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Desiccation Tolerance in the Tardigrade *Richtersius coronifer* Relies on Muscle Mediated Structural Reorganization

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Abstract

Life unfolds within a framework of constraining abiotic factors, yet some organisms are adapted to handle large fluctuations in physical and chemical parameters. Tardigrades are microscopic ecdysozoans well known for their ability to endure hostile conditions, such as complete desiccation -- a phenomenon called anhydrobiosis. During dehydration, anhydrobiotic animals undergo a series of anatomical changes. Whether this reorganization is an essential regulated event mediated by active controlled processes, or merely a passive result of the dehydration process, has not been clearly determined. Here, we investigate parameters pivotal to the formation of the so-called "tun", a state that in tardigrades and rotifers marks the entrance into anhydrobiosis. Estimation of body volume in the eutardigrade *Richtersius coronifer* reveals an 87 % reduction in volume from the hydrated active state to the dehydrated tun state, underlining the structural stress associated with entering anhydrobiosis. Survival experiments with pharmacological inhibitors of mitochondrial energy production and muscle contractions show that i) mitochondrial energy production is a prerequisite for surviving desiccation, ii) uncoupling the mitochondria abolishes tun formation, and iii) inhibiting the musculature impairs the ability to form viable tuns. We moreover provide a comparative analysis of the structural changes involved in tun formation, using a combination of cytochemistry, confocal laser scanning microscopy and 3D reconstructions as well as scanning electron microscopy. Our data reveal that the musculature mediates a structural reorganization vital for anhydrobiotic survival, and furthermore that maintaining structural integrity is essential for resumption of life following rehydration.


Introduction

Anhydrobiosis is defined as a reversible entry into a latent state of life in response to desiccation [1]. This phenomenon is widespread across life kingdoms; among animals it is known from rotifers, nematodes and tardigrades as well as certain species of arthropods [2]. In the anhydrobiotic state, metabolic activities come to a reversible standstill, and the organism displays an increased resistance to physicochemical extremes [3]. Tardigrades are microscopic ecdysozoans [4-7] that can remain in this dehydrated state for up to 20 years [8], yet once external conditions again become favorable they resume life unaffected [9-11]. Many anhydrobiotic organisms are known to rely on specific bioprotectants, such as certain saccharides and proteins as well as antioxidant enzymes, in order to offset the damages associated with complete desiccation, e.g. [12-19]; however, a unifying theory on how "life without water" is biologically feasible can still not be claimed.

Upon sensing an as yet unidentified cue associated with a decrease in external water potential, anhydrobiotic animals undergo a series of anatomical changes. Rotifers and tardigrades contract in the anterior-posterior direction, and their extremities invaginate, resulting in a compact body shape called a “tun” [20,21]. Nematodes, incapable of a corresponding longitudinal contraction, coil into a tight spiral [20]. The functional significance of these changes has been suggested to be a reduced rate of evaporative water-loss, as well as a controlled packaging of organs, cells and organelles during the desiccation process [21-24]. Studies on anhydrobiotic rotifers [21,25] and nematodes [26] suggest that this reorganization of internal anatomy is coordinated and necessary for maintaining structural integrity and for anhydrobiotic survival. However, experiments on the
Exposure to toxins

°C, for a maximum of 2 weeks, until experimentation. PLOS ONE | www.plosone.org

Here, we investigate the anatomical changes that occur during anhydrobiosis in the tardigrade Richtersius coronifer (Richters, 1903), a species well known for its ability to enter anhydrobiosis [10,28,29]. We show that mitochondrial energy production and a functional musculature are prerequisites for the formation of the tun state. We furthermore present a detailed analysis of the musculature involved in tun formation.

Materials and Methods

Ethics Statement

Specimens of the tardigrade Richtersius coronifer (Figure 1) were collected from mosses on Öland, Sweden. Collection of specimens was approved by Station Linné (Porten till Alvaret).

Storing of tardigrades

Active animals were sorted from water soaked moss using a dissection microscope, and kept in ddH2O at 4 °C for two to three days to ensure that they remained active. Groups of 20-25 tardigrades, cleaned of debris, were transferred to, and dehydrated on small pieces of Whatman 3 filters (diameter app. 5 mm; see 28). Filters with dehydrated Richtersius coronifer were rinsed several times in ddH2O, and subsequently stored at 4 °C. For 24 h, both dehydrated groups were rehydrated, and allowed to revive over a further 72 h. One additional group kept in ddH2O during the five-day experimental period provided an estimate of baseline mortality. Survival was subsequently assessed for all groups with animals responsive to tactile stimuli being considered alive. Concentrations of 0.1 mM and 1.0 mM DNP were tested, with four to five experimental repeats conducted for each concentration.

We further investigated how pre-incubation in unlabeled phalloidin affected anhydrobiotic survival in Richtersius coronifer. Phalloidin is a bicyclic heptapeptide that selectively binds and stabilizes actin filaments (F-actin), which blocks nucleotide exchange [32] and consequently inhibits cross-bridge cycling and muscle contractions. Preliminary phalloidin incubation experiments, using fluorescent phalloidin, revealed that the primary entry site of the toxin was through the mouth and cloaca of the tardigrade. In these experiments, phalloidin would similarly be observed staining the muscles, as visualized by the fluorescent signal (data not shown), thus confirming that the toxin had access to the muscles during incubation experiments. The experimental procedure for pre-incubating animals in unlabeled phalloidin was as described above for the DNP-experiments, but with concentrations of 0.01, 0.1, 0.5 and 1.0 mg/ml phalloidin tested instead of DNP. Five experimental repeats were conducted at each concentration.

Following the dehydration protocol described above, and excluding animals damaged during placement on Whatman filters, an average of 97 ± 5 % animals survived induction of anhydrobiosis based on all the control experiments (A→W, Figure 2A-B). This notable survival rate, which is comparable to that reported previously [28], is not significantly different from the baseline survival, i.e. animals kept in ddH2O (W, Figure 2A-B; Tables S1, S2), demonstrating that anhydrobiosis is not associated with increased mortality in Richtersius coronifer using this protocol.

Microscopy

Fluorescent labeling of muscles and cell nuclei were performed in order to investigate morphological changes (e.g. rearrangement of organs and cells) occurring during anhydrobiosis in Richtersius coronifer. Active tardigrades were relaxed using CO2-enriched water, whereas dehydrated specimens placed on Whatman filters were “dry fixed” (i.e. placed over the fumes of a 3 % paraformaldehyde fixative) for 30 min, prior to fixation. Both hydrated and dehydrated animals were subsequently fixed for 20 min at RT in 3 %
Figure 1. Rearrangement of organs and cells during anhydrobiotic tun formation in *Richtersius coronifer*. Light microscopy of *A*. active, hydrated animal (lateral view) and *B*. a tun (ventral view) showing the rearrangement of major anatomical structures during tun formation. Note the compact body shape of the tun. Dashed circles indicate areas of the midgut (mg), gonads (go) and pharyngeal bulb (pb), respectively. The degree of longitudinal contraction is ultimately limited by the length of the rigid stylets (st). The pharyngeal bulb is for the most part repositioned in the dorsomedian plane. Maximum projection image of a confocal z-series of *C*. hydrated DAPI stained specimen (lateral view), and *D*. DAPI stained tun (ventral view) demonstrating the reposition of cell nuclei during tun formation. Scanning electron micrograph of *E*. a hydrated specimen (lateral view) and *F*. a tun (dorsal view) revealing the extensive changes in external morphology associated with formation of the tun. A→P, anterior-posterior axis; br, brain; gl-gIIV, ventral ganglia; mo, mouth. Scale bars = 100 μm.

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Figure 2. Effect of DNP and phalloidin on anhydrobiotic survival. A. Pre-incubation in DNP prior to dehydration, and attempting to induce anhydrobiosis (D→A→W), significantly reduces survival to 4 ± 9 % (0.1 mM) and 2 ± 4 % (1.0 mM). B. Incubating tardigrades in phalloidin (P→W) did not decrease survival at 0.01 mg/ml (97 ± 7 %), 0.1 mg/ml (96 ± 4 %) and 0.5 (95 ± 5 %) mg/ml. At 1 mg/ml, survival was significantly reduced to 26 ± 7 % (P<0.001; Table S2). Pre-incubation in phalloidin (P→A→W) reduced post-anhydrobiotic survival at concentrations of 0.1 mg/ml (82 ± 6 %), 0.5 mg/ml (77 ± 6 %) and 1.0 mg/ml (0 ± 0%) (P<0.001; Table S2). Significant differences between treatments are indicated by asterisks, with the significance levels P>0.05 (not significant) and P<0.001 (significant, ***).

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were dehydrated through a graded series of ethanol and
thickness of app. 12 nm; JEOL JFC-2300 HR sputtercoater)
(OriginLab, Northampton, MA, USA). Significant difference
(Figure 1A, 1C, 1E). As in other tardigrades, the complex
developed nervous and muscular systems, a complex feeding
visualize nuclei. Specimens were mounted on microscope
slides in Vectashield (Vector Laboratories Inc., CA, USA).
Image acquisition was performed on a Leica DM RXE 6 TL
inverted microscope equipped with a Leica TCS SP2 AOBS
confocal laser scanning unit, using the 488 nm argon/crypton
laser or the 405 nm UV-laser. A maximum projection or normal
shading of the z-series image was processed and edited in the
3D reconstruction software IMARIS (Bitplane AG, Zürich,
Netherlands) and incubated in PBS with 1 % Triton X-100, 0.1
% Na2S2O4 and a 1:20 dilution of Alexa Fluor 488 conjugated
phalloidin (Invitrogen, CA, USA) for up to 48 h. Afterwards, the
specimens were rinsed three times in PBS. Additional
preparations were washed with PBS and incubated in PBS with
1 % Triton X-100 and 1 % sodium citrate (pH 7.4). Following
fixation, the specimens were rinsed three times in PBS. Additional
comparisons of means (Table S1 and S2). The statistical tests
were performed using the data analysis program OriginPro 7.5
(OriginLab, Northampton, MA, USA). Significant difference
between treatments is indicated by asterisks, with significance
values (see Figure 3).

In order to visualize the external morphology of Richtersius
coronifer, we used scanning electron microscopy. Active
specimens were relaxed in CO2-enriched water and subsequently
fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer
(pH 7.4). Following fixation, the specimens were dehydrated through a graded series of ethanol and acetone. The specimens were then critical point dried (Bal-Tec CPD 030 critical point dryer), mounted on aluminum stubs, sputter-coated with palladium (65-70 s corresponding to a thickness of app. 12 nm; JEOL JFC-2300 HR sputtercoater) and examined in a JEOL JSM-6335F Field Emission scanning electron microscope. Both anhydrobiotic tuns and dehydrated collapsed specimens were mounted directly on aluminum stubs, sputter-coated with palladium and examined.

Statistics
Data are expressed as means ± s.d. The statistical significance
of differences between the various exposures was tested using one-way ANOVA followed by a Tukey’s multiple
corrections (see Figure 3).

Results and Discussion

Gross morphology
*Richtersius coronifer* is a large tardigrade species measuring
up to more than 1000 µm [33]. It has an elongated body outline
typical of eutardigrades with few visible sensory appendages,
and four pairs of legs each equipped with two double claws
(Figure 1A, 1C, 1E). As in other tardigrades, the complex
internal organ systems include a large brain and well
developed nervous and muscular systems, a complex feeding
apparatus with a muscular pharynx (the pharyngeal bulb) and
associated stylets for puncturing food particles, a subdivided
alimentary tract, as well as reproductive and osmoregulatory
organs. The specimens of *R. coronifer* from Öland were yellow
and laid round eggs ornamented with heavy spines.

Anatomical changes during anhydrobiosis
Anhydrobiotic tardigrades respond to removal of external
water by contracting in the anterior-posterior direction, and at the
same time withdrawing head and limbs, forming the
compact body shape called a tun (Figure 1B, 1D, 1F; Movie S1). According to our observations of *Richtersius coronifer*, this process is initiated when the animal senses a cue associated
with change in external water potential. The process can be
divided into three separate stages: I) active and hydrated; II)
dehydrating and ‘tucking in’; III) anhydrobiotic tun state (see
Movie S1). Our estimations of body volume in *R. coronifer*
reveal an 87 ± 5 % (n=17) reduction in volume from the
hydrated active state to the dehydrated tun state (Table 1). This
dramatic change in body volume is larger than the 60 %
reduction reported from bdelloid rotifers [25] and further
underlines the structural stress associated with entering
anhydrobiosis. Conversely, hydrated, active specimens of the
marine tardigrade *Halobiotus crispae* have been shown to
 tolerate above 60 % increase in body volume, during
exposures to hypotonic solutions, thus emphasizing the
amazing ability of tardigrades to withstand physical stress
[34,35]. The degree of longitudinal contraction during tun
formation in *R. coronifer* varies between individual tardigrades,
but is ultimately limited by the length of the rigid stylets (Figure
1A-B). The pharyngeal bulb is for the most part repositioned in
the dorsoventral plane of the animal (Figure 1B), its relocation
relying on a flexible esophagus. During the longitudinal
contraction, and in concert with the evaporative loss of the fluid
filled body cavity, organs and cells seem to be tucked in place
by undulatory movements of the trunk (stage II – ‘tucking in’,
Movie S1; Figure 1C-D).

Tun formation relies on mitochondrial energy
production
Animals exposed to DNP for 24 h became passive and
bloated, but regained activity following transfer to double
distilled water (D→W, Figure 2A) with only a small decrease in
survival, as compared to water controls (W, Figure 2A), at the
highest DNP dose (survival decreased to 80 ± 11 %, P<0.05;
Table S1). However pre-incubating specimens in DNP for 24 h
prior to inducing anhydrobiosis almost completely abolished
survival (D→A→W, Figure 2A). These DNP exposed animals
failed to form a tun and collapsed into an irregular flattened
shape upon dehydration (Figure 3G), indicating that successful
dehydration and the ability to form a tun is dependent on
mitochondrial energy production. This observation is supported
by an earlier report stating that eutardigrades, of the species
*Paramacrobiotus areolatus*, failed to form tuns under anoxic
conditions [22].
Figure 3. Myoanatomical changes in *Richtersius coronifer* during tun formation. **A-D.** 3D reconstructions of the tardigrade musculature as visualized by fluorescent phalloidin. **A.** Lateral view of active (hydrated) state showing details of the dorsal, lateral and leg musculature. **B.** Ventral view showing details of the ventral longitudinal musculature in the active state. **C.** Dorsal view of the myoanatomy of the tun (dehydrated) state. **D.** Ventral view of the myoanatomy of the tun. **E-F.** Scanning electron microscopy (SEM) of animals in the tun state showing the corresponding external morphology of the myoanatomy presented in **C** and **D.** **E.** Dorsal view of the tun. **F.** Ventral view of the tun. **G.** SEM of an animal incubated in 1.0 mg/ml phalloidin for 24 h and subsequently dehydrated. The animal failed to form a tun upon dehydration, and collapsed into a flattened shape. A similar collapse was seen in DNP exposed animals upon dehydration. **H.** Corresponding maximum projection image of a confocal z-series of the musculature of a specimen incubated in 1.0 mg/ml phalloidin for 24 h before dehydration. A↔P, anterior-posterior axis; A-W, dorsal attachment sites; τ₀-τ₄, lateral attachment sites; la-g, ventral intermediate attachment sites; 1-7, ventromedian attachment sites; pb, pharyngeal bulb; ω₁-Ω, attachment sites of muscles associated with the pharyngeal bulb; stm, stylet muscles. Solid circles indicate lateral attachment sites, solid squares show ventral intermediate attachment sites, while dashed circles indicate areas of the legs. Scale bars = 100 μm.

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Figure 2B), did not decrease survival significantly, though muscle system was rendered non-functional, collapsed in a manner similarly to the DNP-treated animals upon drying, and did not revive following rehydration.

No tardigrades survived anhydrobiosis after pre-incubation in 1 mg/ml phalloidin (Figure 2B; Table S2). Notably, animals in which the withdrawal of the legs during tun formation (Figure 4C). Thus a range of muscles direct – in a predictable and coordinated manner – the structural rearrangements necessary for formation of the tun state.

**Table 1.** Reduction in volume (%) of *Richtersius coronifer* from active (hydrated) to tun (dehydrated) state.

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<th>Hydrated Length (μm)</th>
<th>Hydrated Width (μm)</th>
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<th>Reduction in volume (%)</th>
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Mean ± s.d. reduction in volume (%) 87 ± 5

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A functional musculature is a prerequisite for anhydrobiotic survival

Incubating animals in respectively 0.01, 0.1 and 0.5 mg/ml phalloidin for 24 h, before transferring them to water (P→W, Figure 2B), did not decrease survival significantly, though survival was reduced at 1 mg/ml (Figure 2B; Table S2). However, pre-incubating animals in 0.1 and 0.5 mg/ml phalloidin, prior to inducing anhydrobiosis, significantly reduced post-anhydrobiotic survival (P→A→W, Figure 2B), indicating that a functional muscle-system is indeed vital for anhydrobiotic survival. No tardigrades survived anhydrobiosis after pre-incubation in 1 mg/ml phalloidin. Notably, animals in which the muscle system was rendered non-functional, collapsed in a manner similarly to the DNP-treated animals upon drying, and did not revive following rehydration.

We subsequently investigated the myoanatomy of *Richtersius coronifer* in active, tun and dehydrated collapsed states with the aid of fluorophore-conjugated phalloidin (Figure 3). In tardigrades, the body musculature is composed of structurally independent muscle fibers that can be assigned to ventral, dorsoventral, dorsal, lateral, and leg musculature [36,37]. The ventral musculature of *R. coronifer* is dominated by seven ventromedian attachment sites (labeled 1-7, Figure 3B, 3D, 3F) from which leg muscles, dorsoventral muscles and lateral muscles originate. In addition, a ventral longitudinal musculature with intermediate attachment sites (labeled c-g, Figure 3A-B, 3F) extends along the anterior-posterior axis. The dorsal longitudinal musculature consists of an outer and an inner muscle strand that both extend the length of the trunk. Both strands are repetitively interrupted by attachment sites (labeled A-W, Figure 3A, 3C, 3E) mainly associated with the legs. Nine lateral sites (labeled t0-t4 and T0-T3, Figure 3A) serve as attachments for the lateral musculature and the dorsoventral muscles. Leg muscles in *R. coronifer* originate from the dorsal, lateral and ventral side of the animal (see 36,37 for further information). The confocal images show that the muscles are contracted in the tun state in comparison to the active, hydrated specimens (Figure 3A-D). Animals that were exposed to 1 mg/ml phalloidin, and subsequently dehydrated, collapsed into a flattened shape and revealed a more disordered muscle organization, in which individual structural elements were difficult to recognize (Figure 3G-H).

**Analysis of the Musculature Involved in Tun Formation**

As previously shown in rotifers [21], and also suggested for tardigrades, e.g. [22], our study confirms that proper tun formation is essential for anhydrobiotic survival. Our results show that uncoupling mitochondrial energy production and inhibiting muscle contraction interferes with formation of the anhydrobiotic tun, thereby respectively abolishing and reducing the ability of *Richtersius coronifer* to survive desiccation. We propose that the dorsal and ventral longitudinal muscles are responsible for contraction of the animal during entry into the tun state (Figure 4A-B), while the lateral musculature assists in the longitudinal contraction, and generates undulatory movements of the trunk that facilitate reorganization of internal structures (stage II – ‘tucking in’, Movie S1). Furthermore, the muscles associated with each leg are activated in the withdrawal of the legs during tun formation (Figure 4C). Thus a range of muscles direct – in a predictable and coordinated manner – the structural rearrangements necessary for formation of the tun state.
Figure 4. Schematic representation of the muscles involved in tun formation in *Richtersius coronifer*. Schematic representation illustrating contraction of muscles during the transition from the active (hydrated) to the tun (dehydrated) state. **A.** Ventral longitudinal musculature. **B.** Dorsal longitudinal musculature. **C.** Leg musculature. The dorsal longitudinal, ventral longitudinal, as well as lateral musculature are involved in reshaping the whole body during anhydrobiosis, and are consequently responsible for generating the compact body shape of the tun. Tun formation is moreover characterized by the withdrawal of the legs into the body cavity. Letters and numbers indicate specific muscle attachment sites (see Figure 3).

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Supporting Information

Movie S1. Anhydrobiotic tun formation in Richtersius coronifer. The most obvious morphological changes associated with tun formation are the anterior-posterior contraction of the trunk and retraction of legs. According to our observations of the behavior of animals during entrance into anhydrobiosis, this process is initiated when the animals sense a decrease in external water potential. Entrance into and exit out of anhydrobiosis can be divided into four separate stages (I, active hydrated; II, dehydrating, ‘tucking in’; III, anhydrobiotic tun state; IV, rehydration) the completion of which is an active process orchestrated by the muscle system. The movie was made using an Infinity X Digital Camera (DeltaPix, Denmark) mounted on a Leica MZ 16 microscope (x80 magnification). High resolution AVI files recorded with the camera software were imported into Windows Movie Maker for the creation of the final video sequence.

(WMV)

Table S1. Statistical analyses of the DNP data.

References


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Author Contributions

Conceived and designed the experiments: KAH AJ NM. Performed the experiments: KAH AJ NM. Contributed reagents/materials/analysis tools: AJ NM. Wrote the manuscript: KAH NM. Edited and commented on the manuscript: KAH AJ NM.


