The LINNEAN SOCIETY of London ZOOLOGICAL Journal of the Linnean Society

Zoological Journal of the Linnean Society, 2016, **178**, 907–911. With 2 figures

Tun formation is not a prerequisite for desiccation tolerance in the marine tidal tardigrade *Echiniscoides sigismundi*

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Received 30 August 2015; revised 14 March 2016; accepted for publication 13 April 2016

The so-called 'tun' state is best known from limno-terrestrial tardigrades and rotifers that rely on this compact body shape for anhydrobiotic survival. Little is known of tun formation in marine species and the evolutionary origin of the state is presently unknown. Here, we investigate desiccation tolerance and tun formation in the marine tidal echiniscoidean tardigrade, *Echiniscoides sigismundi* (M. Schultze, 1865). Groups of approximately 20 *E. sigismundi* sampled from Lynæs (Denmark) were dehydrated on filter paper from seawater as well as ultrapurified water and kept for 48 h at 5 °C, after which they were rehydrated in seawater. The activity and behaviour of the tardigrades was examined under a light microscope, whereas scanning electron microscopy was used for high-resolution three-dimensional imaging. When dehydrated from seawater, *E. sigismundi* enters a tun, however, when exposed to ultrapurified water, the tardigrade swells and becomes incapable of movement, and thus incapable of tun formation. Nonetheless, *E. sigismundi* tolerates being dehydrated from ultrapurified water, revealing an exceptional and unparalleled resilience towards losing structural integrity. Our results confirm previous investigations, which suggest that tun formation relies on a functional musculature. They further suggest that tun formation may have evolved as a response to elevated external pressure rather than desiccation per se.

doi: 10.1111/zoj.12444

ADDITIONAL KEYWORDS: cryptobiosis – *Echiniscoides* – marine – osmotic pressure – Tardigrada.

INTRODUCTION

Tardigrades depend on having a surrounding film of water to be in their active and reproductive state. They are, nevertheless, found in a range of habitats worldwide (e.g. McInnes, 1994; Hansen, Jørgensen & Kristensen, 2001; Nelson, 2002; Convey & McInnes, 2005), including moss beds, which frequently dry out, leaving the tardigrades in a dehydrated, ametabolic state known as anhydrobiosis (e.g. Rebecchi, Altiero & Guidetti, 2007; Guidetti, Altiero & Rebecchi, 2011; Wełnicz *et al.*, 2011). Moreover, these microscopic metazoans are well known for their ability to tolerate a range of other extreme stresses (Møbjerg *et al.*, 2011); however, significant variations in stress tolerance between species complicate investigations into the underlying processes associated with extreme stress tolerance. Notably, the specifics of how tardigrades enter and exit states of latent life, collectively referred to as cryptobiosis (Keilin, 1959), remain elusive.

Tardigrades are an ideal model for studies on cryptobiosis because of their abundance in extreme habitats (e.g. moss beds), as well as their extraordinary stress tolerances when compared with other cryptobiotic animals. The tardigrade lineage divides into two main

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extant branches: eutardigrades and heterotardigrades (Sands *et al.*, 2008; Jørgensen *et al.*, 2010). The eutardigrade branch consists primarily of limnoterrestrial species, whereas heterotardigrades divide into limnoterrestrial/marine–tidal echiniscoideans and the exclusively marine arthrotardigrades. Most studies on cryptobiosis have been performed on eutardigrades (e.g. Jönsson & Rebecchi, 2002; Schill, Steinbrück & Köhler, 2004; Hengherr, Brümmer & Schill, 2008; Rebecchi *et al.*, 2009; Yamaguchi *et al.*, 2012), whereas comparatively fewer studies have been performed on heterotardigrades (e.g. Persson *et al.*, 2011; Jørgensen & Møbjerg, 2015).

It has been a commonly held belief, based on earlier observations made in eutardigrades, that anhydrobiosis (cryptobiosis induced by desiccation) is accompanied by a morphological transition into a so-called 'tun state' in tardigrades, and that this transition is a necessary response for post-anhydrobiotic survival (e.g. Crowe & Madin, 1974; Crowe, 1975; Wright, Westh & Ramløv, 1992; Sømme, 1996). This assumption is supported by recent experimental data showing that tun formation is mediated by the musculature, and an essential process for anhydrobiotic survival in the eutardigrade *Richtersius coronifer* (Halberg, Jørgensen & Møbjerg, 2013). Limnoterrestrial echiniscoideans also form tuns, and we have recently shown that tun formation occurs in the arthrotardigrade *Styraconyx haploceros* (Jørgensen & Møbjerg, 2015). Experimental data on heterotardigrades are scarce, however, and whether tun formation is essential for anhydrobiotic survival in tardigrades in general remains to be elucidated. In the present study we present data on tun formation and desiccation tolerance in the marine tidal echiniscoidean, *Echiniscoides sigismundi* (M. Schultze, 1865), sampled from Lynæs, Denmark.

MATERIAL AND METHODS

Tardigrade sampling

Echiniscoides sigismundi was collected in autumn (September–November 2013) from barnacles in the tidal zone of North Sealand (Lynæs, Denmark). During sampling, the water temperature and salinity at the locality were in the ranges 9–13 °C and 18–20‰, respectively. After collection, the tardigrades were kept at 5 °C for 3–4 weeks on barnacle shells in seawater from the locality.

Experimental procedures

Prior to experimentation, *E. sigismundi* was isolated from the barnacle shells under a microscope (Zeiss Stemi 2000). Only clearly active animals were used in the experiments. The animals were pooled into groups of approximately 20 specimens and placed in watch glasses with either filtered seawater from the locality or ultrapurified water (Barnstead EASYpure UV/UF; Dubuque, IA, USA) for 1 h. The tardigrades were subsequently transferred to a piece of filter paper in a small volume of appropriate water and desiccated under ambient conditions (22–23 °C). The point of complete desiccation was defined as the moment when *E. sigismundi* exhibited an easily visible colour change, i.e. the animals would go from transparent to rusty yellow. Following complete desiccation the tardigrades were kept dehydrated on filter paper for 48 h at 5 °C, after which they were rehydrated in seawater. Their activity was monitored at 5 min, 30 min, 2 h, 24 h, and 48 h post rehydration. The tardigrades were considered active and alive if they exhibited clear movement or responded to a tactile stimulus. Dehydration was performed on two different dates, with three replicates in both seawater and ultrapurified water on each date. Relative humidity was 62% during dehydration and 56% during rehydration in the first experiment, whereas it was 39% and 44%, respectively, in the second experiment. Survival rates did not vary between the two dates.

Scanning electron microscopy

For scanning electron microscopy (SEM), fully hydrated specimens were fixed for 2 h at room temperature in 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.4). The specimens were subsequently washed in ultrapurified water, dehydrated through a graded series of ethanol, critical-point dried (Autosamdri-815; Tousimis, Rockville, USA), and mounted on aluminium stubs. In addition, unfixed specimens dried on filter paper were also mounted on stubs. The stubs were subsequently coated for 80 s with platinum–palladium using a JFC-2300HR sputter coater (Jeol, Tokyo, Japan). SEM images were obtained using a JSM-6335F scanning electron microscope (Jeol, Tokyo, Japan).

Statistics

Data are expressed as means ± standard errors of the mean (SEM). For statistical analysis, OriginPro 9.1 (OriginLab, Northampton, MA, USA) was used to compute a one-tailed, two-sample Student's *t*-test at a significance level of $P \leq 0.05$. Data sets were tested for normality and variance before computing the *t* statistic.

RESULTS AND DISCUSSION

During dehydration, limnoterrestrial eutardigrades contract along the anterior–posterior axis, while at the same time retracting their legs, thereby forming

the body shape termed a tun. This tun state seems to be a prerequisite for anhydrobiotic survival in eutardigrades (Crowe & Madin, 1974; Crowe, 1975; Wright *et al.*, 1992; Sømme, 1996; Halberg *et al.*, 2013).

Our present study reveals that the marine heterotardigrade *E. sigismundi* dehydrated from its natural habitat (seawater) contracts into a tun that is comparable with the well-known eutardigrade tuns (Fig. 1A, B). When exposed to ultrapurified water, *E. sigismundi* swell and become incapable of contraction, and when subsequently transferred to filter paper and dehydrated, they collapse rather than form tuns (Fig. 1C).

Our experimental data reveal that tardigrades dehydrated from seawater for 48 h have a mean ± SEM activity of 99 ± 1% and 95 ± 2% (N = 6), 24 and 48 h post-rehydration, respectively (Fig. 2). Remarkably, *E. sigismundi* dehydrated for 48 h from ultrapurified water has an activity of 99 ± 1% and 92 ± 3% (N = 6) at 24 and 48 h post-rehydration (Fig. 2).

Echiniscoides sigismundi thus seems to handle short-term desiccation from seawater and ultrapurified water equally well. Yet there is a difference in the recovery rates, with animals dried from seawater recovering significantly faster than those dried from ultrapurified water (Fig. 2), i.e. at 30 min and 2 h post-rehydration there is a significant difference in activity between seawater and ultrapurified water (P = 0.02 and 0.001, respectively). At 48 h the post-rehydration activity of animals exposed to seawater and ultrapurified water did not significantly differ (P = 0.19). Hence, this species seems less dependent on maintaining structural integrity compared with limnoterrestrial eutardigrades.

Our results suggest that the processes underlying desiccation tolerance in echiniscoideans are different from those of eutardigrades. A possible implication is that anhydrobiosis has evolved several times within tardigrades or, alternatively, that the fundamental processes underlying cryptobiosis are an inherent feature of tardigrades and that these processes

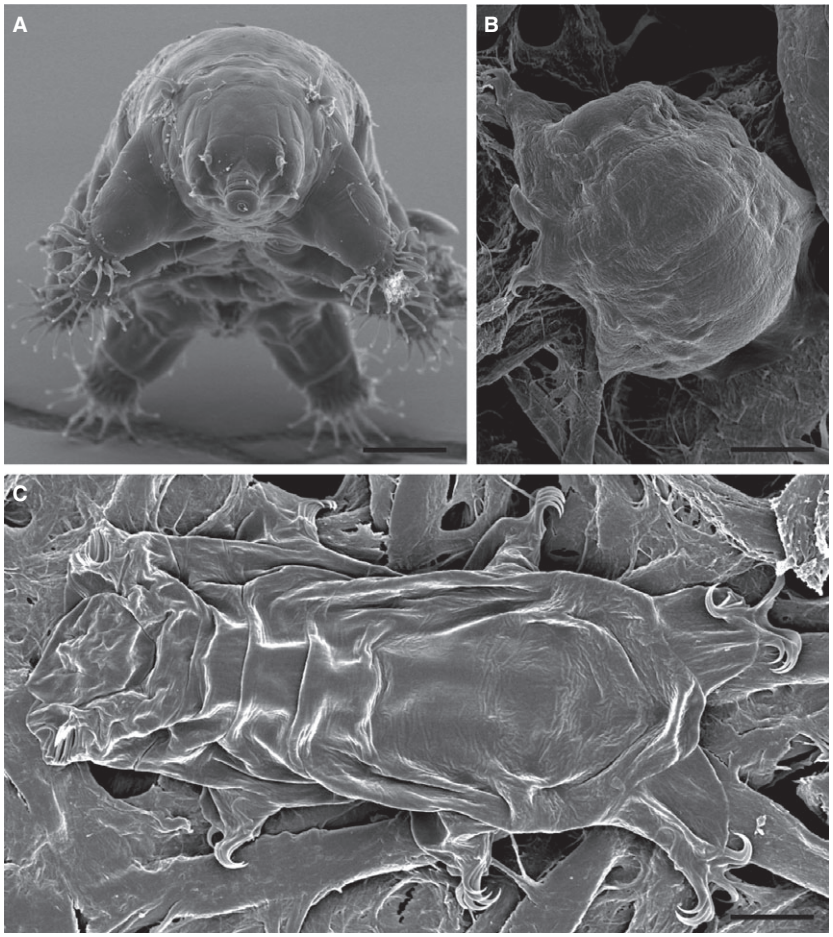

Figure 1. Scanning electron micrographs: A, active hydrated *Echiniscoides sigismundi* (frontal view); B, tun (dorsal view) formed during dehydration from seawater; C, *E. sigismundi* dried from ultrapurified water (dorsal view). Scale bars: 20 μm.

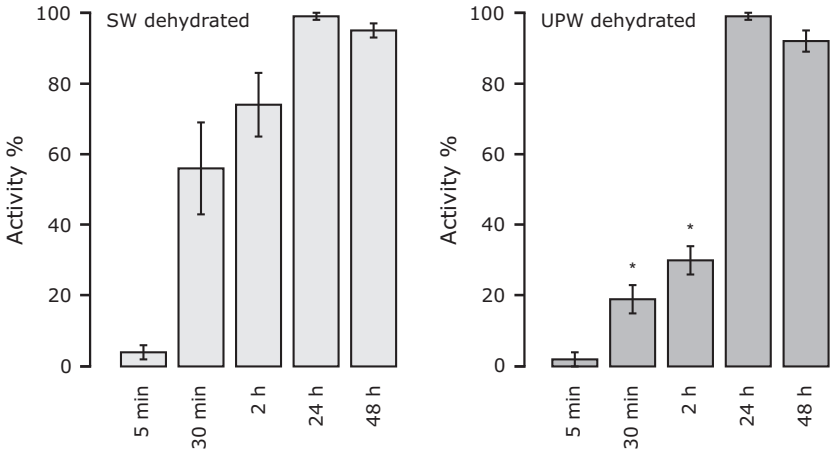

Figure 2. Desiccation tolerance of *Echiniscoides sigismundi*. Tardigrades were desiccated for a period of 48 hours from seawater (SW) and ultrapurified water (UPW). They were subsequently rehydrated in seawater from the locality and monitored at 5 min, 30 min, 2 h, 24 h, and 48 h post-rehydration. Mean ± SEM activity ($N = 6$) for SW-dehydrated tardigrades: 4 ± 2% (5 min post-rehydration); 56 ± 13% (30 min post-rehydration); 74 ± 9% (2 h post-rehydration); 99 ± 1% (24 h post-rehydration); 95 ± 2% (48 h post rehydration). Mean ± SEM activity ($N = 6$) for UPW-dehydrated tardigrades: 2 ± 2% (5 min post-rehydration); 19 ± 4% (30 min post-rehydration); 30 ± 4% (2 h post-rehydration); 99 ± 1% (24 h post-rehydration); 92 ± 3% (48 h post-rehydration). *Significant difference ($P \leq 0.05$) between activity of SW- and UPW-desiccated tardigrades, at the given time point.

subsequently have been modified and refined in the different evolutionary lineages. The latter hypothesis is supported by the observation of desiccation tolerance and tun formation within marine arthrotardigrades (Jørgensen & Møbjerg, 2015). The presence of tun formation among all extant lineages, including marine echiniscoideans and arthrotardigrades, implies that the tun is an ancient and homologous trait, which evolved within the marine environment. If this is the scenario, the tun evolved as an adaptation to something other than anhydrobiosis. A characteristic of the marine environment is the presence of salts that build osmotic pressure. Tun formation may thus have evolved as a response to increases in salt concentrations associated with fluctuating marine habitats near land. Notably, *E. sigismundi* only forms tuns when dehydrated from seawater, supporting the hypothesis that tun formation originally was a response to elevated external osmotic pressure rather than desiccation per se. This hypothesis would further explain the ability of the tardigrade tun to withstand extraordinarily high hydrostatic pressures (Seki & Toyoshima, 1998).

ACKNOWLEDGEMENTS

The present study was supported by the Carlsberg foundation (grant no 2011_01_0539) and the Danish Council for Independent Research (grant no. DFF-4090-00145).

REFERENCES

Convey P, McInnes SJ. 2005. Exceptional tardigrade-dominated ecosystems in Ellsworth Land, Antarctica. *Ecology* **86:** 519–527.

Crowe JH. 1975. The physiology of cryptobiosis in tardigrades. *Memorie Dell'Istituto Italiano Di Idrobiologia* **32:** 37–59.

Crowe JH, Madin KA. 1974. Anhydrobiosis in tardigrades and nematodes. *Transactions of the American Microscopical Society* **93:** 513–524.

Guidetti R, Altiero T, Rebecchi L. 2011. On dormancy strategies in tardigrades. *Journal of Insect Physiology* **57:** 567–576.

Halberg KA, Jørgensen A, Møbjerg N. 2013. Desiccation tolerance in the tardigrade *Richtersius coronifer* relies on muscle mediated structural reorganization. *PLoS One* **8:** e85091.

Hansen JG, Jørgensen A, Kristensen RM. 2001. Preliminary studies of the tardigrade fauna of the Faroe Bank. *Zoologischer Anzeiger* **240:** 385–393.

Hengherr S, Brümmer F, Schill RO. 2008. Anhydrobiosis in tardigrades and its effects on longevity traits. *Journal of Zoology* **275:** 216–220.

Jönsson KI, Rebecchi L. 2002. Experimentally induced anhydrobiosis in the tardigrade *Richtersius coronifer*: phenotypic factors affecting survival. *Journal of Experimental Zoology* **293:** 578–584.

Jørgensen A, Møbjerg N. 2015. Notes on the cryptobiotic capability of the marine arthrotardigrades *Styraconyx haploceros* (Halechiniscidae) and *Batillipes pennaki* (Batillipedidae) from the tidal zone in Roscoff, France. *Marine Biology Research* **11:** 214–217.

Jørgensen A, Faurby S, Hansen JG, Møbjerg N, Kristensen RM. 2010. Molecular phylogeny of Arthrotardigrada (Tardigrada). *Molecular Phylogenetics and Evolution* **54:** 1006–1015.

Keilin D. 1959. The problem of anabiosis or latent life: history and current concept. *Proceedings of the Royal Society of London. Series B* **150:** 149–191.

McInnes SJ. 1994. Zoogeographic distribution of terrestrial/freshwater tardigrades from current literature. *Journal of Natural History* **28:** 257–352.

Møbjerg N, Halberg KA, Jørgensen A, Persson D, Bjørn M, Ramløv H, Kristensen RM. 2011. Survival in extreme environments – on the current knowledge of adaptations in tardigrades. *Acta Physiologica Scandinavia* **202:** 409–420.

Nelson DR. 2002. Current status of the Tardigrada: evolution and ecology. *Integrative and Comparative Biology* **42:** 652–659.

Persson D, Halberg KA, Jørgensen A, Ricci C, Møbjerg N, Kristensen RM. 2011. Extreme stress tolerance in tardigrades: surviving space conditions in low earth orbit. *Journal of Zoological Systematics and Evolutionary Research* **49:** 90–97.

Rebecchi L, Altiero T, Guidetti R. 2007. Anhydrobiosis : the extreme limit of desiccation tolerance. *Invertebrate Survival Journal* **4:** 65–81.

Rebecchi L, Cesari M, Altiero T, Frigieri A, Guidetti R. 2009. Survival and DNA degradation in anhydrobiotic tardigrades. *Journal of Experimental Biology* **212:** 4033–4039.

Sands CJ, McInnes SJ, Marley NJ, Goodall-Copestake WP, Convey P, Linse K. 2008. Phylum Tardigrada: an "individual" approach. *Cladistics* **24:** 861–871.

Schill RO, Steinbrück GHB, Köhler HR. 2004. Stress gene (*hsp 70*) and quantitative expression in *Milnesium tardigradum* (Tardigrada) during active and cryptobiotic stages. *Journal of Experimental Biology* **207:** 1607–1613.

Seki K, Toyoshima M. 1998. Preserving tardigrades under pressure. *Nature* **395:** 853–854.

Sømme L. 1996. Anhydrobiosis and cold tolerance in tardigrades. *European Journal of Entomology* **93:** 349–357.

Wełnicz W, Grohme MA, Kaczmarek Ł, Schill RO, Frohme M. 2011. Anhydrobiosis in tardigrades – the last decade. *Journal of Insect Physiology* **57:** 577–583.

Wright JC, Westh P, Ramløv H. 1992. Cryptobiosis in Tardigrada. *Biological Reviews* **67:** 1–29.

Yamaguchi A, Tanaka S, Yamaguchi S, Kuwahara H, Takamura C, Imajoh-Ohmi S, Horikawa DD, Toyoda A, Katayama T, Arakawa K, Fujiyama A, Kubo T, Kunieda T. 2012. Two novel heat–soluble protein families abundantly expressed in an anhydrobiotic tardigrade. *PLoS One* **7:** e44209.