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Cyclomorphosis in Tardigrada: adaptation to environmental constraints

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SUMMARY

Tardigrades exhibit a remarkable resilience against environmental extremes. In the present study, we investigate mechanisms of survival and physiological adaptations associated with sub-zero temperatures and severe osmotic stress in two commonly found cyclomorphic stages of the marine eutardigrade *Halobiotus crispae*. Our results show that only animals in the so-called pseudosimplex 1 stage are freeze tolerant. In pseudosimplex 1, as well as active-stage animals kept at a salinity of 20 ppt, ice formation proceeds rapidly at a crystallization temperature of around –20°C, revealing extensive supercooling in both stages, while excluding the presence of physiologically relevant ice-nucleating agents. Experiments on osmotic stress tolerance show that the active stage tolerates the largest range of salinities. Changes in body volume and hemolymph osmolality of active-stage specimens (350–500 μm) were measured following salinity transfers from 20 ppt. Hemolymph osmolality at 20 ppt was approximately 950 mOsm kg^{-1}. Exposure to hypo-osmotic stress in 2 and 10 ppt caused (1) rapid swelling followed by a regulatory volume decrease, with body volume reaching control levels after 48 h and (2) decrease in hemolymph osmolality followed by a stabilization at significantly lower osmolalities. Exposure to hyperosmotic stress in 40 ppt caused (1) rapid volume reduction, followed by a regulatory increase, but with a new steady-state after 24 h below control values and (2) significant increase in hemolymph osmolality. At any investigated external salinity, active-stage *H. crispae* hyper-regulate, indicating a high water turnover and excretion of dilute urine. This is likely a general feature of eutardigrades.

Key words: cyclomorphosis, environmental stress, freeze tolerance, *Halobiotus crispae*, invertebrate, osmoregulation, tardigrade, volume regulation.

INTRODUCTION

The phylum Tardigrada comprises a group of hydrophilous micrometazoans, exhibiting close affinities to the euarthropod complex (Garey et al., 1996; Giribet et al., 1996; Mallatt et al., 2004). They occupy a range of niches in terrestrial, freshwater and marine environments from continental Antarctica (Convey and McInnes, 2005) to the icecap of Greenland (Grøngaard et al., 1999) yet are especially abundant in mosses and lichens, where they constitute a major component of the cryptic fauna. Along with nematodes and rotifers, selected species of tardigrades exhibit a remarkable resilience against physical extremes, including low and high temperatures (–253°C to +151°C), ionizing radiation (up to 6000 Gy), vacuum, high pressure (up to 600 MPa) and extreme desiccation (Ramløv and Westh, 1992; Westh and Kristensen, 1992; Ramløv and Westh, 2001; Schill et al., 2004; Horikawa et al., 2006; Jönson and Schill, 2007; Hengherr et al., 2008; Hengherr et al., 2009). However, the underlying physiological and biochemical mechanisms mediating these unique tolerances are still largely unidentified and represent an exciting challenge to contemporary biology.

The marine eutardigrade *Halobiotus crispae* Kristensen 1982 (Fig. 1) colonizes tidal and subtidal habitats at numerous localities throughout the northern hemisphere (Møbjerg et al., 2007). This species is characterized by the appearance of seasonal cyclic changes in morphology, i.e. cyclomorphosis (Kristensen, 1982). Three distinct cyclomorphic stages have been recognized: (1) the active stage, (2) the pseudosimplex 1 (P1) stage and (3) the pseudosimplex 2 (P2) stage (Møbjerg et al., 2007). The defining physiological and biochemical characteristics of the individual stages are largely unknown but most likely correlate with dominant abiotic factors. A ubiquitous factor in all tidal and subtidal habitats is the large temporal and spatial fluctuations in external salinity. Yet, additional adaptations are necessary at high latitudes to ensure winter survival due to prolonged exposure to subzero temperatures. In the present study, we focus on the adaptive significance of the two main cyclomorphic stages in *H. crispae*, i.e. the active stage corresponding to the reproductive stage of other tardigrades and the P1 stage, a hibernation stage, which is comparable to the cysts found in other tardigrades (e.g. Guidetti et al., 2008). We do not deal with the P2 stage, which is a sexual maturation stage that has not yet been reported from other tardigrades. Our preliminary data, however, suggest that this stage has a unique osmoregulatory profile. We show that the transition between the active and P1 stages is associated with profound changes in the physiology of the animal. The P1 stage is the only stage at which *H. crispae* survives internal ice formation. The active stage tolerates large shifts in ambient salinity and we investigate in detail the volume and osmoregulatory capacity of this stage. Our study presents the first detailed analysis of osmoregulation in tardigrades. The data show that active-stage *H. crispae* hyper-regulate at any investigated external salinity, which would indicate excretion of dilute urine. This is likely to be a general feature of eutardigrades, which all possess Malpighian tubules.

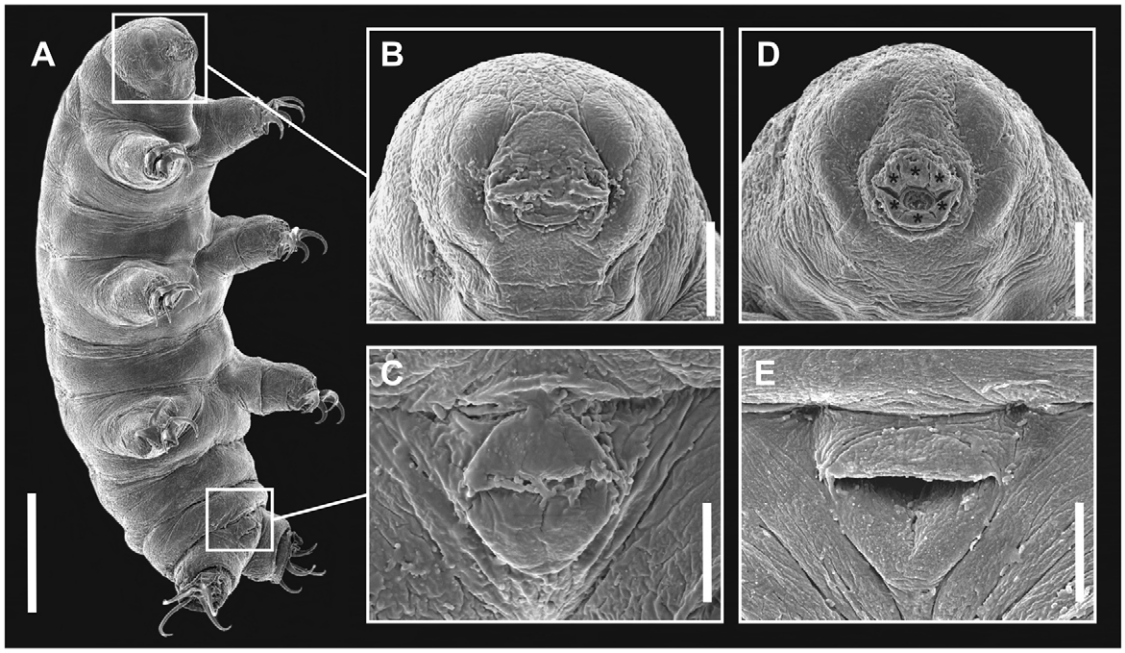

Fig. 1. SEM investigation of *Halobiotus crispae* from Vellerup Vig, Denmark. (A) Overview of P1 stage indicating the areas shown in B and C. The thick outer cuticle functionally isolates the animal from the surroundings (scale bar=100 μm). (B) Close-up of the head region of P1. Notice that the mouth is closed by cuticular thickenings (scale bar=25 μm). (C) Close-up of the posterior area of the P1 stage. As shown for the mouth, the cloaca is closed (scale bar=10 μm). (D) Close-up of the head region of the active stage. Note the six peribuccal sensory organs (*) that surround the open mouth (scale bar=25 μm). (E) Close-up of the posterior area of the active stage, revealing the open tri-lobed cloaca (scale bar=10 μm).

MATERIALS AND METHODS

Tardigrade sampling

Specimens of *Halobiotus crispae* were collected at regular intervals in the period 2005 to 2008 at Vellerup Vig, Isefjord, Denmark (55°44.209′N, 11°51.365′E) and at Nipisat Bay, Disko Island, West Greenland (69°25.934′N, 54°10.768′E) in August 2006. At Vellerup Vig, bottom samples were collected at an approximate depth of 1.0–2.5 m, while samples from Nipisat Bay were taken in the subtidal zone 3–4 cm below low tide. With the exception of the data on osmotic stress tolerance presented in Fig. 3, the obtained results are based entirely on animals collected at Vellerup Vig. Detailed descriptions of the two localities can be found elsewhere (Kristensen, 1982; Møbjerg et al., 2007). Collected samples were freshwater-shocked, decanted into a conical net (mesh size 62 μm) and transferred to Petri dishes. These dishes were supplied with fresh seawater (SW; 18–20 ppt; pH 8–9) and substrate from the locality. Tardigrades were localized using a Leica MZ16 stereomicroscope (Leica Microsystems, Wetzlar, Germany), and primarily found on the haptera of various filamentous algae present in the substrate. Isolated tardigrades from Vellerup Vig were kept at 4°C in SW for periods of up to 6 months by regularly supplying fresh substrate from the locality. Different cyclomorphic stages (see Fig. 1) were identified using an Olympus BX 51 interference-contrast microscope (Olympus, Tokyo, Japan).

Scanning electron microscopy

For scanning electron microscopy, specimens were fixed in 2.5% glutaraldehyde in 0.1 mol l^{-1} sodium cacodylate buffer (pH 7.4), rinsed in the buffer and subsequently postfixed in 1% OsO_4 in 0.1 mol l^{-1} sodium cacodylate buffer (pH 7.4). Following fixation, the specimens were dehydrated through a graded series of ethanol and acetone. They were critical point dried (Bal-Tec CPD 030 critical point dryer, Bal-Tec Union, Balzers, Liechtenstein), mounted on aluminum stubs, sputter-coated with platinum–palladium (thickness ~12 nm) using a JEOL JFC-2300 HR (JEOL, Tokyo, Japan) and examined in a JEOL JSM-6335F Field Emission scanning electron microscope (JEOL, Japan).

Cold hardiness

Six groups of 10 animals in both the active and P1 stage were transferred to Eppendorf tubes containing 1.5 ml of SW from Vellerup Vig (20 ppt). The samples were cooled to a constant temperature of –20°C at a cooling rate of approximately 1°C min^{-1} (Block, 1991) and held at the target temperature for a period of 24 h. The animals were thawed at room temperature and the survival assessed successively over the course of 96 h. Animals retaining locomotory function or responsive to tactile stimuli following this period were considered alive.

As subzero temperatures may be experienced for longer periods of time in Arctic habitats, the long-term survival at subzero temperatures was investigated. An additional six groups of 10 specimens in each cyclomorphic stage were frozen to –20°C at a cooling rate of approximately 1°C min^{-1} and kept frozen for a total of 36 days. Survival was assessed as described above.

Differential scanning calorimetry

The quantity and kinetics of ice formation associated with cooling of *H. crispae* from Vellerup Vig (20 ppt) in active and P1 stages were studied by differential scanning calorimetry (DSC). Groups of 40–75 animals in each respective stage were transferred to 30 μl aluminum DSC pans. In order to avoid dehydrating the animals during the removal of external water, the tardigrades were clumped in the central part of the DSC pan and excess water was subsequently removed with small pieces of delicate task wipes. Sample mass was

determined gravimetrically to the nearest 0.01 mg using a fine-scale AT261 Deltarange (Mettler-Toledo, Columbus, OH, USA), yielding a total mass of 0.16–0.39 mg (wet mass). The pans were sealed and transferred to a calorimeter (Perkin Elmer DSC 7 equipped with an Intercooler II mechanical cooling device), with an empty pan as reference. The calorimeter was calibrated with gallium [melting point, T_m=29.78°C; melting enthalpy, ΔH_m=80.1 J g^{-1}], water (T_m=0°C) and n-decane (T_m=–29.66°C). All scans involved cooling from 5°C to –40°C and subsequent reheating to 5°C at a cooling rate of 5°C min^{-1}. Samples were reweighed following the DSC run to ensure that no water loss had occurred. In order to determine the water content of samples following the freeze/thaw cycle, pans were punctured and dried at 80°C to a constant mass (dry mass). A minimum of three groups of animals in each stage was used (see Table 1). The obtained thermograms (heat flows *vs* temperature) were analyzed with respect to crystallization temperature (T_c), amount of ice formed during the freezing exotherm (assuming that the latent heat of crystallization is the same as for pure water), T_m and the osmotic pressure of the extracellular fluids as calculated by the standard DSC 7 software. Ice contents were calculated using the water content and the enthalpy of the freeze exotherm. The temperature dependence of the enthalpy of crystallization of water was taken into account as previously described (Kristiansen and Westh, 1991). For hemolymph osmolality calculations, the onset melting point measured by the DSC 7 software was determined according to the approach of Nicholajsen and Hvidt (Nicholajsen and Hvidt, 1994), in which the established melting point of the body fluid was derived from a standard curve made from predetermined NaCl solutions.

Preparation of experimental solutions

Salt water solutions of different osmotic pressure were made by successive dilution with distilled water or by evaporative reduction of 100% SW from the locality. Measurements of osmotic pressure were made in parallel on a Vapro 5520 vapor pressure osmometer (Wescor, Logan, UT, USA) and on a refractometer (S-1 Shibuya Land, Tokyo, Japan).

Osmotic stress tolerance

Animals collected from the Danish as well as the Greenlandic population were used for the current experiment. Groups of 20 specimens were transferred to small glass vials containing 4 ml of 100% SW at 4°C, and specimens were either exposed to a gradual increase or decrease in salinity. The gradual changes in salinity were performed over the course of 4–5 h by periodically replacing small volumes of SW with prefixed solutions of either a higher or lower salinity. Animal activity was concomitantly assessed. Animals were allowed a period of 20–40 min of acclimatization following a salinity change prior to assessment. Individuals responsive to tactile stimuli were considered active. Five groups of specimens in active and P1 stages were assessed at both hypo- and hyperosmotic salinities.

Volume measurements

Individual adult active-stage specimens of *H. crispae* (size 300–500 μm) from Vellerup Vig (20 ppt) were visualized in an Olympus BX 51 microscope (Olympus), photographed using a digital camera (C-5050, Olympus) and subsequently exposed for set time periods of 30 min, 1, 2, 4, 24 and 48 h to saltwater solutions with salinities of 2 ppt, 10 ppt and 40 ppt. The osmotic treatments were conducted in small glass vials containing 4 ml of SW at 4°C. At the end of each time interval, individuals were transferred, in a drop of the appropriate solution, to glass microscope slides and photographed under cover slips for subsequent estimations of body volume. During photography, great care was taken to minimize the time spent by the animals under the cover slips, in order to avoid evaporative water loss, which would alter the osmotic pressure of the solution. The animals were ensured total freedom of movement. Following photography, individuals were returned to the respective salinities until the end of the next set time period, when the process was repeated. At each of the time intervals, 10–14 individuals were photographed at each of the SW treatments. Images were analyzed using DP-soft^{TM} (Olympus), and total body volume was calculated according to the equation: $V_{total}=\pi(r^2_{body}h_{body}+2r^2_{leg}h_{leg})$, where V is the volume of the specimen, r is the measured radius, and h is the measured length of the body and hind legs, respectively.

In order to assess the behavioral response of *H. crispae* during osmotic shock and to quantify potential mortality related to the respective treatments, a separate experiment was performed. Specimens (N=10) were transferred directly to 2 ppt, 10 ppt, 20 ppt (control) and 40 ppt, respectively, and animal activity was subsequently monitored over the course of 48 h at 4°C. Individuals responsive to tactile stimuli at the above-mentioned set time periods were considered active and alive (see Fig. 7). Three groups exposed to each treatment were assessed.

Measurement of hemolymph osmolality

Hemolymph osmolality was measured in individual tardigrades following exposure for 30 min, 4 and 48 h to the experimental solutions of 2 ppt (62 mOsm kg^{-1}), 10 ppt (311 mOsm kg^{-1}) and 40 ppt (1245 mOsm kg^{-1}). Six animals were used for osmolality determination in each of the experimental solutions. Six animals kept at 20 ppt (623 mOsm kg^{-1}) served as a control. Hemolymph

Table 1. Post-freeze survival and data obtained from differential scanning calorimetry on *H. crispae* from Vellerup Vig, Denmark (20 ppt) in the active and P1 stages

Sample	Post-freeze survival (%) (frozen for 24 h)	Post-freeze survival (%) (frozen for 36 days)	Crystallization temp. (°C)	Melting temp. (°C)	Water content (%)	Body-water frozen during freezing exotherm (%)	Osmolality of extracellular fluids (mOsm kg^{-1})
Pseudosimplex 1	53.3±15 (6)	12.7±7 (6)	–19.6±3.1 (6)	–4.29±0.79 (5)	67±4 (5)	59±3 (4)	928±77 (5)
Active	0 (6)	0 (6)	–21.6±2.1 (3)	–4.48±0.15 (3)	68±4 (3)	69±5 (3)	975±36 (3)
t-test (P<0.05)	*	*	NS	NS	NS	*	NS

All values are expressed as means ± s.d. Parentheses indicate the number of replicate groups examined, each group containing 40–75 animals. The first column refers to the type of cyclomorphic stage investigated. Second and third columns show the survival following cooling to –20°C at 1°C min^{-1} for 24 h and 36 days, respectively. The temperatures in the fourth and fifth columns are the onsets of the peaks as calculated by the DSC 7 software. Water content was determined gravimetrically using the equation: (wet mass – dry mass)/wet mass. Ice content (seventh column) was calculated using the water content and the enthalpy (ΔH) of the freezing exotherm. The final column indicates the osmolality of the extracellular fluids determined by melting point depression. Significance level was P>0.05 (NS, not significant), P<0.05 (*, significant).

samples (2–3 nl) were collected by piercing individual specimens under immersion oil (type A; 150 centistoke; Cargille Laboratories, Cedar Grove, NJ, USA) using hand-pulled glass capillary tubes (capacity 1 µl; Micro-caps, Drummond Scientific Company, Broomall, PA, USA). Hemolymph samples were acquired through capillary action and subsequently ejected into immersion oil. Care was taken to ensure that the measurements were made on fluid originating from hemolymph alone and samples containing gut contents were discarded. Prior to sample collection, immersion oil was collected into the capillary tube in order to avoid any evaporative water loss. Using an Irvin loop, samples were immediately transferred in a drop of immersion oil into sample oil wells (type B; 1250 centistoke; Cargille Laboratories) of a calibrated nanoliter osmometer (Clifton Technical Physics, Hartford, NY, USA), and the osmolality (mOsm kg^{-1}) was determined by melting point depression (MDP=1.858°C Osm^{-1}).

Statistics

Significant differences between experimental and control conditions were tested using unpaired, two-tailed *t*-tests, and a significance level of $P \leq 0.05$.

RESULTS

Survival at sub-zero temperatures

The external morphology of *H. crispae* in the active and the P1 stage is shown in Fig. 1. Notably, the P1 stage is characterized by a conspicuous double cuticle, in which both the mouth and cloaca are closed by cuticular thickenings (Fig. 1A–C). Post-freeze survival following both short- and long-term exposure to subzero temperatures is listed in Table 1. An example of the quantitative kinetics of ice formation associated with the freeze/thaw cycle of P1-stage *H. crispae* kept at 20 ppt is illustrated in Fig. 2. The collective DSC analysis of the onsets and areas of peaks after the freeze/thaw cycles, together with the water content of the samples, provide the remaining results listed in Table 1.

Whereas animals in the active stage and P2 stage (data not shown) were intolerant of freezing, animals in the P1 stage were demonstrated to be freeze-tolerant. Post-freeze survival following 24 h exposure to –20°C was 53.3±15%; however, when prolonging the period spent at subzero temperatures to 36 days, the survival of animals in the P1 stage decreased to 12.7±7% (Table 1). Animal recovery was monitored over a period of 96 h following the freeze/thaw cycle; however, the majority of animals had resumed activity after a period of 48 h. No additional recovery was monitored beyond 96 h following any of the investigated treatments.

Substantial ice formation proceeded rapidly following the first ice nucleation in the freeze-tolerant P1 stage with a mean of –19.6±3.1°C; as indicated by the large exotherm in Fig. 2. At the given salinity, the amount of ice formed during the freezing exotherm amounted to approximately 60% of the body water (Table 1). The absence of additional small exothermic peaks during the subsequent cooling to –40°C indicated that no additional ice formation occurred following the initial large freezing exotherm. The freeze exotherm lasted less than one minute. The initial separation of ice (T_c) occurred in the temperature range of –15.4 to –23.2°C.

Only marginal differences in T_c of animals in the P1 and active stage were observed (Table 1), suggesting an absence of seasonal variations in ice-nucleating activity in *H. crispae*. In spite of invariant water contents between the two stages, the amount of water crystallizing during cooling in animals in the active stage was significantly higher than in animals in the P1 stage; however, the melting points of the two stages remained largely unaltered. The latter indicates that body fluid osmolality at a given external salinity is unaffected by the animal's transition from the active to the P1 stage.

Volume- and osmoregulatory capacity

Fig. 3 shows the percentage of active animals of *H. crispae* following the exposure to gradual changes in the external salinity. When comparing Danish P1 and active-stage *H. crispae*, the active stage displayed a larger tolerance towards the more concentrated SW solutions and were slightly more tolerant of the very dilute solutions. Indeed, a significantly higher percentage of active-stage specimens

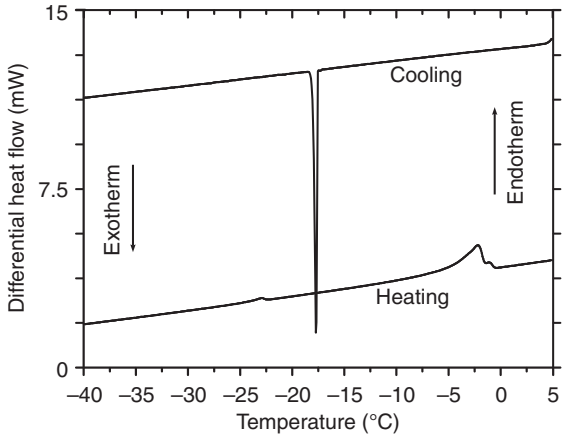

Fig. 2. Representative thermogram displaying the exothermic and endothermic events associated with the cooling and heating of a sample containing P1-stage *Halobiotus crispae* from Vellerup Vig, Denmark (20 ppt). The crystallization and melting temperatures were estimated as the onsets of peaks (–17.5°C and –4.5°C, respectively) using the DSC 7 software (see also Table 1). The very low crystallization temperatures measured exclude the presence of physiologically relevant ice-nucleating agents.

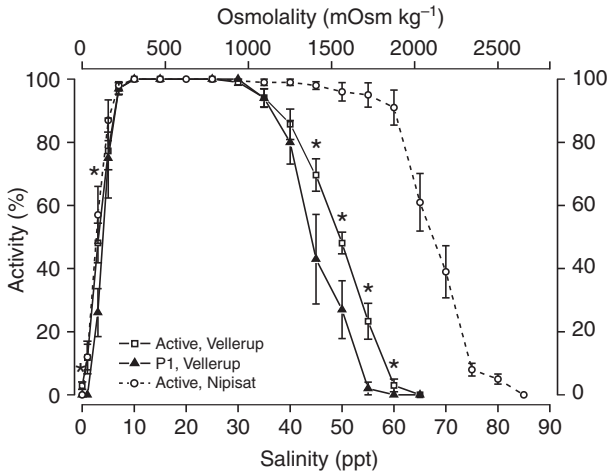

Fig. 3. Osmotic stress tolerance of *Halobiotus crispae* in the P1 and active stages. Active-stage (□) and P1-stage (▲) specimens from the Danish population at Vellerup Vig. Active-stage (○) specimens from the Greenlandic population at Nipisat Bay. Data are means ± s.e.m. from five independent experiments. *, significantly different from Vellerup Vig P1 stage (P<0.05).

remained active in the salinity spectrum of 0–3 ppt and 45–60 ppt, as compared with the animals in the P1 stage, and in general seemed less affected by the impositions of osmotic stress. Our preliminary data on P2 from Vellerup Vig show that this stage tolerates very dilute solutions better than the other stages, yet is the least tolerant of increases in salinity, becoming inactive at around 50 ppt. As a comparison, the active stage from Greenland (Nipisat Bay), living in a more exposed habitat compared with the Vellerup Vig population, displayed an even higher tolerance to concentrated SW, with observed activity at 80 ppt.

In the following, we investigate in detail volume and osmoregulation in active-stage *H. crispae* from Vellerup Vig kept at a control salinity of 20 ppt (Figs 4–8). When exposed to a severe hypo-osmotic shock of 2 ppt (63 mOsm kg^{-1}), the animals exhibited a large significant increase in body volume, resulting in a total body volume of 127±11% after merely 0.5 h of exposure (Fig. 4A,B). During this time period, animals became bloated and rigid and most specimens lost locomotory functions (Fig. 7A). This passive uptake of water continued during the initial 2 h of the exposure, culminating in a total body volume of 162±17%. However, after this period of time, a regulatory volume decrease (RVD) was observed. The total body volume of specimens was considerably reduced to 114±14% following 48 h immersion and was not significantly different from the controls (Fig. 4B). Additionally, an increase in the number of active animals was observed, yet a considerable number of specimens remained passive throughout the treatment (Fig. 7A). Nevertheless, following a gradual return to 20 ppt, all animals regained locomotory functions. No mortality was observed in any of the treatments.

Upon immersion of individual specimens into a less severe hypo-osmotic media of 10 ppt (311 mOsm kg^{-1}) a similarly significant increase in total body volume was observed; reaching a mean value of 132±11% after 0.5 h exposure (Fig. 5A,B). However, following this initial increase, total body volume stabilized, and a RVD was observed after 1–2 h exposure. After 4 h incubation, total body volume was 110±8%, which was not significantly different from the control situation. An effect on the locomotory functions was observed initially, as some animals displayed sluggish movements, yet only a limited number of animals were passive during this experiment (Fig. 7B).

When transferring *H. crispae* to a hyperosmotic solution of 40 ppt (1245 mOsm kg^{-1}), a significant decrease in total body volume was observed (Fig. 6A,B). Total body volume was significantly reduced to 66±9% following the first 0.5 h of immersion and remained largely unaltered during the following hours of the treatment. After 24 h, most specimens had displayed a regulatory volume increase, resulting in a mean total body volume of 82±9%, yet total body volume remained significantly different from the control situation even after 48 h. Nevertheless, animal motility was little affected by the hypertonic shock, neither at the initial transfer nor throughout the rest of the experiment (Fig. 7C).

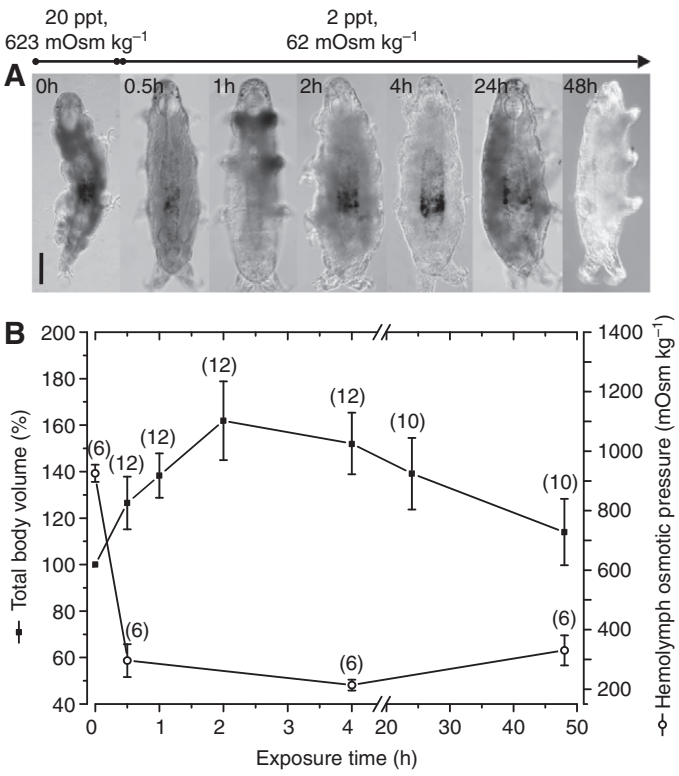

Fig. 4. (A) *Halobiotus crispae* in active stage from Vellerup Vig, Denmark (20 ppt). Light-microscopical images at different time points following exposure to 2 ppt (62 mOsm kg^{-1}) of a single specimen (scale bar=100 μm). (B) Changes in total body volume (■) and measured internal osmolality (○) over a period of 48 h following exposure to an external salinity of 2 ppt. Data are expressed as means ± s.d. Numbers in parentheses indicate the number of animals used for assessment of body volume and hemolymph osmolality at each time point.

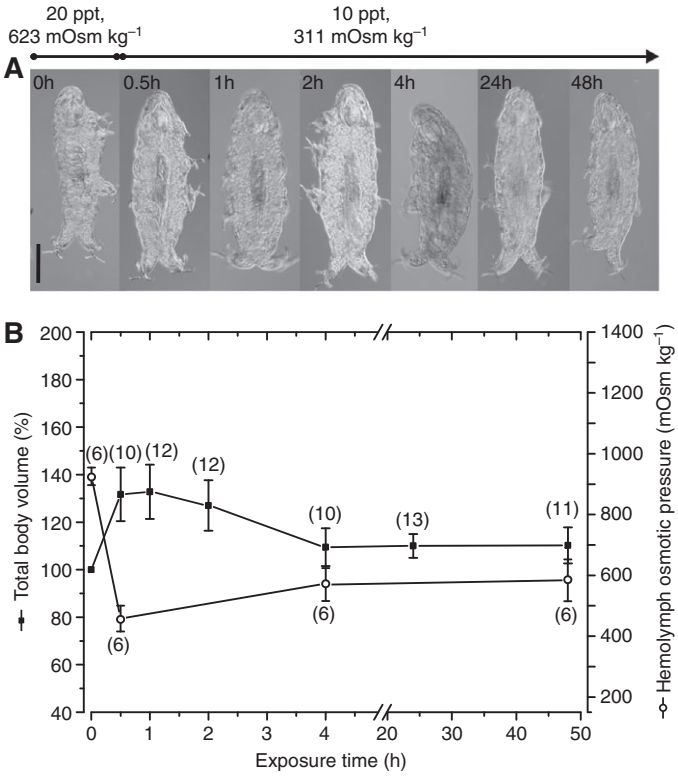

Fig. 5. (A) *Halobiotus crispae* in active stage from Vellerup Vig, Denmark (20 ppt). Light-microscopical images at different time points following exposure to 10 ppt (311 mOsm kg^{-1}) of a single specimen (scale bar=100 μm). (B) Changes in total body volume (■) and measured internal osmolality (○) over a period of 48 h following an exposure to an external salinity of 10 ppt. Data are expressed as means ± s.d. Numbers in parentheses indicate the number of animals used for assessment of body volume and hemolymph osmolality at each time point.

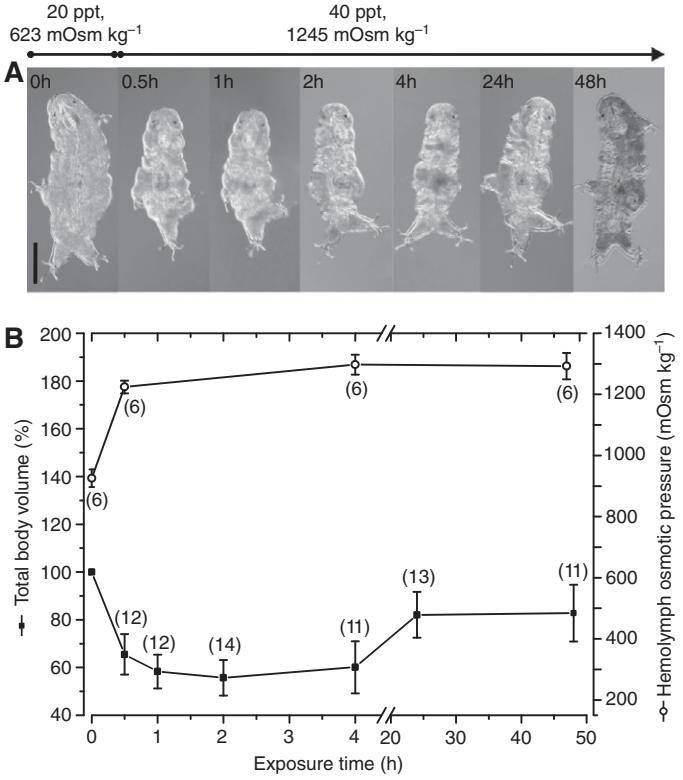

Fig. 6 (A) *Halobiotus crispae* in active stage from Vellerup Vig, Denmark (20 ppt). Light-microscopical images at different time points following exposure to 40 ppt (1245 mOsm kg^{-1}) of a single specimen (scale bar=100 μm). (B) Changes in total body volume (■) and measured internal osmolality (○) over a period of 48 h following an exposure to an external salinity of 40 ppt. Data are expressed as means ± s.d. Numbers in parentheses indicate the number of animals used for assessment of body volume and hemolymph osmolality at each time point.

Notably, hemolymph osmotic pressure differed significantly from the control condition at all time points examined during the various salinity treatments (Figs 4–6). Hemolymph osmolality of *H. crispae* varied in proportion to the gradient set up by the salinity transfer. The body fluids of animals kept under control conditions (20 ppt, 623 mOsm kg^{-1}) had an osmolality of 926±29 mOsm kg^{-1}, indicating that *H. crispae* hyper-regulates during steady-state conditions. This hyperosmotic regulation was independently confirmed by the DSC investigation in which the hemolymph osmolality was measured at 975±36 mOsm kg^{-1} (Table 1). The two measurements are not significantly different (*t*-test, P<0.01). Upon immersion into a hypo-osmotic media of 2 ppt (63 mOsm kg^{-1}), a large rapid decrease was observed, as hemolymph osmolality was reduced to 296±55 mOsm kg^{-1} following 0.5 h exposure. This value remained largely constant throughout the rest of the treatment, resulting in an osmolality of 330±51 mOsm kg^{-1} after 48 h (Fig. 4B). A similar pattern was associated with a less severe (10 ppt, 311 mOsm kg^{-1}) hypo-osmotic treatment. The osmolality of the extracellular body fluids changed to 458±42 mOsm kg^{-1} after 0.5 h immersion, yet a slight increase to 584±69 was detected following 48 h incubation (Fig. 5B). Conversely, hyperosmotic stress (40 ppt, 1245 mOsm kg^{-1}) induced an initial increase in hemolymph osmolality to 1224±21 mOsm kg^{-1} after 0.5 h (Fig. 6B). A steady-state value of 1293±43 mOsm kg^{-1} was obtained after 48 h. Our study reveals that the active stage tolerates large shifts in hemolymph osmolality, with a final osmolality ranging from 330±51 mOsm kg^{-1} to 1293±43 mOsm kg^{-1} during the investigated treatments. The initial changes in hemolymph osmotic pressure are very fast (maximally within 0.5 h), suggesting a limited resistance to cross-cuticular movement of osmotically active solutes and water.

The osmotic performance after 48 h acclimation as a function of the external salinity is summarized in Fig. 8. Notably, during control and steady-state conditions following exposure to diluted media, *H. crispae* maintains a consistent osmotic gradient of 270–330 mOsm kg^{-1} above that of the external environment. This capacity to hyper-regulate becomes less pronounced in the high-osmolality solution, in which the hemolymph osmolality is merely sustained 50 mOsm kg^{-1} above that of the surroundings. In summary, our data show that *H. crispae* maintains an osmotic pressure gradient between the internal and external environment, remaining hyper-osmotic during all investigated salinities. This hyper-regulation would indicate (1) the excretion of dilute urine and/or (2) the adaptive synthesis of organic osmolytes and/or (3) the active uptake of salts from the external medium.

DISCUSSION

In Greenland (Nipisat Bay), the transformation of *H. crispae* into the P1 stage is correlated with the approach of the long Arctic winter, and this stage is thus considered a true hibernation stage, which is

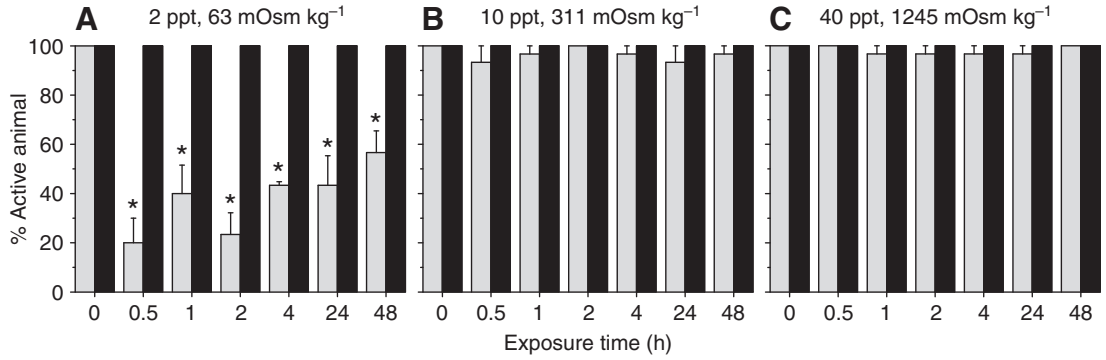

Fig. 7. *Halobiotus crispae* in active stage from Vellerup Vig, Denmark. Percentage of animals responsive to tactile stimuli following exposure to (A) 2 ppt (62 mOsm kg^{-1}), (B) 10 ppt (311 mOsm kg^{-1}) and (C) 40 ppt (1245 mOsm kg^{-1}) over a period of 48 h. Black columns indicate control condition at a salinity of 20 ppt (623 mOsm kg^{-1}). Gray columns indicate osmotic shock exposure. Three groups of 10 animals were used for the assessment at each salinity. Data are expressed as means ± s.e.m. (*, significantly different from control condition at P<0.05).

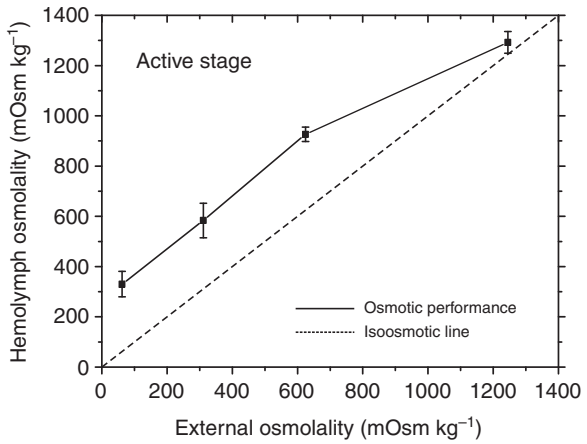

Fig. 8. Measured hemolymph osmolality of *Halobiotus crispae* (active stage from Vellerup Vig, Denmark) during steady-state conditions after 48 h acclimation to 2 ppt (63 mOsm kg^{-1}), 10 ppt (311 mOsm kg^{-1}), 20 ppt (623 mOsm kg^{-1}) and 40 ppt (1245 mOsm kg^{-1}), respectively. Each point represents the mean ± s.d. of the individual experiments. The broken line indicates the isoosmotic line at which no osmoregulation occurs.

functionally characterized as a movable cyst (Kristensen, 1982; Møbjerg et al., 2007; Guidetti et al., 2008). In Denmark (Vellerup Vig), this stage is dominant during the summer months, presumably enabling *H. crispae* to withstand heat stress and oxygen depletion. The active stage, the only stage at which active feeding and sexual reproduction occur, is the dominant stage during the Greenlandic summer, whereas this stage is present during late winter and the spring months in Denmark (Møbjerg et al., 2007).

Freeze avoidance and freeze tolerance

Winter temperatures are frequently below the equilibrium freezing point of the surrounding seawater at least in some portions of the natural environments of *H. crispae*, and certain habitats may even become completely frozen for extended periods of the year (Kristensen, 1982). Enduring such hostile surroundings requires corresponding cold-tolerance strategies that enable long-term survival. Traditionally, two main options are exploited by ectothermic animals when faced with subzero temperatures, i.e. freeze avoidance and freeze tolerance (Lee, 1991). When exposing animals in the P1 stage of *H. crispae* to temperatures below the equilibrium freezing point (T_c) of their body fluids, freeze tolerance is demonstrated, indicating that winter survival could involve extracellular ice formation in this species. However, the finding that mortality increases with prolonged exposure to subzero temperatures suggests that the consequent damages accumulate in proportion to the time spent exposed to freezing conditions. This observation is likely explained by the depletion of essential metabolites and is of particular interest in Arctic habitats in which subfreezing temperatures have to be endured for long periods of time.

In freeze-tolerant organisms, ice formation is usually promoted at relatively high subzero temperatures (–2 to –10°C) by ice-nucleating agents present in the extracellular fluid (Zachariassen, 1985; Block, 1991; Westh and Kristensen, 1992). The adaptive advantage of such a strategy is that the process of ice formation proceeds relatively slowly at relatively high temperatures, enabling the organism to maintain the damage associated with freezing within tolerable boundaries. Indeed, both the localization and the amount of ice formed in freeze-tolerant organisms are usually under tight control. Surprisingly, our calorimetric investigation of the freeze-tolerant P1 stage reveals that ice crystallization occurs at approximately –20°C, excluding the presence of any physiologically relevant ice-nucleating agents in this stage. In fact, the very low crystallization temperatures measured in both stages suggest that the capacity for supercooling is maintained throughout the majority of the year, which is in general contrast to the pattern observed in most other freeze-tolerant invertebrates (Block, 1991; Westh and Kristensen, 1992; Ramløv et al., 1996). However, in spite of an invariant melting point and water content between the two stages, the amount of water crystallized during cooling to –40°C appears to be about 60% for animals in the P1 stage and 70% for animals in the active stage (see Table 1). Consequently, the cellular dehydration induced by freezing is expected to be much higher in animals in the active stage, compared with that of specimens in the P1 stage. This reduction in ice accumulation in specimens in the P1 stage could potentially be explained by an increased production of macromolecules [which kinetically inhibits ice formation but has negligible effect on the melting temperature (see Westh and Kristensen, 1992)], as compared with the active stage. Whether this observation alone explains the observed freeze tolerance is difficult to determine. Selected cryptobiotic species of tardigrades, nematodes as well as some freeze-tolerant insects tolerate as much as 80% of the body water being converted into ice (Westh and Kristensen, 1992; Ramløv and Westh, 1993; Wharton and Block, 1997; Hengherr et al., 2009).

The apparent morphological difference between the two stages is similarly relevant in regard to the observed freeze tolerance. The P1 stage is formed from an incomplete molt in which both the mouth and cloaca become sealed by cuticular thickenings (see Fig. 1), and the gut content is often shed prior to this transition. In nature, ice nucleation can be initiated by a wide range of exogenous substances (Wharton and Worland, 1998). Consequently, the additional layer of cuticle could increase the capacity to avoid inoculative freezing in animals in the P1 stage, as has been demonstrated for eggs of the nematode *Panagrolaimus davidi* Timm 1971 (see Wharton, 1994). Indeed, the extensive capacity for supercooling in both the active and P1 stage, along with the additional layer of cuticle and the clearing of gut contents in P1, would indicate that *H. crispae* preferentially seek to avoid internal ice formation. Nevertheless, animals in the P1 stage tolerate internal ice formation for both shorter and longer periods of time.

Volume and osmoregulation

Our results indicate that active-stage *H. crispae* is the most tolerant of changes in external salinity. Specimens from the population from Nipisat Bay, Greenland exhibit an increased tolerance towards concentrated SW solutions as compared with animals from Vellerup Vig, Denmark, suggesting that the ability to tolerate large increases in salinity is potentiated by living in a more-exposed habitat. The observed volume regulatory response of active-stage *H. crispae* during hypo- and hypertonic treatments differs in a significant way. When exposed to the hypo-osmotic solutions, the initial increase in total body volume was regulated to a new steady state, which was not significantly different from the control condition. In fact, a new steady-state value was demonstrated after merely 4 h immersion in the external medium of 10 ppt. Conversely, during acute exposure to concentrated seawater (40 ppt), a partial recovery to normal levels was demonstrated; however, total body volume remained significantly different from the control condition even after 48 h immersion. These data suggest that the body volume of *H. crispae* is more tightly regulated during exposure to dilute as compared with

more concentrated saltwater solutions. Interestingly, this observation seems reflected in an evolutionary context. According to our previous study, *Halobiotus* has evolved within the freshwater genus *Isohypsibius*, thus potentially explaining the enhanced volume regulatory response during exposure to dilute media (Møbjerg et al., 2007).

When submitted to osmotic shock of 10 ppt and 40 ppt, respectively, our data show that *H. crispae* experience few limitations in terms of motility. Active-stage specimens from the Nipisat population even retain activity when exposed to a gradual salinity increase to 60 ppt (Fig. 3). However, upon direct transfer to an extreme seawater dilution of 2 ppt, animal activity is markedly reduced, probably due to the pronounced increase in hydrostatic pressure and concomitant reduction in hemolymph osmotic pressure. Proper locomotory function in tardigrades relies on the hydrostatic pressure of the body cavity (Kinchin, 1994); thus, maintaining an appropriate body volume is essential to normal coordination of movement. In addition, as the membrane potentials of animal cells are highly dependent upon extracellular ionic strength (Spyropoulos and Teorell, 1968), the concomitant changes in hemolymph osmotic pressure could influence animal motility due to inhibition of neuromuscular activity. Indeed, the fact that a significant number of animals were observed passive during exposure to 2 ppt, while animals remained largely unaffected during exposure to 10 ppt, in spite of experiencing comparable average changes in body volume (compare Fig. 4B and Fig. 5B after 0.5 h immersion), suggests that not only total body volume but also hemolymph osmolality is an important factor in maintaining locomotory functions.

Exposing *H. crispae* to severe osmotic stress reveals that this species is a euryhaline osmoconformer, in which the hemolymph osmotic pressure is largely governed by the external environment. However, when analyzing hemolymph osmotic pressure at steady state following 48 h exposure to the various salinity treatments as a function of the external osmolality, an interesting pattern emerges. *H. crispae* maintains a large osmotic pressure gradient between the internal and external environment, thus distinctly hyper-regulating during all investigated salinity treatments – albeit markedly less in concentrated seawater. This would imply a large water turnover in this animal, with osmotic water uptake being balanced by the excretion of dilute urine.

Hyperosmoregulation is known in other euryhaline invertebrates. The crayfish *Procambarus clarkii* Girard 1852 (Arthropoda) produces highly dilute urine and is a strong hyperosmoregulator in freshwater (Sarver et al., 1994). However, the excreted urine becomes progressively more concentrated in media of higher ionic strength and is nearly isoosmotic when exposed to an external concentration of 750 mOsm kg^{-1}, at which *P. clarkii* cease to hyper-regulate (Sarver et al., 1994). Moreover, similar osmoregulatory responses have been reported from nematodes (Fusé et al., 1993; Forster, 1998). Indeed, the internal osmolality of the parasitic nematode *Pseudoterranova decipiens* (Krabbe, 1878) was maintained 90 mOsm kg^{-1} above that of the external environment during exposure to media of widely varying osmolality (Fusé et al., 1993).

Three glands positioned at the transition zone between the midgut and rectum of eutardigrades are traditionally ascribed an osmoregulatory function. The term used for these structures, i.e. Malpighian tubules, was introduced more than a century ago (Plate, 1889). The positional conformity of the Malpighian tubules in eutardigrades and in hexapods has been used as a strong argument in favor of a homology between these structures (Greven, 1982; Møbjerg and Dahl, 1996). However, at present, no functional data exist relating the Malpighian tubules of tardigrades to an osmoregulatory role. Nevertheless, several detailed morphological investigations of the tubules support the hypothesis. These studies have provided ultrastructural data, which are in agreement with an active transporting epithelium involved in solute and fluid transport (Greven, 1979; Weglarska, 1987a; Weglarska, 1987b; Møbjerg and Dahl, 1996; Peltzer et al., 2007). As holds for insects, the Malpighian tubules of tardigrades are considered secretion–reabsorption kidneys. In light of the ultrastructural data available on tardigrade Malpighian tubules, it seems reasonable to assume that the first steps in urine formation take place across initial segment cells, characterized by a conspicuous basal labyrinth, numerous mitochondria and an enlarged apical surface. There is, moreover, ultrastructural support for assigning the tardigrade rectum an osmoregulatory function (Dewel and Dewel, 1979). A possible mode of urine formation was outlined by Dewel and Dewel (Dewel and Dewel, 1979). They suggest that isoosmotic urine produced by the Malpighian tubules is modified in the rectum through the active reabsorption of solutes, leading to the excretion of hypo-osmotic urine. Our data seem in favor of such a mechanism of urine formation. Interestingly, preliminary and unpublished data (H.R. research group) on *Richtersius coronifer* (Richters 1903) indicate that this species also remains hyperosmotic during exposures to a range of external salinities and it is therefore likely that hyper-regulation, and possibly hypo-osmotic urine formation, is a general feature of eutardigrades. However, until functional studies at the cellular and molecular level are performed, the exact mechanisms involved in osmoregulation in tardigrades remain to be elucidated.

In conclusion, we show that the transition between the individual cyclomorphic stages of *H. crispae* is associated with profound changes in the physiology of the animal. Our results show that animals in the active stage tolerate large changes in the external osmotic pressure by regulating their total body volume and by enduring large concomitant changes in hemolymph osmotic pressure. *H. crispae* remains hyperosmotic at any investigated external salinity, suggesting that this species is a strong hyper-regulator. Our study is the first to provide evidence for the volume and osmoregulatory capacity in Tardigrada. Whereas animals in the active stage are intolerant of freezing, the P1 stage is demonstrated to be freeze tolerant. The relatively low crystallization temperature reveals that extensive supercooling of the body fluids takes place during cooling and that no physiologically relevant ice-nucleating agents are present.

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LIST OF ABBREVIATIONS

DSC	differential scanning calorimetry
MDP	melting point depression
RVD	regulatory volume decrease
SW	seawater
T_c	crystallization temperature
T_m	melting temperature

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