Pro gradu -tutkielma

Gene expression patterns during anhydrobiosis in *Hypsibius exemplaris*

Tiia Nikupaavola

Jyväskylän yliopisto

Bio- ja ympäristötieteiden laitos

Solu- ja molekyylibiologia

05.10.2018

JYVÄSKYLÄN YLIOPISTO, Matemaattis-luonnontieteellinen tiedekunta
Bio- ja ympäristötieteiden laitos
[Solu- ja molekyylibiologia]

Tiia Nikupaavola:	Geeniekspressio anhydrobioosin aikana karhukaisella *Hypsibius exemplaris*
Pro gradu -tutkielma:	38 s., 0 liitettä
Työn ohjaajat:	Professori Emily Knott, Sara Calhim
Tarkastajat:	Professori Emily Knott, akatemiatutkija Matti Jalasvuori
08/2018	

Hakusanat: Aquaporiini, ddPCR, HSP, *Hypsibius exemplaris*, LEA

Yksi evoluutiobiologian kysymyksistä on tunnistaa ja ymmärtää geenit, jotka vaikuttavat sopeutumista edistäviin piirteisiin. Anhydrobioosi on fenotyyppinen sopeuma, joka mahdollistaa yksilön selviytymisen ajoittaiselta kuivumiselta muuten sen luontaisesti akvaattisessa ympäristössä ja se on yleinen useilla pienselkärangattomilla, erityisesti karhukaisilla. Geenien säätelyn tutkiminen mahdollistaa ymmärtämään anhydrobioosin eri vaiheita molekyylitasolla. Lämpöshokkiproteiinien (HSPs), aquaporiinien (AQPs) ja myöhäis-alkionkehityksen runsaat proteiinien (LEAs) on esitetty vaikuttavan anhydrobioosiin. Valitettavasti aikaisemmat tutkimukset ovat pääasiassa keskittyneet vain yhteen proteiiniryhmään, ja/tai vertaillut ilmentämisen eroja anhydrobioosin pääpisteissä, kuten eroja aktiivisten ja kuivuneiden yksilöiden välillä. Tutkimuksessani mittaan samanaikaisesti kolmen eri geenin ilmentymistä edellä mainitusta proteiiniryhmistä ja vertailen ilmentämisen tasoja peräkkäisistä anhydrobioosin vaiheista (aktiivinen, esivaikutus, siirtymä ja kuiva) karhukaisella *Hypsibius exemplaris*. Ilmentämisen erojen tarkka mittaaminen onnistui käyttämällä digital droplet PCR (ddPCR) tekniikkaa, joka on optimoitu pienille näytemäärille. Tuloksissa kohdegeenien ekspressio osoitti erilaisia ilmentämisen malleja tutkituille geeneille. *HSP70-like 1* geenin ilmentyminen laski 80% siirtymävaiheen ja kuivavaiheen välillä, kun taas ilmentyminen esivaikutus-, siirtymä- ja kuiva-vaiheiden välillä pysyi samanlaisena. *HSP70-like 1* geenin ekspressiotasot ovat samankaltaiset kuin mitä *HSP70-3* geenillä on mitattu karhukaisella *M. tardigradum*. Samankaltaisuus voi viitata siihen, että *HSP70-like 1* geenillä on samankaltainen rooli anhydrobioosissa karhukaisella *H. exemplaris*. *AQP10* ilmentyminen laski kolmasosaan aktiivisten ja siirtymävaiheen yksilöiden välillä, mutta esivaikutusvaiheen aikana ilmentyminen oli viisinkertainen verrattuna siirtymävaiheen yksilöihin. Korkea ekspressio esivaikutusvaiheen aikana viittaa *AQP10* geenin mahdollisesti vaikuttavan anhydrobioosiin. Myös *LEA1* geenin ilmentymisen kasvu esivaikutusvaiheen aikana viittaa geenillä olevan rooli anhydrobioosissa. Tutkimukseni osoittaa, kuinka tärkeää on tutkia lajispesifisiä mekanismeja karhukaisten sopeutumiseen muuttuvia ympäristöoloja kohtaan, jonka lisäksi tutkimus tarjoaa vertailukohdan nykyisille ja tuleville tutkimuksille.

UNIVERSITY OF JYVÄSKYLÄ, Faculty of Mathematics and Science
Department of Biological and Environmental Science
Cell and molecular biology

Tiia Nikupaavola:	Gene expression patterns during anhydrobiosis in *Hypsibius exemplaris*
MSc thesis:	38 p., 0 appendices
Supervisors:	Professor Emily Knott and Sara Calhim
Inspectors:	Professor Emily Knott, Academy Research fellow Matti Jalasvuori

08/2018

Identifying and understanding the role of genes underlying adaptive phenotypes is a major goal of modern evolutionary ecology. Anhydrobiosis is phenotypic adaptation that allows individuals to withstand the temporary desiccation of their otherwise aquatic environment. It is common across microinvertebrate groups, namely tardigrades. Gene expression analyses provide a window to the molecular mechanisms that enable organisms to survive and recover from these events. Proteins of three major groups have been suggested to have a key role in such adaptations: heat-shock proteins (HSPs), aquaporins (AQPs) and late embryogenic abundant proteins (LEAs). However, previous studies have largely focused on only a small subset of these proteins, and/or only compared effects between the end points of the anhydrobiosis process (i.e. dry vs. active individuals). In my study, I combine for the first time (i) the simultaneous estimate of expression levels in all three key protein groups, and (ii) a comparison across consecutive anhydrobiosis states (active, transitioning and dry), using the tardigrade *Hypsibius exemplaris.* Moreover, by using digital droplet PCR (ddPCR), a novel approach that is optimized for small sampling units, I could obtain more precise expression measures. The results show different patterns of gene expression in all target genes. The expression level of *HSP70-like 1* dropped by 80% in the dry state compared to the transition state, while the transcript copies halved between active and transitional states. *HSP70-like 1* transcript numbers stayed similar between active and transitional states. *AQP10* expression levels were 3 time more in the active state and 5 times more in the preconditioning state, while the dry state had 3 times more transcript copies compared to the transitional state. *LEA1* showed induction during the preconditioning state, while the expression levels in active, transition and dry states stayed similar. The findings from *HSP70-like 1* are similar to expression changes in *HSP70-3* observed previously in the tardigrade *Milnesium tardigradum*. This implies that *HSP70-like 1* might have a similar role in *H. exemplaris*. The induction of *AQP10* in preconditioning suggests this gene is important for *H. exemplaris* when entering anhydrobiosis. The induction of *LEA1* in the preconditioning state also suggests this gene has a role in entering anhydrobiosis. In conclusion, this study illustrates the importance of studying species-specific mechanisms of tardigrade adaptation to changing limnoterrestrial conditions as well as provides a benchmark for comparative studies and future experimental research.

TABLE OF CONTENTS

TABLE OF CONTENTS 4

1 INTRODUCTION 1

1.2 Heat-shock proteins 2

1.3 Aquaporins 3

1.4 Late Embryogenesis Abundant proteins 3

1.5 Tardigrades 4

1.6 Study aim 5

1.6.1. Hypotheses 5

2 MATERIALS AND METHODS 6

2.2 Materials 6

2.3 Methods 8

3 RESULTS 14

4 DISCUSSION 20

5 SUMMARY 27

THANKS 28

LITERATURE 28

GLOSSARY AND ABBREVIATIONS

GLOSSARY

Cryptobiosis	Biological state, in which metabolism is reversibly suspended by environmental factors
Anhydrobiosis	A form of cryptobiosis induced by desiccation
Reference gene	Gene that is assumed to be constitutively and stably expressed so that it can be used to remove variation from the target gene (normalization)
Vitrification	A process in which a substance transform into a non-crystalline amorphous glass

ABBREVIATIONS

AIC	Aikake information criterion, which estimates the relative quality of statistical model for a given set of data
AQP	Aquaporin
CAHS	Cytoplasmic Abundant Heat Soluble proteins
ddPCR	Digital droplet PCR
HSPs	Heat-shock proteins
LEAs	Late embryogenic abundant proteins
Nb	Negative binomial error distribution model
Pois	Poisson error distribution model
SAHS	Secretory Abundant Heat Soluble proteins

1 INTRODUCTION

Different forms of cryptobiotic states exist, in which metabolic activity is reversibly attenuated due to exogenous factors (Rebecchi et al., 2007; Møjberg et al. 2011). Anhydrobiosis is the most studied cryptobiotic state (Møjberg et al. 2011) that is induced by desiccation (Wright et al., 1992). It represents survival strategy to periods of severe dehydration (Bertolani et al., 2004) in some microorganisms, plants and invertebrate animals, such as rotifers, nematodes and tardigrades (Møjberg et al. 2011; Rebecchi et al., 2007). Usually desiccation causes severe cellular damage (Rebecchi et al., 2007), but in anhydrobiosis organisms tolerate extremely low (< 0.1 g H_2O/g dry weight) water concentrations inside cells (Crowe, et al., 1991; Alpert, 2005). This allows the animals to survive desiccation that could otherwise kill the individual, without sustaining damage (Nelson, 2010; Wright, 1989; Rebecchi et al., 2007).

Entering and exiting anhydrobiosis is thought to be energetically costly for the organism (Ricci and Manuela, 1998). Metabolic, molecular, tissue, organ and DNA damage caused by oxidative processes that occur when entering and exiting anhydrobiosis are known be detrimental (Neuman et al., 2006; Møbjerg et al., 2011). Persisting for a longer time in a suspended anhydrobiotic state requires more energy for repairing any occurring cellular damages (Jönsson, 2005). Indeed, the tardigrade *Richtersius coronifer* in longer anhydrobiotic states showed decreased survivability (Czernekova and Jönsson, 2016), longer recovery rates (Neumann et al., 2009) and increased DNA damage (Bertolani et al., 2004; Neumann et al., 2009) than those in anhydrobiotic states for shorter times. During anhydrobiosis, an organism lacks metabolic features that are present in active beings, namely replication and translation processes (Clegg, 2001; Mali et al., 2010), which is why the production of new mRNA is halted and the regulation of existing mRNA becomes important (Förster et al., 2009). These regulative elements are believed to be important for an organism's ability to transition between active

and anhydrobiotic states (Förster et al., 2009). Several metabolic changes in bioproducts, such as carbohydrates, proteins, enzymes and free radicals are also thought to be required while entering and exiting anhydrobiosis (Mali et al., 2010; Møjberg et al., 2011; Wang et al., 2014; Kondo et al., 2015). These bioproducts can work as osmolytes, compounds affecting cell fluid balance during osmotic stress (Crowe et al., 1992) or dehydration stress by directly influencing structures of macromolecules, including membranes, DNA and proteins (Møjberg et al., 2011). Transcriptomes have been compared from several tardigrade species to define common adaptations against stress (Mali et al., 2010; Förster et al., 2012; Wang et al., 2014). Such stress related pathway proteins found in the tardigrade *Hypsibius dujardini* consist of genes from three major groups, namely heat shock proteins (HSPs), aquaporins (AQPs) and late-embryogenesis abundant proteins (LEAs) (Förster et al., 2012).

1.2 Heat-shock proteins

Heat-shock (HSP) proteins are molecular chaperones that help to fold newly synthesized proteins, regulate their intracellular locations, protect against stress related denaturation and partake in intracellular stress responses (Feder and Hofmann, 1999; Clegg 2001; Sun and Macrae, 2005; Förster et al., 2009). Genes encoding these proteins are conserved in all of the organisms they have been found (Feder and Hofmann, 1999), including tardigrades (Adhikari et al., 2009; Mali et al., 2010; Reuner et al., 2010; Yoshida et al., 2017). Due to their function in the cell, HSPs are believed to enhance tolerance in cryptobiosis (Møjberg et al. 2011). Schill et al. (2004) observed different levels of expression of HSP70 genes across consecutive states of cryptobiosis in the tardigrade *Milnesium tardigradum* and from the three recognized HSP70 isoforms, *HSP70-II* was the most relevant in (recovering from) anhydrobiosis. *HSP70* induction in rehydrating tardigrade *Richtersius coronifer* (Jönssön and Schill, 2007) shows a similar gene expression pattern as observed in *M. tardigradum* (Schill et al., 2004). In contrast, Reuner et al. (2010) found slight upregulation of *HSP70-II* in transitional state in *M. tardigradum* while entering anhydrobiosis, suggesting that the role of HSPs is to prepare the

organism for rehydration before the actual anhydrobiotic state. Differences in HSP expression in tardigrades can be due to their possible species-specific regulation (Møjberg et al., 2011), raising the importance to study species-specific gene regulation patterns of heat-shock proteins.

1.3 Aquaporins

Aquaporins (AQPs) are hydrophobic, integral membrane proteins (Verkman, 2005) residing mainly in the cell membrane (Grohme et al., 2013). Common among plants and animals (Verkman, 2005), these water-channel proteins are divided into two types depending upon whether they selectively transport just water or transport water and small uncharged molecules (King et al., 2004; Kikawada et al., 2008; Gomes et al., 2009; Mali et al., 2010). In addition, AQPs are thought to also facilitate cell migration, although the mechanism for this is still unknown (Verkman, 2005). With other transmembrane proteins, aquaporins have been associated with water transport when diffusion is restricted, such as during anhydrobiosis (Mali et al., 2010). Resistance to freezing temperatures has been suggested to be due to transport of water and glycerol via aquaporins in moth *Chilo suppressalis* larvae (Izumi et al., 2007). While aquaporin transcripts have been found in several tardigrade species (Mali et al., 2010), no significant AQP expression levels were found in *M. tardigradum* (Grohme et al., 2013), and overall low expression levels in *H. exemplaris* were found in both active and inactive stages (Yoshida et al., 2017).

1.4 Late Embryogenesis Abundant proteins

Late-embryogenesis abundant proteins (LEAs) are generally hydrophilic (Hand et al., 2011), heat-soluble proteins that facilitate stress tolerance by preventing protein aggregation during dehydration in anhydrobiotic organisms (Garcia, 2011; Yamaguchi et al., 2012; Wang et al., 2014). LEAs have also showed signs of serving as “hydration buffer”, increasing water retention in the plant *Arabidopsis thaliana* (Hand et al., 2011). The function of LEA proteins in anhydrobiosis is still unclear,

although studies with nematodes (Browne et al., 2004; Goyal et al., 2005; Adhikari et al., 2009), chironomids (Kikawada et al., 2006) and plants (Förster et al., 2012), and even tardigrades (Shockarie et al., 2010; Förster et al., 2012; Yoshida et al., 2017) support their role in anhydrobiotic processes. Since LEAs are also present in seeds sensitive to dehydration (Tunnacliffe et al., 2007), the expression of LEAs alone do not protect an organism against dehydration and other factors are required to successfully undergo anhydrobiosis.

1.5 Tardigrades

Tardigrades are invertebrate micrometazoans (Nelson, 2010) that inhabit marine, freshwater and terrestrial habitats worldwide (Møjberg et al., 2011). Tardigrades are especially common in limnoterrestrial habitats, such as mosses, lichens and in leaf waste (Nelson, 2010), where they are dependent on water in their active state (Nelson, 2010; Møjberg et al., 2011). In recent years, tardigrades have gained interest due to their high tolerance against extreme environmental stressors, such as UV-radiation (Horikawa et al., 2013), gamma and heavy ion radiation (Horikawa et al., 2006), subzero temperatures (Hengherr et al., 2009), desiccation (reviewed in Rebecchi et al., 2007), high salinity (reviewed in Møjberg et al., 2011), and even cosmic radiation and microgravity (Persson et al., 2011). Anhydrobiosis represents survival strategy for tardigrades to periods of severe dehydration (Bertolani et al., 2004). While entering anhydrobiosis, tardigrades show anterior-posterior body contraction and limb invagination as it enters to a form called “tun” (Rebecchi et al., 2007). In this state, tardigrades are able to survive under unfavorable conditions (Wang et al., 2014), and when water is again available they can return into their active state (Crowe et al., 1992).

Anhydrobiotic/Cryptobiotic capacity has shown interspecific (Förster et al., 2012) and intraspecific variation in tardigrades (Horikawa & Higashi, 2004), and this variation is thought to be based on genetics. Among tardigrades, the majority of genes related to cryptobiotic capacity are those associated with stress responses (e.g. mRNA regulation and redox-protection) (Förster et al., 2009). Because

genome sizes of tardigrade species vary greatly and many of their genes are still unknown, there is not much information about tardigrade physiology and the genetic mechanisms associated with extreme adaptive ability (Mali et al., 2010; Møbjerg et al., 2011). Further studies are required to better understand the genetic adaptations regarding tolerance against changing environmental conditions. However, the mechanisms allowing cryptobiosis have gained a lot of interest in recent years, such as how an organism's metabolic state is normalized after both short-term cryptobiosis or years of suspension in a cryptobiotic state (Møbjerg et al., 2011).

1.6 Study aim

This study aimed to compare gene expression in the tardigrade *Hypsibius exemplaris* when entering anhydrobiosis. I measured simultaneous estimation of expression levels in *HSP70-like 1*, *AQP10* and *LEA1* genes across consecutive anhydrobiosis states (active, preconditioning, transitioning and dry). These target genes were selected since they have been studied already previously with anhydrobiotic animals, and thus it was possible to formulate hypotheses. I used digital droplet PCR (ddPCR), which is a technique optimized for precise detection of even miniscule expression differences in a sample (Hindson et al., 2011). Because of its higher precision and the lower quantity of template required, ddPCR was chosen over qPCR. The focus of this analysis was to assess expression level changes between the transitional stage and the other anhydrobiosis states. Since there are no previous studies that have assessed consecutive expression pattern in *H. exemplaris*, the following hypotheses have been based on the assumption that the target genes used in this study behave similarly across species.

1.6.1. Hypotheses

I expected to observe downregulation in *HSP70-like 1* gene in the transitional state compared to other observed anhydrobiosis states (active, preconditioned and dry).

Previous studies with anhydrobiotic tardigrade *M. tardigradum* showed downregulation of *HSP70 isoform 1* in the transitional state, suggesting the gene having no direct role in anhydrobiosis (Schill et al., 2004; Reuner et al., 2010). In addition, *HSP70* showed downregulation in the transitional stage in tardigrade *R. coronifer* (Jönsson and Schill, 2007).

The expression of *AQP10* was assumed to show induction in the transitional state compared to other anhydrobiosis states observed. Even though AQPs have been suggested to be important while entering and/or exiting anhydrobiosis (Mali et al., 2010), previous studies have omitted the transitional state in their observations, showing no significant expression changes in anhydrobiotic *M. tardigradum* (Grohme et al., 2013) or contradictory expression patterns between anhydrobiotic tardigrades *R. varieornatus* and *H. dujardini* (Yoshida et al., 2017).

LEA1 was also assumed to show higher expression levels in the transitional state, as an induction of *Aav-lea-1* was observed to show an increase in response to desiccation in the nematode *Aphelenchus avenae* (Browne, et al., 2004) and in the chironomid *Polypedilum vanderplanki* while transitioning to anhydrobiosis (Kikawada et al., 2006).

2 MATERIALS AND METHODS

2.2 Materials

Tardigrade culture and sampling

The Z151 strain from a single parthenogenetic female of tardigrade *Hypsibius dujardini* (later named *H. exemplaris*, Gasiorek et al., 2018) was purchased from Sciento (UK) was maintained at 16 °C with 2h light period per day. Tardigrades were reared on plastic plates (45 mm diameter) in mineral water (Rainbow, Finland) containing *Chlorococcum* sp (Sciento, UK) as food *ad libitum*. Cultures were checked every 2 weeks and food replaced as needed.

Species-specific PCR primers

In this study, primers to specific target genes were used for PCR (Table 1). Primers were designed by my supervisor Emily Knott. Primers were designed with Primer3 software (Untergasser et al., 2012) based on partial gene sequences present in NCBI GenBank (HSP70-like isoform 1, accession no. OQV24176.1; AQP10, accession no. OQV21572.1; LEA1, accession no. OQV16121.1) and for actin (accession no. HM238268.1). Actin was selected as the reference gene. Reference gene is used to normalize the expression result of the target genes, thus it is expected to be continuously and stably expressed despite the experimental treatment. Because of this, the variation in the reference gene expression due to methodological errors can be removed from the variation in the target gene. Actin was selected as a reference gene for this study as previous gene expression studies on tardigrades (Schill et al., 2004; Reuner et al., 2010) have also used actin as a reference gene. The primers purchased from TAG Copenhagen are summarized in Table 1a-d.

Table 1. Primers used to amplify sequences of actin, hsp70, aquaporin and LEA genes.

Primer	Sequence
(a) *Hsp70-like isoform 1*	for 5′-AATGGTCGTTGTGAGGCTGT-3′
	rev 5′-TAGCGGTGTCCCAGAATTCG-3′
(b) *Aquaporin 10*	for 5′-AGCCAGCTTATTCCGCCATC-3′
	rev 5′-CGTCACTAGTCCGCTGTACA-3′
(c) *LEA 1*	for 5′-GAGCGCGACCTTTCTACAGA-3′
	rev 5′-CTTCACTTCCGCCGTCTTCT-3′
(d) *Actin*	for 5′-ATCAGTCAGTTCCAGCACCG-3′
	rev 5′-TCCATTGTCCACGACCAAGG-3′

for, forward; rev, reverse

2.3 Methods

Desiccation protocol

Procedures were performed at 18ºC without light using a climate chamber (Panasonic, MLR-352H). Protocol followed the one established in Kondo et al. (2016) with slight modifications (Figure 1) optimized for the equipment used in my study, as Kondo (2016) used different solutes to equilibrate relative humidity instead of a climate chamber. In short, tardigrades kept in their rearing conditions were transferred onto a glass plate with mineral water for the preconditioning period for 24 hrs. at 90% relative humidity (RH). Kondo et al. (2010) showed the necessity of a preconditioning period for successful anhydrobiosis in *H. exemplaris* and that a 24 hour preconditioning period was enough to ensure high survivability of the tardigrades, which is why I used one day long period in this study. After the preconditioning period, two tardigrades were placed on the same nylon net filter (Millipore, USA; pore size 11 µm, 25 mm diameter) 'island' (meshes were cut in eight pieces) placed in a plastic petri dish (45 mm diameter) with 15 µl mineral water. Each 'island' was considered a sample. The desiccation protocol was optimized to obtain a reliable 'transition' stage timing in this species. For desiccation, animals were first exposed to 80% RH for 24 hours following a further 24 hours at 60% RH. Transitional stage was defined after the first 24 hours of desiccation similarly as in Schill et al. (2004), in which the tardigrade's legs were drawn in, but the body still showed distinct activity, e.g. movement. The desiccation protocol was repeated three times (sequential weeks) with four replicates each. Thus, the total sample size was 48, 12 samples per anhydrobiosis state.

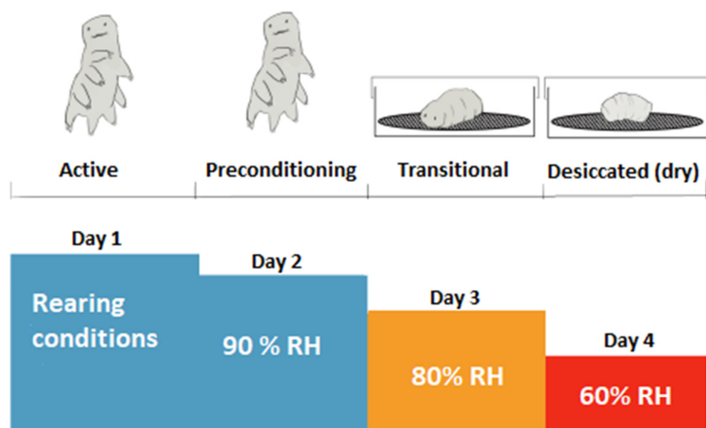

Figure 1 Scheme of desiccation protocol.

Total RNA extraction from samples

Extractions were performed from the 'island' samples, each holding two tardigrade individuals. In total 12 replicates, each of active, preconditioned, transitional and anhydrobiotic states was analyzed. The method for RNA extraction employed a protocol previously optimized for *Hypsibius exemplaris* (Arakawa et al., 2016). Each island was placed in a low-binding PCR tube (Starlab, Germany, E1405-2600, lot. 16183). For active and preconditioning samples, two tardigrades were placed in a low-binding PCR tube with minimal water carryover (< 2 µl) and the mesh island was added separately. Pipette tips were used to crush animals by pressing them against the tube wall, followed by immediate lysing in 200 µl TriZol reagent (ThermoFisher Scientific, USA, 15596026, lot. 13902002). RNA was extracted using Direct-Zol RNA microprep kit (Zymo Research, USA, Cat. R2062, lot. ZRZ189215) following the manufacturer's instructions. RNA quantity was checked using the Tape Station 2200 and the High Sensitivity RNA assay (Agilent Technologies, USA).

cDNA preparation

Reverse transcription (RT) for cDNA preparation was performed with the total RNA of each sample. Concentrations of RNA between samples were not standardized. The iScript kit (Bio-Rad Laboratories, USA) reagents and

instructions were used to prepare a master mix with 4 µl of RNA template. Reverse transcription was performed with following PCR protocol: 5 min at 25 C, 20 min at 46C and 1 min at 95 C.

Gene expression measurement with Droplet Digital PCR (ddPCR)

Gene expression was measured using the QX200 ddPCR platform and reagents from Bio-Rad. All reagents were thawed to room temperature. Samples and reagents were mixed by vortexing to ensure homogeneity and centrifuged briefly to collect all contents at the bottom of the tube. Reaction recipe for one sample was prepared as in Table 2. The recipe was multiplied by the number of samples, controls and reference gene used.

Table 2. Recipe used for the ddPCR reaction mix

EvaGreen Supermix	11 µl
Primer forward (1 uM)	1.1 µl
Primer reverse (1 uM)	1.1 µl
dH20 (Hyclone)	6.6 µl
Template cDNA (undiluted)	2.2 µl
Total volume	22 µl

After adding the template last, the reactions were mixed by pipetting and briefly centrifuged. For the control samples, dH_2O was added in equal volume instead of template. Once the reaction mixtures were ready, 20 µl of each reaction mix was loaded into a sample well of DG8 Cartridge for QX200 Droplet Generator (BioRad, #1864008) followed by adding 70 µl of QX200 Droplet Generation Oil for EvaGreen (BioRad, #1864006) into the oil wells, and droplets were made using the QX200 Droplet Generator following the manufacturer's instructions..

After Droplet generation, droplets were carefully transferred into a 96-well plate (BioRad, #12001925). The plate was sealed for 5 seconds at 180ºC using the PX1

PCR Plate Sealer (BioRad). Then, samples proceeded to thermal cycling (see protocol in Table 3)

Table 3. Cycling conditions for Bio-Rad's C1000 PCR

Cycling Step	Temperature, C	Time	Ramp Rate	Number of Cycles
Enzyme activation	95	5 min	2 C/sec	1
Denaturation	95	30 sec		39
Annealing	58	1 min		
Extension	72	45 sec		
Signal stabilization	4			1
	90			1
Hold (optional)	12	Infinite		1

After thermal cycling, the plate was transferred in the QX200 Droplet Reader (BioRad) and QuantaSoft Software (v.1.7.4.0917, Bio-Rad Laboratories, USA) was used to set up the plate layout and for analysis of positive (successful amplification) and negative (no amplification) droplets as well as calculation of copy numbers.

Statistics

Output of copy numbers were used as the raw data in statistical analyses using R program (v. 3.4.3, R Core Team, 2017). Transcript copy number was multiplied by 100 to have full numbers to model data as counts. The number of reference gene copies were then used so that the effect size would be the transcript amount of the gene of interest per unit copy of the reference gene. Expression levels in transitional state was then compared to active, precondition and dry states. The

effect of weeks was not included as a random effect due to the small number of repeats (n=3). Possible differences in expression levels were measured with Generalized linear models, where six different count data distributions were compared: Poisson (pois), Negative Binomial (nb), pois zero-inflated, nb zero-inflated, pois hurdle (zero-augmented), and nb hurdle. The best model for a given dataset was selected based on the AIC value, which estimates the quality of each model relative to each of the other models used (Table 4). The statistical significance of differences in transcript levels of all target genes were tested using zero-inflated Negative Binomial error distribution model. Despite the fact that the Hurdle nb and Zero inflated nb models do not differ by at least 2 delta AIC, I chose the latter model because the lack of biological reason to expect different biological processes behind zero and non-zero values. The total amount of samples per state was 12, but since in some cases the copy number of actin=0, values in these samples were excluded from the analyses as the zero values were assumed to be due to methodological errors. I also checked if actin was a good reference gene by testing if its copy number changes with different anhydrobiosis state.

Table 4. The AIC estimates of value fit for given set of data. The selected model show the AIC value in bold font.

AIC value	Gene		
	HSP70-like 1	AQP10	LEA1
Poisson	1142.6648	1552.3142	1190.5705
Nb	666.6060	764.8423	1192.5220
Zeroinfl_pois	611.2978	655.4430	238.1854

Zeroinfl_nb	**254.9675**	**286.4457**	**174.8959**
Hurdle_pois	612.5571	655.9807	238.6894
Hurdle_nb	257.1820	287.8362	175.7759

I analyzed the number of zeros and how they were spread across different states in the target genes as well as in actin. Table 5 shows that actin had zeros in 12 samples, which were excluded from the subsequent analyses as in these transcript copy number for actin=0 was assumed to be due to methodological errors. For the target genes, *LEA1* had most zeros (~42%) evenly spread across anhydrobiosis states. *HSP-like 1* and *AQP10* had 9 and 6 zeros, respectively, mainly in samples from the transitional and the dry states.

Table 5. Zero count and spread across anhydrobiosis states.

Gene	Number of zeros	Anhydrobiosis states			
		Active	Precond	Trans	Dry
Actin	12	1	3	4	4
HSP70-like 1	9	NA	2	3	4
AQP10	6	1	NA	2	3
LEA1	20	5	4	6	5

3 RESULTS

The expression levels of actin stayed similar across states entering anhydrobiosis (Table 6), which gives statistical justification for actin as reference gene.

Table 6. Actin count model statistical output

State	Estimate Std.	Error	Z value	Pr (>|z|)
Intercept (Transition)	3.16231	0.25656	12.326	<2e-16 ***
Active	-0.25690	0.33886	-0.758	0.448
Precondition	-0.29694	0.35491	-0.837	0.403
Dry	-0.04879	0.36320	-0.134	0.893

I found different patterns of gene expression in each target gene. *HSP70-like 1* expression levels (Figure 2) showed no significant change in expression between the active state (~1.98 transcripts) and the transitional state (~1.43 transcripts). The number of transcripts decrease by half from the active to the preconditioning state (~0.46 transcripts). The expression levels drop significantly by 80% from the transitional state to the dry state (~0.3 transcripts) (Table 7).

Table 7. HSP70-like 1 count model statistical output

State	Estimate Std.	Error	Z value	Pr (>|z|)
Intercept (Transition)	0.3281	0.4323	0.759	0.4478
Active	0.3552	0.5209	0.682	0.4952

Precondition	-1.1031	0.5818	-1.896	0.0580
Dry	-1.5747	0.6577	-2.394	0.167 *
Log (theta)	0.1504	0.3143	0.478	0.6324

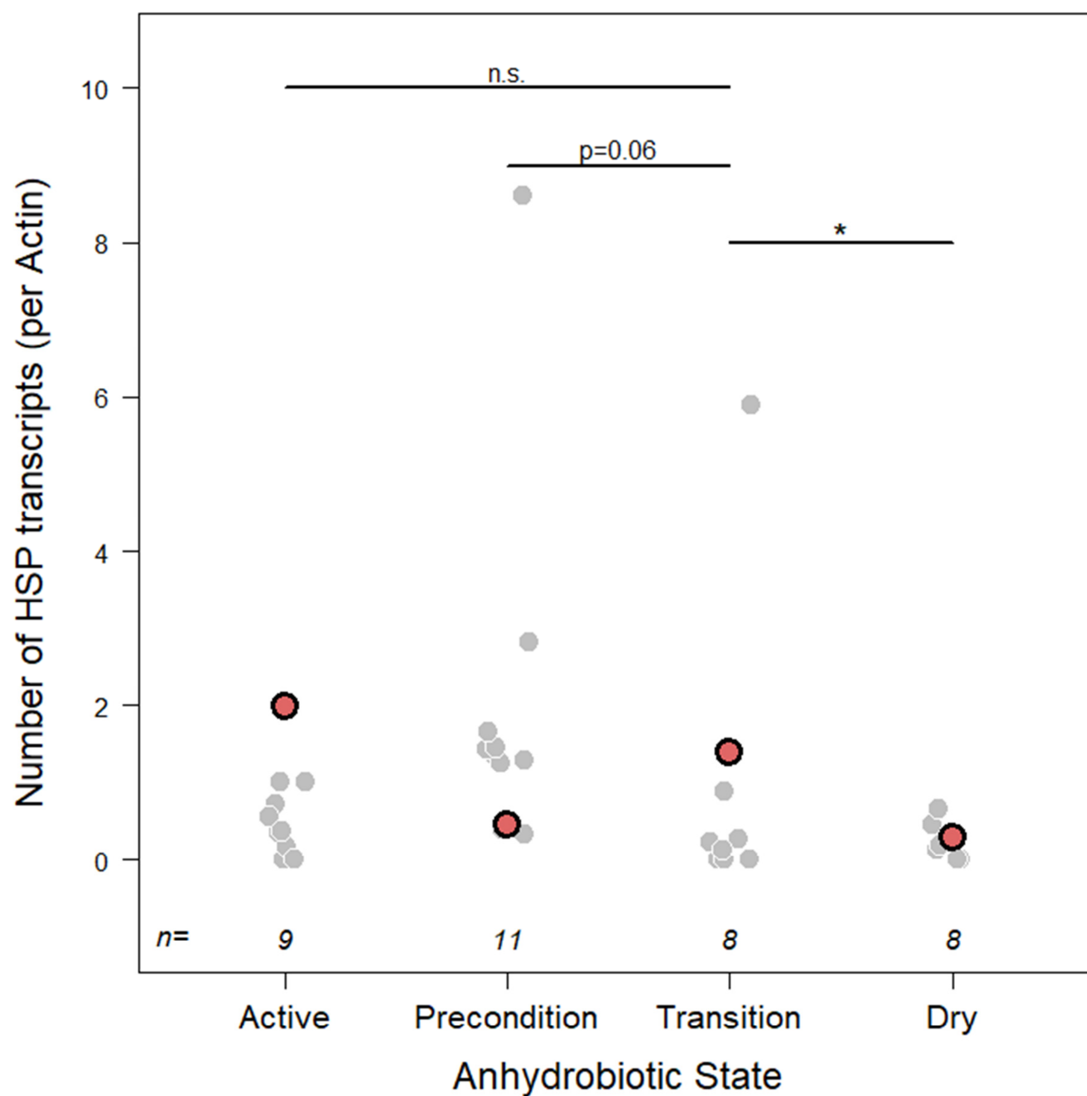

Figure 2. Expression patterns of Hsp70-like 1 gene across anhydrobiosis states. Grey dots represent the raw data and red dots represent the model estimate of expression levels controlled for actin copy number. The mRNA copy number was calculated based on actin reference gene as described in materials and methods. N= the number of samples included in analysis.

The expression of *AQP10* (Figure 3) show the estimate number of *AQP10* transcript in transition state to be approximately 0.49 transcripts. The transcript number decreased by three-fold between the active (1.34) and the transition states, while the expression levels in the preconditioning state (~2.63 transcripts) was higher by five-fold compared to the expression levels in the transitional state. In the dry state, the expression levels showed three times more transcript (1.35) compared to the transitional state (Table 8).

Table 8. AQP10 count model statistical output

State	Estimate Std.	Error	Z value	Pr (>|z|)
Intercept (trans.)	-0.7232	0.3603	-2.007	0.044732 *
Active	1.0170	0.4430	2.296	0.021686 *
Precondition	1.0233	0.5072	2.018	0.043629 *
Dry	1.6894	0.4454	3.793	0.000149 ***
Log (theta)	0.5528	0.3017	1.832	0.066924

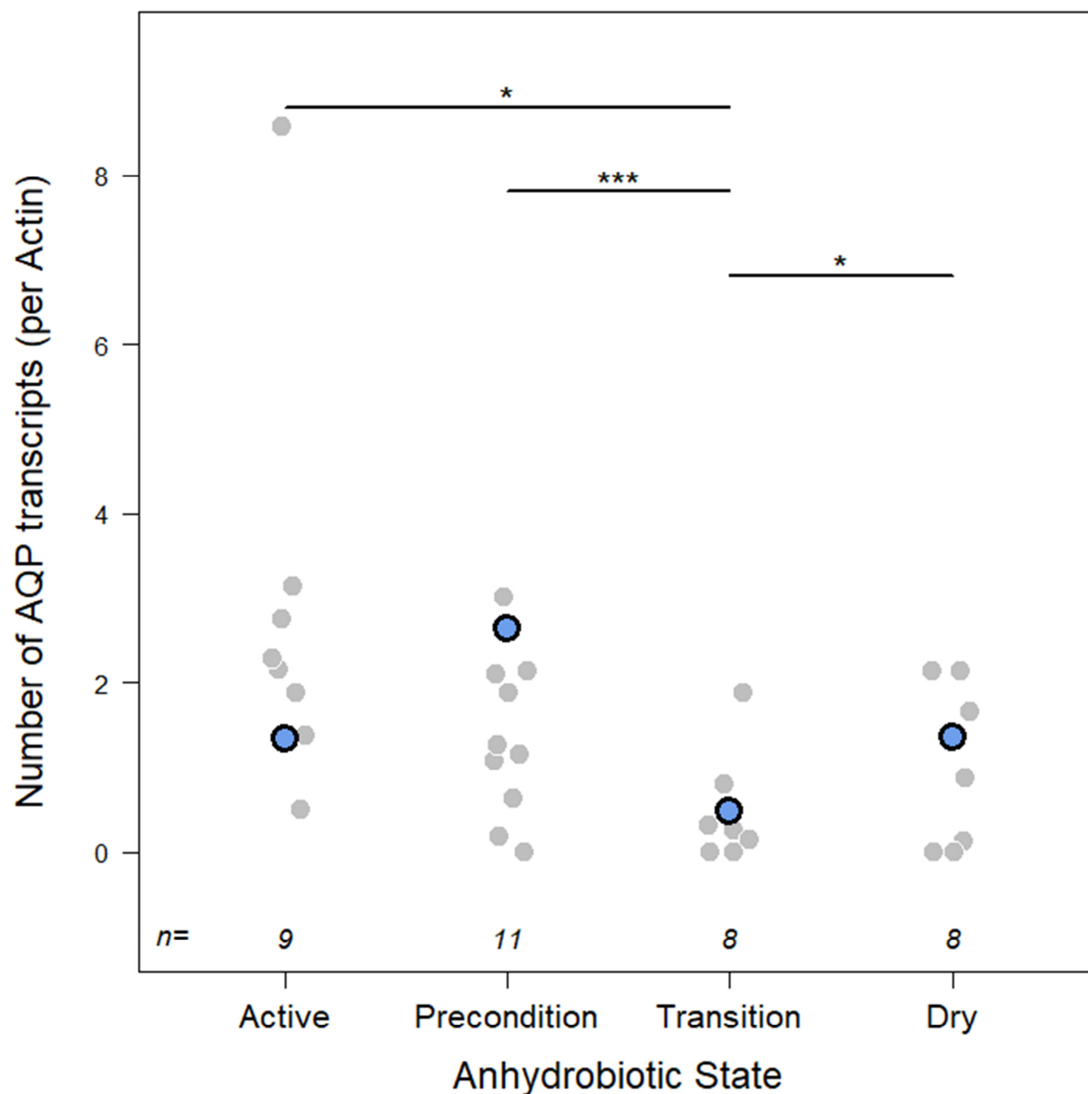

Figure 3. Expression patterns of Aquaporin 10 gene across anhydrobiosis states. Grey dots represent the raw data and blue dots represent the model estimate of expression levels that accounted for the actin gene copy number. The mRNA copy number was calculated based on actin reference gene as described in materials and methods. N= the number of samples included in analysis.

The expression pattern of *LEA1* (Figure 4) showed no significant difference in expression between the active state (0.57 transcripts) and the dry state (0.49 transcripts) compared to the transitioning state (0.37 transcripts). However, the transcript number increased in the preconditioning state (1.32 transcripts) showing 3,5 times more transcript compared to the transitional state (Table 9).

Table 9. LEA1 count model statistical output

State	Estimate Std.	Error	Z value	Pr (>|z|)
Intercept (trans.)	-0.9862	0.4930	-2.001	0.0454 *
Active	0.4297	0.5797	0.741	0.4586
Precondition	1.2623	0.5881	2.146	0.0318 *
Dry	0.2679	0.6662	0.402	0.6875
Log (theta)	0.8731	0.4474	1.951	0.0510

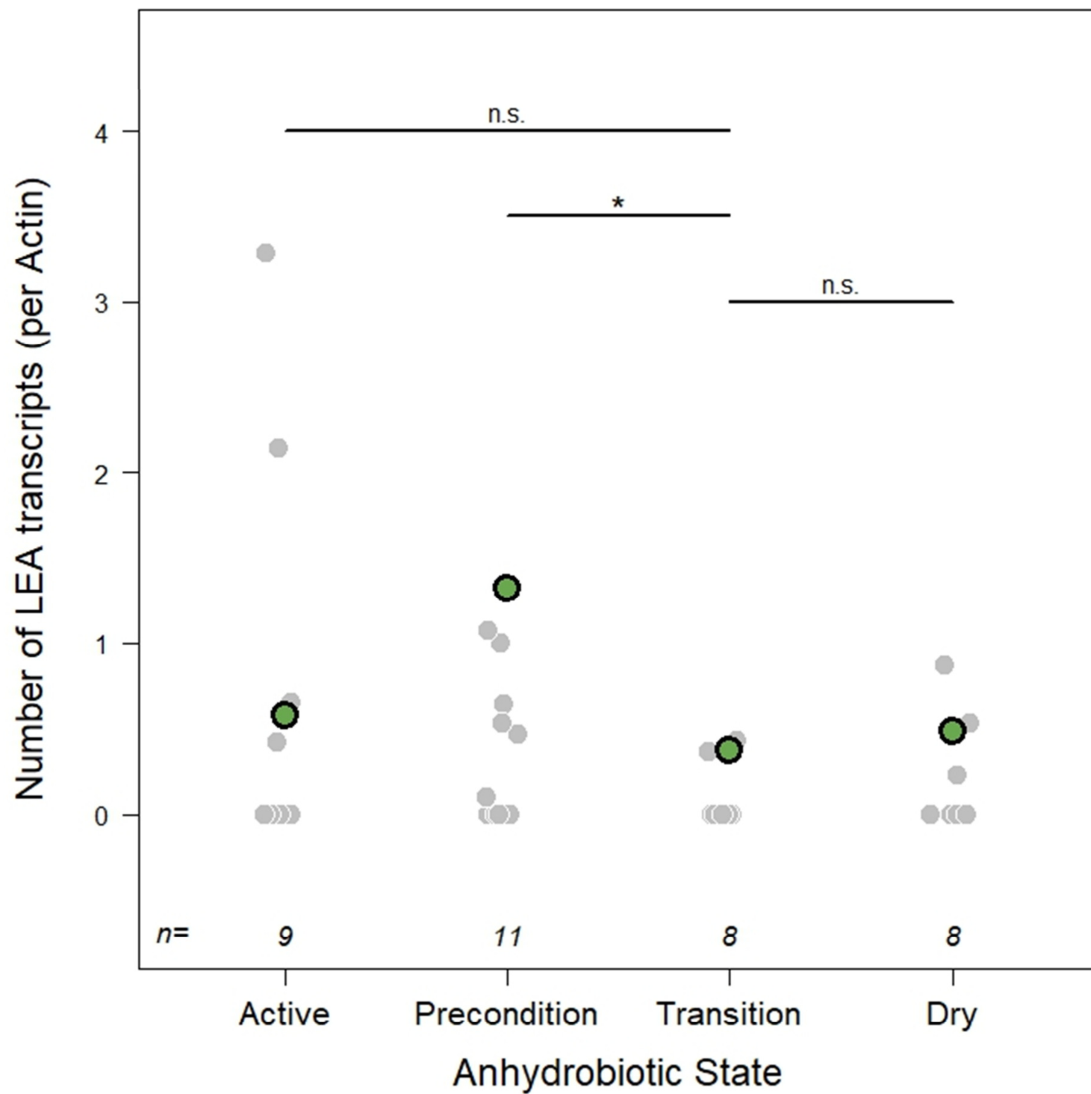

Figure 4. Expression patterns of LEA 1 gene across anhydrobiosis states. Grey dots represent the raw data and green dots represent the model estimate of expression levels that accounted for the actin gene copy number. The mRNA copy number was calculated based on actin reference gene as described in materials and methods. N= the number of samples included in analysis.

4 DISCUSSION

Hsp70

The results from *HSP70-like 1* gene show that downregulation is taking place at the onset of the preconditioning state with transcript levels halving compared to active state. The expression pattern does not completely correspond to hypotheses stated in the introduction as *HSP70-like 1* did show, in addition to slight upregulation in the transitional state, the highest expression levels in the active state. Surprisingly, *HSP70-like 1* expression levels showed no similar pattern to *HSP70 isoform 1* expression levels (active-transition-dry states expression levels significantly different) observed in previous studies with the tardigrade *M. tardigradum* (Schill et al., 2004; Reuner et al., 2010, respectively). Expression differences in *M. tardigradum* observed in the Shill et al. study (2004) might be due to lower sample size (N=5) used in the study, and also in differences due to using different tardigrade species (Møjberg et al., 2011). In addition, the different expression patterns observed in *M. tardigradum* between the Schill et al. (2004) and the Reuner et al. (2010) studies may also be due to different sample sizes (N=5 and N=50 respectively). However, *HSP70-like 1* expression patterns in active, transition and dry states show similar expression pattern to *HSP70 isoform 3* in the Schill et al. study (2004). The observed expression levels drop between the active and the precondition states is unclear. It would be interesting to repeat this study with larger sample size to see whether *HSP70-like 1* would also show expression patterns similar to *HSP70 isoform 3* as seen in the Reuner et al. study (2010). Nevertheless, the slight induction of *HSP70-like 1* in the transitional state suggests a possible role of *HSP70-like 1* in entering anhydrobiosis in *H. exemplaris*, whether it be by preparing individuals for anhydrobiosis by preventing protein aggregation (Sun and Macrae, 2005) or by assisting proteins intracellular locations (Feder and Hofmann, 1999). However, future studies should use similar desiccation protocols along with same anhydrobiosis states to confirm the relationship between *HSP70-3* and *HSP70-like 1.* It might also be that expression of

some heat-shock proteins are tightly regulated, as studies with *Drosophila* fly larvae with excess copies of *HSP70* showed greater larva-to-adult mortality (reviewed in Feder and Hofmann, 1999). Thus, even small expression differences might yield biologically significant outcomes. In addition, the role of *HSP70-like 1* during recovery from anhydrobiosis in *H. exemplaris* is still questionable. Future comparative studies should include rehydration processes to assess expression changes across anhydrobiosis and include gene expression measures from other heat-shock proteins associated with anhydrobiosis in tardigrades. *HSP90*, *HSP60* (Reuner et al., 2010) and different isoforms of *HSP70*, especially isoform 2 (Schill et al., 2004) and III (Reuner et al., 2010) have previously shown upregulation in transitional state. In conclusion, the different expression patterns of HSPs between tardigrade species found in this and other studies highlight the importance of species-specific studies to further understand the importance and regulation of heat-shock proteins in Tardigrada.

AQP10

Results of AQP10 expression presented in this study do not corroborate the hypotheses, as the highest expression levels were assumed in the transitional state, which contradicts with the upregulation that was observed during preconditioning and dry states. However, the expression pattern seen in active and dry states matches with previous work on *M. tardigradum* (Grohme et al., 2013) and *H. exemplaris* (Yoshida et al., 2017). Their observations of low levels in *AQP*s expression only analyzed active and dry states omitting changes occurring between these states, which is why the upregulation I found from *AQP10* in the preconditioning and the dry states suggest that this gene might have a role in entering anhydrobiosis in *H. exemplaris*. Since the function of AQPs is to maintain water homeostasis inside the cells, the upregulation of *AQP10* in the dry state suggests increased need for efficient transport of water and uncharged solutes during excessive drying (Mali et al., 2010). On the contrary, the expression levels drop by 67% between the active and the dry states opposes the predicted AQP role in water transport. Since there should be little metabolic activity in the desiccated

(dry) individuals (Clegg, 2001; Mali et al., 2010), the observed upregulation from the transitional state to the dry state illustrates a further need for closer inspection of the expression changes during entering anhydrobiosis, e.g. increasing time interval steps between extractions. However, the earlier upregulation of *AQP10* in the precondition state may have a biological relevance. *AQP10* was previously classified as aquaglyceroprotein, which enhances the transport of glycerol and water across membranes (Verkman, 2005). Previous studies have shown an increase of free glycerol at the onset of anhydrobiosis in the nematode *A. avenae* (Crowe, 1979), suggesting glycerol may be used as a membrane protectant during desiccation. Simon and Wiebe (1975) observed how high humidities slowed glycerol leakage from plant seeds before hydration in water, which Crowe (1979) also observed with *A. avenae*. Glycerol content increased in anhydrobiotic *A. avenae* the longer they were kept in high humidity prior desiccation (Madin and Crowe, 1975). Madin and Crowe (1975) also observed correlation between the increase in glycerol and nematode survival. Notably, high humidities before desiccation were shown to be important for high survival for *H. exemplaris* (Kondo et al., 2010). It could be that the preconditioning period prepares *H. exemplaris* for anhydrobiosis by facilitating the transport of glycerol via AQPs, assisted by high relative humidity. Browne et al. (2004) observed higher positive correlation with preconditioning time and carbohydrate content and nematode survival in *A. avenae*, which supports this suggested role of the preconditioning period. However, tardigrades in my study had no food available during the desiccation protocol. Thus, the observed upregulation of *AQP10* gene in the preconditioning state might just be a response to starvation (Hibuse et al., 2006), and may not represent early preparation to anhydrobiosis. Future studies should mimic more natural conditions, e.g. include food for the animals throughout the study, to assess the response-to-starvation hypothesis. The induction of *AQP10* in the dry state may correspond to increased synthesis of glycerol that is used to stabilize membranes and enable cellular survival in extreme desiccation. However, Crowe (1984) noticed how, even at low concentrations, glycerol is able to cause fusion of membranes, which leads no non-resuspension upon rehydration, disrupting both

the structure and function of membranes. It might be that inadequate desiccation might cause cellular components to rupture due to excess glycerol, leading to inability to rehydrate animals from anhydrobiosis. Crowe (1984) also noticed how glycerol interacted strongly with water leading to increased thermal stability of proteins. Thus, glycerol transported by *AQP10* may also stabilize protein structures under low water concentrations by substituting water under desiccation. Studies with brine shrimp *Artemia* further support the water-replacement hypothesis of glycerol (Clegg, 1982).

Sugars or sugar-alcohols, such as glycerol, are also known to stabilize anhydrobiotic organisms by forming glasses inside the cells (also known as vitrification) (Crowe, 1998). This vitrification hypothesis is supported by observations of an increase in glass formation as water amount decreases in plants and *Artemia* cysts (Crowe, 1998; Clegg, 1982 respectively), and the absence of glass transition in desiccation-intolerant embryos of soybean (Koster, 1991). The high viscosity of glasses is able to slow chemical reactions and even stop them altogether and prevent membrane fusion (Crowe, 1998). Sugars that replace water maintain membranes in crystalline phase (solid structure) and prevent membranes from going through a liquid crystalline phase during rehydration (Crowe, 1998). Though at the time vitrification lacked experimental evidence, Miyata et al. (2012) showed glycerol promoting a clathrate-like structure of water molecules around each glycerol molecule, and this bound water is associated with protein, nucleic acid and membrane structural integrity (Womersley, 1981). Thus, it could be that the increased uptake of glycerol by induction of *AQP10* might maintain these integrities during anhydrobiosis, but further studies are required to test this hypothesis in tardigrades. Similar correlation between disaccharides (e.g. trehalose) and anhydrobiosis survival are known. For example, studies have shown high concentrations of disaccharides, such as trehalose, in anhydrobiotic organisms (Crowe et al., 1992; Carpenter et al., 1998). Consequently, increasing trehalose content in the cells is suggested to be an important adaptive strategy for survival during anhydrobiosis (Crowe et al., 1998). However, studies on trehalose synthesis have shown that this disaccharide is not ubiquitously required for

tardigrades (Hengherr et al., 2007; Neumann et al., 2009; Förster et al., 2012; Yoshida et al., 2017). Other carbohydrates, such as lactose, maltose and cellobiose have also shown to provide substitution to water under severe desiccation (Crowe, 1984), which may contribute to anhydrobiosis in tardigrades.

LEA1

The expression pattern observed in *LEA1* differed from the hypotheses, and showed a trend of downregulation as tardigrades progressed into anhydrobiosis. Expression pattern in *H. exemplaris* differed from those observed in the nematode *A. avenae* (Browne et al., 2004) and in the chironomid *P. vanderplanki* (Kikawada et al., 2006), which showed an increase in expression when exposed to desiccation and an elevated expression until reaching anhydrobiosis, respectively. Regarding the role of LEAs in the cells, namely protection against protein aggregation (Garcia, 2011; Hand et al., 2011; Yamaguchi et al., 2012; Wang et al., 2014), the increase of *LEA1* in the preconditioning state is rather interesting and suggests that this gene in *H. exemplaris* has some role in entering anhydrobiosis. A review by Hand et al. (2011) discusses how LEAs (in plants) can stabilize vitrified sugar glasses. The induction of *LEA1* during the preconditioning period could then possibly be in response to increased intake of glycerol via AQPs in preparation to anhydrobiosis. However, there were several samples in which LEA1-expression was null (N=20, Table 5) that should be considered. Future studies should also include observations of *LEA1* expression patterns in rehydration processes to obtain coherent perspective of its function in anhydrobiosis, as well as comparative studies to see possible interactions of *LEA1* with other genes during anhydrobiosis. Other LEAs or LEA-like genes are also suggested to have a prominent role in anhydrobiosis. Yamaguchi et al (2012) noticed five heat-soluble proteins in the tardigrade *Ramazzottius varieornatus*, from which none were LEA-proteins. Instead, the proteins belonged to two other protein families (Secretory Abundant Heat Soluble proteins and Cytoplasmic Abundant Heat Soluble proteins), which all were unique and conserved in tardigrades. Secretory Abundant Heat Soluble (SAHS) proteins contain secretory signal and are

suggested to protect extracellular components, while Cytoplasmic Abundant Heat Soluble proteins (CAHS) did not contain secretory signals and were mainly located the in cytoplasm or in mitochondria, in which they are believed to employ their protective functions (Yamaguchi et al., 2012). *SAHS* and *CAHS* loci have been also found in *H. exemplaris* (Yoshida et al., 2017), showing low resting expression and up-regulation during anhydrobiosis induction, and reached overexpression levels in anhydrobiotic state (Yoshida et al., 2017). Dehydration caused similar conformational changes in CAHS and SAHS proteins as in LEA proteins, and SAHS and CAHS encoding genes had LEA-motifs (sequences that have biological significance), which suggests these three protein families may share similar biological functions in tardigrades (Yoshida et al., 2012). Previous studies have shown how LEA proteins are able increase the glass-transition temperature of vitrified sugars (Hand et al., 2011), strengthening the glassy state. It could be that both CAHS and SAHS may serve similar purpose in tardigrades, but further studies comparing measurements of LEAs or other LEA-like protein concentrations against sugar levels should be done to assess whether these genes show contribution to vitrification in tardigrades.

Overall, these results give more insight to how the studied genes may be involved in anhydrobiosis in *H. exemplaris*, but there is still room for further development. For example, crushing the animals with pipette tip turned out to be quite troublesome and an alternative method is suggested to be developed, or instead using multiple individuals per sample to ensure sufficient template yield. Also, it is advised to use defined amounts of RNA in digital PCR experiments (Huggett et al., 2013), which was omitted in this particular study and should be considered in future experiments. This could to some extent circumvent the problem of variable extraction yield among samples. Methodologically, the use of two individuals per sample made the extraction process more feasible, although using multiple individuals in a single sample hinders the study of adaptation on individual level.

My results describe only the direct response of target genes to desiccation. Gene expression studies alone do not illustrate the amount of protein made from the

transcripts (Evans, 2015) and thus may not reveal all biological significances. Some genes are required to be constitutively expressed, while in others, even the smallest changes in expression might lead to biologically relevant changes, even though statistically it might not be apparent (Evans, 2015). In addition, I did not assess interactions among target gene expression levels during anhydrobiosis. Thus, complementary studies including protein concentrations should be done to assess the biological relevance of gene patterns observed. There is also one other thing to be cautious about regarding this particular study. Since the success rate of anhydrobiosis in other studies have been assessed by the survival rate after rehydration, and this study only concentrated on processes while entering anhydrobiosis, the question whether the individuals in this study were "dying" and "dead" in "transitioning" and "dry" states stays unanswered. Rehydration processes should be included to assess this problem, but unfortunately, due to the lack of time, I had to concentrate only in dehydration states.

Regarding the specific PCR method used, ddPCR suited this study better for several reasons in addition to those already mentioned in the introduction. Partitioning of PCR reaction into oil droplets prior amplification and the use of reference gene in ddPCR corrects for differential loading of cDNA between samples, which offers more precise quantification and more reproducible method compared to qPCR methods (Taylor et al., 2017). In addition, Taq polymerase (the protein used in PCR to amplify the template) is more likely to be inhibited in qPCR, which poses a problem when template amounts are miniscule, especially when cDNA is used (Taylor et al., 2017). Last, during optimization and actual data collection, the inclusion of MIQE guidelines (Taylor et al., 2017) for ddPCR should be considered to improve future analyzes and comparability.

5 SUMMARY

This study shows different gene expression patterns in three observed target genes across consecutive anhydrobiosis states in *H. exemplaris*. *HSP70-like 1* expression only showed significant difference between the dry and the transitioning states, suggesting this gene is not directly involved in anhydrobiosis. Similarities in expression patterns between *HSP70-like 1* and *HSP70 -3* in *H. exemplaris* and *M. tardigradum* respectively suggests that these two genes act similarly across different tardigrade species. However, studies with standardized desiccation protocols need to be done to give more coherent illustration of HSP expression patterns across different tardigrade species. Expression of *AQP10* showed significant upregulation in both the preconditioning and in the dry states, suggesting it has a role in entering anhydrobiosis. My results showed different expression patterns from previous studies with *M. tardigradum* and *H. exemplaris*, emphasizing the importance to study transitional states in gene expression level studies. The induction of *AQP10* in the preconditioning state might be in response to starvation and may not illustrate preparation to anhydrobiosis. However, *AQP10* upregulation in the dry state suggest its importance in anhydrobiosis, whether due to the elevated need of glycerol for protection of membranes (replacing water inside the cells to stabilize structures during extreme dehydration) or by forming glass structures to help slow down metabolism inside the cells and prepare for anhydrobiosis. *LEA1* expression showed no significant changes as the copy number decreased from the transitioning to the dry state, whereas the highest expression levels were observed in the preconditioning state, which showed slightly higher expression levels compared to the transitioning state. The induction if the preconditioning state suggests *LEA1* having a role in anhydrobiosis and should be looked into in future research. *LEA1* expression pattern observed differed from nematode *A. avenae* and chironomid *P. vanderplanki*, illustrating the importance of studying LEA expressions in tardigrades for better understanding the role of LEAs in anhydrobiosis. Closer inspection to other LEAs and LEA-like genes should also be included, as in recent

years, proteins in two families that have been found to be unique and conserved in tardigrades show association in anhydrobiosis.

In conclusion, this study, as far as I know, is the first to simultaneously assess gene expression of three different genes associated in anhydrobiosis across consecutive anhydrobiosis states. This study also illustrates the need of studying gene expression of tardigrade adaptation in anhydrobiosis across different tardigrade species to better understand species-specific mechanisms behind it. This study shows the importance of studying species-specific mechanisms of adaptation. In addition, this study sets the foundations for future tardigrade studies in the University of Jyväskylä.

THANKS

Thanks to my supervisors Emily Knott and Sara Calhim for their helpful advice and critical discussion throughout the experiment, data analysis and Thesis writing. I also wish to thank Betty Väänänen foundation (Kuopio, Finland) for funding this study.

LITERATURE

Adhikari B. N., Wall D. H. & Adams B. J. (2009). Desiccation survival in an Antarctic nematode: molecular analysis. *BMC Genomics, 10*(69). doi:10.1186/1471-2164-10-69

Alpert P. (2005). The Limits and Frontiers of Desiccation-Tolerant Life. *INTEGR. COMP. BIOL*(45), 685-695.

Bertolani R., Guidetti R., Jönsson I., Altiero T., Boschini D. & Rebecchi L. (2004). Experiences with dormancy in tardigrades. *J. Lmnol., 63*, 16-25.

Browne J. A., Dolan K. M., Tyson T., Goyal K., Tunnacliffe,A. & Brunell A. M. (2004). Dehydration-Specific Induction of Hydrophilic Protein Genes in the. *EUKARYOTIC CELL, 3*(4), 966-975. doi:10.1128/EC.3.4.966–975.2004

Carpenter J. F., Crowe L. M., Crowe J. H. (1998). The role of vitrification in anhydrobiosis. *Annual review of physiology*, 73-103. doi:10.1146/annurev.physiol.60.1.73.

Clegg J. S., Seitz P., Seitz W. & Hazlewood C. F. (1982). Cellular responses to extreme water loss: The water-replacement hypothesis. *Cryobiology, 19*, 306-316.

Clegg J. S. (2001). (Review) Cryptobiosis - a peculiar state of biological organization. *Comparative Biochemistry and Physiology Part B*(128), 613-624.

Crowe J. H., O'Dell S. J. & Armstrong D. A. (1979). Anhydrobiosis in Nematodes: Permeability during rehydration. *J. Exp. Zool., 207*, 431-438.

Crowe L. M., Mouradian R., Crowe J. H., Jackson S. A. & Womersley C. (1984). Effects of carbohydrates on membrane stability at low water activities. *Biochimica et Biophysica Acta, 769*, 141-150.

Crowe J. H., Hoekstra F. A. & Crowe L. M. (1991). ANHYDROBIOSIS. *Annual Reviews Physiology* (54), 579-599.

Crowe J. H., Hoekstra F. A. & Crowe L. M. (1992). Anhydrobiosis. *Annu. Rev. Physiol.*, 579-5799.

Crowe J. H., Carpenter J. F. & Crowe L. M. (1998). The role of vitrification in anhydrobiosis. *Annu. Rev. Physiol., 60*(73), 73-103.

Czernekova M. & Jönsson I. K. (2016). Experimentally Induced Repeated Anhydrobiosis in the Eutardigrade Richtersius coronifer. *PLoS ONE, 11*(11). doi:10.1371/journal.

Evans T. G. (2015). Considerations for the use of transcriptomics in identifying the 'genes that matter' for environmental adaptation. *The Journal of Experimental Biology, 218*, 1925-1935. doi:10.1242/jeb.114306

Feder M. E. & Hofmann G. E. (1999). HEAT-SHOCK PROTEINS,MOLECULAR CHAPERONES, AND THE STRESS RESPONSE: Evolutionary and Ecological Physiology. *Annu. Rev. Physiol., 61*, 243-282.

Förster F., Liang C., Shkumatov A., Beisser D., Engelmann J. C., Schnölzer M.,. . . 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). doi:10.1186/1471-2164-10-469

Förster F., Beisser D., Grohme, M. A., Liang C., Mali B., Siegl,A. M.,. . . Dandekar T. (2012). Transcriptome Analysis in Tardigrade Species Reveals Specific. *Bioinformatics and Biology Insights, 6*. doi:10.4137/BBI.S9150

García A. H. (2011). (Review) Anhydrobiosis in bacteria: From physiology to applications. *J. Biosci., 36*, 939-950. doi:10.1007/s12038-011-9107-0

Gasiorek P., Steck D., Morek W. & Michalczyk L. (2018). An integrative redescription of Hypsibius dujardini (Doyére, 1840), the nominal taxon for Hypsibioidea (Tardigrada: Eutardigrada). *Zootaxa*. doi:https://doi.org/10.11646/zootaxa.0000.0.0

Gomes D., Agasse A., Thiébaud P., Delrot S., Gerós H. & Chaumont F. (2009). (Review) Aquaporins are multifunctional water and solute transporters highly divergent in living organisms. *Biochimica et Biophysica Acta*, 1213-1228. doi:10.1016/j.bbamem.2009.03.009

Goyal K., Walton L. J., Browne J. A., Burnell A. M. & Tunnacliffe A. (2005). Molecular Anhydrobiology: Identifying Molecules Implicated in Invertebrate Anhydrobiosis. *INTEGR. COMP. BIOL., 45*, 702-709.

Grohme M. A., Mali B., Wełnicz W., Michel S., Schill R. O. & Frohme M. (2013). The Aquaporin Channel Repertoire of the Tardigrade Milnesium. *Bioinformatics and Biology Insights, 7*, 153-165. doi:10.4137/BBI.S11497

Hand S. C., Menze M. A., Toner M., Boswell L. & Moore D. (2011). LEA proteins during water stress: Not just for plants anymore. *Annu. Rev. Physiol., 73*, 115-134. doi:10.1146/annurev-physiol-012110-142203

Hengherr S., Heyer A. G., Köhler H. R. & Schill R. O. (2007). Trehalose and anhydrobiosis in tardigrades – evidence for. *The FEBS Journal, 275*, 281-288. doi:10.1111/j.1742-4658.2007.06198.x

Hengerr S., Worland M. R., Reuner A., Brümmer F. & Schill R. (2009). Freeze tolerance, supercooling points and ice formation: comparative studies on the subzero temperature survival of limno-terrestrial tardigrades. *The Journal of Experimental Biology, 212*, 802-807. doi:10.1242/jeb.025973

Hibuse T., Maeda N., Nagasawa A. & Funahashi T. (2006). Aquaporins and glycerol metabolism. *Biochimica et Biophysica Acta*, 1004-1011. doi:10.1016/j.bbamem.2006.01.008

Hindson B. J., Ness K. D., Masquelier D. A., Belgrader P., Heredia N. J., Makarewicz A. J.,. . . Colston B. W. (2011). High-Throughput Driplet Digital PCR system for Absolute Quantitation of DNA Copy Number. *Analytical Chemistry*, 8604-8610. doi:dx.doi.org/10.1021/ac202028g

Horikawa D. D. & Higashi S. (2004). Desiccation Tolerance of the Tardigrade Milnesium tardigradum Collected in. *Zoological Science, 21*(8), 813-816.

Horikawa D. D.,Sakashita T., Katagiri C., Watanabe M., Kikawada T., Nakahara, Y.,. . . Kuwabara M. (2006). Radiation tolerance in the tardigrade Milnesium

tardigradum. *Int. J. Radiat. Biol., 82*(12), 843-848. doi:10.1080/09553000600972956

Horikawa D. D., Cumbers J., Sakakibara I., Rogoff D., Leuko S., Harnoto R.,. . . Rothschild L. J. (2013). Analysis of DNA Repair and Protection in the Tardigrade Ramazzottius varieornatus and Hypsibius dujardini after Exposure to UVC Radiation. *PLoS ONE, 8*(6).

Huggett J. F., Foy C. A., Benes V., Emslie K., Garson J. A., Haynes R.,. . . Bustin S. A. (2013). The digital MIQE Guidelines: Minimum information for publication of Quantitative digital PCR experiments. *Clinical Chemistry, 59*(6), 892-902. doi:10.1373/clinchem.2013.206375

Izumi Y., Sonoda S., Yoshida H., Danska H. V. & Tsumuki H. (2007). Role of membrane transport of water and glycerol in the freeze tolerance of the rice stem borer, Chilo suppressalis Walker (Lepidoptera: Pyralidae).

Jönsson I. K. (2005). The Evolution of Life Histories in Holo-anhydrobiotic Animals: A First Approach. *INTEGR. COMP. BIOL.*(45), 764-770.

Jönsson I. K. & Schill R. O. (2007). Induction of Hsp70 by desiccation, ionising radiation and heat-shock. *Comparative Biochemistry and Physiology, Part B, 146*, 456–460. doi:10.1016/j.cbpb.2006.10.111

Kikawada T., Nakahara Y., Kanamori Y., Iwata K.-i., Watanabe M., McGee B.,. . . Okuda T. (2006). Dehydration-induced expression of LEA proteins in an anhydrobiotic chironomid. *Biochemical and Biophysical Research Communications, 348*, 56-61. doi:10.1016/j.bbrc.2006.07.003

Kikawada T., Saito A., Kanamori Y., Fujita M., Śnigórska K., Watanabe M. & Okuda T. (2008). Dehydration-inducible changes in expression of two aquaporins in the sleeping chironomid, Polypedilum vanderplanki. *Biochimica et Biophysica Acta, 1778*, 514-520. doi:10.1016/j.bbamem.2007.11.009

King L. S., Kozono D. & Agre P. (2004). (Review) FROM STRUCTURE TO DISEASE: THE EVOLVING TALE OF AQUAPORIN BIOLOGY. *Nature, 5*, 687-698.

Kondo K., Kubo T. & Kunieda T. (2015). Suggested Involvement of PP1/PP2A Activity and De Novo Gene Expression in Anhydrobiotid Survival in a Tardigrade, Hypribius dujardini, by Chemical Genetic Approach. *PLOS ONE*, 1-18. doi:10.137/journal.pone.0144803

Koster K. L. (1991). Glass formation and desiccation tolerance in seeds. *Plant Physiol., 96*, 302-304.

Madin K. A. & Crowe J. H. (1975). Anhydrobiosis in Nematodes: Carbohydrate and Lipid metabolism during dehydration. *J. Exp. Zool.* , 335-342.

Mali B., Grohme M. A., Förster F., Dandekar T., Schnölzer M., Reuter D.,. . . Frohme M. (2010). Transcriptome survey of the anhydrobiotic. *BCM Genomics, 11*(168). doi:10.1186/1471-2164-11-168

Miyata K., Hayakawa S., Kajiwara K. & Kanno H. (2012). Supercooling and vitrification of aqueous glycerol solutions at normal and high pressures. *Cryobiology*, 113-116. doi:http://dx.doi.org/10.1016/j.cryobiol.2012.05.002

Møbjerg N., Halberg K. A., Jørgensen A., Persson D., Bjørn M., Ramløv H. & Kristensen R. M. (2011). (Review) Survival in extreme environments – on the current. *Acta Phydiologica, 202*, 409-420. doi:10.1111/j.1748-1716.2011.02252.x

Nelson D. R., Guidetti R. & Rebecchi L. (2010). *Tardigrada. Ecology and Classification of North American Freshwater Invertebrates*. Elsevier Inc. doi:10.1016/B978-0-12-374855-3.00014-5

Neuman Y. (2006). (Review) Cryptobiosis: A new theoretical perspective. *Progress in Biophysics and Molecular Biology, 92*, 258-267. doi:10.1016/j.pbiomolbio.2005.11.001

Neumann S., Reuner A., Brümmer F. & Schill R. O. (2009). DNA damage in storage cells of anhydrobiotic tardigrades. *Comparative Biochemistry and Physiology, Part A, 153*, 425-429. doi:10.1016/j.cbpa.2009.04.611

Persson D., Halberg K. A., Jorgensen A., Ricci C., Mobjerg N. & Reinhardt K. M. (2011). Extreme stress tolerance in tardigrades: surviving space conditions in low earth. *J Zool Syst Evol Res, 49*, 90-97. doi:10.1111/j.1439-0469.2010.00605.x

Rebecchi L., Altiero T. & Guidetti R. (2007). (Review) Anhydrobiosis: the extreme limit of desiccation tolerance. 65-81.

Reuner A., Hengherr S., Mali B., Förster F., Arndt D., Reindardt R.,. . . Schill R. O. (2010). Stress response in tardigrades: differential gene expression. (15), 423-430. doi:10.1007/s12192-009-0158-1

Ricci C. & Manuela C. (1998). Stress during dormancy: effect on recovery rates and life-history traits of anhydrobiotic animals. *Aquatic Ecology, 32*, 353-359. doi:https://doi.org/10.1023/A:1009905404868

Schill R. O., Steinbrück G. H. & Köhler H. R. (2004). Stress gene (hsp70) sequences and quantitative expression in Milnesium. *The Journal of Experimental Biology, 207*, 1607-1613. doi:10.1242/jeb.00935

Schokarie E., Hotz-Wagenblatt A., Warnken U., Mali B., Frohme M., Förster F.,. . . Schnölzer M. (2010). Proteomic Analysis of Tardigrades: Towards a Better. *PLoS ONE, 5*(3). doi:10.1371/journal.pone.0009502

Simon E. W. and Wiebe H. H. (1975). Leakage during imbibition, resistance to damage at low temperature and the water content of peas. *New Phytol., 74*, 407-411. doi:https://doi.org/10.1111/j.1469-8137.1975.tb01351.x

Sun Y. and MacRae T. H. (2005). (Review) Small heat shock proteins: molecular structure and. *Cell. Mol. Life Sci., 62*, 2460–2476. doi:10.1007/s00018-005-5190-4

Taylor S. C., Laperriere G., Germain H. (2017). Droplet Digital PCR versus qPCR for gene expression analysis with low abundant targets: from variable nonsense to publication quality data. *Scientific Reports*. doi:https://doi.org/10.1038/s41598-017-02217-x

Team R. Core (2017). R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. Noudettu osoitteesta https://www.R-project.org

Tunnacliffe A. & Wise M. J. (2007). (Review) The continuing conundrum of the LEA proteins. *Naturwissenschaften, 94*, 791-812. doi:10.1007/s00114-007-0254-y

Untergrasser A., Cutcutache I., Koressaar T., Ye J., Faircloth B. C., Remm M. & Rozen S. G. (2012). Primer3—new capabilities and interfaces. *Nucleic Acids Research, 40*(15), 115-115. doi:10.1093/nar/gks596

Wang C., Grohme M. A., Mali B., Schill R. O. & Frohme M. (2014). Towards Decrypting Cryptobiosis—Analyzing. *PLoS ONE, 9*(3), 1-15. doi:10.1371/journal.pone.0092663

Verkman A. S. (2005). More than just water channels: unexpected cellular. *Journal of Cell Science, 1*(18), 3225-3232. doi:doi:10.1242/jcs.02519

Womersley C. (1981). Biochemical and physiological aspects of anhydrobiosis. *Comp. Biochem. Physiol., 70B*, 669-678.

Wrigth J. C. (1989). DESICCATION TOLERANCE AND WATER-RETENTIVE MECHANISMS IN TARDIGRADES. *J. exp. Biol., 142*, 267-292.

Yamaguchi A., Tanaka S., Yamaguchi S., Kuwahara H., Takamura C., Ohmi-Imajoh S.,. . . Kunieda T. (2012). Two Novel Heat-Soluble Protein Families Abundantly. *PLoS ONE, 7*(8). doi:10.1371/journal.pone.0044209

Yoshida Y., Koutsovoulos G., Laetsch D. R., Stevens L., Kumar S., Horikawa, D. D.,. . . Arakawa K. (2017). Comparative genomics of the tardigrades Hypsibius dujardini and Ramazzottius varieornatus. *PLOS Biology, 15*(7), 1-40. doi:https://doi.org/10.1371/journal.pbio.2002266