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Extreme freeze-tolerance in cryophilic tardigrades relies on controlled ice formation but does not involve significant change in transcription

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ABSTRACT

Subzero temperatures are among the most significant factors defining the distribution of organisms, yet, certain taxa have evolved to overcome this barrier. The microscopic tardigrades are among the most freeze-tolerant animals, with selected species reported to survive milli-Kelvin temperatures. Here, we estimate survival of fully hydrated eutardigrades of the species Ramazzottius varieornatus following exposures to −20 °C and −80 °C as well as −196 °C with or without initial cooling to −80 °C. The tardigrades easily survive these temperatures, yet with a significant decrease in viability following rapid cooling by direct exposure to −196 °C. Hence, post-freeze recovery of R. varieornatus seems to rely on cooling rate and thus controlled ice formation. Cryophilic organisms are renowned for having cold-active enzymes that secure appropriate reaction rates at low temperatures. Hence, extreme freeze-tolerance in R. varieornatus could potentially involve syntheses of cryoprotectants and de novo transcription. We therefore generated a reference transcriptome for this cryophilic R. varieornatus population and explored for differential gene expression patterns following cooling to ~80 °C as compared to active 5 °C controls. Specifically, we tested for fast transcription potentially occurring within 25 min of cooling from room temperature to a supercooling point of ca. −20 °C, at which the tardigrades presumably freeze and enter into the ametabolic state of cryobiosis. Our analyses revealed no evidence for differential gene expression. We, therefore, conclude that extreme freeze-tolerance in R. varieornatus relies on controlled extracellular freezing with any freeze-tolerance related genes being constitutively expressed.

1. Introduction

Life on Earth is constrained by physicochemical factors, among which temperature and the availability of liquid water are the most significant determinants of global biodiversity. Some species have nevertheless adapted to living in both extreme temperatures, and under large fluctuations in water availability (Rothschild and Mancinelli, 2001; Wharton, 2002; Feller and Gerday, 2003). Tardigrades are microscopic animals that are renowned for their extreme stress tolerance and their ability to enter a state of suspended animation, which is known as cryptobiosis (Clegg, 2001; Hibshman et al., 2020; Møbjerg and Neves, 2021). In this state, tardigrades can, for example, tolerate exposure to the vacuum and cosmic radiation of space, very high hydrostatic and osmotic pressures, complete desiccation, and exposure to extremely low temperatures (e.g. Rahm, 1924; Becquerel, 1950; Jönsson et al., 2008; Hengherr et al., 2009; Møbjerg et al., 2011; Halberg et al., 2013; Hashimoto et al., 2016; Heidemann et al., 2016; Ono et al., 2016; Lee et al., 2021). Here, we focus on cryobiosis, a form of cryptobiosis that allows exposure to low temperatures with an apparent absence of a lower lethal temperature (Wright, 2001).

Sub-zero temperatures are a common occurrence in polar, alpine and temperate climate zones, and many organisms thus endure temperatures well below the freezing point of pure water. Freezing of body and cell water is, nevertheless, fatal to organisms not adapted to these situations. Among cold tolerant ectotherms, three general strategies have been recognized that prevent cellular damage following exposure to sub-zero temperatures: freeze-tolerance, freeze-avoidance and cryoprotective dehydration (Zachariassen, 1985; Storey and Storey, 1988; Block, 1991; Storey and Storey, 1996; Holmstrup et al., 2002; Ramlow and Frits, 2020). Freeze-tolerant organisms survive various amounts of
extracellular ice formation, whereas freeze-avoiding organisms do not – the latter supercool, thus keeping body water in its liquid phase (Ramløv and Friis, 2020). Cryoprotective dehydration involves rapid water evaporation through highly permeable integuments of selected soil invertebrates, which thereby avoid internal ice formation (Holmstrøp et al., 2002). It should be noted that desiccated tardigrades in the so-called anhydrobiotic tun state are highly tolerant of extremely low temperatures and that they, as such, are cryoprotectively dehydrated. In addition to the three above-mentioned strategies, a fourth strategy, i.e., cryobiosis seems to have evolved among cryptobiotic organisms. Cryobiosis involves a relatively high supercooling capacity and tolerance towards extracellular ice formation (Hengherr and Schill, 2018).

Tardigrades may, thus, survive freezing temperatures both in their desiccated (anhydrobiotic) tun state and in their hydrated state – the latter inducing the state of cryobiosis (e.g. West and Kristensen, 1992; Wright et al., 1992; Somme and Meier, 1995; Hengherr et al., 2009; Guidetti et al., 2011; Møbjerg et al., 2011; Hengherr and Schill, 2018). Tardigrades in cryobiosis often have a partly contracted body, but the state does not depend on formation of the so-called tun (Wright et al., 1992; Møbjerg and Neves, 2021). Rahm (1921, 1924) reported that limno-terrestrial tardigrades (Ramazzottius oberhaeuseri, Milnesium tardigradum and Macrobiotus sp.) can survive exposures to −253 °C for 45 min in what may have been a fully hydrated state. More recent studies have shown that limno-terrestrial Antarctic tardigrades endure freezing from the active hydrated state for extended periods of time at temperatures ≥ −80 °C (Somme and Meier, 1995; Tsujimoto et al., 2016). The tardigrades (Barbaria jenningsi, Mesobiotus furciger and Diphascon chilenense), however, seem to die if exposed to −180 °C for periods of 1–2 days (Somme and Meier, 1995). The ability to tolerate freezing at −80 °C from the active, fully hydrated state was also confirmed in a study on selected temperate region eutardigrades collected from different habitats (Guidetti et al., 2011). Furthermore, investigations on freezing of active, fully hydrated Richtersius coronifer, a temperate region limno-terrestrial eutardigrade, indicate that this species tolerates 15–120 min at −196 °C (Ramløv and Westh, 1992; Persson et al., 2011), but not prolonged exposures of up to 24 h (Persson et al., 2011). Survival depends on cooling rate, which could indicate that cryoprotectants need to be synthesized during the cooling phase in order for the tardigrades to survive extreme subzero temperatures (Ramløv and Westh, 1992; Hengherr et al., 2009; Guidetti et al., 2011; Hengherr and Schill, 2018).

In order to provide new insight into cryobiosis and its underlying mechanisms, we investigated subzero temperature tolerance in the eutardigrade Ramazzottius varieornatus following freezing for 24 h at −20 °C, −80 °C and −196 °C, with tardigrades being exposed to −196 °C either directly or following initial cooling to −80 °C. We hypothesized that genes involved in cryoprotective survival are either constitutively expressed or up-regulated during entrance into the ametabolic state. In order to address whether cryobiosis relies on de novo transcription, we generated a de novo reference transcriptome from this Danish population of R. varieornatus and quantified differential gene expression following cooling from room temperature to −80 °C as compared to control conditions (i.e., 5 °C). Specifically, we tested for fast transcription potentially occurring during cooling involving genes related to extreme freeze-tolerance (e.g., Guzowski et al., 1999; Lau et al., 2001; Feller and Gerday, 2003; Spruessel et al., 2004; Kamilari et al., 2019). To the best of our knowledge, the current study presents the first differential expression analysis of cryophilic tardigrades.

2. Methods

2.1. Collection of tardigrades

Sediment samples containing R. varieornatus Bertolani and Kinchin, 1993 (Eutardigrada, Ramazzottiiidae) were collected in November 2016 and February 2018 from a roof gutter in Nivå, Denmark (55°56′36.53″ N, 12°30′00.90″ E). The samples were either diluted immediately in ultrapure water (Millipore Milli-Q® Reference, Merck, Darmstadt, Germany) and kept refrigerated at ca. 5 °C or stored at −20 °C for later use. Frozen samples were thawed, diluted in ultrapure water and acclimated at 5 °C before use (see Neves et al., 2020a). Highly active, adult R. varieornatus (Fig. 1) were identified from the refrigerated samples under a stereomicroscope, and transferred to embryo dishes at room temperature (RT; 21–25 °C) using hand-pulled glass pipettes. Approximately 3275 specimens were used for the freezing experiments and RNA sequencing as described below (Fig. 2).

![Fig. 1. Light microscopic image of Ramazzottius varieornatus in its fully hydrated and active state.](image-url)
The activity level of single tardigrades from each experimental and control group was assessed under a stereomicroscope and quantified 24 h after the subzero exposures, i.e., 48 h after start of the experiments. Tardigrades were considered active, and alive, if they exhibited spontaneous movement of legs and body or responded to water currents generated by a pipette. Any tardigrade that appeared bloated or ruptured, or did not show clear movement and remained inactive following gentle tactile stimuli, was considered inactive. As inactive

Fig. 2. Graphical representation of the methods used to evaluate the effect of exposing fully hydrated and active tardigrades to subzero temperatures. a. Method used for assessment of tardigrade activity (compare to Fig. 3). Tardigrades acclimated at 5 °C were randomly pooled into groups (5 × ca. 20) at RT and either transferred (i) into cryotubes and subjected to different subzero temperatures or (ii) to a well of a 12-well plate and kept as controls (48 h at ca. 5 °C). Tardigrades in cryotubes were either (i) exposed to subzero temperatures (−20, −80, and −196 °C) for 24 h or (ii) kept for 2 h at −80 °C and subsequently exposed to −196 °C for a 22 h period. The cryotubes were subsequently thawed for 2 h before tardigrades from each group were transferred to a well of a 12-well plate and kept at 5 °C for another 22 h. Tardigrade activity in each experimental and control group was assessed under stereomicroscope 48 h after start of the experiments. b. Method used for assessment of transcriptomic response. Groups (3 × ca. 180–190) of isolated and cleaned tardigrades were either kept at 5 °C or frozen at −80 °C for 2 or 24 h and used to extract total RNA for differential gene expression analyses. Sequenced reads were mapped against a reference transcriptome assembled de novo from specimens (N = 510) processed at RT or kept at −80 °C for several weeks. Note: *refers to the boiling point of liquid nitrogen.

The activity level of single tardigrades from each experimental and control group was assessed under a stereomicroscope and quantified 24 h after the subzero exposures, i.e., 48 h after start of the experiments. Tardigrades were considered active, and alive, if they exhibited spontaneous movement of legs and body or responded to water currents generated by a pipette. Any tardigrade that appeared bloated or ruptured, or did not show clear movement and remained inactive following gentle tactile stimuli, was considered inactive. As inactive

Fig. 3. Tardigrade activity following freezing and control conditions. Tardigrade activity, i.e., the proportion of active animals, was estimated following four different freezing experiments, and for 5 °C controls (n = 5 replicate groups each containing ca. 20 specimens, compare to Fig. 2a). Horizontal lines represent medians. Boxes and whiskers represent interquartile ranges and 1.5 × interquartile ranges, respectively, whereas single data points (●) represent the observed proportion of active tardigrades in each group.
tardigrades are not necessarily dead, the activity data likely represent an underestimation of survival rates.

### 2.3. Data analyses and presentation of subzero temperature experiments

Tardigrade activity, given in proportions, was calculated as the number of active specimens divided by the total number of specimens in each group (Fig. 3). The data are presented with medians and inter-quartile ranges. A test of equal or given proportions in R (two-sided Z-test) was used to evaluate whether activity was significantly different between tardigrades exposed directly to −196 °C, or following two hours at −80 °C. Light microscopic images of R. varieornatus were acquired with an Olympus DP27 digital microscope camera mounted on an Olympus BX53 compound microscope.

### 2.4. Assessment of transcriptomic response: Overview of RNA extractions and sequencing

Samples collected in February 2018 were stored frozen and cultivated for at least 2 months at ca. 5 °C (see Neves et al., 2020b) before highly active, adult tardigrades were thoroughly cleaned in ultrapure water at room temperature and processed for a reference transcriptome or differential expression analyses as described below (Figs. 2b). A total of approximately 2760 specimens were used for total RNA extractions. RNA samples were sent to BGI Europe A/S for library preparation and sequencing using the DNA nanoball sequencing platform (DNBSEQ-G400). At BGI sample quantity and quality were evaluated (Agilent 4200, Agilent Technologies, CA, USA) before mRNA was selected and reverse transcribed.

### 2.5. De novo assembly of a reference transcriptome

A de novo reference transcriptome was generated from this Danish population of R. varieornatus. The reference transcriptome was prepared from total RNA extracted from approximately 510 tardigrades, including specimens processed immediately after sorting at RT as well as specimens frozen at −80 °C for several weeks (Fig. 2b). The transcriptome assembly is based on sequencing of short pair-end reads (100 bp). Specifically, the collected RNA, a total of 1.8 μg, was sequenced using DNBseq technology and the data were subsequently processed using a standard bioinformatics pipeline at BGI. In brief, sequencing adapters were trimmed from the reads, and reads with >5% Ns or >20% low quality bases (Phred score ≤ 15) were discarded. Filtered reads were used to generate a de novo transcriptome assembly using Trinity version 2.0.6 (Grabherr et al., 2011), with the following parameters: -min_contig_length 150 -min_kmer_cov 3 -min_glue 3 -bflx_opts '-V5-edge-thr=0.1-stder' (assembly statistics can be found in Table 1). Assembled transcripts were clustered into ‘Unigenes’, i.e., uniquely assembled transcripts that correspond to an isoform(s) or a gene, using Tgicl v.2.0.6 (Pertea et al., 2003) with the following parameters: -l 40 < l 10 -v 25 -O ‘repeat_stringency 0.95 -minmatch 35 -minscore 35’. A functional annotation of the transcriptome through homology search was performed using Blast v.2.2.23 (Altschul et al., 1990), Diamond v.0.8.31 (Buchfink et al., 2015), Blast2GO v.2.5.0 (Conesa et al., 2005) and InterProScan v.5.11–51.0 (Quevillon et al., 2005) together with the following databases: NR, NT, GO, KOG, KEGG, Swiss-Prot and InterPro. Finally, TransDecoder v.3.0.1, getorf (EMBOSS:6.5.7.0) and hmmsearch v.3.0 (Mistry et al., 2013) were used to identify coding sequences and transcription factors within the Unigenes. Overall, 21,153 out of the 27,054 identified Unigenes, i.e., 78%, were identified and functionally annotated across the seven databases (Table 2).

### 2.6. RNA sequencing for differential expression analyses

Highly active, clean tardigrades were transferred in 1 μl ultrapure water to BeadBug™ tubes (Benchmark Scientific, Sayreville, NJ). A total of twelve tubes, each containing between 164 and 280 tardigrades, were prepared (Fig. 2b; Table 3). Six tubes were cooled to −80 °C for either 2 or 24 h with 3 replicates at each time interval. For comparison, the same numbers of replicates were kept for 2 and 24 h at 5 °C. Active control tardigrades were kept clean without food in order to minimize any potential contamination during RNA extraction. The two time-series, i.e., 2 h and 24 h, were conducted in order to reveal any time-dependent factor that potentially could influence differential transcription. Tubes removed from the −80 °C freezer, were thawed at RT for approximately 25 min, before water was removed from the sample, ultimately leaving the tardigrades covered by a film of cold water (ca. 0 °C) with little or no ice left. Assuming a melting point close to 0 °C for this limno-terrestrial species (Hengherr et al., 2009), the tardigrades would have had little or no time to restart metabolism before the RNA extraction. Specifically, total RNA was immediately extracted from these post-cryobiotic tardigrades and from the controls using an RNAasy® Plus Universal Kit (Qiagen, Hilden, Germany). The kit protocol was strictly followed, except for the first step, which was altered by adding glass beads to the tubes before homogenization (see Neves et al., 2022). Total RNA was eluted in RNase free water, and quantity and quality were evaluated with a NanoDrop® ND-1000 spectrophotometer (Pقوlab Biotechnologie GmbH, Erlangen, Germany). As outlined above, the extracted RNA was evaluated and subsequently sequenced by BGI Europe A/S.

An initial FastQC quality check (Andreas, 2010) of the raw RNAseq data produced by BGI revealed no apparent contaminations or other faults, and we therefore proceeded with trimming the adapters and low-quality stretches of the reads using AdapterRemoval (Schubert et al., 2016). Additionally, the quality of the reads was assessed using FastQC (Ewels et al., 2016) on both pre- and post-trimmed reads (Table 3).

### 2.7. Differential expression analyses

Salmon version 1.1.0 (Patro et al., 2017) was used to quantify the number of read counts for each transcript. The NumRead metric estimated by Salmon was used for subsequent differential expression

---

**Table 1**

RNA-Seq summary data for *Ramazzottius varieornatus* reference transcriptome (data from BGI).

<table>
<thead>
<tr>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bases</td>
<td>6.71 Gb</td>
</tr>
<tr>
<td>Raw reads</td>
<td>72.69 M</td>
</tr>
<tr>
<td>Clean reads</td>
<td>67.09 M</td>
</tr>
<tr>
<td>Q20 (Clean reads)</td>
<td>97.69%</td>
</tr>
<tr>
<td>Number of transcripts</td>
<td>32,975</td>
</tr>
<tr>
<td>Mean length of transcripts</td>
<td>1381 bp</td>
</tr>
<tr>
<td>NS0(transcripts)</td>
<td>2438</td>
</tr>
<tr>
<td>GO (transcripts)</td>
<td>49.77%</td>
</tr>
<tr>
<td>Number of Unigenes</td>
<td>27,054</td>
</tr>
<tr>
<td>Mean length of Unigenes</td>
<td>1518 bp</td>
</tr>
<tr>
<td>NS0(Unigenes)</td>
<td>2517 bp</td>
</tr>
<tr>
<td>GO(Unigenes)</td>
<td>49.80%</td>
</tr>
</tbody>
</table>

**Table 2**

Annotation of *Ramazzottius varieornatus* reference transcriptome (data from BGI).

<table>
<thead>
<tr>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Unigenes</td>
<td>27,054</td>
</tr>
<tr>
<td>Unigenes with hits in NR database</td>
<td>21,047</td>
</tr>
<tr>
<td>Unigenes with hits in NT database</td>
<td>2773</td>
</tr>
<tr>
<td>Unigenes with hits in Swiss-Prot database</td>
<td>13,678</td>
</tr>
<tr>
<td>Unigenes with KEGG pathways</td>
<td>13,648</td>
</tr>
<tr>
<td>Unigenes with hits in KOG database</td>
<td>13,154</td>
</tr>
<tr>
<td>Unigenes with hits in InterPro database</td>
<td>13,485</td>
</tr>
<tr>
<td>Unigenes with GO terms</td>
<td>2341</td>
</tr>
<tr>
<td>Unigenes annotated by all seven databases</td>
<td>701</td>
</tr>
<tr>
<td>Total annotated Unigenes</td>
<td>21,153</td>
</tr>
</tbody>
</table>
analyses. Genes that were not expressed (a total of 187) in more than half of the samples were excluded from further analyses. Three Bioconductor packages were used to perform differential expression analyses: DESeq2, EdgeR and Limma. For EdgeR, the quasi-likelihood F-test was used as it is best suited for experiments with few replicates and is effective at controlling the false discovery rate. A threshold of false discovery rate (FDR) or adjusted p-value of \( \leq 0.05 \) was used to determine whether a gene was up- or down-regulated. The terms up-regulated and down-regulated refers to the level of gene expression of the experimental group being either significantly higher or lower than that of the control. MA-Plots and Venn diagrams (Fig. 4) were made in R and final assemblage of graphs was conducted in CorelDraw (Corel Corporation).

3. Results

3.1. Subzero temperature tolerance of hydrated tardigrades

We estimated the survival of active, fully hydrated \( R. \) varieornatus (Fig. 1) following exposure to subzero temperatures as compared to active controls kept at 5 \( ^\circ \)C (Fig. 2a). Tardigrades were exposed to three different subzero temperatures, i.e., 20 \( ^\circ \)C, 80 \( ^\circ \)C, and 196 \( ^\circ \)C, respectively, for 24 h and then retransferred to 5 \( ^\circ \)C for 24 h before their activity was assessed (Fig. 2a). In addition to direct transfer into 196 \( ^\circ \)C, we also tested the effect on tardigrade survival of initial cooling to 80 \( ^\circ \)C prior to transfer into liquid nitrogen. Survival estimates are based on tardigrade activity, i.e., the proportion of active animals, 24 h after exposure to a given temperature.

High levels of activity were found among the tardigrades cooled to Table 3

<table>
<thead>
<tr>
<th>Samples</th>
<th>Approx. specimen no.</th>
<th>Total RNA (( \mu )g)</th>
<th>No. of raw reads</th>
<th>No. of clean reads</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 2 h,1</td>
<td>180</td>
<td>0.7</td>
<td>55,601,572</td>
<td>49,829,250</td>
</tr>
<tr>
<td>Control 2 h,2</td>
<td>180</td>
<td>0.6</td>
<td>65,618,412</td>
<td>51,473,260</td>
</tr>
<tr>
<td>Control 2 h,3</td>
<td>180</td>
<td>0.5</td>
<td>66,263,116</td>
<td>51,435,648</td>
</tr>
<tr>
<td>- 80 °C 2 h,1</td>
<td>280</td>
<td>1</td>
<td>58,234,036</td>
<td>49,135,648</td>
</tr>
<tr>
<td>- 80 °C 2 h,2</td>
<td>180</td>
<td>1</td>
<td>55,216,458</td>
<td>50,953,274</td>
</tr>
<tr>
<td>- 80 °C 2 h,3</td>
<td>164</td>
<td>0.8</td>
<td>57,000,784</td>
<td>49,049,154</td>
</tr>
<tr>
<td>Control 24 h,1</td>
<td>180</td>
<td>0.9</td>
<td>56,928,272</td>
<td>49,559,704</td>
</tr>
<tr>
<td>Control 24 h,2</td>
<td>180</td>
<td>0.7</td>
<td>62,207,110</td>
<td>52,118,148</td>
</tr>
<tr>
<td>Control 24 h,3</td>
<td>180</td>
<td>0.8</td>
<td>59,143,574</td>
<td>49,760,720</td>
</tr>
<tr>
<td>- 80 °C 24 h,1</td>
<td>180</td>
<td>0.7</td>
<td>62,356,654</td>
<td>49,767,914</td>
</tr>
<tr>
<td>- 80 °C 24 h,2</td>
<td>180</td>
<td>0.6</td>
<td>61,916,738</td>
<td>49,762,362</td>
</tr>
<tr>
<td>- 80 °C 24 h,3</td>
<td>180</td>
<td>0.4</td>
<td>68,020,914</td>
<td>49,911,012</td>
</tr>
</tbody>
</table>

Fig. 4. Differential gene expression between 5 °C controls and tardigrades cooled to –80 °C for 2 or 24 h (compare to Table 4). a and b) MA-Plots of DESeq2 data, showing the log2 fold change against the mean of normalized counts. Differentially expressed genes are highlighted in blue. DESeq2 found 47 up-regulated and 81 down-regulated genes in tardigrades kept at –80 °C for 24 h as compared to controls (5 °C), and only 2 up-regulated genes following exposure to –80 °C for 2 h. c and d) Venn diagrams of the 2-h and 24-h freezing datasets as determined by three different differential gene expression analyses: DESeq2, EdgeR and Limma. The red circle represents the DEGs identified by EdgeR, the blue represents Limma+voom and the green represents DESeq2. Numbers in overlapping zones give the DEGs identified in more than one analysis. Note that only two DEGs (both down-regulated) were identified across all three methods for the 24-h freezing dataset. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
−20 °C and −80 °C, and in controls kept at 5 °C during the entire experiment (Fig. 3). Specifically, the mean ± se activity of control tardigrades was 99.1 ± 0.9%, whereas the activity of tardigrades cooled to −20 °C and −80 °C for 24 h was 96.9 ± 1.9% and 97.1 ± 1.1%, respectively. *R. variornatus* is furthermore capable of surviving submergence into liquid nitrogen for at least 24 h (Fig. 3). The proportion of active specimens was, however, significantly affected by initial cooling to −80 °C (χ²(1) = 96.1, *p* < 2.2×10⁻¹⁶). Indeed, the mean ± se activity of the tardigrades after direct transfer into liquid nitrogen was 19.9 ± 3.3%, whereas it increased to 87.9 ± 3.1% with cooling to −80 °C for 24 h, prior to exposure to −196 °C.

### 3.2. Differential gene expression analyses of RNA-Seq datasets

We subsequently addressed the observed effect on survival of cooling to −80 °C prior to the −196 °C exposure (Fig. 3). Specifically, we generated a de novo reference transcriptome from this cryophilic population of *R. variornatus* (*Tables 1 and 2*) and assessed the transcriptomic response of *R. variornatus* subjected to −80 °C for 2 and 24 h (Fig. 2b). A total of twelve samples were sequenced, with three RNA-Seq replicates generated for each experimental and control condition (Table 3). The pair-end reads (2 × 100 bp) were subsequently trimmed of adapter and low-quality sequences, resulting in a total of ~603 million clean reads, with an average number of reads per sample of ~50.2 million (~25.1 million pairs per sample).

To look for differentially expressed genes, we compared gene expression levels of the three 5 °C controls with those of their corresponding experimental groups, i.e., the three 2-h 5 °C control groups were compared with the three 2-h −80 °C experimental groups and the three 24-h 5 °C control groups with the three 24-h −80 °C experimental groups (Table 4). The time it takes for tardigrades to regain activity following extreme stress depends on exposure time and the total amount of damage obtained during the extreme stress exposure (e.g. Crowe and Higgins, 1967; Neumann et al., 2009; Rebecchi et al., 2009). Thus, time spent in the frozen state could potentially have influenced transcription of genes in the early recovery phase before RNA extraction. Therefore, we also compared gene expression levels between 2-h and 24-h 5 °C experimental groups (Table 4). Moreover, the initial cleaning of the tardigrades as well as food deprivation of 24-h 5 °C controls could potentially have influenced gene expression patterns. Hence, we also compared expression between the 2-h and 24-h 5 °C controls (Table 4).

The three differential expression analysis methods, DESeq2 (Love et al., 2014), EdgeR (Robinson et al., 2016; McCarthy et al., 2012; Chen et al., 2016), and Limma (Ritchie et al., 2015) revealed little evidence for differentially expressed genes (DEGs) in any of the comparisons (considering a FDR ≤ 0.05) (Table 4, Fig. 4). Specifically, as compared to controls, the total number of DEGs identified by DESeq2 was 128 for the 24-h freezing experiments, whereas only two were identified in the 2-h freezing experiment. An overview of fold changes between control and freezing conditions, as a function of the average expression level, is depicted in Fig. 4a and b based on the DESeq2 datasets. Only two of the 128 DEGs identified by DESeq2, for the 24-h datasets, were found across all three methods (Fig. 4d; Table 4). These two DEGs, which were both down-regulated, were the only two predicted by Limma in any of the analyses (Table 4, Figs. 4c,d). Both genes are not annotated in the transcriptome and no homolog was found in public databases, i.e., no hits were identified for any of the two genes through a BLAST search.

### 4. Discussion

We show that the limno-terrestrial eutardigrade *Ramazzottius variornatus* tolerates freezing from the active hydrated state, likely entering cryobiosis, a state characterized by the reversible cessation of metabolism (Clegg, 2001; Wright, 2001; Mobjerg et al., 2011). Specifically, *R. variornatus* tolerates temperatures down to at least −196 °C, where no or negligible amounts of water is present in the liquid state. The tardigrades can remain viable when stored in liquid nitrogen (ca. −196 °C), if they are initially cooled to −80 °C for 2 h. Tardigrade activity following freezing is dependent on cooling rate, with very high rates (> 100 °C min⁻¹), as introduced by direct submergence into liquid nitrogen, affecting post-freeze activity (Fig. 3). It follows that control of freezing events is a key factor underlying the extreme subzero temperature tolerance.

Tardigrades are microscopic animals (<1.2 mm) with a high surface area to volume ratio, and highly permeable integuments (Mobjerg et al., 2018). Water will therefore leave the tardigrades during freezing of the surroundings, potentially enforcing a massive osmotic shock on the animals (Westh and Kristensen, 1992). Importantly, tardigrades are capable of tolerating extreme fluctuations in osmotic pressure (e.g. Heidemann et al., 2016; Halberg et al., 2009; Hygum et al., 2016; Sorensen-Hygum et al., 2018). Moreover, low molecular weight organic osmolytes (e.g. polyols, sugars and amino acids) as found in the active state of cryptobiotic tardigrades (Halberg et al., 2013), will slow down and reduce extracellular ice formation, while ensuring cellular dehydration. While freezing occurs in the surroundings, and likely eventually in the tardigrade hemocoel, cells will dehydrate, leaving no or very small amounts of liquid water within the intracellular compartment. Very high cooling rates, as introduced by direct submergence into liquid nitrogen, will impair establishment of new osmotic equilibria, promoting intracellular freezing (Ramlov and Friis, 2020)—cooling the tardigrades too fast will thus prevent the cells from dehydrating sufficiently, promoting intracellular ice formation. It follows that extreme freeze tolerance in tardigrades likely relies on controlled extracellular ice formation accompanied by movement of water out of the cells (likely through aquaporin water channels; Gromh et al., 2013).

To investigate whether cryobiosis relies on a change in transcriptional activity, we extracted RNA and performed differential gene expression analyses on tardigrades cooled to −80 °C as compared to controls kept at 5 °C. The experiment was designed to detect transcription potentially occurring within the 25 min of cooling from room temperature to a supercooling point of around −20 °C, when the tardigrades presumably freeze and thus enter into the ametabolic state of cryptobiosis. The latter would entail fast transcription as is known to occur within advanced eukaryotes (e.g., Guzowski et al., 1999; Spruessel et al., 2004), but at low temperatures characteristic for psychrophile organisms (e.g. Lau et al., 2001; Feller and Gerday, 2003). Our analyses of the RNA-seq data revealed little evidence for differentially expressed genes (Fig. 4), suggesting that most (if not all) genes involved in the extreme freeze tolerance of the tardigrades are constitutively expressed. Specifically, two unannotated DEGs were identified for the 24-h freezing experiment (Fig. 4d). Both transcripts were down-regulated in the tardigrades cooled to −80 °C for 24 h as compared to the controls kept at 5 °C. For comparison, no DEGs were identified across the three analyses method for the 2-h freezing experiment (Fig. 4c). The latter suggests that the two unannotated DEGs are not involved in preparation for freezing. The two transcripts could potentially be related to the early post-freeze recovery phase or, more likely, a result of food deprivation in the 24-h 5 °C controls. Specifically, food deprivation of the active 5 °C controls
will likely influence transcription in a time-dependent manner and, depending on the nutritional status of individual tardigrades, may contribute to variance in gene expression patterns among control replicates. Thus, to recapitulate, preparation for extreme sub-zero temperature exposure of hydrated \( R. \) varieornatus does not seem to involve a significant change in transcription. The latter observation is in clear contrast to the major transcriptomic shift, involving 9634 differentially expressed genes, observed in a parallel 24-h experiment, in which RNA was extracted from specimens of \( R. \) varieornatus heated to 35 °C as compared to 5 °C controls (Neves et al., 2022).

In summary, our results show that \( R. \) varieornatus tolerates freezing to at least –196 °C from its active and fully hydrated state, while presumably entering the ametabolic state of cryobiosis. Post-freeze recovery seems to rely on controlled extracellular ice formation and thus osmotic dehydration of cells, avoiding or at least minimizing intracellular ice formation. The cryophilic tardigrades are always ready to enter cryobiosis with essential genes likely being constitutively expressed. Thus, genes that have been indicated to play a role in the cryptobiotic cryobiosis with essential genes likely being constitutively expressed.

**References**


