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RESEARCH ARTICLE

Effect of repeated freeze–thaw cycles on geographically different populations of the freeze-tolerant worm *Enchytraeus albidus* (Oligochaeta)

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ABSTRACT

Freeze-tolerant organisms survive internal ice formation; however, the adaptations to repeated freeze–thaw cycles are often not well investigated. Here we report how three geographically different populations of *Enchytraeus albidus* (Germany, Iceland and Svalbard) respond to three temperature treatments – constant thawed (0°C), constant freezing (−5°C) and fluctuating temperature (0 to −5°C) – over a period of 42 days. Survival varied between treatments and populations such that enchytraeids from arctic locations had a higher survival following prolonged freeze periods compared with temperate populations. However, enchytraeids from temperate locations had the same survival rate as arctic populations when exposed to repeated freeze–thaw events. Across all populations, metabolic rate decreased markedly in frozen animals (−5°C) compared with thawed controls (0°C). This decrease is likely due to the lower temperature of frozen animals, but also to the transition to the frozen state per se. Animals exposed to repeated freeze–thaw events had an intermediate metabolic rate and freeze–thaw events were not associated with pronounced excess energetic costs. Overwintering under either condition was not associated with a decrease in lipid content; however, during exposure to constant freezing and repeated freeze–thaw events there was a noticeable decrease in carbohydrate stores over time. Thus, animals exposed to constant freezing showed a decrease in glycogen stores, while both glucose and glycogen content decreased over time when the organisms were exposed to repeated freezing. The results therefore suggest that carbohydrate resources are important as a fuel for *E. albidus* during freezing whereas lipid resources are of marginal importance.

KEY WORDS: Freeze–thaw cycles, Glucose, Glycogen, Metabolic rate, Cold tolerance

INTRODUCTION

Many cold-tolerant invertebrate species, including several insects and annelids, are known to survive extensive ice formation within their body fluids (Block, 1982; Ramløv, 2000; Holmstrup, 2003; Sinclair et al., 2003; Slotsbo et al., 2008). An oligochaete representative of such freeze-tolerant organisms is the enchytraeid *Enchytraeus albidus* (Henle 1837), which inhabits shorelines and organically rich soils of northern Europe, Greenland and Svalbard. *Enchytraeus albidus* remains near the surface of soils and in rotting seaweed on beaches year round where it plays an important role in the decomposition of dead organic material (Didden, 1993; Laurén et al., 2012). Because this species does not seek thermal refuge during cold periods it is highly exposed to fluctuating temperatures including repeated freeze–thaw events.

Although many animal species are able to survive freezing, it is clear that the transition between the frozen and unfrozen states can have profound physiological consequences. These changes include loss of mobility, loss of internal convective transport, synthesis of cryoprotectants and antifreeze proteins to protect cells and inhibit recrystallization, and establishment of metabolic depression that may be important to reduce the winter energy consumption (Ramløv, 2000; Bale, 2002; Calderon et al., 2009; Marshall and Sinclair, 2012b). Oligochaetes, including *E. albidus*, are no exception to these generalized responses. Thus, freeze-tolerant oligochaetes are characterised by a large build-up of glycogen reserves prior to winter, a large accumulation of cryoprotectants following freezing and a (moderate) metabolic depression during the frozen period (Berman and Leirikh, 1985; Holmstrup et al., 1999; Rasmussen and Holmstrup, 2002; Holmstrup and Overgaard, 2007; Holmstrup et al., 2007; Overgaard et al., 2007; Slotsbo et al., 2008; Calderon et al., 2009; Overgaard et al., 2009; Silva et al., 2013; Fisker et al., 2014). The relationship between winter survival and accumulation of glycogen and glucose is, however, more complex than first assumed, as accumulated glucose can serve both as a cryoprotectant and as a fermentable fuel (Calderon et al., 2009; Fisker et al., 2014).

Despite an impressive array of adaptations to overcome freezing, freeze-tolerant organisms may still experience physical damage during freezing (particularly if freezing is severe, rapid or prolonged) (Bale, 2002; Lee, 2010; Marshall and Sinclair, 2012a). Furthermore, in some freeze-tolerant animals it has been found that the freeze–thaw events carry energetic costs associated with re-establishment of osmotic and ionic gradients, and re-synthesis of damaged proteins and cryoprotectants (Churchill and Storey, 1989; Marshall and Sinclair, 2011; Sinclair et al., 2013). Accordingly, the damage and energetic costs are expected to be especially great during repeated freeze–thaw events. For example, it was reported that energetic consumption was increased during freezing and following thawing in the wood frog (*Rana sylvatica*) (Sinclair et al., 2013), and for the woolly bear caterpillar (*Pyrrharctia isabella*) it was found that the energetic costs of repeated freeze–thaw events outweighs the stress of prolonged freezing (Marshall and Sinclair, 2011). Nonetheless, it is possible that some freeze-tolerant species and/or populations may be well adapted to repeated freeze-thaw events, especially those from temperate habitats (Sinclair et al., 2003; Sinclair and Chown, 2005).

Enchytraeus albidus has a wide distribution covering both temperate and arctic climates (Table 1). Different populations are

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Table 1. Selected climatic characteristics of the locations from which the enchytraeids were collected (data from www.worldclimate.com)

Country/ID	Location	Coordinates	Average temperature (°C)	Average temperature of coldest month (°C)[a]	Sub-zero degree months[b]
Germany	Germany	53°32.992′N, 8°34.983′E	9.5	1.5	0
Iceland	Mosfellsbær	64°10.525′N, 21°43.393′W	4.6	–0.3	–0.3
Svalbard	Svalbard	78°13.115′N, 15°38.925′E	–6.4	–15.9	–90.7

[a]24 h average temperature of the coldest month of the year.
[b]Sum of the 24 h average temperatures of all months where this temperature was below 0°C. This provides an index of severity and duration of the winter at the location.

therefore likely to be locally adapted to different scenarios of daily, seasonally and annual temperature variation (Sinclair et al., 2003; Sinclair and Chown, 2005). Earlier studies have demonstrated that such differences have led to local adaptation in the freeze tolerance of oligochaetes, where arctic populations are more freeze tolerant than their sister populations from more temperate locations (Rasmussen and Holmstrup, 2002; Holmstrup et al., 2007; Slotsbo et al., 2008; Fisker et al., 2014). These differences are manifested with respect to both the lowest tolerable temperature and the duration of freezing that the populations can endure. Worms experiencing different microclimate conditions during the winter will be exposed not only to differences in the duration and temperature of the frozen period, but also to different occurrences of freeze–thaw events. However, for oligochaetes, little is known regarding the fitness or energetic cost of freeze–thaw cycles, nor is it known whether there are local adaptations in populations' ability to tolerate repeated freeze–thaw events.

The aim of the present study was to examine the effect of repeated freeze–thaw cycles on *E. albidus*. This was explored by exposing three geographically distinct populations (Germany, Iceland and Svalbard) to three different temperature treatments: constant thawed (0°C), constant freezing (−5°C) and fluctuating temperature (0 to −5°C). The different populations were followed over a period of 42 days while measuring mortality, glucose, glycogen and lipid content, as well as metabolic rate (MR) to test the following hypotheses: (1) repeated freezing and thawing is energetically more costly to *E. albidus* than constant conditions; (2) enchytraeids from temperate locations are better adapted than arctic populations to repeated freeze–thaw cycles; (3) enchytraeids from arctic locations are better adapted than temperate populations to prolonged freeze periods, and (4) the energy used for metabolism during freezing is primarily produced from the available glucose, and not from lipid stores.

RESULTS

Freeze mortality

Survival of the three *E. albidus* populations was clearly dependent on temperature treatment. Thus, survival varied significantly between temperature treatments (0°C, −5°C and fluctuating; chi-square test, $P<0.001$). All populations had practically no mortality when exposed to a constant benign temperature (0°C; Fig. 1A). However, mortality was observed when the populations were exposed to the constant freezing treatment at −5°C (Fig. 1B), and under this condition mortality developed faster in the Germany population compared with populations from Iceland or Svalbard. Thus, there was a significantly lower survival in the Germany population compared with the Iceland and Svalbard populations following 22.5 days at −5°C (chi-square test, $P<0.001$). The variation in survival between populations was smaller when the populations were exposed to fluctuating temperatures (repeated exposure to 0 and −5°C; Fig. 1C) such that there were no significant differences in mortality between populations when tested after exposure to fluctuating temperatures for either 21.5 or 41.5 days (~14 and 25 days as frozen, respectively; chi-square test, day 21.5: $P=0.16$; day 41.5: $P=0.40$).

Metabolic rate

Mass-corrected MR was estimated from the rate of CO_2 production ($\dot{V}_{CO_2}$). The MR of *E. albidus* measured at the constant benign temperature (0°C) varied between 150 and 275 µl CO_2 h^{-1} g^{-1} dry mass (DM) (Fig. 2). We found significant differences associated with time, which were manifested as an increase in MR over time (linear regression; $P<0.01$ for all populations) and an overall difference between populations (one-way ANOVA on ranks, $P<0.01$), where the Svalbard population had 15% higher metabolic rate than the other populations. The MR of frozen animals (−5°C) was much lower than that of thawed and fluctuating animals, ranging from 25 to 60 µl CO_2 h^{-1} g^{-1} DM (Fig. 3), resulting in an apparent thermal dependency of MR over a 10°C span (Q_{10}) of 41-, 16- and 18-fold for the Germany, Iceland and Svalbard populations, respectively (note, however, that this temperature change also represents a transition from the thawed to the frozen state). MR did not change significantly over the duration of the frozen period at constant −5°C (linear regression), but the Germany population had a CO_2 production rate that was ~38% lower than that of the other populations (one-way ANOVA on ranks; $P<0.05$). Repeated freezing and thawing (0 to −5°C) resulted in a MR that fluctuated between values similar to those reported under constant conditions (Fig. 4). For the fluctuating treatment, the average MR was calculated across the different freeze–thaw cycles. In cases where measurements were absent, we estimated the MR (Fig. 4, hatched areas). This estimation was performed to avoid bias associated with particular time points missing and to have two complete freeze–thaw cycles from which to average the MR during freeze–thaw events. At days 2–4, three measurements were missing at the beginning and we assumed that the MR was the same as during the following three measurements. In other cases where measurements were absent, the MR was estimated as an average of those preceding and following the lacking measurement. Because the use of intermittent closed respirometry does not allow for a fine time resolution, we cannot quantify the specific costs of freezing and thawing, but instead we explored whether there were additional costs associated with freeze–thaw exposure by testing whether the average metabolic cost during the freeze–thaw cycle was different from a weighted average of the $\dot{V}_{CO_2}$ measured at 0°C and −5°C, respectively (Table 2). This weighted 'average metabolic rate' used the simple assumption that MR is an average of $\dot{V}_{CO_2}$ from frozen (−5°C) and unfrozen (0°C) animals, and here we assumed that animals were frozen 60% of the time and thawed for 40% of the time, as this approximates the time where the enchytraeids should be frozen during the fluctuating conditions (we added a small ice crystal

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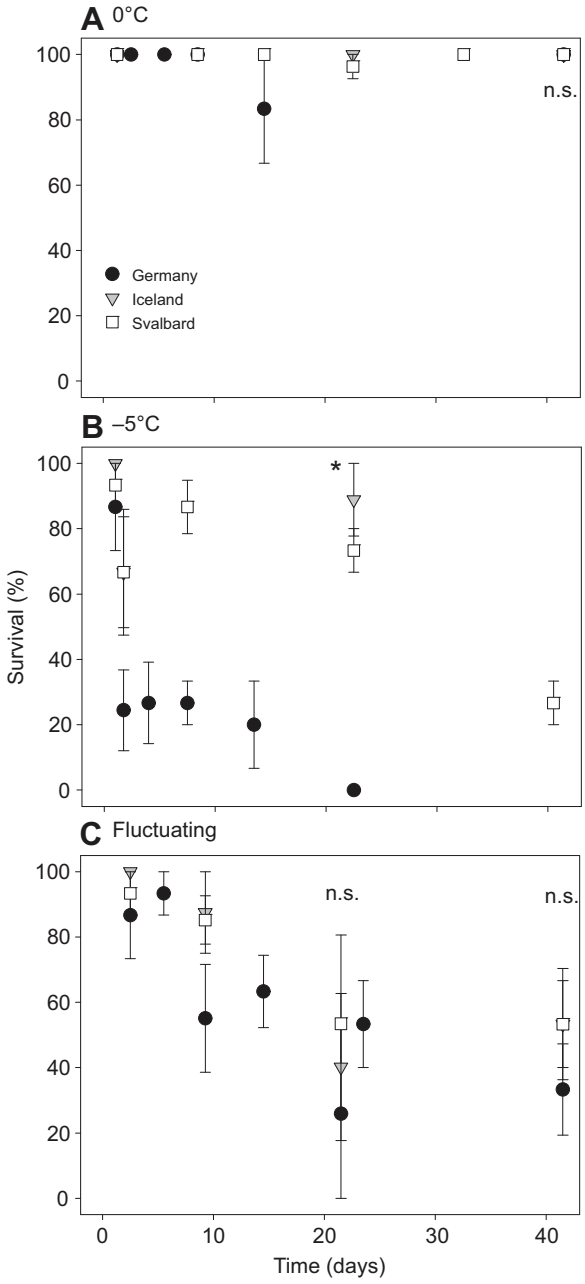

Fig. 1. Survival (mean ± s.e.m., *N*=3–5) of *Enchytraeus albidus* exposed to three different experimental temperature treatments. (A) Constant thawed (0°C); (B) constant freezing (−5°C); (C) fluctuating temperature (0 to −5°C). Significant differences between populations at specific time points are marked with asterisks and non-significant differences are indicated by n.s. (all populations were sampled at these time points).

each time the temperature reached −1.5±0.3°C). The outcome of this analysis revealed a significant interaction between treatment (estimated and measured) and population (P<0.05; Table 2). The measured values in all populations were higher than the estimated values. This was, however, only significant for the Iceland population (difference ~23%), suggesting that the weighted average was a good estimate of $\dot{V}_{CO_2}$ under fluctuating conditions. In other words, the MR measured during freeze–thaw cycles was only slightly elevated above the estimate, which assumes that the animals switch between thawed and frozen states with no major costs associated with these transitions.

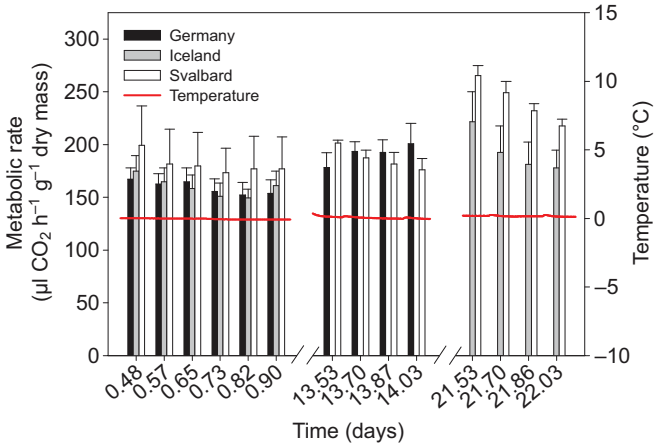

Fig. 2. Mass-corrected metabolic rate (mean ± s.e.m., *N*=3) of *E. albidus* estimated from CO_2 production at a constant thawed temperature (0°C). The metabolic rate was measured during three periods (start, 13 days and 21 days) of the experiment (the metabolic rate was measured in different replicates during the three different periods). Red lines show the observed temperature during the measurements.

Glucose accumulation, glycogen reserves and total lipid content

Initial glucose levels were generally low in unfrozen *E. albidus* (+2°C) but the levels varied significantly between populations (one-way ANOVA, $F_{2,11}$=6.967, P<0.05; Table 3), such that the Svalbard population had a significantly higher glucose level in the unfrozen state compared with the other populations. There was also a significant difference in initial glycogen reserves (+2°C) between the three populations (one-way ANOVA, $F_{2,12}$=94.838, P<0.001; Table 3), where the Germany population had the lowest glycogen content (55.3 µg mg^{-1} DM) and the Svalbard population the highest (133.7 µg mg^{-1} DM). The initial lipid content (215.8 to 281.5 µg mg^{-1} DM) did not vary significantly between populations (Table 3).

During the experiments, glucose levels in unfrozen animals varied significantly in the Svalbard population, with a small increase in the glucose levels over time, while it remained stable in the two other populations (linear regression, P<0.05; Fig. 5A). Frozen worms maintained at constant −5°C rapidly accumulated large amounts of glucose (60–110 µg mg^{-1} DM; Fig. 5B), but there were no significant decreases or increases in the glucose concentration over time. Glucose

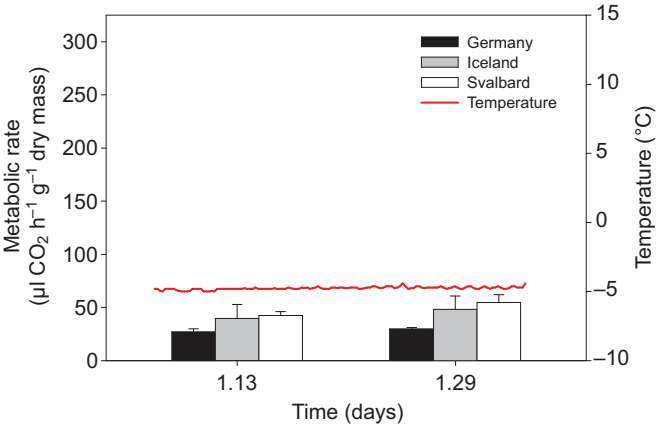

Fig. 3. Mass-corrected metabolic rate (CO_2 production rate; mean ± s.e.m., *N*=2–3) of *E. albidus* during freezing (−5°C). Red line shows the temperature during the measurements.

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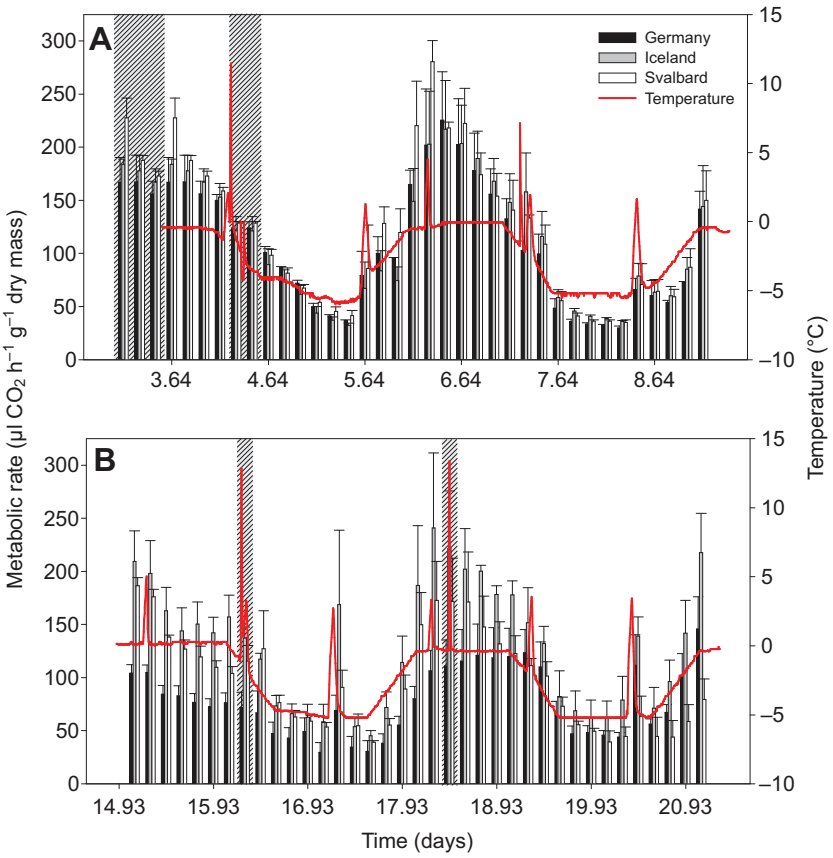

Fig. 4. Mass-corrected metabolic rate of *E. albidus* (mean ± s.e.m., *N*=3) was estimated from CO_2 production rate at fluctuating temperatures (0 to −5°C). The metabolic rate was measured (A) from day 3.5 to day 9 (cycles 2–4) and (B) from day 14 to day 21 (cycles 6–8) during exposure to repeated freeze–thaw cycles (metabolic rate was measured in different replicates during the two different periods). Values based on calculated estimates are marked as hatched (see Materials and methods for further explanation). Red lines show the observed temperature during the measurements (sudden short-term deviations in temperature were due to random defrosting events of the cooling cabinet).

also accumulated in animals exposed to fluctuating temperatures, but here values were generally lower (20–60 µg mg^{-1} DM) and the levels of accumulated glucose during freezing decreased significantly over time in all populations (Fig. 5C).

There was no consistent tendency for glycogen levels to decrease with time when animals were maintained at constant 0°C, and levels even increased significantly in the Germany and Iceland populations (Fig. 5D). During constant exposure to −5°C there was a significant decrease in glycogen over time in the Svalbard population (linear regression, P<0.05; Fig. 5E). The glycogen reserves also decreased significantly over time in all populations when exposed to the fluctuating temperature treatment (linear regression, P<0.05; Fig. 5F). After 41.5 days, the glycogen reserves ended up as low as 30–60 µg mg^{-1} DM when the populations were exposed to the fluctuating treatment.

The total lipid content was relatively stable in all populations and treatments (Fig. 5G–I), although it did decrease slightly (but significantly) with time in the Svalbard population exposed to the constant thawed treatment (linear regression, P<0.05).

DISCUSSION

Freeze mortality

Terrestrial and littoral habitats are exposed to both daily and seasonal changes in temperature, and in temperate as well as arctic regions such changes may involve repeated freeze–thaw events (e.g. Coulson et al., 1995; Irwin and Lee, 2003; Calderon et al., 2009; Coulson, 2013; Sinclair et al., 2013). Freeze–thaw events may entail energetic costs as the re-establishment of osmotic and ionic gradients after freezing could be costly. Further effects such as re-synthesis of damaged proteins and the repeated synthesis and reconversion of cryoprotectants could also be energetically costly (Churchill and Storey, 1989; Irwin et al., 2003; Marshall and Sinclair, 2011; Sinclair et al., 2013). If there are particular costs associated with the transition to and from the frozen state, then these

Table 2. Average CO_2 production of *Enchytraeus albidius* estimated from measured values

Treatment	CO_2 production (µl CO_2 h^{-1} g^{-1} dry mass)		
	Germany	Iceland	Svalbard
Constant thawed (0°C)	172.2±3.5^{a,*}	173.3±0.9^{a,*}	200.0±1.5^{b,*}
Constant freezing (−5°C)	28.6±0.7^{a,*}	41.9±7.6^{a,b,*}	50.6±3.1^{b*}
Measured freeze–thaw (0 to −5°C)	94.0±8.4^{a}	122.5±5.8^{b,‡}	111.1±4.5^{a,b}
Estimated freeze–thaw [60% frozen (−5°C)–40% thawed (0°C)]	86.0±1.1^{a}	94.5±4.6^{a,b,‡}	110.3±1.5^{b}

Constant thawed values were averaged over three periods of measurements, constant freezing values were averaged over one period, and fluctuating values were averaged over measurement periods that each covered two freeze–thaw cycles. Estimated values were calculated from constant thawed and freezing treatments in the stated proportions. Differences between constant thawed and constant freezing were tested with a two-way ANOVA (Tukey's *post hoc* test) and significant effect of treatment within populations is marked with asterisks (*P<0.05). A similar test was used to test for differences between measured freeze–thaw and estimated freeze–thaw, and significant effect of treatment within populations is marked with a double dagger (‡P<0.05). Different superscripted letters indicate significant differences between populations (P<0.05). Data are means ± s.e.m.

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Table 3. Initial level of glucose, glycogen and total lipid content in the three populations of *E. albidius*

	Glucose (μl CO_2 h^{-1} g^{-1} dry mass)	Glycogen (μl CO_2 h^{-1} g^{-1} dry mass)	Total lipid (μl CO_2 h^{-1} g^{-1} dry mass)
Germany	1.5±0.5[a]	55.3±3.0[a]	281.5±20.8[a]
Iceland	1.9±0.4[a]	72.1±4.8[b]	218.5±18.1[a]
Svalbard	4.0±0.7[b]	133.7±4.7[c]	215.8±24.3[a]

Significant differences (one-way ANOVA, Tukey's *post hoc* test, $P<0.05$) among glucose, glycogen or total lipid content between populations are indicated with different superscripted letters. Data are means ± s.e.m., N=4–5.

costs would be expected to accumulate under repeated freeze–thaw events. Accordingly, it has been suggested that organisms that experience freeze–thaw cycles on a regular basis in their habitat are likely to be well adapted to repeated freeze periods, whereas organisms that only experience prolonged freeze periods will be better adapted to long-term freezing (Sinclair et al., 2003; Sinclair and Chown, 2005).

The putative benefits or costs of repeated freeze exposure have previously been investigated in the insects *Pyrrharctia isabella* and *Pringleophaga marioni* (Sinclair and Chown, 2005; Marshall and Sinclair, 2011), and this issue has also been discussed with regard to the energetic cost of freeze–thaw cycles in freeze-tolerant frogs (Sinclair et al., 2013). The present study extends this line of research by examining a similar problem in the annelid *E. albidus*. Importantly, however, the present study also examines the putative importance of local adaptation as we compare populations of the same species that originate from habitats expected to experience varying degrees and durations of freezing as well as possible differences in the frequency of freeze–thaw events. We have previously established that the populations from Svalbard, Iceland and Germany vary greatly with respect to long-term freeze tolerance (Fisker et al., 2014). A similar observation was also made in the present study where the Germany population succumbed after 22.5 days of exposure to −5°C while worms from both Iceland and Svalbard had relatively high survival rates at the same time point (70–90%). The present study therefore supports the hypothesis that the temperate population is less well adapted to constant freezing compared with the two arctic populations. Interestingly, at fluctuating temperatures there was no significant difference between population survival rate after either 21.5 or 41.5 days (all populations had a survival of 25 to 50%). By the end of the freeze–thaw exposure (41.5 days), the enchytraeids had been frozen for a total of ~25 days (i.e. 60% of the time). Enchytraeids from arctic locations are therefore better adapted to prolonged freezing periods compared with temperate populations, while there seems to be little difference in the populations' ability to tolerate repeated freezing and thawing events. Unfortunately, we do not have good records of microclimatic thermal conditions from the origin of the populations, and are therefore unable to assess whether freeze–thaw events are more pronounced in the more temperate or the more arctic climates (Table 1). However, for the present study, it is our working hypothesis that freeze–thaw events are at least as frequent in the more mild winter temperate conditions as in the colder arctic areas, where temperatures are often found to remain below 0°C for extended periods (Calderon et al., 2009).

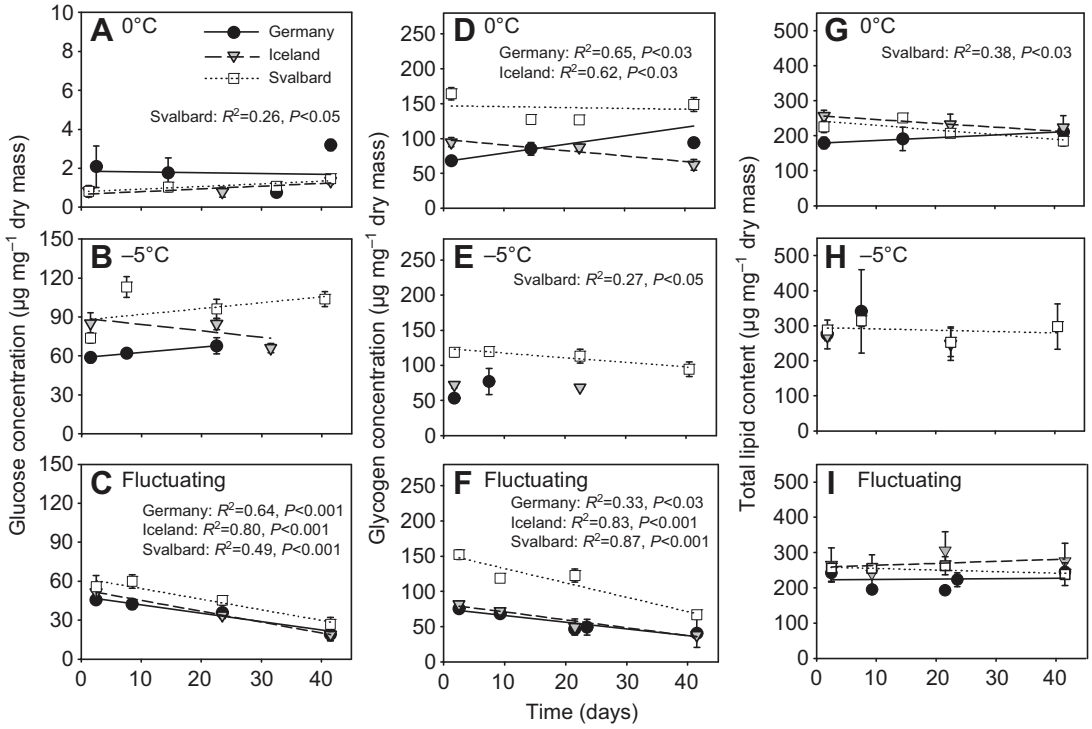

Fig. 5. Glucose accumulation, glycogen reserves and total lipid content (mean ± s.e.m., N=1–5) in different populations of *E. albidus* during exposure to different cold treatments. (A–C) Glucose accumulation during exposure to (A) constant thawed (0°C), (B) constant freezing (−5°C) and (C) fluctuating (0 to −5°C) temperature treatments. (D–F) Glycogen reserves during exposure to (D) 0°C, (E) −5°C and (F) 0 to −5°C. (G–I) Total lipid content during exposure to (G) 0°C, (H) −5°C and (I) 0 to −5°C. Lines show linear regressions (see supplementary material Table S2 for detailed statistics).

To our knowledge, this is the first study that has directly tested for population differences in response to freezing and thawing cycles, although some previous studies have focused on variation in population responses to fluctuating temperatures on species that do not freeze (Ragland and Kingsolver, 2008; Kingsolver et al., 2009; Cooper et al., 2012; Marshall and Sinclair, 2012a). As reviewed by Marshall and Sinclair (Marshall and Sinclair, 2012a), the interpretation of the results of repeated cold exposure is often dependent on experimental design and therefore often difficult to compare across studies. This is also the case for comparisons across populations with regard to repeated cold exposure. Thus, Kingsolver et al. (Kingsolver et al., 2009) found that a field population of *Manduca sexta*, which usually experiences fluctuating temperatures, performed less well under constant thermal conditions than a laboratory population maintained for 250 generations at a constant temperature, whereas under daily temperature fluctuations the field and laboratory populations performed similarly. By contrast, a study of *Drosophila melanogaster* lines raised under contrasting temperatures (including fluctuating) indicated that selection favoured plasticity of thermal tolerances equally between populations (Cooper et al., 2012). The variation in these findings underlines the need to gain a better understanding of the geographic variation in physiological responses to fluctuating temperatures in general, including local adaptation to freeze–thaw events. In the present study, we found evidence for local adaptation of *E. albidus* populations, but this is related primarily to differences associated with prolonged or severe cold exposures and not to the tolerance to repeated freeze–thaw cycles, which was similar between populations. An alternate interpretation of this result is that the strong differences found in freeze resistance between temperate and arctic populations of oligochaetes (Rasmussen and Holmstrup, 2002; Holmstrup et al., 2007; Slotsbo et al., 2008; Fisker et al., 2014) are not manifested in adaptations to repeated freeze–thaw events, possibly because the occurrence of such events is more similar between these environments.

Energetic costs of constant freezing and repeated freezing

Metabolic rate (measured as CO_2 production rate) increased slightly with time in the *E. albidus* exposed to a constant thawed temperature at 0°C. However, there was no difference between populations and the MR was also similar to that measured in a previous study (~175 µl CO_2 h^{-1} g^{-1} DM) (Fisker et al., 2014). Fisker et al. (Fisker et al., 2014) reported that MR was reduced by almost 50% when the organisms were frozen at −2°C (60–85 µl CO_2 h^{-1} g^{-1} DM) as compared with unfrozen animals at the same temperature (120–140 µl CO_2 h^{-1} g^{-1} DM). The depression varied between populations (Svalbard and Germany), where the Germany population had the smallest metabolic depression associated with freezing. In the present study we found an even lower MR at −5°C (25–60 µl CO_2 h^{-1} g^{-1} DM), but surprisingly the MR of the Germany population was lowest under these conditions compared with the other populations. The high thermal dependency found over this small temperature interval (apparent thermal dependency of MR over a 10°C span of 41-, 16- and 18-fold for the Germany, Iceland and Svalbard populations, respectively) indicates that some form of metabolic depression/quiescence has taken place during freezing, which could be due to cellular dehydration and/or immobilization of the animals. Moreover, the results indicate that the dynamics of a freeze-induced metabolic depression differs between populations. An alternate explanation for the low measurements of $\dot{V}_{CO_2}$ during freezing could be related to increased reliance on anaerobic metabolism. Interestingly, however, we found no suggestions of anaerobic metabolism during freezing. Firstly, we performed a range of pilot experiments that qualitatively demonstrated that metabolic rate was aerobic during freezing (see supplementary material Fig. S1, Table S1) (Fisker et al., 2014). Secondly, the measurements during fluctuating conditions also indicate that metabolism is aerobic. Thus, we observed that $\dot{V}_{CO_2}$ fluctuated between the values reported under constant conditions and any anaerobic metabolism would likely result in acidification, which would titrate the animals' endogenous HCO_3^- stores and then cause an extra release of CO_2 during freezing, while the reverse would be true if the animal's acid–base balance were to be re-established during the thaw period. Previous studies on freeze-tolerant insects and vertebrates have also suggested that metabolism can remain (partially) aerobic during freezing (Voituron et al., 2002; Sinclair et al., 2004; Sinclair et al., 2013), while a study of the freeze-tolerant worm *D. octaedra* indicated that metabolism was a mixture of aerobic and (mostly) anaerobic metabolism (Calderon et al., 2009). In the case of *E. albidus*, the aerobic metabolism is likely to be dependent on the relatively low ice content found in this species at −5°C (Patrício Silva et al., 2013).

Earlier studies on the metabolic costs of freeze–thaw processes in different organisms have yielded contradicting results (e.g. Churchill and Storey, 1989; Sinclair et al., 2004; Sinclair and Chown, 2005; Marshall and Sinclair, 2011; Teets et al., 2011; Sinclair et al., 2013). Some studies measured the actual cost of freezing during the freezing and thawing process, whereas others simply measured the effects of previous freezing on the MR of thawed animals. In invertebrates, Teets et al. (Teets et al., 2011) found a decrease in energy stores when *Belgica antarctica* was exposed to repeated freezing, while Marshall and Sinclair (Marshall and Sinclair, 2011) did not find any change in MR or energy reserves after exposing *P. isabella* to repeated freezing. In frogs, the thawing process has been shown to be more energetically costly than the freezing process (Layne, 2000; Voituron et al., 2009; Sinclair et al., 2013). The temporal resolution of the respirometry system used in the present study does not allow for a detailed analysis of the costs associated with the freezing or thawing process; however, the MR of *E. albidus* exposed to freeze–thaw events fluctuated between values similar to those found under constant conditions at 0 and −5°C, which suggests that large costs of freezing or thawing are absent. As seen in Fig. 4, there were some incidents during which the thermal cabinet reheated, and it is unclear whether these events lead to a complete defrosting of the animals, but if this was the case it would only mean that our conclusions were extraordinarily conservative. Thus, we tested the hypothesis that freeze–thaw events are costly; if these spikes represent an 'additional event', then this would potentially incur extra costs, which we did not find.

As an alternative way to examine whether freeze–thaw cycles were associated with extraordinary costs, we used the simple assumption that the average metabolic expenditure during freezing and thawing should equal a weighted average of that found under the two constant regimes. Specifically, we assumed the MR to be equal to that found at 60% of the time being frozen and 40% of the time being thawed. This approach is based on a simplification that might suffer from a slight underestimation of the 'estimated' metabolic rate because of a 'reverse Jensen's inequality', where a transition to the frozen state elicits a hypo-metabolic state that is greater than a 'normal' exponential temperature–MR relationship. However, a more detailed description of the temperature–MR relationship is needed to control appropriately for this effect. Using this analysis, we found a significant interaction between estimated/measured respiration rate and population. This was

manifested in the Iceland population, where we measured a significant higher CO_2 production compared with that estimated from the 'constant regimes', while there was no difference between estimated and measured costs in the two other populations (Table 2). These results indicate that the costs of repeated freeze–thaw cycles are dependent on population, such that some populations (Iceland) may have a small additional energetic cost of repeated freeze–thaw cycles while others (Germany and Svalbard) do not. The absence of major costs associated with the fluctuating treatment (freeze–thaw events) was backed by analysis of the temporal loss of lipid and glycogen stores (see supplementary material Fig. S2), and a similar conclusion was reached by Marshall and Sinclair (Marshall and Sinclair, 2011) and Sinclair et al. (Sinclair et al., 2004), who also failed to find energetic costs of repeated freezing in the caterpillars *P. isabella* or *P. marioni*. Thus, repeated freeze–thaw events are less costly than being constantly thawed at 0°C, and our results therefore suggest that mild winters may cause quicker energy depletion unless worms are able to feed and replenish their energy resources when exposed to the cold (non-freezing) conditions.

Changes in metabolic energy sources during freezing

Energy reserves are known to be important during winter because the frozen state immobilizes the organism and because *E. albidus* use their glycogen stores to synthesize glucose for cryoprotection during freezing (Slotsbo et al., 2008; Fisker et al., 2014). Further, previous studies (Fisker et al., 2014; Calderon et al., 2009) proposed that the mobilized glucose stores are also important as a fuel when the animals are frozen. An earlier study of freeze-tolerant oligochaetes (*D. octaedra*) found that the ability to survive winter frost correlates with the size of glycogen reserves (Holmstrup et al., 2007), but for *E. albidus* the relationship between winter survival and accumulation of glucose is generally weak (Slotsbo et al., 2008; Silva et al., 2013; Fisker et al., 2014). Here, we investigated whether the energy used for metabolism during the different treatments is primarily correlated to catabolism of glucose/glycogen or lipids.

Overall, there were few changes in the proportional lipid content between treatments and populations, suggesting that the rate of lipid catabolism is not particularly affected by the different cold treatments or populations. During constant thawed temperature we found no overall trend in the glycogen reserves or glucose accumulation; however, during constant freezing we found that the glycogen reserves decreased significantly in the Svalbard population while there was no decrease in glucose accumulation. This is a different pattern to that which Calderon et al. (Calderon et al., 2009) found in *D. octaedra*, and suggests that the use of energy reserves during freezing is species specific. For example, it is possible that the reliance on glycolytic metabolism is less profound in *E. albidus* because of the larger reliance on aerobic metabolism during freezing (see above). Nevertheless, the drastic depletion of carbohydrates in the freeze–thaw treatment (most pronounced in the Svalbard population) may be due to the use of glucose for metabolism since the organisms did not feed during the experiment. Another possibility is that the organisms simply leak their cryoprotectants (glucose) because of osmotic challenges occurring during freezing and thawing or that some glucose is lost by urine production, as known from studies of freeze-tolerant frogs (e.g. Layne et al., 1996).

Conclusions

To summarize, we did not find general support for our first hypothesis that repeated freezing and thawing is energetically costly for *E. albidus*. Thus, freeze–thaw events were not associated with pronounced excess energetic costs, although the population from Iceland had a small increase in overall energy consumption when compared with a weighted average estimated from animals kept at constant conditions. We found partial support for our second and third hypotheses investigating whether enchytraeids from temperate locations are primarily adapted to repeated freeze–thaw cycles while enchytraeids from arctic locations are better adapted to prolonged freeze periods. Survival did vary between treatments and populations such that enchytraeids from arctic locations have a higher survival rate at prolonged freeze periods compared with temperate populations. However, enchytraeids from temperate locations have the same survival rate as that of arctic populations when exposed to repeated freeze–thaw events. Finally, our last hypothesis was that the energy used for metabolism during freezing is primarily produced from the available glucose, and not from lipid stores. While there were no consistent changes in the relative lipid, glycogen or glucose contents during constant conditions, we found a decrease in the relative size of the glucose and glycogen stores in worms exposed to fluctuating temperature. This result indicates an increased reliance on carbohydrate energy reserves during the freeze–thaw treatment or alternatively that some of the accumulated glucose is lost during the freeze–thaw transition. Irrespective of the origin of this loss, this result could indicate that repeated freeze–thaw events can lead to a relative depletion of glycogen and/or glucose stores, which are important both as cryoprotectants and as fuel for metabolism during frost exposure.

MATERIALS AND METHODS

Experimental design

The three *Enchytraeus albidus* populations tested originated from Germany, Iceland and Svalbard (for details, see Table 1). Founder Iceland and Svalbard populations were collected from decaying seaweed near the seashore at sampling sites. A founder population from Germany was obtained from a commercial supplier (Tierfischfutter, Jena, Germany). Originally these worms were collected from garden compost near Jena, Germany. All populations were maintained at 5°C for at least 6 months so that progeny of the founder populations were used in tests (for more information, see Fisker et al., 2014).

All populations were acclimated to 2°C for 3 months prior to cold exposure and then distributed in 9 ml vials containing moist filter paper (three individuals in each), after which they were randomly placed in one of three custom-made programmable cooling cabinets (accurate to ±0.2°C). The animals were exposed to three different temperature treatments (Fig. 6): (1) constant thawed at 0°C ± 0.2°C; (2) constant freezing at −5°C, where the animals were initially placed at 0°C and within 12 h the temperature was gradually lowered to −5±0.2°C (a small ice crystal was added once the temperature reached −1°C to ensure controlled freezing at a high sub-zero temperature); and (3) freeze–thaw cycle, where the temperature fluctuated sequentially between 0 and −5°C (±0.2°C) over a period of 3 days [0°C for 24 h, cooling period (0 to −5°C) for 12 h, −5°C for 24 h, and heating period (−5 to 0°C) for 12 h]. Every third day (during the cooling period), a small ice crystal was added to ensure controlled freezing (at a temperature of −1 to −2°C). The total number of vials (three individuals in each) was as follows: constant thawed: Germany, 36; Iceland, 15; Svalbard, 33; constant frozen: Germany, 30; Iceland, 15; Svalbard, 27; fluctuating: Germany, 39; Iceland, 24; Svalbard, 30.

All three populations were exposed to the three different treatments, but the duration of the treatment and time points where samples were collected varied according to the total number of available animals and expected tolerance between populations (Fig. 6) (Fisker et al., 2014). Animals from all three populations were sampled until the end of the experiment in all treatments except for the constant frozen treatment, in which organisms from Iceland and Germany were only sampled until day 22.5 (due to mortality and total number of available animals). Survival was assessed after placing the worms in a 2°C room for 48 h, where enchytraeids were considered alive if they reacted to tactile stimuli, had normal locomotor activity, and showed

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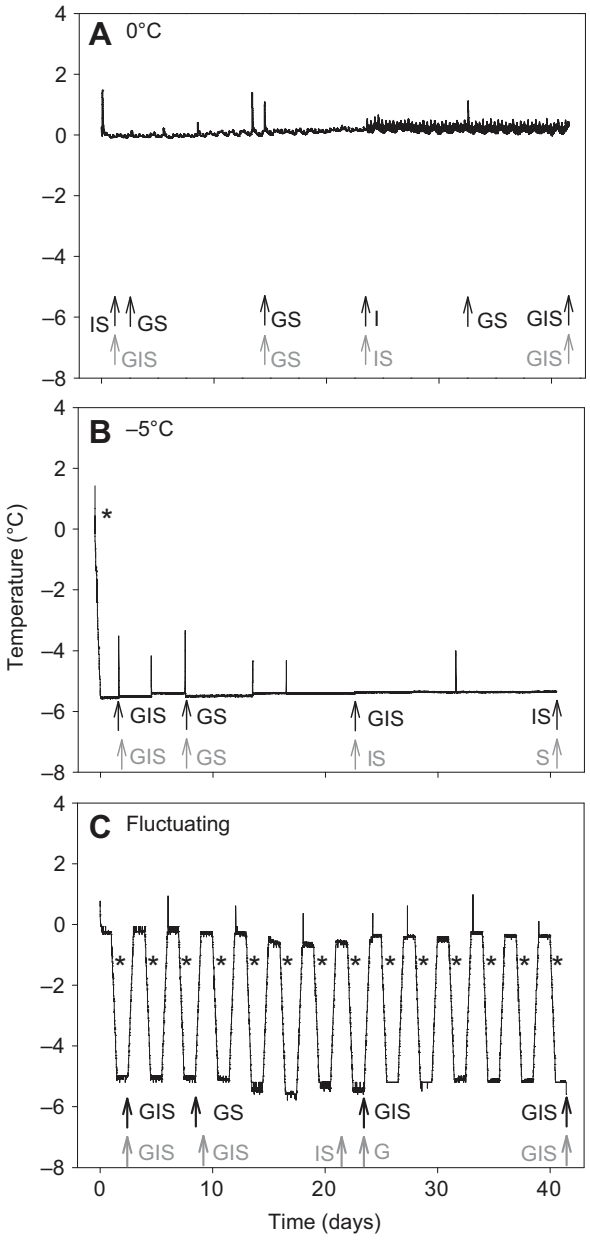

Fig. 6. Data plots of the experimental temperature treatments. (A) Constant thawed (0°C); (B) constant freezing (−5°C); (C) fluctuating (0 to −5°C). The addition of ice crystals to initiate freezing is marked with asterisks. Sampling of worms for glucose (black) and glycogen and lipid (grey) analysis is marked with arrows. G, Germany; I, Iceland; S, Svalbard.

no visible signs of freezing damage (deformations and/or rupture of the skin). The surviving animals from the survival test were subsequently quickly frozen at −80°C and were later used for measurement of total glycogen and total lipid content. In parallel, we sampled animals directly from the three temperature treatments to assess glucose concentrations. These samples were frozen at −80°C within 5 min of collection. In the fluctuating temperature treatment, animals used for glucose determination were sampled just before the increasing temperature period when worms were still frozen (Fig. 6).

Metabolic rate

The MR of *E. albidus* was estimated from the CO_2 production rate ($\dot{V}_{CO_2}$) using intermittent closed respirometry (stop/flow respirometry) while the animals were exposed to one of the three different temperature treatments. The experimental protocol is similar to the one described in Fisker et al. (Fisker et al., 2014). In brief, the protocol sequentially measures CO_2 production in 16 parallel respirometry chambers during exposure to the different experimental treatments such that continuous and independent estimates of $\dot{V}_{CO_2}$ were obtained from each sample every fourth hour. For each measurement we had three replicates per population with each replicate consisting of approximately seven to 10 individuals.

The animals were placed in cylindrical glass chambers (20 mm diameter, 70 mm length) containing a small piece of moistened filter paper to maintain 100% relative humidity. The metabolic chambers were moved to a separate programmable freezer cabinet where the measurements of $\dot{V}_{CO_2}$ took place. Here we measured CO_2 production rate from the CO_2 produced during a closing period using two eight-channel multiplexers (RM Gas Flow Multiplexer, Sable Systems, Las Vegas, NV, USA) to sequentially open and close the 16 respirometry chambers. The chambers were closed for a fixed period (3 h 45 min), after which the chamber air was flushed over a 15 min period such that the CO_2 produced during the closed phase could be quantified. Before the air flow entered the metabolic chambers it passed through a soda lime column to remove atmospheric CO_2. Further, to remove water vapour the air flow passed through a calcium chloride column after leaving the respirometry chamber. Air flow was fixed at a rate of 150 ml min^{-1} using an adjustable mass flow meter (Side-Trak®, Sierra Instruments, Monterey, CA, USA) controlled by a flow controller (MFC 2-channel v. 1.0, Sable Systems), and the fractional CO_2 concentration was measured using a Li-6251 CO_2 analyzer (LI-COR Environmental, Lincoln, NE, USA). Data were sampled every second using a Sable Systems interface (UI-2 Data Acquisition Interface) and ExpeData software (ExpeData-UI2). For all experimental rounds, some respirometry chambers were left empty such that measurements could be corrected for any instrumental leakage or CO_2 diffusion across the tubing. This correction was performed by subtracting the value of the empty chambers from the recorded CO_2 production in the chambers with animals. We generally found little difference between technical replicates, indicating that the experimental system quickly established a steady state. All MRs were calculated on a dry mass basis, measurements of which were obtained from the experimental animals after the measurements were terminated. Further, MR was mass-corrected to a dry mass of 1 mg using the formula:

$$(\dot{V}_{CO_2} / M_c) = (\dot{V}_{CO_2} / M_i) \times \left(M_c / M_i\right)^{b-1}, \quad (1)$$

where $(\dot{V}_{CO_2}/M_c)$ is the mass-corrected CO_2 production rate as it would be at a mass of M_c, $(\dot{V}_{CO_2}/M_i)$ is the uncorrected mass-specific MR of an enchytraeid with mass M_i, and b–1 is the scaling exponent for the mass-specific CO_2 production rate (here we use b–1=−0.25).

In the fluctuating treatment, the frozen state was initiated by adding a small ice crystal to each chamber in the beginning while the temperature was 0°C (during the application of ice, we opened the chambers and therefore there are missing values). Visual inspection clearly confirmed that no individuals were frozen at 0°C even though the ice was added and all animals froze during the temperature decrease to −5°C.

Glucose accumulation

The enchytraeids were freeze-dried for 24 h to obtain dry mass. Glucose was extracted by homogenizing the sample in 0.3 ml of 6% perchloric acid. This was done using a Tissue-Lyser II with a steel bead at 30 Hz for 30 s (Qiagen, Copenhagen, Denmark). After homogenizing, the samples were neutralised by adding 79 µl 2 mol l^{-1} K_2CO_3. Directly after the neutralisation samples were put on ice for 10 min and centrifuged at 10,000 ***g*** for 10 min to separate the precipitate from the supernatant. The supernatant was used to measure the glucose concentration spectrophotometrically (Glucose Gluc-DH kit, Diagnostic Systems GmbH, Holzheim, Germany). All concentrations were calculated relative to glucose standards of known concentration that had been subjected to the same extraction procedure as tissue samples.

Total lipid and glycogen content

Lipid and glycogen content were measured on the same animal samples. To obtain dry mass, the animals were freeze-dried for 24 h and a Soxhlet apparatus were used for the analysis of total lipid content. This apparatus consisted of a round-bottomed boiling flask containing petroleum ether

(boiling point 40–60°C) that was heated in an oil bath. This system was connected to an extraction chamber with a bypass sidearm conducting solvent vapor and a siphon arm refluxing the organic solvent. At the top there was a condenser with water as coolant. Each replicate was put in a piece of aluminum foil and weighed, after which it was separately placed inside a small tin container with small holes. Twenty containers at a time were placed inside the extraction chamber and refluxed for ~72 h (>60 rounds of reflux). All samples were left for 1 day in the fume hood to allow most of the petroleum ether to evaporate, after which the samples were dried for 3–4 days at 60°C before the lipid-free samples were re-weighed to the nearest 0.01 mg. The total lipid content was then calculated as fraction of initial mass.

Glycogen content was measured as described by Overgaard et al. (Overgaard et al., 2007). For this analysis, we extracted glycogen in 0.4 ml 1 mol l^{-1} NaOH for 3 h at 75°C, thus we extracted all the glycogen while the free glucose was degraded. Of this extract, 100 µl was transferred to 0.9 ml acetate buffer (0.25 mol l^{-1}, pH 4.75) containing 400 mg l^{-1} amyloglucosidase (EC 3.2.1.3, Sigma-Aldrich Denmark A/S, Copenhagen, Denmark). The sample was placed at 25°C for 1.5 h to ensure that all glycogen was cleaved to glucose. Glycogen was measured spectrophotometrically as glucose units using the method described above. Glycogen content was calculated relative to glycogen standards of known concentration that had been subjected to the same procedure as tissue samples.

Statistical analyses

The survival data were tested at specific time points across the three treatments: 0°C at 41.5 days, −5°C at 22.5 days, and fluctuating at 21.5 and 41.5 days (equals 22 days as frozen). First, within each temperature treatment, a chi-square test compared survival of all populations between treatments. Second, a chi-square test was used to compare population survival among the different treatments.

Changes in MR over time at 0°C and −5°C were tested by linear regression, and one-way ANOVAs were used to test for differences between populations (Kruskal–Wallis one-way ANOVA on ranks was used when data did not conform to the requirements of normality or equal variance). To test whether the average MR of worms exposed to fluctuating temperature was higher than expected, we used a two-way ANOVA [with populations and estimate (measured versus estimated) as factors]. For this test, the estimated MR was assumed to be the weighted average of that measured at −5°C and 0°C (60% of the time assumed to be frozen: $\dot{V}_{CO_2}$ as at −5°C; 40% of the time unfrozen: $\dot{V}_{CO_2}$ as at 0°C).

One-way ANOVA was used to test for differences between populations with regard to the initial content of lipid, glucose and glycogen (+2°C). To detect changes in lipid, glucose and glycogen content over time and between temperature treatments, linear regressions were performed.

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Competing interests

The authors declare no competing financial interests.

Author contributions

K.V.F., J.O. and M.H. designed and conceived the research; K.V.F., H.M. and J.O. performed the experiments; K.V.F. and H.M. described and analyzed the data; K.V.F. and J.O. drafted the manuscript; and all authors revised the manuscript.

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Supplementary material

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