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RESEARCH ARTICLE

Induced cold-tolerance mechanisms depend on duration of acclimation in the chill-sensitive *Folsomia candida* (Collembola)

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SUMMARY

During cold periods ectotherms may improve low temperature tolerance *via* rapid cold hardening (RCH) over a period of hours and/or long-term cold acclimation (LTCA) during days, weeks or months. However, the effect of duration and the major underlying mechanisms of these processes are still not fully understood. In the present study, the molecular and biochemical responses to RCH (1–3 h) and LTCA (1–3 days) and the corresponding benefits to survival were investigated using the chill-sensitive collembolan *Folsomia candida*. We investigated osmolyte accumulation, membrane restructuring and transcription of candidate genes as well as survival benefits in response to RCH and LTCA. RCH induced significant upregulation of targeted genes encoding enzymes related to carbohydrate metabolic pathways and genes encoding small and constitutively expressed heat shock proteins (Hsps), indicating that the animals rely on protein protection from a subset of Hsps during RCH and probably also LTCA. The upregulation of genes involved in carbohydrate metabolic processes initiated during RCH was likely responsible for a transient accumulation of myoinositol during LTCA, which may support the protection of protein and membrane function and structure. Membrane restructuring, composed especially of a significantly increased ratio of unsaturated to saturated phospholipid fatty acids seems to be a mechanism supplementary to activation of Hsps and myoinositol accumulation in LTCA. Thus, the moderate increase in cold shock tolerance conferred by RCH seems to be dominated by effects of Hsps, whereas the substantially better cold tolerance achieved after LTCA is dominated by post-transcriptional processes increasing membrane fluidity and cryoprotectant concentration.

Key words: cold acclimation, cryoprotection, gene expression, heat shock protein, homeoviscous adaptation, membrane phospholipid, rapid cold hardening.

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INTRODUCTION

Low temperature is an important factor determining the fitness, survival and distribution of arthropods (Andrewartha and Birch, 1954). Species of polar and temperate regions will often be exposed to subzero temperatures during cold seasons that may cause freezing of their body fluids. Within these cold-hardy species, two major cold-tolerance strategies – freeze avoidance and freeze tolerance – have evolved that effectively ensure survival (Lee, 2010; Zachariassen, 1985). Species from milder climates often have a low cold tolerance, and can suffer injury or death at temperatures above their supercooling point. These species are termed chilling intolerant or chill sensitive (Lee, 2010).

At low temperatures (but not necessarily subzero), chill-sensitive species are prone to direct chilling injury, which can be associated with cold denaturation of proteins, loss of membrane function and dissipation of trans-membrane gradients of Na^+ and K^+, causing depolarization of the membrane and, ultimately, cell death (Ramløy, 2000; Koštál et al., 2004). However, chilling injury can be partly or fully avoided by preceding cold acclimation of shorter or longer duration. Thus, most species show the capacity to improve cold tolerance by pre-exposure to a non-lethal low temperature either rapidly within minutes to hours [termed rapid cold hardening (RCH)] (Meats, 1973; Lee et al., 1987; Czajka and Lee, 1990; Powell and Bale, 2004; Bahrndorff et al., 2009a; Nyamukondiwa et al., 2011; Teets et al., 2012) and/or during long-term cold acclimation (LTCA) during days, weeks or months. Of these acclimation strategies, RCH is probably of ecological significance for arthropods experiencing considerable diurnal temperature fluctuations in their habitat (Kelty and Lee, 2001), while LTCA might be more related to seasonal adaptations.

Collembola are mainly inhabitants of the soil, which may buffer temperature changes. However, temperature fluctuates considerably in the upper soil layers, and many species of Collembola have the capacity to rapidly cold harden (Worland and Convey, 2001; Bahrndorff et al., 2009a). In the present study, we use the chill-sensitive collembolan *Folsomia candida* Willem 1902 (Isotomidae) as a model organism to investigate the role of duration and temperature in cold acclimation physiological processes. *Folsomia candida* possess RCH capacity, and most likely also cold acclimate on a seasonal basis. However, to date, LTCA capacity and the mechanisms enabling short- and long-term cold acclimation have only been sparsely studied in this organism (de Boer et al., 2010).

The biochemical and molecular mechanisms underlying cold adaptation in chill-sensitive ectotherms remain to be fully elucidated, although it is broadly agreed that they can involve upregulation of heat shock proteins (Hsps), accumulation of cryoprotectants and changes of membrane phospholipid composition (Lee and Denlinger, 2010).

During LTCA, membrane compositional changes are expected to increase membrane fluidity at low temperature and thus reduce perturbation of ion homeostasis, which is imperative for chill tolerance (MacMillan and Sinclair, 2011). However, this process might also occur on much shorter time scales. Thus, some studies suggest that reorganization of membrane phospholipids is involved in RCH (Overgaard et al., 2005; Overgaard et al., 2006; Michaud and Denlinger, 2006; Lee et al., 2006). However, other studies suggest that membrane changes are of little or no importance in RCH and LTCA (MacMillan et al., 2009; Koštál et al., 2011), suggesting species-specific mechanisms.

Chill-sensitive organisms may benefit from the accumulation of cryoprotectants, especially due to their stabilising properties with protein and membrane structures (Carpenter and Crowe, 1988; Crowe et al., 1988). Low or moderate concentrations of cryoprotectants such as glycerol, glucose or sucrose are accumulated in chill-sensitive insects during LTCA and RCH and it has been proposed that they play a role even at low concentrations (Chen et al., 1987; Overgaard et al., 2007; Colinet et al., 2012). *Folsomia candida* accumulates compatible osmolytes such as myoinositol and glucose in response to drought (Bayley and Holmstrup, 1999; Sjursen et al., 2001). It is possible that these osmolytes are also of importance for cold tolerance in this species, as low concentrations of osmolytes seem to expand fully hydrated membranes laterally (Andersen et al., 2011). Such change will allow increased disordering of lipid chains [e.g. increased ratio of the conic phosphatidylethanolamines and unsaturated phospholipid-derived fatty acids (PLFAs)] in membranes of cold-acclimated organisms. Additionally, the expression of genes encoding enzymes involved in the synthesis of these osmolytes (and other presumed cryoprotectants) may be upregulated during cold acclimation.

Hsps function as molecular chaperones and form temporary complexes with unfolded or misfolded polypeptides. In their interaction with other Hsps, these proteins protect cytoskeleton, cell structure and function. Hence, Hsps can halt or prevent irreversible protein denaturation induced by various biotic and abiotic stresses, including thermal stress (Lindquist and Craig, 1988; Parsell and Lindquist, 1993; Schlesinger, 1994; Feder and Hofmann, 1999). In some species, Hsps may be induced by both short- and long-term cold exposure and have a major impact on cold tolerance. For instance, multiple genes encoding Hsps appear to be involved in the RCH process in *Drosophila melanogaster* (Doucet et al., 2009). However, this is not a generally occurring phenomenon in ectotherms, in either RCH (Joplin et al., 1990; Kelty and Lee, 2001) or LTCA (Nielsen et al., 2005; de Boer et al., 2010). Recent studies have suggested that Hsps are probably major components in the signalling pathway for cold sensing and for the RCH response (Yi et al., 2007; Yi and Lee, 2011). In *F. candida*, LTCA (3 days at 0°C) induces *hsp70* upregulation, indicating that this Hsp affects cold tolerance in *F. candida* (de Boer et al., 2010). Because Hsps interact, other genes encoding a suite of Hsps in *F. candida* may also respond to lowered temperatures.

The aim of this study was to investigate and compare the molecular and biochemical responses in *F. candida* and the following induction of cold tolerance during different types of cold acclimation. We tested the effects of RCH (1–3 h) and LTCA (1–3 days), and hypothesized that both would improve cold tolerance, although the latter response has not previously been shown in this species. Furthermore, we hypothesized that the short- and long-term acclimation responses involved different molecular and physiological mechanisms. The responses studied were osmolyte accumulation, homeoviscous adaptation and transcription of genes encoding Hsps and metabolic enzymes expected to be involved in cold adaptation.

MATERIALS AND METHODS

Animals

Folsomia candida originating from a laboratory culture were kept at 20±1°C (12 h:12 h light:dark) on Petri dishes with water-saturated plaster of Paris mixed with charcoal. The collembolans were fed dried baker's yeast and were 6–8 weeks old with a fresh mass in the range 150–200 µg when used for experiments.

RCH, LTCA and cold-shock treatments

A full factorial experimental setup was used to investigate the effect of acclimation temperature and duration on cold tolerance. Temperature treatments were 20 (control), 15, 10, 5 and 0°C. Hardening/acclimation periods were 1, 2 or 3 h (RCH), and 24, 48 or 72 h (LTCA). These treatments were initiated in sequence, enabling simultaneous cold-shock exposure of all collembolans. RCH was carried out with collembolans placed in 2 ml centrifuge tubes to allow fast and consistent temperature manipulation, whereas collembolans for LTCA were kept on Petri dishes during cold treatment to prevent effects of long-term storage in tubes. Collembolans for controls were treated similarly and kept at 20°C. Cold-shock tolerance was evaluated by determining survival after exposure to −6°C for 90 min. Immediately before cold shock, LTCA and control collembolans were quickly transferred to centrifuge tubes, and all tubes were submerged in a temperature-controlled water bath (Hetofrig, Birkerød, Denmark) containing 70% ethanol. Measurements of temperature in the tubes were made using thermocouples (Gemini Data Loggers, Chichester, UK), showing that the temperature in tubes transferred from 20°C reached −6°C within 10 min, and that the programmed temperature was constant with a precision of ±0.1°C. After cold shock, the tubes were quickly dried with paper tissue. Collembolans were transferred to Petri dishes and cold-shock survival was assessed after 24 h recovery at 20°C. Collembolans walking in a coordinated manner after gentle tactile stimulation with a brush were considered as survivors. For each treatment, six replicates with 12 collembolans per tube were used.

Samples used for measurements of cryoprotectants, PLFA composition and gene expression were snap frozen in liquid nitrogen after RCH or LTCA treatment (i.e. just before cold shock). These samples were stored at –80°C until analyses were carried out. Cryoprotectant content and PLFA composition were determined for *F. candida* subjected to 5°C for 1, 2, 3, 24, 48 and 72 h. Likewise, gene expression was analysed in collembolans exposed to these treatments and additionally to 20, 15, 10 and 0°C for 1 h (RCH) and for 24 h (LTCA). For gene expression, 20 collembolans were pooled in each sample and three to four replicates per treatment were run. For analyses of PLFA composition (N=6), each sample consisted of 10 collembolans. Samples used for analysis of cryoprotectants consisted of 15 collembolans (N=5–6) and were dried at 60°C for 24 h and weighed to the nearest 0.01 mg using a Sartorius Ultra Microbalance SC-2 (Bradford, MA, USA) in order to quantify cryoprotectant contents on a dry mass basis.

Analysis of PLFAs

Sample tissue was crushed in 0.5 ml PO_4 buffer using a TissueLyser II (Qiagen, Copenhagen, Denmark) at 30 Hz for 2×15 s, and 1 ml PO_4 buffer was added to each sample, for a total volume of 1.5 ml. A crude extraction of all lipids was conducted by adding 3 ml methanol and 1.5 ml chloroform to the samples in 12 ml glass centrifuge tubes, which were whirly-mixed for 1 min and incubated

at room temperature for 2.5 h. Another 1.5 ml chloroform and 1.5 ml PO_4 buffer were added to each sample, which were again whirly-mixed for 1 min and left at room temperature overnight. Centrifugation at 1600 ***g*** for 10 min separated the organic compounds dissolved in chloroform from the more polar compounds in a watery phase. The watery phase was discarded and chloroform was evaporated from the organic phase under nitrogen flow. To separate neutral lipids and lipids of medium polarity (e.g. cholesterol) from polar lipids, the samples were redissolved in 900 µl chloroform and then slowly vacuum-filtered through solid-phase silica columns (100 mg; Bond Elute, Agilent Technologies, Santa Clara, CA, USA) which were pre-conditioned with 1.5 ml chloroform. The neutral and medium polarity lipids were eluted with 1.5 ml chloroform and 6 ml acetone, respectively, and discarded. Polar lipids (mainly phospholipids) were eluted with 1.5 ml methanol, which afterwards was evaporated under gentle nitrogen flow and then transesterified by a mild alkaline methanolysis (Dowling et al., 1986). To this end, 500 µl methanol, 500 µl toluene and 1 ml 0.2 mol l^{-1} KOH in methanol were added to the samples, which were briefly whirly-mixed and incubated at 37°C for 15 min. After trans-methylation, 2 ml heptane, 300 µl 1 mol l^{-1} acetic acid and 2 ml Elga water were added to each sample, which was whirly-mixed for 1 min and centrifuged at 400 ***g*** for 5 min. From the resultant two-phase mixture, the upper organic phase containing the fatty acids was removed and the extraction was repeated by adding 2 ml heptane to the acetic acid/water phase. Total heptane volume was evaporated under nitrogen flow and the fatty acid methyl esters (FAMEs) were re-dissolved in 1 ml heptane for gas chromatographic analysis coupled with mass spectrometry (GC-MS) on a Shimadzu GCMS-QP2010 Plus equipped with an auto-sampler (Columbia, MD, USA). The FAMEs were separated on a Supelco Omegawax 320 column (length 30 m, inner diameter 0.32 mm; Sigma-Aldrich, Stockholm, Sweden) using helium as carrier. Samples of 1 µl were injected in split mode (split ratio 10:1) at 220°C and the oven was programmed to hold the temperature at 60°C for 2 min, then increase it to 280°C over 26 min and hold it at the final temperature for 2 min. Column flow was 1.79 ml min^{-1} at a pressure of 90.7 kPa. The GC-MS inter-phase temperature was 200°C, and the ion source temperature was 220°C. The mass spectrometer was operated in the electron ionization mode. Identification of individual fatty acids was based on mass spectra of known FAME standards (Nu-check Prep, Elysian, MN, USA). Fatty acids were designated as $X{:}Y\omega Z$, where X indicates the number of C atoms, Y the number of double bonds and Z the position of the first double bond counting from the methyl end of the molecule, if known. Areas of identified peaks were quantified using external standards, and mol% distributions were calculated. The degree of unsaturation was calculated as: Σ(% monoenes + 2×% dienes + 3×% trienes....)/100 (Kates, 1986). In addition, the unsaturation ratio, Σ unsaturated fatty acids/Σ saturated fatty acids (UFA/SFA), was calculated. Finally, the average number of C atoms in the fatty acid chains was calculated.

Analysis of cryoprotectants

Freeze-dried collembolans were crushed in 0.5 ml 70% ethanol using a TissueLyser II (Qiagen) at 30 Hz for 2×15 s. The samples were diluted in another 1.0 ml ethanol and placed under a gentle nitrogen flow for evaporation to dryness at room temperature (~120 min). Silylation was conducted by adding 900 µl pyridine, 90 µl hexamethyldisilizane and 10 µl chlorotrimethylsilane (all from Sigma-Aldrich, Brøndby, Denmark) and incubated in darkness for 2–3 h at room temperature. Finally, samples were centrifuged for 3 min at 3900 ***g*** (Centrifuge 5430, Fisher Scientific, Slangerup, Denmark) and the resulting supernatants were transferred to analysis tubes before GC-MS analysis. Standard solutions of myoinositol, glucose and trehalose were treated as described. Samples of 2 µl were injected in split mode (split ratio 70:1) at 280°C and the oven was programmed to hold the temperature at 50°C for 1 min, then increase it to 260°C over 2.5 min and hold it at the final temperature for 5 min. Column flow was 0.6 ml min^{-1} at a pressure of 90.7 kPa. The GC-MS inter-phase temperature was 200°C, and the ion source temperature was 220°C. The mass spectrometer was operated in the electron ionization mode.

Gene expression

Total RNA was extracted using NucleoSpin RNA II (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions, and the resulting RNA concentration was determined using an Implen NanoPhotometer (AH Diagnostics, Aarhus, Denmark). cDNA was synthesized from 1 µg total RNA using the Omniscript Reverse Transcriptase Kit (Qiagen) and an Anchored Oligo(dT)20 primer (Invitrogen A/S, Naerum, Denmark) in a total volume of 13 µl according to the manufacturer's instructions. Sequences for genes analysed were obtained from http://www.collembase.org (Timmermans et al., 2007). Primers were designed using Primer3 (Rozen and Skaletsky, 2000) and synthesized by MWG Eurofins (Ebersberg, Germany; Table 1). qPCR was conducted on a Stratagene MX3005P (AH Diagnostics) using Stratagene Brilliant II SYBR Green qPCR Mastermix (AH Diagnostics). Each sample was measured in duplicate and contained 5 µl of cDNA template (equivalent to 20 ng total RNA) along with 900 nmol l^{-1} primers in a final volume of 15 µl. DNA amplification was initiated with 95°C for 10 min to activate the DNA polymerase, followed by 40 cycles of 95°C for 10 s and 60°C for 60 s. Finally, a single cycle with gradually increasing temperature was performed to measure the melting points of the products. Melting curves were visually inspected to verify a single amplification product with no primer-dimers.

Statistical analyses

Survival proportions were arcsine square root transformed to improve homogeneity of variance. Subsequently, two-way ANOVA testing for temperature and time effects within and between RCH and LTCA were performed to analyse the effects of RCH and LTCA on cold shock tolerance. *Post hoc t*-tests (Dunn's) with incorporated correction for multiple testing were applied to all two-way ANOVAs. Additionally, one-way ANOVA with *post hoc t*-tests were performed on 5°C data to evaluate the effect of treatment time on cold-shock tolerance within RCH and LTCA. All *t*-tests included Bartlett's test of homogeneity of variances.

The change of PLFA composition in response to RCH and LTCA was analysed as: (1) individual changes in mol% for each of the seven fatty acids included in the analysis; and as overall changes in (2) degree of unsaturation, (3) UFA/SFA and (4) average length of fatty acids. Bonferroni *t*-tests were performed to test for the effect of cold treatment duration on PLFA composition at 5°C. All tests included Bartlett's test of homogeneity of variances. When data were not normally distributed, non-parametric Kruskal–Wallis one-way ANOVAs on ranks were performed.

Temporal control samples were compared and pooled as no differences were found. Following this, the effect of RCH and LTCA on glucose and myoinositol concentration in *F. candida* was analysed with one-way ANOVA using treatment (control and temporal cold treatments) as a fixed factor. *Post hoc* tests (Fisher's

Table 1. Clone ID, primer sequences (F, forward; R, reverse) and amplification efficiency for genes investigated by qPCR

Gene	Collembase ID	Primer sequence	Efficiency
Heat Shock Protein 70 (*hsp70*)	Fcc02719	F: CGAAGGATGACTTGGAGAGG R: CGAACCACTGCTCAATCTCA	2.2
Heat Shock Cognate 70 (*hsc70*)	Fcc05668	F: CGAAGATTTGACGATGCTGA R: CCATTGGAGGCTTTGACAAT	2.3
Heat Shock Protein 40 (*hsp40*)	Fcc020305	F: CCTTGGTGGAAGTACACGGATC R: TTTACCCCTAAGACGCATTCGA	2.3
Heat Shock Protein 23 (*hsp23*)	Fcc01918	F: GAAACATGAGCACGACGATG R: CTGAACGTCTTCGGGAATGT	2.3
Heat Shock Protein 90 (*hsp90*)	Fcc01660	F: AGCGGCCAGTAACAGTCCTA R: AAATGAGCGACCAACCATTC	2.2
Heat Shock Protein 10 (*hsp10*)	Fcc03153	F: AGGTCCTGGTCACAGGAATG R: CCTTCGTTCCTCCATACTCG	1.8
Peroxisomal Acyl-CoA Oxidases (*percoa*)	Fcc00071	F: ACCAAGGTGGATATGCCAAA R: TCCTGCTTCAGGATTTCACA	1.6
Enoyl-CoA hydratase (*enocoa*)	Fcc02456	F: GAAGGACTTCTTTGCGAACG R: TCATTCCTTCTGCTCGGTCT	2.3
Z9-desaturase (*z9des*)	Fcc0901	F: ATACCCAGAGCTGCGATTTG R: AAGTCGAGCCTTGAACGTGT	2.3
Delta-6-desaturase (*d6des*)	Fcc1007	F: CCTTGTCCGCCTATCTGTTC R: ATGTGCAGTGTGCAGGAGTG	2.5
Delta-5-desaturase (*d5des*)	Fcc1740	F: GTGGATTCGTTTCTGGTCGT R: ATCGGAGAATGCACGTTTCT	2.3
Delta-9-desaturase (*d9des*)	Fcc5159	F: TATGCTCCAGCAAACCAGTG R: CGTTGGTGTCGGTGTACTTG	2.1
Ribose 5-Phosphate Isomerase (*rpi*)	Fcc02130	F: TGATCACATCGAGGATGGAA R: GTGTTCTTTCCGCAATTCTTTC	2.0
Glucose-6-Phosphate Dehydrogenase (*g6pd*)	Fcc03100	F: ATGAACGCTTGATCCTCGAC R: CAAGAGCGGCGTAAAGATTC	2.2
Glucose-6-Phosphate Isomerase (*gpi*)	Fcc00680	F: ATCGCCATGTATGAGCACAA R: GCCAACTCTTTGCCAAGTTC	2.2
Glyceraldehyde-3-Phosphate Dehydrogenase (*gpd*)	Fcc05377	F: CACAAATCCCGGGTACATTT R: CTCCAATATGCGTGATCAACTT	2.4
Sorbitol Dehydrogenase (*sor*)	Fcc06503	F: AGTGCTGCTGGGTAGCAGTT R: TCCTGTTGTTAACGCTGCTG	1.6

LSD) were performed to investigate the effect of RCH and LTCA on glucose and myoinositol concentration in *F. candida*.

The effect of RCH and LTCA on gene expression was investigated for 17 target genes related to the general stress response or carbohydrate and fatty acid metabolism. Raw qPCR data were analysed with a programmed Excel workbook, Data Analysis for Real-Time PCR (DART-PCR) (Peirson et al., 2003), which enables calculation of threshold cycles and amplification efficiencies for every gene (Table 1). Prior to further analysis, outliers were omitted in the few cases where they were identified by DART-PCR. Gene expression data obtained from qPCR were normalised using the algorithm NORMA-gene, which calculates a normalisation factor without the use of reference genes (Heckmann et al., 2011). Normalisation with NORMA-gene was conducted separately for two independently synthesized batches of cDNA, used for expression analysis of either 'desaturase genes' or 'other genes'. To compare temperature and time effects of RCH and LTCA on gene expression, a two-way ANOVA was conducted. Furthermore, the effect of temperature on gene expression within RCH and LTCA was evaluated separately by linear regression.

All statistical tests were performed in SAS Enterprise Guide 4.3 (SAS Institute, Cary, NC, USA) using a significance level at 5%.

RESULTS

Effect of RCH and LTCA on cold-shock tolerance

In all RCH and LTCA treatments, cold-shock survival (−6°C, 90 min) was increased compared with controls kept at 20°C up until cold shock (Fig. 1). Thus, pre-treatment at decreasing temperatures (two-way ANOVA, $P<0.0001$, $F_{4,172}=52.2$) and increasing treatment duration (two-way ANOVA, $P<0.0001$, $F_{5,172}=18.6$) had significant positive effects on cold-shock tolerance compared with controls in *F. candida*. Across temperatures, mean survival was significantly higher after LTCA than after RCH treatments (Fig. 1A). Thus, LTCA at 5 to 15°C raised cold-shock tolerance more than RCH did. However, this difference was eliminated at 0°C, which explain the significant temperature–time interaction (two-way ANOVA, $P<0.0001$, $F_{20,172}=7.1$).

For collembolans pre-treated at 5°C, 3 h RCH improved cold-shock tolerance significantly more than 1 h RCH (one-way ANOVA, $F_{2,16}=10.0$, $P=0.002$; Fig. 1B). Maximum cold-shock tolerance (98% survival) was achieved after 24–72 h exposure. Within LTCA treatments at 5°C, exposure time (24, 48 and 72 h) did not affect cold tolerance significantly (one-way ANOVA, $F_{2,17}=1.2$, $P=0.34$; Fig. 1B).

Composition of PLFAs and expression of related genes

Low temperature acclimation (5°C at 1–3 days) induced a slight and significant increase of the UFA/SFA ratio (Kruskal–Wallis, $H_3=8.49$, $P=0.037$; Table 2). This change was a result of a decrease in the mol% of SFA (16:0 and 18:0) and a concomitant increase in the mol% of mono- and polyunsaturated fatty acids, with 3 days LTCA showing the biggest increase in the UFA/SFA ratio. These changes were not statistically significant when the fatty acids were analysed individually (Table 2). Finally, the average length of fatty acid chains and degree of unsaturation increased slightly, but insignificantly, after cold exposure. No significant changes in response to RCH were detected (Table 2).

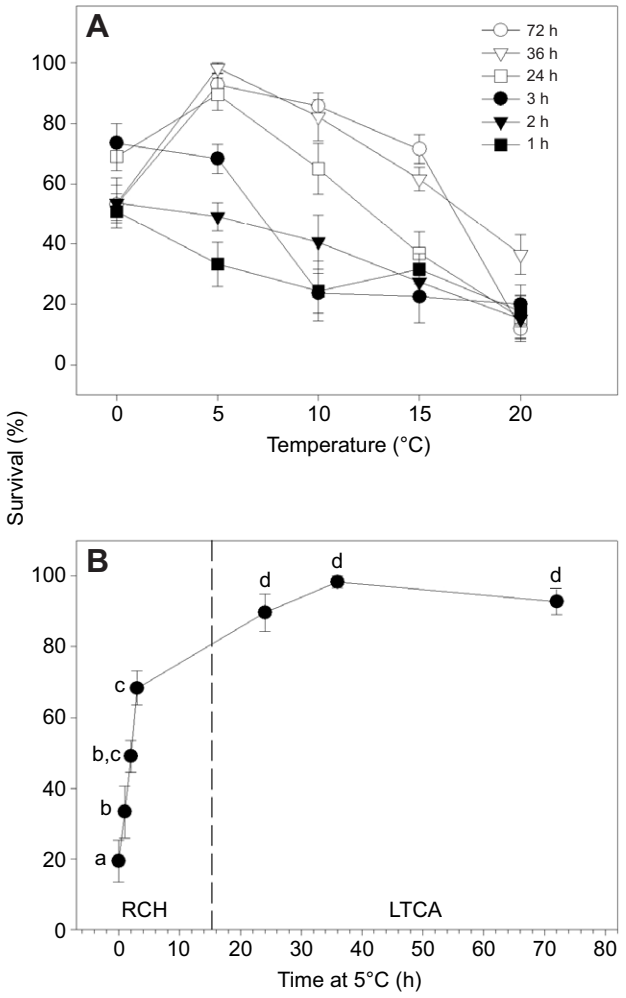

Fig. 1. Temperature- and duration-dependent cold-shock survival (90 min at −6°C) in *Folsomia candida*. (A) Collembolans were pre-exposed to 0, 5, 10, 15 and 20°C for periods ranging from 1 to 72 h (N=6, 12 collembolans per replicate). (B) Duration-dependent cold-shock survival in *F. candida* exposed to 5°C. In collembolans given this pre-treatment, changes in gene expression, phospholipid-derived fatty acid composition and osmolyte concentration were also studied (see Figs 2–4, Table 2). Different letters indicate significant difference between treatments (P<0.05). Error bars indicate ±s.e.m.

The expression of six of the genes encoding enzymes involved in fatty acid metabolism (*enocoa*, *percoa*, *z9des*, *d6des*, *d5des* and *d9des*; Table 1) were measured to investigate whether the activity of these enzymes changed in parallel with PLFA changes. Expression of most of the targeted genes related to fatty acid metabolism seemed unaffected by RCH and LTCA (Fig. 2A). However, the expression of *d9des* was significantly affected by duration of exposure to 5°C (Kruskal–Wallis, H_5=12.74, P=0.03), with expression after 48 h fivefold higher than at other times studied (Fig. 2B).

Accumulation of cryoprotectants and expression of related genes

The detected concentrations of glucose and myoinositol are shown in Table 2. One-way ANOVA analyses on concentrations of myoinositol and glucose showed non-significant changes after 1–3 h at 5°C (myoinositol: $F_{3,20}$=1.68, P=0.21; glucose: $F_{3,20}$=0.36, P=0.78). LTCA at 5°C had a significant effect on myoinositol ($F_{3,29}$=4.44, P=0.012), with a transient accumulation of myoinositol peaking at 48 h after treatment with a concentration approximately twofold higher than control levels. Concomitantly, glucose concentration varied non-significantly ($F_{3,29}$=0.99, P=0.42) compared with controls at 20°C (Table 2).

The effect of short- and long-term cold-exposure on carbohydrate metabolism was further investigated by measuring the expression of genes encoding some of the enzymes related to the polyol pathway [sorbitol dehydrogenase (*sor*)], the glycolytic pathway [glucose 6-phosphate isomerase (*gpi*) and glyceraldehyde-3-phosphate dehydrogenase (*gpd*)], and the pentose phosphate pathway [glucose-6-phosphate dehydrogenase (*g6pd*) and ribose 5-phosphate isomerase (*rpi*)].

Expression of these genes in 5°C-acclimated *F. candida* was significantly affected by exposure time; RCH induced a significantly increased expression of all of these genes in comparison to both the control (20°C) and LTCA (Fig. 3).

Heat shock proteins

Genes coding for small Hsps (*hsp40*, *hsp23* and *hsp10*) and the constitutively expressed *hsc70* were significantly influenced by exposure time at 5°C; RCH significantly increased gene expression compared with the control (20°C) and LTCA (Fig. 4). *hsp70* and *hsp90* were not significantly influenced by cold acclimation in *F. candida* in this study (data not shown).

DISCUSSION

Cold tolerance in *F. candida*

RCH improved cold-shock tolerance in *F. candida*, which confirms the previous findings of adaptive low temperature responses in this species (Holmstrup et al., 2008; Bahrndorff et al., 2009a). In addition, cold tolerance was increased by LTCA, which has not previously been described in this species. Thus, despite living in soil where sudden temperature changes are relatively buffered, this springtail may benefit from acclimation to decreased temperatures on both an hourly (RCH) and a daily basis (LTCA), as observed for most arthropods living above ground (Lee et al., 1987). This corroborates recent indications of *F. candida* relying on physiological adaptation to cold rather than the previously assumed behavioural avoidance (Boiteau and MacKinley, 2012).

PLFA composition and metabolism

Homeoviscous adaptation, where membrane fluidity is sustained as temperature decreases and thereby improves cold tolerance (Hazel, 1995), is a universal cellular mechanism that has been described in a wide variety of organisms ranging from bacteria to vertebrates (Hazel and Williams, 1990). Such changes in membrane fluidity can arise from a number of biochemical changes. In some chill-sensitive dipterans, the increased unsaturation of PLFAs has been suggested as one of the underlying mechanisms of their RCH capacity (Overgaard et al., 2005; Overgaard et al., 2006; Michaud and Denlinger, 2006). However, other studies indicate that this trait seems to be treatment sensitive and is not of significant mechanistic importance to RCH capacity (MacMillan et al., 2009), but it is for LTCA capacity in *D. melanogaster* larvae (Koštál et al., 2011). In the present study, increased unsaturation of PLFAs does not seem to be a major component of RCH in *F. candida*. In contrast, the PLFA UFA/SFA ratio increased significantly after LTCA in *F. candida*. Thus, our results corroborate other studies showing that softening of membranes achieved from altered composition of

Table 2. Composition of phospholipid-derived fatty acids (PLFAs; mol%) and concentration of targeted cryoprotectants (mg g^{-1} dry mass) in *Folsomia candida* subjected to rapid cold hardening (RCH) at 5°C for 1, 2 or 3 h or long-term cold acclimation (LTCA) at 5°C for 24, 48 or 72 h

	RCH				LTCA			
	Control	1 h	2 h	3 h	Control	24 h	48 h	72 h
PLFAs								
16:0	8.02±0.14	8.23±0.18	7.78±0.13	7.47±0.27	6.81±0.24	6.67±0.24	6.78±0.22	6.29±0.41
16:1ω7	2.36±0.10	2.39±0.06	2.37±0.04	2.25±0.13	2.60±0.52	2.36±0.74	1.17±0.69	1.89±0.83
18:0	21.64±0.35	21.03±0.29	20.77±0.30	20.69±0.29	18.18±0.47	17.24±0.27	17.52±0.31	16.59±0.76
18:1ω9	16.46±0.11	16.60±0.26	17.04±0.17	16.63±0.27	19.26±0.62	19.80±0.50	18.87±0.56	19.71±0.59
18:2ω6	6.46±0.05	6.35±0.11	6.38±0.11	6.32±0.07	6.79±0.27	7.07±0.23	7.19±0.29	7.62±0.40
20:4ω6	30.37±0.30	30.80±0.47	30.76±0.33	31.87±0.50	31.70±0.99	31.62±0.58	32.88±0.67	32.35±0.96
20:5ω3	14.69±0.31	14.60±0.22	14.90±0.25	14.78±0.21	14.67±0.35	15.24±0.56	15.58±0.47	15.5±0.53
Degree of unsaturation	2.27±0.01	2.28±0.02	2.30±0.02	2.33±0.01	2.36±0.04	2.39±0.04	2.44±0.03	2.44±0.05
UFA/SFA	2.37±0.03	2.42±0.05	2.51±0.05	2.55±0.03	**3.00±0.05**[a]	**3.19±0.05**[a,b]	**3.12±0.04**[a,b]	**3.41±0.21**[b]
Average chain length	18.69±0.01	18.70±0.02	18.71±0.01	18.74±0.02	18.74±0.03	18.76±0.03	18.81±0.03	18.79±0.04
Cryoprotectants								
Myoinositol	0.62±0.15	0.74±0.26	0.40±0.18	0.20±0.12	**0.29±0.04**[a]	**0.39±0.10**[a,b]	**0.63±0.14**[b]	**0.25±0.02**[a]
Glucose	1.08±0.19	0.81±0.28	0.85±0.09	0.95±0.20	1.02±0.09	0.79±0.12	1.09±0.13	1.07±0.07

Mean values (±s.e.m.) are shown; PLFA: N=6; osmolytes: N=5–6.
Bold font indicates significant effect of treatments, and within each analysis letters indicate significant difference between treatments (P<0.05).

PLFAs underlies LTCA in chill-sensitive and cold-tolerant arthropods (Overgaard et al., 2008; Koštál et al., 2011).

Unsaturation of PLFAs during cold acclimation can be achieved by substituting SFAs with UFAs recruited from the neutral lipid storage, as seen in two other collembolans (van Dooremalen and Ellers, 2010; Purać et al., 2011). Cold acclimation may also induce a conversion of phosphatidylcholines to phosphatidylethanolamines, which, due to their more conical shape, increase membrane fluidity (Hazel and Williams, 1990). Indeed, LTCA causes this conversion in both *Sarcophaga similis* (Goto and Katagiri, 2011) and in larvae of *D. melanogaster* (Koštál et al., 2011). Further and more detailed analysis of PLFA speciation is needed to show such mechanisms in *F. candida*. Lastly, an increased unsaturation may be a result of enzymatic desaturation of existing saturated and monounsaturated PLFA molecules of the membrane, as described in plants and various animal groups including some, but not all, insect species studied (Cripps et al., 1986; Tocher et al., 1998; Nakamura and Nara, 2004). In the house cricket, *Acheta domesticus*, a gradual temperature decrease from room temperature to 15°C during a 72 h period induces an increased Δ^{12} desaturase activity (Batcabe et al., 2000),

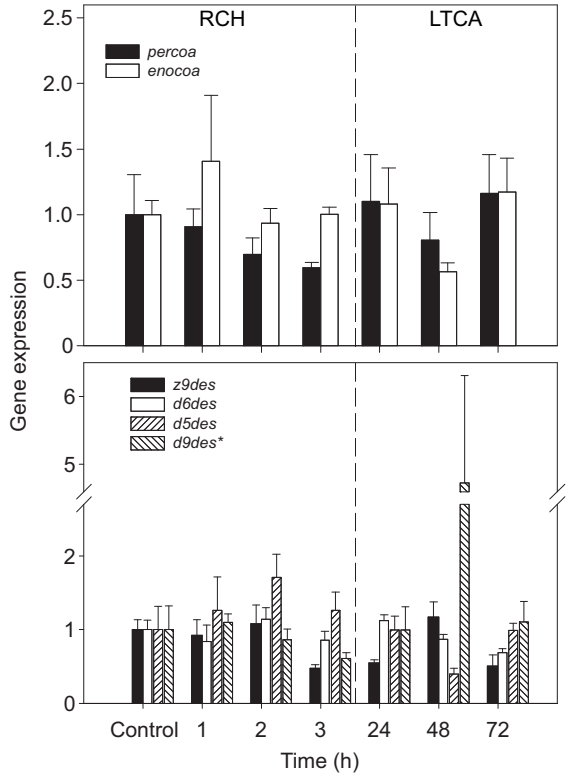

Fig. 2. Expression of genes encoding enzymes related to fatty acid metabolism (means ± s.e.m.) in *Folsomia candida* exposed to 5°C for 1 to 72 h (N=3–4, 20 collembolans per replicate). (A) *percoa*, *Peroxisomal Acyl-CoA Oxidase*; *enocoa*, *Enoyl-CoA hydratase*. (B) *z9des*, *Z9-desaturase*; *d6des*, *Delta-6-desaturase*; *d5des*, *Delta-5-desaturase*; *d9des*, *Delta-9-desaturase*. Gene expression is normalised to mean expression of control samples (20°C, 24 h). Asterisk indicates a significant effect of treatment (P≤0.05). RCH, rapid cold hardening; LTCA, long-term cold acclimation.

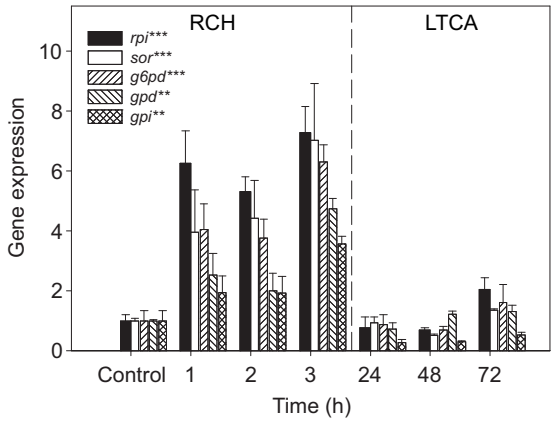

Fig. 3. Expression of genes encoding enzymes related to carbohydrate metabolism (means ± s.e.m.) in *Folsomia candida* exposed to 5°C for 1–72 h (N=3–4, 20 collembolans per replicate). *rpi*, *Ribose 5-Phosphate Isomerase*; *sor*, *Sorbitol Dehydrogenase*; *g6pd*, *Glucose-6-Phosphate Dehydrogenase*; *gpd*, *Glyceraldehyde-3-Phosphate Dehydrogenase*; *gpi*, *Glucose-6-Phosphate Isomerase*. Gene expression is normalised to mean expression of control samples (20°C, 24 h). Asterisks indicate significant effects of treatments: **P≤0.01, ***P≤0.005.

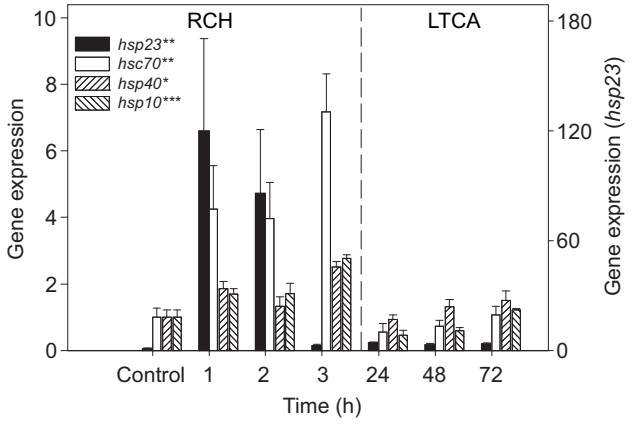

Fig. 4. Expression of responsive genes encoding heat shock proteins (means ± s.e.m.) in *Folsomia candida* exposed to 5°C for 1–72 h (*N*=3–4, 20 collembolans per replicate). *hsp23*, *Heat Shock Protein 23* (gene expression is plotted on right-hand *y*-axis with different scale); *hsc70*, *Heat Shock Cognate 70*; *hsp40*, *Heat Shock Protein 40*; *hsp10*, *Heat Shock Protein 10*. Gene expression is normalised to mean expression of control samples (20°C, 24 h). Asterisks indicate significant effects of treatments: **P*≤0.05, ***P*≤0.01, ****P*≤0.005.

which may convey increased cold tolerance by increasing PLFA unsaturation. Another enzyme, Δ^9 desaturase, can synthesize oleic acid (18:1) from desaturation of stearic acid (18:0). The relationship between Δ^9 desaturase activity and low temperature has been well studied in fish and nematodes (Tiku et al., 1996; Murray et al., 2007). However, Δ^9 desaturase has not previously been related to low temperature physiology in arthropods. In the present study, expression of *d9des* was upregulated fivefold after 48 h at 5°C. After 72 h at 5°C the mol% of 18:0 was reduced compared with controls, and the mol% of 18:1 was likewise slightly increased. These results indicate a link between the abruptly increased expression of *d9des*, membrane restructuring and increased cold tolerance in response to LTCA in *F. candida*. However, further studies are needed to fully describe and understand the importance of Δ^9 desaturase in arthropod cold tolerance, as this enzyme seems to respond differently to modest, moderate and extreme cooling (Trueman et al., 2000). In some invertebrates, *d9des* has been related to pheromones (Choi et al., 2002; Matousková et al., 2008). Thus, the observed upregulation of *d9des* after 48 h at 5°C could indicate a response related to reproduction in *F. candida*. However, this response is most likely only active at the transcriptive level, because collembolan reproduction usually is arrested at exposure to 5°C.

Fatty acid metabolism involves several pathways, whereby oxidation of fatty acids in the peroxisome results in fatty acid shortening and, like desaturation, enhances fatty acid fluidity. Peroxisomal acyl-CoA oxidase and enoyl-CoA hydratase participates in this catalyzation of FA chain shortening. In the present study, expression of *percoa* and *encoa* was unaffected by cold treatments, consistent with the unchanged average PLFA carbon chain length.

Compatible osmolytes and carbohydrate metabolism

Cryoprotection can be partly achieved through the accumulation of compatible osmolytes such as sugars and polyols. These osmolytes may protect membranes and proteins and/or reduce enzyme activities for energy conservation during long-term cooling (Zachariassen, 1985). At high concentrations, compatible osmolytes increase cold tolerance by depression of melting and supercooling points enabled by the osmolyte's colligative effects; however, this is not of relevance to chill-sensitive arthropods, where the role of cryoprotectants is less obvious. Nevertheless, low concentrations of cryoprotectants tend to improve cold tolerance of chill-sensitive arthropods. For example, the flesh fly, *S. crassipalpis*, accumulates small amounts of glucose, sorbitol, glycerol and pyruvate during RCH (Chen et al., 1987; Michaud and Denlinger, 2007), and in adult *D. melanogaster* the total (although low) sugar concentration correlates with increased cold-shock tolerance following RCH (Overgaard et al., 2007). In the present study, LTCA induced a twofold increase in myoinositol concentration (from 0.29 to 0.63 mg g^{-1} dry mass) in *F. candida*. This concentration is much less than that found in some winter acclimated beetles and flies (Koštál et al., 1996; Soudi and Moharramipour, 2012; Vesala et al., 2012) and also to the concentration induced in response to 72 h mild drought in *F. candida* and *F. fimetaria* (40 mg g^{-1} dry mass). Thus, the previous indications that drought and cold tolerance can be achieved by overlapping adaptations in *F. candida* (Bayley and Holmstrup, 1999; Holmstrup et al., 2002) seem not connected to high levels of myoinositol alone. The accumulation of myoinositol in *F. candida* in response to cold acclimation seems to be transient, as the concentration is back to control levels after 3 days. Thus, it is unknown to what degree myoinositol accumulation could support increased cold tolerance (in a non-colligative way), for example by stabilising proteins at low temperature and allowing increased membrane lipid disordering and fluidity (Carpenter and Crowe, 1988; Crowe et al., 1988; Andersen et al., 2011). In contrast to the role of myoinositol for drought tolerance, the temporal nature of the induction by cold could suggest that the increased concentration is only needed until other physiological adaptations have fully kicked in. However, this needs further investigation.

RCH in *F. candida* induced an upregulation of some genes encoding enzymes in the polyol pathway (*sor*), the glycolytic pathway (*gpi*, *gpd*) and the pentose phosphate pathway (*g6pd*, *rpi*). These upregulations indicate an increased biosynthetic activity related to organismal rearrangement of carbohydrate metabolites, including accumulation of cryoprotectants, which seem to play a role in tolerance to longer cold exposures. Additionally, it is suggested that the initial upregulation of genes encoding enzymes related to the carbohydrate metabolic pathways is due to an increased energy requirement for protein production to compensate for cold-induced protein denaturation (Gething and Sambrook, 1992).

Heat shock proteins

Low temperature denaturation of proteins is a general phenomenon, and the subsequent induction of transcription and translation of Hsps have been known to be induced by cold exposure for decades (Ananthan et al., 1986; Burton et al., 1988). However, since then the induction of Hsps in relation to cold has been shown to be both species and/or treatment dependent in several cases (Joplin et al., 1990; Hoffmann et al., 2003; Li and Denlinger, 2008; Doucet et al., 2009). In the present study, the most distinct gene expression pattern in response to RCH *versus* LTCA was the rapid upregulation of *hsc70*, *hsp40*, *hsp23* and *hsp10* within 1–3 h followed by stabilisation at control levels after 24 h (at 5°C). Such a temporal gene expression pattern has also been described for *hsp70* in another collembolan (*Orchesella cincta*). In these collembolans, genomic and proteomic responses of Hsp70 were displaced; *hsp70* upregulation preceded Hsp70 expression, and only the latter correlated with increased heat tolerance in *O. cincta*, which peaked 49 h after heat hardening (Bahrndorff et al., 2009b). A similar temporal difference in gene

and protein expression may also be found in Hsps of *F. candida* in response to low temperatures, allowing continuous protection from Hsps through LTCA even if the gene is no longer upregulated.

Hsc70 is constitutively expressed and present under most conditions, and may be downregulated (Sonoda et al., 2006) or unchanged during cold stress in arthropods (Colinet et al., 2010; Zhang and Denlinger, 2010). In the firebug, *Pyrrhocoris apterus*, *hsc70* is upregulated sixfold 6–8 h after LTCA (–5°C for 5 days). However, no upregulation of Hsc70 protein was detected in *P. apterus* after cold stress (Koštál and Tollarová-Borovanská, 2009). This is probably because the expression of inducible and constitutive Hsp70s may change during development and various stages of the cell cycle. In the moth *Manduca sexta*, *hsc70* expression is sensitive to ecdysteroids and is therefore suggested to be involved in regulation of the ecdysone receptor assembly (Rybczynski and Gilbert, 2000). This may also be the case in *F. candida*, where the rapidly upregulated *hsc70* expression may be related to a cold-induced moult cease, as the thermal limit for moulting activity in temperate Collembola is approximately 5°C (Hopkin, 1997).

Hsp40, Hsp23 and Hsp10 belong to the small Hsps. *hsp40* is cold induced after 3 days at 0°C in *F. candida* (de Boer et al., 2010). Thus, along with results of the present study, it seems that Hsp40 may be important to *F. candida* in both RCH and LTCA. An important function of Hsp40 is to assist Hsp70 in polypeptide binding and release in an interdependent manner (Fan et al., 2003; Kampinga and Craig, 2010).

In the present study, RCH induced *hsp23* in *F. candida*. Recent studies have shown that *hsp23* is also upregulated in response to heat (Nota et al., 2010) and prolonged severe drought and rehydration in *F. candida* (Waagner et al., 2012). Thus, Hsp23 may play a central role in response to climatic stress in general. In other invertebrates, *hsp22* and *hsp23* are upregulated in response to cold and seem to be related to organism dormancy (Yocum et al., 1998; Rinehart et al., 2007) or protection of cells related to moulting and/or reproduction (Zimmerman et al., 1983; Qin et al., 2005; Burns et al., 2010). The latter may also be the case in *F. candida* because moulting and reproduction in these collembolans are sensitive to both drought and extreme temperatures.

Hsp10 has, to our knowledge, not previously been related to cold tolerance. However, Hsp10 is an essential component of the mitochondrial protein folding apparatus (Höhfeld and Hartl, 1994), where it interacts with Hsp60, which is upregulated on the mRNA level in cold recovering *C. variipennis sonorensis* (Gething and Sambrook, 1992; Nunamaker et al., 1996). Thus, the present upregulation of *hsp10* in response to short-term temperature decrease may be related to the protection of mitochondrial compartments in *F. candida*.

CONCLUSIONS

RCH induces upregulation of some genes encoding enzymes important in carbohydrate metabolism and small and constitutively expressed *hsp*s in *F. candida*. This indicates that although RCH improves cold tolerance in these organisms, RCH induces a high stress level rather than a high protection level in *F. candida*, as discussed by Sørensen (Sørensen, 2010). In contrast, the high protection level is induced in *F. candida* by LTCA, where increased degree of PLFA unsaturation, possibly assisted by myoinositol accumulation, may serve as protection of membrane structure and function during longer-term cold exposure. Additionally, the RCH-induced Hsps may continue to play a protective role during LTCA, although gene transcription was mostly a transient response. In conclusion, the moderate increase in cold-shock tolerance conferred by RCH seems to be dominated by effects of Hsps, whereas the substantially better cold tolerance achieved after LTCA is dominated by post-transcriptional processes, increasing membrane fluidity and cryoprotectant concentration.

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AUTHOR CONTRIBUTIONS

D.W., M.H., M.B. and J.G.S. designed the research; D.W. and J.G.S. performed the research; D.W., M.H. and J.G.S. analyzed the data; and D.W., M.H., M.B. and J.G.S. wrote the paper.

COMPETING INTERESTS

No competing interests declared.

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