

RESEARCH ARTICLE

Osmoregulation and salinity-induced oxidative stress: is oxidative adaptation determined by gill function?

Georgina A. Rivera-Ingraham^{1,*}, Kiam Barri¹, Mélanie Boël¹, Emilie Farcy¹, Anne-Laure Charles², Bernard Geny² and Jehan-Hervé Lignot¹

ABSTRACT

Osmoregulating decapods such as the Mediterranean green crab *Carcinus aestuarii* possess two groups of spatially segregated gills: anterior gills serve mainly respiratory purposes, while posterior gills contain osmoregulatory structures. The co-existence of similar tissues serving different functions allows the study of differential adaptation, in terms of free radical metabolism, upon salinity change. Crabs were immersed for 2 weeks in seawater (SW, 37 ppt), diluted SW (dSW, 10 ppt) and concentrated SW (cSW, 45 ppt). Exposure to dSW was the most challenging condition, elevating respiration rates of whole animals and free radical formation in hemolymph (assessed fluorometrically using C-H₂DFFDA). Further analyses considered anterior and posterior gills separately, and the results showed that posterior gills are the main tissues fueling osmoregulatory-related processes because their respiration rates in dSW were 3.2-fold higher than those of anterior gills, and this was accompanied by an increase in mitochondrial density (citrate synthase activity) and increased levels of reactive oxygen species (ROS) formation (1.4-fold greater, measured through electron paramagnetic resonance). Paradoxically, these posterior gills showed undisturbed caspase 3/7 activity, used here as a marker for apoptosis. This may only be due to the high antioxidant protection that posterior gills benefit from [superoxide dismutase (SOD) in posterior gills was over 6 times higher than in anterior gills]. In conclusion, osmoregulating posterior gills are better adapted to dSW exposure than respiratory anterior gills because they are capable of controlling the deleterious effects of the ROS production resulting from this salinity-induced stress.

KEY WORDS: Antioxidant defense, *Carcinus aestuarii*, ROS production, Osmoregulation

INTRODUCTION

The generation of reactive oxygen species (ROS) is a natural process derived from membrane-linked electron transport and normal metabolic processes (Fridovich, 1995). However, environmental changes such as increases/decreases in temperature, oxygen and pH, but also salinity, are often accompanied by dramatic increases in ROS formation and changes in antioxidant capacity. An increase in ROS formation surpassing the cellular antioxidant defenses leads to increased cellular damage, mutagenesis and ultimately cell death. Estuarine animals can represent interesting experimental models for

oxidative stress (OS)-related research, as these organisms must cope with large cyclic (daily and seasonal) fluctuations in temperature, salinity, UV radiation or air exposure that characterize these habitats (Freire et al., 2012). These drastic physicochemical changes require estuarine organisms to set in place cellular mechanisms (often at the level of specialized tissues) in order to cope with such environmental variations and which frequently result in an increase of ROS production. Salinity is, among the natural stressors, one of the most determinant factors affecting the distribution and physiology of estuarine animals (Peterson and Ross, 1991; Henry et al., 2012; McNamara and Faria, 2012). While OS in aquatic ecosystems is an increasing field of study (see Lushchak, 2011; Abele et al., 2012), there is, still, a significant lack of information regarding how estuarine invertebrates cope with large salinity changes that occur in their environment, notably in terms of ROS production and the mechanisms that are put in place to avoid their deleterious effects (e.g. Freire et al., 2012).

Green crabs from the *Carcinus* genus (Crustacea, Decapoda) are one of the most widespread osmotically tolerant species in estuaries (Fulton et al., 2013) and thus represent an interesting physiological model in osmoregulation and salinity-tolerance studies. These robust organisms are of special interest, showing high tolerance to environmental and physiological changes. It is this tolerance to starvation (Matozzo et al., 2011), cold temperature (Kelley et al., 2013), hypoxia (Simonik and Henry, 2014), water alkalization (Cripps et al., 2013) and a wide diversity of pollutants (Henry et al., 2012) that has allowed, for example, the invasive Atlantic green crab *Carcinus maenas* to colonize many different regions of the world, and the Mediterranean green crab *Carcinus aestuarii* to invade Japan (Carlton and Cohen, 2003).

For these carcinoid osmoregulating crustaceans, two groups of spatially segregated gills are found: anterior gills serve mainly respiratory purposes, but also have a pronounced capacity for active ammonia excretion (Weihrach et al., 1998), which occurs in crabs acclimating to low environmental salinity (e.g. Mangum et al., 1976); posterior gills contain osmoregulatory structures (e.g. Copeland and Fitzjarrell, 1968; Gilles et al., 1988; Péqueux, 1995) and constitute the important ion-regulating organs (e.g. Siebers et al., 1982). These decapod crabs possess nine phyllobranchiate gills in each branchial chamber: the anterior pairs (1–6) have an epithelial layer typical of gas-exchanging tissues, composed of pillar cells and thin pavement epithelial cells (<1 µm) with sparse mitochondria (Compere et al., 1989); the last three pairs (7–9) are involved in respiration and osmoregulation (Compere et al., 1989) and exhibit the highest Na⁺/K⁺-ATPase activity (Siebers et al., 1982). Within these posterior gills, some lamellae are involved in respiration and show a structure as described above, while others share both functions and present an osmoregulatory tissue as well (Goodman and Cavey, 1990), located proximal to the afferent vessel (Compere et al., 1989). These specialized

¹Groupe fonctionnel AEO (Adaptation Ecophysologique et Ontogénèse), Université de Montpellier, UMR 9190 MARBEC, Place Eugène Bataillon, Montpellier 34095, France. ²EA 3072, Fédération de Médecine Translationnelle de Strasbourg, Université de Strasbourg, 11 rue Humann, Strasbourg 67000, France.

*Author for correspondence (georgina.rivera-ingraham@univ-montp2.fr)

List of symbols and abbreviations

CAT	catalase
C-H ₂ DFFDA	5-(and-6)-carboxy-2',7'-difluorodihydrofluorescein diacetate
CS	citrate synthase
cSW	concentrated seawater
DCF	2',7'-dichlorofluorescein
DM	dry mass
dSW	diluted seawater
EPR	electron paramagnetic resonance
M _b	body mass
OP	osmotic pressure
OS	oxidative stress
P _{O₂,crit}	critical oxygen partial pressure
P _{O₂}	oxygen partial pressure
RLU	relative light units
ROS	reactive oxygen species
RR	respiration rate
RR _{or}	respiration rate in the oxyregulatory interval
SOD	superoxide dismutase
SW	seawater

tissues present a thick prismatic epithelium (10 µm) composed of mitochondria-rich cells with numerous membrane infoldings, characteristic of salt-transporting tissues. Unlike the anterior gill epithelium, these posterior gills suffer significant structural changes, notably at the level of the prismatic epithelium responsible for transepithelial salt transport, when animals are exposed to diluted seawater, indicating their role in osmoregulation (see Compere et al., 1989). Thus, having in part different functions, anterior and posterior gills in crabs constitute an interesting model for studying function-related differential adaptation to environmentally induced OS.

Aside from these ultra-morphological studies, there is a large amount of biological, ecological and molecular knowledge available for *C. maenas*, and it is thus considered to be a suitable experimental model in areas such as ecotoxicology (reviewed by Rodrigues and Pardal, 2014). Here, a closely related species has been studied, the Mediterranean green crab *C. aestuarii*. This species has been significantly less studied from an ecophysiological point of view than its Atlantic counterpart *C. maenas*. The objective of our study was not only to contribute to the ecophysiological knowledge of the Mediterranean green crab but also to use it as a model to study how specialized organs such as gills can manage enhanced ROS formation resulting from different functions (osmoregulation versus respiration). We compared anterior (respiratory) and posterior (osmoregulatory) gills to determine the degree of stress suffered by these two functionally different tissues under different environmental salinity and to determine which mechanisms, if any, are put in place to minimize such stress. Studies of this type on crustacean species are scarce (e.g. Paital and Chainy, 2010; Van Horn et al., 2010; Freire et al., 2012) but those independently analyzing anterior and posterior gills are even scarcer (e.g. Freire et al., 2011). We approached this subject by carrying out an energetic study in conjunction with a thorough analysis of the oxidative metabolism of the two types of gills after exposing *C. aestuarii* to different environmental salinity (ranging from hypersaline to hyposaline seawater) in order to set the basis for further studies focused on disentangling the mechanistic pathways in which similar tissues with slightly different functions are able to cope with differential OS levels.

MATERIALS AND METHODS**Animal collection and maintenance**

Mediterranean green crabs *C. aestuarii* (Nardo 1847) were collected in January 2015 from Palavas-les-Flots and Sète lagoons, located on the Mediterranean coast of France. As previous studies have shown that male and female crabs respond differently to salinity changes (Neufeld et al., 1980), only females were considered in the study (being the most abundant from the animals collected). Crabs, with an average size of 3.52±0.07 cm and a mass of 16.5±4.3 g, were transported to the laboratory where they were disinfected by dipping them in seawater (SW; 1090 mOsm kg⁻¹, ~37 ppt) with 500 ppt KMnO₄ for 5 min (as in Paital and Chainy, 2012). The animals were rinsed twice in clean SW, then allowed to acclimate to laboratory conditions (SW at 19°C) for 1 week. No mortality was recorded during this period of time.

Osmoregulation

After acclimation, 72 animals were used for analysis of the osmoregulatory capacity of the species. In quadruplicate, groups of three animals were exposed to six different salinities: 150 (~5 ppt), 300 (~10 ppt), 500 (~17 ppt), 750 (~25 ppt), 950 (~32 ppt) and 1300 mOsm kg⁻¹ (~44 ppt). Animals were maintained in isolated boxes for a minimum of 72 h to avoid stress due to interindividual interactions, which is sufficient time for osmoregulation to be achieved in similar species such as *C. maenas* (Lovett et al., 2001). After this time, a hemolymph sample was taken using a 1 ml hypodermic syringe. Osmotic pressure (OP) of hemolymph samples was immediately quantified by freezing point depression osmometry (Model 3320, Advanced Instruments, Inc., Norwood, MA, USA). Values were plotted against the osmolality of the external medium. Mortality rates for each treatment were also recorded.

Experimental setup

Three systems were established for the main study, each composed of five groups of at least six interconnected boxes of maximum dimension 20×13×10 cm (l×w×h). All of them were supplied with water through a flow-through system of 150 l of SW equipped with two aeration and two filtration systems. A minimum of 30 individuals were introduced into individual, numbered boxes for individual follow-up. Crabs were fed thawed mussels every 48 h and were allowed to eat for a period of 6 h. After this time, all remaining food was removed in order to avoid the deterioration of water quality. During the first 4 days of isolation, animals were exposed to SW to allow them to acclimate to the captivity conditions. After this time, the water was changed for each of the three systems: one to diluted SW (dSW; 295 mOsm kg⁻¹, ~10 ppt), the second to hypersaline or concentrated SW (cSW; 1320 mOsm kg⁻¹, ~45 ppt) and the third to clean SW (control). Because of the characteristics of the flow system used, salinity change occurred gradually and equilibration to the target salinity values occurred in a maximum time of 2 h.

Every 48 h, water quality was checked using Quantofix[®] nitrate/nitrite test strips (Macherey-Nagel GmbH and Co., Duren, Germany), and water temperature and salinity were registered using a YSI 85 handheld meter (YSI Incorporated, Yellow Springs, OH, USA). Water was changed weekly unless nitrate and/or nitrite values increased over 10 and 1 mg l⁻¹, respectively.

Sampling

Two weeks after salinity changes, all animals were sampled. To minimize animal suffering, crabs were placed at -20°C for 2 min and all further manipulations were conducted on ice to maintain anesthetic conditions. Animals were first measured and weighed. Immediately after, and for control purposes, a hemolymph sample was taken for OP quantification following the same procedure described above; 18 of these hemolymph samples (6 per treatment) were used for ROS determination as explained below. For a minimum of 10 animals per treatment, gills were carefully dissected: the second and third pairs of gills (as representatives of respiratory gills) were preserved for ROS formation/caspase and antioxidant activity analyses, respectively; the seventh and eighth pairs of gills (as representatives of gills involved in osmoregulation) were preserved for the same purposes. All samples were stored at -80°C until further analyses.

Respiration measurements

An additional 20 animals (5 per treatment) were used for respiration measurements. To avoid any possible interference in the measurements related to nutritional stress (Herreid, 1980), animals were fasted for 48 h prior to the start of the experiment. Crabs were introduced into 750 ml chambers provided with a magnetic stirrer (to homogenize O_2 concentration over the water column) and equipped with an oxygen sensor spot (OXSP5, PyroScience GmbH, Aachen, Germany) glued to the chamber wall using silicon paste. Because we recorded large interindividual variation in activity (which had a clear effect on respiration rate, RR), animals within the chambers were enclosed in a $6 \times 6.6 \times 3$ cm ($l \times w \times h$) box of 1 mm^2 plastic mesh in order to exclude the perturbing effect of animal activity. This structure was raised from the bottom of the chamber to avoid disturbance of the crab by the magnetic stirrer. Measurements were conducted in filtered SW ($0.2 \mu\text{m}$ Whatman) at each of the three selected salinities (10, 37 and 45 ppt). Animals were allowed to acclimate for 30 min to avoid the influence of manipulation on RR. After this time, and in fully oxygenated medium, chambers were air-tight sealed and RR was recorded as a function of declining oxygen partial pressure (P_{O_2}) over time. Measurements were carried out with a non-invasive 4-channel FireSting O_2 meter (PyroScience). Four parallel measurements were obtained at a time (including a blank). Data were recorded at 3 s intervals at 20°C , and the experiments were stopped when O_2 was completely consumed from each of the chambers containing an animal. RR is expressed as $\text{nmol } O_2 \text{ h}^{-1} \text{ g}^{-1}$ body mass (M_b). A critical environmental oxygen partial pressure ($P_{O_{2,crit}}$) was determined based on section-wise linear regression of each respiration curve (Dugleby, 1984), above which animals respire in an oxyregulating manner. Below this point, specific RR decreases with environmental P_{O_2} and animals switch to oxyconforming respiration, usually coinciding with the onset of anaerobic metabolism (see Herreid, 1980; Pörtner and Grieshaber, 1993).

Respiration measurements were also carried out on freshly dissected anterior and posterior gills. Tissues were immersed in an individual well of a 96-well microplate, equipped with an oxygen sensor spot (as mentioned above) glued to the bottom of the well using silicon paste. Measurements were conducted in sterile filtered SW ($0.2 \mu\text{m}$ Whatman) at each of the three selected salinities and supplemented with 25 mmol l^{-1} sodium Hepes to avoid pH changes during the measurement. In fully oxygenated medium, chambers were air-tight sealed using a coverslip and data were recorded as mentioned above. Gills were allowed to respire until O_2 reached $<10\%$ in each well containing a tissue, which never took more than 3.7 h. Only normoxic O_2 consumption rates were considered in the study. These were calculated as the slope of decreasing P_{O_2} in all cases above 30% air saturation, before the onset of oxyconformity. After each experiment, gills were rinsed in MilliQ water and dried at 55°C for a minimum of 48 h. For these measurements, RR is expressed as $\text{nmol } O_2 \text{ h}^{-1} \text{ g}^{-1}$ dry mass (DM).

Mitochondrial density

Citrate synthase (CS) activity, frequently used as an indicator of mitochondrial density, was measured in anterior and posterior gill homogenates following Srere et al. (1963). Gill samples were homogenized 1:10 (w:v) in a buffer composed of 20 mmol l^{-1} Tris, 1 mmol l^{-1} EDTA, 0.1% Tween 20 at a pH 7.4. Tissue homogenization was achieved using four stainless steel milling balls (Retsch, no. FR0120) in a Mixer Mill MM 400 (Retsch GmbH, Haan, Germany; 1 min at 30 beats s^{-1}). CS activity was measured in duplicate in $6.5 \mu\text{l}$ of supernatant and using a final volume of $165 \mu\text{l}$. Baseline activity (in the absence of oxaloacetic acid) and free Co-A production (unchained by the addition of oxaloacetic acid) were read for each sample as changes in absorbance at 412 nm for at least 3 min at 20°C using a microplate reader (Tecan Infinite M200, Tecan, Männedorf, Switzerland). Results are expressed as units ($U, \mu\text{mol min}^{-1}$) per mg protein, quantified after Bradford (1976).

Metabolite analyses

Glucose and lactate concentrations were assessed in anterior and posterior gills, previously homogenized following the same procedure as described for the CS measurements. Metabolite analyses were conducted on the

resulting supernatants using the Glucose RTU kit (ref. 61 269) and the Lactate PAP kit (ref. 61 192), with the Calimat kit (ref. 62 321) as a reference and following the instructions provided by the manufacturer (Biomérieux, Marcy l'Etoile, France). Values are expressed per mg protein.

Assessment of the general redox state

As recommended by Forman et al. (2015), in the present paper we use the term ROS to name a group of substances containing one or more activated atoms of oxygen (radicals or not) that are generated by oxygen reduction. The redox state of the animals was assessed fluorometrically by using the membrane-permeable 5-carboxyl-2',7'-difluorodihydrofluorescein diacetate (C-H₂DFFDA, Molecular Probes C-13293) on fresh hemolymph samples, which is converted to 2',7'-dichlorofluorescein (DCF). Samples were diluted 1:100 (v:v) in isotonic SW (obtained for each treatment as the average of the OP values obtained for 10 previously analyzed animals; 622.5, 1006 and 1350 mOsm kg^{-1} for dSW, SW and cSW treatments, respectively). In order to account for possible differences in DCF fluorescence related to differences in salinity, three H₂O₂ reference curves (one for each corresponding salinity) were analyzed in parallel to the samples, ranging from 0% to 0.7% H₂O₂ in 0.1% intervals. Even though it has been shown that C-H₂DFFDA is not a specific indicator of H₂O₂ formation for various reasons (Grisham, 2012; Kalynaraman et al., 2012), this molecule was chosen for the reference curve because it is known to be the most stable ROS. Samples were incubated for 10 min in $10 \mu\text{mol l}^{-1}$ in C-H₂DFFDA (excitation 488 nm, emission 525 nm) in a flat-bottomed black microplate. All samples were analyzed in duplicate. Fluorescence readings were taken every 5 min for 30 min using a microplate reader.

Taking into account the complex redox reactions of C-H₂DFFDA and its limitations for directly measuring H₂O₂ (see review by Kalynaraman et al., 2012), in order to validate and complement the measurement described above, ROS generation was also assessed on gill tissues through electron paramagnetic resonance (EPR). Frozen anterior and posterior gills were homogenized on ice for 1 min using a manual potter at a ratio 1:10 (w:v) in a buffer (50 mmol l^{-1} Tris, 34 mmol l^{-1} SDS, 10% glycerol; G. Meyer, personal communication) supplemented with $1 \mu\text{g ml}^{-1}$ of each of the protease inhibitors leupeptin, pepstatin and aprotinin. Homogenates were centrifuged at $17,000 \text{ g}$ for 10 min at 4°C . The experimental protocol for ROS detection was adapted from Mrakic-Sposta et al. (2012). A $25 \mu\text{l}$ sample of the resulting supernatants was mixed with spin probe CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine HCl, $200 \mu\text{mol l}^{-1}$). The mixed solution was introduced into a glass EPR capillary tube (NoxygenScience Transfer, Diagnostics, Elzach, Germany), and then placed inside the cavity of the e-scanspectrometer (Bruker, Rheinstetten, Germany) for data acquisition. The analysis temperature was maintained at 37°C by the temperature and gas controller Bio III unit, interfaced to the spectrometer. Detection of ROS production was conducted using a BenchTop EPR spectrometer-SCAN under the following EPR settings: center field $g=2.011$; field sweep 60 G; microwave power 20 mW; modulation amplitude 2 G; conversion time 10.24 ms; time constant 40.96 ms, number of scans: 10. The EPR signal is proportional to the unpaired electron number. The results are expressed in $\mu\text{mol min}^{-1} \text{ mg}^{-1}$ tissue.

Antioxidant defense activity

Catalase (CAT) and superoxide dismutase (SOD) activities were measured spectrophotometrically in anterior gills (pair 3) as well as in posterior gills (pair 7). All samples were diluted 1:5 and 1:7 (w:v) for CAT and SOD measurements, respectively, in a 50 mmol l^{-1} KPi buffer with 120 mmol l^{-1} KCl (supplemented with 0.1% Triton X-100 for CAT measurements). Tissue homogenization was achieved as previously described for CS activity measurements. CAT activity was measured in supernatants as the decomposition of a 0.3 mol l^{-1} H₂O₂ solution in a 50 mmol l^{-1} KPi buffer (after Aebi, 1984) while SOD activity was determined using the cytochrome oxidase assay following the protocol described by Livingstone et al. (1992). Values were related to protein content measured after Bradford (1976). All measurements were carried out in triplicate in a microplate reader.

Cell damage

Apoptosis was assessed as the activity of caspases 3 and 7, involved in apoptotic cell disintegration. This was determined in frozen gill samples (pairs 2 and 8) previously homogenized at 4°C at a ratio 1:100 (w:v) in a lysis buffer composed of 25 mmol l⁻¹ Hepes, 5 mmol l⁻¹ MgCl₂, 1 mmol l⁻¹ EGTA and 1 µl ml⁻¹ of each of the protease inhibitors leupeptin, pepstatin and aprotinin (Strahl and Abele, 2010; Rivera-Ingraham et al., 2013). Homogenization was conducted following the same procedure as for the CS analyses. Apoptosis level in supernatants was measured using the Caspase-Glo[®] 3/7 kit (Promega Corporation, Madison, WI, USA) at 20°C, according to the manufacturer's instructions. Values were recorded in a Tecan microplate reader and expressed as relative light units (RLU) referred to mg protein content (Bradford, 1976).

Statistical analyses

All values are expressed as means±s.e.m. Data sets were analyzed using SPSS 15.0 (SPSS Inc., USA). When data complied with the assumptions for parametric analyses, the resulting data were compared using ANOVA (followed by Student–Newman–Keuls *a posteriori* multiple comparison test). When this was not the case, Kruskal–Wallis tests were conducted followed by Mann–Whitney pair-wise comparisons (*U*-tests).

RESULTS

Osmoregulation

Carcinus aestuarii is an osmoconformer species down to 750 mOsm kg⁻¹ of environmental salinity (Fig. 1). Below this point, the species weakly osmoregulates the osmolality of its internal medium. The difference between external medium and hemolymph osmolality increases as salinity decreases, ranging from an average of 146 mOsm kg⁻¹ to 311 mOsm kg⁻¹ at environmental salinities of 500 and 150 mOsm kg⁻¹, respectively. Mortality rates were highest for animals immersed in 150 mOsm kg⁻¹ medium (41.67%) and null at 750 mOsm kg⁻¹, a salinity that roughly corresponds to the point at which animals switch from an osmoconforming to an osmoregulating physiology.

Whole-animal respiration

In whole-animal measurements, all respiratory profiles showed two different phases regardless of the environmental salinity: at higher

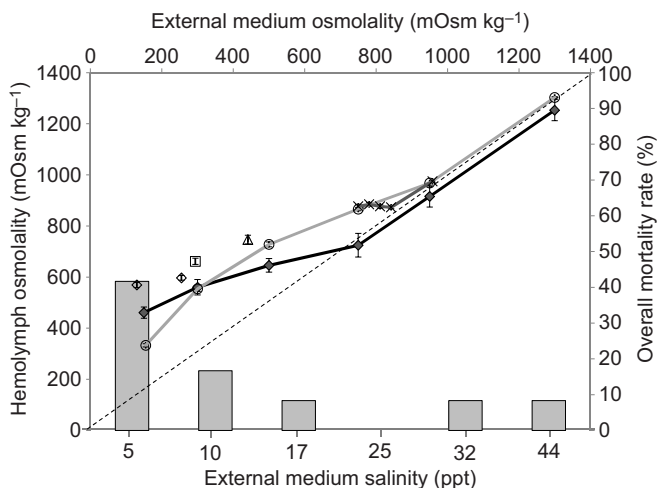


Fig. 1. Variations in hemolymph osmolality in relation to the salinity of the external medium for *Carcinus aestuarii*. Data are shown for *C. aestuarii* (present study; black line and filled diamonds) and for *Carcinus maenas* juveniles (light gray line and circles; adapted from Cieluch et al., 2004) and adults (dark gray line with crosses; adapted from Henry, 2005); triangles, adapted from Jillette et al., 2011; square, adapted from Henry et al., 2002; and diamonds, adapted from Lovett et al., 2001). Mortality rates registered for *C. aestuarii* (present study) are represented by the gray bars.

oxygen concentration, organisms were oxyregulating until reaching $P_{O_{2,crit}}$, the point at which they switch to oxyconforming respiration (Fig. 2A). Data analyses indicate that as the salinity of the environmental medium decreased, there were significant increases in $P_{O_{2,crit}}$ ($P<0.001$), which was displaced towards higher $P_{O_{2,crit}}$. Decreased salinity also produced significant increases in the average RR above $P_{O_{2,crit}}$ (oxyregulatory respiration, RR_{or} ; $P<0.001$; Fig. 2B).

Excised-gill respiration, mitochondrial density and metabolite content

The respiratory profiles of isolated gills showed that RR decreased in an oxyconforming manner and no $P_{O_{2,crit}}$ was detected. RR showed large interindividual deviations, which determined that no significant differences were registered among salinities for anterior ($P=0.241$) or posterior gills ($P=0.591$). There were no differences between these two types of gill at 10 ppt ($P=0.111$), 37 ppt ($P=0.564$) or 45 ppt ($P=0.444$) (Fig. 3A). Nevertheless, when analyzing the results for each crab individually (by obtaining a ratio between the RR of posterior and anterior gills), we observed that osmoregulation (exposure to 10 ppt) caused a significant imbalance in the respiration rates of both types of gills due to a slight increase in posterior gill RR but mainly to a significant decrease in anterior gill metabolism ($P<0.01$; Fig. 3A).

CS activity remained constant in anterior gills, regardless of environmental salinity ($P=0.365$) and always significantly lower than in posterior gills. However, in posterior gills we recorded a significant increase upon dSW exposure ($P<0.01$), reaching 1.8-fold higher than in SW or cSW (Fig. 3B).

Glucose concentration did not vary in either anterior ($P=0.292$) or posterior ($P=0.638$) gills (data not shown). However, lactate concentration showed a significant increase in anterior gills of crabs exposed to dSW ($P<0.05$; Fig. 3C).

Free radical formation

ROS formation, measured as DCF fluorescence in hemolymph samples, showed that as salinity of the external medium decreases, ROS formation increases ($P<0.001$; Fig. 4). EPR analyses revealed that while anterior gills showed no differences in ROS formation ($P=0.561$), posterior gills showed a significant increase when exposed to 10 ppt ($P<0.05$; 10 ppt>37 ppt=45 ppt). However, only at 10 ppt did we observe significantly higher values in posterior gills as compared with anterior gills ($P<0.01$).

Antioxidant activity

Among salinity treatments, anterior gills showed no significant differences in CAT ($P=0.383$) or SOD activity ($P=0.494$). For posterior gills, however, decreasing environmental salinity significantly increased both CAT ($P=0.038$; 10 ppt>37 ppt=45 ppt; Fig. 5A) and SOD ($P=0.023$; 10 ppt>37 ppt>45 ppt; Fig. 5B) activity. Among gill types, the greatest differences were recorded at 10 ppt, and enzymatic activity in posterior gills was 1.6- and 6.3-fold higher than in anterior gills for CAT and SOD, respectively.

Cell damage

The decrease in environmental salinity resulted in significant increases in caspase 3/7 activity in anterior gills ($P=0.036$), while for posterior gills, no differences among treatments were observed ($P=0.068$; Fig. 6). The largest difference among gills types was detected at 10 ppt (even though this did not reach significance; $P=0.168$), with anterior gills showing an average of 2.2-fold higher caspase 3/7 activity than posterior gills.

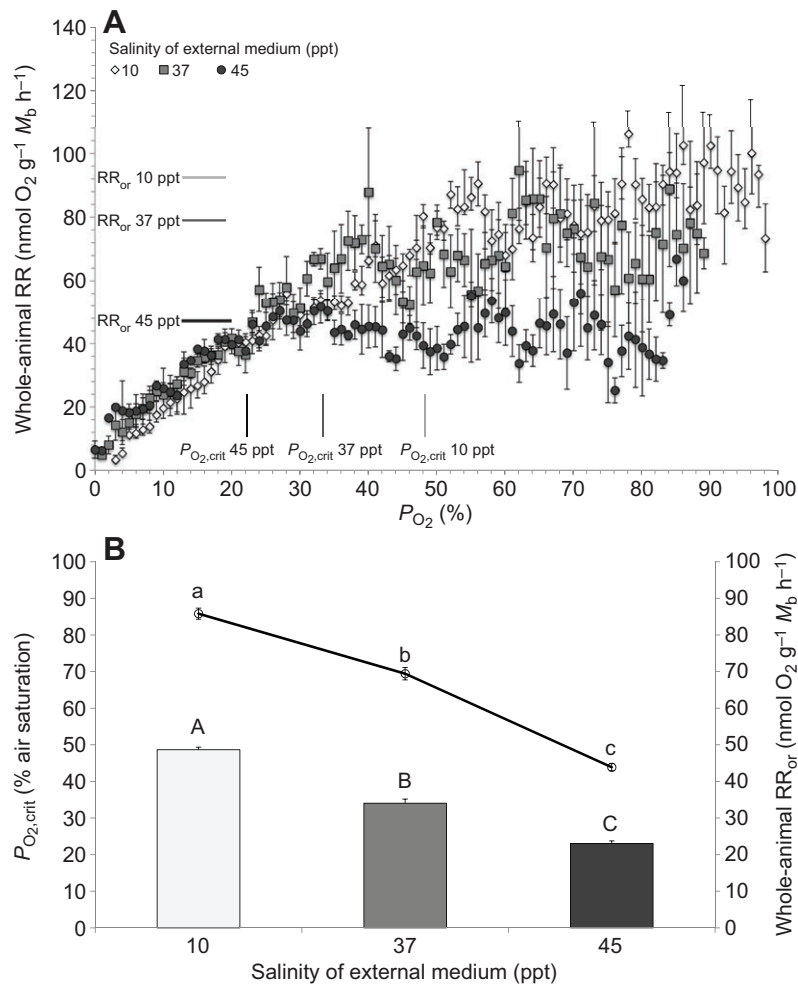


Fig. 2. Whole-animal respirometry results under different environmental salinities. (A) Respirometry profiles showing the average critical oxygen partial pressure ($P_{O_2,crit}$) and respiration rate during the oxyregulating period (RR_{or}) for each salinity treatment. (B) Differences in $P_{O_2,crit}$ (bars) and in RR_{or} (black line) for whole-animal respirometry. Different letters represent significantly different values based on a Student–Newman–Keuls *post hoc* multiple comparison test. M_b , body mass.

DISCUSSION

This study not only provides new ecophysiological information regarding *C. aestuarii* but also highlights the value of dissociating anterior and posterior gills as physiological models for the study of function-related oxidative metabolism.

Carcinus aestuarii: adaptations to subtropical estuarine environments?

Even though the overall osmoregulation pattern of *C. aestuarii* is similar to that of its sibling species *C. maenas*, showing a clear osmoconforming physiology in SW and cSW, there are, however, some notable differences concerning the hyperosmoregulating capabilities of the two species. Firstly, the point at which animals start osmoregulating hemolymph osmotic pressure differs between species: for *C. aestuarii*, this point is established at between 500 and 750 mOsm kg⁻¹ (~17–25 ppt; present study) while for *C. maenas* it has been reported to be around 800 mOsm kg⁻¹ (~27 ppt; Lovett et al., 2001), 840 mOsm kg⁻¹ (~28 ppt; Henry, 2005), 900 mOsm kg⁻¹ (~31 ppt; Thurberg et al., 1973), or even up to 950 mOsm kg⁻¹ (~32 ppt) for *C. maenas* juveniles (Cieluch et al., 2004) (Fig. 1). We also observed differences regarding the degree of hyperosmoregulating capability. From the studies by Henry et al. (2002), Lovett et al. (2001) or Jillette et al. (2011) (values shown in Fig. 1), we conclude that *C. maenas* is a stronger regulator than its Mediterranean counterpart, as adult crabs immersed in dSW maintain overall hemolymph osmolality higher than that of

C. aestuarii. Altogether, we suggest that by adopting these less energetically expensive osmoregulation strategies, *C. aestuarii* may be better adapted to the low environmental salinities that it frequently encounters from January to June in Mediterranean coastal lagoons and estuaries.

The respirometric analyses ($P_{O_2,crit}$ values) may also provide interesting insights into differences among species or acclimation/tolerance to other key variables such as temperature (e.g. Butler and Taylor, 1975). When exposing Atlantic green crabs to temperatures of 10°C, Taylor et al. (1977) observed a salinity-dependent shift in $P_{O_2,crit}$ in *C. maenas*: when crabs were immersed in SW, $P_{O_2,crit}$ was established at 25% air saturation while at 50% SW this value increased to 38% air saturation. The same authors reported that increasing temperature to 18°C caused $P_{O_2,crit}$ to shift towards higher oxygen concentrations, as would be expected (see review by Herreid, 1980) but the salinity-dependent change disappeared and $P_{O_2,crit}$ remained constant at 70 mm Hg (~45% air saturation). In our study, at temperatures as high as 19–20°C, we still registered salinity-dependent differences in $P_{O_2,crit}$ in *C. aestuarii*, which in SW is established at 34%, lower than the reported value for *C. maenas*. Taken together, our results support the hypothesis that *C. aestuarii* may also be tolerating higher water temperatures than *C. maenas*. These wider physiological ranges may be necessary qualities in order to live in the changing environmental conditions occurring within the Mediterranean coastal lagoons. Ecologically speaking, this would be coherent with the estuarine distribution of

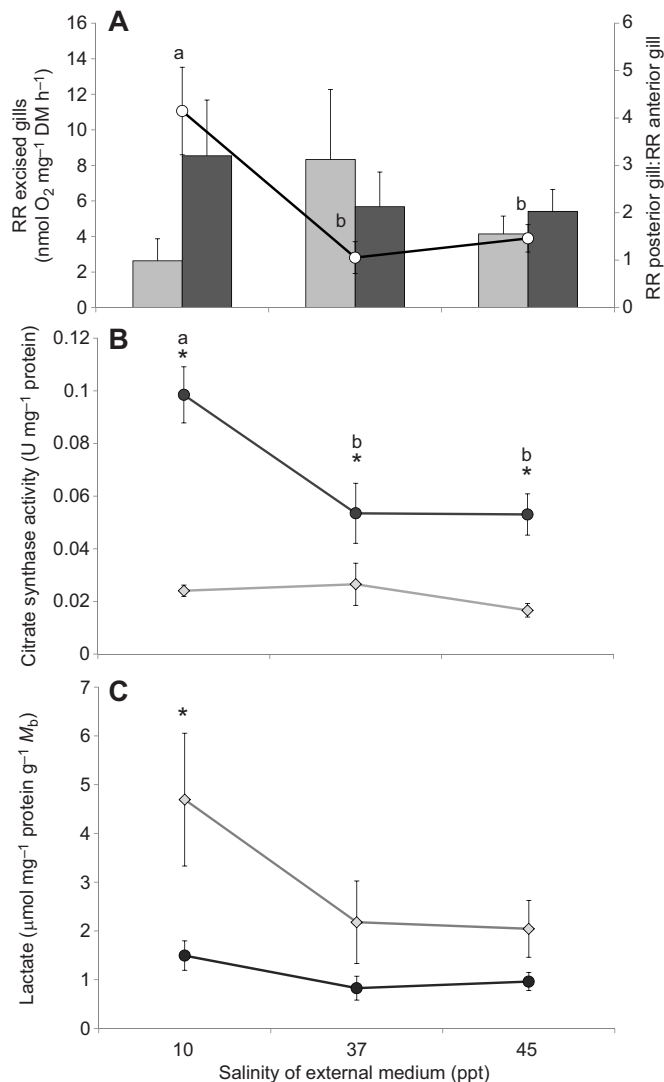


Fig. 3. Energetics of excised gills. (A) Respiration rates of isolated anterior gills (light gray bars) and posterior gills (dark gray bars). Because of the important interindividual differences recorded, this graph also includes the average ratio for each salinity treatment (obtained as RR posterior gill:RR anterior gill) for each single individual. Different letters represent significantly different values based on pairwise Mann–Whitney *U*-test comparisons. (B,C) Citrate synthase activity and lactate content, respectively, corresponding to anterior gills (light gray diamonds and lines) and posterior gills (dark gray circles and lines). Different letters represent significantly different values among salinity treatments based on Student–Newman–Keuls *a posteriori* multiple comparison test. *Significantly different values among gill types. DM, dry mass.

C. aestuarii because, given the transitional characteristics of estuaries, organisms inhabiting these areas are subject to high salinity variations. In areas such as Mediterranean lagoons, where *C. aestuarii* is commonly present, water salinity can oscillate between 5 and 40 ppt and temperature can reach 25–27°C (e.g. Souchu et al., 2001; Akin et al., 2005).

But how do these Mediterranean crabs physiologically respond to a decrease in water salinity?

Hypo-osmotic stress results in high ROS production and enhanced energy expenditure

Regardless of the species, osmoregulation is an energetically expensive process due to active ion pumping in specialized

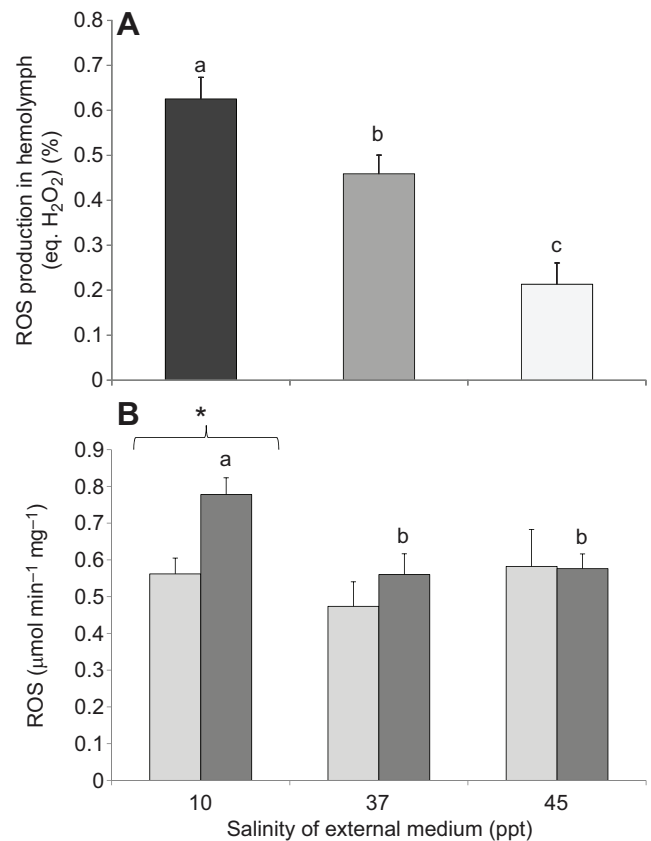


Fig. 4. Formation of free radicals. (A) ROS in hemolymph, determined fluorometrically using the ROS-dependent dye C-H₂DFFDA. (B) ROS in gill tissues, determined by electron paramagnetic resonance (EPR). Values with the same letter belong to the same subset based on Mann–Whitney *U*-test pairwise comparison (A) and Student–Newman–Keuls *a posteriori* multiple comparison test (B). *Significant difference among gill types.

epithelial cells that are rich in mitochondria, and *C. aestuarii* is no exception. When undergoing exposure to dSW, a number of ultrastructural changes occur within the apical and basolateral areas of the posterior gill cells, as detailed by Compere et al. (1989) and Charmantier (1998) for similar species such as *C. maenas*. Between 6 and 24 h after transfer to dSW, there is an upregulation of the transcripts encoding many of the membrane transporters involved in osmoregulation (Na⁺/K⁺-ATPase, carbonic anhydrase, sodium/glucose co-transporter, etc.; Towle et al., 2011). Additionally, there is a significant increase of the specific activity of the Na⁺/K⁺-ATPase in posterior gills, regulating the uptake of Na⁺ and Cl⁻ across gill epithelia. This explains the increase we registered in whole-animal respiration upon salinity decrease. A previous study by King (1965) also reported an increase of 33% in the RR of *C. aestuarii* (referred to as *C. mediterraneus* in the original publication) immediately after they were submerged in dSW (~19 ppt). Our experimental crabs, acclimated for 2 weeks to dSW, showed a much lower increase in RR (24% over SW-acclimated crabs). This may be due to the fact that, as in *C. maenas*, RR is maintained constantly higher than in animals acclimated to SW, but significantly lower than during the first 2–3 h of hyposmotic shock (Taylor, 1977), a time when a burst in RR occurs, likely enabling the animals to fuel the first compensatory mechanisms. For other crabs species such as *Scylla serrata*, also a hyperosmoregulator (Chen and Chia, 1997), long-term acclimation to 10 ppt SW results in an increase of 55% of their RR (Paital and

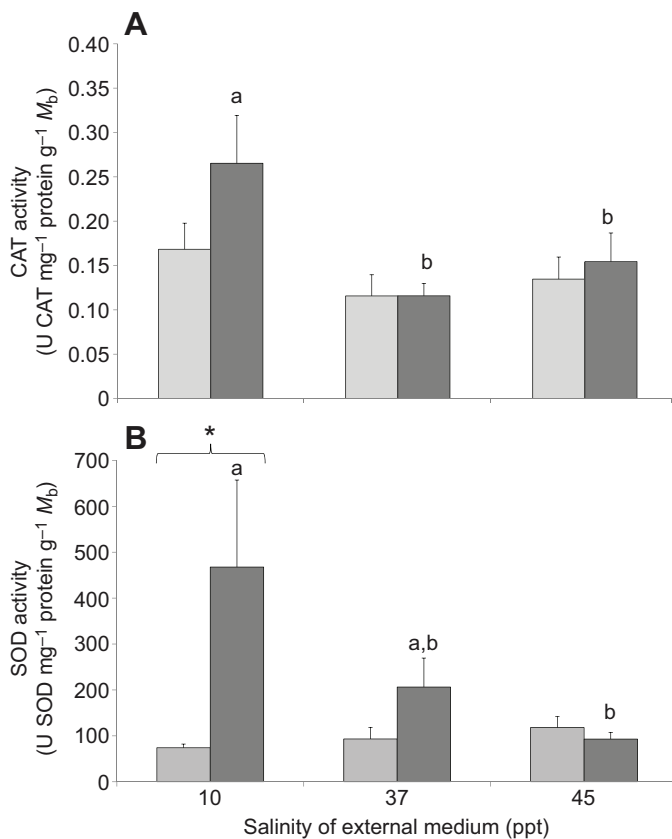


Fig. 5. Antioxidant activity in *C. aestuarii* gills. (A) Catalase (CAT). (B) Superoxide dismutase (SOD). Light gray bars represent anterior gills (gill pair 3) while dark gray bars correspond to posterior gills (gill pair 7). *Significant difference among gill types.

Chainy, 2010). This would indicate that even though the most energetically dependent processes have already been achieved (namely upregulation of osmoregulation-associated genes, morphological changes in osmoregulating tissues, etc.), there is still a need to actively fuel certain processes such as active ion transmembrane pumping. Based on our increased ROS levels

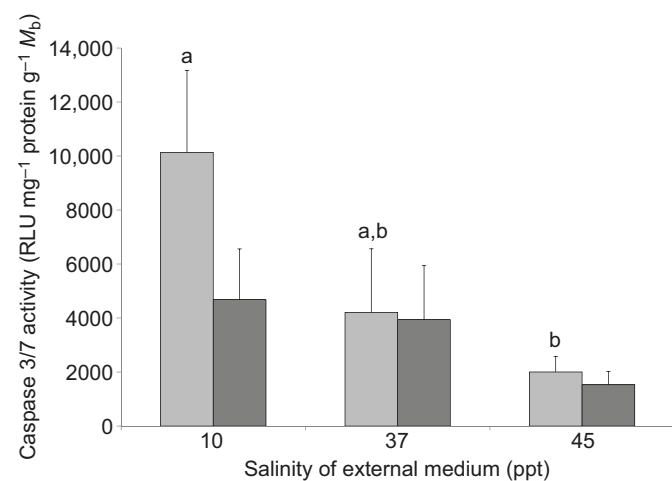


Fig. 6. Caspase 3/7 activity in anterior and posterior gills of *C. aestuarii*. Light gray bars represent anterior gills (gill pair 2) while dark gray bars correspond to posterior gills (gill pair 8). Error bars represent s.e.m. RLU, relative light unit.

in hemolymph samples (Fig. 4A) and in posterior gills upon dSW exposure, we can establish a clear relationship between energy expenditure and ROS production upon hemolymph hyperosmoregulation. However, there is no consensus as to how environmental salinity affects free radical metabolism of estuarine organisms. The concentration of NaCl is intimately correlated with ROS formation in mammalian (Zhou et al., 2005) and plant models (see Miller et al., 2010 and references therein). However, studies on other hyperosmoregulating estuarine crab species are controversial: De Martinez Gaspar Martins and Bianchini (2009) and Van Horn et al. (2010) reported an increase in antioxidant defense upon hypo-osmotic stress, in agreement with our results. Others report activation of the antioxidant system upon hypersalinity exposure (Freire et al., 2011), showing increased oxidative damage under similar circumstances (Paital and Chainy, 2010).

Two functions and two different responses: hypo-osmotic stress reveals differential oxidative stress adaptation between gill types

The respirometric analyses carried out on excised gills expose sharp differences between gill types in terms of adaptation to hypo-osmotic stress. Upon salinity decrease, anterior gills have a tendency to decrease their RR (Fig. 3A) while mitochondrial density remains unchanged (Fig. 3B). These observations, along with the significant increase in lactate concentration recorded in these tissues, suggests that aerobic metabolism in anterior gills may be impaired upon dSW exposure. While maintaining stable antioxidant defenses, these gills also suffer from a significant degree of cell damage (caspase activity). Lipofuscin-like pigment accumulation (as defined by Katz and Robinson, 2002), another relevant marker of oxidative stress (e.g. Rocchetta et al., 2014), also showed an increase on histological sections analyzed in the context of another study (G.A.R.-I. and J.H.L., manuscript in preparation), leading us to hypothesize that this general increase in damage markers may arise from the increased ROS resulting from exposure to dSW and the failure to put in place a clear defense mechanism as a response to dSW-derived ROS formation. However, the idea that the increased apoptosis levels could be the result of the lack of an effective cell volume regulation in anterior gills should not be discarded.

Posterior (osmoregulatory) gills behave much more protectively than anterior gills upon a decrease in salinity; this is probably an adaptive response as these tissues fuel energy-dependent processes related to dSW exposure. These tissues carry a part of the burden of ion transport upon hypo-osmotic stress, and have therefore increased RR and greater potential for producing ATP, as has been demonstrated in other decapod species (see review by Jiménez and Kinsey, 2015). The small increase we registered in RR of *C. aestuarii* posterior gills is likely due to the 1.8-fold increase in the number of mitochondria (as derived from the CS measurements shown in Fig. 3B). In agreement with our results, Machado Lauer et al. (2012) showed in *Neohelice granulata* how exposure to dSW (2 ppt) results in an increase in mitochondrial density, but, more interestingly, these mitochondria in turn decrease their membrane potential upon hypo-osmotic stress: whether this decrease in mitochondrial membrane potential is due to intense respiration or to other pathological (or not) states and how these changes affect ROS formation deserves further attention. A deeper analysis on this subject would help to determine the origin of the dSW-related ROS formation within gill tissues. Traditionally, mitochondria are considered as the major ROS producers, especially under stress, being ROS-formation rate dependent on mitochondrial membrane

potential (reviewed by Abele et al., 2007). Based on these premises, such a significant mitochondrial enrichment occurring within posterior gills may very well be a double-edge sword: while being essential for correctly acclimating to dSW, they may also be responsible for the increased ROS formation we quantified in these tissues through the EPR analysis (Fig. 4B) and likely contribute to the increased DCF fluorescence measured in the hemolymph (Fig. 4A). But other hypotheses consider that healthy mitochondria have a limited contribution to ROS formation (see Nohl et al., 2004), and that these might be mainly involved in signaling pathways as will be further discussed below.

Regardless of whether these ROS originated from mitochondrial respiration or from other extramitochondrial sources [e.g. activity of certain oxidases such as xanthine oxidase, NAD(P)H oxidases, etc.; e.g. Bedard and Krause, 2007], taken together, we conclude that exposure to dSW causes posterior gills to increase their number of mitochondria, increase ROS production (as seen through EPR measurements; Fig. 4B) but also enhance their antioxidant activity (CAT and SOD; Fig. 5), avoiding significant cell damage and demonstrating a clear ‘preparation for oxidative stress’ behavior consisting of a notable upregulation of their antioxidant defenses.

Is the increase in ROS formation involved in enhancing antioxidant activity in posterior gills?

In the present study, regardless of the increase in DCF fluorescence that exposure to dSW induces, cell damage levels in posterior gills remain unchanged, allowing us to hypothesize that the induction of antioxidant enzymes (notably SOD) may occur before the dSW-derived ROS formation overwhelms the basal antioxidant defense of the cells. Is this a case of oxidative stress-induced antioxidant enzyme up-regulation? While ROS has long been known to have the potential to cause oxidative damage during exposure to abiotic stressors (reviewed by Lushchak, 2011), including salinity (e.g. Pinto Rodrigues et al., 2012), ROS are far from being solely harmful molecules. A growing number of studies demonstrate that ROS-mediated signaling is involved in maintaining redox balance and avoiding ROS-induced damage in a wide diversity of organisms and systems: within the kidney, for example, reports show how NaCl-induced ROS formation is involved in the activation of transcription factors upregulating transcription of osmoprotective genes (Zhou et al., 2005). Other studies in plants show that ROS may also be involved in signaling salt-induced stress responses. Some authors suggest that salt-induced oxidative stress in plants may be required to induce the necessary acclimation mechanisms. The review by Miller et al. (2010) provides a large compendium of evidence that supports the hypothesis of an oxidative stress-induced acclimation to abiotic stress. Exposure to other stressors such as hypoxia results in increased ROS formation, which has a protective effect on different cell types as demonstrated by many (see, for example, review by Welker et al., 2013; Hermes-Lima et al., 2015). The increased ROS formation activates the antioxidant defense in many species, ranging from flatworms (Zelck and Von Janowsky, 2004) to plants (Guan et al., 2000; Jiang and Zhang, 2003), and this increase in antioxidants has been coined ‘preparation for oxidative stress’ (Welker et al., 2013). In our study, we did indeed see that high ROS production in posterior gills was accompanied by increased antioxidant activity. A study by Lucu et al. (2008) also showed that long-term acclimation of Mediterranean green crabs to 10 ppt resulted in a significant increase of metallothionein content in posterior gills, which may have a further antioxidant role. These reports highlight that while posterior gill tissues support the osmoregulation procedure, they are clearly capable of putting in

place the necessary mechanisms to minimize the OS derived from enhanced ROS formation.

Conclusions and future perspectives

The evidence provided in this text shows how exposure to low salinity induces significant oxidative stress in *C. aestuarii*, affects hypoxia tolerance but also notably compromises the functional viability of anterior gills. Taken together, these results point to the fact that anterior and posterior gills not only differ in function but also have different strategies for coping with the oxidative stress derived from the decrease in environmental salinity. Posterior gills, containing mitochondria-rich cells, significantly enhance antioxidant defenses, namely SOD, in order to account for intense ROS production likely derived from osmoregulation. Further analyses dissociating these two gill types and their mitochondria will certainly open interesting perspectives on the evolution of antioxidant defense and the adaptive response to oxidative stress in functionally different tissues. For example, could the physiological costs of replacing osmoregulatory mitochondria-rich cells determine the fact that posterior gills should be protected at the expense of purely respiratory tissues? Also, this study raises the question as to how and when this upregulation of antioxidant defense occurs. Future research will benefit from the experimental use of anti- and pro-oxidants to disentangle the answers to these two questions.

Acknowledgements

The authors would like to thank Gregory Meyer (Université d'Avignon, France) for his useful advice on the EPR protocol and Evelyse Grousset, Caroline Ferrat and Romain Gros for their help in the sampling process. Additional thanks go to Guy Charmantier and two anonymous referees for their interesting comments and suggestions on the original manuscript.

Competing interests

The authors declare no competing or financial interests.

Author contributions

G.A.R.-I.: expertise in oxidative stress, experimental design, sampling, laboratory and data analyses, manuscript preparation. K.B.: laboratory and data analyses. M.B.: laboratory and data analyses. E.F.: laboratory and data analyses and manuscript preparation. A.-L.C.: expertise in oxidative stress, EPR measurements. B.G.: expertise in oxidative stress, EPR measurements. J.-H.L.: expertise in osmoregulation, sampling, experimental design, manuscript preparation.

Funding

This work was supported by Marie Curie Actions EU grant (FP7-PEOPLE-2013-IEF) [grant number: 622087-‘IAS-Life’].

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