF generates ROS during its degradation by xanthine oxidation metabolic pathway (Gagné et al., 2007; Nunes et al., 2022), which may lead in part to the oxidative stress as reported herein.

The mRNA levels of *Cu/Zn-sod* and *Mn-sod* were herein only increased at T12 (except C4 at T5). These results corroborate partially with those reported by De Marco et al. (2022a), in which CAF was found to induce an increase of SOD activity in gills of *M. galloprovincialis* after 3, 5 and 12 days. In this study, the apparent lack of effect at T3 and T5 may be attributed to the effectiveness of other antioxidant and detoxicant pathways, It is plausible that the SOD pathway in the DG is activated following a more prolonged stress in respect, for instance, to a "first tissue exposure" like the gills (Lutz, 2004). The results herein provided are also in line with Cruz et al. (2016), whose findings indicate the activation of SOD in *R. philippinarum* after a long-termed exposure to 0.5–18 μg/L of CAF.

Despite the similar biochemical role of their proteinic products, *Cu/Zn-sod* and *Mn-sod* gene respond differently to CAF exposure. Indeed, all the herein tested concentrations (C1-C5) showed an inductive effect for the cytosolic SOD related gene (*Cu/Zn-sod*), whilst solely the highest concentration C5 induced an up-regulation of the gene encoding for the mitochondrial SOD (*Mn-sod*). As well, a higher fold induction was observed for *Cu/Zn-sod* than *Mn-sod* in treated organisms compared with control. This pattern is likely due to the higher sensitivity of cytosolic SOD (activated at 5 ng/L) to pharmaceuticals compared to mitochondrial SOD. Thus, cytosolic SOD is probably the enzyme isoform playing the most important role (Kim et al., 2015). Interestingly, our findings are consistent with previous researches reporting that *Cu/Zn-sod* was found to be induced to a higher extent than *Mn-sod* in vertebrates and invertebrates such as polychaetes (Rhee et al., 2011), copepods (Kim et al., 2011), pufferfish (Kim et al., 2010), the rotifer *Brachionus koreanus* (Kim et al., 2015), the bivalve *M. galloprovincialis* (Giannetto et al., 2017) and the ciliate *Pseudocohnilembus persalinus* (Wang et al., 2017), in response to several stressors including pharmaceuticals (e.g., oxytetracycline).

In regard to CAT, CAF exposure provoked an increase in mRNA levels and enzyme activity at both T5 and T12 with respect to control group. Our findings are in agreement with those reported by De Marco et al. (2022a) and Cruz et al. (2016) in marine bivalves on the induction of CAT activity at early stages of exposure (3 d) and at prolonged exposure (28 d), emphasising its role in reducing oxidant species, mainly H_2O_2. A greater effect both in terms of CAT gene expression and enzymatic activity obviously occurred after a prolonged exposure to CAF, suggesting its persistence in mussel DG and a stronger impact at elevated concentrations, as elucidated by a gradual up-regulation at T12. Likewise, a higher activity of CAT was recorded in *R. philippinarum* (Cruz et al., 2016) and in the polychaete *Hediste diversicolor* (Pires et al., 2016) after being exposed to CAF (up to 18 μg/L) for 28 d (Cruz et al., 2016).

4.3.2. Effects of CAF on biotransformation

The activity of GST and the transcript levels of *gst* were significantly up-regulated after 5 and 12 days following the exposure to the three highest dosages of CAF (C3, C4 and C5). GSTs isoenzymes are involved in the phase II detoxification pathway (Habig et al., 1974). and act also as antioxidants in tissues (Manduzio et al., 2004; Trute et al., 2007; Birben et al., 2012). Hence, changes in GSTs denote the presence of metabolic and antioxidant disorder in response to chemical stress (Halliwell and Gutteridge, 2015). The increases in *gst* fold induction and GST activity suggests an additional challenge to the metabolism of individuals exposed to at higher CAF dosages to detoxify CAF toxic metabolites and remove resulting ROS. The activation of GSTs in response to different pharmaceuticals including CAF was recently documented (Lin et al., 2014; Aguirre-Martínez et al., 2015; Oliveira et al., 2015; Nunes et al., 2020). In marine bivalves, Capolupo et al. (2016) reported an increment of GST activity in DG rather than in gills of *M. galloprovincialis* exposed to 0.05 and 0.5 μg/L of CAF for 7 days. This biological trend, along with the results of De Marco et al. (2022a), are in line with those herein reported, arguing a higher involvement of this enzyme in DG than in gills for the detoxification processes of this PhAC. Inductive effects of CAF were also reported in the DG of *R. philippinarum* (Aguirre-Martínez et al., 2016; Cruz et al., 2016) and *C. fluminea* (Aguirre-Martínez et al., 2015) at concentration range from 0.1 μg/L to 50 μg/L. Hence, all these data corroborate the molecular and enzymatic changes herein observed emphasising the importance of this metabolic pathway in the detoxification process of CAF within mussel DG.

4.3.3. Effects of CAF on lipid peroxidation

It well is known how free radicals and ROS can produce lipid peroxidation (LPO), destabilizing the cell membranes (Su et al., 2019). In this paper, LPO levels raised in the DG of mussels exposed to the four highest concentrations of CAF (C2-C5), following only 12 days of exposure. Considering other studies (Aguirre-Martínez et al., 2016; Capolupo et al., 2016; Cruz et al., 2016; De Marco et al., 2022a), it is possible to argue that the antioxidant and detoxification system of DG, compared to other tissues such as the gills (De Marco et al., 2022a), appears to be effective in counteracting LPO at least during the early

stages of exposure to toxicants (Capolupo et al., 2016), whereas it is weak during prolonged exposure (Aguirre-Martínez et al., 2016; Cruz et al., 2016; De Marco et al., 2022a).

Overall, the IBR provides an highly effective way of approaching environmental toxicology (Perussolo et al., 2019; Nayak and Patnaik, 2021). In this study, the results achieved for CAF exposure highlighted a time- and dose-dependent increase of IBR, corroborating observations made in previous works concerning the effects of drugs in aquatic invertebrates (Trombini et al., 2019).

4.4. Effects of SA on mussel DG: oxidative stress, biotransformation and LPO

The effects of SA on oxidative stress-related gene expression in DG of mussels from the same experimental design were previously described in Afsa et al. (2022b), therefore this work focuses mostly on the oxidative stress-related enzymatic activities and biotransformation. An increase in GST activity combined with an inhibition of CAT was recorded, and these results are in line with what observed at the molecular level in Afsa et al. (2022b). Furthermore, the lack of alterations in LPO suggests the ability of SA to inhibit the mitochondrial activity and ROS production, as previously stated in De Marco et al. (2022b). In fact, SA was reported to inhibit ferrochelatase, a crucial enzyme for the catalysis of the terminal step in heme biosynthesis, which results in the inhibition of several hemoenzymes synthesis including those involved in ROS generation and cellular respiration (e.g., cytochromoxidases) (Gupta et al., 2013; Zivna et al., 2016). Therefore, the intracellular ROS formation could be dropped by SA exposure, mainly in the mitochondria, participating in the anti-inflammatory effect of this NSAI drug (Gupta et al., 2013). Interestingly, the mitochondrial dysfunction is likely favoured by the chelating properties of salicylates on Fe (III) (Pozdnyakov et al., 2015), resulting in the reduction of Fe (III) availability and/or the inactivation of hemoproteins by complex formation. Therefore, this explanation could justify the inhibitory effects in terms of antioxidant enzymatic activities, as observed in non-target aquatic organisms, both invertebrates and vertebrates, exposed to SA (Zivna et al., 2015; Freitas et al., 2019; Freitas et al., 2020a; Freitas et al., 2020b; De Marco et al., 2022b) and ASA (Gómez-Oliván et al., 2014).

The ability of SA to inhibit ROS production by acting on mitochondrial activity appears to be supported by biomarker star plots and IBR. In fact, except for the biomarkers related to GST (enzymatic activity and gene expression), the other biomarkers mainly linked to the antioxidant response displayed lower values compared to those recorded in control. Thus, these results prove the utility of IBR not only in field (Pain-Devin et al., 2014; Samanta et al., 2018), but even in laboratory studies (García-Medina et al., 2022; Fu et al., 2022). Otherwise, another possible explication could be connected to the observed rise in GST activity in mussels exposed to SA (Freitas et al., 2019; De Marco et al., 2022b) that, due to its role as a class II biotransformation enzyme is capable, alongside the presence of haemocytes (Afsa et al., 2022b; De Marco et al., 2022b), to triggering the elimination of the most harmful metabolites of SA, thereby avoiding oxidative damage such as LPO.

4.5. Effects of CAF+SA on mussel DG

4.5.1. Effects of CAF+SA on oxidative stress and biotransformation

Interestingly, following the exposure to the mixture CAF+SA, different patterns have been observed for all the chosen end-points, compared to the individual effect of CAF and SA. About SOD, a significant and remarkable down-regulation was noticed for both SOD encoding genes, only at T12.

Regarding CAT and GST at molecular and biochemical level, the exposure to the mixture provoked an early response compared to CAF alone. In fact, an increase in CAT enzymatic activity and up-regulation of *cat* and *gst* genes was noticed at T3. These results suggest a tendency for mixtures of contaminants to cause more intense and earlier effects than single exposures, as seen previously in De Marco et al. (2022a) and herein highlighted by the IBR. The CAT inhibition both at molecular and biochemical level observed at T5 is likely to be linked to the effects of SA, as supported by previous works (Freitas et al., 2019; Freitas et al., 2020a; Freitas et al., 2020b; Afsa et al., 2022b; De Marco et al., 2022b). The lack of effects at T12 is probably accounted by the different effects of the two PhACs (Aguirre-Martínez et al., 2016; Capolupo et al., 2016; Cruz et al., 2016; De Marco et al., 2022a).

A similar trends can be drawn for GST. In fact, although an increase of GST gene expression and ~~CAT~~ enzyme activities was observed in the early stages of exposure (T3, T5) at the highest concentrations (C3-C5), a significant drop both at the molecular and enzyme level was then recorded following 12 days of exposure. A comparable decline in GST was documented in gills of mussels from the same experimental design (De Marco et al., 2022a), as well as in mollusks exposed to other PhACs such as paracetamol (Brandão et al., 2014) and ibuprofen (Gonzalez-Rey and Bebianno, 2012), thus prompting claims on how exposure to CAF+SA mixture may lead to the formation of metabolites able to impair the activity of the detoxification system. CAT and GST followed by a decrease/inhibition at T5 and T12, together with the lack of responsiveness of antioxidant enzymes observed mainly at the end of the exposure to CAF+SA may drive to different explanations. In one hand, the observed molecular and biochemical profile under the exposure to the mixture could be attributed to the different effects of CAF and SA, when tested separately. In fact, we have previously shown that the exposure to SA, individually, resulted in a significant decrease in the mRNA amounts of oxidative stress related genes, namely *Cu/Zn-sod, Mn-sod* and *cat* in *M. galloprovincialis*, under the same conditions (Afsa et al., 2022b). Likewise, a decrease in the fold induction of *sod* was observed at the early life stages of *M. galloprovincialis* treated with diclofenac, another well-known NSAI drug (Balbi et al., 2018). Overall, the herein observed transcription and biochemical patterns seem to be mostly affected by the inhibitory effect of SA, as reinforced by its ability in targeting and interfering several pathways. This effect was more prominent at T5 and more intense at T12, in particular for *gst* and *sod* genes.

4.5.2. Effects of CAF+SA on lipid peroxidation

Overall, the onset of oxidative damage in the presence of both compounds cannot be ruled-out. The data on LPO appear to corroborate the lack of a synergic effect at the level of mussel DG, but rather highlight a predominance of the effect of one metabolite upon the other. In fact, referring to previous studies, although SA and CAF, separately, were able to induce lipid peroxidation in invertebrates like bivalves (Gagné et al., 2007; Martín-Díaz et al., 2009; Aguirre-Martínez et al., 2016; De Marco et al., 2022a; De Marco et al., 2022b), crustaceans (Gómez-Oliván et al., 2014) and in vertebrates (Doi and Horie, 2010; Zivna et al., 2015), in this study no significant variation was found for LPO levels in mussel DG, contrarily to what previously documented in mussel gills in which SA and CAF, in binary mixture, induced an increment of lipid peroxides (De Marco et al., 2022a). Therefore, these data suggest the ability of CAF+SA mixture to differentially induce oxidative damage in different tissues. The apparent absence of significant inactivation of CAT at enzymatic and molecular level do not allow us to properly assess the capacity of SA to trigger mitochondrial impairment despite the occurrence of CAF, albeit the stars plot and IBR trend, comparable to those observed for SA alone, seem to enforce this condition. On the other hand, according to the data for GST, it may be hypothesized a possible impairment in the detoxification system. Nevertheless, further studies are needed.

5. Conclusion

To conclude, findings from this study indicate that CAF individually, at environmentally relevant doses, may induce oxidative stress in mussel DG, whereas SA alone may reduce cellular ROS production acting on

mitochondrial activity. Therefore, transcription modulation represents, coupled with fluctuations in enzymatic activity, a part of the early defense response activated to avoid CAF-induced oxidative stress. The exposure to the mixture CAF+SA enhanced a notable different transcriptional and biochemical response compared to CAF alone, which is mostly attributed to SA effects. The IBR has been confirmed as an excellent additional indicator for environmental biological toxicity assessment. Overall, these results enlarge the knowledge of the effects of these emerging contaminants on non-target aquatic organisms as short-term adaptive and defensive strategies, and emphasize the need to properly assess the environmental risk of pharmaceuticals.

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CRediT authorship contribution statement

Sabrine Afsa: Methodology, Formal analysis, Investigation, Writing – original draft. **Giuseppe De Marco:** Methodology, Formal analysis, Investigation, Writing – original draft. **Antonio Cristaldi:** Formal analysis. **Alessia Giannetto:** Formal analysis, Data curation, Visualization. **Mariachiara Galati:** Formal analysis. **Barbara Billè**: Formal analysis. **Gea Oliveri Conti:** Methodology, Formal analysis. **Hedi ben Mansour:** Formal analysis. **Margherita Ferrante:** Methodology, Formal analysis. **Tiziana Cappello:** Conceptualization, Supervision, Resources, Methodology, Visualization, Validation, Writing – review & editing.

Declaration of Competing Interest

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

Data Availability

Data will be made available on request.

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