The Journal of Experimental Biology 216, 1235-1243

doi:10.1242/jeb.075531

RESEARCH ARTICLE

Inorganic ion composition in Tardigrada: cryptobionts contain a large fraction of unidentified organic solutes

Kenneth Agerlin Halberg[1,*], Kristine Wulff Larsen[2], Aslak Jørgensen[3], Hans Ramløv[2] and Nadja Møbjerg[1]

[1]Department of Biology, The August Krogh Centre, University of Copenhagen, Universitetsparken 13, DK-2100 Copenhagen Ø, Denmark, [2]Department of Nature, Systems and Models, University of Roskilde, Universitetsvej 1, DK-4000 Roskilde, Denmark and [3]Laboratory of Molecular Systematics, Natural History Museum of Denmark, University of Copenhagen, Sølvgade 83, DK-1307 Copenhagen K, Denmark

*Author for correspondence (kahalberg@bio.ku.dk)

SUMMARY

Many species of tardigrades are known to tolerate extreme environmental stress, yet detailed knowledge of the mechanisms underlying the remarkable adaptations of tardigrades is still lacking, as are answers to many questions regarding their basic biology. Here, we present data on the inorganic ion composition and total osmotic concentration of five different species of tardigrades (*Echiniscus testudo*, *Milnesium tardigradum*, *Richtersius coronifer*, *Macrobiotus* cf. *hufelandi* and *Halobiotus crispae*) using high-performance liquid chromatography and nanoliter osmometry. Quantification of the ionic content indicates that Na^+ and Cl^- are the principal inorganic ions in tardigrade fluids, albeit other ions, i.e. K^+, NH_4^+, Ca^{2+}, Mg^{2+}, F^-, SO_4^{2-} and PO_4^{3-} were also detected. In limno-terrestrial tardigrades, the respective ions are concentrated by a large factor compared with that of the external medium (Na^+, ×70–800; K^+, ×20–90; Ca^{2+} and Mg^{2+}, ×30–200; F^-, ×160–1040, Cl^-, ×20–50; PO_4^{3-}, ×700–2800; SO_4^{2-}, ×30–150). In contrast, in the marine species *H. crispae*, Na^+, Cl^- and SO_4^{2-} are almost in ionic equilibrium with (brackish) salt water, while K^+, Ca^{2+}, Mg^{2+} and F^- are only slightly concentrated (×2–10). An anion deficit of ~120 mEq l^{-1} in *M. tardigradum* and *H. crispae* indicates the presence of unidentified ionic components in these species. Body fluid osmolality ranges from 361±49 mOsm kg^{-1} in *R. coronifer* to 961±43 mOsm kg^{-1} in *H. crispae*. Concentrations of most inorganic ions are largely identical between active and dehydrated groups of *R. coronifer*, suggesting that this tardigrade does not lose large quantities of inorganic ions during dehydration. The large osmotic and ionic gradients maintained by both limno-terrestrial and marine species are indicative of a powerful ion-retentive mechanism in Tardigrada. Moreover, our data indicate that cryptobiotic tardigrades contain a large fraction of unidentified organic osmolytes, the identification of which is expected to provide increased insight into the phenomenon of cryptobiosis.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/216/7/1235/DC1

Key words: cryptobiosis, inorganic ions, ion chromatography, nanoliter osmometry, organic osmolytes, tardigrade.

Received 1 June 2012; Accepted 5 December 2012

INTRODUCTION

Tardigrades are minute animals (50–1200 μm), many of which are capable of tolerating extreme environmental stress (Guidetti et al., 2011; Møbjerg et al., 2011). This capacity derives mainly from their ability to enter a latent state of life, i.e. cryptobiosis, in which their resistance to adverse environmental conditions is greatly increased (Keilin, 1959; Clegg, 2001). Indeed, their extremophilic nature has favored them as model organisms in astrobiology and space research (Jönsson et al., 2008; Rebecchi et al., 2009; Persson et al., 2011). In recent years, considerable effort has been devoted to the field, as the translational output associated with a detailed understanding of the complex stress biology of tardigrades is expected to include new methods for preserving and stabilizing biological materials (Wełnicz et al., 2011). Several advances have been made in our understanding of tardigrade stress responses, especially regarding (i) the role of selective carbohydrates (i.e. trehalose), (ii) the differential expression of stress proteins (heat shock proteins and late embryogenesis abundant proteins), and (iii) the identification of anti-oxidant defenses and DNA repair mechanisms (Guidetti et al., 2011; Wełnicz et al., 2011; Møbjerg et al., 2011). In addition, the field of 'omics' points to several new exciting aspects of tardigrade adaptations (Förster et al., 2009; Schokraie et al., 2010; Förster et al., 2012); however, a general understanding of the mechanisms explaining the extreme adaptations found among tardigrades is still lacking. Consequently, new approaches that may provide further insight into the superior stress adaptations of tardigrades are greatly needed.

Knowledge of the composition as well as concentrations of dissolved particles in internal fluids is fundamental to the understanding of basic physiological processes, such as fluid and electrolyte homeostasis, signal transduction and solute transport. Accordingly, such data have long been available for most major groups of animals (e.g. Macallum, 1910; Robertson, 1949; Robertson, 1954; Schmidt-Nielsen and Fänge, 1958; Sutcliffe, 1962; Hronowski and Armstrong, 1977). However, nothing is known about body fluid composition of tardigrades, which has been a major obstacle to the understanding of fluid and solute dynamics in these animals (Halberg et al., 2009; Møbjerg et al., 2011; Halberg and Møbjerg, 2012). It is especially important to address questions relating to this area of research if we wish to unravel the biological

mechanisms mediating the profound tolerance to extreme desiccation (anhydrobiosis) – the most widespread form of cryptobiosis in Tardigrada and among other phyla.

Here, we used a combination of high-performance liquid chromatography (HPLC) and nanoliter osmometry, to identify and quantify inorganic cations and anions present in tardigrade homogenates, and to measure the total osmotic concentrations of five different species of tardigrades. Our study indicates that tardigrades possess powerful ion-retentive and osmoregulatory capacities, and that cryptobiotic species contain a large fraction of unidentified organic solutes.

MATERIALS AND METHODS

Tardigrade sampling

Specimens of *Richtersius coronifer* (Richters 1903), *Macrobiotus* cf. *hufelandi* and *Milnesium tardigradum* Doyère 1840 were extracted from moss collected at Öland, Sweden, while *Echiniscus testudo* Doyère 1840 was found in moss collected at Nivå, Denmark. These species were extracted by washing the respective moss samples with tap water, either conventionally using a 'mermaid bra' (mesh size 60 μm) or through six different sieves of progressively smaller mesh size. Specimens of *Halobiotus crispae* Kristensen 1982 were isolated from marine algae and sediment collected from Vellerup Vig, Denmark, according to a previously described method (Halberg et al., 2013). The animals collected were in the so-called pseudosimplex 2 stage (Kristensen, 1982; Møbjerg et al., 2007; Halberg et al., 2009; Halberg et al., 2013). The limno-terrestrial species extracted from moss samples were kept in ddH_2O, while *H. crispae* was kept in salt water (SW, 20‰) from the locality until experimentation. The total number of animals used in the experiments was 2220 *R. coronifer*, 326 *M. tardigradum*, 630 *M.* cf. *hufelandi*, 426 *E. testudo* and 268 *H. crispae*.

Inorganic cation and anion analysis

The dominant cations and anions present in the different tardigrade species were determined by HPLC using a Metrohm chromatography system (830 IC interface, 818 IC pump, 819 IC conductivity detector, columns C4-150/4.0 (cations) and A supp 5 150/4.0 (anions); Metrohm, Herisau, Switzerland). The eluents (mobile phases) were made according to the manufacturer's instructions: for cations, the eluent consisted of 0.7 mmol l^{-1} dipicolinic acid ($C_7H_5NO_4$) and 1.7 mmol l^{-1} (65%) nitric acid (HNO_3); for anions, it consisted of 3.2 mmol l^{-1} sodium carbonate (Na_2CO_3) and 1.0 mmol l^{-1} sodium bicarbonate ($NaHCO_3$). The eluents were filtered (mesh size 45 μm) prior to use. The analysis settings employed were a flow rate of 0.9 ml min^{-1} (cations) and 0.7 ml min^{-1} (anions) with a pressure of ~6.4 MPa. Cation analyses were performed using a conductivity detector (non-suppressed), whereas anion detection was conducted using chemical suppression in combination with conductivity detection. Chemical suppression reduces background (i.e. mobile phase) conductivity while increasing the conductivity of the analytes, thus increasing signal to noise ratio. However, significant changes in signal to noise ratio using chemical suppression could not be achieved for cation detection and this was therefore omitted. Fluka multi-element cation and anion standards (Sigma-Aldrich, St Louis, MO, USA) were used to construct calibration curves for the respective ions, bracketing the concentration range of interest. Based on these calibration curves, the HPLC software (IC Net 2.3, Metrohm) calculated the ion concentrations of all subsequent samples (mg l^{-1}), which were recalculated to mmol l^{-1} and adjusted according to the appropriate dilution factor (see below). Representative chromatograms of both the cationic and anionic fractions are shown for all investigated species in Figs 1, 2. The empirically determined elution order and retention times (t_R) of the investigated ions were Na^+ (t_R=5.37 min), NH_4^+ (t_R=6.03 min), K^+ (t_R=7.73 min), Ca^{2+} (t_R=18.12 min), Mg^{2+} (t_R=23.17 min), F^- (t_R=4.05 min), Cl^- (t_R=6.01 min), PO_4^{3-} (t_R=14.74 min) and SO_4^{2-} (t_R=16.58 min).

Sample preparation

Following extraction, specimens were washed repeatedly with ddH_2O (*H. crispae* was washed in filtered SW), and subsequently transferred, using an Irwin loop, to sample tubes containing cation eluent (50–175 μl); dissolving samples in cation eluent allows a more precise quantification of cations as a result of the increased signal to noise ratio (Kurt Jensen, Metrohm Nordic, Denmark, personal communication). To avoid unwanted dilution of the samples, surface water was removed by blotting the animals with tissue paper prior to transfer. Pilot experiments revealed that this process was crucial to acquire reproducible data, and was accordingly performed in as fast and uniform a way as possible. A total of 40–225 animals were transferred to each test tube and the sample was subsequently homogenized using a sterile plastic pestle; great care was taken to ensure complete homogenization (visually confirmed at ×50 magnification), and the pestle was subsequently rinsed with a small volume (40–100 μl) of cation eluent to ensure total transfer of ions to the test tube. The number of animals per sample (N) varied according to species size and availability (Tables 1, 2). The entire sample was then centrifuged (10 min at 5600 r.p.m.) to remove solid particles, and the supernatant was filtered (mesh size 0.20 μm) using

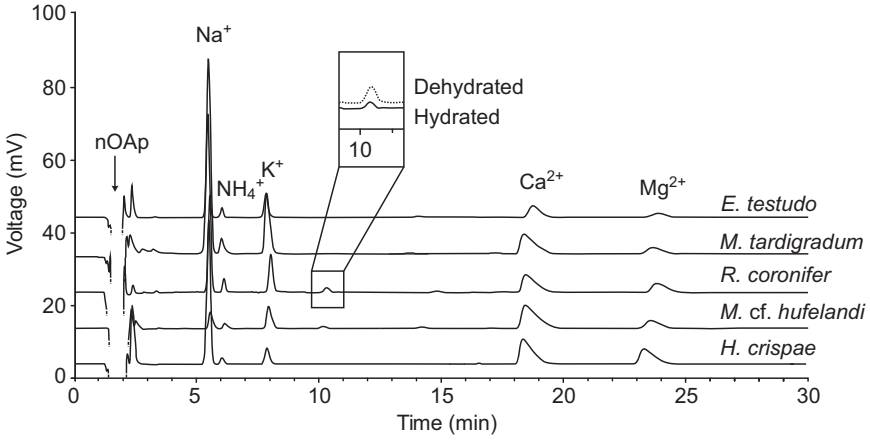

Fig. 1. Representative chromatograms showing the principal cations present in hydrated animals of each investigated species: *Echiniscus testudo*, *Milnesium tardigradum*, *Richtersius coronifer*, *Macrobiotus* cf. *hufelandi* and *Halobiotus crispae*. Inset shows an unidentified compound (retention time t_R=10.36 min) that increases more than twofold in absolute concentration in dehydrated *R. coronifer*. For details of cation column, eluent and flow rate, see Materials and methods; injection volume, 60 μl. nOAp, negative organic acid peak.

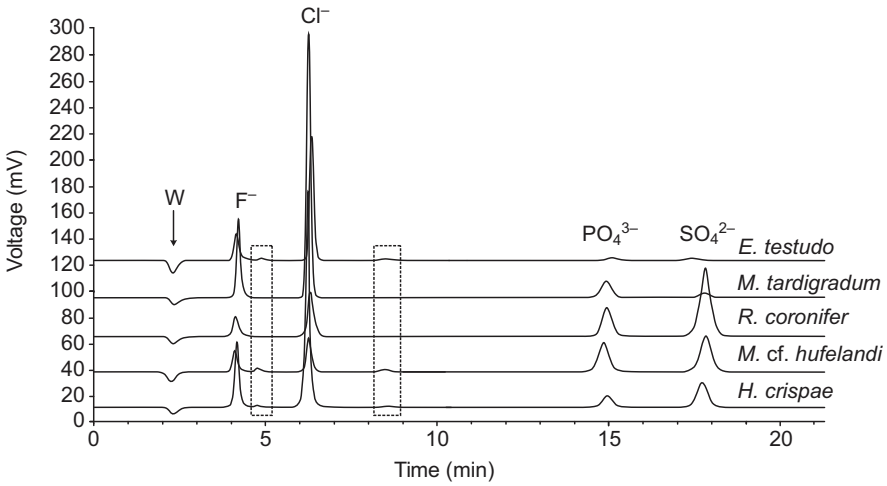

Fig. 2. Representative chromatograms showing the principal anions present in hydrated animals of each investigated species. Dashed rectangles indicate unidentified compounds (t_R=4.74 min and t_R=7.24 min). For details of anion column, eluent and flow rate, see Materials and methods; injection volume, 60 µl. W, negative water peak.

a disposable syringe filter (Sartorius AG, Göttingen, Germany). The samples were subsequently frozen at –20°C if not quantified immediately. A total of five to seven samples (*n*) were prepared for each species (Table 2).

The ionic composition of the external media, i.e. moss water (MW) and SW, was additionally determined. Moss samples were rehydrated in ddH_2O for several hours, and MW samples were subsequently collected from between the leaf-covered stems. SW samples were prepared by diluting SW (1:200) collected at the locality. Samples were analyzed in triplicate using both vapor pressure osmometry (Vapro 5520, Wescor Inc., UT, USA) and HPLC (Table 3).

In order to document changes in inorganic ion content between hydrated, active and dehydrated anhydrobiotic animals, samples of dehydrated *R. coronifer* were additionally prepared. Six groups each consisting of 75 animals (Table 2) were transferred to 1.5 ml Eppendorf tubes (Eppendorf AG, Hamburg, Germany), and excess water was removed by blotting the animals with tissue paper. The animals were subsequently allowed to dehydrate in the tubes over the ensuing 24 h at room temperature and humidity. Following complete dehydration, tissue paper saturated with ddH_2O was used to rinse the surface of the animals to remove potential solutes extruded on the surface of the animals, and dry tissue paper was used to remove excess moisture. A volume of 90 µl of cation eluent was added to each sample, and the samples were immediately homogenized. The samples were then prepared as described above. Data (mg l^{-1}) from this experiment were directly compared with those of hydrated animals (Table 4, Fig. 3B), as the two sets of samples contained a near-identical number of animals per unit volume (i.e. 0.82 and 0.83 animals µl^{-1} eluent, respectively), which circumvented the need for recalculation (see below). In order to test whether *R. coronifer* actually produced viable tuns during the above-mentioned conditions, post-anhydrobiotic survival was assessed. Using four groups of 50 specimens, a survival rate of 94±4% (mean ± s.d.) was observed, which is comparable to previous reports on the survival rate of *R. coronifer* (Persson et al., 2011).

Calculation of ion concentration

The volume of each investigated species was calculated according to an modified version of a previously described method (Halberg et al., 2009). In brief, micrographs of *N*=20 animals of each species were acquired, using a digital camera (C-5050, Olympus, Tokyo, Japan) mounted on an Olympus BX 51 microscope, and length (*h*) and width (2*r*) of the trunk and hindlegs (measurements on the hindlegs were used as representative for all legs) were measured along the middle plane for each specimen, using the Olympus DP-soft imaging software (DP-soft, Soft Imaging System, Münster, Germany). The fluid volume of an individual tardigrade of each species was then calculated using Eqn 1, approximating the geometric shape of the trunk and legs as a cylinder and adjusting the volume of liquid according to the gravimetrically measured water content:

$$V_{\text{individual}} = \pi\,(r^2_{\text{trunk}} h_{\text{trunk}} + 8r^2_{\text{leg}} h_{\text{leg}}) \times W\,, \qquad (1)$$

where $V_{\text{individual}}$ is the volume of an individual tardigrade, r is the radius and h the length of the trunk and hindlegs, while W (0.72, mean gravimetrically measured fractional water content of *R. coronifer* and *H. crispae*) (Westh and Kristensen, 1992; Halberg et al., 2009) is an estimate of the fractional water content. Using these data (Table 1), the total tardigrade test volume was calculated by multiplying the calculated average volume of N = 20 individuals

Table 1. Volume estimations

	Trunk (µm)		Legs (µm)		$V_{\text{individual}}$ (nl) (Eqn 1)
	h	2*r*	*h*	2*r*	
Echiniscus testudo	287±46	133±20	34±9	32±5	3.1±1.3
Milnesium tardigradum	687±79	178±16	52±9	48±8	13.1±3.7
Richtersius coronifer	605±68	188±17	57±13	44±7	12.2±2.6
Macrobiotus cf. *hufelandi*	517±47	147±16	45±6	43±5	6.4±1.8
Halobiotus crispae	408±56	132±12	57±10	40±6	4.1±1.3

Average values of length (*h*) and width (2*r*) of the trunk and legs of *N*=20 animals, as well as the calculated average volume (Eqn 1), of each investigated species.
Data are expressed as means ± s.d.

Table 2. Sample data

	n	*N*	*n*×*N*	V_{total} (µl) (Eqn 2)	*F* (µl)	*D* (Eqn 3)
Echiniscus testudo	5	80	400	0.25	140.25	561
Milnesium tardigradum	6	50	300	0.63	140.63	223
Richtersius coronifer (H)	7	225	1575	2.75	277.75	101
Richtersius coronifer (D)	6	75	450	–	–	–
Macrobiotus cf. *hufelandi*	6	100	600	0.64	140.64	219
Halobiotus crispae	6	40	240	0.17	90.17	530

The number of samples (*n*), number of animals per sample (*N*), total number of animals used (*n*×*N*), total tardigrade test volume (V_{total}, Eqn 2), final volume (*F*), as well as the resulting dilution factor (*D*, Eqn 3) are listed.

Data from both hydrated (H) and dehydrated (D) samples of *R. coronifer* are shown; however, as dehydrated animals have no measurable water content, values for V_{total}, *F* and *D* cannot be listed.

with the number of animals included in the sample according to Eqn 2:

$$V_{total} = V_{individual} \times N , \quad (2)$$

where V_{total} is the total tardigrade test volume and *N* is the number of animals included in the sample. Lastly, the concentrations of the dominant cations and anions in the investigated species of tardigrades were calculated by multiplying the measured ion concentrations by the dilution factor, which was calculated according to Eqn 3:

$$D = F / V_{total} , \quad (3)$$

where *D* is the dilution factor and *F* is the final volume (i.e. volume of cation eluent the tardigrades were transferred to + V_{total}). Sample information for the respective species is listed in Table 2.

Nanoliter osmometry

The total osmotic concentration of tardigrades from each investigated species was measured using nanoliter osmometry. This was done in order to measured the fraction of the total osmotic concentration that the identified inorganic ions constitute in each species (Table 3). Using the same procedure for removing excess water as described above, individual specimens were transferred into sample wells (loaded with oil type B, viscosity 1250 cSt ±10%; Cargille Laboratories, Cedar Grove, NJ, USA) of a calibrated nanoliter osmometer (Clifton Technical Physics, Hartford, NY, USA), and the osmolality (mOsm kg^{-1}) was determined by freezing point depression (FPD, –1.86°C kg mol^{-1}). The nanoliter osmometer was calibrated with ddH_2O (0 mmol kg^{-1}) and with 290 and 1000 mmol kg^{-1} standards (Wescor, Logan, UT, USA) prior to use. Six to 10 animals of each species were used in this experiment (Table 3).

Statistics

Differences in the respective ion concentrations as well as in total solutes between species were tested using one-way ANOVA followed by Tukey's multiple comparisons of means, with significance levels as indicated (*P<0.05, **P<0.01 and ***P<0.001; P>0.05 was not significant, n.s.) (see supplementary material Table S1). In addition, significant changes in the individual inorganic ion concentrations between active and anhydrobiotic *R. coronifer* were tested using an unpaired, two-sample *t*-test with

Table 3. Ionic composition and total osmotic concentration of the investigated species of tardigrades

	MW	*Echiniscus testudo*	*Milnesium tardigradum*	*Richtersius coronifer*	*Macrobiotus* cf. *hufelandi*	SW	*Halobiotus crispae*
Cations (mmol l^{-1})							
Na^+	0.19±0.03	86±12	150±25	29±5	14±3	280±15	257±36
NH_4^+	0.38±0.07	21±4	15±6	11±5	6±2	–	31±7
K^+	0.86±0.12	36±4	73±12	22±4	19±2	7±1	42±12
Ca^{2+}	0.68±0.10	26±6	59±9	23±3	32±3	10±3	102±9
Mg^{2+}	0.06±0.06	2±1	13±2	7±1	8±1	31±5	64±17
Total cations (mmol l^{-1})	2.17	171	310	92	79	328	496
Total cations (mequiv l^{-1})	2.91	199	382	122	119	369	662
Anions (mmol l^{-1})							
F^-	0.05±0.00	30±6	52±16	7±3	8±4	0.3±0.1	39±16
Cl^-	2.58±0.40	140±19	126±11	48±15	42±8	278±11	280±19
PO_4^{3-}	0.01±0.01	7±2	25±5	16±5	28±4	–	61±11
SO_4^{2-}	0.10±0.01	3±2	3±1	15±4	12±2	17±2	22±10
Total anions (mmol l^{-1})	2.74	150	154	79	82	295	363
Total anions (mequiv l^{-1})	2.86	197	259	133	158	312	546
Ionic conc. (mmol l^{-1})	4.91 (3)	321(5)	464 (6)	171 (7)	161 (6)	623 (3)	859 (6)
Total osmotic conc. (mOsm kg^{-1})	4.33±0.58 (3)	507±36 (6)	769±33 (6)	361±49 (10)	524±31 (10)	632±6 (3)	961±43 (8)
Osmotic deficit (mmol l^{-1})	0.58	186	305	190	363	9	102
Charge deficit (mequiv l^{-1})	0.05 [–]	2 [–]	123 [–]	11 [+]	39 [+]	57 [–]	116 [–]

Concentrations of cations and anions detected in each investigated species, as well as in moss water (MW) and 20‰ salt water (SW) samples, and the corresponding total osmotic concentration. In addition, the osmotic deficit (calculated as the difference between ionic concentration and total osmotic concentration) and the calculated charge deficit (calculated as the difference between positive and negative charges) are listed; the polarity of the charge deficit is indicated in square brackets.

Numbers in parentheses indicate the number of samples tested. Data are expressed as means ± s.d.

Table 4. Changes in ionic composition during dehydration of *Richtersius coronifer*

	Hydrated	Dehydrated	
Cations			
Na^+	6.3±1.2	6.9±1.6	n.s.
NH_4^+	1.8±0.9	0.6±0.1	**
K^+	8.1±1.7	6.6±0.6	*
Ca^{2+}	8.7±1.2	8.9±2.4	n.s.
Mg^{2+}	1.7±0.2	1.9±0.6	n.s.
Total cations (mg l^{-1})	26.6	25.0	
Anions			
F^-	1.2±0.5	0.6±0.4	n.s.
Cl^-	16.4±5.3	19.8±2.0	*
PO_4^{3-}	15.0±4.2	12.3±2.5	n.s.
SO_4^{2-}	13.8±3.9	13.0±3.4	n.s.
Total anions (mg l^{-1})	46.4	45.8	
Ionic conc. (mg l^{-1})	73.0 (7)	70.8 (6)	

Concentration of the respective cations and anions measured in hydrated, active *R. coronifer* specimens compared with dehydrated anhydrobiotic *R. coronifer*.

Numbers in parentheses indicate the number of samples tested. Data are expressed as means ± s.d.

Significant differences in the concentrations of the respective inorganic ion concentrations were tested using an unpaired, two-sample *t*-test; significance levels are indicated (*P<0.05, **P<0.01; n.s., not significant, P>0.05).

corresponding significance levels (see Table 4). The statistical analyses were performed using the data analysis software OriginPro 7.5 (OriginLab, Northhampton, MA, USA).

RESULTS

Ionic composition and total osmotic concentration in tardigrades

Our HPLC and nanoliter osmometry analyses revealed that there are significant differences in the concentrations of the different ions as well as in the total osmotic concentrations between the species (supplementary material Table S1). Total osmotic concentration ranges from 361±49 mOsm kg^{-1} in *R. coronifer* to 961±43 mOsm kg^{-1} in *H. crispae* (Table 3), indicating that both limno-terrestrial and marine species are hyperosmotic with respect to the external medium. Comparison of the total osmotic concentrations with the calculated total ionic concentrations (Table 3) reveals that there is a large 'osmotic deficit', especially in the limno-terrestrial cryptobiotic species *E. testudo*, *M. tardigradum*, *R. coronifer* and *M.* cf. *hufelandi* (Fig. 3A, Table 3). Furthermore, in most species the measured positive and negative charges do not entirely maintain electroneutrality (Table 3). This charge deficit indicates that ionic components remain unidentified, especially in *M. tardigradum* and *H. crispae*, and that these unidentified ions contribute to the observed osmotic deficit. The charge deficit appears to account for the entire osmotic deficit in *H. crispae* (Table 3); however, even when taking the charge deficits into account, a large fraction of unidentified osmolytes remains unaccounted for (~37–69% of the total osmotic concentration) in the limno-terrestrial cryptobiotic species (Fig. 3A, Table 3). These differences are likely to be even greater than indicated in Table 3, as our ionic data are not corrected according to the osmotic activity coefficients of the respective ions.

Na^+ and Cl^- are the principal inorganic ions of tardigrade fluids, accounting for 11–56% of the total osmotic concentration in all investigated species; *M.* cf. *hufelandi* and *H. crispae* contain the lowest and highest concentrations, respectively (Fig. 3A, Table 3).

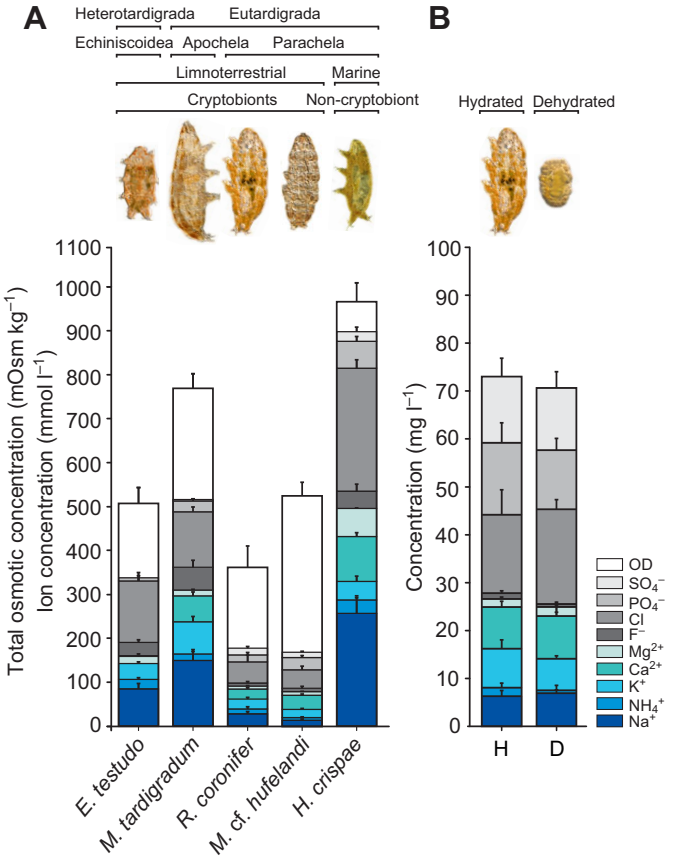

Fig. 3. Graphical representation of the respective ion contributions to total osmotic concentration in the investigated species of tardigrades.
(A) Concentration of the respective cations and anions measured in each investigated species by HPLC (mmol l^{-1}), as well as the corresponding total osmotic concentration (mOsm kg^{-1}), as measured by nanoliter osmometry. OD represents the osmotic deficit. The phylogenetic position and habitat preference of each species is listed. The light micrographs of the animals are shown to scale (see Table 1 for average species size).
(B) Concentration of the respective cations and anions measured in hydrated (H), active specimens compared with dehydrated (D) anhydrobiotic *R. coronifer*. Data are expressed as means ± s.d.

There are notable differences in the Na^+/Cl^- ratio among the species, i.e. the ratio is less than unity in the limno-terrestrial herbivores *E. testudo* (0.61), *R. coronifer* (0.60) and *M.* cf. *hufelandi* (0.33), higher than unity in the limno-terrestrial predator *M. tardigradum* (1.19) and close to unity in the marine herbivore *H. crispae* (0.92).

Compared with Na^+ and Cl^-, the $[K^+]$ is relatively low in all species, ranging from 19 to 73 mmol l^{-1} (Table 3). The Na^+/K^+ ratio is higher than unity in *E. testudo* (2.39), *M. tardigradum* (2.05), *R. coronifer* (1.32) and *H. crispae* (6.12), but lower than unity in *M.* cf. *hufelandi* (0.77). The highest absolute $[K^+]$ is found in the predator *M. tardigradum*, which is ~2–3 times the concentration of herbivorous species (Table 3).

In general, a high $[Ca^{2+}]$ seems characteristic of tardigrade fluids, ranging from 23 to 102 mmol l^{-1}, with the highest concentration measured in the marine tardigrade *H. crispae* (Table 3). In contrast, $[Mg^{2+}]$ is comparatively low, especially in the limno-terrestrial species, with concentrations of 2–64 mmol l^{-1} detected (Table 3). Interestingly, the $[Mg^{2+}]$ does not differ between the limno-terrestrial species, but it is significantly higher in *H. crispae* (F=63.06, P<0.001, d.f.=4, N=30; supplementary material Table S1). The high Mg^{2+} content of *H. crispae* probably relates to the high $[Mg^{2+}]$ of

SW (Table 3). NH_4^+ also contributes to the total osmotic concentrations measured in all species, with values of 6–31 mmol l^{-1} detected (Table 3).

Substantial [F^-] was detected in all tardigrade samples, ranging from 7 to 52 mmol l^{-1} (Table 3). Considerable [PO_4^{3-}] and [SO_4^{2-}] were also measured in all tardigrade species, ranging from 7 to 61 mmol l^{-1} and 3 to 22 mmol l^{-1}, respectively, with the highest concentrations detected in the marine species *H. crispae* (Fig. 3A, Table 3).

In addition to the identified inorganic ions, several unidentified peaks were observed (Figs 1, 2). An unidentified cationic compound having a t_R of 10.36 min was observed in both *R. coronifer* and *M.* cf. *hufelandi* (Fig. 1), which was shown to more than double in concentration in dehydrated *R. coronifer* (Fig. 1, inset). Two additional unidentified anionic compounds, having a t_R of 4.74 and 7.24 min, respectively, were observed in *E. testudo*, *M.* cf. *hufelandi* and *H. crispae* (Fig. 2).

Changes in ionic composition during dehydration

The concentrations of the examined ions showed little variation from the active to the anhydrobiotic state in specimens of *R. coronifer* (Fig. 3B, Table 4). Excluding small yet significant changes in the K^+ and Cl^- content (Table 4), only NH_4^+ was significantly reduced from the hydrated to the dehydrated state (t=3.44, d.f.=10, P=0.0063). NH_4^+ is a waste product of protein catabolism; hence, the observed reduction in NH_4^+ could reflect the general shut-down of metabolic processes during the dehydration process. However, because NH_4^+ is a weak acid that easily converts to NH_3 depending on the pH of the solution, the measured biological concentration is no more than tentative.

DISCUSSION

Ionic composition in tardigrades

In the present study, we obtained data on the ionic composition of five different species of tardigrades, representing members of Heterotardigrada (Echiniscoidea) and Eutardigrada (Apochela and Parachela), four evolutionarily distant families (Echiniscidae, Milnesiidae, Macrobiotidae and Isohypsibiidae), as well as both limno-terrestrial and marine habitats. Our analyses of osmotic and ionic concentrations were performed on homogenates of the investigated species, and provide a much needed indication of osmolyte composition in Tardigrada, fundamental to our understanding of tardigrade physiology. In the following, we will discuss the ionic composition of the respective species in relation to their systematic position and habitat preference, and also in relation to data from representatives of phylogenetically related groups (i.e. other members of Panarthropoda) living in habitats with comparable osmotic pressure (Table 5).

Echiniscus testudo (Heterotardigrada: Echiniscidae) belongs to a different evolutionary lineage from the other species included in our study. Compared with the limno-terrestrial parachelan eutardigrades, the ionic composition of this heterotardigrade is characterized by a large contribution of Na^+ and Cl^- (~45%), and a very low contribution of Mg^{2+} (0.4%), SO_4^{2-} (0.6%) and PO_4^{3-} (1.4%) (Table 5). The large contribution of Na^+ and Cl^- to total osmotic concentration, which is comparable to that seen in the secondarily marine eutardigrade *H. crispae* (Table 5), could reflect the marine origin of tardigrades (Jørgensen et al., 2010).

The family Milnesiidae, represented by the predator *M. tardigradum*, is currently considered the sister group of all other eutardigrades (Guidetti et al., 2009). *Milnesium tardigradum* contains the highest total osmotic as well as ionic concentration among the limno-terrestrial species, with conspicuously high levels of both K^+ and F^- (Table 3). The high [K^+] in *M. tardigradum* compared with that of the phytophagous species was somewhat surprising, as e.g. carnivorous insects generally contain low levels of K^+ (Sutcliffe, 1962). Conversely, phytophagous tardigrades are known to feed on bryophytes that are typically high in K^+ and low in Na^+ (Brown and Buck, 1979), and were therefore, analogous to phytophagous insects (Sutcliffe, 1962), expected to reflect this

Table 5. Comparison of ion contributions in tardigrades and related phyla

Species	External osmotic conc. (mOsm kg^{-1})	Internal osmotic conc. (mOsm kg^{-1})	Osmotic contribution (%)								Reference
			Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	SO_4^{2-}	PO_4^{3-}	HCO_3^-	
Halobiotus crispae (Tardigrada)	632	961	26.7	4.4	10.6	6.7	29.1	2.3	6.3	–	This study
Gammarus oceanicus (Crustacea)	646	940	32.9	2.7	5.4	2.1	31.2	–	–	–	Normant et al., 2005
Echiniscus testudo (Tardigrada)	4.91	351	17.0	7.1	5.1	0.4	27.6	0.6	1.4	–	This study
Milnesium tardigradum (Tardigrada)	4.91	769	19.5	9.5	7.7	1.7	16.4	0.4	3.3	–	This study
Richtersius coronifer (Tardigrada)	4.91	361	8.0	6.1	6.4	1.9	13.3	4.2	4.4	–	This study
Macrobiotus cf. *hufelandi* (Tardigrada)	4.91	524	2.7	3.6	6.1	1.5	8.0	2.3	5.3	–	This study
Macrobrachium rosenbergii (Crustacea)	8.00	425	75.3	2.2	5.6	0.8	–	–	–	–	Wilder et al., 1998
Aeshna grandis – larvae (Insecta)	2.25	395	36.7	2.3	1.9	1.9	27.8	–	1.0	3.8	Sutcliffe, 1962
Peripatus acacioi (Onychophora)	TR	198	46.9	1.7	1.7	0.2	45.0	–	2.3	3.0	Campiglia, 1976

Total osmotic concentration of the external and internal fluids, as well as the osmotic contribution of the respective ions to the total internal concentration in each of the investigated species of tardigrades.

Corresponding data on hemolymph concentration and composition of selected species of crustaceans, insects and onychophorans are included for comparative purposes.

TR, terrestrial; –, not measured.

relative ion composition in their extracellular body fluids. Notably, K^+ contributes 3.6–9.5% of total hemolymph concentration in tardigrades (Table 5), which is similar to the 2–10% observed in insects (Sutcliffe, 1963).

Milnesium tardigradum is also the species in which the highest [F^-] was detected. This ion is typically found in hard tissue, such as bones of vertebrates and exoskeletons of invertebrates, and is found in extremely high levels in the cuticle of marine crustaceans, for example (Adelung et al., 1987; Sands et al., 1998). In contrast, the [F^-] in blood/hemolymph and soft tissue is negligible (Adelung et al., 1987; Sands et al., 1998). Therefore, the [F^-] of our samples likely reflect ions bound to cuticular structures, and is accordingly not an ion that contributes to the dissolved anionic fraction. Indeed, high levels of dissolved F^- are deleterious to enzymatic function (Eagers, 1969). In line with this suggestion, the observed differences in [F^-] could relate to differences in cuticle composition, with a lower F^- content in members of Macrobiotiidae (*R. coronifer* and *M.* cf. *hufelandi*) compared with other species (Table 3). Interestingly, the relative ion contributions to total osmotic concentration suggests that *M. tardigradum* resembles the heterotardigrades more than the other limno-terrestrial eutardigrades (e.g. high Na^+ and Cl^- concentrations).

Sutcliffe argued that definitive types of hemolymph in insects are related to the systematic position within Insecta (Sutcliffe, 1962; Sutcliffe, 1963). Apart from a significant difference in [PO_4^{3-}] (F=63.30, P<0.05, d.f.=4, N=33), the concentrations of the remaining ions in *R. coronifer* and *M.* cf. *hufelandi* (Eutardigrada: Macrobiotidae) are not significantly different (supplementary material Table S1). Moreover, the ionic contributions to the total osmotic concentrations are remarkably similar (Table 5). As a testable hypothesis, these similarities suggest that the relative ion composition among the species at least in part relates to phylogeny and the systematic position in Tardigrada (although habitat preference evidently is also important). As compared with the other groups of limno-terrestrial tardigrades, the inorganic ion content of *R. coronifer* and *M.* cf. *hufelandi* is characterized by a relatively small concentration of Na^+ (~2–7 times lower) and Cl^- (~2–3 times lower), and, conversely, a relatively large contribution of SO_4^{2-} (~4–10 times higher).

In general, the ionic composition of the marine tardigrade *H. crispae* (Eutardigrada: Isohypsibiidae) is distinct from those of the limno-terrestrial species. Specifically, Na^+ and Cl^- account for more than 50% of the total osmotic concentration, and the divalent cations (Ca^{2+} and Mg^{2+}) were detected in conspicuously high concentrations (Tables 3, 5). Although correspondingly high [Mg^{2+}] have also been reported for marine crustaceans (Tentori and Lockwood, 1990), it should be taken into account that some ions (e.g. Ca^{2+} and Mg^{2+}) are known to form complexes with proteins and other macromolecules *in vivo*. For example, the stylet and stylet supports of tardigrades are known to be composed of $CaCO_3$ (Bird and McClure, 1997; Guidetti et al., 2012), which suggests that the [Ca^{2+}] in tardigrade fluids is likely to be lower than indicated (Table 3).

The highest total osmotic and ionic concentrations were measured in *H. crispae* (Table 3). In contrast to the limno-terrestrial species, the total osmotic concentration of *H. crispae* is almost exclusively accounted for by the measured total ionic concentration (Fig. 3, Table 3). In fact, the negative charge deficit observed in *H. crispae* (and *M. tardigradum*) indicates that additional ionic components remain unidentified in this species. It is therefore unlikely that *H. crispae* contains large amounts of organic solutes. In most animals, HCO_3^- is important for pH regulation of the extracellular fluid (e.g. Melzner et al., 2009); hence, this ion is likely to account for some of the negative charge deficit observed in *H. crispae*.

The contribution of the total diffusible ions to the total osmotic concentration of tardigrades appears roughly similar to that of the hemolymph of arthropods and, to a lesser extent, that of onychophorans (Table 5). Albeit this comparison suffers from a paucity of data, it indicates that tardigrades possess a hemolymph roughly similar in inorganic ion composition to that of closely related groups, i.e. other members of Panarthropoda. This is increasingly evident when considering that Na^+ (predominantly an extracellular ion) is expected to make a larger contribution, whereas K^+ and Ca^{2+} (mainly intracellular ions) are expected to make a smaller contribution to the total osmotic concentration of the hemolymph compared with that of the entire animal (Table 5).

It is relevant to compare concentrations of ions in tardigrade body fluids with those of the respective external media (Table 3). The strongest ability to concentrate ions is seen in the limno-terrestrial species, which are hyperosmotic with respect to the external environment by as much as ~350–750 mOsm kg^{-1} (Fig. 3A, Table 3). The marine *H. crispae* maintains a hemolymph osmotic pressure ~300 mOsm kg^{-1} above that of the environment, yet does so over a much larger range of external salinities compared with the limno-terrestrial *R. coronifer* (Halberg et al., 2009; Møbjerg et al., 2011). Tardigrade locomotion depends on the antagonistic hydrostatic pressure of the body cavity (Kinchin, 1994); hence, tardigrades must maintain internal osmotic pressure above that of the surroundings to facilitate locomotion. It should be emphasized that the MW samples were acquired by rehydrating moss samples with ddH_2O, and not as precipitation from the locality; however, as atmospheric precipitation generally contains few dissolved particles, with an osmotic concentration of less than 400 µmol l^{-1} (Granat, 1972), this is unlikely to be of any consequence. Accordingly, Na^+ is concentrated by a factor of ×70–800, K^+ by ×20–90, Ca^{2+} and Mg^{2+} by ×30–200, whereas F^- is concentrated by ×160–1040, Cl^- by ×20–50, PO_4^{3-} by ×700–2800 and SO_4^{2-} by ×30–150 in limno-terrestrial tardigrades. In contrast, Na^+, Cl^- and SO_4^{2-} are basically in ionic equilibrium with respect to the external SW in *H. crispae*, while K^+, Ca^{2+}, Mg^{2+} and F^- are concentrated by a factor of ×2–10. The large osmotic and ionic gradients maintained by both limno-terrestrial and marine species are indicative of powerful ion-retentive mechanisms in Tardigrada – functions that presumably are maintained by such organ systems as the Malpighian tubules (only found in eutardigrades) (e.g. Møbjerg and Dahl, 1996) and the gut system (Halberg et al., 2009; Halberg and Møbjerg, 2012).

Changes in ionic composition during dehydration

Desiccation is the most severe form of osmotic stress, and tardigrades are among the most desiccation-tolerant animals on Earth (Møbjerg et al., 2011). As anhydrobiotic tardigrades dehydrate, liquid water is slowly reduced to immeasurable levels (Westh and Ramløv, 1991), which leads to a dramatic increase in dissolved particles and osmotic pressure of the body fluids. In general, the basis for osmotic stress tolerance involves the active extrusion of inorganic ions combined with the accumulation of organic ‘compatible’ osmolytes, thus serving the function of protecting the integrity of DNA and proteins (Yancey, 2005). With this in mind, it is surprising that *R. coronifer* does not exclude inorganic ions during dehydration (Fig. 3B, Table 4). Evidenced by the large osmotic deficit that appears to be restricted to cryptobiotic tardigrades (Fig. 3A, Table 3), it is tempting to suggest that a large quantity of organic osmolytes are constitutively synthesized in these species, thus enabling the animals to respond quickly to decreases in external water potential. Organic

osmolytes can be accumulated in large amounts without perturbing cellular function, hence the term 'compatible', and are known to stabilize macromolecular structures by direct interaction with proteins and membrane lipids (Crowe et al., 1987; Hincha and Hagemann, 2004; Yancey, 2005). In the light of the continuous dehydration–rehydration cycles that may occur in limno-terrestrial habitats, such a strategy therefore seems favorable, and is additionally supported by the short time span (<10 min) with which tardigrades can enter the tun stage successfully. However, *de novo* synthesis of solutes during dehydration of cryptobiotic tardigrades has also been reported (Westh and Ramløv, 1991; Hengherr et al., 2008; Jönsson and Persson, 2010), and is indirectly confirmed in our study by the observed doubling in absolute concentration of an (unidentified) solute following dehydration of *R. coronifer* (Fig. 1, inset).

Cryptobiotic species contain a large fraction of unidentified solutes

As the osmotic deficits noted above appear restricted to cryptobiotic species, it is reasonable to assume that this fraction of unidentified organic osmolytes includes compounds associated with cryptobiotic survival. Organic osmolytes fall into a few major categories (sugars, polyols, amino acids and various derivatives), and are universally exploited by both plants and animals (Yancey, 2005). Several chemical compounds belonging to these groups have been shown to provide protective functions during osmotic stress (Hincha and Hagemann, 2004; Yancey, 2005). The demonstration of survival during extreme osmotic conditions makes tardigrades especially interesting for the identification of osmolytes with protective functions, as well as for the study of their physio-chemical mechanisms (Halberg et al., 2009; Møbjerg et al., 2011). The non-reducing sugar trehalose has already received much attention (Westh and Ramløv, 1991; Hengherr et al., 2008; Jönsson and Persson, 2010). However, trehalose is only present in minute quantities in active tardigrades (e.g. 0.1% dry mass in *R. coronifer*), and is maximally accumulated to 2.9% of dry mass in *Macrobiotus islandicus* (Jönsson and Persson, 2010). Accordingly, trehalose cannot account for the observed osmotic deficits demonstrated in the present study, although it may be sufficient to confer protection against dehydration in the investigated members of Macrobiotidae. In fact, cell concentration of trehalose for maximal cell survival during dehydration of murine fibroblasts is about 5.3×10^{10} molecules cell^{-1} (Chen et al., 2001). Assuming that this also applies to tardigrade cells and that the cell number of *R. coronifer* is comparable to that of *H. crispae* (1058±53 cells animal^{-1} excluding gametes) (Møbjerg et al., 2011), the estimated number of trehalose required would be 5.6×10^{13} molecules animal^{-1}. Trehalose is accumulated to 2.3% of dry mass in *R. coronifer* (Westh and Ramløv, 1991), and using the measured dry mass of 2.9 μg animal^{-1} (Westh and Ramløv, 1991), a single individual accumulates on average 6.67×10^{-8} g trehalose during desiccation. With a molecular mass of 342.3 g mol^{-1} this corresponds to 1.96×10^{-10} mol animal^{-1}, and by multiplying by Avogadro's constant (6.023×10^{23} molecules mol^{-1}) this equals 1.18×10^{14} trehalose molecules animal^{-1}, which is twice the estimated number of molecules required for protection against dehydration. In fact, the calculated number of trehalose molecules is probably higher than that required, as tardigrade cells are much smaller than murine fibroblasts, and therefore have a smaller total area of membrane to protect. However, as previously mentioned, trehalose is only accumulated in significant amounts in selected species (Hengherr et al., 2008; Jönsson and Persson, 2010), which suggests that a diverse pattern of dehydration mechanisms has evolved in Tardigrada. The mechanisms for protection of cells and tissues during other forms of cryptobiosis (e.g. cryobiosis) may moreover be distinct from those of anhydrobiosis (Møbjerg et al., 2011). Accordingly, these as yet unidentified biochemical pathways would have to be recognized and characterized in order to obtain a detailed insight into the metabolic and chemical mechanisms of cryptobiosis.

ACKNOWLEDGEMENTS

We would like to thank Anne Lise Maarup for technical assistance and Reinhardt Møbjerg Kristensen (Natural History Museum of Denmark) for loan of the Olympus BX 51 microscope. Emma Tinnert and Dave Karlsson, Station Linné (Porten till Alvaret), Ölands Skogsby, Sweden, are warmly thanked for accommodation during sampling of tardigrades.

FUNDING

Funding came from the Carlsberg Foundation and the Freja-Programme (Faculty of Science, University of Copenhagen).

REFERENCES

Adelung, D., Buchholz, F., Culik, B. and Keck, A. (1987). Fluoride in tissues of krill *Euphausia superba* Dana and *Meganyctiphanes norvegica* M. Sars in relation to the moult cycle. *Polar Biol.* **7**, 43-50.

Bird, A. F. and McClure, S. G. (1997). Composition of the stylets of the tardigrade, *Macrobiotus* cf. *pseudohufelandi*. *Trans. R. Soc. S. Aust.* **121**, 43-50.

Brown, D. H. and Buck, G. W. (1979). Desiccation effects and cation distribution in bryophytes. *New Phytol.* **82**, 115-125.

Campiglia, S. (1976). The blood of *Peripatus acacioi* Marcus & Marcus (Onychophora) – III. The ionic composition of the hemolymph. *Comp. Biochem. Physiol.* **54A**, 129-133.

Chen, T., Acker, J. P., Eroglu, A., Cheley, S., Bayley, H., Fowler, A. and Toner, M. (2001). Beneficial effect of intracellular trehalose on the membrane integrity of dried mammalian cells. *Cryobiology* **43**, 168-181.

Clegg, J. S. (2001). Cryptobiosis – a peculiar state of biological organization. *Comp. Biochem. Physiol.* **128B**, 613-624.

Crowe, J. H., Crowe, L. M., Carpenter, J. F. and Aurell Wistrom, C. (1987). Stabilization of dry phospholipid bilayers and proteins by sugars. *Biochem. J.* **242**, 1-10.

Eagers, R. Y. (1969). *Toxic Properties of Inorganic Fluoride Compounds*. New York, NY: Elsevier.

Förster, F., Liang, C., Shkumatov, A., Beisser, D., Engelmann, J. C., Schnölzer, M., Frohme, M., Müller, T., Schill, R. O. and Dandekar, T. (2009). Tardigrade workbench: comparing stress-related proteins, sequence-similar and functional protein clusters as well as RNA elements in tardigrades. *BMC Genomics* **10**, 469.

Förster, F., Beisser, D., Grohme, M. A., Liang, C., Mali, B., Siegl, A. M., Engelmann, J. C., Shkumatov, A. V., Schokraie, E., Müller, T. et al. (2012). Transcriptome analysis in tardigrade species reveals specific molecular pathways for stress adaptations. *Bioinform. Biol. Insights* **6**, 69-96.

Granat, L. (1972). On the relation between pH and the chemical composition in atmospheric precipitation. *Tellus* **24**, 550-560.

Guidetti, R., Schill, R. O., Bertolani, R., Dandekar, T. and Wolf, M. (2009). New molecular data for tardigrade phylogeny, with the erection of *Paramacrobiotus* gen. nov. *J. Zoolog. Syst. Evol. Res.* **47**, 315-321.

Guidetti, R., Altiero, T. and Rebecchi, L. (2011). On dormancy strategies in tardigrades. *J. Insect Physiol.* **57**, 567-576.

Guidetti, R., Altiero, T., Marchioro, T., Amadé, L. S., Avdonina, A. M., Bertolani, R. and Rebecchi, L. (2012). Form and function of the feeding apparatus in Eutardigrada (Tardigrada). *Zoomorphology* **131**, 127-148.

Halberg, K. A. and Møbjerg, N. (2012). First evidence of epithelial transport in tardigrades: a comparative investigation of organic anion transport. *J. Exp. Biol.* **215**, 497-507.

Halberg, K. A., Persson, D., Ramløv, H., Westh, P., Kristensen, R. M. and Møbjerg, N. (2009). Cyclomorphosis in Tardigrada: adaptation to environmental constraints. *J. Exp. Biol.* **212**, 2803-2811.

Halberg, K. A., Persson, D., Jørgensen, A., Kristensen, R. M. and Møbjerg, N. (2013). Ecology and thermal tolerance of the marine tardigrade *Halobiotus crispae* (Eutardigrada: Isohypsibiidae). *Mar. Biol. Res.* doi: 10.1080/17451000.2013.765577.

Hengherr, S., Heyer, A. G., Köhler, H. R. and Schill, R. (2008). Trehalose and anhydrobiosis in tardigrades – evidence for divergence in response to dehydration. *FEBS J.* **275**, 281-288.

Hincha, D. K. and Hagemann, M. (2004). Stabilization of model membranes during drying by compatible solutes involved in the stress tolerance of plants and microorganisms. *Biochem. J.* **383**, 277-283.

Hronowski, L. and Armstrong, J. B. (1977). Ion composition of the plasma of *Ambystoma mexicanum*. *Comp. Biochem. Physiol.* **58A**, 181-183.

Jönsson, K. I. and Persson, O. (2010). Trehalose in three species of desiccation tolerant tardigrades. *Open Zool. J.* **3**, 1-5.

Jönsson, K. I., Rabbow, E., Schill, R. O., Harms-Ringdahl, M. and Rettberg, P. (2008). Tardigrades survive exposure to space in low Earth orbit. *Curr. Biol.* **18**, R729-R731.

Jørgensen, A., Faurby, S., Hansen, J. G., Møbjerg, N. and Kristensen, R. M. (2010). Molecular phylogeny of Arthrotardigrada (Tardigrada). *Mol. Phylogenet. Evol.* **54**, 1006-1015.

Keilin, D. (1959). The problem of anabiosis or latent life: history and current concept. *Proc. R. Soc. Lond. B Biol. Sci.* **150**, 149-191.
Kinchin, I. M. (1994). *The Biology of Tardigrades*. London: Portland Press Ltd.
Kristensen, R. M. (1982). The first record of cyclomorphosis in Tardigrada based on a new genus and species from Arctic meiobenthos. *J. Zoolog. Syst. Evol. Res.* **20**, 249-270.
Macallum, A. B. (1910). The inorganic composition of the blood in vertebrates and invertebrates, and its origin. *Proc. R. Soc. Lond B* **82**, 602-624.
Melzner, F., Gutowska, M. A., Hu, M. and Stumpp, M. (2009). Acid-base regulation capacity and associated proton extrusion mechanisms in marine invertebrates: an overview. *Comp. Biochem. Physiol.* **153A**, S80-S80.
Møbjerg, N. and Dahl, C. (1996). Studies on the morphology and ultrastructure of the Malpighian tubules of *Halobiotus crispae* Kristensen, 1982 (Eutardigrada). *Zool. J. Linn. Soc.* **116**, 85-99.
Møbjerg, N., Jørgensen, A., Eibye-Jacobsen, J., Halberg, K. A., Persson, D. and Kristensen, R. M. (2007). New records on cyclomorphosis in the marine eutardigrade *Halobiotus crispae* (Eutardigrada: Hypsibiidae). *J. Limnol.* **66 Suppl. 1**, 132-140.
Møbjerg, N. M., Halberg, K. A., Jørgensen, A., Persson, D., Bjørn, M., Ramløv, H. and Kristensen, R. M. (2011). Survival in extreme environments – on the current knowledge of adaptations in tardigrades. *Acta Physiol. (Oxf.)* **202**, 409-420.
Normant, M., Kubicka, M., Lapucki, T., Czarnowski, W. and Michalowska, M. (2005). Osmotic and ionic haemolymph concentration in the Baltic Sea amphipod *Gammarus oceanicus* in relation to water salinity. *Comp. Biochem. Physiol.* **141A**, 94-99.
Persson, D., Halberg, K. A., Jørgensen, A., Ricci, C., Møbjerg, N. and Kristensen, R. M. (2011). Extreme stress tolerance in tardigrades: Surviving space conditions in low earth orbit. *J. Zoolog. Syst. Evol. Res.* **49**, 90-97.
Rebecchi, L., Altiero, T., Guidetti, R., Cesari, M., Bertolani, R., Negroni, M. and Rizzo, M. A. (2009). Tardigrade resistance to space effects: first results of the experiment of LIFE – TARSE mission on FOTON-M3 (September 2007). *Astrobiology* **9**, 581-591.
Robertson, J. D. (1949). Ionic regulation in some marine invertebrates. *J. Exp. Biol.* **26**, 182-200.
Robertson, J. D. (1954). The chemical composition of the blood of some aquatic chordates, including members of the Tunicata, Cyclostomata and Osteichthyes. *J. Exp. Biol.* **31**, 424-442.
Sands, M., Nicol, S. and McMinn, A. (1998). Fluoride in Antarctic marine crustaceans. *Mar. Biol.* **132**, 591-598.
Schmidt-Nielsen, K. and Fänge, R. (1958). Salt glands in marine reptiles. *Nature* **182**, 783-784.
Schokraie, E., Hotz-Wagenblatt, A., Warnken, U., Mali, B., Frohme, M., Förster, F., Dandekar, T., Hengherr, S., Schill, R. O. and Schnölzer, M. (2010). Proteomic analysis of tardigrades: towards a better understanding of molecular mechanisms by anhydrobiotic organisms. *PLoS ONE* **5**, e9502.
Sutcliffe, D. W. (1962). The composition of haemolymph in aquatic insects. *J. Exp. Biol.* **39**, 325-343.
Sutcliffe, D. W. (1963). The chemical composition of haemolymph in insects and some other arthropods, in relation to their phylogeny. *Comp. Biochem. Physiol.* **9**, 121-135.
Tentori, E. and Lockwood, A. P. M. (1990). Haemolymph magnesium levels in some oceanic crustaceans. *Comp. Biochem. Physiol.* **95A**, 545-548.
Wełnicz, W., Grohme, M. A., Kaczmarek, L., Schill, R. O. and Frohme, M. (2011). Anhydrobiosis in tardigrades – the last decade. *J. Insect Physiol.* **57**, 577-583.
Westh, P. and Kristensen, R. M. (1992). Ice formation in the freeze-tolerant eutardigrades *Adorybiotus coronifer* and *Amphibolus nebulosus* studied by differential scanning calorimetry. *Polar Biol.* **12**, 693-699.
Westh, P. and Ramløv, H. (1991). Trehalose accumulation in the tardigrade *Adorybiotus coronifer* during anhydrobiosis. *J. Exp. Zool.* **258**, 303-311.
Wilder, M. N., Ikuta, K., Atmomarsono, M., Hatta, T. and Komuro, K. (1998). Changes in osmotic and ionic concentrations in the hemolymph *of Macrobrachium rosenbergii* exposed to *v*arying salinities and correlation to ionic and crystalline composition of the cuticle. *Comp. Biochem. Physiol.* **119A**, 941-950.
Yancey, P. H. (2005). Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *J. Exp. Biol.* **208**, 2819-2830.