Osmoregulation, bioenergetics and oxidative stress in coastal marine invertebrates: raising the questions for future research

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ABSTRACT

Osmoregulation is by no means an energetically cheap process, and its costs have been extensively quantified in terms of respiration and aerobic metabolism. Common products of mitochondrial activity are reactive oxygen and nitrogen species, which may cause oxidative stress by degrading key cell components, while playing essential roles in cell homeostasis. Given the delicate equilibrium between pro- and antioxidants in fueling acclimation responses, the need for a thorough understanding of the relationship between salinity-induced oxidative stress and osmoregulation arises as an important issue, especially in the context of global changes and anthropogenic impacts on coastal habitats. This is especially urgent for intertidal/estuarine organisms, which may be subject to drastic salinity and habitat changes, leading to redox imbalance. How do osmoregulation strategies determine energy expenditure, and how do these processes affect organisms in terms of oxidative stress? What mechanisms are used to cope with salinity-induced oxidative stress? This Commentary aims to highlight the main gaps in our knowledge, covering all levels of organization. From an energy-redox perspective, we discuss the link between environmental salinity changes and physiological responses at different levels of biological organization. Future studies should seek to provide a detailed understanding of the relationship between osmoregulatory strategies and redox metabolism, thereby informing conservation physiologists and allowing them to tackle the new challenges imposed by global climate change.

KEY WORDS: Hyper-/hypo-osmoregulator, Hyper-/iso-osmoregulators, Osmoconformers, Antioxidants, Free radicals, Hypometabolism, Mitochondria

Introduction

Marine invertebrates, such as mollusks and crustaceans, living in coastal lagoons, mangrove swamps, deltas, estuaries or intertidal areas are frequently exposed to large changes in their physical environment. These changes are due to regular (daily or seasonal) patterns (Helmuth, 1999; Zhang et al., 2010) but can also be induced by sudden events (e.g. Williams et al., 2011). The changing physical parameters include temperature, UV radiation and oxygenation; however, in this Commentary, we focus on changes in salinity. This constitutes one of the major challenges to shallow coastal invertebrates (Freire et al., 2012), and the abrupt and unpredictable variations in salinity determine the distribution and physiology of

these invertebrates (Henry et al., 2012; McNamara and Faria, 2012; Peterson and Ross, 1991). Heavy rains and run-off waters, as well as low tides in conjunction with high environmental temperatures, can have a significant impact on salinity range – coastal salinity can fluctuate from that of freshwater (near 0 ppt) to hypersaline seawater levels, reaching maximum values in habitats such as supratidal pools (>150 ppt) (McAllen et al., 1998). Anthropogenic intensification (leading to urban/industrial wastewater or stormwater discharges) and the consequent climate change-associated events also increase the frequency and extent of these changes.

To correctly acclimate to salinity fluctuations, organisms must make finely tuned adjustments at the cellular level in order to compensate for and control ion and water flux across biological membranes (e.g. Havird et al., 2013). In the event of increased environmental salinity, the osmolality (see Glossary) of internal media must be increased (through active uptake or synthesis of osmolytes) in order to avoid water loss, dehydration and loss of turgor pressure (Hoffman et al., 2009; Wehner et al., 2003), because changes in cell volume can potentially lead to protein denaturation (e.g. Gutierre et al., 2014), breakdown of cell volume regulatory capacities and subsequent apoptosis (e.g. Gómez-Angelats and Cidlowski, 2002). The opposite reaction is observed in the case of a hypotonic challenge; mechanisms to control water fluxes into cells include: (1) decreases in membrane permeability to water, (2) changes in the concentration of osmotic effectors (amino acids and organic ions) (reviewed by Pierce, 1982) to decrease internal osmolality, (3) changes in the expression of channels or active membrane carriers – such as Na^+/K^+-ATPase (NKA) or $Na^+/K^+/Cl^-$ cotransporters, or carbonic anhydrase (Henry et al., 2002; Lovett et al., 2006; Lv et al., 2016) – and (4) the production of ammonia (Rosas et al., 1999), among others (Łapucki and Normant, 2008).

Animals exposed to salinity stress must increase their energy expenditure to successfully acclimate to the stressor and ensure cellular protection (Sokolova et al., 2012b). We may thus consider osmoregulation as a costly process, which is probably why it has been extensively studied in terms of bioenergetic costs, namely respiration and aerobic metabolism (e.g. Gilles, 1973; Goolish and Burton, 1989). In this sense, bioenergetic approaches have been considered as a common denominator for predicting tolerance limits when organisms are exposed to stress (Sokolova et al., 2012a). But aerobic metabolism (and thus mitochondrial activity) inevitably entails the production of reactive oxygen and nitrogen species (ROS and RNS, respectively), although the relationship between mitochondrial functioning and reactive species production is highly variable (Barja, 2007). However, given the lack of studies linking osmoregulation, energetics and oxidative stress in estuarine/coastal marine invertebrates (Freire et al., 2012), this Commentary aims to highlight key questions and potential ideas for future investigation in this area. We begin by considering osmoregulation

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List of abbreviations

AER	aniosmotic extracellular osmoregulation
FAA	free amino acids
GST	glutathione S-transferase
IIR	isosmotic intracellular regulation
NKA	Na$^+$/K$^+$-ATPase
POS	preparation for oxidative stress
PUFA	polyunsaturated fatty acids
RNS	reactive nitrogen species
ROS	reactive oxygen species

and ROS and RNS production in more detail. We then turn to the field of biomedicine to discuss current knowledge of how ROS affect osmoregulatory capacities in mammals because, to our knowledge, there is no information available to date for marine organisms. Finally, special attention is paid to cases in which hypo-osmotic shock induces metabolic arrest, a situation in which active mechanisms are energetically limited, and which may require preparation for subsequent tissue re-oxygenation.

Osmoregulatory strategies

Osmoconformers

When exposed to salinity changes, many marine invertebrates do not invest energy in transport mechanisms; thus, the osmolality of their internal medium fluctuates according to the osmolality of the environment. These organisms are termed osmoconformers (see Glossary), and they include many bivalves (e.g. Carregosa et al., 2014a; Shumway, 1977), polychaetes (e.g. Freitas et al., 2015; Shumway and Davenport, 1977), crustaceans (e.g. McAllen et al., 1998; Svetlichny et al., 2012) and echinoderms (e.g. Castellano et al.,

2016a,b). Osmoconformers are mostly stenohaline (see Glossary) and are normally restricted to marine waters (Lignot and Charmantier, 2015). They are not able to perform osmotic regulation of their extracellular fluid and rely solely on isosmotic intracellular regulation (of volume) (IIR; see Glossary) (as defined by Florkin, 1962). This involves (1) increasing or decreasing the concentrations of osmotically active solutes [e.g. ninhydrin-positive substances, K$^+$ and free amino acids (FAA)] to achieve cell volume regulation, and (2) modifying membrane-bound transporters (Gilles, 1987; Kirschner, 1991; Péqueux, 1995). Osmosensing is achieved through a wide variety of internal mechanisms (e.g. Ca^{2+} gradients, transient receptor potential ion channels, cell volume sensors) (Kültz, 2007) and is often controlled by specific hormones (Lignot and Charmantier, 2015).

Osmoregulators

As opposed to osmoconformers, other species (termed 'osmoregulators'; see Glossary) perform not only IIR, but also anisosmotic extracellular osmoregulation (AER; see Glossary) when exposed to variations in environmental salinity (Florkin, 1962). These organisms, when exposed to dilute seawater or freshwater, initiate a series of (energetically costly) mechanisms that allow them to hyper-regulate, i.e. to maintain their extracellular fluids at a higher osmolality than that of their surrounding medium. This is thought to represent a selective advantage when dealing with fluctuating salinities (e.g. estuaries) (Barnes, 1967). However, at higher salinities, osmoregulators behave as either iso- or hypo-regulators, i.e. they maintain their body fluids at the same or lower osmolalities compared with those of the surrounding medium, respectively. Thus, osmoregulators can be hyper-/iso- or hyper-/hypo-osmoregulators. Hyper-/iso-osmoregulation is mostly seen in freshwater species but is also common in many estuarine invertebrates such as some

Glossary

Anisosmotic extracellular osmoregulation (AER)
Mechanisms acting to maintain body (extracellular) fluid volume, osmotic pressure and ionic composition despite environmental salinity changes.

DCFH-oxidizing species
Reactive oxygen or nitrogen species that are capable of oxidizing the redox-sensitive fluorophore DCFH [5-(and-6)-carboxy-2′,7′-difluorodihydrofluorescein], a commonly used fluorescent dye for determining ROS/RNS formation in vivo or ex vivo.

Euryhaline species
Species that are able to tolerate large shifts in environmental salinity.

Free radical
An atom or molecule with unpaired electrons. Some highly reactive free radicals include those derived from oxygen (ROS) (e.g. superoxide anion, hydroxyl radical or singlet oxygen). However, other ROS, such as hydrogen peroxide or peroxynitrite, although highly oxidizing, are not free radicals.

Hormesis
The process through which a compound that is toxic or deleterious at high doses or concentrations produces a beneficial effect at lower quantities.

Isosmotic intracellular regulation (IIR)
Cellular mechanisms that, upon a change in surrounding salinity, lead to the adjustment of the intracellular osmotic pressure to meet that of the environment, thus minimizing variations in cell hydration.

Isosmotic point
For hyper-/hypo-osmoregulating species, environmental salinity at which the osmolality of body fluids and environmental media are equal.

Osmoconformers
Species that maintain their internal medium isosmotic to their environment, minimizing water fluxes across membranes. Energetically, osmoconformation is considered to be the cheapest strategy of osmoregulation, and it is the most common within marine invertebrates.

Osmolality
Osmotic pressure of a solution, i.e. measurement of the amount of osmotically effective solutes in a given solvent. It is commonly expressed in osmols per kilogram of solvent.

Osmoregulators
Species that carry out anisosmotic extracellular regulation when exposed to extracellular osmolality changes. This is achieved through several mechanisms involving various permeability and salt transport properties within different ion-transporting epithelia. Thus, it is often an energetically expensive strategy.

Redox balance
The balance between cellular antioxidants and pro-oxidants to avoid a surplus of the latter; a vital physiological requirement for homeostasis.

Stenohaline species
Species that may only tolerate small shifts in environmental salinity.

decapods (e.g. Lynch et al., 1973; Rivera-Ingraham et al., 2016a; Young, 1979), isopods (e.g. Charmantier and Charmantier-Daures, 1994; Łapucki and Normant, 2008), amphipods (Morritt and Spicer, 1995) and cladocerans (Aladin, 1991). Hyper-/hypo-osmoregulatory behavior is more frequent in organisms that experience frequent changes in environmental salinity (e.g. estuarine invertebrates) (Lignot and Charmantier, 2015), such as shrimp (e.g. Castille and Lawrence, 1981; Chen et al., 1995), but also other isopods (e.g. Kelley and Burbanck, 1972) and decapods (e.g. Anger and Charmantier, 2000; Charmantier et al., 2002; Thurman, 2003). Osmoregulation is achieved by controlling ionic fluxes, mostly those of Na^+ and Cl^- ions; this control makes use of both limiting and compensatory processes (e.g. control of membrane permeability or epithelial leaks, and active pumping, respectively).

As mentioned above, osmoregulation is considered to be an energetically costly process and the maintenance of ion gradients is one of the most ATP-consuming processes (reviewed in Hand and Hardewig, 1996; Sokolova et al., 2012a). For example, from studies using arthropods, the theoretical cost of producing active solutes such as proline and alanine has been estimated to represent as much as 11.6% of daily energy use (Goolish and Burton, 1989). There are, however, exceptions to this rule; for example, mollusks carry out osmolyte (alanine) synthesis for IIR during high salinity acclimation using anaerobic pathways of glucose degradation (Baginski and Pierce, 1975; De Zwaan and Van Marrewijk, 1973), thus reducing the costs of this process.

Fueling energetically expensive mechanisms: a double-edged sword

In any healthy and undisturbed situation, the aerobic ATP production necessary to fuel any active process will always involve the formation of ROS and RNS. These species are derived from membrane-linked electron transport and normal metabolic processes (Fridovich, 1995). ROS and RNS play key roles in cellular homeostasis (Palumbo, 2005; Viña et al., 2013), but are mostly known for their deleterious effects on cellular compounds, also known as oxidative stress (Sies, 1997). Dramatic increases in ROS and RNS production often accompany exposure to stressors, whether biotic (e.g. toxins, immune challenges) (Behrens et al., 2016; Ciacci et al., 2010; Gómez-Mendikute and Cajaraville, 2003) or abiotic (e.g. temperature, salinity, oxygenation) (Abele et al., 2002; Paital and Chainy, 2014; Rivera-Ingraham et al., 2013, 2016b). Therefore, organisms must set up cellular mechanisms (often located in specialized tissues) to re-establish cellular redox balance (see Glossary), thus permitting the organism to respond to the disturbance while maintaining homeostasis (Fig. 1). Thus, it is likely that upon exposure to a stressor (here, salinity), energy expenditures are not solely related to, in our case, osmoregulation, but are also required to fuel the active mechanisms needed to restore cellular redox balance. Thus, salinity change, osmoregulation, energetic balance and redox equilibrium are deeply intertwined, and although this has been well studied in plants, it is not always reflected in heterotroph-related literature.
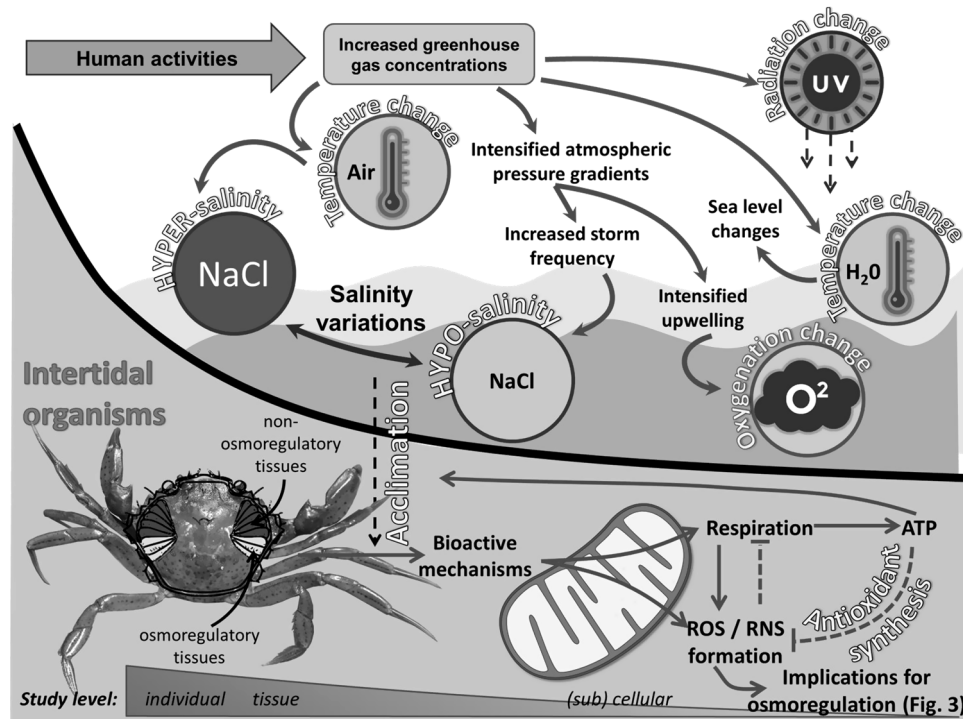

Fig. 1. The main environmental factors altered by anthropogenic climate change, to which intertidal organisms must acclimate. Human-induced changes to the climate are affecting coastal environments such as lagoons, estuaries and mangrove swamps. The increase in greenhouse gas concentrations is causing air and water temperature to rise, making intertidal organisms more likely to encounter progressive increases in environmental salinity. The increased frequency of extreme meteorological events is another good example of how climate change affects coastal organisms, obliging animals to deal with increasingly frequent exposures to diluted seawater. Thus, in this Commentary, special focus has been given to salinity variations in the intertidal environment and the energetic and redox consequences of dealing with such changes. Osmoregulatory and non-osmoregulatory tissues (which are represented here by the crab's posterior and anterior gills, respectively) show different means of acclimation to these changes. Osmoregulation is an energetically costly process requiring ATP production, namely by means of mitochondrial activity. As shown in the diagram, this has numerous implications in terms of cellular redox metabolism.

Lessons from coastal marine environments

At the whole-organism level, much work has been done on the osmoregulatory capacity of a wide range of coastal invertebrates (Bückle et al., 2006; Chen and Chia, 1997; Deaton et al., 1989; Webb et al., 1971) at different stages of development (e.g. Charmantier, 1998) and even under the influence of physiological (e.g. Lignot et al., 1999) and physical constraints (e.g. Goolish and Burton, 1989; LaMacchia and Roth, 2015). However, the question remains of whether there is an advantage in terms of energetics or redox balance of being a hyper-/hypo-osmoregulator compared with a hyper-/iso-osmoregulator or an osmoconformer. Most importantly, is there a link between osmoregulation strategy, environmental salinity and oxidative adaptation (i.e. the ability to sense and neutralize pro-oxidant conditions)?

Energetic costs associated with osmoconformity

Hypothetically, respiration rates should vary according to the degree of osmoregulation or osmoconformity (Williams, 1984), and osmoconforming marine invertebrates should have lower energy requirements (Willmer, 2001). However, as shown in Fig. 2, the relationship between osmoregulatory strategies and energy-redox parameters [e.g. respiration, production of free radicals (see Glossary), antioxidant defenses and oxidative damage markers] is not straightforward. Throughout this Commentary, we highlight some of the possible sources for the large interspecific differences. The large variability in physiological mechanisms leading to cell volume regulation, and their associated energetic costs, could be a source of difference. In osmoconformers (i.e. some intertidal copepods), hypo-osmotic shock is associated with increased
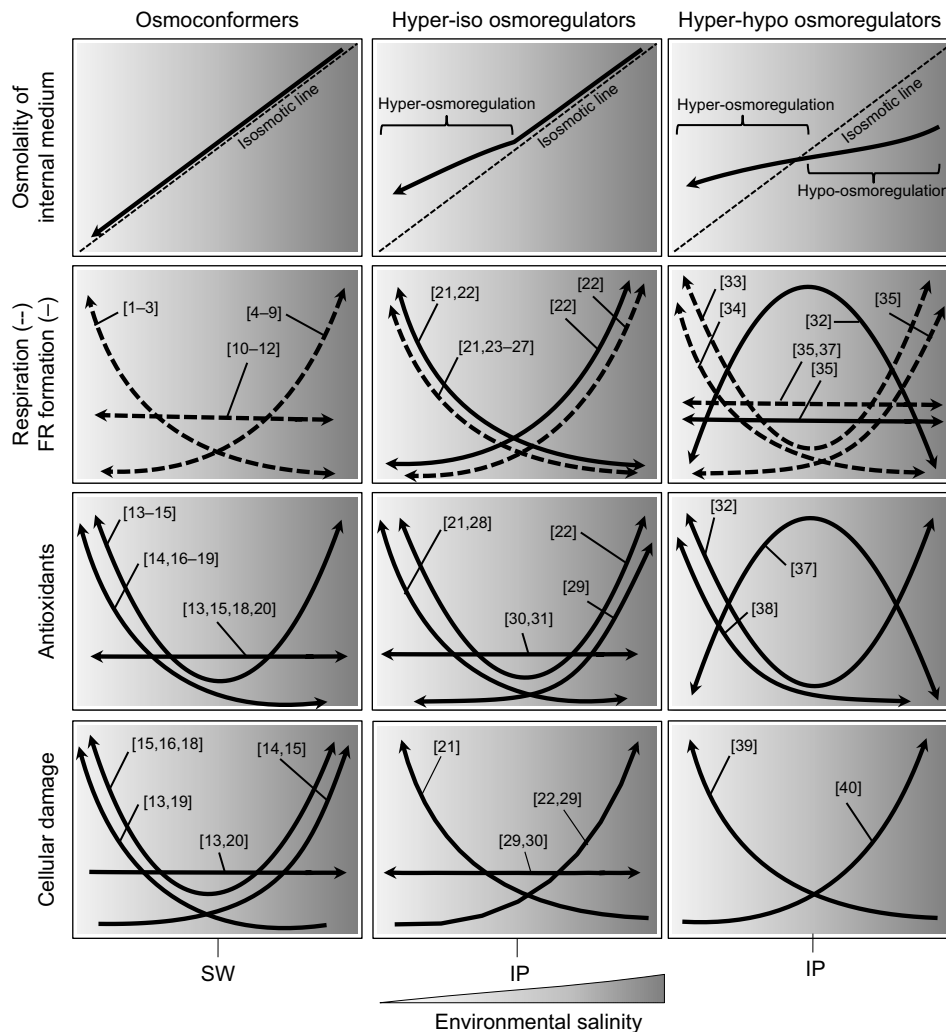

Fig. 2. Representation of the patterns of physiological response according to osmoregulation strategy and environmental salinity in marine invertebrates. This compilation of the literature shows the enormous complexity of responses, demonstrating that environmental salinity must not be the sole factor affecting respiration and oxidative status. IP, isosmotic point; SW, seawater; FR, free radical. Numbers correspond to relevant references, as follows: [1] (Goolish and Burton, 1989); [2] (McAllen and Taylor, 2001); [3] (Kim et al., 2001); [4] (Bouxin, 1931); [5] (Navarro and González, 1998); [6] (Sarà et al., 2008); [7] (Shin et al., 2011); [8] (Widdows, 1985); [9] (Yu et al., 2013); [10] (Begum et al., 2009); [11] (Shumway, 1978); [12] (Svetlichny et al., 2012); [13] (Bertrand et al., 2016); [14] (Freitas et al., 2015); [15] (Velez et al., 2016b); [16] (Carregosa et al., 2014b); [17] (De Zoysa et al., 2009); [18] (Moreira et al., 2016a); [19] (Velez et al., 2016a); [20] (Zanette et al., 2011); [21] (Rivera-Ingraham et al., 2016a); [22] (Rivera-Ingraham et al., 2016b); [23] (Dehnel, 1960); [24] (Gilles, 1973); [25] (King, 1965); [26] (Vlasblom et al., 1977); [27] (Sabourin and Stickle, 1980); [28] (De Martinez Gaspar Martins and Bianchini, 2009); [29] (Freire et al., 2011); [30] (Pinto Rodrigues et al., 2012); [31] (Rosas et al., 1999); [32] (Fernandes, 2010) [33] (Rowe, 2002); [34] (Chen and Lin, 1992); [35] (D. Theuerkauff, G.A.R.-I., J. Roques, L. Azzopardi, M. Lejeune, E. Farcy, J.-H.L. and E. Sucré, unpublished); [36] (Edwards, 1982a); [37] (Liu et al., 2007); [38] (D. Theuerkauff, G.A.R.-I., Y. Mercky, M. Lejeune, E. Sucré and J.-H.L., unpublished); [39] (Pallavi et al., 2012); [40] (Paital and Chainy, 2010).

respiration rates (Goolish and Burton, 1989; McAllen et al., 2002). This was interpreted as an attempt to oxidatively deaminate FAA (such as proline or alanine) in order to decrease the osmolyte pool, allowing the organism to control water and ion fluxes and to successfully cope with hypo-osmotic shock (Gilles, 1987). It is possible that an individual's behavior during exposure to an environmental salinity change could further contribute to observed differences in energetic costs. Some authors have suggested that the increased energetic cost may be linked to increased activity (McAllen and Taylor, 2001) as the animal attempts to escape from unfavorable conditions (Gross, 1957).

In other osmoconforming copepods, environmental salinity does not influence oxygen consumption rates (Svetlichny et al., 2012). However, most sources indicate that osmoconformers have low energetic requirements when exposed to decreased salinity. As salinity increases, their respiration rates also increase (Bouxin, 1931; Navarro and González, 1998; Sarà et al., 2008; Shin et al., 2011; Widdows, 1985; Yu et al., 2013), probably because of the active production of methylamines, FAA and derivates. These organic osmolytes are used by marine osmoconforming molluscs, polychaetes, crustaceans (Goolish and Burton, 1989) and other marine invertebrates such as sipunculids (Peng et al., 1994; Virkar, 1966) to increase intracellular osmolality. This is most probably a widespread and conserved ancestral strategy.

Energetic costs associated with osmoregulation

A wide variety of estuarine invertebrates behave as hyper-/iso-regulators, and thus only osmoregulate at lower environmental salinities. The physiological mechanisms of hyper-regulation come at an energetic cost, which usually translates into higher respiration rates (Dehnel, 1960; Rivera-Ingraham et al., 2016a; Sabourin and Stickle, 1980) required to fuel the catabolism of osmotically active amino acids (Gilles, 1973) and the active uptake of salts (Willmer, 2001). As shown in the hyper-regulating isopod *Idotea chelipes*, metabolic rates increase linearly with increasing difference between the osmolality of the medium and the hemolymph (Łapucki and Normant, 2008). However, this is far from a general trend; for example, the marine intertidal flatworm *Macrostomum lignano* is able to regulate its body volume at extremely low salinities while appearing to enter a state of metabolic arrest (Rivera-Ingraham et al., 2016b). So how can organisms control water and ion fluxes with limited energetic resources? In cases of limited oxygen, and thus energy availability, nematodes such as *Caenorhabditis elegans* can reduce the permeability of their cellular membranes through the downregulation of aquaporin water channels, thus reducing the energetic cost of countering hypo-osmotic effects (LaMacchia and Roth, 2015). These mechanisms that allow the maintenance of ion homeostasis under conditions of reduced energy availability have been well described in various animal and cellular models (reviewed by Hand and Hardewig, 1996), but the use of highly tolerant sessile intertidal organisms may open up new perspectives in the study of energetic trade-offs.

As opposed to osmoconforming and hyper-/iso-osmoregulating species, some invertebrates regulate extracellular fluids at both low and high salinities. As shown in Fig. 2, the physiological responses are significantly more variable among these hyper-/hypo-osmoregulating species. In some species, hyper-regulation induces an increase in respiration rates (Chen and Lin, 1992), whereas, for others, hypo-regulation is more energetically costly (D. Theuerkauff, G.A.R.-I., J. Roques, L. Azzopardi, M. Lejeune, E. Farcy, J.-H.L. and E. Sucré, unpublished). One exception is the case of saltwater mosquito larvae, which are nearly perfect

osmoregulators (e.g. Edwards, 1982b; Nayar and Sauerman, 1974) (although they represent only 5% of all mosquito species; Bradley, 1987). Results by Edwards (1982a) show that respiration rates of these larvae are not altered by changes in environmental salinity. Thus, at this point, we can only highlight the large range of responses existing among the different osmoregulating strategies in terms of whole-animal respiratory patterns. Even if respiration rate measurements are traditionally used as a marker for energy metabolism, they do not necessarily correlate with ATP production, a matter that may partly explain the diversity of respiratory patterns shown in Fig. 2.

Current research on fundamental mitochondrial functioning indicates the need to analyze mitochondrial efficiency – that is, the amount of ATP generated per molecule of O_2 consumed by mitochondria – in order to correctly address energetic studies (Salin et al., 2015a). In fact, much of the O_2 consumption by mitochondria can be explained through H^+ pumping and leaking across internal membranes, a subject that has received much attention in the field of biomedical research. Data on rodents indicate that the contribution of H^+ leak-associated respiration can account for an average of 20% of the total standard metabolic rate (Rolfe and Brand, 1997). Thus, could variations in mitochondrial efficiency partially explain the discrepancies in whole-animal respiration rates across organisms with different osmoregulation strategies? To address this question, mitochondrial respiratory control could be assessed by looking at mitochondrial respiration rates in response to ADP from isolated osmoregulatory tissues. The cellular rate of ATP production, proton leak rate, coupling efficiency, maximum respiratory rate, respiratory control ratio and spare respiratory capacity, along with the mitochondrial membrane potential, could be measured from isolated ion-transporting cells (ionocytes) of individuals kept at different salinities. But could these functional differences also explain the differences in salinity-induced patterns of ROS formation?

Redox metabolism

There are very few reports analyzing ROS formation during salinity changes in marine organisms, which is hardly surprising given the technical difficulty of quantifying free radicals due to the limitations of the most accessible tools (Kalyanaraman et al., 2012) and the extremely low half-life of these particles. The latter range from 10^{-9} s for OH• (Karogodina et al., 2011) to ~1 ms for H_2O_2 (Bak and Weerapana, 2015), thus requiring *in vivo* or *ex vivo* measurements. But again, in marine invertebrates these reports are not only scarce, but also contradictory.

From two examples of hyper-/iso-regulators, we see that respiration rates may increase at higher salinities (e.g. in the intertidal flatworm *M. lignano*) (Rivera-Ingraham et al., 2016b) or at lower salinities (e.g. the Mediterranean green crab *Carcinus aestuarii*) (Rivera-Ingraham et al., 2016a). Live-imaging techniques and *in vivo* analysis of free radical production can be used with *M. lignano*, as this organism is small and transparent. We recently revealed that when exposed to hypersalinity, these flatworms increase respiration rates, which is accompanied by a dramatic increase in superoxide anion ($O_2 \cdot^-$) production while H_2O_2 and other ROS/RNS (specifically DCFH-oxidizing species; see Glossary) decreased (Rivera-Ingraham et al., 2016b). This hypersaline exposure is accompanied by upregulation of the gene expression of various antioxidants, although this does not always enable organisms to avoid increased apoptosis following exposure to high environmental salinities, a failure that is most probably due to dysfunctioning, worn-out cells. However, some of the FAA synthesized during exposure to hypersaline environments can also play a direct or indirect role in redox balance (see Yancey, 2005 and references

therein). In *C. aestuarii*, increased respiration rate at low salinity is accompanied by increased ROS formation in hemolymph and tissues (Rivera-Ingraham et al., 2016a). But, as highlighted by Salin et al. (2015b), respiration and ROS production are not necessarily linked.

In contrast to *M. lignano* and *C. aestuarii*, the hyper-/hypo-osmoregulating estuarine crab *Neohelice granulata* increases H_2O_2 formation under hyperosmotic shock (H_2O_2 measured in the surrounding medium) (Fernandes, 2010). In *Uca urvillei*, a mangrove crab with similar osmoregulating strategies, increased salinity causes an increase in respiration rates, which is not accompanied by changes in ROS formation in freshly collected hemolymph (D. Theuerkauff, G.A.R.-I., J. Roques, L. Azzopardi, M. Lejeune, E. Farcy, J.-H.L. and E. Sucré, unpublished).

Could the variety of results on the relationship between ROS-formation patterns and salinity changes be explained by the lack of fundamental mitochondrial data? The relationship among mitochondrial respiration rates, ATP production and ROS formation has been a subject of debate for some time now, and most of the available data were collected in mammalian models. It is generally accepted that there is a fixed percentage of ROS produced per unit of O_2 consumption (Nicholls, 2004) at the level of complex I and III in the mitochondrial electron transport chain (Turrens, 2003). However, the contribution of complex III to ROS formation is determined by mitochondrial membrane potential ($\Delta\Psi_m$) and, consequently, by the processes modifying this parameter, i.e. ATP production (Adam-Vizi and Chinopoulos, 2006), H^+ leak (Brookes, 2005) and expression of uncoupling proteins (Mattiasson et al., 2003; Speakman et al., 2004). An elegant study by Salin et al. (2015b) showed that H_2O_2 formation in fish mitochondria is negatively correlated with standard metabolic rates. Once again, this highlights the need to complement whole-animal respiration measurements with analysis at lower levels of organization (e.g. cellular or molecular levels).

It is also worth noting an issue that is rarely discussed in the literature: the physiological cost of osmoregulation may greatly vary among tissues. Thus, another possible source of variation in the patterns shown in Fig. 2 could be related to differential oxidative adaptation between osmoregulatory and non-osmoregulatory organs. In Rivera-Ingraham et al. (2016a), we used an estuarine hyper-/iso-osmoregulating decapod crab as a study model to demonstrate that non-osmoregulating (anterior) and osmoregulating (posterior) gill tissues facing hypo-osmotic shock can clearly differ in their metabolic and oxidative response. Posterior gills with osmoregulatory function respond by generating new mitochondria, thus producing the energy needed to fuel osmoregulatory structures as well as the antioxidant defenses (namely superoxide dismutase) that are required to counteract stress-induced ROS production. However, anterior gills (which are mainly respiratory) enter a state of metabolic arrest on exposure to hypo-osmotic shock, and are affected by a higher apoptotic rate. Further analyses dissociating the functional partitioning between osmoregulatory and non-osmoregulatory tissues will certainly open interesting new perspectives on antioxidant defense evolution and adaptive responses to oxidative stress at the tissue level. For example, in hyper-/hypo-osmoregulating crabs, could the physiological cost of replacing osmoregulatory ionocytes lead to an optimized protection of the posterior gills at the expense of purely respiratory anterior gills? Also, when – and how – does upregulation of the antioxidant defense occur?

Effects of oxidative stress on osmoregulation capacities: implications from mammalian work

As reviewed above, radical and non-radical reactive species are generated by mitochondrial respiration and other processes during salinity stress, and these can attack virtually all types of biomolecules. Among these, polyunsaturated fatty acids (PUFA), which are essential components of membrane phospholipids, are especially prone to oxidation. The two or more carbon-to-carbon double bonds in PUFA render these molecules vulnerable to ROS interactions, resulting in lipid radicals which, in the presence of molecular oxygen, result in lipid peroxyl radical formation (Fig. 3). In turn, these can react with other fatty acids to produce lipid peroxides and additional fatty acid radicals. These processes have been well documented in mammals (reviewed by Yin et al., 2011), as they have been suggested to be associated with diverse pathologies ranging from cancer (e.g. Gönenç et al., 2001) to Alzheimer's disease (e.g. Montine et al., 2004). As we discuss above, the literature shows that both high and low environmental salinities can be associated with increased ROS production (Fig. 2) (Freire et al., 2012), which can, in turn, interfere with osmoregulation through different pathways.

As discussed above, under hypo-osmotic conditions, some weak osmoregulators (e.g. euryhaline brachyuran crabs; see Glossary) respond by overexpressing and enhancing the activity of membrane-bound ion channels, co-transporters/exchangers and ATPases (e.g. Havird et al., 2013; Towle et al., 2011). This can be accompanied by mitochondrial biogenesis to meet the energy requirements to fuel osmoregulation (Rivera-Ingraham et al., 2016a). While small amounts of ROS are required to activate osmoregulatory pathways (Wagner et al., 2013), larger and uncontrolled quantities lead to the degeneration or inactivation of the membrane-bound ion-transporting pumps such as NKA (Kim and Akera, 1987; Kukreja et al., 1990), thus reducing osmoregulatory capacities. Invertebrates have rarely been the subject of this type of study, but many works using different mammalian cells show how pro-oxidant conditions not only induce lipid peroxidation, but also lead to a decrease in the activity of NKA (Dobrota et al., 1999; Ostadal et al., 2004; Thomas and Reed, 1990) or Ca^{2+}-ATPase (Kaneko et al., 1989; Lee and Okabe, 1995), both of which are essential for osmoregulation in marine invertebrates.

For some species, hyperosmotic stress can equally result in increased ROS formation (e.g. Rivera-Ingraham et al., 2016b), and these pro-oxidant conditions, if not properly controlled, may also interfere with correct acclimation. The mechanisms used by renal cells to counteract hypersalinity are well studied; in this system, the synthesis or accumulation of FAA and other osmolytes is essential. However, it has been reported that in renal cells, ROS may inhibit the activity of enzymes involved in osmolyte synthesis. Rosas-Rodríguez and Valenzuela-Soto (2010) show this for betaine aldehyde dehydrogenase (catalyzing glycine betaine production), aldose reductase (converting glucose to sorbitol) and glycerophosphochiline:choline phosphodiesterase (involved in the synthesis of glycerophosphocholine), all of which produce important solutes necessary for correct renal cell osmoregulation. In the context of anthropogenic effects on shallow aquatic environments, the study of the impact that exposure to ROS-generating stressors may have on osmoregulatory capacities is a relevant line of research.

Salinity-induced metabolic depression and recovery: does the 'preparation for oxidative stress' hypothesis apply for hypo-osmotic shock?

A common response to environmental stressors is the induction of metabolic depression (Hand and Hardewig, 1996), a state in which animals decrease their basal metabolic rate to minimize energy expenditure (Guppy and Withers, 1999), thus maximizing survival
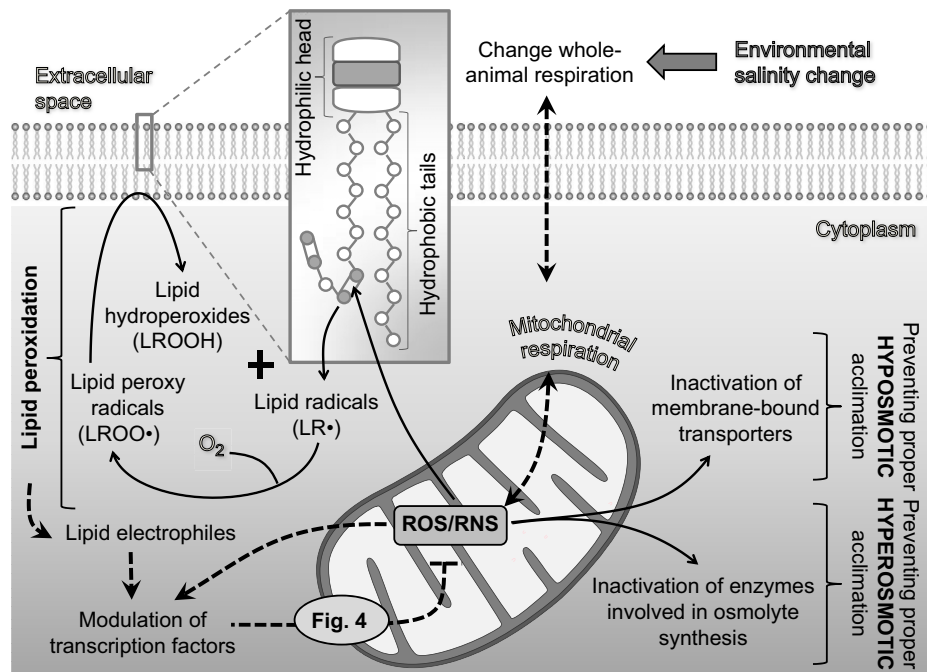
Fig. 3. Schematic diagram representing how environmental salinity may cause changes in the invertebrate energy-redox axis. When exposed to stressful conditions, organisms must increase their energy expenditure in order to fuel the mechanisms required for successful acclimation. Mitochondria, as the main energy producers in eukaryotic cells, play a central role in acclimation processes. However, they also represent the main source of reactive oxygen and nitrogen species (ROS/RNS), although the relationship between mitochondrial respiration and ROS/RNS formation is not fully understood. ROS/RNS can potentially lead to the peroxidation of lipids (such as those composing cellular membranes), as well as damaging other cellular molecules; ROS/RNS potentially have negative consequences for correct acclimation to hyper- and hypo-osmotic conditions. However, the lipid electrophiles resulting from such processes can have, along with ROS/RNS themselves, a role in the activation of transcription factors leading to upregulation of cellular defenses (i.e. a hormetic effect; see Fig. 4). Solid arrows represent well-described processes, while dashed arrows indicate those pathways that are less well understood.

time until the return of favorable conditions. Although this is most commonly attributed to exposure to low temperature or hypoxia, for example, a review of the literature shows that some marine invertebrates – ranging from flatworms (Rivera-Ingraham et al., 2016b) and echinoderms (Yu et al., 2013) to mollusks (Morritt et al., 2007; Sokolova et al., 2000; Stickle and Sabourin, 1979) – enter a state of metabolic depression when exposed to hypo-osmotic conditions or freshwater (Fig. 4). This environmentally induced metabolic depression may predominantly affect specific tissues in a wide variety of species (e.g. Flanigan et al., 1991; Lewis and Driedzic, 2007; Smith et al., 1996).

The physiological and biochemical processes triggering metabolic depression and arousal are complex but have been the focus of much scientific attention (Biggar and Storey, 2010; Storey, 1988; Storey and Storey, 2004). Physiologically, mitochondrial recovery is one of the most challenging processes; cell re-oxygenation commonly leads to large peaks in ROS and RNS formation, a process known as the 'oxidative burst', which, if not controlled, can have deleterious consequences for cellular compounds and for survival. Thus, it may be of critical importance for organisms frequently encountering environmental or functional hypoxia (e.g. many shallow coastal invertebrates, which are mainly sessile or have low motility and are thus unable to escape from such conditions), to prepare adequate mechanisms to counteract the pro-oxidant conditions of arousal and re-oxygenation. When facing hypoxia, freezing, starvation or other environmental constraints inducing metabolic arrest, tolerant species are indeed capable of 'preparing for oxidative stress' (POS) (Hermes-Lima et al., 2015, 1998). This requires such organisms to increase their antioxidant defenses before triggering metabolic shutdown in order to counteract the burst of ROS generated upon

reperfusion. A recent literature review by Moreira et al. (2016b) shows that the diversity of organisms capable of POS is phylogenetically broad, suggesting that this is an old and relatively conserved mechanism.

Could a hypo-osmotically induced metabolic decrease lead to a similar preparation for oxidative stress? As recently shown in Rivera-Ingraham et al. (2016b), flatworms under hyposaline conditions not only decrease their activity and respiration rates, but also significantly increase very specific antioxidant defenses, namely the level of glutathione S-transferase (GST). Could this be interpreted as a POS mechanism? If animals are indeed under metabolic depression, how would investing in GST upregulation benefit the individuals except as a preparatory step for the expected return to pro-oxidant conditions? If this is the case, what are the triggering signals? Current hypotheses consider that small quantities of ROS are involved in this process (i.e. it is an example of hormesis; see Glossary) and may, thus, be essential for correct homeostasis (Fig. 4).

Over the last decades, the ROS-induced hormetic effect in acclimation to environmental challenges has been increasingly highlighted by numerous studies (e.g. Russell and Cotter, 2015), although mainly in plants (Carmody et al., 2016; Suzuki et al., 2012). As reviewed by Hermes-Lima et al. (2015), there is considerable indirect evidence supporting the role of ROS (and products of biomolecule oxidation) in activating transcription factors in animals, leading to antioxidant upregulation. Regarding salinity changes, in human renal cells exposed to high salinity stress, for example, ROS are required for the transactivation of specific transcription factors leading to the transcription of osmoprotective genes (Zhou et al., 2005). However, direct measurement of ROS
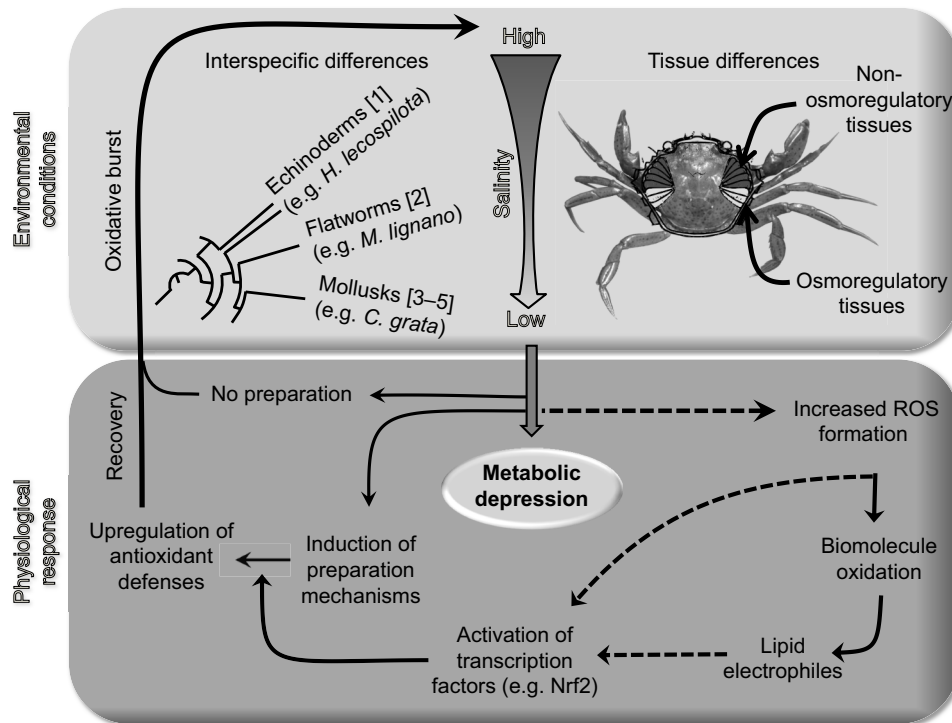
Fig. 4. Is the preparation for oxidative stress (POS) hypothesis relevant to marine invertebrates exposed to hypo-osmotic shock? Hypo- and hyper-osmotic stress may induce metabolic depression in certain species across phyla (example species are shown), but also in specific tissues or cells. However, before decreasing metabolic rates, some tolerant species/tissues or specialized cells may upregulate antioxidant defenses, a behavior that has been interpreted as a preparation for the oxidative stress (free radical burst) occurring upon recovery of standard metabolic rates and tissue reoxygenation. As reviewed by Hermes-Lima et al. (2015), these processes are suggested to be directly (hormetic pathway) or indirectly mediated by ROS formation (via lipid electrophiles derived from ROS peroxidation of lipids), through the activation of transcription factors leading to the upregulation of protective mechanisms. Solid arrows represent well-described processes, while dashed arrows indicate those pathways that are less well understood. [1] (Yu et al., 2013); [2] (Rivera-Ingraham et al., 2016b); [3] (Morritt et al., 2007); [4] (Sokolova et al., 2000); [5] (Stickle and Sabourin, 1979).

formation under *in vivo* or *ex vivo* conditions is challenging, and thus not often performed in marine eco-physiological studies, and there is no clear evidence of when this signaling could occur. Shallow coastal invertebrates that respond to large salinity changes by entering metabolic depression could constitute interesting study models to analyze the applicability of POS under osmotic stress. This would allow us to understand (1) the energetic trade-offs between energy savings and upregulation of antioxidant and osmoprotective genes required for recovery from metabolic depression, (2) the role of ROS in signaling and triggering such processes and (3) the consequences of anthropogenically derived changes in ROS-mediated signals and homeostasis.

Ecological relevance

It is worth considering any integrated physiological approach such as the one described in this Commentary in a broader ecological context. Could the highlighted differences between species be linked to evolutionary adaptations to distinct habitats? Above, we highlighted that particular osmoregulatory strategies are normally associated with certain habitats. Moreover, numerous studies have shown how zonation in shallow coastal environments is related to differential physiological responses to environmental factors such as salinity. In the literature, one can find examples relating to differential 'horizontal' distribution, such as a salinity gradient in estuaries that transitions from seaward stenohaline to landward euryhaline conditions (e.g. Giménez, 2003; Pinkster and Broodbakker, 1980).

An interesting study by Freire et al. (2011) compares two estuarine crab species of the genus *Callinectes* with different distributions along a salinity gradient. Despite their similar osmoregulatory behavior, the two species show significant differences in their redox metabolism. *Callinectes danae*, a more euryhaline species inhabiting more unstable environments, shows higher antioxidant defenses than the congeneric species *C. ornatus*, which lives in more stable conditions. There are other cases, such as the recent work of Theuerkauff et al. (unpublished) using two tropical crabs (*Neosarmatium meinerti* and *U. urvillei*), but in this case, the species inhabit the same area of a mangrove swamp. The authors demonstrate that despite having the same osmoregulatory strategy and similar isosmotic points (see Glossary), the two species have significantly different physiological responses to salinity changes. While *N. meinerti* does not show significant change in respiration rates, *U. urvillei* increases its respiration during hypo-regulation. Other examples of intraspecific variation can also be found. Two recent studies show, for example, how the mollusk *Ruditapes philipinarum* displays a completely different redox response to the same environmental salinity fluctuations depending on whether the animals come from a coastal lagoon (Velez et al., 2016b) or an estuary (Velez et al., 2016a). These variations were interpreted as differences relating to their ecological background (C. Velez, personal communication). Differential physiological responses are not restricted to 'horizontal' distributions (e.g. river–ocean gradient across an estuary). There are similar examples with 'vertical' distributions (e.g. different intertidal levels), showing how congeneric species (e.g. subantarctic limpets) living at different heights/depths from zero-tide also show different responses in terms of redox

imbalances (e.g. Malanga et al., 2004). The same also applies to congeners distributed at different tidal levels, where mussels located in the upper areas (and thus exposed to longer cycles of air exposure) present greater antioxidant defenses compared with populations located lower in the intertidal zone (e.g. Letendre et al., 2008).

Conclusions and future perspectives

Intracellular and extracellular osmoregulation mechanisms have been widely studied in most phyla, as have the biotic and abiotic factors influencing this capacity. The associated energy requirements have also been quantified through classical research methods. But the biomedical field is shedding new light on this domain, revealing the need to examine these mechanisms at the subcellular (namely mitochondrial) level to fully understand the energetic requirements (and consequences) of these processes. Undoubtedly, the large diversity of marine invertebrates and the great variability in osmoregulatory strategies, life histories, evolutionary backgrounds, tissue functions, etc., are responsible for the enormous variability of energy-redox responses upon changes in environmental salinity that we have briefly highlighted here. Benefiting from such a large biodiversity, we suggest applying a functional mitochondrial approach (analysis of mitochondrial morphology, number and efficiency, i.e. oxygen consumption, electron transport chain activity and H^+ leak and associated ROS/RNS formation), which may help us to disentangle patterns linking energy, redox and salinity-related responses. Most importantly, understanding such subcellular processes may help us to elucidate different evolutionary adaptations to different marine environments as well as to predict the role of ROS/RNS in promoting or preventing essential physiological responses leading to stress acclimation. The field of plant biology may provide key clues to aid in deciphering the mechanisms of salinity-induced ROS and RNS formation, as well as hinting at their role in acclimation to hypersaline conditions. This Commentary highlights the need to integrate the methodological approach, working hypotheses and future research directions from these two fields. In the wider context of global climate change and the anthropogenic alteration of coastal habitats, there is an increasing need to apply these research directions to the study of how changes in ROS/RNS balance may affect salinity acclimation in marine intertidal organisms. These organisms represent ideal models that can be used to make important advances in the field of animal conservation physiology.

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