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Salinity stress from the perspective of the energy-redox axis: Lessons from a marine intertidal flatworm

Georgina A. Rivera-Ingraham [a,*], Aude Nommick [a], Eva Blondeau-Bidet [a], Peter Ladurner [b], Jehan-Hervé Lignot [a]

[a] Groupe fonctionnel AEO (Adaptation Ecophysiologique et Ontogenèse), UMR 9190 MARBEC, University of Montpellier, 34095 Montpellier, France
[b] Institute of Zoology and Center for Molecular Biosciences Innsbruck, University of Innsbruck, A-6020 Innsbruck, Austria

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ABSTRACT

In the context of global change, there is an urgent need for researchers in conservation physiology to understand the physiological mechanisms leading to the acquisition of stress acclimation phenotypes. Intertidal organisms continuously cope with drastic changes in their environmental conditions, making them outstanding models for the study of physiological acclimation. As the implementation of such processes usually comes at a high bioenergetic cost, a mitochondrial/oxidative stress approach emerges as the most relevant approach when seeking to analyze whole-animal responses. Here we use the intertidal flatworm *Macrostomum lignano* to analyze the bioenergetics of salinity acclimation and its consequences in terms of reactive oxygen/nitrogen species formation and physiological response to counteract redox imbalance. Measures of water fluxes and body volume suggest that *M. lignano* is a hyper-/iso-regulator. Higher salinities were revealed to be the most energetically expensive conditions, with an increase in mitochondrial density accompanied by increased respiration rates. Such modifications came at the price of enhanced superoxide anion production, likely associated with a high caspase 3 upregulation. These animals nevertheless managed to live at high levels of environmental salinity through the upregulation of several mitochondrial antioxidant enzymes such as superoxide dismutase. Contrarily, animals at low salinities decreased their respiration rates, reduced their activity and increased nitric oxide formation, suggesting a certain degree of metabolic arrest. A contradictory increase in dichlorofluorescein fluorescence and an upregulation of gluthathione-S-transferase pi 1 (GSTP1) expression were observed in these individuals. If animals at low salinity are indeed facing metabolic depression, the return to seawater may result in an oxidative burst. We hypothesize that this increase in GSTP1 could be a "preparation for oxidative stress", i.e. a mechanism to counteract the production of free radicals upon returning to seawater. The results of the present study shed new light on how tolerant organisms carry out subcellular adaptations to withstand environmental change.

1. Introduction

Among other factors, organisms located in estuarine and intertidal environments are frequently exposed to high changes in environmental salinity. These changes are particularly noticeable in the interstitial habitat, and can occur over very short periods of time: the association of tidal influences with high temperatures and intense evaporation common during summer months may quickly increase water salinity. Similarly, heavy rains and/or freshwater run-off waters can drastically decrease salinity levels. Thus, salinity is a crucial factor determining population structures of intertidal and estuarine free-living meiofaunal organisms [1,2]. In order to survive in these habitats, organisms must successfully implement osmoregulatory mechanisms that will allow them to regulate their water content [1].

Based on their osmoregulatory responses, marine invertebrates can be considered as osmoconformers or osmoregulators (which in turn can be hyper-/iso-regulators or hyper-/hypo-regulators), depending on whether they maintain the osmotic pressure of their internal fluids higher, lower or at the same osmotic pressure as their environmental salinity [3]. In osmoconformers, hemolymph osmolality changes mirror those of the external medium. The same is true of hyper-/iso-regulators at higher salinities, although they are capable of controlling hemolymph osmolality through

Abbreviations: ASW, artificial seawater; C-H_2DFFDA, 5-(and-6)-carboxy-2′,7′-difluorodihydrofluorescein diacetate; DCF, dichlorofluorescein; DHE, dihidroethidium; $\Delta_{\Psi m}$, mitochondrial membrane potential; NO, nitric oxide; P_{c2}, lower critical pO_2; P_{c1}, upper critical pO_2; ROI, region of interest; ROS, reactive oxygen species; RNS, reactive nitrogen species; SW, seawater; VO_2, respiration rates

* Corresponding author.
E-mail address: georgina.rivera-ingraham@umontpellier.fr (G.A. Rivera-Ingraham).

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hyper-regulating processes when immersed in diluted seawater (SW). Hyper-/hypo-regulators maintain the osmolality of their internal medium at a relatively constant level, regardless of the environmental salinity and thus hypo-regulate under higher salinity concentrations and hyper-regulate in diluted SW. The absence of a circulatory system in meiofaunal organisms such as free-living Platyhelminthes, which deal with all physiological adjustments to cope with changes in ionic concentrations on an intracellular level, results in initial whole body swelling or dehydration when experiencing hypo or hyper-tonic stress, respectively. These mechanisms usually involve the accumulation or catabolism of organic osmolytes and derivatives that contribute to intracellular osmolality and control of water and ion fluxes across membranes. A great number of works have described osmoregulatory capacity and mechanisms at different life stages e.g. [4,5], according to nutritional status e.g. [6,7] or even under the effect of different chemical or physical stressors e.g. [8,9]. Very few studies investigate osmoregulation in meiofaunal organisms, and mainly concentrate on nematodes [1,10,11], largely ignoring the flatworm model. reviewed by [12–15].

The energetic costs of osmoregulation in invertebrates have been described in a number of publications which have commonly addressed this mechanism through measurements of respiration rates e.g. [16,17]. Part of this respiration leads to the ATP production required for fueling osmoregulation processes, and inevitably results in reactive oxygen and nitrogen species (ROS/RNS) formation at the mitochondrial level. Although these compounds play a necessary role in cellular homeostasis, they are also known for their deleterious effects on cellular compounds such as proteins, lipids or even nucleic acids. These reactions, commonly known as "oxidative stress" (OS) can be partially counteracted by the upregulation of antioxidant defenses, another active process demanding additional energy expenditures. Yet few studies have examined the effects of changes in environmental salinity on mitochondrial activity, free radical production and the management of antioxidant defenses [18,19], and fewer still have sought to identify the link between these processes and osmoregulation e.g. [20,21]. This question is crucially important in the light of recent studies showing that whole animal respiratory rates are not necessarily a proxy of energetic metabolism [22].

This study concentrates on the energy-redox axis to understand the physiological and behavioral responses of animals facing environmental salinity changes within a changing environment where intertidal organisms are subjected to increasing episodes of hyper- and hypo-osmotic shocks. We contribute to literature with our use of a novel study model, the upper intertidal free-living flatworm, *Macrostomum lignano* (Rhabditophora: Macrostomorpha) [23]. This is an interesting species to study physiological adaptation to environmental change [24], but also a good model for wide variety of studies, ranging from sexual selection [25] to stem-cell research [26], ageing [27] or bioadhesion [28]. Our main goal is to analyze how hyper- or hypotonic stress affects animal energetic balance, mitochondrial function and thus, ROS/RNS levels, and thus evaluate the costs of acquiring an acclimation phenotype and the ability of these animals to counteract ROS overproduction with scavenging enzymes. This model is a small and transparent organism, providing a unique opportunity for studying the effects of hyper and hypo-osmotic shocks on free radical formation and mitochondrial functioning through the application of live-imaging techniques *in-vivo*.

2. Materials and methods

2.1. Animal culturing and experimental treatments

Cultures of *M. lignano* (DV-1 line) [29] were reared in artificial SW (ASW) (SeaSalts, Sigma S-9883) (35 ppt). Animals were placed in petri dishes on which the diatom *Nitszchia curvilineata* previously grown in Guillard's F/2 medium (Sigma G0154) for a minimum of 3 weeks. Both diatom and worm cultures were maintained at room temperature (RT, 20 °C) with a 16:8 h (day: night) photoperiod. All animals used in this study were adults and thus synchronized for size and also age (<1.5 months old).

We considered 4 different salinity values for which no mortality rates had been observed in preliminary experiments: 5 ppt, 15 ppt, 35 ppt (considered here as control conditions) and 55 ppt. Animals were exposed to the environments for 6 h in all cases except for gene expression analyses, where treatments were prolonged to 24 h to ensure the induction of significant changes in stress-related mRNA abundance [30–32]. All analyses were carried out in ASW.

2.2. Volume measurements

With an average length of 0.8 mm, *M. lignano* individuals are too small for osmotic pressure measurements through the use of common techniques. Internal osmotic concentration was therefore indirectly inferred through body volume measurements, a common procedure for these or similar organisms such as free-living nematodes [33]. Worms acclimated to 35 ppt were imaged with a Leica Diaplan microscope equipped with a Leica DC300F camera (Leica Microsystems, Wetzlar, Germany) using a ≈ 200 μm-deep slide (as described in Schärer et al. [83]) and 3 μl medium, all covered with a coverslip. These conditions ensured a standardized measurement, where animals could only move in the X-Y axis while staying in focus under the microscope [34]. Each individual was imaged before (T=0) and after salinity change at five-minute intervals (T=5 to T=60 min). Given that this is a 2D measurement where muscle contractions are likely to induce changes in area (independent of water content), animals were, when possible, photographed when moving about. For the same reason, three images where taken for each animal and time point. The calculation of animal total area was calculated using ImageJ software (NIH, Rasband WS). For each of the experimental worms, total animal area was averaged using the three corresponding pictures. Values were expressed as relative body volume compared to their size at 35 ppt (T=0). Thus, values above 1 and below 1 indicate a gain or loss of body volume, respectively.

2.3. Animal activity

Five animals that had previously been acclimated to different salinities were stained with Mitotracker Deep Red 633 (Ex: 633 nm; Em: 660 nm) to facilitate animal tracking. The fluorophore was added to experimental mediums at a concentration of 0.33 μM at least 1 h before the analysis and were kept in this medium until the end of the experiment. Animals were observed individually in the same conditions as for the body volume analyses. They were individually scanned for 2 min at a frequency of 200 msec using a confocal spinning disk W1 Andor coupled with an inverted microscope Nikon equipped with a Neo sCMOS camera. This technique, along with the use of deep red staining was used to minimize the impact of the laser on animal activity. Resulting images were stacked using ImageJ software and then imported into Imaris and analyzed using the "Imaris Track" module (BitPlane). All parameters taken into account (e.g. average or maximum animal speed) were calculated using the different