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Two faces of one coin: Beneficial and deleterious effects of reactive oxygen species during short-term acclimation to hypo-osmotic stress in a decapod crab

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ABSTRACT

Exposure to environmental changes often results in the production of reactive oxygen species (ROS), which, if uncontrolled, leads to loss of cellular homeostasis and oxidative distress. However, at physiological levels these same ROS are known to be key players in cellular signaling and the regulation of key biological activities (oxidative eustress). While ROS are known to mediate salinity tolerance in plants, little is known for the animal kingdom. In this study, we use the Mediterranean crab *Carcinus aestuarii,* highly tolerant to salinity changes in its environment, as a model to test the healthy or pathological role of ROS due to exposure to diluted seawater (dSW). Crabs were injected either with an antioxidant [*N*-acetylcysteine (NAC), 150 $mg{\cdot}kg^{-1}$] or phosphate buffered saline (PBS). One hour after the first injection, animals were either maintained in seawater (SW) or transferred to dSW and injections were carried out at 12-h intervals. After ≈48 h of salinity change, all animals were sacrificed and gills dissected for analysis. NAC injections successfully inhibited ROS formation occurring due to dSW transfer. However, this induced 55% crab mortality, as well as an inhibition of the enhanced catalase defenses and mitochondrial biogenesis that occur with decreased salinity. Crab osmoregulatory capacity under dSW condition was not affected by NAC, although it induced in anterior (non-osmoregulatory) gills a 146-fold increase in $Na^+/K^+/2Cl^-$ expression levels, reaching values typically observed in osmoregulatory tissues. We discuss how ROS influences the physiology of anterior and posterior gills, which have two different physiological functions and strategies during hyper-osmoregulation in dSW.

1. Introduction

As a consequence of aerobic metabolism, all cells produce reactive oxygen and nitrogen species (ROS, RNS). However, exposure to environmental changes such as hypoxia or salinity (e.g. Rivera-Ingraham et al., 2016a; Carregosa et al., 2014; Abele et al., 1998), pollutants [e.g. Espinosa and Rivera-Ingraham, 2016; Freitas et al., 2015; Torres et al., 2002), or infections (reviewed by Schwarz, 1996) may lead to increased (supraphysiological) ROS levels. From a pathological perspective, the resulting increase in ROS can deplete antioxidant defenses of the cell, leading to oxidative distress: cells, unprotected against ROS see key cellular components (such as protein, lipids, or even nucleic acids) damaged, which further exacerbates ROS formation through a number of processes such as impaired mitochondrial function (Regoli and Giuliani, 2014). Thus, scavenging ROS (e.g. by supplementing diet with antioxidants) has been suggested in some cases to improve cytoprotective defenses, survival, and fitness upon stress induced by heavy metal poisoning (e.g. Gurer and Ercal, 2000), exposure to pesticides (e.g. Peña-Llopis et al., 2003; Al-Harbi et al., 2014), herbicides (Behrens et al., 2016), or hydrocarbons (Cantrell et al., 1996). However, in other cases, antioxidant treatment failed to provide protection (e.g. Lygren et al., 2000; Olsen et al., 1999), and even had a negative effect (reviewed by Bjelakovic et al., 2007). The reason behind this may be that, in an hormetic fashion, physiological amounts of ROS, and namely hydrogen

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peroxide (H_2O_2), play key roles in cellular (redox) signaling (Schieber and Chandel, 2014), essential for the maintenance of cellular homeostasis (i.e. oxidative eustress) (Sies and Jones, 2020). In the context of cellular stress, ROS mediates numerous key biological processes which include inducing tolerance to environmental changes as supported by many studies in a wide variety of organisms including plants, bivalves, and mammals (e.g. De Zoysa et al., 2008; Neill et al., 2002; Röhrdanz and Kahl, 1997). These ROS may be released during stress conditions and act in retrograde (organelle-to-nucleus) signaling and subsequently modulate anterograde (nucleus-to-organelle) responses (Woodson and Chory, 2008). Therefore, and contrarily to the pathological situations described above, when ROS may be necessary for correctly inducing a stress acclimation response, ROS formation inhibition (through antioxidant supplementation) may inhibit the response to correctly acclimate to environmental change (Ristow and Zarse, 2010).

ROS involvement in triggering tolerance to salinity variations in the environment have been mostly addressed in plants (Tanveer and Ahmed, 2020). Coastal animals which are frequently exposed to large variations in environmental salinity have less frequently been the subject of study. Previous works from our group have shown in different marine invertebrates that ROS formation accompanies exposure to environmental salinity changes, while antioxidant and other cytoprotective enzymes are upregulated (Rivera-Ingraham et al., 2016a; Rivera-Ingraham et al., 2016b). Thus, in this work we aimed to determine to which extent salinity-induced ROS formation is due to a pathological situation, and whether these ROS are involved in stress acclimation. In this case, we hypothesize that antioxidant supplementation to animals should hamper the acclimation response, and thus should have a negative effect on the organism.

To test our hypothesis, we used the Mediterranean green crab *Carcinus aestuarii* Nardo, 1847 (Crustacea: Portunidae) as a study model. Being an estuarine species, this decapod is frequently exposed to large environmental changes, notably in environmental salinity and, thus, it is an interesting organism in which to study adaptations to cope with such changes. We focused on gill tissues, for two main reasons: i) because of them being one of the animals' first barriers with their environment and, ii) because these decapod crustaceans possess two metabolically different gill types. *C. aestuarii* in particular possesses 9 phyllobranchiate gills in each branchial chamber. These can be divided into two gill types which, in terms of function, redox balance, metabolism, and salinity-induced ultrastructural reorganization, respond very differently to variations of environmental salinity as demonstrated previously by our group (Rivera-Ingraham et al., 2016b). Anterior gills (pairs 1–6) serve mainly respiratory purposes, and are thus composed of fine epithelial layers typical of gas-exchanging tissues with sparse mitochondria (Compere et al., 1989). When exposed to decreased environmental salinity, these do not suffer significant ultra-structural modifications, and in the long term, they enter a state of reduced metabolic rate and are affected by cellular damage (Rivera-Ingraham et al., 2016b). Contrarily, posterior gills (pairs 7–9) do not only conduct respiratory processes but have also gill lamellae involved in osmoregulation (Compere et al., 1989). These lamellae have much thicker epithelia, typical of salt-transporting tissues. When exposed to low salinities they exhibit high Na^+/K^+-ATPase activity and a large number of mitochondria to fuel all the energetically expensive osmoregulatory processes (Compere et al., 1989; Siebers et al., 1982). As opposed to anterior gills, these posterior gills respond to decreasing salinity by increasing their antioxidant defenses and their metabolic rates despite the large amount of ROS detected in previous studies (Rivera-Ingraham et al., 2016b).

To test if ROS are involved in the acclimation response to dilution of environmental media or if they are just a consequence of a pathological situation, we exposed crabs to either seawater (SW) or diluted seawater (dSW) with or without treatment with an antioxidant (with good H_2O_2 scavenging properties). Our goal was to analyze, in both anterior (purely respiratory gills) and posterior gills (respiratory and osmoregulatory), the effect of antioxidant treatment on the redox metabolism and osmoregulatory function to investigate the possible role of ROS in the early stages of the acclimation process.

2. Materials and methods

2.1. Animal collection

All Mediterranean green crabs (*C. aestuarii*) used in this study were simultaneously collected during the summer period at the Ingril lagoon, located on the Mediterranean coast of France (43° 26′ 14.23″N; 3° 46′ 45.037″E), presenting at the time of collection a salinity of 35 ppt. Previous studies have shown that male and female crabs respond differently to changes in environmental salinity (Neufeld et al., 1980), and thus only males were considered in this study. Animals, with an average weight of 36.23 ± 2.11 g, were transported to the laboratory and were allowed to acclimate to seawater (SW, 35 ppt) at 19 °C for 1 week in a flow-through system of 150 l equipped with aeration and filtration systems. Every 48 h, animals were fed thawed mussels and were allowed to eat for approximately 6 h. After this time, all food particles were removed to avoid deterioration of water quality, which was assessed daily using Quantofix ® nitrate/nitrite test strips (Macherey-Nagel GmbH and Co. Duren, Germany). Water temperature and salinity was also recorded daily using an YSI 85 handheld meter (YSI Incorporated, Yellow Springs, OH, USA).

2.2. Experimental setup

Two independent 150-l flow-through systems were used for this study, each composed of five groups of at least six interconnected boxes of a maximum dimension of 20x13x10 cm (lxwxh). Such boxes were supplied with fully air-saturated water through two aeration systems and maintained clean using two filters. To minimize social-induced stress, 48 h prior to the beginning of the experiment all animals were individually identified, weighted and introduced in an individual box, where they were maintained throughout the complete experiment. The 48 h acclimation to the individual boxes was carried out in SW. After this time, all animals (total N = 45) were randomly attributed to one of the following treatments: i) injection of an antioxidant, here 4 μl 0.25 M *N*-acetylcysteine (NAC) per g crab fresh weight (CFW) (150 mg NAC · kg^{-1} CFW). This concentration was fixed based on previous works (e.g. López Conesa et al., 2001; Zhang et al., 1994; Matuszczak et al., 2005; Víctor et al., 2003) and this particular antioxidant was selected for it being most commonly used in studies addressing the role of ROS in biological or pathological processes (Zafarullah et al., 2003) for its good ROS scavenging properties (except for superoxide) (Aruoma et al., 1989); ii) injection of 4 μl phosphate buffered saline (PBS) per g CFW, serving as control of the impact that injection may have on crabs, due, for example, to handling stress. To reduce such stress, all injections were carried out on ice, a process which lasted a maximum of 1 min. Injections were in all cases carried into the sinus of the 5th right-sided pereiopod (e.g. Wilkens et al., 1985). Animals were then maintained emerged at RT for at least 10 min to allow puncture wounds to heal before reintroducing animals to water. Injections were carried out ensuring that approximately half of the crabs in each of the two flow-through systems were injected with NAC. After 1 h of first injection, water in the first system was replaced by clean SW. In the second system, water was progressively diluted to 10 ppt (dSW, ≈300 mOsm · kg^{-1}). Because the characteristics of the flow system used, target salinity values were achieved in a maximum time of 2 h. This salinity was chosen based on previous studies where we determined that *C. aestuarii* has an isosmotic point of around 750 mOsm · kg^{-1} (≈25 ppt), and, while it behaves as an osmoconformer above this point, it hyperosmoregulates under lower salinities. It is under these conditions of reduced environmental salinity that we may determine the consequences of altering redox balances on osmoregulating capacities.

To ensure animals received equivalent doses of NAC, the ROS-scavenger was here supplied through injections, obliging to regularly

renew the doses. Thus, after water was changed in each of the systems, both NAC and PBS injections were carried out at 12-h intervals to ensure that ROS was effectively scavenged throughout the experiment. This dose-renewal time has been previously demonstrated to maintain ROS scavenging when NAC is supplied through injection (Víctor et al., 2003). Mortality rates were recorded at the time of each injection. A total of 5 injections were administered to each individual. All injected solutions were adjusted to the osmotic pressure (OP) of crab hemolymph at SW (35 ppt, ≈1030 mOsm · kg^{-1}) or dSW (10 ppt, ≈300 mOsm · kg^{-1}) to avoid causing an osmotic shock on the crab. OP values for animals exposed to dSW were obtained from the time-dependent osmoregulation curve for *C. aestuarii* by Barri (2015) to avoid over-stressing organisms by obtaining a hemolymph sample at each injection time.

All animals were euthanized after 60 h of transfer to dSW, time at which acute hemolymph osmolality stabilizes in *C. aestuarii* (Barri, 2015) and maximum gene expression of osmoregulatory-related genes is often achieved (e.g. Luquet et al., 2005). For this, animals were rendered insensitive to stimuli by placing them at −18 °C for about 2 min and then euthanized following the spiking procedure (i.e. using a sharp metal spike to destroy their nerve centre) in agreement with the recommendations of the different animal wellbeing (e.g. RSPCA). For each crab, a hemolymph sample was taken using a 1 ml hypodermic syringe. Hemolymph OP was immediately quantified by freezing point depression osmometry (Model 3320, Advanced Instruments, Inc., Norwood, MA, USA). For a minimum of 5 crabs per treatment and salinity, both anterior gills (pairs 1–4, with a purely respiratory function) and posterior gills (pairs 5–8, with respiratory as well as osmoregulatory function) were dissected and preserved to conduct the following analyses: left-sided gill pairs 2 and 8 were preserved in TRIzol® Reagent (Invitrogen) for gene expression quantification and stored at −80 °C until further quantification. Right-sided pair gills 3 and 7 were immersed in SEI buffer (0.3 M sucrose, 0.02 M Na_2EDTA, 0.1 M imidazole) and stored for Na^+/K^+-ATPase, citrate synthase (CS) and caspase activity measurements. Left-sided pair gills 3 and 7 were flash frozen in liquid nitrogen and preserved at −80 °C for antioxidant quantifications. All animals used in the study were in inter-molt stage (C4) as confirmed through postmortem epipodite examination.

2.3. Assessment of ROS appearance in hemolymph

We will here refer to ROS as those molecules (radical or not) containing one or more activated atoms of oxygen and which are a result of oxygen reduction (Forman et al., 2015) but also H_2O_2. ROS formation in crab hemolymph (obtained as previously described for OP analyses) was conducted as in Rivera-Ingraham et al. (2016b). Briefly, samples were diluted 1:100 (v:v) in isotonic medium, to avoid the effects of osmotic shock on any hemolymph circulating particles. Values for such media were previously established as the average of the OP measurements of all animals composing each treatment. For animals that remained under SW, these values were fixed at 1069 and 1098 mOsm kg^{-1} for control (PBS) and NAC-treated animals, respectively. For those crabs transferred to dSW, hemolymph samples were diluted in 696 and 651 mOsm kg^{-1} media for PBS and NAC-injected crabs, respectively. ROS was then fluorometrically quantified using 5-carbonyl-2′,7′-difluorodihydrofluorescein diacetate (C-H_2DFFDA, Molecular Probes C-13293), which is converted to its fluorescent form 2′,7′-dichlorofluorescein (DCF) upon reaction with a wide variety of ROS (except superoxide). Since previous studies have shown a salinity-dependent fluorescence of DCF (Rivera-Ingraham et al., 2016b), relative fluorescence values were normalized using a H_2O_2 curve (ranging from 0 to 0.7% H_2O_2) for each salinity treatment. Even though C-H_2DFFDA is not a H_2O_2-specific dye (e.g. Grisham, 2012), this ROS was chosen for this task because it is one of the most stable species. All samples were incubated for 10 min at 20 °C with the dye and were analyzed in a flat-bottom black microplate. Fluorescence signals (excitation: 488 nm; emission: 525 nm) were obtained with a Tecan Infinite M200 (Tecan, Männendof, Switzerland).

2.4. Antioxidant quantification

Antioxidant activity was assessed as catalase (CAT) and superoxide dismutase (SOD) activities, which were determined spectrophotometrically in anterior and posterior gills. Samples were weighed and diluted to a 1:7 and 1:14 ratio (w:v) for CAT and SOD measurements, respectively, in a 50 mM KPi buffer with 120 mM KCl. Tissue homogenization was achieved using four stainless steel milling balls (Retsch, no. FR0120) in a Mixer Mill MM400 (Retscher GmbH, Haan, Germany) (30 sec at 30 beats · sec^{-1}). CAT activity was measured in resulting supernatants as the decomposition of a 0.3 mM H_2O_2 solution in a 50 mM KPi buffer following the protocol established by Aebi (1984). SOD activity was determined using the cytochrome oxidase assay as described in Livingstone et al. (1992). All measurements were carried out in triplicate using a microplate reader (Tecan Infinite M200, Tecan, Männedorf, Switzerland). Values were expressed as units of activity (UCAT and USOD) per g CFW and per mg protein, quantified according to Bradford (1976).

2.5. Na^+/K^+-ATPase (NKA) activity measurements

Quantifications were carried out on gills that had been previously preserved in SEI buffer at −80 °C. Samples were homogenized in MIMM solution (250 mM sucrose, 5 mM $MgCl_2$, 1 μg · ml^{-1} of each of the following protease inhibitors: 0.5 mg · ml^{-1} leupeptin in ddH_2O, pepstatin in 90:10 ethanol:acetic acid and aprotinin in PBS, pH 7.4) using the same method as described for antioxidant quantifications. Samples were then centrifuged to obtain a supernatant on which protein concentration was assessed using the method described by Bradford (1976). All samples were diluted with MIMM solution to achieve a sample containing 0.1 mg prot · ml^{-1}. NKA activity was then determined following the protocol originally described by Flik et al. (1983) and modified by Lorin-Nebel et al. (2013). Briefly, NKA activity was estimated as the differential hydrolysis of ATP in a ATP- medium (100 mM NaCl, 10 mM $MgCl_2$, 100 mM Hepes, 5 mM ATP, pH 7.4) supplemented either with 15 mM KCl or with 10 mM ouabain. In a microplate and in triplicate, released inorganic phosphorous (Pi) was assessed colorimetrically against a certified standard (Sigma, France) and results were expressed as μmol Pi · mg prot $^{-1}$ · h^{-1} · g CFW^{-1}. All quantifications were conducted a minimum of two different days in order to confirm the accuracy of results.

2.6. Citrate synthase (CS) activity

To indirectly quantify mitochondrial density, CS activity was assessed on MIMM-homogenized samples and following the same procedure described in Rivera-Ingraham et al. (2016b) and using a microplate reader. Free Co-A production, resulting from the addition of oxalacetic acid to samples, was quantified by recording changes in absorbance at 412 nm in a microplate reader. Results were expressed as units (mU, nmol · min^{-1}) · mg $prot^{-1}$ · g CFW. Protein content was quantified by Bradford (1976).

2.7. Cellular damage assessments

Apoptosis was determined as the specific activity of caspase 3 and 7, involved in programmed cell death. Activities were assessed on previously homogenized samples, which were diluted to a final ratio 1:100 (w:v) using a lysis buffer composed of 25 mM Hepes, 5 mM $MgCl_2$, 1 mM EGTA and a cocktail of protease inhibitors as described for NKA measurements (Rivera-Ingraham et al., 2016b). Caspase activities were determined using the Caspase-Glo® 3/7 kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Values were recorded using a microplate reader and expressed as relative light units (RLU) per mg protein (Bradford, 1976) and per g CFW.

2.8. Reference genes and primer design

Forward and reverse primer sequences used for the qPCR are shown in Table 1.

The 18-S ribosomal and elongation factor-2 (EF-2) were selected as possible reference genes (housekeeping genes). The choice of reference genes was based on closely related species such as *Carcinus maenas* (McDonald et al., 2011) as well as for other crabs (e.g. Covi et al., 2008; Goff et al., 2017). Furthermore, we focused our gene expression study on the mitochondrial manganese superoxide dismutase (mnSOD), Na^+/K^+-ATPase alpha subunit (nka), and $Na^+/K^+/2Cl^-$contransporter (nkcc). All primers in this study were designed based on the sequences information obtained from the Gene Bank (https://www.ncbi.nlm.nih.gov). PCR primers were designed using a Primer3 online program (Rozen and Skaletsky, 1999). For each primer pair a melting curve was performed to control the amplification specificity. Standard curves prepared from serial dilutions of quantified amplicons for each gene were included in all qPCR plates to calculate efficiency, which were between 1.7 and 1.90.

2.9. RNA extraction

Gill samples previously stored in TRIzol®Reagent and frozen at −80 °C were used for isolation of RNA. To do this, about 20 mg of sample was homogenized in 1 ml of the same reagent and using the same procedure described for the antioxidant quantification. RNA was isolated following the instructions provided by the manufacturer, and then quantified using a Nanodrop®ND-1000 V3300 spectrophotometer (Nanodrop Technology Inc., USA). Quality of RNA products was determined by microfluidic electrophoresis using an Agilent Bioanalyzer 2100 equipped with a RNA Nano Chip (Agilent Technologies, CA, USA). A DNAase treatment (DNAase I Amp Grade, Invitrogen) was carried out for each sample and cDNA was obtained using M-MLV reverse transcriptase and random primers (Invitrogen, France).

2.10. Gene expression quantification by Real-time qPCR

Gene expression levels were studies by real-time PCR. Using an Echo®525 liquid handling system (Labcyte Inc., California, USA), 2.5 μl of LightCycler-FastStart DNA Master SYBR-Green I™ Mix (Roche, Mannheim, Germany), 0.25 μl of each primer (forward and reverse at 0.5 μM final concentration), and 2 μl of cDNA were dispensed into a waiting 384-well reaction plate, and each sample was tested in triplicate. The qPCR amplification program consisted of denaturation at 95 °C for 10 min, followed by 40 cycles of repeat amplification (95 °C, 15 s), hybridization (60 °C, 5 s) and elongation (72 °C, 10 s) and a final step at 40 °C for 30 s.

The stability of the candidate reference genes was tested with the macros Normfinder (Andersen et al., 2004) and gNorm (Vandesompele et al., 2002).

2.11. Statistical analyses

All results are presented as means ± standard errors of the mean (S.E.M.). The Kolmogorov-Smirnov test was used to test normality while homoscedasticity was tested by the Levene test. When the requirements for parametric analyses were met (i.e. ROS formation, CAT activity and mnSOD expression), anterior and posterior gill data was separately evaluated using a two-way analysis of variance (ANOVA) with salinity (2 levels: 10 or 35 ppt) and treatment (2 levels: PBS or NAC) as fix factors. A Student-Newman-Keuls post-hoc test was used to compare the different conditions. For all other parameters for which the assumptions for parametric analyses were not met, a Kruskal-Wallis test was applied followed by U-Mann-Whitney pairwise comparisons. The gene expression correlation matrix was obtained using the Pearson correlation coefficient, which measured the linear dependence between two variables. The level of statistical significance was in all cases established at $p < 0.05$. Statistical analyses were conducted using SPSS 15.0 (SPSS Inc., Chicago, IL, USA) and RStudio software (version 2023.03.0 + 386).

3. Results

3.1. Mortality rates, osmotic pressure (OP) and ROS formation in the hemolymph

No mortality was registered during the 7-day acclimation period. No crabs died as a consequence of injections (PBS or NAC) under SW conditions (Fig. 1a). Transfer to dSW did not induce mortality in PBS-injected crabs, but those treated with NAC suffered 55% mortality rates. ROS formation in the hemolymph was significantly affected by salinity and treatment ($F = 5.828$; $p < 0.01$; see Table 2 for 2-way ANOVA results). The transfer to dSW caused control animals (PBS-injected) to produce 1.4-fold more ROS than crabs maintained in SW. However, this response was not observed in NAC-injected animals, for which ROS formation levels remained similar to those registered in animals maintained in SW (Fig. 1a). Regarding hemolymph OP, statistical differences were recorded among treatments ($K = 21.005$; $p < 0.001$). However, these were only salinity-associated differences. Crabs in the SW condition maintained hemolymph OP at an average of 1084 ± 12 mOsm · kg^{-1}; in dSW condition, values decreased to 679 ± 15 mOsm · kg^{-1} (Fig. 1b).

3.2. Antioxidant defenses

Significant differences were registered among treatments for both SOD ($K = 24.097$; $p = 0.001$) (Fig. 2A) and CAT activities ($F = 6.046$; $p < 0.001$, see Table 2 for two-way ANOVA results) (Fig. 2B). The transfer to dSW caused SOD activity to decrease in both anterior and posterior gills, while CAT activity increased in both tissues compared to SW values. However, the increase in CAT activity was not observed in NAC-injected animals. For crabs remaining in SW, NAC-injected crabs did not show differences with controls in terms of CAT activities, while their SOD activities were decreased by 2.3- and 6-fold in anterior and

Table 1
List of primers used for the gene expression anlaysis.

Gene acronym	Gene name	GenBank accession number	Primer sequence 5′ to 3’ Forward/reverse	Amplicon length (bp)
18-S	18-S ribosomal	AY919107.1	TGGCCGGCAGAGTTTACTTT TTTCACCTCTAGCGTCGCAA	182
ef2	Elongation factor-2	GU808334.1	TTCAAGGTTCCTGCTGGCAA CGGTCTTCACTGGTCAGCTT	587
mnSOD	Mitochondrial manganese superoxide dismutase	FM213479.1	GCT GCC CTG GAG AAG AAG TT ACC TCG CTC CTC ACC TAG TT	228
nka	Na^+/K^+-ATPase alpha subunit	AY035550.1	GGA GTT GGG TCT TGG AG CTT GGC AGT GAT GGG GTG AT	247
nkcc	$Na^+/K^+/2Cl^-$contransporter	AY035548.1	TGA TCT CTC GGT CCC TTG GT TAA GGG TTG CTG TGC CTA CG	198

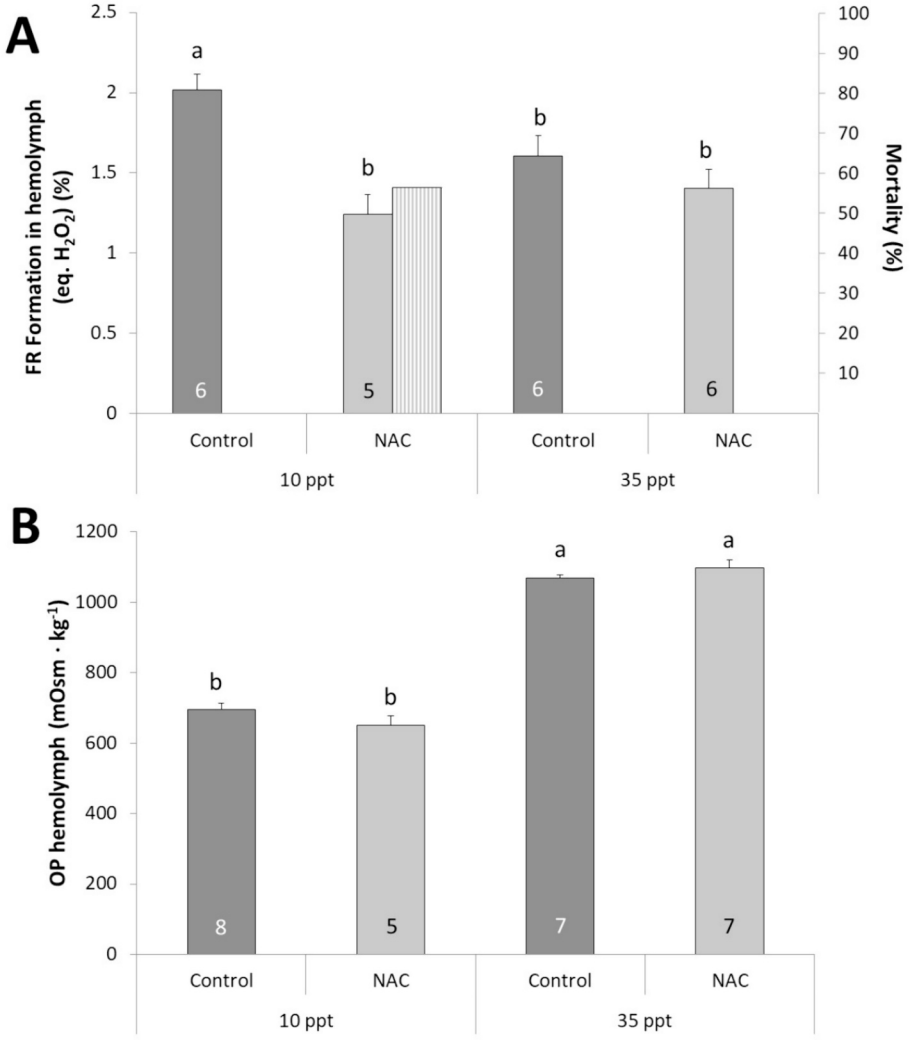

Fig. 1. Hemolymph parameters for crabs exposed to each of the experimental treatments. A) Free radical formation (plain colored bars) and mortality rates (stripped bars). B) Hemolymph osmotic pressure. Values associated with the same letter belong to the same subgroup according to a Student-Newman-Keuls test (A) and U-Mann-Whitney pairwise comparisons tests (B). Numbers associated to each bar indicate the number of replicates.

Table 2
Two-way ANOVA results testing the effects of salinity (10 ppt or 35 ppt) and treatment (PBS or NAC injection) on ROS formation in the hemolymph and CAT activity and mnSOD expression levels in anterior and posterior gills. Significant differences are identified by asterisks ($p < 0.05$*). All other biochemical parameters considered did not meet the ANOVA assumptions and were thus analyzed using non-parametric tests.

	Conditions	Salinity		Treatment		Interaction	
Tissue	Biochemical parameters	F	*p*-value	F	p-value	F	p-value
Hemolymph	ROS formation	1.162	0.295	17.998	<0.001*	6.234	0.022*
Anterior gills	CAT activity	26.602	<0.001*	2.839	0.101	10.257	0.004*
Posterior gills		4.296	0.051	1.614	0.219	1.541	0.229
Anterior gills	mnSOD expression	18.692	<0.001*	1.619	0.216	0.477	0.020*
Posterior gills		1.510	0.232	2.374	0.137	1.003	0.327

posterior gills, respectively.

3.3. NKA activities

We recorded tissue-specific significant differences in NKA activity ($K = 25.571$; $p = 0.001$), with posterior gills showing between 2.2 and 2.7-fold higher values than anterior gills under SW conditions and between 4.6 and 5.7-higher under dSW (Fig. 3). Neither salinity change nor NAC injections showed an impact on NKA activities.

3.4. Citrate synthase (CS) results

CS activity showed significant differences among tissues and treatments ($K = 26.631$; $p < 0.001$) (Fig. 4). Under SW conditions, posterior gills showed 1.8-fold higher CS activity than anterior gills in control conditions. These differences were not observed in dSW. Regardless of the environmental salinity, both anterior and posterior gills responded to NAC injections by decreasing their CS activities. Under SW conditions, values decreased by 2- and 4-fold while under dSW, CS activity was reduced by 3- and 1.5-fold (anterior and posterior gills, respectively).

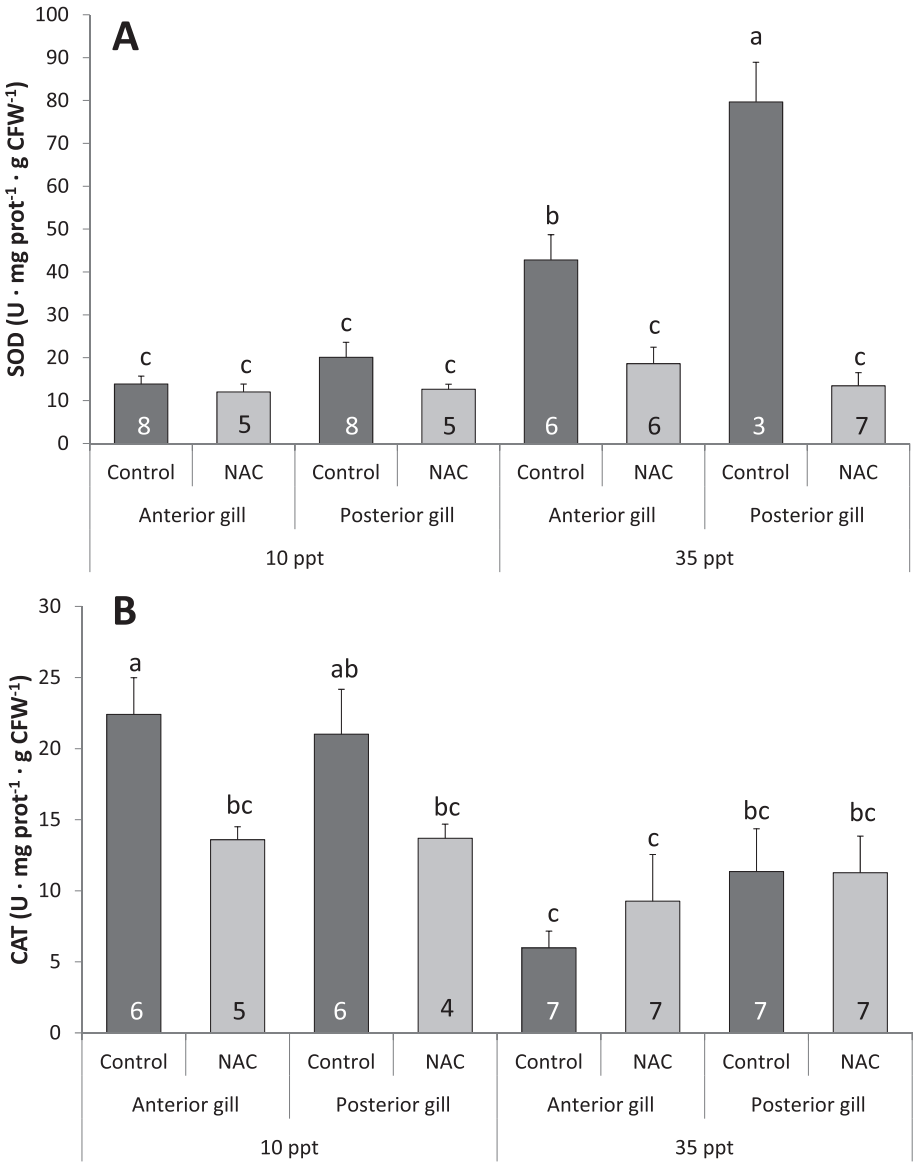

Fig. 2. Effect of NAC on antioxidant defenses in anterior and posterior gills of crabs exposed to SW and dSW. A) Superoxide dismutase (SOD) activity. B) Catalase (CAT) activity. Values associated with the same letter belong to the same subgroup according to U-Mann-Whitney pairwise comparisons (A) and a Student-Newman-Keuls test (B). CFW = crab fresh weight. Numbers associated to each bar indicate the number of replicates.

3.5. Cellular damage

Results show that significant differences exist among treatments (K = 25.929; $p < 0.01$) (Fig. 5). Under SW conditions, both anterior and posterior gills showed similar caspase activities, with an average of 9.9 RFU · mg prot^{-1} · g CFW^{-1}. For both tissues, these values were significantly reduced by NAC treatment. For crabs exposed to dSW, anterior gills showed 6.9-fold higher caspase activity values than posterior gills, but NAC injections had no effect on either gill tissue. However, NAC-treated crabs under dSW had often blackened gill tissues.

3.6. Gene expression

Elongation factor 2 (EF-2) was selected as an internal control (housekeeping gene) since it showed the most consistent expression levels among treatments. Experimental treatments had a significant effect on the expression of all the genes tested (Fig. 6): manganese superoxide dismutase (*mnsod*) expression was generally higher in posterior gills than anterior gills, however there was not statistically difference upon transfer to dSW. Contrarily, gene expression levels in anterior gills significantly increased upon transfer to dSW (F = 6.482; $p < 0.001$, see Table 2 for two-way ANOVA results). However, NAC did not alter gene expression levels. Following a similar pattern, the expression of *nka* was equally affected by gill type and environmental salinity and NAC solely affected anterior gills under dSW by decreasing *nka* expression (K = 36.190; $p < 0.001$). Finally, *nkcc* expression showed large differences among treatments and tissues (K = 41.887; $p < 0.001$): posterior gills showed significantly higher values than anterior gills at 35 ppt. However, the transfer from SW to dSW caused values to increase in both gill types with and increase considerably in posterior gills compared to anterior gills (4.2-fold increase in anterior gills and 88.9-fold increase in posterior gills) for controls. NAC solely affected anterior gills under dSW, causing a 146-fold upregulation over control values, reaching levels of posterior (osmoregulatory) gills. A correlation matrix (Fig. 7a, b) revealed that in SW *nkcc* expression is positively correlated with *nka* and *mnsod* in anterior gills while for posterior gills, these are negatively co-expressed. While under these conditions, NAC does not affect anterior gills, it does posterior gills, namely through *nkcc* expression.

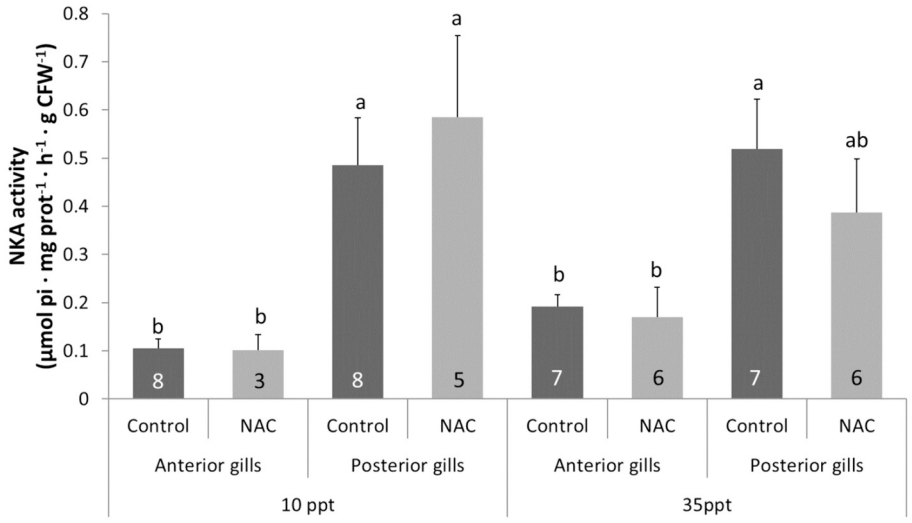

Fig. 3. Effects of NAC on Na^+/K^+-ATPase (NKA) activity in anterior and posterior gills of crabs exposed to SW and dSW. Values associated with the same letter belong to the same subgroup according to U-Mann-Whitney pairwise comparisons. CFW = crab fresh weight. Numbers associated to each bar indicate the number of replicates.

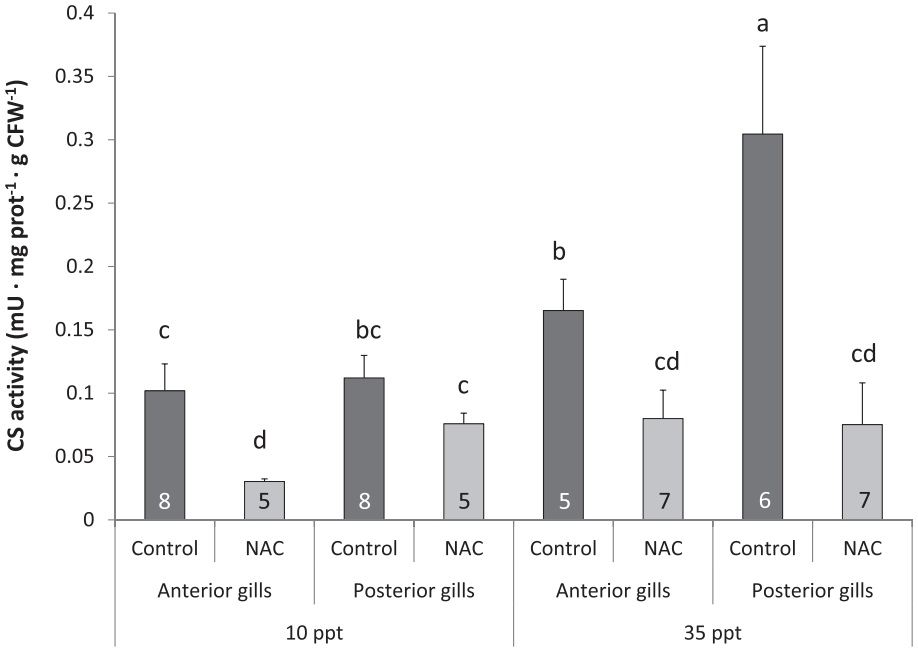

Fig. 4. Effects of NAC on citrate synthase (CS) activity in anterior and posterior gills of crabs exposed to SW and dSW. Values associated with the same letter belong to the same subgroup according to U-Mann-Whitney pairwise comparisons. CFW = crab fresh weight. Numbers associated to each bar indicate the number of replicates.

Transfer of animals into dSW accentuated the *nkcc* correlation with *nka* and *mnsod* expression in posterior gills, and these were again quenched by NAC injections.

4. Discussion

It is known that acclimation to both biotic (e.g. energy or nutrient stress) and abiotic stressors (e.g. hypoxia) are under the control of redox signaling (Sies and Jones, 2020). To the authors knowledge, this study provides for the first time evidence that salinity stress in crustaceans, as for plants, may also be mediated through ROS signaling. The coastal crab *C. aestuarii*, being an estuarine organism frequently exposed to significant variations in environmental temperature, salinity or oxygenation, has proven to be a good model in studying the role of ROS in acclimation to osmotic shock. Furthermore, benefiting from the fact that it possesses gills with and without osmoregulatory function, we further studied how organs with different metabolic capacities differentially respond (in terms of redox homeostasis and osmoregulation) to alterations of the redox balance.

4.1. NAC-injected crabs fail to upregulate their antioxidant defenses upon osmotic stress

Environmental changes, including salinity variations, affect ROS formation in tissues as shown by many (reviewed by Rivera-Ingraham and Lignot, 2017). In hyper-iso osmoregulators such as *C. aestuarii* free radical formation has been reported to increase with decreasing environmental salinity (Rivera-Ingraham et al., 2016b). We here used NAC

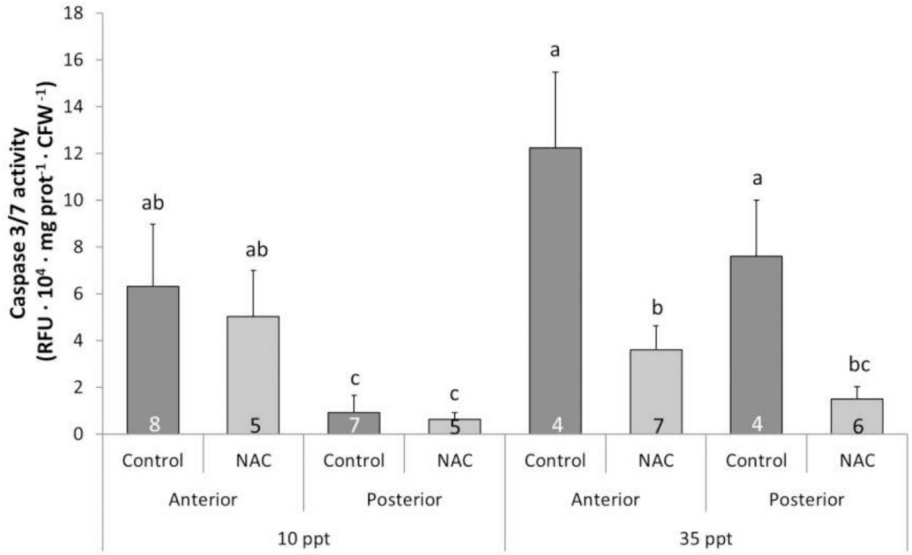

Fig. 5. Effects of NAC on caspase 3/7 activity in anterior and posterior gills of crabs exposed to SW and dSW. Values associated with the same letter belong to the same subgroup according to U-Mann-Whitney pairwise comparisons. CFW = crab fresh weight. Numbers associated to each bar indicate the number of replicates.

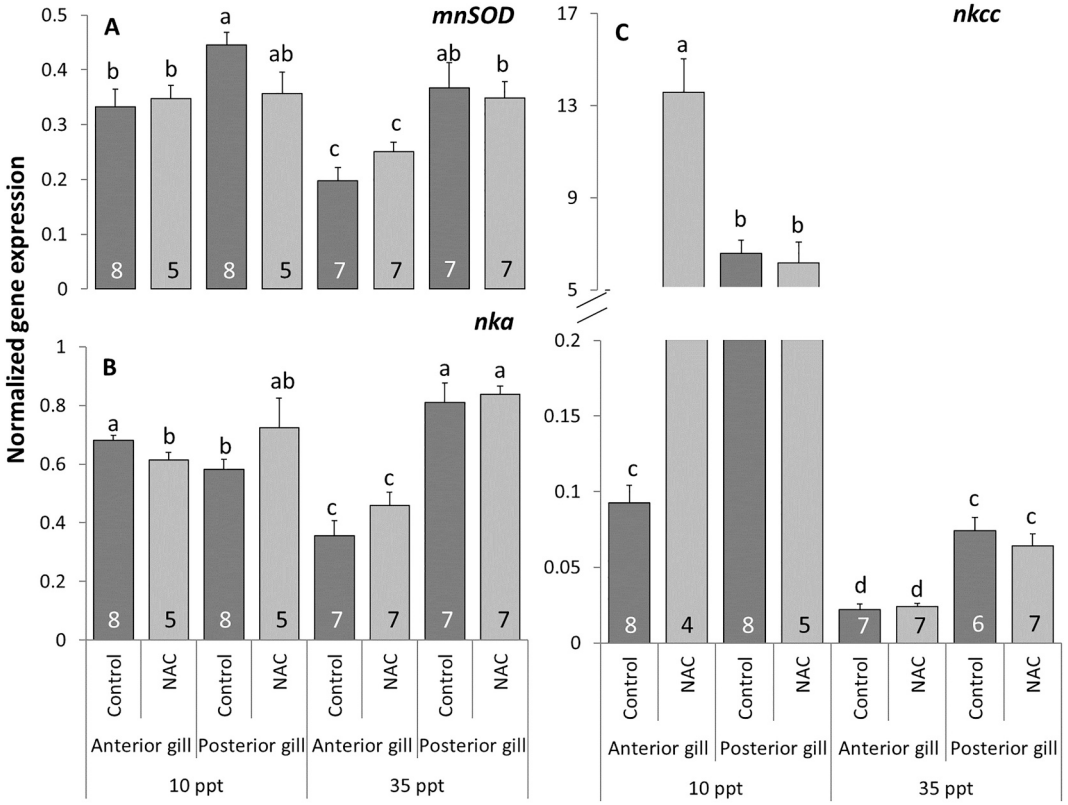

Fig. 6. Quantitative gene expression results. A) mitochondrial manganese superoxide dismutase (*mnSOD),* B) Na^+/K^+-ATPase (*nka*) and C) $Na^+/K^+/2Cl^-$ cotransporter (*nkcc*) in *C. aestuarii* under different experimental conditions. Values associated with the same letter belong to the same subgroup according to a Student-Newman-Keuls test (A) and U-Mann-Whitney pairwise comparisons (B,C). Numbers associated to each bar indicate the number of replicates.

to quench the ROS formation occurring upon the transition to dSW to infer on the role that these ROS have in acclimation. Our ROS measurements in hemolymph samples confirmed that NAC injections were efficient against the dSW-derived increase in ROS formation. However, in doing so, a 55% mortality rate was induced. Even if NAC has been shown to have toxic effects at high concentrations (Johnston et al., 1983), the low doses used here have been reported to have beneficial effects in many other studies (e.g. López Conesa et al., 2001; Zhang et al., 1994; Matuszczak et al., 2005). The fact that NAC solely affected the animals that were immersed in dSW, leads us to hypothesize that quenching ROS formation affects crab acclimation success to dSW and, thus, leads to the high mortality observed in the present study. Indeed, we observed that gill tissues in NAC-treated crabs under dSW were often blackened, which could be indicative of loss of osmoregulatory capacity

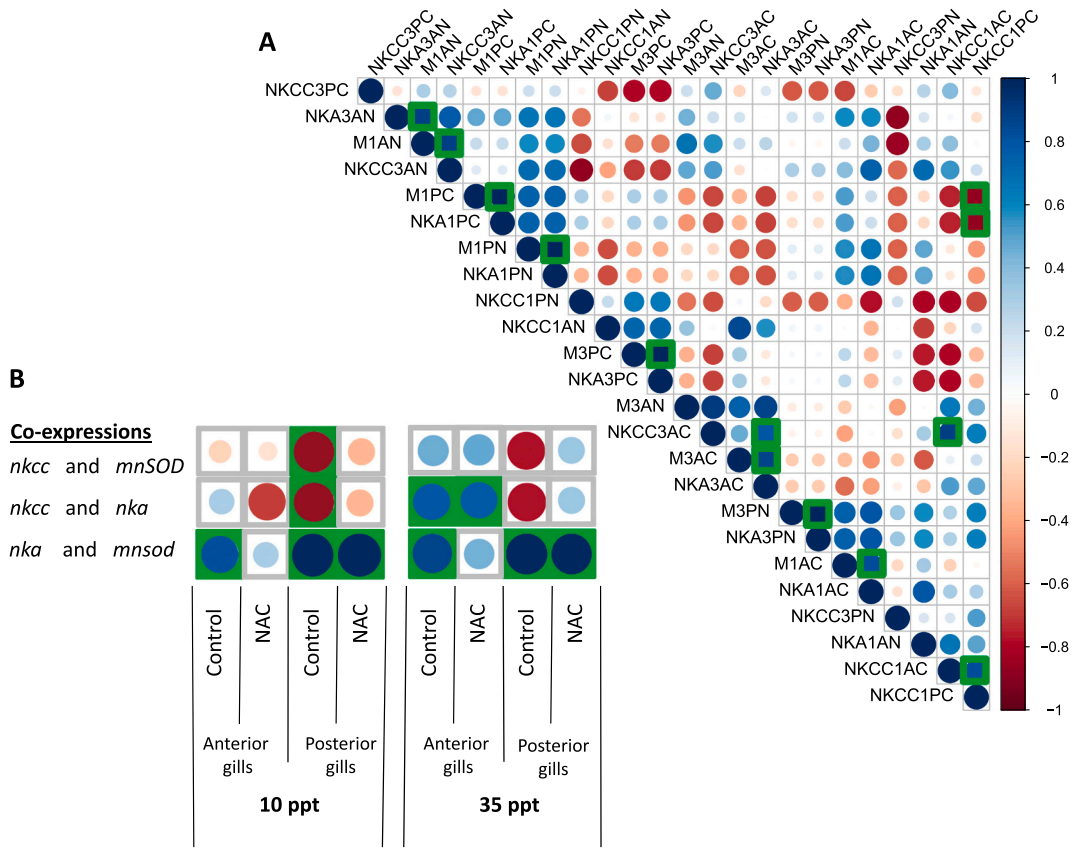

Fig. 7. Gene expression correlations among treatments and tissues. A) Correlation matrix; B) Correlation summary. Positive correlations are displayed in blue while negative correlations are shown in red. Color intensity and size of the circle are proportional to the correlation coefficients. Legend on the right side of the correlogram shows correlation coefficients. Green squares show statistically significant correlations ($p < 0.05$). Cases are coded as follows: first letters correspond to the gene tested (NKA, NKCC, and M for mnSOD), followed by the salinity condition (1 or 3 for10 and 35 ppt, respectively), followed by the tissue analyzed (A or P for anterior and posterior gills, respectively), and the injection treatment (C or N, corresponding to controls and NAC-injected animals). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(e.g. Spicer, 2013) or even necrosis (reviewed by Frischer et al., 2022).

ROS quenching under dSW impacted crab antioxidant defenses. Under natural conditions, transfer to dSW caused an increase in CAT activity, supporting the importance of this defense mechanism against such hypo-osmotic stress (as previously seen in Rivera-Ingraham et al., 2016b), especially in a short-term period (present study). This increase in CAT was not observed in NAC-injected crabs. However, this inhibitory effect of NAC on CAT activity does not come as a surprise, given that low doses of ROS are known to mediate the enhanced expression and/or activity of cytoprotective genes/enzymes. It also explains how pro-oxidant conditions such as exposure to bacterial infection (e.g. Zheng et al., 2010; Dubovskiy et al., 2008), pollutants (e.g. Krøvel et al., 2008; Trevisan et al., 2014), and abiotic factors (Rivera-Ingraham et al., 2016a; Rivera-Ingraham et al., 2016b) may lead to increased antioxidant defenses. Most studies addressing the various pathways linking increased ROS and antioxidant upregulation have been done using mammalian models or cell lines (e.g. Gómez-Cabrera et al., 2008; Mustafi et al., 2009), and information on aquatic organisms is quite scarce. With most of the available studies in the aquatic environment concentrating on fish, evidence shows that exposure to ROS can upregulate antioxidant genes (Zheng et al., 2010; Giuliani and Regoli, 2014), mainly through the Nrf2-Keap1 system (Giuliani and Regoli, 2014). Very briefly, under stressing conditions, Nrf2 dissociates from Keap-1, allowing the former to enter the nucleus, dimerize with small proteins, and transcriptionally upregulate numerous antioxidant and cytoprotective genes (Osburn and Kensler, 2008). H_2O_2 has been directly related to the activation of the Nrf2-Keap1 system (e.g. Yin et al., 2015; Wilson et al., 2005) and the good H_2O_2 - scavenging properties of NAC could explain the reduced antioxidant response. However, the fact that NAC affects different antioxidants at different environmental salinities indicates a very complex regulation system, known to be affected not only in a dose-dependent manner (as expected for salinity changes), but also by many biological factors (reviewed by Veal et al., 2007). These complex mechanisms deserve further attention.

4.2. *Impact of NAC injections on bioenergetics*

The lack of appropriate acclimation to dSW is also patent from a bioenergetic perspective. In order to successfully acclimate to dSW, a large ultrastructural reorganization of the posterior (osmoregulatory) gills is necessary (Compere et al., 1989), and the increase in mitochondrial mass is essential to fuel the ATP-consuming processes, ensuring correct water and salt homeostasis (Péqueux, 1995). For *C. aestuarii,* our previous studies demonstrated that such a CS increase occurs in posterior gills with a reduced environmental salinity (Rivera-Ingraham et al., 2016b). Even if we do not observe such an increase here, likely due to the shorter term exposure, we see that with the NAC treatment mitochondrial biomass is significantly reduced both in SW and dSW conditions and in the two gill types. The consequence of such a loss in energetic capacity under SW conditions would be interesting to confirm and pursue as it may be of critical importance in order to comply with the energy-consuming processes required for proper acclimation in dSW. We interrogate ourselves as well about how much this may contribute to the high mortality rate observed for the NAC-treated group. The impact of NAC may be related to the role of ROS in mitochondrial biogenesis. Mitochondrial biogenesis control is a complex

biological process, involving mitochondrial and nuclear genes. The communication between these two organelles, known as the mitochondrial retrograde signaling pathway (reviewed by Suzuki et al., 2012), is in great part mediated by mitochondrial ROS, redox state, and calcium homeostasis (Lee and Wei, 2005; Barbour and Turner, 2014). As mentioned above, NAC is a good H_2O_2 scavenger (Aruoma et al., 1989), and this ROS in particular, has been identified as inducing an increase in mitochondrial mass in mammalian cells (Lee et al., 2000). The quenching of H_2O_2 production by NAC may explain our results. Studies on human senescent cells or those affected by defects in the mitochondrial respiratory chain, commonly affected by increased levels of oxidative stress (e.g. Sohal and Sohal, 1991; Richter et al., 1995), have been reported to have increased mitochondrial mass (Lee et al., 1998; Lee et al., 2002).

4.3. Osmoregulatory effects are determined by gill type

Transferring crabs from SW to dSW caused hemolymph OP, as expected for an hyper-osmoregulator such as *C. aestuarii* (Rivera-Ingraham et al., 2016b), to reach about 700 mOsm · kg^{-1}, well over the environmental osmolality (300 mOsm · kg^{-1}). We also observed the NKCC and NKA (negative) correlation expressions expected for an estuarine species: increased NKA activity under low salinities occur for the purpose of ionoregulation, while low levels of NKCC expression ensures conferring hyposmoregulatory endurance, just as it happens in many other estuarine crustaceans (Maraschi et al., 2021; Xu et al., 2023) and other estuarine species including fish (Kang et al., 2012; Kang et al., 2010; Su et al., 2022). NAC treatment did not affect hemolymph OP or NKA activities. NAC may actually be protecting the activity of this and other membrane pumps involved in osmoregulation, as they are targets of ROS, and H_2O_2 in particular (Rohn et al., 1996; Rohn et al., 1993; Kurella et al., 1997). ROS are known to interfere with the activity of membrane pumps through the modification of the enzyme's oligomeric structure, consequently reducing enzyme hydrolysis activity (Dobrota et al., 1999). Interestingly, even if no changes occurred in enzymatic activities, NAC did have an effect on gene expression levels: the *nka-nkcc* expression correlation, for example, was hampered in posterior gills by NAC treatment, making us to hypothesize that this process is regulated by ROS signaling pathways. Anterior gills too showed altered gene expression results: *nkcc* gene expression level of NAC-injected crabs under dSW was 146-fold higher than their respective controls. This integral membrane protein, responsible for the simultaneous transport of one Na^+, one K^+ and two Cl^- in epithelial cells and generally recognized as a key player in cell osmoregulation and volume homeostasis, is strongly upregulated in osmoregulatory (posterior) gill pairs of crabs exposed to decreased environmental salinity (present study, e.g. Luquet et al., 2005; Martins et al., 2011; Lv et al., 2016). While anterior gills show a much smaller upregulation (present study, e.g. Luquet et al., 2005), the fact that under the influence of NAC anterior gills respond to low salinity by increasing *nkcc* expression (to match and even overpass posterior gill levels) under conditions of decreased ROS, leads us to hypothesize that the strong oxidative distress usually experienced by anterior gills under dSW (Rivera-Ingraham et al., 2016b) may be hampering them from having an osmoregulatory function as posterior gills do. However, to ensure that NAC treatment actually results in an increase of NKCC protein content in anterior gills, our gene-expression results must be corroborated with a quantitative analysis at the protein level (Western Blot analysis).

5. Conclusions and future perspectives

In a dose-dependent manner, ROS may have a diverse range of effects, from signaling to negative effects on physiology. Although the mechanisms through which ROS are involved in homeostasis have been well addressed in mammalian models, direct experimentation in marine organisms is scarce and especially regarding salinity changes. Studies like the one presented here can: a) contribute to identifying the main mechanisms and determine similarities with mammalians, b) help us understand how conserved this role is throughout phylogeny, and ultimately, c) allow for a better use of these mechanisms for conservation/production purposes. The results of this study highlight the complex effects of ROS scavenging at different environmental salinities and, of special interest is the fact that these differ between gill types. Despite this complexity, we conclude from this study that by scavenging dSW-induced ROS formation, we are hampering energy-redox acclimation to low salinity environments in both anterior and posterior gills, which is likely responsible for the high mortality rate observed. Nevertheless, this deserves further attention and experimentation, using other ROS scavengers or using small doses of pro-oxidant conditions with animals kept in SW, to corroborate some of the conclusions presented here. It should also be taken into account that this study focused on the short-term acclimation to salinity changes, but it would be of additional interest to compare these results with those obtained with different populations, exposed to different environmental conditions, to investigate the cross-effect of long-term acclimation or adaptation changes. Further research should also attempt to elucidate the molecular mechanisms involved in this acclimation response and to confirm the possible role of ROS as a triggering signal for the upregulation of cytoprotective genes (through, for example, pathways such as the Nrf2-Keap1 system) or osmoregulatory genes (through AMP-activated protein kinase signaling). Finally, given the antioxidant protection that anterior gills can develop, and the specific osmoregulatory tissue characteristics, an ultrastructural analysis on these specialized cells would be necessary to confirm that these changes are induced at the morphological level.

Animal ethics

No permits were required to conduct this study. However, all animals were captured, manipulated and euthanized humanely and following the International Guiding Principles for Biomedical Research Involving Animals, issued by the Council for the International Organizations of Medical Sciences.

Declarations of AI usage

The authors declare not to have used any AI tools for any purpose in this study.

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CRediT authorship contribution statement

Georgina A. Rivera-Ingraham: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Diana Martínez-Alarcón:** Writing – review & editing, Investigation, Formal analysis. **Dimitri Theuerkauff:** Methodology, Investigation, Formal analysis. **Aude Nommick:** Methodology, Investigation, Formal analysis. **Jehan-Hervé Lignot:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

No competing interests declared.

Data availability

All data will be made public upon request.

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