



The brine shrimp *Artemia franciscana* as a model for astrobiological studies: Physiological adaptations to Mars-like atmospheric pressure conditions

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ABSTRACT

Understanding the adaptation mechanisms of extremophiles to extreme environments is fundamental to predicting organisms' capacity to survive in space and plan future space exploration missions. This study explores the physiological and metabolic adaptations of nauplii of a eukaryotic organism, *Artemia franciscana*, hatched from cysts exposed to Mars-like pressure conditions (6 mbar) by analyzing aerobic and anaerobic metabolism, mitochondrial function, and oxidative stress in nauplii. Mars-like pressure did not inhibit nauplii's hatching or *in vivo* respiration, indicating that the fundamental metabolic functions are preserved but affected cellular metabolism. The lower lactate levels suggested reduced anaerobic metabolism, and the reduction in the activity of Complex I of the electron transport chain, resulting in reduced *in vitro* respiration supported by pyruvate plus malate, suggested an effect on aerobic metabolism. However, the succinate-supported respiration remained stable according to unchanged Complex II activity. Changes in aerobic metabolism could affect Reactive Oxygen Species (ROS) production and management. We did not observe changes in ROS levels according to the unchanged activity of NADPH oxidase, a source of ROS in the early development stages of nauplii. A total antioxidant capacity reduction and increased susceptibility to oxidants were observed despite this. However, lipid and protein oxidative stress markers levels remained unchanged, likely due to the increased activity of antioxidant enzymes. Our results underscore the resilience of the cysts to Mars-like pressure conditions, indicating the potential of *Artemia franciscana* as a model organism in astrobiological research, opening new avenues for exploration in astrobiology.

1. Introduction

The climate change we have inevitably witnessed in recent decades has posed new challenges to the study of life, shifting attention toward the boundaries within which life, as we know it, is possible (Jorquera et al., 2019). Extremophile organisms, *i.e.* organisms capable of living in

Earth's extreme environments, embrace evidence of tools to counteract climate change with the exploration of their promiscuous enzymes and metabolic pathways, furnishing the possibility to control many environmental stresses (Panda et al., 2022). The extremophile category includes organisms belonging to all three domains of life: Archaea, Bacteria, and Eukarya (Mastascusa et al., 2014), even if more recent

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studies suggest a two-domain model of life represented by Archaea and Bacteria, with Eukarya included in Archaea (Doolittle, 2020; Nobs et al., 2022). Understanding how extremophiles respond to environmental perturbations is crucial for predicting their resilience and assessing the potential implications for ecosystem dynamics (Rampelotto, 2024). Furthermore, extremophiles' great adaptability allows us to consider them as potential life forms to be found elsewhere in the Universe (Merino et al., 2019). The research on the origin, evolution, and potential distribution of life beyond Earth represents the most fascinating goals of the multidisciplinary science of Astrobiology (Cockell, 2020). Astrobiological studies need to fully understand extremophiles' adaptation mechanisms in hostile space environmental conditions. An ideal biological form for astrobiological studies should be able to be transported alive over long distances in space and undergo dormancy until it reaches its destination. The reduced metabolism during dormancy would increase the survival rate during interstellar travel, providing additional protection against the space environment. Among all the organisms capable of dormancy, only a small group, which includes brewer's yeast, most seeds, tardigrades, the nematode *Caenorhabditis elegans*, Chironomidae insects, and brine shrimps, can tolerate space-flight (Lantin et al., 2022). Recent studies demonstrated that these organisms can cope with space conditions due to their higher radiation tolerance, lower content of nuclear material in the cells, DNA repair, and the ability to regenerate tissue and organs (Lantin et al., 2022). In particular, we focused our attention on the brine shrimp belonging to the *Artemia* species (subclass Branchiopoda, order Anostraca), an ancient species that appeared on Earth ~400 million years ago (Chabet Dis et al., 2024). *Artemia* are classified into six bisexual categories, each prevalent in numerous parthenogenic populations (Morris and Afzelius, 1967). They thrive across approximately 500 natural and artificial salt lakes spread over tropical, subtropical, and temperate climatic zones (Van Stappen, 1996). When the environmental conditions can endanger the species' survival, they produce some resistant cysts in the form of gastrulae, identified by a yolk-filled embryo enveloped by a dark and robust capsule full of lipoproteins drenched with chitin and haematin with approximately 4000 nuclei (Morris and Afzelius, 1967), arrested at the G₂/M phase with a complete turning off of RNA and protein synthesis that can reversibly return to active life (Hand et al., 2016). This structure offers protection from the environment and UV sources through phenolic tanning or melanization processes (Speight and Deneault, 2015). This particular state is called "cryptobiosis", a state of desiccation characterized by dormancy with a lower metabolic activity (Clegg, 1965). Thanks to its cyst form, *Artemia franciscana* (*A. franciscana*) exhibits extraordinary tolerance to a variety of environmental stressors, i.e. salinity fluctuations, temperature variations, and radiation exposure (Cockell, 2020). In experiments carried out on the Russian orbital segment of the ISS and in the Biorisk experiment, *Artemia* showed the greatest resistance among all the aquatic organisms used (Lantin et al., 2022). Another study reported that they can also resist radiation and temperature space conditions (Aleksiev et al., 2019). However, limited data on *A. franciscana*'s adaptation mechanisms to extreme environments is available. Changes in environmental conditions can affect cellular metabolism and redox homeostasis of aerobic life (Napolitano et al., 2022a). A recent study showed a redox response in salt-stressed *Artemia* aimed at controlling Reactive Oxygen Species (ROS) production by the up-regulation of catalases, peroxidases, and heme enzymes involved in ROS metabolism (De Vos et al., 2021). The authors also reported metabolic alteration linked to an enhanced mitochondrial function under anoxia conditions with significant upregulation of genes involved in ATP production in the mitochondrial membrane, cristae morphogenesis, mitochondrial transport, and inter-membrane chaperoning (De Vos et al., 2021).

Among the extreme conditions to be investigated for astrobiological purposes, little information is available on Mars-like pressure conditions. This study aims to evaluate the aerobic and anaerobic metabolism and redox homeostasis of *A. franciscana* nauplii hatched from cysts

exposed to pressure conditions mimicking those on the surface of Mars. The results reported here are relevant to broaden knowledge on the adaptations of eukaryotic organisms to extreme conditions. Moreover, our study furnishes the basis for future investigations since the research on extremophiles that tolerate conditions similar to those found on Mars, can gain valuable insights into biosignatures of potential life forms that might exist on the red planet (Rothschild and Mancinelli, 2001; Matsubara et al., 2017; Merino et al., 2019).

2. Materials and methods

2.1. Experimental design

Artemia cysts were purchased from a commercial shopper (Hobby®, Gelsdorf, Germany). The species was identified as *A. franciscana* by Cavion et al., 2020 through PCR sequencing of the cytochrome c oxidase subunit I gene region with BLASTn comparison with *A. franciscana* isolates (GenBank IDs: DQ119645 and MK393317). Cysts were uniformly placed and fixed in an 8 cm diameter Petri dish at room pressure and temperature. Cysts were exposed to 1 h of Mars-like pressure conditions (6 mbar), reproduced using a Planetary Environment Simulation chamber consisting of a 57 L stainless steel chamber equipped with a vacuum pump (HiScroll 12, Pfeiffer), built in the Cosmic Physic Laboratory at the Parthenope University of Naples. Unpublished data from our research group highlighted that exposure of the cysts to low-pressure conditions for longer times (24 h and 7 days) did not influence the nauplii's hatching percentage, which was comparable to the control group. Considering that 1 h is the minimum time required by the Planetary Environment Simulation chamber to reduce the error related to Mars-like pressure fluctuations, we chose to study the metabolic responses of the nauplii that hatched after the minimum exposure time. After the simulation, the cysts were gently removed from the Petri plate, and suspended in 10 mL of artificial seawater to determine the total number. 450 µL of a 1:100 diluted cysts suspension was used for the procedure. The count was determined in 50 µL of the diluted suspension, and the mean of three replicate counts was calculated. Successively, the cysts were placed in hatcheries (Hobby®) and incubated for 72 h at 26 °C under constant aeration and a 16-h light photoperiod in 500 mL of artificial seawater (Instant Ocean, 36 ‰) prepared using sea salt 36 g/L and adjusted to pH 8.5 using 1 N NaOH. Hatching occurred within a few hours of incubation and the nauplii (instar I-II), separated from cysts by exploiting their phototactic response, were collected with a filter net and resuspended in 10 mL of artificial seawater. The total number of nauplii was determined by using a stereomicroscope (Leica Wild Heerbrugg M3B). To fix the animals, a few drops of 4 % formaldehyde were added to 450 µL of a 1:100 diluted nauplii suspension. The nauplii count was determined as previously described for the cyst's procedure. This method was preliminarily proved to be a reliable evaluation of the number of individuals collected (Napolitano et al., 2022b). The total number of cysts and nauplii obtained from seven different controls and exposition to Mars like pressure experiments was used to evaluate the hatching percentage. After counting and collecting, the different living nauplii populations were resuspended in a saline solution containing 220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM Tris, pH 7.4. The suspensions were maintained at 0 °C and homogenized using a Potter-Elvehjem glass homogenizer set to a standard speed (500 rpm) for 1 min. The protein concentration in the homogenates was measured by the colorimetric Bio-Rad Bradford protein assay, using a commercial kit (Bio-Rad, Hercules, CA, USA), and aliquots were used for analytical procedures.

2.2. Metabolic parameters

2.2.1. In vivo oxygen consumption

To gain information on the whole metabolism of the organisms, aliquots of 1 mL of nauplii resuspended in artificial seawater, were used to

evaluate the *in vivo* routine oxygen consumption. The number of nauplii in each aliquot was determined as previously described. Oxygen consumption rates were determined at 26 °C using a Hansatech respirometer (Oxygraph⁺, Hansatech Instruments Ltd., Norfolk, UK) and expressed in nmol O₂ min⁻¹ for each individual.

2.2.2. *In vitro* oxygen consumption

To gain insights into aerobic metabolism, *in vitro* oxygen consumption was measured on four homogenates of nauplii populations for each experimental group (C and 1 h exposure to Mars-like pressure) at 26 °C by a Hansatech respirometer (Oxygraph⁺, Hansatech Instruments Ltd., Norfolk, UK). The measurements were performed by using 0.2 mg of homogenate proteins diluted in 1.0 mL of incubation medium (145 mM KCl, 30 mM Hepes, 5 mM KH₂PO₄, 3 mM MgCl₂, 0.1 mM EGTA, pH 7.4) in the presence of respiratory substrates linked to Complex II (Succinate, 10 mM) or Complex I (Pyruvate plus Malate, 10 and 2.5 mM, respectively), in absence (State 4) and in presence (State 3) of 500 μM ADP. Oxygen consumption was expressed as nmol O₂ min⁻¹ • mg⁻¹ proteins.

2.2.3. Mitochondrial complexes activity

To gain more insight into the metabolic effects of Mars-like pressure exposure we measured the activities of the mitochondrial electron transport chain complexes that play a pivotal role in consuming oxygen to produce ATP through Oxidative Phosphorylation (OXPHOS). Measurements were performed on four homogenates of nauplii populations for each experimental group (C and 1 h exposure to Mars-like pressure).

Complex I activity was detected by evaluating the NADH (5 mM) -dependent reduction of coenzyme Q1 (10 mM) in the presence of 0.02 mg of protein of the sample in 10 mM K₂HPO₄, 2 % lecithin, pH 8.0, according to the method of Ragan et al. (1987) modified for microplates reader (SynergyTM HTX Multimode Microplate Reader, BioTek Santa Clara, CA, USA). The activity of Complex I was followed by measuring the decrease of absorbance at 340 and expressed as μmol NADH min⁻¹ • mg⁻¹ proteins.

Complex II activity determination was detected by evaluating the ubiquinol (Coenzyme Q₂, CoQ₂, 2.5 mM)-induced reduction of 2, 6-dichlorophenylindophenol (DCPIP, 4.65 mM). Coenzyme Q₂ was reduced by 0.02 mg of proteins in the presence of 1 M succinate in 1 M K₂HPO₄, pH 7.4. DCPIP reduction was followed at 600 nm in a microplates' reader (SynergyTM HTX Multimode Microplate Reader, BioTek). Complex II activity was expressed as μmol DCPIP • min⁻¹ • mg⁻¹ proteins (Ragan et al., 1987 with modifications).

The activity of complex III was determined by evaluating the reduction of cytochrome c (0.05 mM) induced by the samples (0.02 mg of protein) due to ubiquinol (CoQ₂, 0.05 mM) in 50 mM K₂HPO₄, pH 7.4. Reduction of CoQ₂ was achieved by incubating ubiquinone (10 mM CoQ₂) with NaBH₄ in H₂O 1:1 (v:v). After the reduction, CoQ₂ was extracted by a mixture of diethyl ether: cyclohexane (2:1, v: v). The sample was preliminarily treated with 50 mM KCN to inhibit cytochrome oxidase activity. Cytochrome c reduction was followed at 550 nm in a UV-Vis Spectrophotometer (Jasco V- 630). Complex III activity was expressed as μmol cytochrome c reduced • min⁻¹ • mg⁻¹ proteins (Ragan et al., 1987).

Complex IV activity was measured by evaluating oxygen consumption of 0.1 mg of proteins. The decrease in oxygen concentration was induced by the reduced form of the cytochrome c (Barré et al., 1987). In brief, 0.1 mg of homogenate proteins were added, after solubilization of the membranes with 1 % Lubrol, to 1.0 mL of buffer solution containing 30 μM Cytochrome C 3131, 10 mM sodium malonate, 75 mM Hepes, 4 μM rotenone, 0.5 mM 2,4-dinitrophenol at pH 7.4. The reaction was started by the addition of a mixture of 30 mM TMPD (*N,N,N',N'*-tetramethyl-1,4-phenylenediamine) and 400 mM ascorbate that reduces the cytochrome c. Oxygen consumption was followed in a Hansatech respirometer (Oxygraph⁺, Hansatech Instruments Ltd., Norfolk, UK) and expressed as nmol O₂ • min⁻¹ • mg⁻¹ proteins.

2.2.4. Lactate content

To gather knowledge on the anaerobic metabolism of *Artemia franciscana* nauplii, homogenate proteins were used to spectrophotometrically evaluate (SynergyTM HTX Multimode Microplate Reader, BioTek Santa Clara, CA, USA) the lactate content following the increased absorbance of NADH at 340 nm, according to Rees et al. (2009) with modifications. Measurements were performed on four homogenates of nauplii populations for each experimental group (C and 1 h exposure to Mars-like pressure). In brief, 0.015 mg of homogenate proteins were added to a buffer solution containing 0.5 M glycine, and 0.4 M hydrazine sulfate, pH 9.5. The L-lactate of the samples was converted into pyruvate by the enzyme L-lactate dehydrogenase (5000 U • mL⁻¹) in the presence of NAD⁺ (25 mM). The pyruvate was irreversibly transformed in hydrazone in the presence of hydrazine, and the whole lactate content was consumed by the enzyme L-lactate dehydrogenase. The lactate content of the samples, calculated by a standard curve of lactate concentration, was expressed as nmol lactate • mg⁻¹ protein.

2.3. Redox state evaluation

2.3.1. Reactive oxygen species (ROS) content and NADPH oxidase (NOX) activity

Since metabolic alterations can be associated with Reactive Oxygen Species (ROS) production, ROS content was spectrofluorimetrically assayed by using a microplate reader (SynergyTM HTX Multimode Microplate Reader, BioTek Santa Clara, CA, USA) with excitation and emission wavelengths of 485 nm and 530 nm, respectively (Driver et al., 2000). Measurements were performed on four homogenates of nauplii populations for each experimental group (C and 1 h exposure to Mars-like pressure). Homogenate aliquots, containing 12.5 μg of proteins and diluted in a 0.1 M monobasic phosphate buffer, pH 7.4, were incubated for 15 min with the non-fluorescent compound 2',7'-dichlorodihydrofluorescein diacetate (10 μM, DCFH-DA). Successively, 100 μM FeCl₃ was added, and the mixture was incubated for 30 min. The conversion of DCFH-DA to the fluorescent product dichlorofluorescein, catalyzed by ROS of the samples, was followed. Background fluorescence (conversion of DCFH to DCF without homogenates) was corrected by including parallel blanks. ROS content was expressed as Relative Fluorescence Units (RFU) • μg⁻¹ proteins.

Since NADPH Oxidase (NOX) enzyme can contribute to ROS production, NOX activity of nauplii homogenates was spectrophotometrically measured (550 nm) by a microplate reader (SynergyTM HTX Multimode Microplate Reader, BioTek Santa Clara, CA, USA) according to Suzuki and Lehrer (1980) and Minkenberg and Ferber (1984) with modifications (Fasciolo et al., 2022). Measurements were performed on 0.2 mg of homogenate proteins by following the reduction of acetylated ferricytochrome c (80 μM) in the presence of NADPH (1 mM) at room temperature (Fasciolo et al., 2022). NOX activity was calculated by the difference of measurements in the presence and the absence of 100 μg/mL superoxide dismutase and expressed as μmol CytC reduced • min⁻¹ • mg⁻¹ proteins.

2.3.2. Oxidative damage to lipids (Hps) and proteins (CO), *in vitro* susceptibility to oxidants (ΔHps), and total antioxidant capacity (TAC)

Lipids and proteins are both macromolecules particularly vulnerable to oxidation by ROS. Measurements were performed on five homogenates of nauplii populations for each experimental group (C and 1 h exposure to Mars-like pressure). The extent of lipid peroxidative processes was spectrophotometrically assessed at 340 nm by measuring the content of lipid hydroperoxides (Hps) in homogenates of the nauplii population (Heath and Tappel, 1976). 10 μg of the homogenate proteins were diluted in 0.2 mM EDTA, 0.124 M Tris-HCl buffer, pH 7.6 (Heath and Tappel, 1976), and Hps content was evaluated by the coupling of two reactions, respectively catalyzed by the enzymes glutathione peroxidase (0.025 U/mL), that neutralizes sample Hps by oxidizing the reduced form of glutathione (0.425 mM GSH), and glutathione

reductase (0.025 U/mL) that reconvert the oxidized form of glutathione (GSSG) into GSH, by oxidizing NADPH. Hps content was expressed as nmol NADPH oxidized $\bullet \text{ min}^{-1} \bullet \text{ mg}^{-1}$ proteins.

The oxidative damage to proteins was detected by evaluating the content of protein-bound carbonyls (CO). CO content was determined by the reaction of CO with 2,4-dinitrophenylhydrazine (DNPH), using a simplified spectrophotometric method (450 nm) adapted to a 96-well standard Nunc plate (Mesquita et al., 2014). Homogenates were diluted in 100 mM NaH_2PO_4 (phosphate buffer), containing 0.2 % digitonin, 80 $\mu\text{g/mL}$ PMSF, 10 $\mu\text{g/mL}$ leupeptin, 14 $\mu\text{g/mL}$ pepstatin, 10 $\mu\text{g/mL}$ aprotinin, pH 7.4, incubated at 0 °C for 10 min and centrifuged (600 g, 4 °C, 10 min). The supernatants were treated with 1 % streptomycin, incubated at room temperature for 10 min, and centrifuged (600 g, 4 °C, 10 min). 40 μL of the supernatants were incubated with an equal volume of 10 mM DNPH (in 2.5 N HCl) at room temperature for 10 min. Successively, 20 μL of sodium hydroxide (6 N) was added. Therefore, 185 μL of phosphate buffer was added to 25 μL of the sample and the absorbance was read at 450 nm. CO content was expressed as $\mu\text{mol CO} \cdot \text{mg}^{-1}$ protein using the extinction coefficient of DNPH ($\epsilon = 22,308 \text{ M}^{-1} \text{ cm}^{-1}$). To determine the total homogenate protein, Bradford reagent was used according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA).

To gain information on the ability of the nauplii to face oxidative stress insult we treated them with a system able to produce ROS and evaluated the increase in oxidative damage. The *in vitro* susceptibility to oxidizing agents was evaluated by the change in Hps (ΔHps) levels induced by treating 0.01 mg of proteins from *Artemia franciscana* homogenates with iron and ascorbate (Fe/As) at a concentration of 100/1000 μM for 10 min at room temperature. Ascorbate reduces iron, and the Fe^{2+} reacts with the lipid hydroperoxides of the sample producing hydroxyl radical that, if not scavenged by the sample's antioxidants, triggers lipid peroxidation. The reaction was stopped by adding 0.2 % 2,6-di-*t*-butyl-*p*-cresol (BHT).

The Total Antioxidant Capacity (TAC) was spectrophotometrically evaluated (734 nm) using a decolorization assay according to Erel (2004) with modifications (Panico et al., 2023). The preformed radical monocation 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($\text{ABTS}^{\bullet+}$) was generated by the oxidation of ABTS (7 mM) in the presence of potassium persulfate (245 mM). The mixture was dark-stored and away from heat sources for 16 h at room temperature. The decolorization of the radical cation was induced by the antioxidants of *Artemia franciscana* homogenates (0.01 mg proteins). For the calibration curve, a stock solution of 2.5 mM 3,5-di-*tert*-4-butylhydroxytoluene (BHT) was utilized. TAC was expressed as BHT equivalents $\bullet \mu\text{g}^{-1}$ proteins. Hps and CO content, *in vitro* susceptibility to oxidants and TAC were all evaluated by using a microplate reader (Synergy™ HTX Multimode Microplate Reader, BioTek Santa Clara, CA, USA).

2.3.3. Activities of the antioxidant enzymes glutathione peroxidase (GPx), glutathione reductase (GR), and superoxide dismutase (SOD)

To assess the capacity of *Artemia franciscana* nauplii to face the harmful effects of ROS content, the antioxidant enzyme activities of Glutathione Peroxidase (GPx), Glutathione Reductase (GR), and Superoxide Dismutase (SOD) were assayed on nauplii homogenates. Measurements were performed on five homogenates of nauplii populations for each experimental group (C and 1 h exposure to Mars-like pressure). GPx activity was measured on 0.02 mg of proteins by following the rate of NADPH oxidation (1.5 mM) in the presence of H_2O_2 (1.5 mM) as substrate, GSH (10 mM) and GR (2.4 U/mL) in 0.1 M KH_2PO_4 , 1 mM EDTA, pH 7.0 (Flohé and Günzler, 1984). GR activity was assayed on 0.02 mg of proteins by measuring the rate of NADPH (2 mM) oxidation after the addition of 20 mM GSSG in 0.2 M KH_2PO_4 , 2 mM EDTA, pH 7.0 (Carlberg and Mannervik, 1985). For both procedures, the rate of NADPH oxidation was monitored at 340 nm, and the results were expressed as $\mu\text{mol NADPH oxidized} \bullet \text{ min}^{-1} \bullet \text{ mg}^{-1}$ proteins.

SOD activity was spectrophotometrically measured (550 nm) by

monitoring the cytochrome *c* reduction decreasing rate due to the neutralization of superoxide radicals induced by the SOD of 0.05 mg of proteins. Superoxide radicals were generated by the xanthine-xanthine oxidase system, in a solution containing 0.1 mM EDTA, 2 mM KCN, 50 mM KH_2PO_4 , 20 mM cytochrome *c*, 5 mM xanthine, and 0.01 U of xanthine oxidase, pH 7.8. The results were expressed as Units of SOD activity $\bullet \text{ mg}^{-1}$ proteins. One unit of SOD activity is defined as the concentration of enzyme that inhibits cytochrome *c* reduction by 50 % in the presence of xanthine + xanthine oxidase (Napolitano et al., 2022b).

GPx, GR, and SOD were all evaluated by using a microplate reader (Synergy™ HTX Multimode Microplate Reader, BioTek Santa Clara, CA, USA).

2.4. Data analysis

Statistics were performed by using GraphPad Prism software, version 9.0 (GraphPad Software Inc., San Diego, CA, USA). Normal distribution and equal variance of the data were evaluated by the Shapiro-Wilk test, and F test, respectively. The unpaired *t*-test was applied for normally distributed data. When equal variance was not given, a non-parametric *t*-test followed by Mann-Whitney (for State 3 respiration with pyruvate plus malate, CO content, and SOD activity) test or Kolmogorov-Smirnov (Complex II activity) test was performed. The *p*-values of $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$ were assigned to indicate statistical significance between the different experimental groups.

3. Results

3.1. Effect of Mars-like pressure on aerobic and anaerobic metabolism

The effect of Mars-like pressure was first investigated by measuring the hatching percentage and the *in vivo* respiration of nauplii hatched from cysts after 1-h exposure.

For the control group, the number of cysts placed in the hatchery and the number of living nauplii collected after 72 h were $22,118 \pm 9550$ and 824 ± 1071 , respectively. For the exposed group, the number of cysts placed in the hatchery and the number of living nauplii collected after 72 h were $28,299 \pm 10,476$ and 1600 ± 894 , respectively. As shown in Fig. 1 (Panel A), the hatching percentage of the exposed sample was comparable to that observed for the control group, thus suggesting that Mars-like pressure exerted no significant effect on the ability of cysts to resume their metabolism ($P = 0.38$).

Similarly, the nauplii *in vivo* respiration (Fig. 1B) which depends upon all the metabolic activities of the nauplii, was not affected after 1 h of exposure to Mars-like pressure of the cysts ($P = 0.72$).

The *in vitro* oxygen consumption showed a significant decrease compared to the control group in the absence (State 4, $P < 0.05$) and in the presence (State 3, $P < 0.05$) of ADP in the pyruvate plus malate-sustained respiration (Fig. 2, Panels C and D). The succinate-sustained

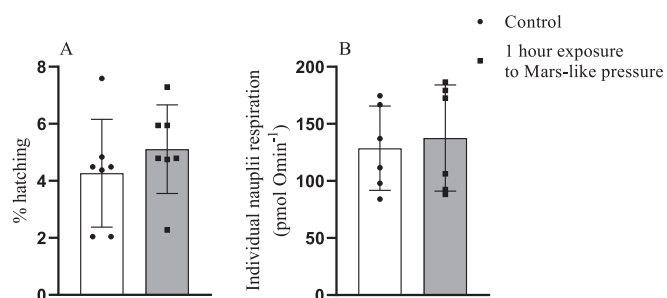


Fig. 1. Percentage of hatching (Panel A) and *in vivo* oxygen (Panel B) consumption of nauplii hatched from cysts exposed to normal and Mars-like pressure for 1 h. Values are means $\pm$ SD of seven different batches of cysts. Data was normally distributed, and unpaired *t*-test was applied.

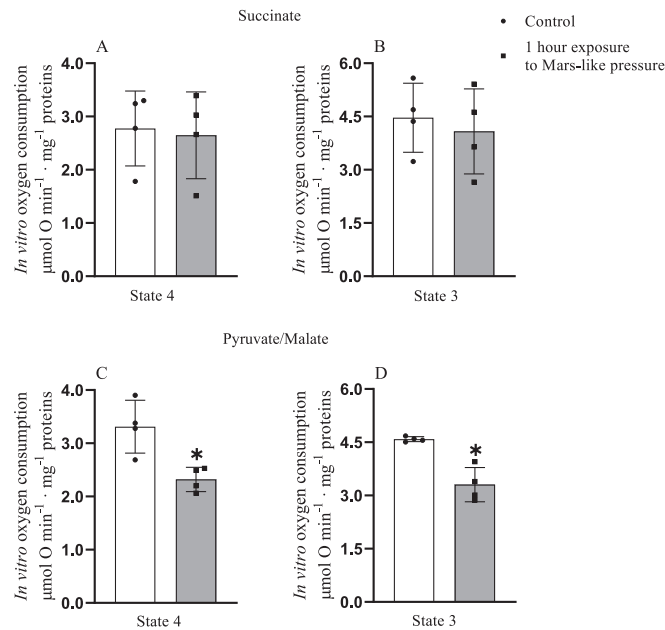


Fig. 2. Oxygen consumption in homogenates of *Artemia franciscana* nauplii hatched from cysts exposed to normal and Mars-like pressure for 1 h. State 3 (in the presence of ADP; Panels B and D) and State 4 (in the absence of ADP; Panels A and C) oxygen consumption is detected in succinate (Panels A and B) and pyruvate/malate (Panels C and D) supplemented homogenates. Values are means $\pm$ SD of four different batches of cysts. State 4 and State 3 with succinate and State 4 with pyruvate plus malate data were normally distributed and unpaired *t*-test was applied. State 3 with pyruvate plus malate data did not have equal variance and a non-parametric *t*-test followed by Mann-Whitney test. **p* < 0.05 significant compared to the control group.

respiration was not affected in nauplii homogenates hatched from cysts exposed to 1 h of Mars-like pressure in both State 3 and 4 (Fig. 2, Panels A and B; *P* = 0.64 and *P* = 0.82, respectively).

Figure 3 shows the mitochondrial complexes activities evaluated in homogenates of nauplii hatched from cysts exposed to normal and Mars-like pressure. A significant decrease in Complex I activity (*P* < 0.05) (Fig. 3, Panel A) was observed in homogenates of nauplii hatched from treated cysts. Complex II (Fig. 3, Panel B, *P* > 1), III (Fig. 3, Panel C, *P* = 0.42), and IV (COX, Fig. 3, Panel D, *P* = 0.07) activities were not affected by Mars-like pressure exposure, even if a tendency toward the reduction was observed for the COX activity.

The anaerobic metabolism, evaluated by the lactate content of nauplii homogenates, resulted in a significant reduction (*P* < 0.001) for animals hatched from cysts exposed to Mars-like pressure, as shown in Fig. 4.

3.2. One hour of Mars-like pressure exposure affected the redox state of *Artemia franciscana* nauplii

The analysis of parameters linked to redox homeostasis showed an influence of Mars-like pressure exposure. As shown in Fig. 5, the treatment did not affect ROS content (Panel A, *P* = 0.20) and NADPH oxidase (NOX) activity (Panel B, *P* = 0.27), an enzyme involved in ROS production, in homogenates of *A. franciscana* nauplii hatched from cysts exposed to Mars-like pressure with respect to the control group, even if a tendency toward the increase was observed for the NOX activity.

Figure 6 shows hydroperoxide levels (HPs) and protein-bound carbonyls content (CO) as markers of oxidative damage to lipids and proteins (Panel A and B, respectively), *in vitro* susceptibility to oxidants (Panel C), and total antioxidant capacity (TAC) (Panel D) of homogenates of control nauplii and nauplii hatched from cysts exposed to Mars-like pressure. One hour of exposure did not affect HPs (*P* = 0.31) and CO

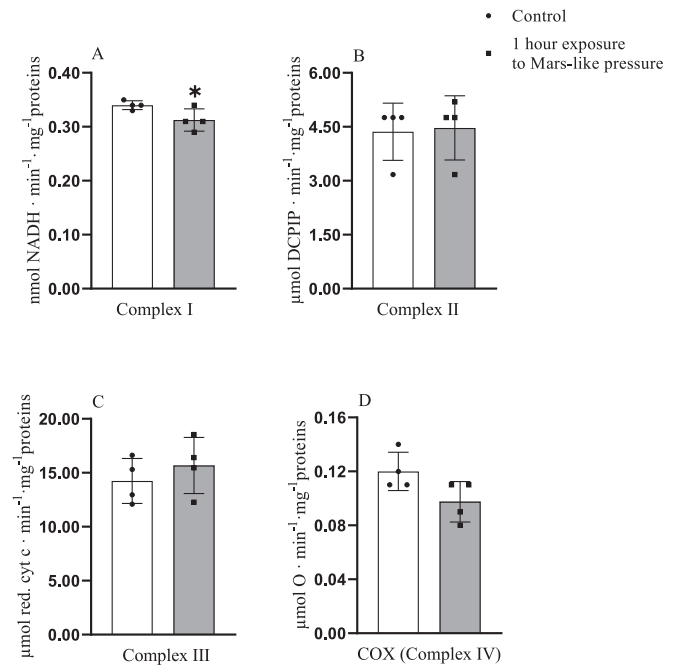


Fig. 3. Complex I (Panel A), Complex II (Panel B), Complex III (Panel C), and Complex IV (or Cytochrome oxidase, COX, Panel D) activities in homogenates of *Artemia franciscana* nauplii hatched from cysts exposed to normal and Mars-like pressure for 1 h. Complex I, III and IV data were normally distributed, and unpaired *t*-test was applied. Complex II data did not have equal variance, and a non-parametric *t*-test was used, followed by Kolmogorov-Smirnov test. Values are means $\pm$ SD of four different batches of cysts. **p* < 0.05 significant compared to the control group.

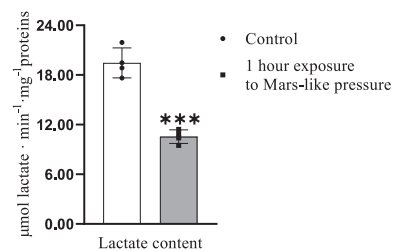


Fig. 4. Lactate content measured in homogenates of *Artemia franciscana* nauplii hatched from cysts exposed to normal and Mars-like pressure for 1 h. Values are means $\pm$ SD of four different batches of cysts. Data were normally distributed, and unpaired *t*-test was applied. ****p* < 0.001 significant compared to the control group.

content (*P* = 0.76). Conversely, the treatment significantly increased the *in vitro* susceptibility to oxidants (*P* < 0.001) and reduced the TAC (*P* < 0.01).

The analysis of the activity of the main antioxidant cellular enzymes showed that one hour of Mars-like pressure exposure induced a significant increase in GPx (*P* < 0.01) and GR activity (*P* < 0.1) (Fig. 7, Panel A and B), while did not affect SOD activity (Fig. 7, Panel C, *P* = 0.73).

4. Discussion

The results reported in this study demonstrated for the first time that exposing *Artemia franciscana* cysts to 1 h of Mars-like pressure does not affect their ability to resume their whole metabolism, as indicated by the hatching percentage and *in vivo* respiration of nauplii hatched from the cysts. However, Mars-like pressure exposure affects both aerobic and anaerobic metabolism, as well as the redox state of the nauplii. We evaluated nauplii homogenates' *in vitro* oxygen consumption to gather

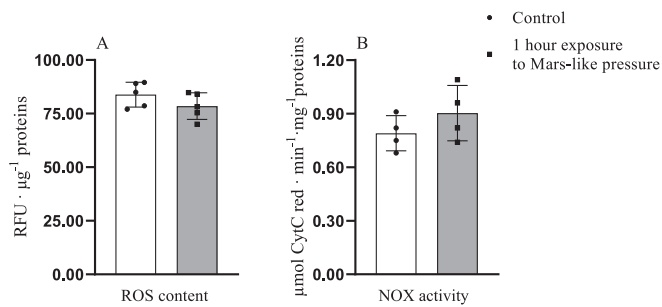


Fig. 5. ROS content (Panel A) and NADPH oxidase activity (Panel B) of homogenates of *Artemia franciscana* nauplii hatched from cysts exposed to normal and Mars-like pressure for 1 h.

Values are means ± SD of five and four different batches of cysts for ROS content and NOX activity, respectively. Data were normally distributed, and unpaired *t*-test was applied.

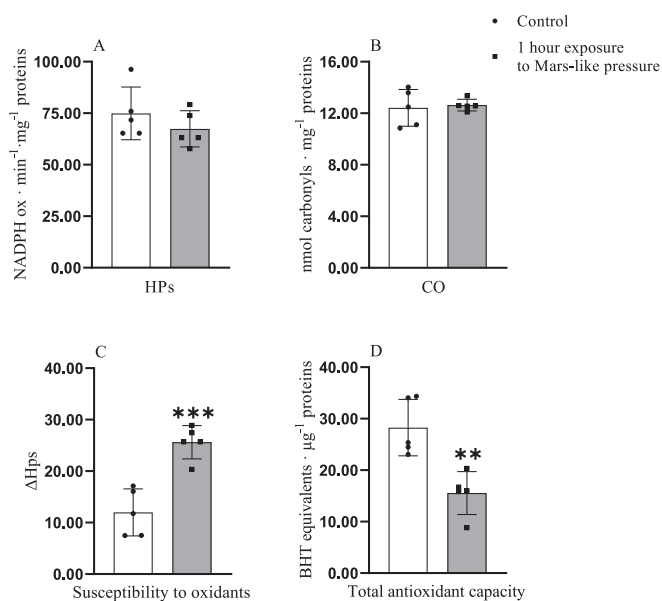


Fig. 6. Oxidative damage to lipids (HPs) and proteins (CO) (Panel A and B, respectively), *in vitro* susceptibility to oxidants (Panel C), and total antioxidant capacity (TAC) (Panel D) of homogenates of *Artemia franciscana* nauplii hatched from cysts exposed to normal and Mars-like pressure for 1 h. Values are means ± SD of five different batches of cysts. HPs, susceptibility to oxidants and total antioxidant capacity data were normally distributed, and unpaired *t*-test was applied. CO data did not have equal variance, and a non-parametric *t*-test was used, followed by the Mann-Whitney test. ***p* < 0.01, ****p* < 0.001 significant compared to the control group.

information about oxidative metabolism. In particular, the respiration rate under non-phosphorylating conditions (State 4) provides information about the uncoupling of respiration from ADP phosphorylation due to proton leak, while ADP-stimulated respiration (State 3) offers information on the respiration capacity.

Our results highlighted a significant reduction in both States of *in vitro* oxygen consumption of nauplii homogenates from Mars-like pressure-exposed cysts when respiration was supported by the mixture of pyruvate plus malate, substrates linked to Complex I of the electron transport chain (ETC), suggesting an effect of exposure on aerobic metabolism.

Analysis of the activity of mitochondrial complexes can explain the observed effects on oxygen consumption. Oxygen consumption with succinate, a Complex II-linked substrate of the ETC, showed no changes in either State 4 or 3 of nauplii homogenates' respiration hatched from

Mars-like pressure-exposed cysts compared to the control group. These results agree with the unchanged activity of Complex II. Conversely, a significant reduction in the activity of Complex I, which exploits NADH's reducing power derived from pyruvate and malate's oxidation to pump electrons through the ETC, was observed in nauplii homogenates from Mars-like pressure-exposed cysts. The reduced Complex I activity could explain the reduction in the pyruvate malate-sustained respiration. Despite the reduced Complex I activity, the maximum oxidative capacity was not compromised, as demonstrated by unchanged COX activity, which correlates with the maximum tissue aerobic capacity *in vitro* (Villani et al., 1998).

On the other hand, since in the early stages of *Artemia* embryos' development, nauplii must maintain their recently activated cellular functions to grow, swim, and molt (Abatzopoulos et al., 2002), high amounts of energy are required. Moreover, nauplii have to face, at the same time, increased oxygen concentrations after cysts' eclosion (Rodríguez-Armenta et al., 2018). At this early stage, the enzymes COX and Alternative Oxidase (AOX) are active to maintain ATP levels and control the oxygen molecule concentration to avoid excessive free radicals and Reactive Oxygen Species (ROS) production, as suggested in other invertebrate species (Abele et al., 2007), decreasing the risk of cell damage by oxidative stress (Korshunov et al., 1997; Tschischka et al., 2000). ROS are a normal by-product of cellular metabolism and, being very reactive molecules, they can oxidatively damage biological macromolecules compromising their functionality when their production increases and overwhelms the antioxidant capacity of the cell (Napolitano et al., 2022a). In our experiments, ROS content and the levels of lipid hydroperoxides and protein-bound carbonyls, markers of oxidative damage to lipids and proteins, respectively, resulted unchanged in the Mars-like pressure exposed nauplii compared to the control group. These results could be explained by the activity of mitochondrial alternative enzymes, including NADH-dehydrogenase type 2 (NDH2), glycerol-phosphate-dehydrogenase (mitGPDH), and AOX in the mitochondrial inner membrane (MIM), which are active during the early stages of *Artemia franciscana* nauplii development (Rodríguez-Armenta et al., 2018). These enzymes are non-pumping alternative redox enzymes in the ETC that decrease the proton gradient in the intermembrane space since they do not pump protons through the MIM, allowing uncoupled ATP synthesis and decreasing ROS production (Cabrera-Orefice et al., 2014). It is possible to hypothesize that the non-pumping alternative redox enzyme systems are magnified in the nauplii hatched from cysts exposed to low pressure and, for this reason, the *in vivo* oxygen consumption and the hatching are not affected notwithstanding *in vitro* mitochondrial respiration is reduced in the nauplii hatched from low-pressure exposed cysts.

The anaerobic metabolism, evaluated by lactate content, was significantly reduced under Mars-like pressure exposure compared to the control condition. Since the anaerobic metabolism is more prevalent than the aerobic one in different developmental stages of *Artemia* nauplii, as shown by the increased lactate dehydrogenase and malate dehydrogenase activities when the embryo emerges from the cysts (Hemamalini and Munuswamy, 1994), our results suggest that Mars-like pressure exposure could affect development and the growth of the nauplii. Martín et al. (2012) also reported that NOX activity increases in the hemocytes of crustaceans during the metamorphic transitions as an adaptative phenomenon developed by crustaceans to protect the larvae exposed to external stimuli. Activation and assembly of the NADPH oxidase are intricate and include different globular proteins involved in signalling pathways to build multi-protein complexes and networks and phosphorylation mechanisms. These processes ensure that oxidase subunits only assemble and form the active enzyme complex when appropriate signals have been received. In our experiments, NOX activity tends to increase under Mars-like pressure exposure compared to the control condition, but not significantly. We can hypothesize that Mars-like pressure affects the biochemical pathways that activate the enzyme, disturbing a physiological response that could affect the future

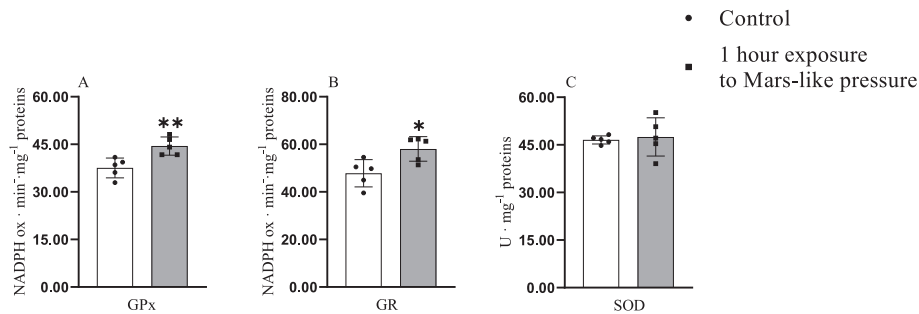


Fig. 7. Glutathione peroxidase (GPx), Glutathione reductase (GR), and Superoxide Dismutase (SOD) activities (Panel A, B, and C, respectively) of homogenates of *Artemia franciscana* nauplii hatched from cysts exposed to normal and Mars-like pressure for 1 h.

Values are means $\pm$ SD of five different batches of cysts. GPx and GR data were normally distributed, and unpaired t-test was applied. SOD data did not have equal variance, and a non-parametric t-test was used followed by Mann-Whitney test. * $p < 0.05$, ** $p < 0.01$ significant compared to the control group.

development of the animals. Further experiments are needed to investigate the metabolism and its modifications in juveniles of *Artemia franciscana* hatched from cysts exposed to Mars-like pressure.

The analysis of the antioxidant enzymes showed increased activity for the GPx and GR enzymes involved in maintaining the ratio between the reduced and the oxidized form of the principal soluble antioxidant, glutathione (GSH). The enzyme GPx neutralizes H_2O_2 into H_2O , taking hydrogens from two GSH molecules, resulting in two H_2O and one oxidized glutathione (GSSG) molecule. Once oxidized, the GSSG is converted into GSH using the enzyme GR (Csiszár et al., 2016). This cellular adaptation could be a compensative response to the increased susceptibility to oxidant molecules and the reduced total antioxidant capacity measured in homogenates of nauplii hatched from cysts exposed to Mars-like pressure. On the other hand, it has been reported that the glutathionylation of complex I subunits, including NDUFS1 and NDUFV1, leads to inhibition of Complex I activity and ROS production (Mailloux et al., 2022), thus suggesting that the maintaining of a balanced GSH/GSSG ratio due to the increased activity of GPx and GR could contribute to preserve the mitochondrial functionality and energy requirement for nauplii development and growth. We did not observe changes in SOD activity that catalyzes the conversion of the radical superoxide (O_2^-) to hydrogen peroxide (H_2O_2), so facing the early production of ROS. This finding aligns with the unmodified ROS content of nauplii homogenates hatched from Mars-like pressure-exposed cysts.

5. Conclusions and future perspectives

Artemia franciscana showed an exciting potential for physiological adaptations, enabling organisms to cope with the environmental challenges they encounter in space.

One h of Mars-like pressure affects the mitochondrial function of nauplii hatched from exposed cysts, reducing the Complex I substrate-bound respiration and Complex I activity. These changes and the increased activity of the antioxidant enzymes GPx and GR could be compensatory responses to the increased susceptibility to oxidative stress, which may, at least in part, depend on the reduced total antioxidant capacity. Nauplii's cells appear to activate responses to avoid mitochondrial dysfunction and continue their growth processes. These adaptation mechanisms highlight *Artemia franciscana*'s resilience and ability to thrive in hostile environmental conditions. The results reported in this study further support the potential use of *Artemia franciscana* for astrobiological purposes, highlighting the animals' metabolic and redox state changes as a response to adaptation to an extreme condition mimicking the space. Moreover, they are prodromal for further studies to evaluate the metabolic adaptations of the cysts to longer exposure times, combinations of different Martian-like environmental conditions, or even studies on the adaptations of the nauplii in other stages of development.

Understanding the mechanisms of *Artemia franciscana* adaptations to

an extreme condition could give new insights into the study of the limit of life besides affording new elements for the search of biosignatures, i.e. traces of past life on other planetary bodies. It could also offer a valid solution to the long-term survival of human missions in space to establish self-sustaining populations in confined space. *Artemia* could provide a renewable food source for astronauts and other organisms facing nutritional challenges during long-duration space missions because of its richness in essential nutrients, including proteins, lipids, and vitamins.

CRediT authorship contribution statement

Maria Teresa Muscari Tomajoli: Writing – review & editing, Writing – original draft, Validation, Software, Methodology, Investigation, Data curation, Conceptualization. **Paola Di Donato:** Writing – review & editing, Methodology, Investigation. **Vincenzo Della Corte:** Methodology, Investigation. **Giovanni Covone:** Writing – review & editing, Methodology. **Gianluca Fasciolo:** Software, Methodology, Investigation. **Eugenio Geremia:** Software, Methodology, Investigation. **Adriana Petito:** Software, Methodology, Investigation. **Luca Toniatti:** Software, Methodology, Investigation. **Laura Inno:** Writing – review & editing, Methodology. **Alessandra Rotundi:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Conceptualization. **Paola Venditti:** Writing – review & editing, Writing – original draft, Supervision, Software, Methodology, Investigation, Data curation, Conceptualization. **Gaetana Napolitano:** Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Methodology, Investigation, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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