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

Georgina A. Rivera-Ingraham¹, Kiam Barri¹, Mélanie Boël¹, Emilie Farcy¹, Anne-Laure Charles², Bernard Geny² and Jehan-Hervé Lignot³


2. EA 3072. Faculté de Médecine, Université de Strasbourg. 11 rue Humann, 67000 Strasbourg, France.
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 coexistence of similar tissues serving partially different functions allows studying differential adaptation, in terms of free radical metabolism, upon salinity change. Crabs were immersed for two weeks in seawater (SW) (37ppt), diluted SW (dSW, 10ppt) and concentrated SW (cSW, 45ppt). Exposure to dSW is the most challenging condition, elevating respiration rates of whole animals and free radical formation in hemolymph (assessed fluorometrically using C-H$_2$DFFDA). Further analyses considered anterior and posterior gills separately, and results evidenced that posterior gills are the main tissues fueling osmoregulatory-related processes since their respiration rates in dSW are 3.2-fold higher than in anterior gills which is accompanied by an increase in mitochondrial density (citrate synthase activity) and increased levels of ROS formation (1.4-fold greater, measured through electron paramagnetic resonance). Paradoxically, these posterior gills showed undisturbed caspase 3/7 activities, used here as a marker for apoptosis. This may only be due to the high antioxidant protection that posterior gills benefit from (SOD in posterior gills is over 6 times higher than in anterior gills). In conclusion, osmoregulating posterior gills are better adapted to dSW exposure than respiratory anterior gills since they are capable of controlling the deleterious effects of the ROS production resulting from this salinity-induced stress.

Summary statement: We here show how gill tissues with different functions (respiratory/osmoregulatory) differentially produce reactive oxygen species and we evidence how they are differently adapted to deal with oxidative stress.

Keywords: Antioxidant defense, *Carcinus aestuarii*, ROS production, osmoregulation.

Abbreviations used in the text:

CAT= catalase; C-H$_2$DFFDA= 5-(and-6)-carboxy-2',7'-difluorodihydrofluorescein diacetate; CS=citrate synthase; cSW= concentrated sea water; dSW= diluted sea water; EPR= electron paramagnetic resonance; OP=osmotic pressure; OS= oxidative stress; $p_c$= critical oxygen partial pressure; $PO_2$= oxygen partial pressure; ROS= reactive oxygen species; RR= respiration rate; RR$_{or}$= respiration rate in the oxyregulatory interval; SOD= superoxide dismutase; SW= sea water;
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, pH but also salinity are often accompanied by dramatic increases in ROS formation and changes in antioxidant capacities. 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, since these organisms must cope with large cyclic (daily and seasonal) fluctuations in temperature, salinity, U.V. radiation or air exposure that characterize these habitats (Freire et al., 2012). These drastic physicochemical changes impose 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 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, an important 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 widely spread 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* (Linnaeus, 1758) to colonize many different regions of the world and the Mediterranean green crab *Carcinus aestuarii* (Nardo, 1847) 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 have also a pronounced capacity for active ammonia excretion (Weihrauch 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 9 phyllobranchiate gills in each branchial chamber, anterior pairs (1-6) having an epithelial layer typical of gas-exchanging tissues, composed of pilaster cells and thin pavement epithelial cells (< 1 µm) with sparse mitochondria (Compere et al., 1989). The last 3 pairs (7-9) are involved in respiration and osmoregulation (Compere et al., 1989) and exhibit the highest Na⁺/K⁺-ATPase activities (Siebers et al., 1982). Within these 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, 1900), located proximate to the afferent vessel (Compere et al., 1989). These specialized tissues present a thick prismatic epithelium (10 µm) composed of mitochondria-rich cells with numerous membrane infoldings, characteristics of salt-transporting tissues. Contrary to the anterior gill epithelium, when animals are exposed to diluted seawater these posterior gills suffer important structural changes, evidencing their role in osmoregulation: these notably take place in the prismatic epithelium responsible for transepithelial salt transport, which develops a system of apical membrane infoldings and establishes a close association between the basolateral membrane infoldings and the numerous mitochondria (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 biological, ecological and molecular knowledge available for C. maenas, and it is thus considered as 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 the study is not only to contribute to the ecophysiological knowledge of the Mediterranean green crab, but to use it as a model to study how specialized organs such as gills can manage enhanced ROS formation resulting from different functions (osmoregulation vs. respiration). We compare 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. Such type of studies are scarce on crustacean species (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 approach 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 different environmental salinity (ranging from hypersaline to hyposaline
sea water) in order to set the bases for further studies focused on disentangling the mechanistic pathways in which similar tissues with partially different functions are able to cope with differential OS levels.
MATERIALS AND METHODS

Animal collection and maintenance

Mediterranean green crabs *C. aestuarii* were collected in January 2015 from Palavas-les-Flots and Sète lagoons, located on the Mediterranean coast of France. Since 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 weight of 16.5 ± 4.3 g were transported to the laboratory where they were disinfected by dipping them in sea water (SW) (1090 mOsm kg\(^{-1}\), ≈37 ppt) with 500 ppt KMnO\(_4\) for 5 min (as in Paital and Chainy, 2012). After rinsing twice in clean SW, animals were allowed to acclimate to laboratory conditions (SW at 19ºC) for 1 week. No mortality rates were recorded during this period of time.

Osmoregulation

After acclimation, 72 animals were used for analyzing the osmoregulatory capacity of the species. In quadruplicate, groups of 3 animals were exposed to 6 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 to avoid stress due to interindividual interactions among them for a minimum of 72, time in which osmoregulation is achieved in similar species such as *C. maenas* (Lovett et al., 2001). After this time, an hemolymph sample was taken using a 1ml 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 5 groups of at least 6 interconnected boxes of a maximum dimension of 20x13x10 cm (lxwxh). All of them were supplied in 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 in individual and numerated boxes for an individual follow-up. Crabs were fed thawed mussels each 48h and were allowed to eat during 6h. After this time all remaining food was removed in order to avoid 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, water was changed for each of the 3 groups: one group was changed to diluted SW
(dSW) (295 mOsm kg⁻¹, ≈10 ppt), the second to hypersaline or concentrated SW (cSW) (1320 mOsm kg⁻¹, ≈45 ppt) while the third one was replaced by clean SW and remained as control. Due to the characteristics of the flow system used, salinity change occurred gradually and equilibrated to the target salinity values in a maximum time of 2h.

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

**Sampling**

Two weeks after salinity changes, all animals were sacrificed. To do this, individuals were introduced at -20°C for 2 min. All further manipulations were conducted on ice to maintain anesthetic conditions.

Animals were first measured and weighted. Immediately after, and for control purposes, an hemolymph sample was taken for OP quantification following the same procedure above described. 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 activities 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 conducting respiration measurements. To avoid any possible interference in the measurements related to nutritional stress (Herreid, 1980), animals were fasting for 48 h prior to the start of the experiment. Crabs were introduced in 750 ml chambers provided with a magnetic stirrer (to homogenize O₂ concentration over the water column) and equipped with an oxygen sensor spot (OXSP5, PyroScience GmbH, Aachen, Germany) glued to its wall using silicon paste. Since we recorded large inter-individual variations in activity (which had a clear effect on respiration rates) (RR), animals within the chambers were enclosed in a 6x6.6x3 cm (LxWxH) box of 1 mm² 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 µm Whatman) at each of the three selected salinities (10, 37 and 45 ppt). Animals were allowed to acclimatize 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 pO$_2$ over time. Measurements were carried out with a non-invasive 4-channel FireSting O$_2$ meter (PyroScience). Four parallel measurements were carried out at a time (including a blank). Data were recorded at 3 sec intervals at 20ºC, and the experiments were stopped when O$_2$ was completely consumed from each of the chambers containing an animal. RR are expressed as nmol O$_2$·h$^{-1}$·g BW$^{-1}$. A critical environmental oxygen pressure ($p_c$) was determined based on section-wise linear regression of each respiration curve (Duggleby, 1984), above which animals respire in an oxyregulating manner. Below this point, specific RR decreases with environmental pO$_2$ 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 its bottom using silicon paste. Measurements were conducted in a sterile filtered SW (0.2 µm Whatman) at each of the three selected salinities and supplemented with 25 mM Na-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 abovementioned. 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 the normoxic O$_2$ consumption rates were considered in the study. These were calculated as the slope of decreasing PO$_2$ in all cases above 30% air saturation, before the onset of oxyconformity. After each experiment, gills were rinsed in MilliQ water and dried at 55ºC for a minimum of 48 h. RR are expressed as nmol O$_2$·h$^{-1}$·g DW$^{-1}$.

**Mitochondrial density**

Citrate synthase (CS) activity, frequently used as an indicator of mitochondrial density, was measured in anterior and posterior gill homogenates after Srere et al. (1963). Gill samples were homogenized 1:10 (w:v) in a buffer composed of 20 mM Tris, 1 mM 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/sec). CS activity was measured in duplicates in 6.5 µl of supernatant and using a final volume of 165 µl. Baseline activity (in the absence of oxaloacetic
acid) and free Co-A production (unchained by the addition of oxaloacetic acid) was read for each sample as changes in absorbance at 412 nm for at least 3 minutes at 20°C using a microplate reader (Tecan Infinite M200, TECAN, Männedorf, Switzerland). Results were expressed as units (U) (µmol 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 were expressed per mg protein.

Assessment of the general redox state

As recommended by Forman et al. (2015), in the present paper we will 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-Carbonyl-2’7’ difluorodihydrofluorescein diacetate (C-H₂DFFDA, Molecular Probes C-13293) on fresh hemolymph samples. 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 analysed 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 the 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 is long proven 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 since it is known to be the most stable ROS. Samples were incubated for 10 minutes in 10 µM in C-H₂DFFDA (ex: 488 nm; em: 525 nm) in a flat bottom black microplate. All samples were analyzed in duplicates. Fluorescence readings were taken each 5 min over 30 min using a microplate reader.
Taking into account the complex redox reactions of C-H$_2$DFDFA and its limitations to directly measure H$_2$O$_2$ (see review by Kalynaraman et al., 2012), in order to validate and complement the measurement above described, ROS generation was also assessed on gills tissues through electron spin resonance (EPR). Frozen anterior and posterior gills were homogenized on ice during 1 min using a manual potter at a ratio 1:10 (w:v) in a buffer (50 mM Tris, 34mM SDS, 10% glycerol) (G. Meyer, pers. com.) supplemented with 1 µg ml$^{-1}$ of each of the protease inhibitors leupeptin, pepstatin and aprotinin. Homogenates were centrifuged at 17,000 g during 10 minutes at 4ºC. The experimental protocol for ROS detection was adapted from Mrakic-Sposta et al. (2012). 25 µl of the resulting supernatants was mixed with spin probe CMH (1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidineHCl, 200 µM). The mixed solution was introduced in 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 37ºC by the Temperature, Gas Controller “Bio III” unit, interfaced to the spectrometer. Detection of ROS production was conducted using BenchTop EPR spectrometer-SCAN under the following EPR settings: center field g = 2.011; field sweep 60 G; microwave power 20 mW; modulation amplitude 2G; conversion time 10.24 ms; time constant 40.96 ms, number of scans: 10. The EPR signal is proportional to the unpaired electron numbers. The result was expressed in µmol · min$^{-1}$ · mg tissue$^{-1}$.

**Antioxidant defense activities**

Catalase (CAT) and superoxide dismutase (SOD) activities were measured spectrophotometrically in anterior (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 mM KPi buffer 120 mM KCl (supplemented with 0.1% Triton X-100 when meant 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 0.3M H$_2$O$_2$ solution in a 50mM KPi buffer after Aebi (1984) while SOD activities were determined using the cytochrome oxidase essay 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 mM HEPES, 5 mM MgCl$_2$, 1 mM 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 intensities in supernatants were measured using the Caspase-Glo® 3/7 kit (Promega Corporation, Madison, WI), according to the manufacturer’s instructions at 20°C. 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 mean ± 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 U-Mann Whitney pair-wise comparisons.

**RESULTS**

**Osmoregulation**

*C. 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 osmoconforming to an osmoregulating physiology.

**Whole-animal respiration**

In whole-animal measurements, all respiratory profiles showed two different phases regardless the environmental salinity: at higher oxygen concentration, organisms were oxyregulating until reaching a $p_c$.
moment in which they switch to oxyconform respiration (Fig. 2A). Data analyses indicate that as the salinity of environmental medium decreases, there are significant increases in $p_c$ which is displaced towards higher $pO_2$ ($p_c < 0.001$). Decreased salinity also produced significant increases in the average RR above $p_c$ (oxyregulatory respiration) ($RR_{or}$) ($RR_{or} < 0.001$) (Fig. 2B).

Excised-gill respiration, mitochondrial density and metabolite content

Respiratory profiles of isolates gills showed that RR decreased in an oxyconforming manner and no $p_c$ were detected. RR showed large interindividual deviations, which determined that no significant differences were registered among salinities for anterior ($p=0.241$) or posterior gills ($p=0.591$). These two types of gills neither showed differences between each other 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 observe that osmoregulation (exposure to 10 ppt) causes an important misbalance in the respiration rates of both types of gills due to a slight increase in posterior gill RR but mainly to an important decrease in anterior gill metabolism ($p<0.01$) (Fig. 3A).

CS activity remained constant in anterior gills, regardless 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 concentrations 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 evidenced that while anterior gills show no differences in ROS formation ($p=0.561$), posterior gills show a significant increase when exposed to 10 ppt ($p<0.05$) (10 ppt > 37 ppt = 45 ppt). On the other hand, only at 10 ppt we observed significant differences between anterior and posterior gills in favor of the later ($p<0.01$).

Antioxidant activities

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

**Cell damage**

Decrease in environmental salinity resulted in significant increases in caspase 3/7 activities in anterior gills ($p=0.036$) while for posterior gills, no differences among treatments were observed ($p=0.068$) (Fig. 6). The largest differences among gills types were detected at 10 ppt, and even though no significant differences were registered ($p=0.168$), anterior gills showed an average of 2.2-fold higher caspase 3/7 activities than posterior gills.
DISCUSSION

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

*C. aestuarii: adaptations to subtropical estuarine environments?*

Even though the overall osmoregulation pattern is similar to that of its sibling species *C. maenas*, showing a clear osmoconforming physiology at SW and cSW, there are, however, some notable differences concerning the hyper-osmoregulating 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 to 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. meanas* juveniles (Cieluch et al., 2004) (Fig. 1). We also observed differences regarding the degree of hyper-osmoregulating 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, since when immersed in dSW adult crabs maintain overall hemolymph osmolalities higher than *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 (\(p_c\) values) may also provide interesting insights on 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 10ºC, Taylor et al. (1977) observed a salinity-dependent shift in \(p_c\) in *C. maenas*: when immersed in SW \(p_c\) 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 18ºC caused \(p_c\) to shift towards higher oxygen concentrations, as it would be expected (see review by Herreid, 1980) but the salinity-dependent change disappeared and the \(p_c\) remained constant at 70 mmHg (=45% air saturation). In our study, at temperatures as high as 19 to 20ºC we still register salinity-dependent differences in \(p_c\) in *C. aestuarii*, which at SW it 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. aestuarii*. 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 *C. aestuarii*, since 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 expenditures**

Regardless the species, osmoregulation is an energetically expensive process due to active ion pumping in specialized epithelial cells that are rich in mitochondria, and in *C. aestuarii* it is not an 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) or Charmantier (1998) for similar species such as *C. maenas*. Between 6-24 h of transfer to dSW, there is an upregulation of the transcripts encoding many of the membrane transporters involved in osmoregulation (Na⁺/K⁺ ATP-ase, carbonic anhydrase, sodium/glucose cotransporter, 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* (mentioned as *C. mediterraneus* in the original publication) immediately after being 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 are maintained constantly higher than animals acclimated to SW, but significantly lower than during the first 2-3h of hyposmotic shock (Taylor, 1977), time when a burst in RR occurs and likely enabling the animals to fuel the first compensatory mechanisms. For other crabs species such as *Scylla serrata*, also an hyperosmoregulator (Chen and Chia, 1997), long-term acclimation to 10 ppt SW results in an increase of 55% of their RR (Paital and 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 trans-membrane pumping. Based on our increased ROS levels in hemolymph samples (Fig. 4A) and in posterior gills upon dSW exposure we can establish a clear relation between
energy expenditures and ROS production upon hemolymph hyper-osmoregulation. However, there is no consensus as to how environmental salinity affects free radical metabolism of estuarine organisms. 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, works 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, agreeing with our results. Others report an activation in 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 evidences differential oxidative stress adaptation between gill types

The respirometric analyses carried out on excised gills evidence the important 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 an important degree of apoptotic activity. Lipofuscin-like pigment accumulation (as defined by Katz and Robinson, 2002), another relevant marker of oxidative stress (e.g. Rocchetta et al., 2014), showed also an increase on histological sections analyzed in the context of another study (ms. in prep.), leading us to hypothesize that this general increase in damage markers may be the result from the increased ROS resulting from the dSW exposure and the failure of putting in place a clear defense mechanism as a response to dSW-derived ROS formation. However, the idea that the increased apoptotic 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, likely an adaptive response since these tissues fuel energy-dependent processes related to dSW exposure. These tissues carry a part of the weight of ion transport upon hypo-osmotic stress, and have therefore increased respiration rates and greater potential for producing ATP, as it 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 derives from the CS measurements shown in Fig. 3B). Agreeing 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 determining 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 an important 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 contributing 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 main be mainly involved in signaling pathways as it will be further discussed below.

Regardless if these ROS are 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 to enhance their antioxidant activities (CAT and SOD) (Fig. 5) avoiding significant cell damage and demonstrating to have a clear “preparation for oxidative stress” behavior consisting of an important upregulation of their antioxidant defenses.

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

In the present study, regardless the increase in DCF fluorescence that exposure to dSW induces, cell damage levels in posterior gills remain undisturbed, allowing us to hypothesize that the induction of antioxidant enzymes (notably SOD) may be occurring 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 is long known to have the potential of causing 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 widely diverse number 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 evidence 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 which support the hypothesis of an oxidative stress-induced acclimation to abiotic stress. Exposure to other stressors such as hypoxia results in increased ROS formation which have protective effects on different cell types as demonstrated by many (e.g. see review by Welker et al., 2013; Hermes-Lima et al., in press). This 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 activities. 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 contents in posterior gills, which may be additionally having a further antioxidant role. These reports highlight that while posterior gill tissues support the weight of the osmoregulation procedure, these 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 evidences how exposure to low salinity induces important oxidative stress in *C. aestuarii*, affects hypoxia tolerance but also notably compromises the functional viability of anterior gills. Taken together, these results evidence the fact that anterior and posterior gills do not only differ in function, but 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 adaptive response to oxidative stress in functionally different tissues. Because, could the physiological costs of replacing osmoregulatory mitochondria-rich cells determine the fact that posterior gills should be uppermost 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 aiming to disentangle the answers for these two questions.
ACKNOWLEDGEMENTS

The authors would like to thank Greg Meyer 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.

FUNDING

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

COMPETING INTEREST STATEMENT

The authors declare they have no competing interests.


Fig. 1: Variations in hemolymph osmolarity in relation with the salinity of the external medium for *C. aestuarii* (present study) (black line and black diamonds), as well as for *C. maenas* juveniles (light grey line and white circles) (adapted from Cieluch et al., 2004) and adults: dark grey line with crosses adapted from Henry (2005), triangles adapted from Jillette et al. (2011), squares from Henry et al. (2002) and diamonds from Lovett et al. (2001). Mortality rates registered for *C. aestuarii* (present study) are represented by the grey bars.
Fig. 2: Whole-animal respirometry results under different environmental salinities. A) Respirometry profiles showing the average $p_c$ and consumption rates during the oxyregulating period ($RR_{or}$) for each salinity treatment. B) Differences in $p_c$ (bars) and in $RR_{or}$ (black line) for whole-animal respirometry. Different letters represent significantly different values based on a post-hoc multiple comparison test Student-Newman-Keuls. BW=body weight.
Fig. 3: Energetics of excised gills. A) Respiration rates of isolated anterior (light grey bars) and posterior gills (dark grey bars). Due to 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 U-Mann Whitney comparisons. B and C) Citrate synthase activities and lactate contents, respectively, corresponding to anterior (light grey diamonds and lines) and posterior gills (dark grey circles and lines). Different letters represent significantly different values among salinity treatments based on Student-Newman-Keuls \textit{a-posteriori} multiple comparison test. * = statistically significantly different values among gill types. BW=body weight; DW=dry weight.
Fig. 4: FR formation results in A) hemolymph, determined fluorometrically using the ROS-dependent dye C-H$_2$DFFDA, and B) gill tissues, by electron paramagnetic resonance (EPR). Values associated to the same letter (a-b-c), belong to the same subset based on pairwise comparison U-Mann Whitney (subfig. A) and a Student-Newman-Keuls \textit{a-posteriori} multiple comparison test (subfig. B). * = statistically significant differences among gill types.
Fig. 5: Antioxidant activities in *C. aestuarii* gills. A) Catalase. B) Superoxide dismutase. Light grey bars represent anterior gills (gill pair 3) while dark grey bars correspond to posterior gills (gill pair 7). * = statistically significant differences among gill types. BW=body weight.
Fig. 6: Caspase 3/7 activities in anterior and posterior gills of *C. aestuarii*. Light grey bars represent anterior gills (gill pair 2) while dark grey bars correspond to posterior gills (gill pair 8). Error bars represent S.E.M. BW=body weight; RLU= relative light unit.