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## Original Articles

## Negative impact of freeze–thaw cycles on the survival of tardigrades

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## ABSTRACT

Global warming effects in temperate and polar regions include higher average temperatures and a decrease in snow cover, which together lead to an increase in the number of freeze–thaw cycles (FTC). These changes could affect the fitness of both terrestrial and aquatic species. In this study, we tested how tardigrades, ubiquitous microscopic invertebrates, face FTC. Tardigrades are amongst the most resistant animals to unfavorable conditions, including long and deep freezing periods, and are an emerging model group for invertebrate ecology and evolution. We used 12 populations of tardigrades, representing different families within order Parachela, inhabiting different ecosystems (glaciers, snow, terrestrial, aquatic), found in various substrates (mosses, sediments in lakes, cryoconite on glaciers, and snow), and originating from different latitudes and altitudes. We estimated the number of cycles required to kill 50% of individuals and tested for its association with ecological characteristics of the natural habitat (e.g., number of months with predicted FTC), while accounting for phylogeny. The most resistant tardigrades to FTC were the ones from mountain areas and glaciers. The estimated number of cycles required to kill 50% of individuals was the highest for mountainous species inhabiting rock pools and cryoconite holes on glaciers (30 and 14 FTC, respectively). Tardigrades from lowlands were the most sensitive to changes, with 50% of individuals dying after three FTC, while lacustrine and subtropical tardigrades required only one FTC to reach 50% mortality. Our study shows that the response to recurrent freezing stress is taxon dependent and related to the local environmental conditions. The predicted increase of FTC cycles will negatively impact tardigrade populations. Considering the abundance and various trophic roles of tardigrades, reduction in population sizes or the disappearance of some fragile species could affect the functioning of both aquatic and terrestrial ecosystems. Tardigrades are candidate indicators of how freeze–thaw cycles impact ubiquitous microscopic metazoans with similar physiological capabilities.

## 1. Introduction

One of the consequences of global warming is the increase in freeze–thaw cycles (FTC) (Harrison et al., 2020; Leuther & Schlüter, 2021; Li et al., 2021). The occurrence of thawing events in temperate and boreal regions during winter and spring time will increase in the next decades, mostly due to the rise of global temperatures and reduced snowfall (Li et al., 2021; Shijin et al., 2022; Harrison et al., 2020). These changes will have an impact on both abiotic and biotic functions of the ecosystem, influencing biogeochemistry, ecology and, consequently,

biodiversity (Larsen et al., 2002; Callaghan et al., 2004; Lawrence et al., 2015; Kreyling et al., 2020). Although microinvertebrates are a crucial element of global soil and freshwater ecosystems, dominating in species richness and biomass, the impact of freeze–thaw events on microscopic invertebrates is poorly documented (Coulson et al., 2000; Callaghan et al., 2004; Sjørnsen et al., 2005; Konestabo et al., 2007; Ávila-Jiménez et al., 2010). Moreover, previous studies focusing on the effects of FTC and prolonged freezing events on soil fauna were conducted on arthropods and annelids (Sulkava & Huhta, 2003; Sjørnsen et al., 2005; Konestabo et al., 2007), while limno-terrestrial (i.e. inhabiting

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bryophytes, lichens or leaf litter) and aquatic invertebrates received little attention (but see: Halberg et al., 2009; Walsh et al., 2014). This knowledge gap limits our ability to develop encompassing, detailed models of the response of microscopic invertebrates to effects of global warming; thus, knowledge on organismal survival under FTC, must be acquired.

Some of the most common microinvertebrates inhabiting both terrestrial and freshwater ecosystems are tardigrades, a phylum also known as water bears. Tardigrades are a cosmopolitan group inhabiting various substrates: bryophytes, lichens, soil, aquatic sediments and even glaciers or snow (Nelson et al., 2015; 2019; Zawierucha et al., 2021). Tardigrades are a significant component of these ecosystems, where they play multiple roles as consumers at different trophic levels (Hohberg & Traunspurger, 2005; Guil & Sanchez-Moreno, 2013; Zawierucha et al., 2022). Due to their cryptobiotic abilities (i.e. tolerance of unfavorable conditions), tardigrades can survive desiccation, high irradiation, osmotic pressure or freezing (Weinicz et al., 2011; Møbjerg et al., 2011; Guidetti et al., 2012; Rebecchi et al., 2020; Møbjerg & Neves, 2021). Although the resistance to freezing is thought to be common across limno-terrestrial tardigrades since they are particularly abundant in polar and boreal ecosystems (Dastych, 1985; Zawierucha et al., 2016), the current knowledge of how they respond to freezing stress is limited (Guidetti et al., 2011a; Sieger et al., 2022).

Research into cryobiosis and freeze tolerance in tardigrades is restricted to a handful of studies (Møbjerg & Neves, 2021). Some tardigrades are able to survive freezing even in liquid nitrogen and 10mK (Lee et al., 2022; Møbjerg et al., 2022). Long term freezing survival was shown by Zawierucha et al. (2018) and Tsujimoto et al. (2016) who recovered tardigrades after 11 and 30 years of being in a frozen state, from cryoconite (biogenic, dark sediment on glaciers) and mosses, respectively. Sømme and Meier (1995) and Guidetti et al. (2011a) found that adaptation to freezing is related to the ability to withstand desiccation (anhydrobiosis). Even though the authors used different sets of temperatures to test how tardigrades respond to freezing, these temperatures are mostly uncommon or do not even occur in ecosystems from where tardigrades were collected (e.g.  $-22\text{ }^{\circ}\text{C}$  to  $-180\text{ }^{\circ}\text{C}$  in Sømme and Meier (1995),  $-9\text{ }^{\circ}\text{C}$  to  $-80\text{ }^{\circ}\text{C}$  in Guidetti et al. (2011a)). Mechanisms responsible for the adaptation to freezing were studied by Halberg et al. (2009), Hengherr et al. (2009) and Møbjerg et al. (2022). Eutardigrades are able to avoid freezing by supercooling to around  $-20\text{ }^{\circ}\text{C}$  (Halberg et al., 2009; Hengherr et al., 2009). Hengherr et al. (2009) studied the supercooling point of limno-terrestrial tardigrades and found that a slower cooling rate increases the chances of survival of animals by preserving the cellular structure from freezing injury. The supercooling point varied between species ranging from  $-23.7 \pm 3.9\text{ }^{\circ}\text{C}$  to  $-11.5 \pm 2.2\text{ }^{\circ}\text{C}$  (Hengherr et al., 2009). Møbjerg et al. (2022) found that the model tardigrade *Ramazzottius varieornatus* can survive freezing under  $-20\text{ }^{\circ}\text{C}$ ,  $-80\text{ }^{\circ}\text{C}$  and  $-196\text{ }^{\circ}\text{C}$ . Extreme freeze-tolerance in *R. varieornatus* is based on a controlled extracellular freezing process (Møbjerg et al., 2022). Although different authors provided findings on the effects of a prolonged or short-term freezing on both hydrated and dehydrated animals, and potential mechanisms controlling survivorship, the effects of subsequent FTC on survivability of tardigrades have been poorly studied (Sieger et al., 2022).

The impact FTC has on survivability of invertebrates is crucial for predicting the effects of climate changes on ecosystems where these animals play a pivotal role. However, such data are missing in tardigrades, with the exception of two studies that focus on their tolerance to high temperature (Giovannini et al., 2018; Neves et al., 2020). Since tardigrades are cosmopolitan and inhabit various niches along latitudes and altitudes, we aimed to quantitatively assess the tolerance to multiple freeze–thaw cycles. We selected a set of tardigrade species representing the order Parachela, inhabiting a wide range of ecosystems, from freshwater reservoirs (cryoconite holes) on glaciers to bryophytes in lowland forests. Our four hypotheses were that (I) FTC tolerance differs between populations belonging to different tardigrade species, (II)

tardigrades inhabiting areas with higher FTC are better adapted to freeze–thaw cycles, (III) increasing FTC numbers negatively impact survivability of tardigrades, and (IV) FTC tolerance has a phylogenetic signal within Parachela.

## 2. Materials and methods

### 2.1. Sampling and species identification

Tardigrades were collected from different altitudes, latitudes, and types of ecosystems. Details of the sampling sites are provided in Table 1. The tardigrades were both extracted directly from samples or obtained from long-term cultures (Table 1). We did not use juveniles for our experiment. The adult tardigrades were originally isolated from different substrates: (i) bryophytes, (ii) leaf litter, (iii) cryoconite, (iv) freshwater sediments and (v) snow surface.

When needed, the tardigrades were extracted from the samples with standard methods under a stereomicroscope (Degma, 2019; Zawierucha et al., 2019a; Ono, 2022) and identified based on external morphology. Specimens for phase contrast microscopy (PCM) and differential interference contrast microscopy (DIC) were mounted on microscope slides in a drop of Hoyer's medium and examined under a microscope at 400x and 1000x magnification. For species identification we used the following keys and species descriptions: Dastych et al. (2003), Pilato & Binda (2010), Schill et al. (2010), Kaczmarek et al. (2017), Zawierucha et al. (2019), Stec et al. (2021), Vecchi & Stec (2021), Ono et al. (2022).

### 2.2. Phylogeny reconstruction

A phylogeny of the tested species was constructed. 18S rDNA (SSU, small ribosome subunit), 28S rDNA (LSU, large ribosome subunit) and CO1 (cytochrome oxidase subunit I) fragments were retrieved from GenBank or produced *de novo*. New sequences were amplified and sequenced according to Stec et al. (2020a), Stec et al. (2020b). Sequencing was performed with an ABI 3130xl sequencer at the Department of Biological and Environmental Science of the University of Jyväskylä (Jyväskylä, Finland). For some species amplification and sequencing failed, thus sequences from the closest species from GenBank were used. Sequences were aligned using the G-INS-I strategy in MAFFT v. 7 (Katoh and Toh, 2008) and concatenated (using the R package “concatpede”; Vecchi & Bruneaux, 2021). ML topology was constructed using the IQ-TREE online server (Nguyen et al., 2015) with automatic model selection 1000 ultrafast bootstrap replicates. GenBank accession numbers of the sequences used for the phylogenetic reconstruction are provided in Table SM.01.

### 2.3. Experimental freeze–thaw treatments

The medium containing the tardigrades was subject to seven consecutive daily freeze–thaw events. Freezing of medium occurred overnight and thawing every morning, to mirror natural conditions in temperate and polar regions during autumn and spring. Tardigrades were kept individually in 24-well plastic plates. Each plastic plate used for the experiment was new, and plates were stored in exactly the same conditions. However, in order to avoid any potential confounding effects of plastic plate identity, each species was represented in at least two distinct plates. Each well was filled with 1 ml of distilled water and a 20  $\mu\text{l}$  of *Chlorella* sp. as food during the thawing period. *Chlorella* was successfully used for culturing tardigrades belonging to the taxonomic groups used in our study (Kosztyla et al., 2016; Bryndová et al., 2020). Before cooling to sub-zero temperatures, the tardigrades were kept for at least for 24 h in  $+3\text{ }^{\circ}\text{C}$  (to allow adaptation to the cold), then over-nighting (14–15 h) between  $-6.1\text{ }^{\circ}\text{C}$  to  $-9.0\text{ }^{\circ}\text{C}$  with a cooling rate 0.19 per minute in a climatic chamber (Binder 9020–0116). This slow cooling process was designed to mimic what a population in nature would experience in northern latitudes (i.e. temperatures above  $0\text{ }^{\circ}\text{C}$  during the

**Table 1**

Details on the species used in present study. % mixed months – percentage of months (from 2010 to 2018) where the average minimum daily temperature was below 0 °C and the average maximum daily temperature was above 0 °C. N – number of specimens used in experiments. Preservation of material before experiment: Fr - Freezing, Dr – dried, Ct – laboratory cultures. ASL – altitude above sea level, ca. – circa. The population of *Hypsibius* sp. Mt Gassan also contained some *Hypsibius nivalis* (~6%) and it was not possible to separate them under stereomicroscopes without permanent slide preparation. However, accounting for the evolutionary proximity and habitat similarity of these two species, the small amount of *H. nivalis* and the precise estimation of the FTC-LD50 (deductible from the narrow 95% CI estimate interval: 3.56 – 5.36), it is safe to assume that this issue did not bias the results.

Species	N	Country	Coordinates	ASL	Ecosystem	Substratum	% mixed months	Fr/Dr/Ct	Date of collection – time in culture (years)
<i>Adropion scoticum</i> (Murray, 1905)	64	Finland	N61°54'19.044" E23°20'44.93"	ca. 150	Peatland	Bryophyte	27.8%	Ct	07.2020 – 1
<i>Cryobiotus klebelsbergi</i> (Mihelčić, 1959)	62	Italy	N46°23'43.0" E10°35'15.0"	Ca. 3000	Glacier	Cryoconite sediment	27.8%	Fr	08.2020 - NA
<i>Dactylobiotus parthenogeneticus</i> Bertolani, 1982	50	UK	N53°33'32" W2°23'48.001"	ca. 60	Lacustrine, lowland	Freshwater sediment	2.8%	Ct	NA - NA
<i>Hypsibius</i> cf. <i>convergens</i>	57	Italy	N44°23'15.907" E10°26'41.161"	ca. 470	Mountainous, forest	Bryophyte	17.6%	Ct	05.2021–0.5
<i>Hypsibius</i> sp.	31	Japan	38° 30' N, 140° 00' E	ca. 770	Mountainous forest	Snow surface	27.8%	Fr	05.2020 - NA
<i>Macrobiotus annewintersae</i> Vecchi & Stec, 2021	34	USA, Mississippi	N32°21'5.479"y W89°56'30.149"	ca. 130	Urban green areas	Leaf litter	6.5%	Ct	12.2019 – 2.5
<i>Macrobiotus ripperi</i> Stec, Vecchi & Michalczyk, 2021	66	Finland	N62°13'24.629" E25°46'20.366"	ca. 90	Forest	Bryophyte	23.1%	Ct	02.2019–3
<i>Paramacrobiotus fairbanksi</i> Schill, Förster, Dandekar & Wolf, 2010	62	Finland	N62°13'48.756" E25°44'36.06"	ca. 100	Urban green areas	Leaf litter	23.1%	Ct	05.2018–4
<i>Paramacrobiotus richtersi</i> (Murray, 1911)	44	Finland	N62°14'35.1" E25°42'55.6"	ca. 10	Forest	Bryophyte	15.7%	Ct	05.2019–3
<i>Ramazzottius</i> sp.	49	Italy	N44°23'46.176" E10°0'14.94"	ca. 1650	Mountainous, rock pool	Freshwater sediment	30.6%	Dr	04.2019 - NA
<i>Ramazzottius</i> sp.	33	Finland	N62°13'39.565" E25°44'23.442"	ca. 140	Forest	Snow surface	23.1%	Dr	12.2021 - NA
<i>Richtersius</i> aff. <i>coronifer</i>	48	Italy	N41°43'11.8" E15°56'01.3"	ca. 790	Mountainous, open landscape	Bryophyte	55.6%	Dr	06.2021 - NA

day but dropping below 0 °C during the night). We note that according to Hengherr et al. (2009) tardigrades likely have supercooling points way below the temperatures used in this study. However, we used different set of species than Hengherr et al. (2009). Therefore, even if the animals in our study were more likely supercooled than literally frozen, they nevertheless experienced the freezing of the surrounding medium. In the morning, the frozen plates were placed at 3 °C (Sanyo MLR-351) for 8–9 h to slowly defrost. Half an hour before observations, each well was aerated by blowing air into the water using a glass Pasteur pipette. Each tardigrade specimen was observed under a stereomicroscope (Zeiss Discovery V8) after each FTC to evaluate its viability: recorded as alive if they were motile; recorded as dead if they were not motile for at least two subsequent observations, stiff and bloated, changed colour, or the epidermis was separated from the external cuticle. After the seventh and last FTC, the tardigrades were checked twice to ascertain their final survival (animals were kept at 3 °C for this period): on the thawing day and on the subsequent day.

#### 2.4. Climatic variables

As a proxy for the natural occurrence of FTCs, we calculated the proportion of months between 2010 and 2018 in which the average maximum daily temperature was above zero, whereas the average minimum daily temperature was below zero – so-called “mixed months”. These data were extracted from the WordClim historical monthly weather data between 2010 and 2018 ([worldclim.org/data/monthlywth.html](http://worldclim.org/data/monthlywth.html)) based on the coordinates from each population sampling location.

#### 2.5. Data analysis

To estimate the number of cycles at which 50% of the individuals of each population died (from here on referred to as FTC-LD50), we fitted the viability data from each cooling cycle using the cumulative function

of a discrete Weibull distribution based on a Bernoulli response distribution. As cold tolerance is a physiological adaptation, it can be hypothesized that the evolution of protective mechanisms can be constrained by what genes/adaptations are already present in that clade. The fitting was performed using Bayesian methods in R v4.2.1 (R Core Team, 2022) with the R package “R2Jags v0.7–1” (Su & Yajima, 2021).

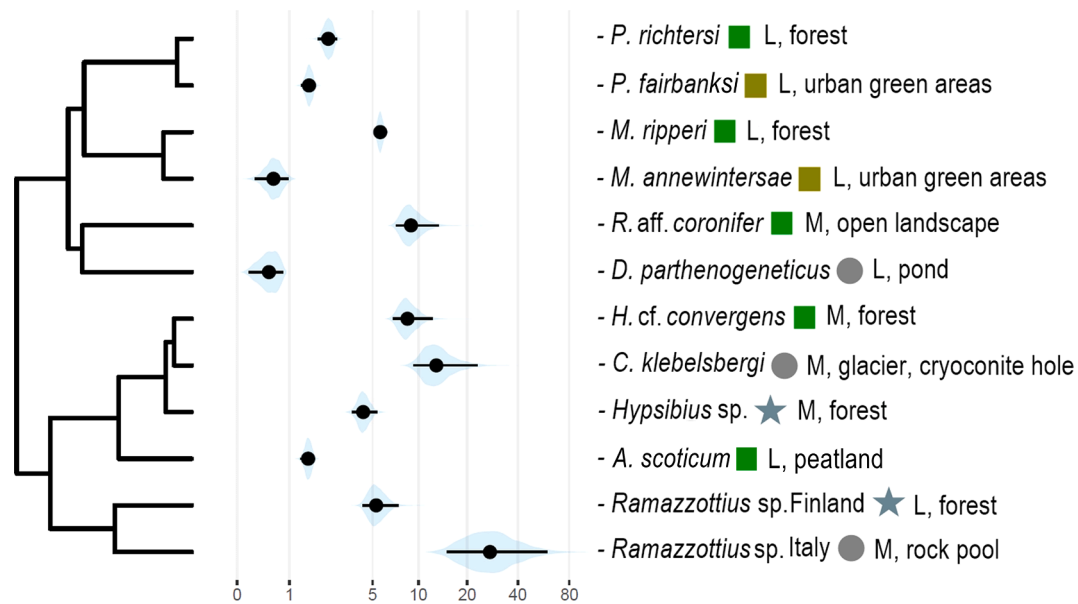
To estimate the phylogenetic signal, Blombergs K (Blomberg et al., 2003) and Pagels  $\lambda$  (Pagel, 1999) were calculated with the R package “phytools v1.2–0” (Revell, 2012).

We tested the effect of the proportion of mixed months on the FTC-LD50 of the analysed populations with a Phylogenetic Linear Model (PLM) using scaled and centered predictor and response variables. Shared evolutionary history between populations was accounted for by including a phylogenetic variance-covariance matrix based on Brownian motion. PLM was implemented in the R package “R2Jags v0.7–1” (Su & Yajima, 2021). The raw data and the R code used for the analysis are publicly available at [https://tardipede.github.io/freezing\\_cycles/](https://tardipede.github.io/freezing_cycles/) and <https://doi.org/10.5281/zenodo.7579151>.

### 3. Results

Our results support the three hypotheses we proposed. Tolerance differs between populations belonging to different tardigrade species corroborating hypothesis I (Fig. 1). The estimated average FTC-LD50 ranged from less than 1 cycle for *Dactylobiotus parthenogeneticus* (lacustrine species) and *Macrobiotus annewintersae* (subtropical species), to almost 14 cycles for *Cryobiotus klebelsbergi* from cryoconite holes on glacier followed by almost 30 cycles for *Ramazzottius* sp. from mountainous rock pools (Table 2, Fig. 1), which is in agreement with our hypothesis II.

The PLM (Table 3) explained 44.8% (average Conditional R<sup>2</sup>) of the variance in the response variable (FTC-LD50), with most of it accounted for by the fixed effect (average Marginal R<sup>2</sup>: 33.8%) compared to phylogeny (average R<sup>2</sup>: 11.0%). The proportion of mixed months (months



**Fig. 1.** Estimated number of FTC (log scaled) to death of 50% of individuals. Legend: squares – limnoterrestrial environments (green – bryophytes, brown – leaf-litter); circle – aquatic ecosystem, freshwater sediments; star – snow surface; L – samples collected at lowlands, M – samples collected at highlands and mountainous regions.

**Table 2**  
Estimated FTC-LD50 average and 95% Credible Interval (C.I.) of the analysed populations.

Population	FTC-LD50 Average [95% C.I.]
<i>Adropion scoticum</i>	1.56 [1.32 – 1.79]
<i>Cryobiotus klebelsbergi</i>	13.75 [9.32 – 23.04]
<i>Dactylobiotus parthenogeneticus</i>	0.52 [0.17 – 0.83]
<i>Hypsibius cf. convergens</i>	8.79 [6.9 – 12.22]
<i>Hypsibius sp.</i>	4.35 [3.56 – 5.36]
<i>Macrobiotus annewintersae</i>	0.62 [0.26 – 0.97]
<i>Macrobiotus ripperi</i>	5.66 [5.22 – 6.18]
<i>Paramacrobiotus fairbanksi</i>	1.59 [1.34 – 1.83]
<i>Paramacrobiotus richtersi</i>	2.34 [1.93 – 2.75]
<i>Ramazzottius sp. Italy</i>	29.73 [14.99 – 59.59]
<i>Ramazzottius sp. Finland</i>	5.45 [4.26 – 7.43]
<i>Richtersius aff. coronifer</i>	9.36 [7.22 – 13.45]

**Table 3**  
Results of PLM.

Parameter	Average	95% C.I.	Bayesian p-value
$\alpha$ Intercept	0.013	[-0.330 – 0.362]	0.944
$\beta$ Mixed months	0.379	[0.069 – 0.678]	0.017
R <sup>2</sup> Conditional	0.448	[0.092 – 0.752]	
R <sup>2</sup> Marginal ( $\alpha + \beta$ )	0.338	[0.018 – 0.636]	
R <sup>2</sup> Phylogeny	0.110	[0.001 – 0.426]	
R <sup>2</sup> Residual	0.552	[0.248 – 0.908]	

with FTC events) had a significant and positive effect on the observed FTC-LD50 (average slope estimate: 0.379; Bayesian p-value: 0.017), as predicted by the hypothesis III.

FTC tolerance shows a negligible phylogenetic signal in Parachela, which does not support the hypothesis IV: the estimated Blomberg's K value was 0.652 with a p-value (against H0: K = 0, based on 1000 randomizations) of 0.145; the estimated Pagels  $\lambda$  was 0.215 with a p-value (against H0:  $\lambda = 0$ , based on Likelihood Ratio test) of 0.767.

#### 4. Discussion

In this study, we show that adaptations to local environmental conditions, specifically the natural occurrence of freeze–thaw cycles (FTC) that are likely shaped by altitude and latitude, could play a crucial role in tardigrade survivability under frequent freeze–thaw cycles. More specifically, we found support for our hypotheses in those tardigrades inhabiting lowlands showed lower survival to FTC when compared to species inhabiting highlands and mountainous regions. Although these minute invertebrates are well known to be resistant to hostile conditions (Guidetti et al., 2012), the possible increase (in frequency) of freeze–thaw events could decrease population size or even eliminate some fragile species inhabiting lacustrine environments in lowland areas.

We obtained good support for the hypothesis I, as we show that survival to FTC varies considerably between species. Guidetti et al. (2011a) suggested that the survival to freezing is linked to local adaptation to specific habitats (moss vs. lichen vs. freshwater sediment). We observed a more diversified response of typical aquatic species to cooling under the current experimental set-up as compared to Guidetti et al. (2011a). The response of the freshwater genus *Dactylobiotus* collected from a temperate pond corroborated the findings of Guidetti et al. (2011a) - the specimens died after one freezing of the medium. However, *Cryobiotus klebelsbergi*, inhabiting cryoconite holes on glaciers (permanently water-filled reservoirs during summer) and *Ramazzottius sp.* inhabiting rock-pools in mountains (ephemeral freshwater bodies) coped very well with repeated FTC. These results suggest that in limnic environments, climatic conditions (influenced by latitude and altitude) are key factors explaining the variation in tardigrade response to FTC corroborating hypothesis II. The geographic origin of the species could be a crucial factor controlling the adaptation to sub-zero temperatures. However, our study does not provide sufficient answer since we used only one cosmopolitan tardigrade species, *P. fairbanksi*, which survivability was lower comparing to mountainous species.

Snow is a unique habitat for invertebrates since it is ephemeral and borderline between aquatic and terrestrial ecosystems. Snow maintains a film of water on its surface when temperature is above zero, providing a colonizable habitat for microinvertebrates (Ono et al., 2021). In this study, we used tardigrades inhabiting green algae blooming on snow in

Japan, as well as tardigrades found among organic particles on snow in a spruce forest in Finland. Tardigrades collected from snow showed intermediate tolerance between the well-adapted – highlands and mountain – and FTC-sensitive – lowland – species. This effect is most likely related to the abiotic features of snow habitats, which are themselves somewhat intermediate between highland and lowland aquatic ecosystems.

The most fragile limnoterrestrial taxa were *Dactylobiotus parthenogeneticus* collected from freshwater sediment and *Macrobotus annewintersae* collected from leaf litter. By living in the sediment on the bottom of ponds and lakes, *D. parthenogeneticus* is naturally buffered from extreme temperature fluctuations and FTC, whereas *M. annewintersae* comes from a geographic area (Mississippi, United States) where FTC are naturally very rare. However, due to ever-more common climatic shifts, the occurrence of events such as FTC in new geographical areas could happen (Bartlein et al., 1998; Vieira et al., 2003; Seager et al., 2010), and pose a serious threat to the survival of both taxa.

Although the surface of peatlands is open and subject to periodic freezing, contrary to our expectations, *Adropion scoticum* was sensitive to repeated FTC. Even widespread species such as *Paramacrobotus fairbanksi* that inhabit various latitudes and altitudes (Kaczmarek et al., 2020; Stec et al., 2020a; Stec et al., 2020b) unexpectedly exhibited low FTC tolerance. The reduced survival to FTC of both aquatic and limnoterrestrial taxa is consistent with the hypothesis III. However, the existence of resting/overwintering eggs (Altiero et al., 2010; Guidetti et al., 2011b) of these species that may survive FTC in nature cannot be excluded. Moreover, avoiding a rapid cooling by migration into the deeper layers of bryophytes could be another adaptation that would allow the persistence of population of these tardigrade species despite FTC (Nelson & Adkins, 2001; Degma et al., 2011).

Our study, due to a low sample size, failed to provide a conclusive answer regarding hypothesis IV regarding a possible link between phylogenetic relations between species representing order Parachela and tolerance adaptation to FTC, even though there is an indication of a small phylogenetic signal. Even congeneric tardigrades had different FTC tolerance (Fig. 1). Moreover, *M. ripperi* collected from Finland was much better adapted to cooling than *M. annewintersae* from southern USA. These two regions differ in latitude and climate.

The ability to withstand freezing was suggested to be related to the ability to withstand desiccation (Guidetti et al., 2011a). We used a wide range of tardigrade taxa inhabiting different environments, which can be subject to both freezing and drying. Indeed, the representatives of *Ramazzottius* species can effectively withstand desiccation (Horikawa et al., 2008; Yagi-Utsumi et al., 2021) and *Ramazzottius* sp. from Italian rock pools was very tolerant to FTC. Previous studies revealed that genus *Ramazzottius* can cope with temperature as low as 10 mK (Lee et al., 2022). Although data on desiccation tolerance for the *Paramacrobotus* or *Macrobotus* species used in this study are unknown, certain species belonging to these genera are known to withstand desiccation (Cesari et al., 2012; Cesari et al., 2022). Representatives of these genera in our study had an unexpectedly low tolerance to FTC. *Cryobiotus klebelsbergi* from cryoconite holes withstands desiccation, only in low temperatures (Zawierucha et al., 2019a) and successfully tolerates FTC as typical psychrophile (Dastyeh et al., 2003; Zawierucha et al., 2019a). Although the temperature of the glacier surface oscillates around 0 °C during summer (Zawierucha et al., 2019b), the repeating FTCs on the ice surface are common diurnally (Fountain et al., 2008; Gardner, 2020), and can be more frequent in mountainous cryospheric habitats in the early spring due to early snowmelt (Vorkauf et al., 2021; Yang et al., 2022). These events could negatively affect glacier metazoans communities in the future.

Sieger et al. (2022) found that the time spent frozen from repeating cooling events did increase the lifespan of the limnoterrestrial tardigrade *Milnesium inceptum* compared to controls. However, the survival after each repeating freezing event also shows a decrease despite a

longer recovery and frozen state periods compared to the current study (7 days vs 24 h). Tardigrade lifespan has been recorded in laboratory conditions for only a handful of species (varying from tens to almost 266 days in permanently active animals; Altiero & Rebecchi 2001; Stone & Vasanathan 2020), and the data are scarcer still for the lifespan of tardigrades entering cryptobiosis (Nagwani et al., 2022; Sieger et al., 2022). Nevertheless, we can assume that higher frequency of FTC could have a stronger negative effect on tardigrade lifespan compared to one prolonged freezing or cooling period. However, this assumption requires further and targeted research.

## 5. Conclusions

Tardigrades are ubiquitous invertebrates, sometimes dominating polar or glacial ecosystems, where they play an important role as consumers and food in their habitats, finally contributing to nutrient cycling (Hohberg & Traunspurger, 2005; Convey & McInnes, 2005; Novotná Jaroměřská et al., 2021). Even though high mountain tardigrades successfully survived FTC, freeze–thaw events of their medium negatively affected their survivability, which may have an impact on the ecosystem functioning of temperate, boreal, and polar ecosystems. Thus, tardigrades could serve as models for studies on the effects of FTC on microscopic metazoans and changes of soil and freshwater ecosystems in lowland and mountainous regions. The mortality of tardigrades associated with FTC could be an indicator of the changes in future taxonomic diversity. For example, greater incidence of FTC as well as higher temperatures could lead to the disappearance of other invertebrate specialists coexisting in these ecosystems, such as nematodes or rotifers, since these share similar physiological capabilities with tardigrades.

## 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.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ecolind.2023.110460>.

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