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

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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

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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

functions of the "Imaris Track" software.

2.4. Respiration measurements

Experimental animals were isolated from their original cultures and fasted for approximately 15 h in ASW to avoid any effect of nutritional stress [35] resulting from dynamic action of feeding as well as the physiological costs of associated processes. Measurements were carried out as previously described in [24], with minor changes: briefly, groups of 20 animals were immersed (in their corresponding salinity) in individual wells of a glass microtiter plate (Mikroglas Chemtech, Mainz, Germany), previously equipped with oxygen sensor spots (OXSP5, sensor code SD7-545-214) (Pyro-Science GmbH, Aachen, Germany). Each well was filled to its maximum capacity (100 ± 0.5 µl) to avoid the formation of air bubbles and the well was sealed with a 18×18 mm coverslip around which silicon was placed to ensure air tightness. Optodes were calibrated to 100% O_2 solubility in air-saturated water and to 0% O_2 water. The latter was chemically achieved using a freshly made 80 mM Na_2SO_3 solution. Oxygen concentration within each of the microchambers was measured using a four-channel fiber-optic oxygen meter (FireSting, Pyro-Science GmbH) and recorded through the Pyro Oxygen Logger software. All measurements started in fully oxygenated water ($>98\%$) and respiration was recorded as a function of declining pO_2 over time. Four measurements were carried out simultaneously (including a blank, containing no animals) and data were recorded in 3 s intervals. After a 6 h acclimation to the different experimental salinities, animals were allowed to respire until the oxygen had been completely consumed in each of the microchambers (4–6 h depending on environmental salinity) and salinity-dependent effects on hypoxia management were assessed. The critical oxygen partial pressure (P_c) as defined by [36], was calculated using the equation described by [37]. Whatever the salinity, an upper critical pO_2 (P_{c1}) and a lower critical pO_2 (P_{c2}) were detected for *M. lignano* between which animals respire in an oxyregulating manner [24], that is, where oxygen consumption rates (VO_2) are maintained constant regardless of environmental O_2 concentration. VO_2 values were calculated using the values between P_{c1} and P_{c2}, corrected for environmental salinity and expressed as nmol O_2 h^{-1} ind^{-1}.

2.5. Mitochondrial parameters

For each of our 4 experimental salinity conditions, mitochondrial density (ρ_m), mitochondrial membrane potential ($\Delta\Psi_m$) and ROS production were measured using the so-called "live-imaging techniques", consisting of the application of specific dyes and *in-vivo* visualization through fluorescence microscopy. In the present study, all analyses were conducted using a Leica DM 2500 confocal microscope (Leica Microsystems). All experiments were carried out using 10 animals per treatment and the entire experimental protocol for all 40 individuals was repeated at least twice to ensure repeatability of results. The dyes in this study (see Table 1) were used individually to avoid any possible interference among them. We also ensured that there was no interference between salt level and fluorescence for each of the dyes. Interaction was only found for 5-(and-6)-carboxy-2′,7′-difluorodihydrofluorescein diacetate (C-H_2DFFDA), with fluorescence increasing with salinity, as previously described [20]. A correction factor was therefore applied for this dye.

Before each experiment, autofluorescence was suppressed by adjusting the threshold. Photobleaching was minimized by conducting short periods (<5 s) of low-resolution (256×256 pixel) scanning for focus adjustments before finally taking a single scan at a resolution of 512×512 pixels for further quantification. All images were processed using Leica LAS Lite software (Leica Microsystems), and as already described in [24] (see Table 1): for non-ratiometric dyes, quantification was carried out by plotting 5–10 transects per animal after checking that they were perpendicular to the longitudinal axis of the body, coinciding on the areas of maximum fluorescence intensity and always lined up on the head of the animal [see Fig. 1 in [24]]. For ratiometric dyes such as dihidroethidium (DHE) and JC-10, quantification was carried out using square regions of interest (ROIs) with a dimension of 40 ± 0.88 µm^2.

2.6. RNA extraction and quantification of transcript levels

Animals were extracted from their original cultures 48 h prior to worm fixation to avoid any possible interference of gut content on gene expression analyses. *M. lignano* were exposed to the different experimental salinities for 24 h, then pools of 4 animals were lysed and their total RNA was extracted using NucleoSpin® RNA XS columns (Macherey-Nagel, Düren, Germany) and following the manufacturer's guidelines. Genomic DNA was eliminated by RNase-free DNase I treatment during the extraction procedure. Pure total RNA was eluted in 15 µl of RNase-free water. RNA concentration and integrity were assessed using an Agilent Bioanalyzer 2100 equipped with an RNA Nano Chip (Agilent Technologies, CA, USA). Reverse transcription was carried out using M-MLV Reverse Transcriptase (Invitrogen, France).

Using an Echo®525 liquid handling system (Labcyte Inc., California, USA), 0.75 µl of LightCycler-FastStart DNA Master SYBR-Green I™ Mix (Roche, Mannheim, Germany), 0.015 µl of each

Table 1
Analysis conditions for each of the dyes used during the study.

Dye	Final conc. used (µM)	Incubation time (min)	Ind/batch	N repetitions (total N ind/treatment)	Excitation		Emission		Calculation
					λ_1 (nm)	λ_2 (nm)	PMT1 (nm)	PMT2 (nm)	
C-H_2DFFDA (in Ethanol)	10.6	30	10	2 (20)	488	–	510–550	–	Maximum Intensity
Dihydroethidium (DHE) (in DMSO)	4	30	10	3 (30)	405	488	400–440	620–660	Ratio PMT2/PMT1
DAF-2DA (in DMSO)	8	30	10	3 (30)	488	–	505–525	–	Maximum intensity
JC-10 (in DMSO)	5	30	10	3 (30)	488	488	500–550	560–600	Ratio PMT1/PMT2
MitoTracker Deep Red 633 (in DMSO)	0.33	60	10	3 (30)	633	–	640–680	–	Maximum intensity
MitoSOX (in DMSO)	5	30	10	2 (20)	510	–	560 – 600	–	Maximum Intensity

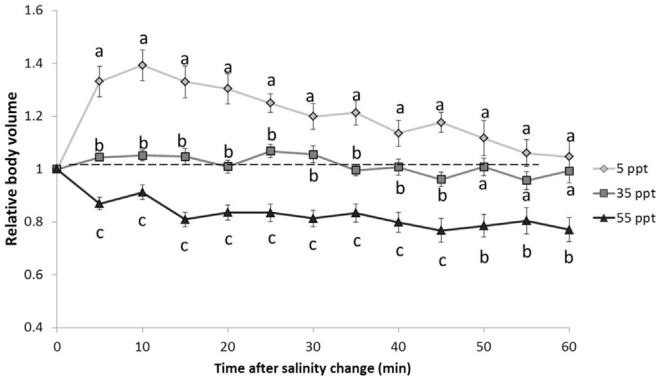

Fig. 1. Relative body volume over time for animals under different conditions of environmental salinity. For each time point, values associated with different letters show significant differences among them.

primer (forward and reverse at 0.2 μM final concentration), 0.22 μl of ultra-pure water and 0.5 μl of cDNA were dispensed into a 384-well reaction plate. Each sample was run in triplicate. The qPCR conditions were as follows: denaturation at 95 °C for 10 min, followed by 45 cycles of repeat amplification (95 °C, 15 s), hybridization (60 °C or 62 °C according to the primer pair used, 5 s) and elongation (72 °C, 10 s) and a final step at 40 °C for 30 s. A melting curve program was performed to control the amplification specificity. Ultra-pure water was used as a no-template control in the qPCR. Efficiencies were between 1.61 and 2.04. Primers were designed based on the *M. lignano* transcriptome assembly (version MLRNA131024) [38]. The primer sequences for each of the genes used are shown in Table 2.

2.7. qPCR data analysis

A total of 6 housekeeping genes were tested to determine the most adequate reference for our analyses: α-tubulin *tuba,* β-tubulin *tubb,* β-actin *actb*, glyceraldehyde 3-phosphate dehydrogenase *gadph*, the ribosomal protein L12 *rpl12* and the GM2 ganglioside activator *gm2a*. Our analyses identified the two last genes as the most adequate in terms of expression stability and Ct values, and *gm2a* had already been highlighted as a good reference gene for *M. lignano* under salinity stress [39]. As proposed by Vandesompele et al [40], relative expression levels were calculated by using the geometric mean of the Ct values of the 2 references genes for each sample and normalizing the target genes to this geometric mean.

2.8. Statistical analyses

Results are presented as means ± standard errors of the mean (S.E.M.). The Shapiro test was used to test normality on data resulting from the mRNA relative level expression. Homoscedasticity was tested by the Bartlett test and the independence of residuals was evaluated using the Durbin-Watson test. For the data resulting from the caspase 3 expression, Box-Cox transformation was applied to achieve normal distribution before performing statistical analysis. Results were evaluated by one-way analysis of variance (ANOVA), followed by a Tukey post-hoc test (HSD: honestly significant difference), with a significance level of $p < 0.05$. All statistical analyses were conducted using the RStudio freeware software (R Core Team, 2014 Version 0.99.891) and SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

Table 2
Genes used in the present study.

Gene acronym	Gene name	Sequence ID database MLRNA131024	Primer sequence 5′ to 3′ Forward/Reverse	Amplicon length (bp)	Annealing temp. (°C)	Amplification efficiency
cox5b	Cytochrome c oxidase subunit 5B	RNA1310_41945	CGCCTGCTAGACCGGTTCC/CTGACGGTACCAGCACGGG	112	60	1.94
casp3	Caspase 3	RNA1310_1479.1	GCCAGAAGACTCCAGCCCC/CTGCGTCTCTCCCTGGCAG	208	62	2.04
gstp1	Glutathione S-transferase pi 1	RNA1310_29579.1	TTCGGTGCCAGGAAGAATCT/ CCCATTTAAAGATGCGGCGA	89	60	1.61
mgst1	Microsomal glutathione S-transferase 1	RNA1310_49001.2	ATGTGCTGCCGTTTGTACTG/CCTACTGGCGGCAAAGATTC	98	60	1.83
prdx6	Peroxiredoxin 6	RNA1310_35269	ACCAGGCCTTGTGATCTTCA/TGTCAATCTGAAGCCGGAGT	229	60	1.94
prdx1	Peroxiredoxin 1	RNA1310_28865.2	CGCGGCCTGTTCATCATCAA/ GCTTGTCGGTCAACTGGAAG	124	60	1.73
sod2	Superoxide dismutase 2, mitochondrial	RNA1310_30405	AAGAACGTTCGCCCACACTA/AGCTTAACTCCGCCACTCTT	101	60	1.81
rpl12	Ribosomal protein L12	RNA1310_36703	GACAAGGTTAACGACGGCTC/ TATAGCAGCCGGTGTGTCAA	81	60	1.87
gm2a	GM2 ganglioside activator	RNA1310_42438	CATCACCCTGCCCCAGATTC/ TCTTCAATGCGACCCTCAAGC	132	62	1.75

BLAST search and sequence alignments of genome- and transcriptome sequences of the genes revealed that, apart from sequences with single nucleotide variations, no paralogs of these transcripts exist.

3. Results

3.1. Volume regulation

Within the first 10 min of exposure to low salinity (5 ppt), *M. lignano* responds by increasing its body volume by an average of 39% (Fig. 1). However, after this time, organisms slowly decrease their volume and 50 min after the first exposure to 5 ppt, no significant differences were observed in comparison with control animals. Contrarily, exposure to hypersaline conditions (55 ppt) caused animals to abruptly lose water content. Animals lost an average of 20% of their total initial volume during the first 15 min of exposure, and did not recover, with significant differences observed throughout the experiment with control animals (35 ppt). Data for these animals was recorded up to 24 h after the first exposure to hyperosmotic conditions, and confirmed the absence of recovery within this time (data not shown).

3.2. Animal activity

Salinity treatments, and particularly exposure to 5 ppt, had a significant impact on animal behavior (see Fig. 2). Animals subjected to the lowest salinity level showed significantly lower activities, and these observations were corroborated through the Imaris analyses, showing these animals had significantly lower maximum ($K=1.621$; $p<0.01$) and average speed ($K=1.484$; $p<0.05$) (Fig. 2A). In these cases, worm behavior was mainly reduced to small contraction movements (see example in Fig. 2B). No significant differences were recorded for these or other parameters for the remaining salinity treatments. However, we observed within the framework of our volume regulation recordings that animals at 55ppt often drastically decreased their activity rates during the first hour of exposure. However, normal activity then returns, as shown by the results in Fig. 2.

3.3. Respiration-related measurements

Average VO_2 values during the oxyregulating phase of respiration (between P_{c1} and P_{c2}) were used for comparisons among treatments. Results indicate that VO_2 increases with increasing salinity ($K=93.632$; $p<0.001$) (see Fig. 3). Animals at 15 ppt showed similar VO_2 and P_c values, while hyposaline exposure causes a decrease in P_{c2} and also impacts VO_2, which decreases 1.7 fold.

3.4. Reactive oxygen species formation

$O_2\cdot-$ formation increases with salinity, as indicated by the 2-OH-E+: DHE ratio results ($K=31.867$; $p<0.001$) (Fig. 4A). Contrarily, dichlorofluorescein (DCF) fluorescence (Fig. 4B) shows a dramatic increase at lower environmental salinities ($K=24.167$; $p<0.001$). As highlighted by DAF-2T fluorescence (Fig. 4C, the presence of nitric oxide (NO) also increased at lower salinities ($F=4.683$; $p<0.01$). In general accordance with 2-OH-E+: DHE ratio results, mitochondrial $O_2\cdot-$ formation (MitoSOX staining) also revealed a significant increase with salinity ($F=20.204$; $p<0.001$) (Fig. 5A).

3.5. Mitochondrial density and energetic state

A small, albeit significant increase was observed in the mitochondrial density of animals that were exposed to the highest salinity (55ppt) over 6 h-period ($F=6.028$; $p<0.01$) (Fig. 5B), reaching an increase of 1.3-fold over control animals. Increasing 55 ppt exposure to 24 h enhanced these differences until reaching 1.8-fold ($K=31.820$; $p<0.001$) (results not shown). On the other hand, $\Delta\Psi_m$ increased significantly at lower salinities ($K=8.530$; $p<0.05$) (Fig. 5C).

3.6. Gene expression quantification

No significant difference was seen in transcript levels of

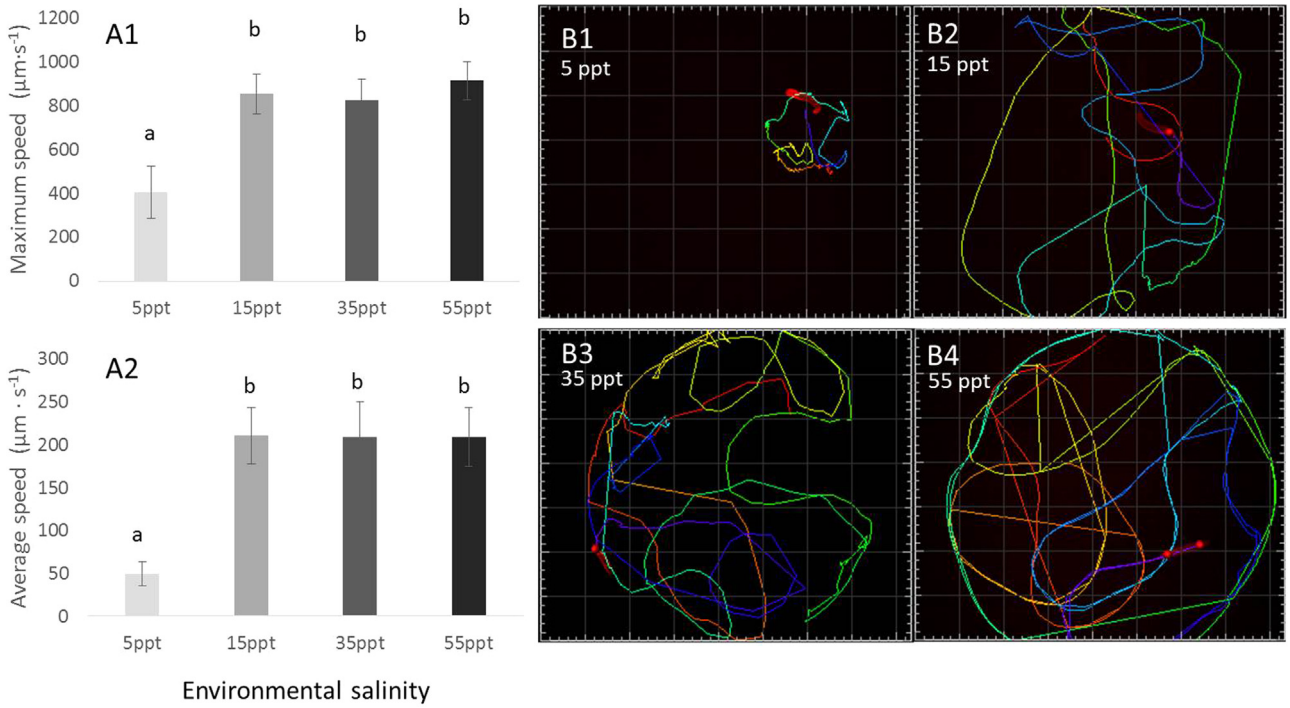

Fig. 2. Activity measurements as recorded through Imaris. A) Quantitative results for animal maximum speed (1) and average speed (2); B) Representative images of animal tracks at 5 ppt (1), 15 ppt (2), 35 ppt (3) and 55 ppt (4).

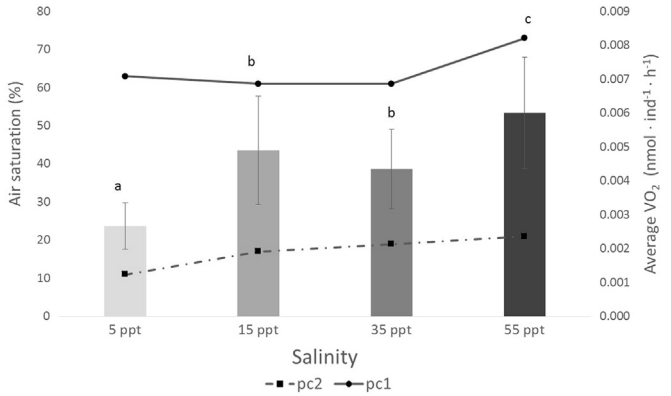

Fig. 3. Results corresponding to the respiration rate measurements conducted on *M. lignano* at different environmental salinities. Bars represent average respiration rates (VO_2) between the upper critical pO_2 (P_{c1}) and lower critical pO_2 (P_{c2}) (continuous and discontinuous lines, respectively).

reference genes *rpl12* and *gm2a* among salinity treatments ($p > 0.062$ and $p > 0.11$, respectively). The qPCR analyses on worms, as shown in Fig. 6 confirmed our Mitotracker Deep Red results, with a significant increase in cytochrome c oxidase (subunit 5B) (*cox5b*) expression with increasing salinity. In the same manner, higher salinities induced a significantly higher expression of caspase 3 (*casp3*), codifying for an enzyme involved in programmed cell death. As expected, exposure to higher salinities induced an increase in many of the antioxidant enzymes analyzed in this study, such as microsomal glutathione-s-transferase mitochondrial (*mgst1*), peroxiredoxin 1 (*prdx1*) or mitochondrial superoxide dismutase (*sod2*). Contrarily, an increase was observed in the levels of two antioxidant enzymes, namely peroxiredoxin 6 (*prdx6*) and gluthathione-s-transferase pi-1 (*gstp1*), when worms were immersed in higher or lower salinities, with higher increases in *prdx6* at higher salinities. GSTP1 was the only redox enzyme showing a clear increase on exposure to low salinity.

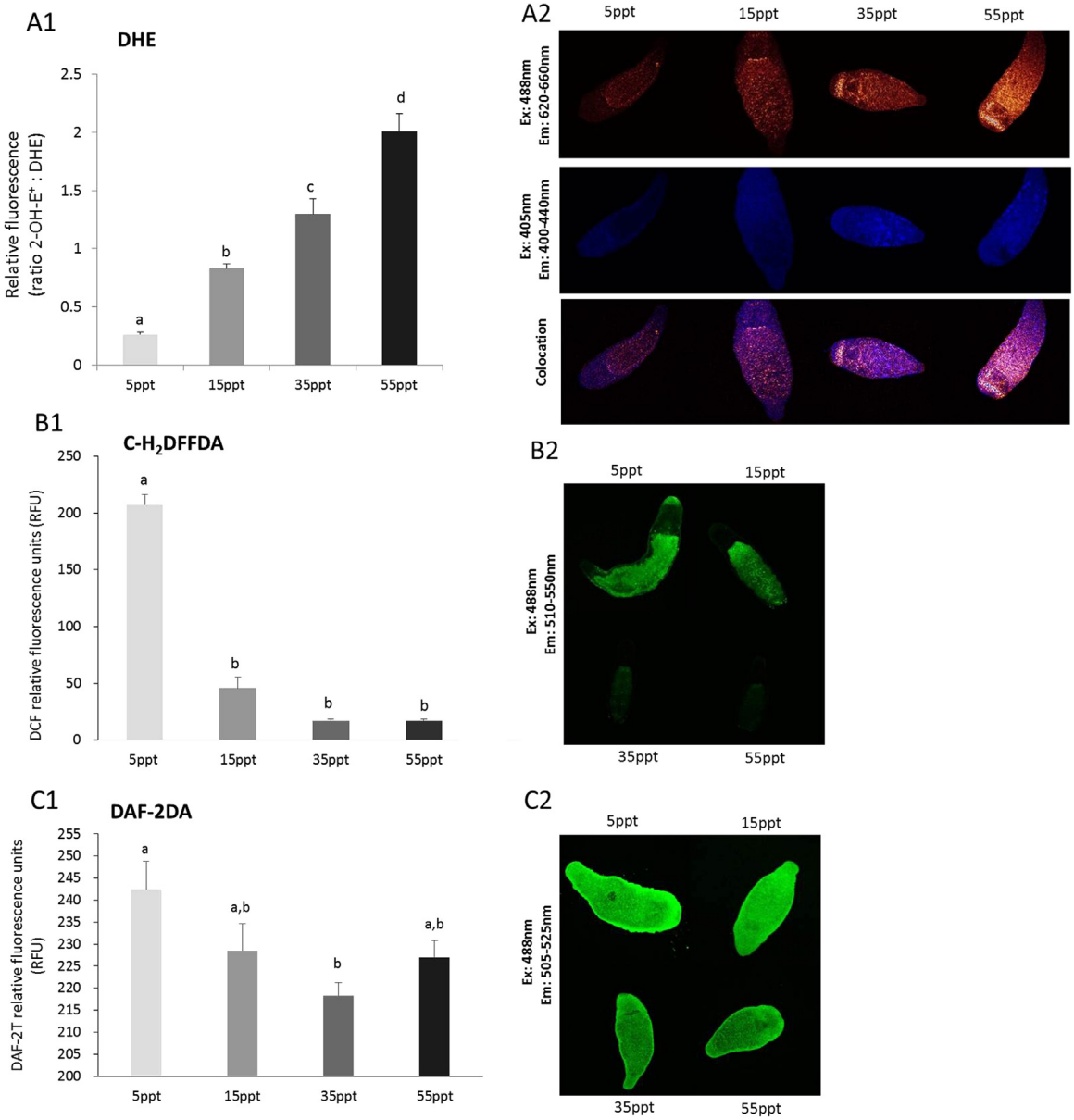

Fig. 4. ROS/RNS formation assessed in living *M. lignano* through a) DHE, b) C-H_2DFFDA and c) DAF staining. Subpanels 1 represent quantitative values of 2-OH-E$^+$: DHE (A), DCF (B) and DAF-2T (C) fluorescence while subpanels 2 show representative images for each of the experiments.

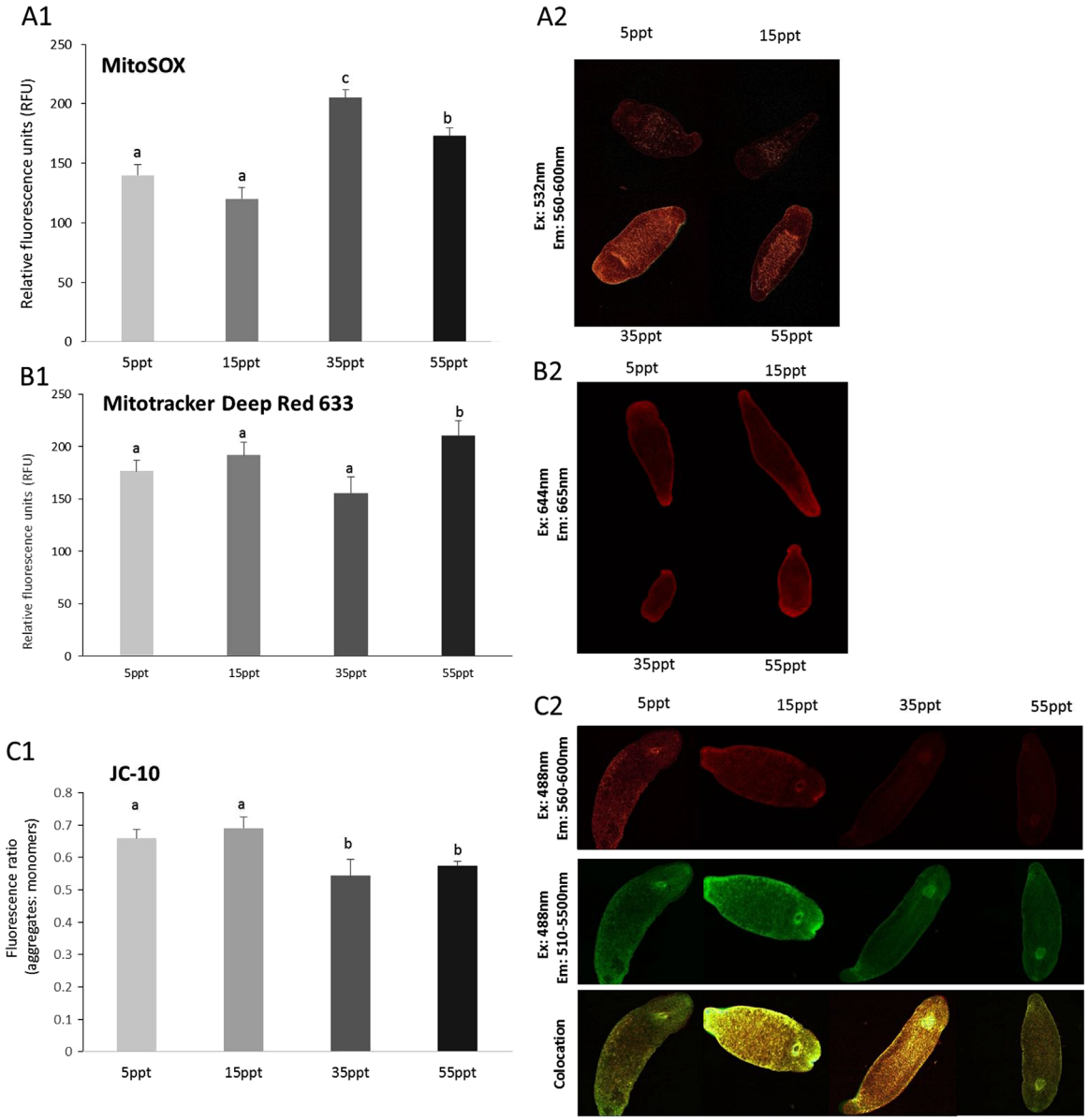

Fig. 5. Mitochondrial analyses carried out in living *M. lignano* using a) MitoSOX, b) Mitotracker Deep Red 633 and b) JC-10 staining, as indicators of mitochondrial $O_2 \cdot -$ formation, ρ_m and $\Delta_{\psi m}$, respectively. Subpanels 1 represent quantitative values while subpanels 2 show representative images for each of the experiments.

4. Discussion

4.1. *M. lignano: osmoconformer or osmoregulator?*

These results show the intertidal flatworm *M. lignano* to be an euryhaline species that easily manages temporary exposure to salinities ranging from freshwater to 80 ppt (Rivera-Ingraham pers. obs). This arises as an essential condition for survival in interstitial waters of coastal marine sediments where they are exposed to air during long periods of time. As reviewed by Wharton and Perry [33], in the absence of osmotic pressure measurements, the regulation of body volume is a reliable tool to determine the osmoregulatory strategy of small meiofaunal organisms. As *M. lignano* can only recover its initial volume under hypo-osmotic conditions, we may conclude that we are dealing with a hyper-/iso-regulator.

The energetic costs of osmoregulation have been addressed in some intertidal macrofaunal invertebrates through the measurement of whole animal respiration rates [e.g. 17]. However, it has been recently been pointed out that these measurements are not necessarily correlated with energy metabolism [22]. This highlights the need to study the mechanistic processes facilitating acclimation to environmental change at the mitochondrial level in terms of ATP production, ROS formation and the maintenance of the redox balance (Rivera-Ingraham & Lignot, subm). What can be learned from *M. lignano*?

4.2. *Hypertonic stress is an energetically costly process accompanied by redox imbalances*

It is likely that *M. lignano* is regularly exposed to hypertonic conditions since this species inhabits upper costal levels that are not regularly bathed by seawater but are frequently exposed to high temperatures and irradiation levels. We may therefore expect *M. lignano* to have developed an optimized cellular and molecular adaptation to withstand the harshness of this environment, as previously evidenced by de Mulder et al [41]. The question remains as to the physiological management of these hypertonic environments by flatworms. It has already been established since the 1960s that euryhalinity in marine invertebrates is largely

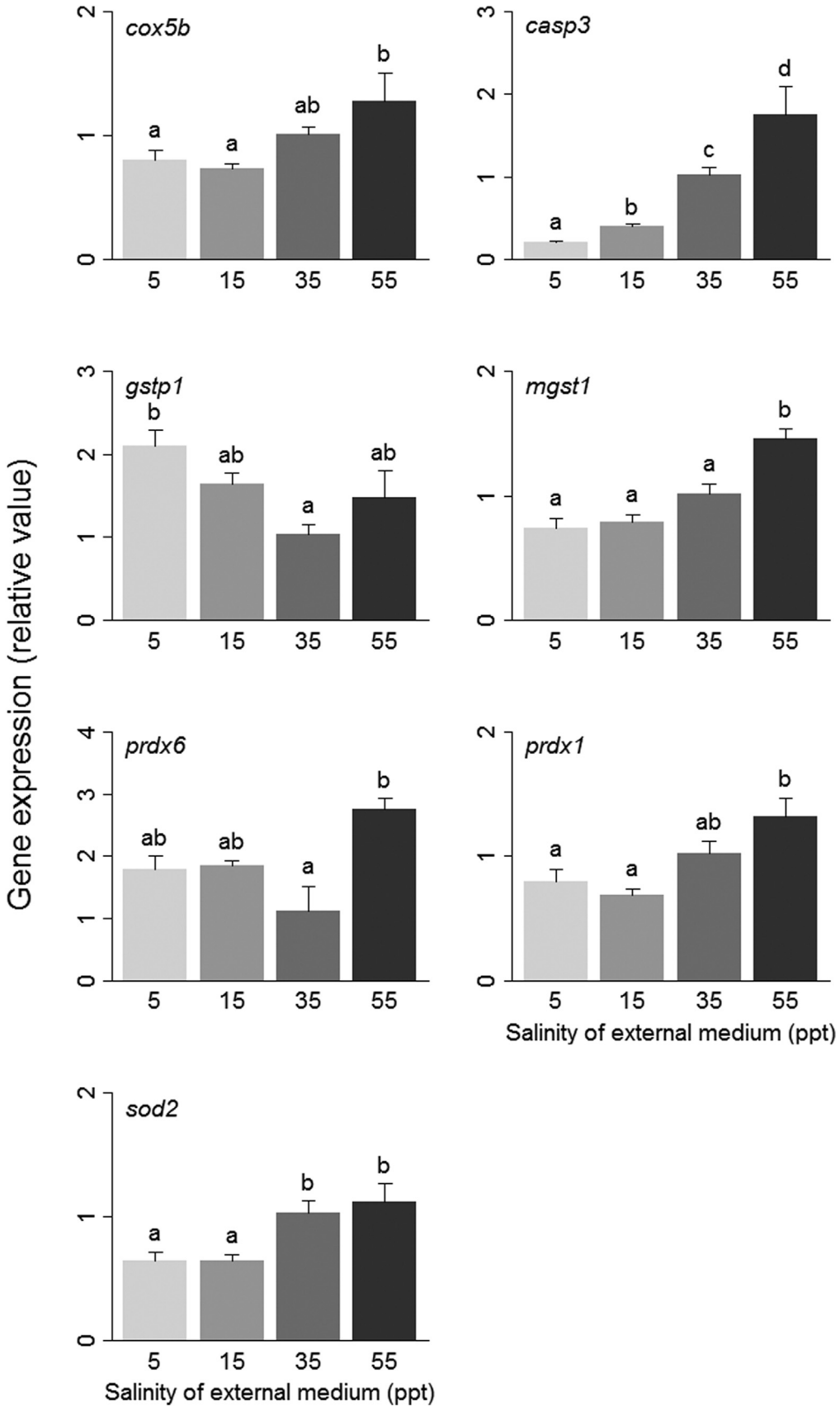

Fig. 6. Quantitative gene expression results for *M. lignano* under different environmental salinities.

determined by their capability to adjust the intracellular concentration of free amino acids [42] and organic ions [43]. Osmotic laws determine that in such hypersaline conditions, water will perfuse out of the worms, leading to dehydration. If these osmolytes are present in the medium, animals can uptake and accumulate them [44]. However, the present study was carried out using artificial seawater, which is supposedly free of organic osmolytes. We can, thus, consider that the worms in our experiment are dealing with hyperosmotic stress through *de novo* synthesis of free amino acids, like in other intertidal invertebrates [45,46] and similar meiofaunal organisms such as nematodes [47]. Since animals are not able to recover their initial volume within 24 h, we may hypothesize that they are osmoconformers at high salinity [48]. This leads to the legitimate question of the energetic costs associated with this osmolyte production.

In our study, animals responded to high salinity by increasing their respiration rates and synthesizing new mitochondria, both likely necessary steps to meet the energetic requirements of this acclimation mechanism. Increased respiration rates have also been observed in other osmoconforming invertebrates ranging from mollusks [49,50] to echinoderms [51]. Although it is traditionally considered that higher respiration rates are a proxy of energy metabolism, recent studies have shown that it this is not always the case [22]. Since we registered decreased $\Delta_{\Psi m}$, we may nevertheless hypothesize that intense ATP production occurs (state 3). Our DHE stainings indicated an intense production of $O_2 \cdot -$ with increasing salinity. At present, it is largely accepted that non-pathological mitochondria presenting higher membrane potential also produce higher amounts of ROS, and vice versa [52–54]. This does not concur with our results. A recent work [55] also suggests that high metabolic rates are associated with low levels of ROS production. However, our results are not the first evidencing hypersalinity-induced $O_2 \cdot -$ formation: studies using the yeast *Saccharomyces cerevisiae* also show that hypertonic stress (0.8 M NaCl) induced a 5-fold increase in $O_2 \cdot -$ that most probably resulted in oxidative stress, given the decrease recorded in antioxidant defenses under these conditions [56]. Likewise, hyperosmolarity stimulation increased 2-OH-E^+ (as well as DCF fluorescence) in mouse hypothalamic cells, but in this case the increase in pro-oxidants was accompanied by an increase in antioxidant enzymes such as CAT or SOD [57]. The same phenomenon was observed with *M. lignano*, in which $O_2 \cdot -$ formation may be controlled through the upregulation of numerous antioxidant enzymes, namely SOD-2, transforming $O_2 \cdot -$ within mitochondria (the main source of $O_2 \cdot -$ within heterotrophs) into H_2O_2. These $O_2 \cdot -$ and the resulting H_2O_2 can each have separate deleterious effects, but the consequences for biological structures are normally greater when these molecules combine with each other or with other reactive species. Whatever the case, these reactive species frequently target unsaturated phospholipids and other components of cell membranes, leading to the formation of lipid peroxides. Among the intermediates of these reactions, we find highly genotoxic species such as lipid hydroperoxides (LOOH) [58]. To counterbalance these compounds, organisms have developed Prdxs, enzymes involved in detoxifying LOOHs by reducing them to their corresponding alcohols. Our results show that the flatworms are indeed upregulating (2-Cys) *prdx 1* and (1-Cys) *prdx6*, which could provide added capacity to detoxify under the pro-oxidant conditions caused by hypersaline exposure, and provide cell protection against lipid peroxidation as previously described in numerous studies e.g. [59,60].

However, our gene expression data indicate that under hypersaline conditions, *casp-3* transcription levels are about 1.7-times higher than under SW conditions (35 ppt) and over 8-times higher than in 5 ppt. Caspases are crucial mediators of apoptosis, and are essential for normal homeostasis [61]. Although the small size of our samples prevents us from analyzing caspase activities in our animals, we have no significant reason to believe that this increase in *casp-3* is an indicator of animal intolerance to high salinity, given that, as it was already mentioned, *M. lignano* is capable of reproducing and withstanding much higher salinity concentrations.

4.3. Dealing with hypo-osmotic stress induces metabolic arrest

Freshwater or near freshwater conditions may occur frequently in intertidal environments due to events such as during rainfall occurring at low tide. In order to survive, animals have to extrude a large amount of metabolites such as free amino acids in order to control their osmolality and body volume. However, free amino acids are not extruded in their current form, but are deaminated [see [16] and references therein], a process which has been associated with the increased VO_2 observed in osmoconforming [16,17] or hyper-osmoregulating invertebrates [20].

Interestingly, *M. lignano* decreases its respiration rates within the first 6 h of exposure. Our measurements reveal that activity rates also decrease significantly as a result of hypo-saline exposure. Animals under these conditions show increased water accumulation and thus increased body volume. We can confidently affirm that these decreased activity rates are not a result of swollen animals not being able to move through our preparations, since observations were corroborated in free animals. Furthermore, a similar decrease in respiration rates and activities has already been observed in other marine platyhelminthes such as *Procerodes littoralis* [15]. This energy-saving strategy is often an adequate mechanism to survive periods of environmental stress, maximizing chances of survival. Hyposmotically-induced metabolic depression has already been observed in other intertidal invertebrates such as periwinkles (*Littorina* spp.). These mollusks are known to enter a dramatic metabolic depression as a response to freshwater exposure [62]. This energy conserving mechanism, characterized by decreased ATP expenditures, may lead mitochondria to build a higher membrane potential. The same decrease in activity rates and increase in $\Delta_{\Psi m}$ was also observed in another study involving *M. lignano* when exposed to acute hypoxia [24], supporting the argument for a hypo-osmotically-induced decrease in metabolic rates.

Exposure to 5 ppt causes *M. lignano* to increase its NO levels. Even though NO is a free radical, this molecule has a relatively low reactivity towards biological molecules, except that it can interact with other compounds such as O_2 and $O_2 \cdot -$ to form more deleterious molecules [63]. NO is mainly known for its signaling properties reviewed by Palumbo [64] and much effort has been made over the last decades to understand the pathways in which this molecule is involved. It has been suggested that in conditions of cellular stress, the NO-complex IV interaction initiates a series of protective mechanisms that result in the prevention of apoptosis [65]. This would concur with the results obtained for transcription levels of *casp-3* in *M. lignano*. As already suggested in the 1990's, NO can lead to metabolic arrest e.g. [66]. Briefly, there are two main pathways in which NO may be involved in the energy-saving strategy observed in *M. lignano*. One possibility is that there is a possible direct interaction of NO with the electron transport chain. It was demonstrated over 40 years ago that NO is capable of linking to the O_2 binding site in the cytochrome oxidase, and thus, to regulate respiration rates [67]. This process would decrease ATP production and increase AMP, ADP and other metabolite concentrations which would in turn regulate other cellular processes such as ion transport and protein synthesis [68]. The effects of NO exposure on respiration rates have already been observed in *M. lignano* [69]. As a lipid-soluble molecule that can easily pass through biological membranes, NO exposure abolishes the

flatworm oxyregulatory capacity. A second theory is that of the stimulation of guanyl cyclase by NO, which may control cellular processes through the cyclic guanosine monophosphate (cGMP) pathway. This mechanism is designed for the regulation of protein kinases and ion channels, among others, but also plays a key role during metabolic arrest in mammalian cells e.g. [70]. Although the cGMP route has been suggested to be important in marine invertebrates (see reviews by [71,72]) as well as vertebrates (e.g. [73]), more information is needed to confirm a link between hypoxia/anoxia-tolerance, cGMP and NO.

Further studies are therefore required to determine and disentangle the relationship between NO production and the induction of metabolic depression, and this flatworm model may be an ideal tool to do so. This would open a new evolutionary perspective on how old and how widespread these pathways are within the phylogeny. Open questions remain concerning a) the mechanism of action of NO and b) the source of this NO. Regarding the latter, previous works have already detected the production and signaling role of this molecule in flatworms [74], where nitric oxide synthase (NOS) was identified as the source. This study and others on *M. lignano* support the hypothesis that NOS is indeed present in this flatworm [69,38]. Yet, DAF-2DA staining under hypoxia is not significantly inhibited by treatment with common NOS-inhibitors such as L-NAME (Rivera-Ingraham et al., unpub), suggesting that most of the NO produced, at least under stress conditions, may have an alternative source.

4.4. Is metabolic depression associated with pro-oxidant cell conditions?

It is equally worth noting that while $O_2 \cdot -$ formation shows a dramatic increase at higher salinities, DCF fluorescence also has an important variation although towards the lowest salinities. As a relatively unspecific dye, DCFH may be oxidized due to numerous parameters ranging from salinity of the medium [20] to a wide variety of intracellular and extracellular conditions e.g. [75,76]. Regardless of the significant flaws of this dye for quantification purposes, we can conclude that hyposaline environments induce a distinct physiological pro-oxidant change in *M. lignano*. However, the question remains of whether this increase in ROS could be related to the generation of NO and its regulatory effects. Qin et al., [70] used rabbit hearts to show that treatment with an NO donor increased cGMP levels, a process which is accompanied by an equal increase in ROS formation, which was suggested to occur through the opening of mito-K_{ATP} channels. Equally, the inhibition of cytochrome oxidase by either NO or molecules with similar inhibitory effects such as CO can lead to a hyperpolarization of the $\Delta\Psi_m$ and an increase in ROS [see [77] and references therein], as observed in our study. These authors also suggest that ROS production resulting from the inhibition of Complex IV may have further benefits, such as anti-inflammatory effects. But what would the significance of such ROS increase be under metabolic depression? And most importantly, how (if we are indeed under restricted energetic conditions) and why does it increase the concentration of certain antioxidant enzymes such as GSTP-1?

4.5. Recovering from hypoosmotic shock: an anticipation for reoxygenation?

If we consider that *M. lignano* significantly reduces its energetic costs (in terms of ATP production, protein turnover and other energy consuming pathways) in order to withstand hypoosmotic shock, it appears contradictory to find an upregulation of the expression of certain genes. However, it is not uncommon to find reports in the literature of cases where organisms show this type of response when dealing with stressors inducing metabolic arrest, such as freezing, fasting or hypoxia. This mechanism has been interpreted as a preparation for the oxidative burst occurring upon recovery and reoxygenation [78,79]. This burst in ROS production, if not properly handled, can eventually lead to cell damage or even death. This mechanism was originally identified in the early 1990's and was called the “preparation for oxidative stress” (POS) hypothesis, a subject that was recently reviewed in detail by Hermes-Lima et al., [78]. But is this the case for *M. lignano*? Our data indicates that this flatworm species is indeed entering a state of “metabolic shutdown” when exposed to low salinity. Also, it is especially interesting to note an increased level of some antioxidant enzymes, namely GSTP1 in this species under these conditions. Are we facing a case of POS? This would be the first time that such a mechanism is described in relation to osmotic challenges. There are numerous publications reporting a negative relationship between the kinetics of GST and respiration rates across invertebrates. To illustrate this, de Oliveira et al. [80] reported increased GST levels in gills of anoxic crabs *Chasmagnathus granulata.* This has been described in insects such as bees which also show GST increased levels when subject to low temperatures and starvation [81]. Likewise, the foot muscle of the land snail *Otala lacteal* increases CAT and SOD activities when facing fasting, but most importantly shows GST defenses between 79% and 108% higher than in active animals [82].

Linking with the open question closing the previous section of the discussion, we consider whether the ROS formation we detected under hypoosmotic shock could be involved in triggering the GST upregulation in our worms. Since the 1990s, an increasing number of works point to the role ROS play under hypoxic or equivalent conditions in the initiation of a series of different pathways oriented to upregulate these antioxidant enzymes required to prepare for reoxygenation or return to favorable conditions (reviewed by [78]).

5. Conclusions and future perspectives

As pointed out in previous ecophysiological studies conducted on this species, many characteristics of this species make it a highly suitable model for studying bioenergetic tradeoffs of environmental changes. As demonstrated in this study, *M. lignano* responds very differently to hypo- and hyper-saline environments in terms of bio-energetics and ROS production. While hypertonic environments induce mitochondrial biogenesis and increase in respiration, these flatworms face hypoosmotic shock by entering metabolic arrest. However, both stressing conditions induce ROS and RNS formation, generating different species for hypo- and hyper-osmotic shocks. Thus, *M. lignano* responds in both cases by increasing their antioxidant defenses, and since the ROS/RNS species vary greatly among conditions, it is not surprising to also find differences in the nature of the antioxidant enzymes generated in each case. However, under metabolic arrest, there should still be an energetic compromise to induce not only osmoregulation processes, but also antioxidant defenses. This leads to the question of why GST is primarily produced, and not other redox enzymes. Is this related to the type of ROS produced or to the bioenergetics cost? Could it be less costly to produce GST than other enzymes such as CAT or SOD?

In order to answer some of these questions opened in this study, future works will consider the use of ROS scavenging products during hypoosmotic shock to reveal whether ROS are actually involved in triggering the signal for GST upregulation, and if so, if the abolishment of the ROS signal threatens animal viability upon return to seawater conditions.

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