Molecular responses of the bdelloid rotifer *Philodina roseola* to dehydration

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To my family

Abbreviations

ABA	abscisic acid
ABREs	ABA-responsive elements
Amp	ampicillin
ARP	Aldehyde Reactive Probe
BLAST	basic local alignment search tool
bp, kb	base pair,kilobase
bZIP	basic Leucine Zipper Domain
cDNA	complementary DNA
DEPC	diethylpyrocarbonate
DMSO	dimethylsulfoxide
dNTP	deoxyribonucleotide
DRE:	dehydration-responsive element
DCF-DA	dichlororofluorescein diacetat
DREBP/CBF1	DRE-binding protein/C-repeat binding factor
dH_2O	distilled water
DNA	deoxyribonucleic acid
DTT	1,4-dithiothreitol
DMSO	dimethylsulfoxide
dNTPs	deoxyribonucleotide triphosphates
EDTA	ethylenediaminetetraacetic acid
GPX	glutathione peroxidase
IPTG	(Isopropyl-ß-D thiogalactopyranoside)
Kan	kanamycin
kb	kilobase
h	hour

LEA	late embryogenesis abundant
mM	millimoles per litre
mRNA	messenger ribonucleic acid
MYC/MYB	myeloblastosis (transcriptionfactor)
NF-kB	nuclear factor –kB kappa Sonderzeichen!
O2-	superoxide anion radical
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNA*i*	RNA interference
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RNase	ribonuclease
RT	room temperature
RT-PCR	reverse transcription-coupled polymerase chain reaction
SOD	superoxide dismutase
sec	second
TAE	tris-acetate-EDTA-buffer
TBE	tris-borate-EDTA-buffer
Tet	tetracycline
5' UTR	5' untranslated region
3' UTR	3' untranslated region
X-gal	5-Bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

Content

1. Introduction

Anhydrobiosis is the ability of certain organism to survive long term exposure to dryness. Under conditions of desiccation the organism dehydrates, whereas resumption of water results in rehydration and thus returning to normal life activities and growth. For a better understanding of this phenomenon desiccation tolerance was analysed in microbes (e.g. yeast, cyanobacteria), (Singh *et al*., 2005; Gao and Ye, 2007) plants, (algae, resurrection plant) (Gupta & Agrawal, 2006; Bernacchia & Furini, 2004) and several invertebrate animals such as nematodes and tardigrades (Higa & Womersley, 1993; Hengherr *et al*., 2008).

In desiccation-tolerant organisms a variety of mechanisms to survive desiccation, e.g. morphological changes (e.g. cyst formation, changes in membrane permeability) and the biosynthesis of stress specific molecules (e.g. trehalose, late abundant embryogenesis (lea) protein) have evolved. Anhydrobiotic organisms can withstand extreme stress like radiation (Watanabe *et al*., 2007), high pressure (Seki &Toyoshima, 1998), heat (Clegg, 2001), and vacuum (Wharton, 1982). Investigating the molecular mechanisms of desiccation tolerance is important to understand the involvement of water in cells (Crowe & Crowe 2000; Wolkers *et al*., 2002).

This work shows a first analysis of the genetic control of desiccation responses in the desiccation-tolerant rotifer *Philodina roseola*. The anhydrobiont bdelloid *Philodina roseola* rotifers are suitable organisms for the research on anhydrobiosis, because they are easy to culture and show morphological changes towards dryness.

1.1. Anhydrobiosis

In 1702 Antoni Van Leeuwenhoek observed the revival of “dead” bdelloid rotifers from a desiccated sample (Wright, 2001). Nearly 250 years later David Keilin was the first who described anhydrobiosis as a particular state of an organism when it shows no visible

sign of life and when its metabolic activity becomes hardly measurable (Keilin, 1959), which he called ‘cryptobiosis’. Cryptobiosis includes anhydrobiosis, cryobiosis,

and anoxybiosis (Clegg, 2001). An organism may enter anoxybiosis under oxygen deficiency. Cryobiosis occurs when exposed to low temperatures and anhydrobiosis when the environment dries out. Anhydrobiosis may confer an extreme stress tolerance. However, the term of desiccation tolerance is used in the literature to describe a wide range of conditions, from partial dryness experienced by algae to complete dryness of truly anhydrobiotic organism like tardigrades (Alpert, 2006) that should be able to tolerate cellular water content of less than 0.1 grams of water per gram dry mass (Clegg, 2001; Hoekstra *et al*., 2001; Carpenter, 1987; Lapinski & Tunnacliffe, 2003)

1.2. Damage occuring during desiccation stress

Desiccation increases the ionic concentration of the cell which can lead to the formation of reactive oxygen species (ROS) (Pereira *et al.,* 2003). ROS is a collective term that designates both oxygen radicals and certain non-radicals that are oxidizing agents and/or are easily converted into free radicals (HOCl, HOBr, O_3, ONOO-, H_2O_2) (Pereira *et al*., 2003). It is formed during respiration in mitochondria. In the dry state, biomolecules become more susceptible to the attack of oxygen (Pereira *et al.,* 2003). The accumulation of intracellular ROS produces an oxidative stress, which can lead to cell death (Hockenbery*et al*., 1993; Kane *et al.,* 1993; Greenlund *et al*., 1995).

The mechanisms of oxidative cellular damage are summarized in figure 1. Free radicals exert deleterious effects on cells through direct attack on proteins and membrane lipids including mitochondrial lipids, resulting in an impaired oxidative phosphorylation (Voss & Siems, 2006; Cardoso *et al.,* 1999; Esterbauer *et al.,* 1991; Levine *et al.,* 1990). Increase of ROS concentration persistently causes oxidative damage in DNA generating various products of base damage, apurinic/apyrimidinic (AP) sites, and strand breaks,

which provoke among others mutagenesis, carcinogenesis and aging (Lindahl *et al*., 1993; Cooke *et al*., 2003).

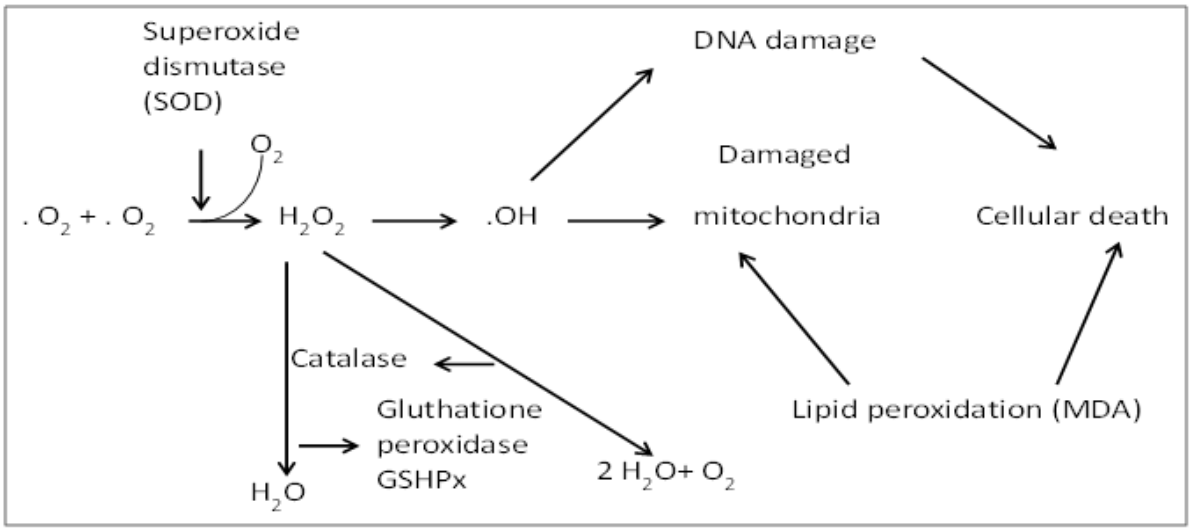

Figure 1: Mechanisms of oxidative cellular damage. Free radicals are reduced into water with the cooperation of the three main antioxidant enzymes: SOD, catalase, and GSHPx. The generation of hydroxyl radicals from hydroperoxide produces the development of oxidative cell injury: DNA damage; carboxylation of proteins; and lipid peroxidation, including lipids of mitochondrial membranes. (Voss & Siems, 2006; Cardoso *et al*., 1999; Esterbauer *et al*., 1991; Levine *et al*., 1990).

Entering anhydrobiosis requires a coordinated series of events to maintain the native structure of biomolecules and to prevent oxidative damage (Oliver *et al*., 2001). Elucidation of the mechanisms that allow some organisms to survive dehydration may lead to development of new methods for preserving biological materials that do not normally support drying.

1.3. Mechanisms involved in desiccation tolerance

Examples of desiccation tolerance can be found in nearly every phyla (Alpert, 2006). Thus, it is suggested that anhydrobiosis has evolved more than once, and that there may not be a conserved, ubiquitous mechanism that allow desiccation tolerance. Many organisms of different taxa such as tardigrades synthetize non-reducing disaccharides such as trehalose during anhydrobiosis, whose increase during desiccation has been

associated with the ability to survive desiccation (Tunnacliffe & Lapinski, 2003; Iling *et al*., 2005; Mali *et al*., 2010; Gal *et al*., 2003).

Trehalose is believed to replace the water molecules on the polar head groups of the membrane-phospholipids upon desiccation (Mali *et al.,* 2010; Reardon *et al.,* 2010). There are at least three different pathways describing the biosynthesis of trehalose. The most widely found pathway involves the transfer of glucose from UDP (uracil-diphosphate glucose)-glucose to glucose 6-phosphate to form trehalose-6-phosphate and UDP. The enzyme catalyzing this reaction is trehalose-phosphate synthase (TPS) (Elbein *et al*., 2003).

Late embryogenesis abundant (LEA) proteins are the best studied proteins associated with desiccation stress. The first LEA protein was discovered in the cotton plant *Gossypium hirsutum* and was found in high abundance in plant seeds (Tunnacliffe & Wise, 2007). They are also synthesized in vegetative tissues in response to drought, cold, and salt stresses (Tunnacliffe & Lapinski, 2003; Pouchkina-Stantcheva*et al*., 2007). LEA proteins show widespread occurrence in prokaryotes and eukaryotes (Garraya-Arroyo *et al.,* 2000; Tunnacliffe & Wise, 2007; Battaglia *et al*., 2008) in association with desiccation tolerance. They have been reported in bacteria, cyanobacteria, yeasts, fungi, nematodes (*Caenorhabditis elegans*, *Steinernema feltiae*and *Aphelenchus avenae*), arthropods such *Artemia fransciscana* cysts, several other collembolan species, larvae of the chironomid insects, and bdelloid rotifers (Hand *et al*., 2007; Solomon *et al*., 2000; Bahrndorff *et al*., 2009; França *et al*., 2007; Storz *et al*., 1987; Jamieson, 1998; Oliver *et al*., 2001), the prokaryotes *Deinococcus radiodurans, Bacillus subtilis*, *Haemophilus influenza* and rotifers (Dure, 2001; Salomon *et al*., 2000; Buchner *et al*., 1998; Srere, 1966). At least six different groups of LEA proteins have been defined on the basis of expression patterns and sequences; the major categories are group 1, group 2 and group 3 (Bray, 1993). In plants the group 3 LEA proteins, comprising two superfamilies (Wise, 2003), are characterized by a

repeated amino acid motif with a defined consensus sequence (Dure, 2001). LEA are assumed to protect cellular or molecular structures from the damaging effects of water loss; a number of putative mechanisms have been proposed, including hydration buffering, direct protection of other proteins or membranes, and renaturation of unfolded proteins (Bray, 1993). LEA proteins were found to prevent protein aggregation *in vitro* (Goyal *et al*., 2005). Also, *in vivo* experiments using the nematode *Aphelenchus avenae* LEA proteins introduced into human cell lines demonstrated that these proteins play a role in anti-aggregation and protein stabilization during desiccation (Chakrabortee *et al*., 2007).

The ability to tolerate the loss of water is also related to the regulation of antioxidant defense (Pereira *et al.,* 2003). Desiccation tolerance and prolonged longevity in the desiccated state depend on the ability to scavenge free radicals, using antioxidants such as glutathione (Kranner & Birtic, 2005), SOD (Shirkey *et al*., 2000; Elstner & Osswald, 1994) ascorbate peroxidase (AP) and other peroxidases, mono- and dehydroascorbate reductases, glutathione reductase (GR) and catalase (Elstner & Osswald, 1994).

Espindola *et al*. (2003) demonstrated that glutathione plays a significant role in protecting cell membranes and in maintaining redox homeostasis under water deficiency, favoring tolerance to the dry state in yeast. SOD scavenge O_2 by converting it to H_20_2, which in turn is broken down to H_20 in the cytoplasm by glutathione peroxidase (GSHPx) (Chance *et al*, 1979) and in peroxisomes by catalase (Aebi, 1984). These enzymes act in concert to provide an important protection mechanism for tissues against oxidative attack. Overexpression of SOD or catalase or treatment with mimetics of these enzymes increased for example the longevity of mice, *Drosophila melanogaster* and *Caenorhabditis elegans* (Orr & Sohal, 1994; Sampayo *et al*., 2003). Overexpression of SOD in *Saccharomyces cerevisiae*increased its tolerance to dehydration (Pereira *et al.,* 2003). Catalase gene expression is regulated by oxidative

and osmotic stresses (Ruis & Hamilton, 1992). In sunflower and bean seeds, as well as in maize leaves, catalase activity increases during dehydration (Bailly *et al.,* 2001, 2004; Jiang & Zhang, 2002), suggesting that this enzyme prevents dehydration-related oxidative damage.

Heat shock proteins (HSP) (Doulias *et al*., 2007; Diller, 2006) and Foxo genes (Tothova *et al*., 2007) have also been linked to desiccation stress in prostata and HeLa cell lines. Aquaporins (Huang *et al*., 2007a; Kikawada *et al*., 2008; Murata *et al*., 2000; Walz *et al*., 1997) and anion channels (Desai *et al.,* 2006; Klebba & Newton, 1998) are specific membrane channels that control cellular osmotic and water homeostasis during desiccation stress. A group of transcription factors that are up-regulated during desiccation stress, belonging to the Leucine-zipper group have also been identified (Deng *et al*., 2006).

Mechanisms involved in desiccation tolerance have been investigated in diverse desiccation tolerant organisms.

Studies on the Cyanobacterium *anabaena* sp. have shown that about 300 genes are differentially expressed during desiccation stress (Garcia-Pichel & Castenholz, 1991) and that the expression mainly involves genes responsible for DNA repair. A proteomic analysis of Cyanobacterium *nostoc commune* under UV stress has identified nearly 500 proteins involved in the UV response (Ehling-Schulz *et al*., 2002). One half of these proteins are involved in an early stress response and the other half belong to the long lasting stress response group. A characteristic stress response, which is also observed in the bacterium *Pseudomonas putida* (Chang *et al*., 2007), is the formation of extrapolysaccharide, which is thought to protect the cell from radiation and rapid water loss and to stabilize cell structures during desiccation (Tamaru *et al*., 2005). Water stress proteins (Scherer & Potts, 1989) and scytone, an ultra-violet light(UV) protective pigment (Garcia-Pichel & Castenholz, 1991), are two unique stress response elements often found in terrestrial cyanobacteria.

Several bacteria of the genus *Deinococcus* exhibit extreme desiccation tolerance (Battista *et al*., 2001; Tanaka *et al*., 2004) as well as resistance to ionizing radiation (IR) (Daly*et al*., 2007; Shukla *et al*., 2007) and UV light (Khairnar *et al*., 2008; Xu *et al*., 2008). The *Deinococcus* DNA repair mechanism appears to function more efficiently than that of any other known organism. Despite recent progress, the molecular pathways responsible for enhanced DNA repair in *Deinococcus* remain still widely unknown (Makarova *et al*., 2007; Daly *et al*., 2007).

Escherichia coli has been investigated for osmotic stress responses. The results showed a pool of 152 genes to be regulated, 107 down- and 45 up-regulated. Most of the upregulated genes are unclassified, unknown or hypothetical (Weber & Jung, 2002).

Cellular arrest is a common stress response of the yeast *Saccharomyces cerevisiae* subjected to desiccation (Singh *et al*., 2005), salt (Alexander *et al*., 2001) and UV stress (Birrell *et al*., 2001). Furthermore, it was shown that trehalose is not necessary for *Saccharomyces cerevisiae* to achieve desiccation tolerance (Ratnakumar & Tunnacliffe, 2006).

The resurrection plants such as *Craterostigma plantagineum* (Bartels & Salamini, 2001) and *Myrothamnus flabellifolia* (Moore *et al*., 2007) are the best studied examples of anhydrobiosis in plants. During desiccation, expression of a large number of transcripts that involve the protein synthetic machinery, ion and metabolite transport and membrane biosynthesis/repair are detected (Oliver *et al*., 2004). Gene products such as LEA protein were identified in the cytoplasm, chloroplasts and other stress induced transcripts, including genes involved in abscisic acid biosynthesis (Leung & Giraudat, 1998), sugar synthesis for structure and vitrification (Vicre *et al*., 2004), regulatory proteins such as protein translation initiation factors, homeodomainleucine zipper genes (Ditzer & Bartels, 2006), superoxide dismutases (SOD) (Veljovic *et al*., 2006) and a gene probably working as a regulatory RNA (Bernacchia and Furini, 2004).

Arabidopsis thaliana as a model system for plants has also been investigated for its response to water stress. Noteworthy here is a study in which *A. thaliana* was exposed to heat and desiccation stress at the same time. The goal was to see whether the response to multiple stresses is a sum of each stress response. *Arabidopsis* was shown to synthesize osmolytes during both stresses. Proline was synthesized as a response to heat stress and sucrose as a response to desiccation stress. The investigators queried whether the plant would produce both osmolytes and only one upon combined desiccation and heat stress. *Arabidopsis* produced only sucrose. This result suggests that one stress, in this case desiccation stress, can also confer tolerance to another stress, and in this case heat shock (Rizhsky *et al*., 2004).

All known anhydrobiotic animals share two common characteristics: they are not larger than 5 mm and have no exoskeleton (Alpert, 2006). Examples of anhydrobiotes are nematodes and tardigrades. Another well studied anhydrobiotic animal is the brine shrimp (e.g. *Artemia salina*), although only the cyst of the animal is desiccation-tolerant. The cyst is a life stage where the organism survives as a latent life form inside a membrane that is sometimes formed by an old cuticle. Research on the cyst has revealed two novel proteins, ferritin and artemin (Chen *et al*., 2003), which are synthesized during desiccation stress, in addition to commonly known proteases (Nithya *et al*., 1996) and heat shock proteins (Liang & Macrae, 1999).

The largest known anhydrobiotic animal is the African chironomid *Polypedilum vanderplanki* (Kikawada *et al*., 2008; Watanabe *et al*., 2002; Watanabe *et al.,* 2007). The only desiccation-tolerant stage of this animal is the larval stage. Anhydrobiosis was shown to be achieved even in the absence of brain function (Watanabe *et al*., 2002), and trehalose biosynthesis could be induced by simply shifting the internal ion concentration (Watanabe *et al*., 2003). These results suggest that anhydrobiosis is directly triggered by environmental conditions and does not require the involvement of a central signaling mechanism triggered by a nervous system.

A comparison between a laboratory grown population of *Drosophila* sp. selected for desiccation tolerance and a population from a desert showed that adaptation to increased desiccation conditions can be achieved by selection (Gibbs, 2002). One particular feature of the laboratory strain that could be preadaptive to desiccation stress was a prolonged larval stage and a higher accumulation of carbohydrates and water in the body (Gefen *et al*., 2006). Another study comparing gene expression analysis between the response to cold and desiccation stress showed differences between the two responses, which supports the idea of specific responses to specific stresses (Sinclair *et al.,* 2007).

Furthermore, studies with the fly *Sarcophaga bullata* indicate temporarily distinct responses to acute stresses like desiccation: an early response and a long term response. This finding stands in agreement with the data from studies with cyanobacteria mentioned above (Yoder *et al*., 2006). In contrast to the studies on the chironomid *Polypedilum vandeplanki* the work on *Sarcophaga* indicates a role for the central nervous system in the stress response.

Bdelloid rotifers are capable of anhydrobiosis – either as eggs or embryos, juveniles or adults – but the capacity differs among species and strains (Ricci, 1998). Entering anhydrobiosis the rotifer contracts into a so called ‘tun’ by withdrawing the cephalic and caudal body portion extremities into the trunk (Ricci & Melone, 1984). This form may help the animal to better control the rate of water loss during the process of desiccation (Dickson & Mercer, 1967; Schramm & Becker, 1987). Caprioli & Ricci (2001) kept samples of anhydrobiotic *Macrotrachela quadricornifera and Adineta oculata* for different periods under controlled conditions and found that the recovery percentages decreased drastically with increasing desiccation duration. Recovery is maximal for adults and late embryos and minimal for juveniles and newly-laid eggs (Ricci *et al.,* 1987; Örstan, 1995; Orsenigo *et al*., 1998).

1.4. Research objectives

Harsh environmental conditions lead to cryptobiosis, which forces an organism to downregulate its cellular functions to a minimum. Anhydrobiosis is one example of cryptobiosis and describes cryptobiosis due to dryness. The effect of water stress shall be discussed in this work by means of morphological and physiological studies of the bdelloid rotifer *Philodina roseola* with a focal point on tolerance mechanisms against desiccation stress.

To characterize the molecular basis of desiccation tolerance a substractive hybridization approach will be established for identification of genes that may be regulated by desiccation stress. Another objective of this work is to define/categorize the proteins LEA, SOD and catalase as proteins regulated by stress. Hence, the final function of LEA, SOD and catalase will be investigated by means of double stranded RNA (dsRNA) and mediated interference RNA (RNAi), in order to assess, whether these proteins participate actively in stress response.

2. Material and methods

2.1. Chemicals and bio-reagent

Ammoniomsulfate	Merck
Agarose	Gibco Life Technologies
Ampicillin	Sigma, Deisenhofen
Bacto Agar	Difco,Becton Dickinson
Bacto trypton	Difco,Becton Dickinson
Bacto yeast extract	Difco,Becton Dickinson
Boric acid	Merck
Bromophenol blue	Biomol
Calcium chloride	Merck
Chloroform	Merck
2,7-Dichlorodihydrofluorescein diacetat	Molecular probes Inc
DNA molecular weight standards	MBI Fermentas
DNA Taq-Polymerase	PE Applied Biosystems
Dnase	Promega
DNTP	Boehringer
DTT (Dithiothreitol)	Sigma Chemie
Dynabeads Oligo(dT)25 magnetic beads	Dynal
Escherichia coli DNA Polymerase	Invitrogen
Escherichia coli Rnase H	Invitrogen

EDTA (ethylenediamine tetraacetic acid)	(Titriplex® III) Merck
Ethanol	Merck
Ethidium Bromide	Invitrogen
Formaldehyde	Sigma-Aldrich
Glycerol	Merck
Glycin	Biomol
IPTG (Isopropyl-ß-D thiogalactopyranoside)	Biosciences
Isopropanol	Merck
Kanamycin	Sigma
Mineral oil	Sigma
M-MulV-reverse transcriptase.	New England Biolab
Natrium hydrogen sulfate	Invitrogen
Oligonucleotides (primers)	MWG Biotech
Paraformaldehyde	Sigma-Aldrich
PCR Mastermix	Invitrogen
Phenol	Carl Roth
Proteinase K	Boehringer
K-acetat	Merck
Potassium phosphate	Merck
Kanamycin	Sigma
Restriction Endonucleases	Roche
Reverse Transcriptase (Superscript II)	Gibco Life Technologies

RNAse H	Roche
Sodium acetate	Carl Roth
Sodium chloride	Merck
Syber Green PCR Master Mix	Qiagen
Taq DNA polymerase	Invitrogen
T4 DNA Polymerase	Invitrogen
T4-DNA Ligase	Promega
Tetracycline	Sigma
TRIzol reagent	Invitrogen
X-gal	Biomol

Salts, acids, bases and solvents not mentioned in the table werequalities from Merck (Darmstadt) or Sigma (Deisenhofen).

2.2. Kits

CloneJET™ PCR Cloning	Fermentas
DNA isolation	Qiagen
DNA quantification	Dojindo
Dynabeads mRNA Direct Micro	Dynal, Oslo
SuperScript first-strand cDNA synthesis	Qiagen
Gel Extraction	Qiagen
Molecular weight marker	Peglab
NucleoSpin™ Reaction Purification	Macherey and Nagel

pcDNA cloning	Promega
PCR product purification	Nucleospin
PCR purification	Qiagen
Plasmid Mini preparation	Fermentas
PCR TOPO cloning	Promega
DNA quantification	Dojindo
Quantitect SYBR Green RT-PCR kit	Qiagen

2.3. *Escherichia coli* strains

BL21-(DE3)	Invitrogen
HT115 (Timmons 2000)	Addgene
TOP10F‘	Strategene

2.4. Extraction and culture of rotifers

Specimens of *Philodina roseola* rotifers were extracted from moss according to the protocol of Peters *et al*. (1993). Moss (*Physcomitrella patens*) was collected in the botanic garden of the University of Düsseldorf and soaked in a vial with 20 ml tap water for 24 h. After that the wet moss was vigorously shaken for 15 sec. and then the water (without the moss) was transferred into a petri dish.

A single rotifer was scaled up for growth for 3 days at room temperature in aerated 250 µl plastic vessel, containing tap water and a suspension of rotifer diet (RS Aquaristik, Schwandorf, Germany) in order to obtain rotifers that are descendants from a single female which was possible because of their asexuality. Bdelloid rotifers reproduce

exclusively by parthenogenesis. For the experiments, rotifers were washed after three days of cultivation, and collected by filtration onto a sieve of mesh size 50 µm.

2.5. Desiccation and survival analysis

The rotifers were dried at 88 %, 76 %, 44 % and 23 % relative humidity (RH). These concentrations were obtained using the saturated salt solutions of barium chloride, sodium chloride, potassium carbonate and potassium acetate respectively (Block, 1953; Eckhoff *et al*., 1982). The RH values were controlled using a hygrometer (Conrad, Hirschau, Germany).

The animals collected from the cultures were randomly subdivided into different groups. The hydrated group served as control. Rotifers were transferred onto 1 cm x 1 cm millipore nitrocellulose filter (5 µm mesh size) and dehydrated at room temperature for 24 h, 48 h or 72 h in a sealed desiccator at the various RHs mentioned above. To slow down the time of dehydration about 50 µl water was remained on the filter containing rotifers. To retard evaporation Millipore filter carrying the rotifers was placed between two pieces of wet Whatman filter paper. After desiccation, the dried rotifers were rehydrated for 24 h and were counted as alive if they were active. Mean survival and standard deviation were calculated depending on the relative humidity value. Each experiment, i.e. dehydration of 50 - 60 specimens per RH-value and period, was replicated 6 times.

2.6. Preparation of genomic DNA

Genomic DNA was prepared from rotifers using the DNeasy Kit (Qiagen, Hilden, Germany). All steps were performed according to the manufacturer’s protocols. Briefly, 150 µg rotifers were crushed under liquid nitrogen using a mortar and resuspended with 1, 5 ml of lysis buffer (100 mM Tris-HCl, pH 8.0, 50 mM NaCl, 50 mM EDTA, 1% SDS, 0.15 mM spermine, 0.5 mM spermidine). Following digestion with proteinase K (100

µg/ml) at 48 °C for 2 h, the preparation was extracted twice with phenol-chloroform-isoamyl (25:24:1) alcohol (2.8). The DNA was then ethanol precipitated, treated with RNAse I (100 µg/ml) and re-extracted with phenol-chloroform-isoamyl alcohol. The purified DNA was precipitated (2.9) with ethanol and resuspended with TE buffer (10 mM Tris-Cl, pH 7.5. 1 mM EDTA). An aliquot of the DNA was quantitatively and qualitatively analysed by spectrophotometric and electrophoretic methods.

The isolated DNA was diluted with distilled water (1:50) and the absorbance at 260 nm against H_2O bidest was measured spectrophotometrically. The calculation of the DNA concentration was based on the following formula: 1 A_{260} = 50 µg/ml for ds DNA; 1 A_{260} = 33 µg/ml for ss DNA. For determination of DNA purity, the $A_{260/280}$ coefficient was photometrically determined. An $A_{260/280}$ < 1.8 indicated contamination of the DNA preparation with protein or aromatic substances such as phenol; while an $A_{260/280}$ > 2.0 indicated possible contamination with RNA.

2.7. Total RNA isolation

Total RNA from mouse tissue was isolated according to Chomczynski and Sacchi (1987). With this method, cell homogenates are directly added to a monophasic acidic phenol guanidine isothiocyanate solution (TRIzol Reagent, Invitrogen). Upon adding chloroform, the RNA remains in the aqueous phase, while proteins and high molecular weight DNA enter the organic phase and the interphase, respectively. Total RNA is recovered by isopropanol precipitation.

Rotifers (200 µg) were lysed in 2 ml TRIzol reagent (Invitrogen, Karlsruhe, Germany). The homogenized sample was first incubated at 37°C for 5 min in order to completely dissociate the nucleoprotein complexes. 400 µl of chloroform per 2 ml of TRIzol were then added to the tube; vigorously mixed for 15 sec and incubated at 30°C for 2 min. The sample was centrifuged at 12.000 xg for 30 min at 4°C. Following centrifugation, the supernatant was separated into a lower red, phenol-chloroform phase, an

interphase, and a colorless upper aqueous phase. RNA is only found in the aqueous phase that was transferred to a fresh tube. The RNA was then precipitated from the aqueous phase by mixing it with 0.5 ml of isopropyl alcohol per 1 ml of TRIzol reagent. The samples were incubated at room temperature for 10 min and then centrifuged at 12,000 x g for 10 min at 4°C. The RNA precipitate formed a gel-like pellet on the side and at the bottom of the tube. The supernatant was then removed carefully and the pellet was washed once with 75 % ethanol, adding at least 1 ml of 75 % ethanol per 1 ml of TRIzol reagent. The sample was mixed by vortexing and centrifuged at 7,500 x g for 5 min at 4°C. The supernatant was removed, the RNA pellet was air-dried for 5 - 10 min, then dissolved in 20 µl of DEPC water, and stored at –70°C until used. RNA yield and purification efficiency was determined with a spectrophotometer (Nanodrop technologies, UK). An OD_{260} of 1 corresponds roughly to 40 µg/ml RNA. A ratio OD_{260}/OD_{280}< 1.8 - 2.0 indicates protein contamination. RNA integrity was assessed by running 0.5 µg of RNA on an agarose gel as described in 2.13.

2.7.1. DNase treatment of extracted RNA

After extraction, RNA was treated with RNase-Free DNase kit (Qiagen, Hilden, Germany) in order to remove genomic DNA following the manufacturer's protocol. All steps were carried out on ice unless otherwise indicated. Per sample 2.5 µg of total RNA was added to a 0.5 ml polypropylene tube and mixed with 5 µl 10 x Incubation Buffer (100 mM Tris-HCl, 25 mM $MgCl_2$, 5 mM $CaCl_2$, pH 7.6). After addition of 10 units DNase I (Roche), DEPC-treated water was added to a final volume of 50 µl and tubes were incubated at 37°C for 30 minutes. Finally, 2 µl of 0.2 M EDTA were added and tubes were heated to 75°C for 10 minutes. Samples were subsequently stored at -80°C or used directly for reverse transcription.

2.7.2. Isolation of mRNA by Dynabeads

The mRNA was isolated from rotifers by using the Dynabeads mRNA Direct Micro Kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. After the cellular components were completely lysed in lysis/binding buffer in a RNase-free 1.5 ml microcentrifuge tube, Dynabeads Oligo(dT)25 were added. These are uniform, superparamagnetic, polystyrene beads with oligo(dT)25 covalently bound to the surface. After thoroughly mixing Dynabeads and the mRNA containing buffer, the tube was placed on a shaker for 5 min at room temperature to allow the mRNA to anneal to the Dynabeads. Subsequently the tube was put in a magnetic particle concentrator. The mRNA loaded Dynabeads were fixed at the tube surface by magnetism allowing to remove the supernatant with protein, rRNA and other debris. According to this protocol, mRNA loaded Dynabeads were washed twice with washing buffer A as well as twice with washing buffer B. Finally, mRNA was dissolved in a 10 µl of DEPC treated H_2O and stored at - 80°C for later use.

2.8. Phenol-chloroform extraction

Phenol extraction was carried out to remove contaminating proteins from a DNA preparation (Köchl *et al*., 2005). The DNA solution was mixed with an equal volume of TE buffer (10 mM Tris.Cl (pH 8.0), 1 mM EDTA) and saturated phenol/chloroform/isoamyl alcohol (25:24:1) in a microcentrifuge tube. The mixture was vortexed for 30 - 60 sec and centrifuged at 14.000 rpm for 5 min at RT to separate the sample into phases. The upper aqueous layer was then carefully removed into a clean tube, avoiding mixture with denatured proteins found at the aqueous/phenol interface. This upper phase was then mixed with an equal volume of the phenol/chloroform/isoamyl alcohol solution mentioned above; the mixture was vortexed and centrifuged (14.000 rpm for 5 min). This step was repeated 2 - 3 times, and the DNA precipitated from the upper aqueous phase through ethanol precipitation.

2.9. Ethanol precipitation

This was carried out to remove contaminating salts from a DNA preparation or to concentrate a DNA preparation (Zeugin & Hartley, 1985). The DNA solution was mixed with 1/10 volume of 3 M sodium acetate and 3 volumes of ethanol and incubated at -70 °C for 30 min. The mixture was centrifuged at 14.000 rpm for 30 min at 4 °C. The supernatant was removed and the DNA pellet was washed with 70 % ethanol and centrifuged at 14.000 rpm for 5 min at 4 °C. The pellet was air-dried and the DNA was resuspended in TE buffer (10 mM Tris-Cl, pH 7.5. 1 mM EDTA) or nuclease free water and stored at -20 °C.

2.10. First strand cDNA synthesis

To synthesize cDNA, reverse transcription was carried out using DNase-treated total RNA. For this purpose, 10 µl aliquots of each DNase-treated sample were added to 0.5 ml polypropylene tubes. All enzymes, buffers and other reagents used in reverse transcription were from a commercial kit (SuperScript First-Strand Synthesis System, Invitrogen, Karlsruhe, Germany). All steps were carried out on ice unless otherwise indicated. To the DNase-treated RNA aliquots (10 µl each), 1 µl Oligo(dt)$_{25}$ primer and 1 µl 10 mM dNTP mix (2.5 mM dATP, 2.5 mM dGTP, 2.5 mM dTTP, 2.5 mM dCTP. Distilled H_2O) were added. Samples were incubated at 65°C for 5 min and then on ice for 2 min. The remaining components were premixed (per sample: 2 µl 10x RT buffer, 4 µl 25 mM $MgCl_2$, 2 µl 0.1 M DTT, 0.5 µl RNase (10 mg/ml), 1 µl SuperScript Reverse Transcriptase [50 U/µl] and 0.5 µl RNase-free water) and 10 µl mix were added per sample. Subsequently, samples were incubated at 25°C for 10 min, 42°C for 50 min, 70°C for 15 min and then quickly chilled on ice. The final reaction volume (22 µl per sample) was diluted 1:2 by addition of 22 µl RNase-free water, and cDNA was stored at - 20°C until use. As a negative control, aliquots of RNA were processed as above, but in the absence of the reverse transcriptase. These so-called -RT (or “minus RT”) controls

allow identification of contamination from genomic DNA or exogenous sources, preventing false-positive results.

2.11. Second strand cDNA synthesis

For the second strand synthesis (D'Alession & Gary, 1988), the following components were added on ice: 45 µl first-strand synthesis reaction; 20 µl of 10 x second-strand buffer (700 mM Tris-HCl (pH 7.4), 100 mM $(NH4)_2SO4$, 50 mM $MgCl_2$, Distilled H_2O); 6 µl of second-strand dNTP mixture (2.5 mM dATP, 2.5 mM dGTP, 2.5 mM dTTP, 2.5 mM dCTP, distilled H_2O); 116 µl of sterile distilled water; 2 µl of RNase H (1.5 U/µl) and 11 µl of DNA polymerase I (9.0 U/µl). The second strand reactions were gently mixed and briefly spun, incubated at 16°C for 2.5 hrs and after addition of T4 Polymerase (20 U) incubated for further 5 min at 16°C.

2.12. Polymerase chain reaction (PCR)

Routine PCRs were performed in 50 µl reaction volumes containing 20 mM Tris-HCl (pH 8.8), 2 mM MgSO4, 10 mM KCl, 10 mM (NH4)2SO4, 200 µM dNTPs, 0.5 U of Taq polymerase, 10 pmol of each oligonucleotide and 100 ng DNA-template (Saiki *et al*., 1988). A total of 35 PCR cycles were performed, consisting of an initial denaturation step at 95°C for 2 min, followed by 35 cycles of: denaturation at 94°C for 45 sec to 1 min, annealing of the oligonucleotide at 48°C to 65°C for 45 sec to 1 min, followed by an extension at 72°C for 1.5 min. After a final 10 min extension step at 72°C and a 4°C hold, the samples were analyzed by agarose gel electrophoresis.

2.13. Agarose gel electrophoresis

After PCR, an aliquot of each sample was used for agarose gel electrophoresis according to Brody and Kern (2004). The electrophoresis standard used was either the DNA molecular weight ladder (Peqlab, Germany) consisting of 15 bands between 0.1

and 10 kilobase pairs with 3 bands (500, 1000 and 1500) at higher concentrations for easy reference or 21 bands between 0,1 and 10 kb with three bands 500,1000 and 3000 bp as reference. Gels were prepared as 1 – 2 % agarose in 1 x TAE buffer (40 mM TRIS, 1 mM EDTA-Na_2-Salz, 40 mM $C_2H_4O_2$), and ethidium bromide was added to the gel for a final concentration of 0.5 µg/ml. Ethidium bromide intercalates into DNA and fluoresces when exposed to ultraviolet light, allowing visualization of bands. Typically, for simple visualization of bands, 15 - 20 µl PCR product were mixed with 2 - 4 µl 6 x Orange DNA loading dye (0.25 % bromophenol blue, 0.25 % xylene - cyanol, 40 % glycerol), carefully dispensed into the gel pockets, and subjected to electrophoresis at 5 V/cm for about 45 - 60 minutes

After exposure to ultraviolet light, specific bands were identified and excised using a scalpel. Slices of agarose gel containing cDNA fragments of interest were processed using a commercially available kit, based on DNA binding to silica membranes and subsequent column purification, according to the manufacturer's instructions (NucleoSpin® Gel and PCR Clean-up, Dueren, Germany). After elution, cDNA concentrations were measured and samples were stored at - 20°C until use.

2.14. Ligation of inserts into a plasmid vector

After extraction from agarose gels or direct purification from PCR mixtures, cDNA fragments were used for ligation (Ferretti & Sgaramella, 1981). During this process, the ends of the cDNA fragment are joined with the open ends of the vector via the enzymatic activity of a DNA ligase. Reactions were assembled in 0.5 ml polypropylene tubes by mixing 5 µl 2 x Rapid Ligation Buffer (100 mM Tris-HCl, 20 mM $MgCl_2$, 2 mM ATP, 20 mM Dithiothreitol, pH 7.5), 1 µl vector (50 ng) (pcDNA, PCR Topo or L4440), 3 µl PCR product and 1 µl T4 DNA Ligase. The molar ratio of vector to insert typically was about 3:1. After incubation at room temperature and 4°C for 1 h each ligated samples were either stored at 4°C or used for transformation of bacterial cells.

2.15. Production of calcium-competent bacterial cells

Bacteria were made competent for transformation by the method of Hanahan (1985). A bacterial colony was inoculated in 10 ml LB-medium (1 % Bacto-tryptone, 0.5 % Bacto-yeast extract and 1 % NaCl) and left to grow overnight at 37°C. The overnight *Escherichia coli* culture (BL21) was diluted 1:100 in 100 ml LB-medium and allowed to grow until OD_{600} = 0.5. The cells were chilled on ice for 15 min and then centrifuged for 10 min at 4000 x g at 4°C. The resulting bacterial pellet was resuspended in ice-cold 80 mM $CaCl_2$ and incubated on ice for 30 min. The cells were then pelleted and resuspended in 10 ml ice-cold 100 mM $CaCl_2$ 20 % glycerol solution, aliquoted at 4°C and frozen at - 70°C.

2.16. Transformation of competent cells

The transformation of the bacterial cells was performed according to the method of Cohen *et al.* (1972). The competent cells were allowed to thaw on ice slowly. 1 µl of each of the ligation reactions (2.14) were used to transform 30 µl of competent *E. coli* cells. The sample was incubated for 30 min at 4°C. The cells were heat-shocked at 42°C for 30 sec and then cooled on ice for 2 min. 250 µl of SOC medium (2 5 % Tryptone, 0.5 % Yeast Extract, 10 mM NaCl, 2.5 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM Glucose) was added and the mixture was agitated at 37°C for 1 h. The cells were then plated on LB agar (LB-medium + 1,5 % Agar) plates containing the appropriate selection antibiotic (Ampicilin (100 µg/ml), Tetracycline (12.5 µg/ml) or Kanamycin (100 µg/ml) for the vector used and incubated overnight at 37°C.

In the case of blue/white selectable vectors, the LB agar plates were spread with 70 µl of 20 mg/ml X-Gal and 70 µl of 0.1 M IPTG. White colonies were selectively picked for further analysis.

2.17. Colony PCR

On the day after plating a colony PCR was done (Ohno *et al*.; 1991) to amplify the DNA fragments cloned into plasmid vectors, using a PCR reaction mixture (2.12), PCR TOPO Vector Primers M13 forward (5'-GTAAAACGACGGCCAG-3') and T7 reverse (5'-CCCTATAGTGAGTCGTATTA-3') or pcDNA Vector Primers T7 forward (5'-TAATACGACTCACTATAGGG-3') and BGH reverse (5'-TAGAAGGCACAGTCGAGG-3') or L4440 Vector Primers T7 forward (5'-TAATACGACTCACTATAGGG-3') and T7 reverse (5'-CCCTATAGTGA-GTCGTATTA-3') to a final volume of 10µl. Single clones were picked using sterile toothpicks and added to the PCR reaction mixture. For the blue white selection, clones that did not take up the vector (PCR TOPO) appeared blue, while clones that successfully incorporated the vector plus insert appeared white. In this case only write clones were added for the PCR reaction. The thermal cycle conditions used to perform the PCR reaction were one cycle of 94°C for 1 min; 94°C for 30 sec; 50 - 60°C for 30 sec; 72°C for 1 min for 25 cycles; and a final elongation step of 72°C for 5 min for 1 cycle. A total of 250 recombinant clones were picked out.

2.18. Culture of *Escherichia coli*

After colony PCR amplification, agarose electrophoresis (2.3) was performed to identify clones that harbor the vector and carry the successfully inserted cDNA fragment of interest. These clones were picked using sterile inoculating loops and transferred to 15 ml conical polypropylene tubes containing 4 ml LB-medium (1 % Bacto-tryptone, 0.5 % Bacto-yeast extract and 1 % NaCl) supplemented with 100 µg/ml ampicillin or Kanamycin or 12,5 µg/ml tetracyclin. Tubes were incubated in a shaker overnight at 37°C and 225 rpm (Reiling*et al*., 1985). On the next morning, cell suspensions were checked for sufficient bacterial growth and absence of obvious contamination, and either stored at 4°C or directly used for preparation of frozen stocks or plasmid DNA extraction.

Frozen stocks of bacterial cell suspension cultures are a convenient means of archiving the recombinant DNA contained in the cells. For this purpose, glycerol was added as a cryoprotectant as follows: the 15 ml tubes containing bacterial cultures were agitated so that the cells were evenly suspended. 200 µl glycerol were dispensed into tubes suitable for freezing at very low temperatures. 800 µl bacterial culture was added, and thorough mixing was achieved by pipetting up and down several times. Tubes were placed on dry ice for immediate freezing and transferred to - 80°C for long-term storage.

2.19. RNAi bacterial cell number determination

2.19.1. Optical density measurements

The optical density (OD) of bacterial cultures was determined at 600 nm using a spectrophotometer. The samples were diluted 1:10 with dH_2O or LB media, which was used as reference as well.

2.19.2. Microscopic count of bacterial cells

200 µl of a bacterial culture were diluted 1:10 with destilled dH_2O and transferred to a Neubauer counting chamber (Armin Baack,Schwerin, Germany). The cells were counted in five fields of the 1 mm^2 center square using a stereo microscope. As each square represents a total volume of 0,004 mm^3 (equivalent to 0,004 µl), the total volume of five squares is 0,02 µl. Hence, the cell concentration per ml was calculated as follows:

$$0{,}02\ \mu l = n \text{ counted cells} \quad |\cdot 50$$

$$1\ \mu l = n \cdot 50 \quad |\cdot 10^3$$

$$1\ ml = 50n \cdot 10^3$$

if diluted:

$$1\ ml = 5 \cdot 10^4 n \cdot D$$

x represents the total amount of counted cells in five squares and D the dilution factor of the bacterial probe.

2.20. Isolation of plasmid DNA

For extraction of plasmid DNA from bacterial cells, a commercial column-based kit was used (Plasmid Miniprep Kit, Fermentas, Leon-Rot, Germany). As starting material, an aliquot of 1.7 ml bacterial cell suspension per culture was transferred to a 2 ml polypropylene tube, and the cells were condensed into a pellet by centrifugation (3 minutes at 12,000 x g). Subsequent steps were carried out according to the manufacturer's instructions, and DNA was eluted with 50 µl nuclease free water. Quality and quantity of DNA in all batches of preparations were monitored as described above (2.7).

2.21. Restriction enzyme digestion of DNA

DNA plasmid, PCR products and vectors constructs were digested with the appropriate restriction endonucleases in the suitable buffer system and at the correct temperature, according to the manufacturer's instructions (MBI, NEB). 1 to 5 µg of DNA (either plasmids or PCR - fragments) were cut by 1 µl restriction endonucleases (10 U/µl) in the presence of 2 µl of the appropriate 10 x restriction buffer (500 mM potassium acetate, 200 mM Tris-acetate, 100 mM Magnesium Acetate, 10 mM Dithiothreitol, pH 7.9) in a final volume of 20 µl. Depending on the endonucleases used, the restriction mix was incubated for 90 min at 37°C or 55°C. In case of plasmids the restriction reaction was controlled by gel electrophoresis (2.13) and the linearised DNA or the insert were

isolated from the agarose gel. Restricted PCR-fragments were directly purified as described in section (2.13).

2.22. Establishment of a subtracted cDNA library

Subtractive hybridization methods provide a means to isolate genes that are specifically expressed in a cell type or tissue (Zhang & Xia 2009; Raghavendra *et al*., 2011), i.e. genes that are differentially regulated during activation or differentiation of cells (Straub*et al*., 2003; Liu *et al*., 2007). The principle of these approaches is to remove the mRNA species common to different cell types or tissues by subtraction, leaving the cell type/tissue-specific mRNAs for further manipulation and analysis. Several subtractive hybridization strategies based on solid-phase hybridization on magnetic dynabeads have previously been described (Zhang & Xia, 2009; Raghavendra et *al*., 2011). In the present study the method used to establish the subtractive hybridization is from Lönneborg *et al.*, (1995). This method take advantage of the properties of magnetic Dynabeads allowing simple and rapid buffer changes required for optimal hybridization and enzymatic reactions.

2.22.1. Substrative hybridization

The subtraction procedures are illustrated in figure 2. Rotifers were pelleted from the culture. After washing (three times) rotifers were concentrated using a sieve (mesh size 50 µm). A 200 µl rotifer pellet was transferred on Millipore filter and allowed to dry for 24 h at 88 % relative humidity (RH) and room temperature. After desiccation rotifers were wept with water for 30 min to release them from the paper. Rotifers were then pelleted and used for RNA and mRNA isolation. Control group of rotifers were maintained hydrated. Poly(A)+ RNA isolated from stressed rotifers was bound to 125 µg of Dynabeads Oligo(dT)25 magnetic beads (Dynal, Lake success, NY) and eluted with 15 µl of diethyl pyrocarbonate (DEPC)-treated dH_2O at 70°C for 2 min as described above.

In the case of the control, the final step of eluting poly(A)+ RNA was omitted. Instead, the control mRNA/Oligo(dT)25 Dynabeads complex was washed twice in 1 x first strand buffer (50 mM Tris-Hcl pH 8,3 mM KCl, 6 MgCl2) and resuspended in 100 µl of cDNA synthesis buffer (1 x first strand buffer, 2 mM DTT, 1 mM dNTPs, 1 µl of RNase inhibitor (40 units/ul) and 2 µl of superscript II reverse transcriptase (200 units/ul; Gibco-BRL, Invitrogen, Karlsruhe, Germany). First strand cDNA was synthesized directly on the magnetic beads using Dynabeads Oligo(dT)25 as primer by reverse-transcriptase polymerase chain reaction (RT-PCR) in a 0,5 ml microcentrifuge tube. The microcentrifuge tube was placed horiziontally in an air incubator with a rotating wheel (40 rpm) at 42°C to keep beads in suspension and incubated for 2 h to insure efficient enzymatic reactions. After synthesis of the first-strang cDNA, the magnetic beads were washed twice with 2 mM EDTA at 95°C for 3 min in order to denaturate the poly(A)+ RNA, which was removed from the first strand cDNA coupled to Dynabeads by magnetic separation. The control first-strand cDNA coupled to dynabeads was hybridized mRNA from stressed rotifers. The control first-strand cDNA coupled to the beads was collected with a magnet and resuspended in 90 µl of hybridization buffer (120 mM NaH_2PO_4 pH 6.8; 820 mM NaCl, 1 mM EDTA, 0.1 % SDS) and 1 µl of RNAse inhibitor(40 U|µl). 15 ml of poly(A)+ RNA isolated from stressed rotifers was added, and the mixture was layered with 100 µl of mineral oil. The cap of the microcentrifuge tube was sealed with parafilm to prevent evaporation. The mass ratio of the control first strand cDNA to the stressed mRNA in this subtraction step was estimated to be approximately 3:1, based on the amount of the control group and stressed rotifers. An initial denaturation step at 95°C for 3 min was followed by hybridization at 65°C for 24 h in a rotary hybridization oven (Stovall Life Science, Greensboro,NC) in order to keep the beads suspended (60 rpm) during the hybridization reaction. The hybridized mRNA/cDNA-Dynabeads complex was captured by magnetic separation and the mineral oil was removed. The supernatant containing unhybridized mRNA was collected

and subjected to a second hybridization reaction with the recycled cDNA-Dynabeads. The magnetic beads were recycled by eluting the hybridized mRNA twice in 1 ml of H_2O at 95°C for 3 min and washing the magnetic beads twice in 1 x washing buffer (150 mM LiCl, 10 mM Tris-HCl pH 8,0, 1 mM EDTA). After three cycles of hybridization, unhybridized stressed mRNA in the final supernatant was capured with fresh oligo(dT)25-Dynabeads (50 µg) and eluted with 5 µl of DEPC-treated water at 70°C for 2 min. The supernatant that contained unhybridized tester mRNAs was carefully collected for the following subtraction library construction process.

2.22.2. Subtracted library construction

Unhybridized mRNAs from stressed from the previous step was reverse transcribed (2.10) and the second- strand cDNAs were synthesized according to 2.11. Synthesized double-stranded cDNAs was purified and precipitated using 7.5 M sodium acetate, glycogen (20 µg/µl), and ice-cold absolute ethanol at - 20°C overnight. The precipitated cDNAs were ligated to pretreated Adaptor primers pairs (P_1; 5'-ATGCTTAGGAATTCC GATTTAGCCTCATA-3' and P_2; 5'-TATGAGGCTAAA-3') by adding 5 U of T_4 DNA ligase and incubating at room temperature for 3 h. The ligated cDNAs was amplified using P_1 and the PCR product was analyzed on a 1,2 % agarose gel. The PCR product of the ligated cDNA was cloned into the clonjet vector as it is described in 2.14. BL21 competent cells were transformed with the cloned cDNAs (2.16). Transformed cells were recovered in 2 ml of Luria - Bertani (LB) medium by incubating at 37°C with gently shaking for 1 h. 1 ml of transformed cells was inoculated in 10 ml of LB/Amp medium for overnight amplification. Glycerol was added to the amplified subtraction library to a final concentration of 20 %. The cDNA library was stored at - 80°C for future applications. Unamplified (300 µl) subtracted cDNA library was plated on LB/Amp plates with 100 µl on each and incubated at 37°C overnight. 250 colonies were randomly picked for individual clone amplification by colony PCR (2.17).

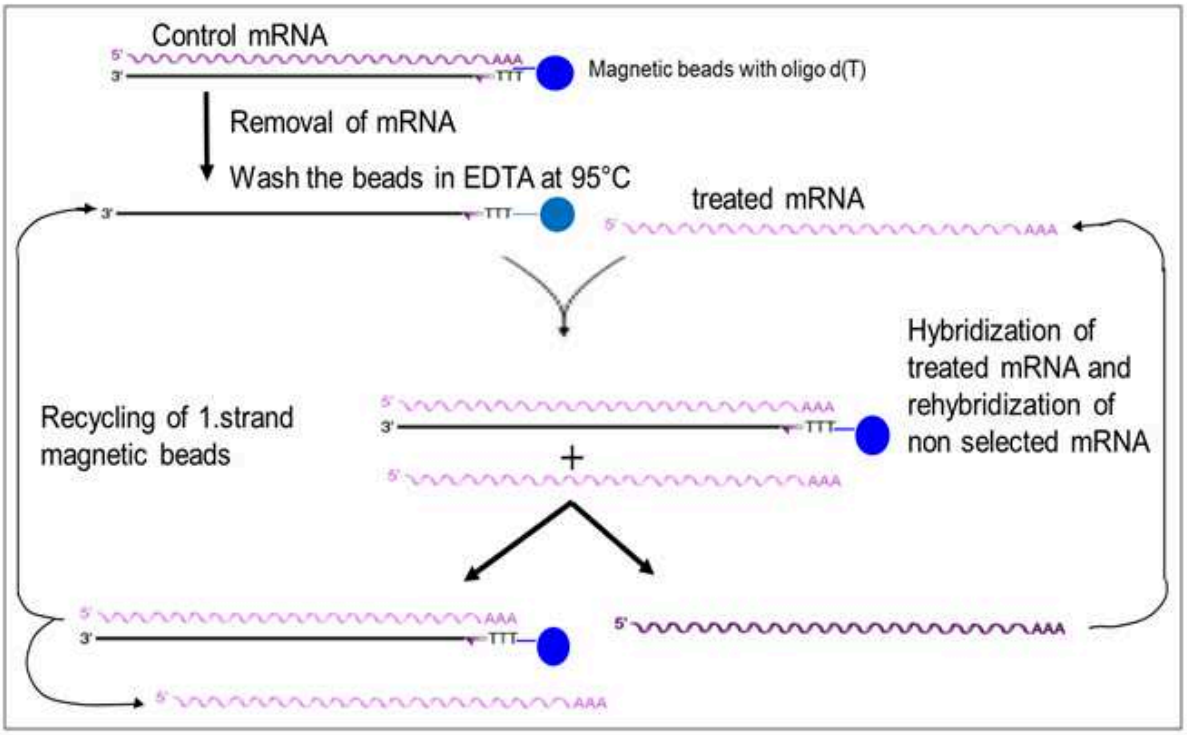

Figure 2: General scheme applied for identifying desiccation stress-specific genes

2.23. Identification of late embryogenesis abundant, superoxide dismutase and catalase genes from the substracted cDNA library

After ligation into Clonjet vector, cloned subtracted cDNA was transformed to competent bacterial cells (2.16). Therefore 1 ml of transformed cell was overnight amplified in LB/Amp media. Plasmid DNA was isolated from the amplified cDNA library (2.20.) and used as template to amplify and isolate *lea, sod* and *cat* cDNA. PCR was carried out according to 2.12. using 10 pmol of specific forward primers 5'-TCAGCHA CWGAACAAGCA-3'; 5'-CATRTBCATSARTTTGGHGA-3' and 5'-TGGACDYTWTACATY CARGTB-3' for *lea, sod* and *cat* cDNA respectively. *Lea* forward primers were designated from the conserved domain between *Adineta ricciae lea*1 A (A8DNR5 EMLB), *Adineta ricciae lea* 1B (A8DNR4, EMLB), *Adineta vaga lea* 1B (D4NWF2, EMLB) and *Adineta vaga lea* 1C (D4NWE1 EMLB). *Sod* forward primers were obtained from the conserved domain between *Schichtosoma mansoni* (FN357906.1, EMBL), *Saccharomyces cerevisiae* (AEHH01000043, NCBI), *Bos Taurus* (EF471304,NCBI) and

Drosophila nasuta (FJ554532.2, EMLB) and *cat* primer used was from the conserved domain of the rotifers *Brachionus plicalitis* (AK301577; NCBI) and *Adineta ricciae* (AB458257.1; NCBI) as well as of *Bos taurus* (AK148961.1; EMLB) and *Mus musculus* (BAE28703; EMBL). Clonjet primer 5'-AAGAACATCGATTTT CCATGGCAG-3' was used as reverse primer. In the second step the remaining *lea*, *sod* and *cat* cDNA fragments were isolated in order to obtain the full sequence. To perform it gene specific reverse primers 5'-ACCAAAATGACCTGCACGTGGAC, 5'-GTTCCAACGCCTTGTTTAG CTG-3' and 5'-GCCCTGCTCCTTGAATGCTTCGT-3' of *lea, sod* and *cat* cDNA respectively were designated from the first isolated cDNA fragment of each gene and Clonjet primer (5'-CGACTCACTATAGGGAGAGCGGC-3') was used as forward primer. PCR reaction was performed as described in 2.12 using plasmid DNA isolated from the subtracted cDNA library as template. PCR products of the first and the second step corresponding to the first and the second fragment cDNAs were resolved on 1,2 % agarose gel. An actin fragment was amplified using the control cDNA as template and the forward 5'-AAYTGGGAYGAYATGGARAA-3' and reverse actin primer 5'-GCCATYTCYTGYTCRAARTC-3' of Fretz and Spindler (1999). Specific bands were than identified and excised from the gel using a scalpel. Slices of agarose gel containing cDNA fragments of interest were extracted and eluted (2.13). Each purified cDNAs species was sent for sequencing to Seqlab (Sequence laboratory Göttingen, Germany).

Matches for the *Philo-lea*, *Philo-sod* and *Philo-cat* sequences were found using BLASTX (Altschul *et al*., 1997) against the National Center for Biotechnology Information (NCBI) non-redundant protein database. Translation to amino acid sequences was done with EMBOSS program TRANSEQ (Rice *et al.,* 2000). Multiple sequence alignments of the deduced amino acid sequences of *Philo-lea*, *Philo-sod* and *Philo-cat* genes and of other proteins were obtained by CLUSTALW (Thompson *et al*., 1994). Hydrophilicity and hydrophobicity of LEA, proteins were predicted online by ProtScale (Lv *et al*., 2011).

2.24. Analysis of *lea*, *sod* and *cat* gene expression

Total RNA from control and dehydrated rotifers was extracted using Trizol (2.7) after exposure at 88 % relative humidity for 24 h. RNA was DNAse treated to remove any genomic DNA (gDNA) contamination (2.7.1) and reverse transcribed using SuperScript First-Strand Synthesis System Kit (2.10 and 2.11). Synthesized cDNA was used as qPCR templates. Control rotifers were maintained hydrated. PCR primers for 5'-CCTTCGTTTGGATTTGGCTGGTCG-3'; rev 5'-GTCACGAACGATTTCACGTTCAGCA-3';for 5'-GTCCACGTGCAGGTCATTTTGGT-3'; rev 5'-GTTGCAGCAGCGAATGGACA-3'; for 5'-CTCGTGCAGCCGGAGGACAA-3', rev 5'-CGGGGTCAGGCTGCCAAGA-3' and for 5' ACGAAGCATTCAAGGAGCAGGGC-3', rev 5'-ACCACGCAGCGTTTC AGCTCT-3' were designated to amplify a small region (approximately 100 - 115 bp) of *Philo-actin, Philo-lea, Philo-sod* and *Philo-cat* respectively from control, and desiccated rotifers. Real-time PCR were performed using a Light Cycler H 2.0 Real-Time PCR apparatus (Roche Diagnostics, Basel, Switzerland) with SYBRH Green PCR Master Mix (Qiagen, Hilden, Germany). Amplifications were performed using a Quantitect SYBR Green RT-PCR kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, with 1 pmol (each) primers, 500 ng of sample RNA template per reaction. Thermal cycling was performed in accordance with the Quantitect kit's instructions for a total of 40 cycles at an annealing temperature of 58°C for each primer pair. The expression of each gene was tested in triplicate in each of three biologically independent experiments. To minimize mRNA quantification errors and genomic DNA contamination, *Philo-actin* cDNA was used as an internal control and the relative expression ratio was based on the expression of a target gene relative to that of actin. The generation of specific PCR products was confirmed by the melting curve analysis and gel electrophoresis. The $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) was employed for relative quantification. The results were based on the mean of triplicate experiments. The products of the qPCR

reactions were visualized on 3 % agarose gels with 10 µl of each RT-PCR product loaded per gel.

2.25. RNA interference: Cloning of the *lea, sod* and *cat* RNA*i*-constructs

The RNA was isolated from *Philodina roseola* and reverse transcribed according to 2.10., which was then used as template for PCR. Fragment cDNAs corresponding to the coding sequence of the *lea, sod* and *cat* genes were PCR amplified using the oligonucleotides primer pair for 5'-ATGATGAAGAAGACATCAACAC-3', rev 5'-ATTCGCCATATTTGATGATATTGC-3'; for 5'-ATGGTAGTGGGTGTTTGGCCTG-3', rev 5'-GCACAGGTCGGGGTCAGG-3' and for 5'-ATGTACATTCTTATTGGTGTCT TGC-3', rev 5'-CGGCTCTAATGTTCAAATTCTG-3' respectively. The PCR conditions were: 95°C for 2 min, 95°C for 45 sec, 48°C for 45 sec, 72°C for 90 sec, and a final step at 72°C for 8 min. Amplified fragments were sequenced and cloned into pcDNA vector for *Philo-lea* and *Philo-cat* and into PCR TOPO vector for *Philo-sod* and digested by HindIII\XhoI for *lea* and *cat* and by SacI\XhoI for *sod*. The digestion efficiency was controlled on an agarose gel (2.13). Fragments containing the desired insert were extracted from the gel (2.13) and recloned into RNAi L4440 vector that was previously digested using the corresponding restriction enzymes. The L4440 constructs were then transformed into the HT115(DE3) RNase III-deficient *E. coli* strain. The cells were then plated on LB agar (LB-medium + 1.5% Agar) plates containing Ampicilin (100 µg/ml) and Tetracycline (12.5 µg/ml). On the day after plating bacterial colonies containing L4440 were selected by colony PCR for those that were cloned with the gene fragments, and single colonies were picked and grown overnight at 37°C in 5 ml LB medium with 12.5 µg/ml tetracycline and 100 µg/ml ampicillin. As a control, bacterial colonies containing a L4440 vector without an insert were grown as described above. The culture was diluted 100 fold and allowed to grow to OD_{600} = 0.4. The induction of the transcription of the dsRNA was initiated by adding Isopropyl β-D-1-thiogalactopyranoside to a final concentration of

0.4 mM and the culture was incubated with shaking for 2 - 4 h at 37°C. The cells were concentrated by centrifugation and used to feed rotifers.

2.25.1. Feeding of rotifers

RNAi experiments were performed by feeding rotifers with transformed bacterial cells HT115 (DE3) containing the RNAi L4440 plasmid with either an insert of *cat*-, *lea*- or *sod*-dsRNA. Control rotifers were fed with bacteria harboring an empty L4440 vector. 2,1 l water supplemented with rotifer diet (RS Aquaristik, Schwandorf, Germany) were inoculated with 21 ml concentrated bacterial cells (OD_{600}: 2,5) and with approx. 300 rotifers per liter. To simultaneously knockdown *sod* and *cat* in rotifers 10,5 ml of each HT115 with *cat*-dsRNA and HT115 with *sod*-dsRNA were mixed and used to feed the rotifers as described previously. For an additive knock down of *lea, sod* and *cat,* 7 ml of each HT115 with *lea*-dsRNA, HT115 with *cat*-dsRNA and HT115 with *sod*- dsRNA were mixed and again used for inoculation of the rotifer culture. After 72 h of growth the rotifers were washed, harvested and either used for survival experiments, the measurement of ROS, a DNA damage assay without an additive knockdown or for RNA isolation.

2.25.2. RNAi efficiency analysis

To investigate the expression patterns of *Philo-lea, Philo-sod* and *Philo-cat* in dsRNA treated rotifers, qRT-PCR was performed (2.24) using total RNA from empty treated group and dsRNA treated rotifers. Empty treated rotifers were fed with bacteria harboring the empty L4440. Total RNA (2.7) was extracted from empty treated and dsRNA-treated rotifers and the integrity of RNA was analyzed on 1,2 % Agarose electrophorese gel. RNA was then DNAse treated (2.8) and reverse-transcribed according to section 2.10. Amplification was performed with a Quantitect SYBR Green RT-PCR kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol as

described by section 2.24. The target gene expression level in empty treated rotifers was compared with that of dsRNA-*lea*, *sod* and *cat* treated rotifers. Primers used for detection of *Philo-lea*, -*sod* and -*cat* transcripts are given in section 2.24. *Philo-actin* cDNA served as an internal standard for data normalization and quantification. The PCR's were performed in triplicate as described in section 2.12. The data were analysed using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen; 2001).

2.26. Rotifers counting after RNAi by feeding

Rotifers were dsRNA treated according to 2.25.1. To start the culture 6000 three days old rotifers were inoculated in each 2,1 l culture vessel and allowed to grow up for 72 h. Prior to counting rotifers , the culture vessels were gently swirled to distribute the animals evenly. To determine their number in the culture vessels 50 ml of the culture was introduced in a petri dish (Diameter 4 cm) and rotifers were counted under microscopic observation and the total number of rotifers present in the culture was calculated. The mean values were calculated from three independent experiments.

2.27. Survival experiments on dsRNA treated rotifers

Experimental rotifers were fed with transformed bacterial cells (2.25.1) and control group rotifers with bacteria carrying an empty L4440 vector. In a separate experiment bacteria containing inserts of *sod*/*cat*-dsRNA and *lea/sod/cat*-dsRNA were also simultaneously used for feeding. Animals were exposed to 88 % relative humidity in a sealed desiccator with 200 ml of saturated salt solution of barium chloride and for 24 h at room temperature. Rotifers were rehydrated for 24 h before the survival rates were calculated. The number of dead animals was counted by continuous absence of movements after rehydration. The survival rate of dsRNA treated rotifers was then compared to control rotifers. The mean values were calculated from five independent experiments using 50 - 60 rotifers for each experiment.

2.28. Morphological analysis during desiccation

Following dsRNA pretreatment, rotifers were dried according to section 2.5. The number of rotifers that had formed a tun was counted following 4 h, 6 h, 8 h, and 10 h of desiccation. The mean values were calculated from three independent experiments using 20 rotifers for each experiment.

2.29. Reactive oxygen species measurement

Dichlorofluorescin diacetate (DCFDA) is a common fluorescence-based probe for reactive oxygen species (ROS) detection *in vitro* and *in vivo.* DCFDA is first deacetylated by endogenous esterases to dichlorofluorescein (DCFH) which can react with several ROS to form the fluorophore DCF (Li *et al*., 2009; 2007).

2.29.1. Measurement of reactive oxygen species (ROS) in control and desiccated rotifers

A fluorescence-based assay was used for the evaluation of oxidative stress in desiccated rotifers with the test compound 2',7'-dichlorofluoresceindiacetate (DCFH-DA). Rotifers were desiccated on Millipore filters according to section 2.5 for 0 h (control), 4 h, 8 h, 14 h and 24 h. Control group rotifers were maintained hydrated during the experiment. Following exposure to desiccation each group of rotifers was homogenized with 500 µl Dimethyl sulfoxide and incubated with H_2DCF-DA (end conc.: 50 µM) for 60 min at room temperature in the dark. H_2DCF-DA is a membrane-permeable substance, which enters the cells and is intracellularly converted to H_2DCF. This non-fluorescent probe can be oxidized by ROS to yield the fluorescent dye DCF. The rise of fluorescence with time indicates the accumulation of ROS. DCF fluorescence was quantified using a fluorescence spectrophotometer (HORIBA, Darmstadt, Germany) (excitation: 485, emission 525 nm).

2.29.2. ROS level measurement in rotifers after dsRNA pretreatment

ROS accumulation was measured in empty treated,*cat-, lea-, sod-, sod/cat- and lea/sod/cat*-dsRNA treated rotifers. Control rotifers used in this case were fed with bacteria carrying an empty vector (L4440). The ROS measurement was performed as described in 2.28.1.

2.30. DNA damage analysis

Oxidative DNA damage can produce a multiplicity of modifications in DNA including base and sugar lesions, AP site (depurine/depyrimidine site), strand breaks and DNA-protein cross-links (Atamna *et al.,* 2000). AP site are one of the major types of damage generated by ROS. Aldehyde Reactive Probe (ARP; N' aminooxymethylcarbonyl-hydrazin-D-biotin) reacts specifically with an aldehyde group present on the open ring form of the AP sites. This reaction makes it possible to detect DNA modifications that result in the formation of an aldehyde group. After treatment with excess ARP reagent, all of the AP sites on DNA are tagged with a biotin residue. These biotin-tagged AP sites can be quantified using the avidin-biotin assay, followed by colorimetric detection with either peroxidase or alkaline phosphatase conjugated to the avidin (figure 3). The ARP method has been used in the present study to detect abasic sites per 1 x 10^5 base pairs (Nucleotides of DNA).

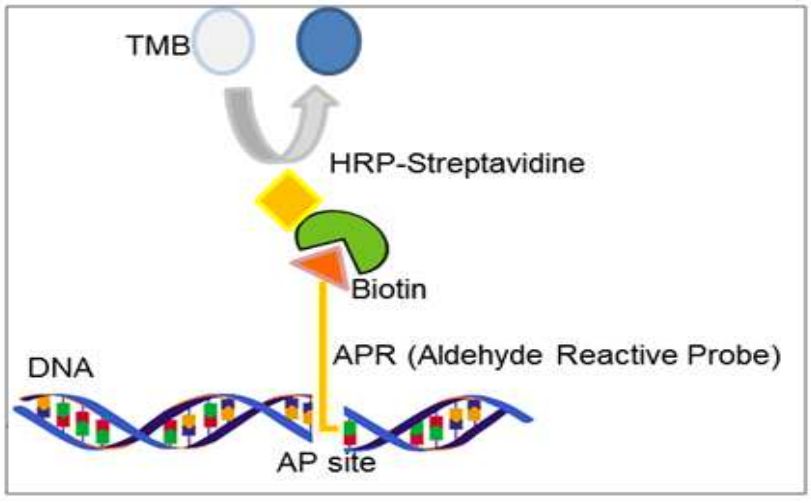

Figure 3: Schematic overview of the ARP reaction with AP

2.30.1. Assay for AP sites in control and desiccated rotifers

Rotifers were exposed to desiccation at 88 % RH for 24 h after which they were shortly hydrated to release them from the Millipore filter. Control rotifers were maintained hydrated. Genomic DNA was extracted from each control and desiccated rotifers and analyzed on a 1 % agarose gel. AP site were determined from control and treated DNA using the DNA quantification Kit (Dojindo Molecular Technologies Inc, Gaithersburg, USA) according to the manufactories instructions. Genomic DNA was incubated with 3 mM Aldehyde Reactive Probe (ARP), which is N'-aminooxymethylcarbonylhydrazino-D-biotin for 60 min at 37°C as previously described (Kubo *et al*., 1992). The DNA was then collected and washed twice with 400 µl TE buffer (10 mM Tris·Cl, pH 8.0; 1 mM EDTA) to remove residual ARP. The DNA was then isolated and immobilized on a 96-well plate with DNA binding solution. The 96-well plate was then incubated with streptavidin-conjugated horseradish peroxidase (HRP) and rinsed with a washing buffer. After adding 100 µl of substrate solution to each well and incubating the microplate at 37 °C for 1 h as the manufacturer suggested, the enzymatic activity of HRP was detected colorimetrically by measuring the absorbance at 650 nm. The number of AP sites was calculated based upon a standard curve generated using ARP standard DNA solutions (Dojindo Molecular Technologies Inc, Gaithersburg, MD) as described previously (Atamna *et al*., 2000).

2.30.2. Assay for AP sites in rotifers after dsRNA pretreatment

Genomic DNA was extracted from empty treated, dsRNA-*lea*, dsRNA-*sod* and dsRNA-*cat* treated rotifers after exposure to 88 % relative humidity and for 24 h. The integrity of DNA was analyzed by the agarose electrophoresis (1 %). Control rotifers used were fed with bacteria harboring the empty RNAi vector L4440. AP sites were colorimetrically measured in desiccated empty treated and dsRNA-treated rotifers according to section 2.29.1.

3. Results

3.1. Effect of dehydration on the survival of rotifers

Survival of the rotifers after anhydrobiosis depended on the relative humidity and duration of dehydration process (figure 4). The rate of survival of rotifers dehydrated only for 24 h was higher than that of the animals stressed for 48 h and 72 h. The rate of recovery was directly related to the RH values. The highest final survival rate (approx. 100 %) was recorded for animals exposed to 88 % RH, while the lowest (12 – 30 %) was recorded for animals exposed to 23 % RH. The recovery rate recorded at 76 % RH was higher than that recorded at 44 % and 23 % RH. The final survival of animals maintained at 76 % RH and 44 % RH for 72h was 60 %, 44 % and 64 % respectively independent of the duration of the desiccation.

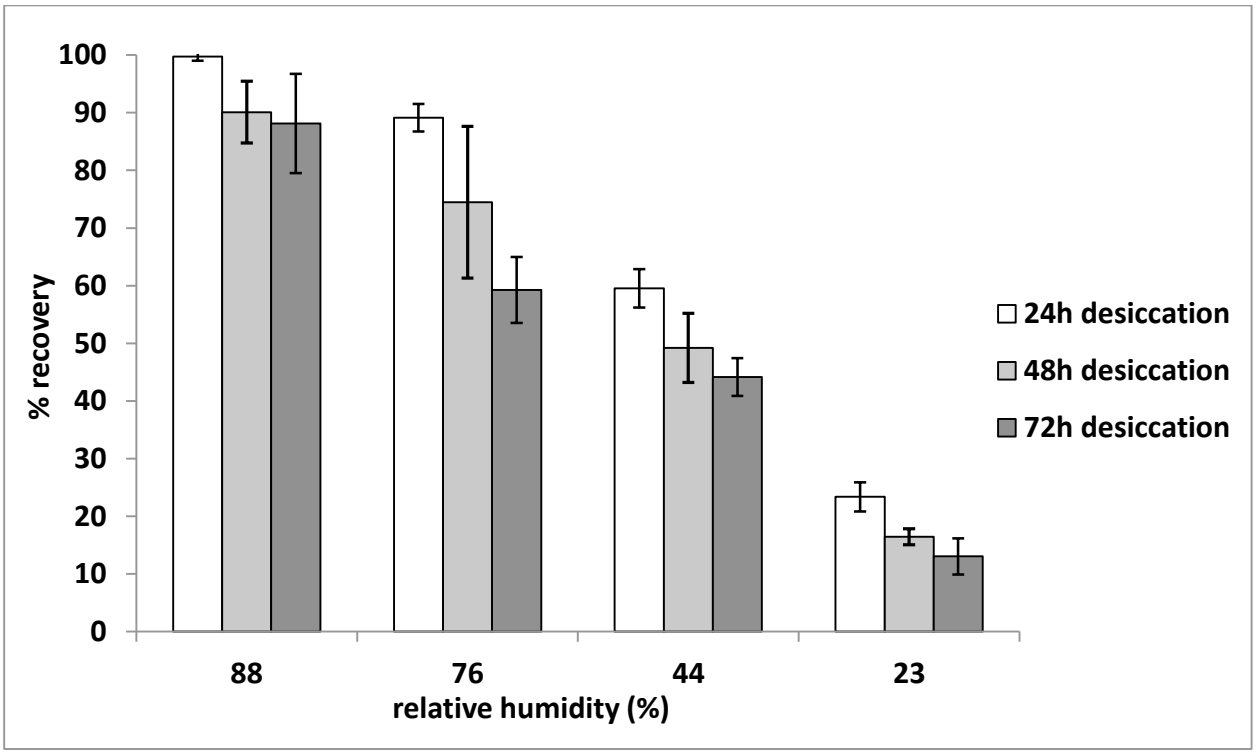

Figure 4: Survival of *Philodina roseola* after exposure to 88 %, 76 %, 44 % and 23 % relative humidity for 24 h, 48 h and 72 h and after 24 h of rehydration. The mean values were calculated from 5 independent experiments. Standard deviation bars are shown.

3.2. Generation of a subtracted cDNA library

Total RNA was prepared from control and desiccated rotifers (figure 5). The quality was analysed by agarose gel electrophoresis. The intact ribosomal RNA bands were visible. Messenger RNA was purified from the total RNA and second strand cDNA was synthesized. The subtraction procedure allowed only molecules in the test population (desiccated) that did not hybridize with molecules in the control population (non-desiccated) to be isolated as mRNA. The subtracted mRNA was synthesized as single and double strand cDNA. Subtracted dscDNA was ligated with Adaptor primer (P_1: 5'-ATGCTTAGGAATTCGATTTAGCCTCATA-3';P_2: 5'-TATGAGGCTAAA-3'). PCR reaction was performed using P_1 primer. Figure 6 shows the electrophoresis of the amplified subtracted cDNA. The PCR product of the subtracted cDNA occurred as a series of fine bands ranging from 150 bp to 1.3 kb. The variation in the banding pattern suggested that different sets of genes were present in the subtracted cDNA population. The subtracted cDNA was cloned into the clonjet plasmid to generate a subtracted cDNA library.

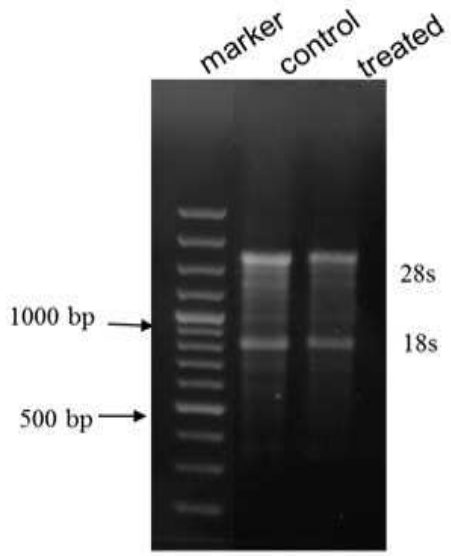

Figure 5: Electrophoretic analysis of the total RNA extraction from control and desiccated rotifers. Lane 1: marker; lane 2: control sample; lane 3: desiccated. In both samples the rRNA bands are clearly visible indicating that the RNA quality was high.

About 250 subtracted clones were yielded with lengths of about 150 bp to 1250 bp. Figure 7 shows as an example the result of the electrophoretic analysis of the subtracted cDNA clones 1 - 18 with lengths of about 200 to 1250 bp. Among the substracted clones Superoxide dismutase (11 clones), catalase (9 clones) and a single group 3 late embryogenesis abundant (Ar-LEA1A) (7 clones), were found by colony PCR experiment.

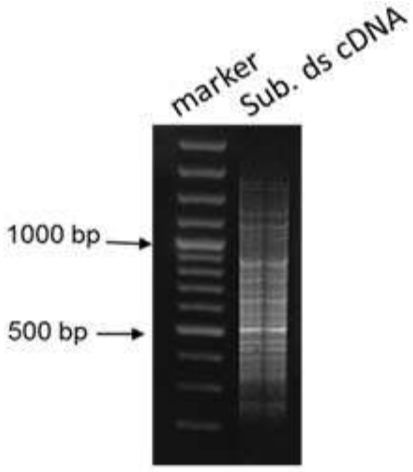

Figure 6: Electrophoretic analysis of the PCR product of the amplified subtracted cDNA. Lane 1: marker; lane 2: substracte dcDNA.

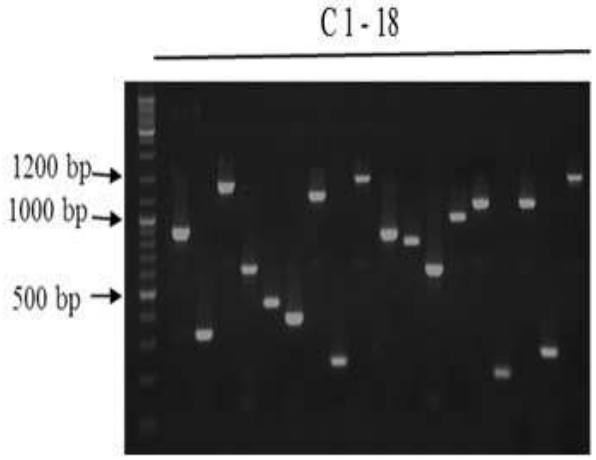

Figure 7: Electrophoretic analysis of the subtracted cDNA clones. Lane 1: marker; lane 2 to lane19: clone1 – 18.

3.3. Identification of *lea, sod* and *cat* from the subtracted cDNA library

The *lea* gene was isolated from the subtracted cDNA library using gene specific forward primer (5'-TCAGCHACWGAACAAGCA-3') made from the conserved domain between

Adineta ricciaelea 1A (A8DNR5,EMBL), *Adineta ricciaelea* 1B (A8DNR4, EMLB); *Adineta vagalea* 1B (D4NWF2, EMBL) and *Adineta vagalea* 1C (D4NWE1,EMBL) and reverse Clonjet vector primer (5'-AAGAACATCGATTTTCCATGGCAG-3'). The first *lea*-cDNA fragment gene contained 775 bp fragments. To obtain the full sequence of *lea*, a reverse primer was made from the first *lea*-cDNA fragment sequence to amplify the subtracted cDNA. Primers from the Clonjet vector were used as reverse primer. The second *lea*-cDNA fragment had a total of 455 bp. Figure 8 shows the full nucleotide sequence of *lea* with the deduced amino acid sequence, which consists of the coding sequence of 645 bp having a potential to encode a protein of 215 amino acids (23 kDa) with 221 bp and 71 bp of 5' and 3'UTR, respectively. The results of the BLAST analysis showed that the genes isolated from *Philodina roseola* share 38 % identity with *Adineta ricciae* group 3 *lea* 1A (A8DNR5 EMBL). Multiple sequence alignments with the amino acid sequence of LEA and other organisms are shown in figure 9. A high identity of LEA rotifers with other organisms was observed. GCAATATCATCAAATATGGCGAAT

Late Embryogenesis Abundant

```
                ATGAACAAATTTCTAACGATACAGATAACTCAGAACTTTAAATGTGTAT
     AATGTGTATCTTTCCCCCCCTACACACACATGTGCACAAAATGAGATGCGAAGGACAGAA
     AGAGACAAAGAGAGAGAAAAGATCCAAGGATCAAAATGCGAAGGCAAATCGTATGCAAAA
     AAAAACGTAAAGTGAAATGATCAGAAGATCAAGTCTTATCATTGCGAAGACATACCGACA
   1  M  M  K  K  T  S  T  Q  T  K  L  D  P  S  F  K  W  T  S  R
   1  ATGATGAAGAAGACATCAACACAAACGAAATTGGATCCATCTTTTAAATGGACTTCACGT
  21  Q  S  A  T  E  Q  A  V  N  A  A  A  D  L  K  D  K  V  K  D
  61  CAATCAGCAACTGAACAAGCAGTTAATGCTGCTGCTGATTTAAAAGATAAAGTTAAAGAT
  41  E  T  C  T  K  S  C  R  R  S  A  P  S  P  R  A  G  H  F  G
 121  GAAACCTGCACCAAAAGTTGTCGAAGGTCTGCACCAAGTCCACGTGCAGGTCATTTTGGT
  61  I  G  S  S  L  R  E  K  L  S  G  Y  A  E  A  A  G  E  A  Y
 181  ATAGGAAGCTCTTTAAGAGAAAAATTGTCTGGCTATGCTGAGGCAGCTGGTGAAGCGTAT
  81  E  N  I  A  P  Q  V  V  A  P  F  A  A  A  T  D  K  A  V  D
 241  GAAAACATCGCTCCGCAAGTCGTTGCTCCATTCGCTGCTGCAACAGACAAAGCTGTCGAC
 101  S  A  T  F  V  K  D  K  A  T  E  A  F  D  N  D  K  A  N  E
 301  AGTGCTACTTTCGTAAAAGATAAAGCTACCGAAGCATTCGACAATGATAAAGCTAATGAA
 121  A  L  K  A  E  A  L  K  D  K  T  V  D  G  A  A  F  V  K  R
 361  GCTTTAAAAGCTGAAGCACTCAAAGACAAGACTGTTGATGGTGCCGCATTTGTCAAACGA
 141  K  V  G  E  A  Y  E  N  I  A  P  Q  V  V  D  G  A  A  F  V
 421  AAAGTTGGTGAAGCATACGAAAATATCGCTCCTCAAGTCGTAGACGGTGCTGCGTTCGTC
 161  K  D  K  A  N  E  A  L  K  A  L  A  P  K  V  A  E  G  A  E
 481  AAAGATAAAGCTAATGAAGCCTTAAAAGCTCTTGCACCAAAAGTAGCAGAAGGTGCCGAA
 181  I  A  F  F  N  V  L  I  L  A  I  L  A  F  Y  L  G  L  K  Q
 541  ATCGCATTTTTTAATGTTTTAATTTTGGCGATCTTAGCCTTTTATCTCGGATTGAAACAA
 201  A  T  E  N  E  E  K  A  I  S  S  N  M  A  N
 601  GCGACAGAAAATGAAGAAAAAGCAATATCATCAAATATGGCGAATTAAATGAAGCATTTG
     AAGATCGAACCATATCCGAAACAAACTCGTTCTAGAAACGTCAAGCCACCGAATTA
```

Figure 8: Nucleotide sequence of the cDNA encoding late embryogenesis abundant and deduced amino acid sequence. Bold/underlined letters indicate the start codon; Bold/italic letters indicate the stop codon. 5'UTR and 3'UTR are indicated in italic letters. Specific forward and reverse primers used to amplify the coding sequence are underlined. Enclosed letters by solid lines indicate forward and reverse primers used for real time PCR to amplify a small region of the coding sequence.

```
LEA_Av_1B       MNKFLTILCLTLFISASLAKQKSATEQAADTAAELKDKVV-ETVKDAYDAAAPKVVEGAE 59
LEA_Av_1C       MNKFLSILCLVLCISATFAKQ-SATEQAVNAAADLKDKVK-DAASAAYDAASPKVAEGAE 58
LEA_Ar_1A       MNKILSILCLILFVSASLAKQKSATEQAVDAAAELKDKVV-ETVKEAYDAAAPKVVEGAE 59
LEA_Ar_1B       MNKIISILCLILFVSASLAKQKSATEQAVDAAAELKDKVV-ETVKEAYDAAAPKVVEGAE 59
LEA_Philo       MMKKTSTQT-KLDPSFKWTSRQSATEQAVNAAADLKDKVKDETCTKSCRRSAPSPRAGHF 59
                * *  :     *  * . :.: ******.::**:*****  :: . :   ::*.   *

LEA_Av_1B       YVKDKTVDGAAFVKQKVGEAYENIAPKVVE----------GAEFVKDKANEAYENLGPK 108
LEA_Av_1C       FIKDK------------------------------------------------------ 63
LEA_Ar_1A       ALKDKTVDGAAFVKQKVGEAYENIAPKVVDGAEYVKDKAVDSATFVKDKATEAFDNLAPK 119
LEA_Ar_1B       ALKDKTIDGAAFVKQKVGEAYENIAPKVVDGAEYVKDKAVDSATFVKDKATEAFDNLAPK 119
LEA_Philo       GIGSS------------------------------------------------------ 64
                 : ..

LEA_Av_1B       VVEGAEFVKDKAVDSANFVKDKANEAYENLAPKVAE----------------------- 144
LEA_Av_1C       ------------------------------------------------------------
LEA_Ar_1A       VVEGAEALKDKTVDGAAFVKQKVGEAYENIAPKVVDGAEYVKDKAVDSATFVKDKATEAF 179
LEA_Ar_1B       VVEGAEALKDKTVDGAAFVKRKVGEAYENIAPQVVD----------------------- 155
LEA_Philo       ------------------------------------------------------------


LEA_Av_1B       --------------------GAAFIKERAGEAYDNLAPKVAEGAEYVKDKAAEAYDAIK- 183
LEA_Av_1C       ------------------------------------------------------------
LEA_Ar_1A       DNLAPKVVEGAEALKDKTVDGAAFVKQKVGEAYENIAPKVAEGTEFVKDKAAEALDAIKP 239
LEA_Ar_1B       --------------------GAAFVKEKAGEAYDNLAPKVAEGAEFVKDKAAEALDAIKP 195
LEA_Philo       ------------------------------------------------------------


LEA_Av_1B       ----------PTVDEAYKTGGKLAGEYAEAAKEKLAKVAEDVKASAQHFAEDTVKAAEQY 233
LEA_Av_1C       ------------AEQAYETGSKVAGEYADVAKEKLAKVADDVKASAQNFANDASKTGQEY 111
LEA_Ar_1A       AVDGAFDTIKPKVEQAYETGSKLAGEYAEVAKDKLAKVAEDVKASAQHFADDAVKAAEQY 299
LEA_Ar_1B       AVDGAFDTIKPKVEQAYETGSKLAGEYAEVAKDKLAKVAEDVKASAQNFADDAVKAAEQY 255
LEA_Philo       ------------------LREKLSG-YAEAAGEAYENIAPQVVAPFAAATDKAVDSAT-F 104
                                  *::* **:.* :   ::* :* *.    ::.: .:.  :

LEA_Av_1B       AKDGVKQGEKLRDDAIELGKDKANEALKAAQKASADAYEASLETAAGLPKKGRKLAEQTV 293
LEA_Av_1C       AQEGLKQGQKLGEQAFEVGKDKANEALKAAQKSGADAYEAALEYGADGVKRAKQLPEQTV 171
LEA_Ar_1A       AKDGVKRGEKLRDDAIELGKEKANEALKAAQQAGADAYEASLETAAGLPKKGRKLAEQTV 359
LEA_Ar_1B       AKDSAKRGEKLRDDAIELGKEKANEALKAAQQAGADAYEASLETAAGLPKKGRKLAEQAV 315
LEA_Philo       VKDKAT-------EAFDN--DKANEALKAEALKDKTVDGAAFVKRKVG-EAYENIAPQVV 154
                .::  .       :*::   :********    .  .  *::       :  .::. *.*

LEA_Av_1B       E---LSRDKANEALKAGRHAGSDALEAAQNYVKESKKQAAKKAKETAEEASETAQKKKRQ 350
LEA_Av_1C       E---LSRDKANEALKAARHAAGDSIDSATEYVQETRKQASKKAKETTEEASEKAQKAKRN 228
LEA_Ar_1A       E---LSRDKANEALKAGRHAGADALEAAQNYVKESRKQAAKKAKETAEEASEVAQKKKRQ 416
LEA_Ar_1B       E---LSRDKANEALKAGRHAGADALEAAQNYVKESRKQAAKKAKETAEEASEVAQKKKRQ 372
LEA_Philo       DGAAFVKDKANEALKALAPKVAEGAEIAFFNVLILAILAFYLGLKQATENEEKAISSN-M 213
                :   : :*********     .:. : *   *      *   . : : * .* * . :

LEA_Av_1B       ATEL 354
LEA_Av_1C       ADL- 231
LEA_Ar_1A       ATEL 420
LEA_Ar_1B       ATEL 376
LEA_Philo       AN-- 215
                *
```

Figure 9: Alignment of the deduced amino acid sequence of *Philo*-LEA with *Adineta ricciae* LEA 1A (A8DNR5 EMLB), *Adineta ricciae* LEA 1B (A8DNR4; EMLB), *Adineta vaga* LEA 1B (D4NWF2, EMLB) and *Adineta vaga* LEA 1C (D4NWE1 EMLB) using Clustal. Asterisk refers to identical amino acids, a double dot refers to a conserved substitution and a single dot refers to a semi-conserved substitution. Enclosed letters by solid lines indicate the conserved domain from which specific primer were made.

The *sod* gene was isolated from the subtracted cDNA library using a gene specific forward primer (5'- CATRTBCATSARTTTGGHGA -3') made from the conserved domain between *Schistosoma mansoni* (FN357906.1, EMBL), *Saccharomyces cerevisiae* (AEHH01000043, NCBI) and *Bos taurus* (EF471304, NCBI). The first *Philo-sod* cDNA fragment gene contained 500 bp fragments. To obtain the complete sequence of *sod*, a reverse primer was designed from the first *sod* fragment sequence to amplify the subtracted cDNA. The primer from the Clonjet vector was used as reverse primer. The second *sod*-cDNA fragment had a total length of 490 bp. Figure 10 shows the full nucleotide sequence of *sod* with the deduced amino acid sequence, which consist of the coding sequence of 388 bp having a potential to encode a protein of 129 amino acids (14 kDa) with 119 bp and 82 bp of 5' and 3'UTR respectively. The expasy bioinformatics tool was used to obtain the deduced amino acid sequence of the isolated gene.

Superoxide Dismutase

```
      TCTAAGAAGAAGAAGTAGAAGAAACTAATTCACCTGAACCACTTATGCTTGCCAAGACG
     TTTTCTGTACAAGTTCCTCAAGTTGCAGGCTATCATCGTCGAGCACAAGAACCCTCTTGG
   1  M  V  V  G  V  W  P  V  L  I  D  S  T  G  I  I  S  E  R  T
   1  ATGGTAGTGGGTGTTTGGCCTGTTTTGATCGACTCTACTGGAATTATCTCTGAGAGAACC
  21  T  K  A  G  S  S  R  K  P  V  Y  G  G  I  E  C  E  L  E  G
  61  ACTAAAGCAGGTTCATCTAGGAAACCAGTCTATGGTGGGATTGAATGTGAGCTTGAAGGT
  41  P  H  I  H  E  F  G  D  P  V  G  L  G  E  N  S  Q  P  G  F
 121  CCACATATTCATGAGTTTGGTGATCCTGTAGGACTTGGAGAGAATAGTCAACCCGGTTTC
  61  P  G  K  T  Q  P  S  D  D  L  K  K  A  I  E  S  Q  W  K  S
 181  CCCGGTAAAACCCAGCCCAGCGATGATCTGAAGAAGGCCATCGAGTCGCAGTGGAAGAGC
  81  I  V  V  V  T  G  D  T  R  A  A  G  G  Q  Q  L  N  K  A  L
 241  ATTGTCGTTGTCACAGGAGATACTCGTGCAGCCGGAGGACAACAGCTAAACAAGGCGTTG
 101  E  L  P  F  T  C  L  K  L  Q  Q  T  T  E  L  E  S  A  D  I
 301  GAACTGCCTTTCACCTGTTTGAAGCTGCAGCAGACGACAGAACTCGAATCGGCTGATATT
 121  S  W  Q  P  D  P  D  L  C
 361  TCTTGGCAGCCTGACCCCGACCTGTGCTAGGCCTGAACTCAAAAGGAGTCAAGAATTCGCT
     GGGTCATCCATTTTTCAGAGGATACTTTTTGCTAATTAATTATTCTTG
```

Figure 10: Nucleotide sequence of the cDNA encoding superoxide dismutase and deduced amino acid sequence. Bold/underlined letters indicate the start codon; Bold/italic letters indicate the stop codon. 5'UTR and 3'UTR are indicated in italic letters. Specific forward and reverse primers used to amplify the coding sequence are underlined. Enclosed letters by solid lines indicate forward and reverse primers used for real time PCR to amplify a small region of the coding sequence.

```
SOD_S.man          --MKAVCVMTGTAGVKGVVKFTQETDNGPVHVHAEFSG-LKAGKHGFHVHEFGDTTNGCT 57
SOD_Dros_na        MVAKAVCVINGDA--KGTVFFEQESSGTPVKVTGEVTG-LAQGLHGFHVHEFGDNTNGCM 57
SOD_S.cer          -MVQAVAVLKGDAGVSGVVKFEQASESEPTTVSYEIAGNSPNAERGFHIHEFGDATNGCV 59
SOD_B.Taurus       ------XAXKGXGPVQGTIHFEAKGDT--VVVTGSITG-LTEGDHGFHVHQFGDNTQGCT 51
SOD_phil           ------MVVGVWPVLIDSTGIISERTTKAGSSRKPVYGGIECELEGPHIHEFGDPVGLGE 54
                           .       .   :             . *      .* *:*:*** .

SOD_S.man          SAGAHFNPTKQEHGAPEDSIRHVGDLGNVVAGADGNAVYNATDKLISLNGSHSIIGRTMV 117
SOD_Dros_na        SSGPHFNPHKKEHGAPTDGERHLGDLGNITASGDGPTAVDITDSQITLFGENSIIGRTVV 117
SOD_S.cer          SAGPHFNPFKKTHGAPTDEVRHVGDMGNVKTDENGVAKGSFKDSLIKLIGPTSVVGRSVV 119
SOD_B.Taurus       SAGPHFNPLSKKHGGPKDEERHVGDLGNVTADKNGVAXVDIVDPLISLSGE--------- 102
SOD_phil           NSQPGFPGKTQPSDDLKKAIESQWKSIVVVTGDTRAAGGQQLNKALELPFTCLKLQQTTE 114
                   .: . *   .:  .   .  .   .   : :.    :  .  :  : *

SOD_S.man          IHENEDDLGRGGHELSKVTGNAGGRLACGVVGLAAE 153
SOD_Dros_na        VHADADDLGKGGHELSKTTGNAGARIGCGVIGIAKI 153
SOD_S.cer          IHAGQDDLGKGDTEESLKTGNAGPRPACGVIGLTN- 154
SOD_B.Taurus       ------------------------------------
SOD_phil           LES-ADISWQPDPDLC-------------------- 129
```

Figure 11: Alignment of the deduced amino acid sequence of the rotifers *Philo*-SOD with SOD from *Schistosoma mansoni* (FN357906.1, EMBL), *Saccharomyces cerevisiae* (AEHH01000043,NCBI*); Bos taurus* (EF471304,NCBI) and *Drosophila nasuta* (FJ554532.2, EMLB) using ClustalW. Asterisk refers to identical amino acids, a double dot refers to a conserved substitution and a single dot refers to a semi-conserved substitution. Enclosed letters by solid lines indicate the conserved domain from which specific primer was made.

The results of the use of ClustalW tool for multiple sequence alignment with the amino acid sequence of SOD with *Schichtosoma mansoni* (FN357906.1, EMBL), *Saccharomyces cerevisiae* (AEHH01000043,NCBI), *Bos tau*rus (EF471304,NCBI) and *Drosophila nasuta* (FJ554532.2, EMLB) is presented in figure 11. The deduced amino acid sequence of the *sod* gene was compared for identity on BLAST. *Philo-sod* showed 30 - 45% identity with other organisms.

Catalase

Figure 12: Nucleotide sequence of the cDNA encoding catalase and deduced amino acid sequence. Bold/underlined letters indicate the start codon; Bold/italic letters indicate the stop codon. 5'UTR and 3'UTR are indicated in italic letters. Specific forward and reverse primers used to amplify the coding sequence are underlined. Enclosed letters by solid lines indicate forward and reverse primers used for real time PCR to amplify a small region of the coding sequence

To isolate the *cat* gene from the subtracted cDNA library gene, specific forward primer (5'-TGGACDYTWTACATYCARGTB-3') were designed from the conserved domain between *Brachionus plicatilis (AK301577, NCBI), Bos taurus (AK148961.1, EMLB), Adineta ricciae (AB458257.1, NCBI)* and *Mus musculus (BAE28703, EMBL)* and reverse Clonjet vector primer (5'-AAGAACATCGATTTTCCATGGCAG-3') were used. The first *cat*-cDNA fragment gene consisted of a 942 bp fragment. To obtain the complete sequence of the *cat* gene, a reverse primer was made from the first *cat*-cDNA

fragment sequence to amplify the subtracted cDNA. A primer from the Clonjet vector was used as reverse primer. The second *cat*-cDNA fragment contained a total of 649 bp. Figure 12 presents the full nucleotide sequence of catalase with the deduced amino acid, which consist of the coding sequence of 586 bp having a potential to encode a protein of 195 amino acids. The 5'UTR and the 3'UTR contain 370 bp and 265 bp, respectively

```
Cat_Bos_tau      --------------------------------MNGYGSHTFKLVNANGEAVYCKFHYKTDQ 29
Cat_Mus          MVWDFWSLRPESLHQVSFLFSDRGIPDGHRHMNGYGSHTFKLVNADGEAVYCKFHYKTDQ 60
Cat_Brapli       --------------------------------MNGYGSHTFKLVNAQGNPVYCKFHFKTDQ 29
Cat_Ar           ------------------------------------------------------------
Cat_phil         ------------------------------------------------------------

Cat_Bos_tau      GIKNLSVEDAARLAHEDPDYGLRDLFNAIATG-NYPSWTLYIQVMTFSEAEIFPFNPFDL 88
Cat_Mus          GIKNLPVGEAGRLAQEDPDYGLRDLFNAIANG-NYPSWTFYIQVMTFKEAETFPFNPFDL 119
Cat_Brapli       GIKNLSAQRASELSGENPDYSTQDLYNAIGCG-NFPSWTLYIQVMSFEEAEKCKFNPFDL 88
Cat_Ar           ------------------------MYNAIAKK-DYPSWTLYIQVVTHEQAQKLPFNPFDL 35
Cat_phil         ------------------------MYILIGVLPVYPSWTLYIQVVNHKQAQKLPFNPFVF 36
                                         ::  *.    :****:****:...:*:   **** :

Cat_Bos_tau      TKVWPHGDYPLIPVGKLVLNRNPVNYFAEVEQLAFDPSNMPPGIEPSPD-KMLQGRLFAY 147
Cat_Mus          TKVWPHKDYPLIPVGKLVLNKNPVNYFAEVEQMAFDPSNMPPGIEPSPD-KMLQGRLFAY 178
Cat_Brapli       TKIWPHGEYPLIPVGRLVLNRNPTNYFAEVEQIAFAPSHLIPGIEPSPD-KMLQGRLFSY 147
Cat_Ar           TKVFSQKDFPLRRVGKMTLNENVENYFAQIEQAGFTPAHMPPGIEASPD-KMLQGRLFSY 94
Cat_phil         LKVFSQKDKPPRRVGKMTLNKGVENYFAQIKLAGFTPAHMPPGIEASPDNEAFKEQGFKF 96
                  *::.: : *   **::.**..  ****:::  .* *::: ****.*** : :: : * :

Cat_Bos_tau      PDTHR---HRLGPNYLQIPVNCP---YRARVANYQRDGPMCMMDNQGGAPNYYPNSFSAP 201
Cat_Mus          PDTHR---HRLGPNYLQIPVNCP---YRARVANYQRDGPMCMHDNQGGAPNYYPNSFSAP 232
Cat_Brapli       ADTHR---HRLGANYLQIPVNCP---FKVR--NYQRDGPQCINDNQDGAPNYYPNSFSGP 199
Cat_Ar           ADTHL---HRLGANYLQIPVNDPETNKHVKVCTYQRDGPMQVGHNFGGCPNYYRNTFRGP 151
Cat_phil         EKMKVKVCTEANDDDMQVGHNFG----GPELKRCVVSEPMQVGHNFGGCPNYYRNTFRES 152
                  . :     . . : :*:  *        .      . *  : .* .*.**** *:*  .

Cat_Bos_tau      EHQ--PSALEHRTHFSGDVQRFNSANDDNVTQVRTFYLKVLNEEQRKRLCENIAGHLKDA 259
Cat_Mus          EQQ--RSALEHSVQCAVDVKRFNSANEDNVTQVRTFYTKVLNEEERKRLCENIAGHLKDA 290
Cat_Brapli       EHN--SVHIESICPVSGDIRRYDSGNEDNFSQVGLFWEKVLNDQEKQRLIENISGHLINA 257
Cat_Ar           EVTDRSKHLEHATFESGMAARHEANDDDNFSQPRVFYQKVLDDRGRAHLIQNIVEHLQQC 211
Cat_phil         EVTDRSKHLEHATFESGMAARHYQRD-------GPFLKETLQN-------LNIRADG--- 195
                 *       :*     :    *.   :          *  :.*::        **  .

Cat_Bos_tau      QLFIQKKAVKNFSDVHPEYGSRIQALLDKYNEEKPKNAVHTYVQHGSHLSAREKANL 316
Cat_Mus          QLFIQKKAVKNFTDVHPDYGARIQALLDKYNAEKPKNAIHTYTQAGSHMAAKGKANL 347
Cat_Brapli       AEFIQERAVTNFGKCHPDYGRRLKEALDLLKNKE--------------ASAIKKSNL 300
Cat_Ar           ------------------------------------------------------------
Cat_phil         ------------------------------------------------------------
```

Figure 13: Alignment of the deduced amino acid sequence of the rotifers *Philo*-catalase with catalase from *Brachionus plicatilis* (AK301577; NCBI), *Bos taurus* (AK148961.1; EMLB); *Adineta ricciae* (AB458257.1; NCBI), and *Mus musculus* (BAE28703; EMBL) using ClustalW. Asterisk refers to identical amino acids, a double dot refers to a conserved substitution and a single dot refers to a semi-conserved substitution. Letters enclosed by a solid line indicate the conserved domain from which specific primer was made.

The deduced amino acid sequence of this gene codes for a protein of 22.3 kDa and was searched for homology on BLASTP. Catalase shared 67 % identity with *Bos taurus* (AK148961.1,EMBL). Alignment of the nucleotide sequence of superoxide dismutase and the deduced amino acid sequence is shown in figure 13. The electrophoresis of the amplified *lea, sod* and *cat* cDNA is presented in figure 14.

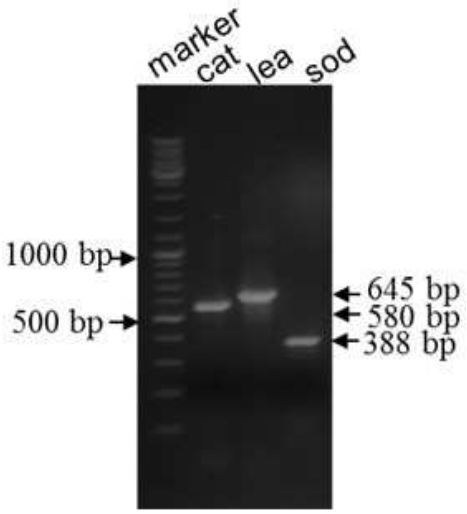

Figure 14: Electrophoretic analysis of the amplified PCR product of the coding sequence of *cat, lea* and *sod* genes using the subtracted cDNA library as template.

ProtScale analysis showed that the number of hydrophilic amino acid residues in *Philo*-LEA is higher than hydrophobic amino acid residues in one peptide chain suggesting that the *Philo*-LEA protein identified in this study is hydrophilic.

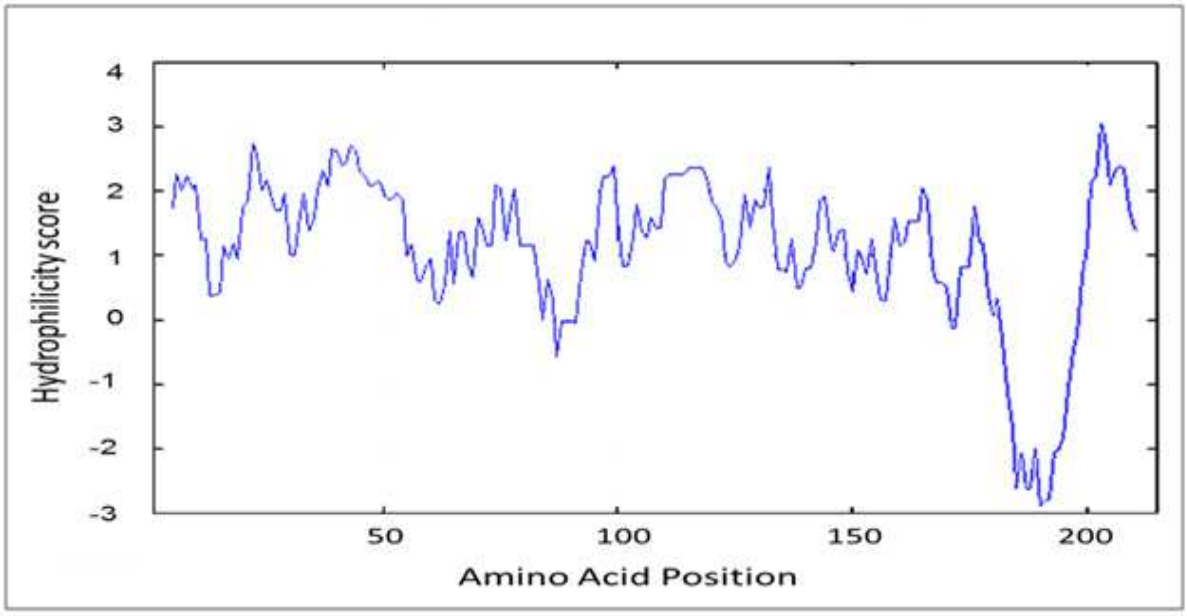

Figure 15: Hydrophilicity/hydrophobicity analysis of protein *Philo* LEA Protein. Those values below the zero line are negative and are therefore hydrophobic.

Comparing rotifer LEA proteins with the LEA sequences from other organisms suggests that *Philodina roseola* LEA proteins probably fit into group 3 LEA proteins. To designate the deduced amino acid sequences for *Philo*-LEA to the LEA major protein groups, the following search for motifs characteristic of LEA domains was performed using BLASTP for the 11-mer domain TAQAAKEKAGE characteristic for group 3 LEA. *Philo*-LEA proteins show 6 tandem repeats with identities ranging from 40% to 50% (figure 16).

```
MOTIF                                  TEQAAKEKAGE
Philo LEA       MMKKTSTQTKLDPSFKWTSRQSATEQAVNAAADLKDKVKDETCTKSCRRSAPSPRAGHFG
60
                                       ****.:  *.

MOTIF                    TEQAAKEKAGE            TEQAAKEKAGE TEQAAKEKAGE
119
Philo LEA       IGSSLREKLSGYAEAAGEAYENIAPQVVAPFAAATDKAVD SATFVKDKATEAFDNDKANE
120
                       . ..  * ***            .  **.:** ::    .*:** *

MOTIF                          TEQAAKEKAGE            TEQAAKEKAGE
179
Philo LEA       ALKAEALKDKTVDGAAFVKRKVGEAYENIAPQVVDGAAFVKDKANEALKALAPKVAEGAE
180
                               .*.*.**                .*:**.*: :*   *..*  :

LEA             IAFFNVLILAILAFYLGLKQATENEEKAISSNMAN------ 215
```

Figure 16: Amino acids sequence of *Philo*-LEA and comparison of the 11-mer motif with the matched repeats found in *Philo*-LEA using BLASTP Programm. The 11-mer LEA motifs are in bold letters and the matched repeats are underlined.

As is shown in table 1 the motif repeat search revealed the presence of three repeat motifs among the bdelloid rotifers *Philodina roseola* LEA, *Adineta ricciae*(Ar-LEA 1A; Ar-LEA 1B; Ar-LEA1C) and *Adineta vaga* (Av-LEA 1B; Av-LEA 1C) with a lenght of eight to nine amino acids and of the form KANEALKA, GEAYENIAP and VDGAAFVK. Each motif occurs 2 to 3 times in the amino acid sequence of LEA

Table 1: Repeat motifs and amino acid positions found in group 3 *Philodina roseola* LEA, *Adineta ricciae* LEA1A, LEA 1B; *Adineta vaga* LEA1B' and LEA 1C.The repeat motifs were searched by using the programs MEME and RADAR.

LEA protein	*Philo*-LEA	*Ar*-LEA 1A	*Ar*-LEA 1B	*Av*-LEA 1B	*Av*-LEA 1C
GEAYENIAP	77-85, 143-152	77-85; 143-152, 209-217	77-85; 143-152	77-85	--
VDGAAFVK	132-139; 154-161	66-73; 132-139; 198-205	132-139; 154-161	66-73	--
KANEALKA	117-124; 163-170	320-327; 365-372	266-273; 321-328	254-261; 298-306	133-140; 177-185

3.4. Confirmation of the induction of *lea, sod* and *cat* genes after desiccation

Lea-, *sod*- and *cat*-cDNA were found to be present in the subtracted cDNA library, indicating that these genes were differentially expressed in the rotifers after dehydration. In order to confirm the induction of *lea, sod* and *cat* genes by dehydration, RT-PCR was used to determine the expression pattern of the *Philo-lea*, -*sod* and -*cat* genes. Amplifications were performed using SYBR Green PCR mix (TaKaRa, Ohtsu, Japan) and gene specific primers. Primers used for the qPCR are illustrated in figure 8, 10 and 12 and were specific for *Philodina roseola* and were made from the non-conserved domain with other organisms. The expression ratio (R) was calculated to compare the relativeexpression between treatments.$R = 2^{-(\Delta CT\ treated\ -\ \Delta CT\ control)}$; ΔCT treated and ΔCT

control are the differences in thresholdcycles for target and reference. *Philo-actin* was used as an internal control group. The expression of *Philo-lea*, *Philo-sod* and *Philo-cat* genes was compared with that of control rotifers by real-time PCR. The DNA agarose gel in figure 17 shows the relative gene expression of each control and treated-form of *Philo-lea*, *Philo-sod* and *Philo-ca*t in relation to *Philo-actin*. A significant increase in the relative expression of *Philo-lea*, *Philo-sod* and *Philo-cat* was observed in dehydrated rotifers. *Lea* had a 40 fold up-regulation in the treated rotifers whereas *sod* gene had a 7 fold and *cat* a 4 fold rise in their expression (figure 18).

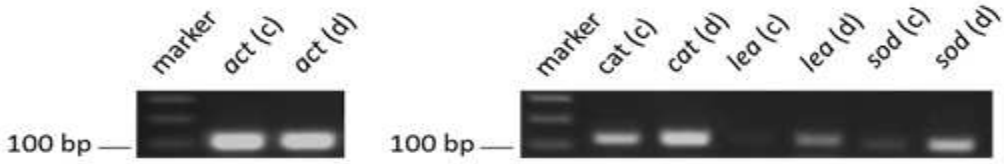

Figure17: Electrophoretic analysis of the amplified RT-PCR product of the *Philo-lea, Philo-sod* and *Philo-cat* genes in control and desiccated rotifers. (A) Transcript levels for the control (lane 2) and treated (lane 3) *Philo-actin* genes, lane 1: marker.(B) lane 1: marker: Transcript levels for the control (lane 2) and treated (lane 3) *Philo-cat* genes in desiccated specimens; lane 4 and lane 5 represent transcript levels for the control and treated *Philo-lea* genes in desiccated; lane 4 and lane 5 represent transcript levels for the control and treated *Philo-sod* genes in desiccated rotifers

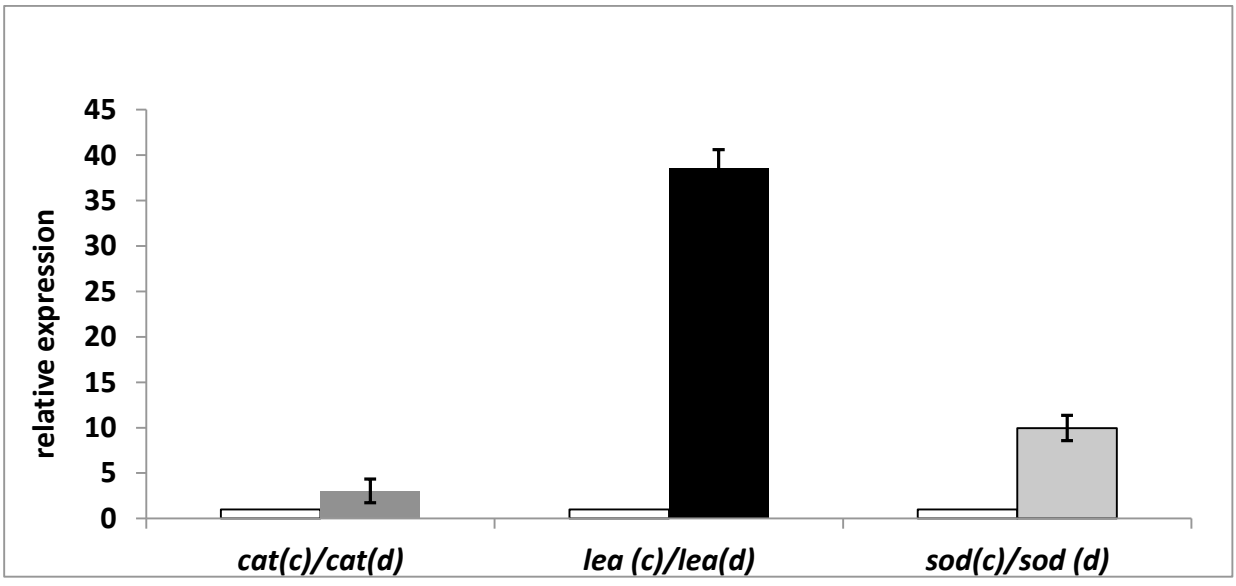

Figure 18: Expression levels of *Philo-lea, Philo-sod* and *Philo-cat* genes in non-desiccated (control) and desiccated rotifers. The expression of each gene was tested in triplicate in each of four independent experiments. Standard deviation bars are shown.

3.5. Construction of plasmids for RNA Interference and *in vitro* transcription of dsRNA

3.5.1. Restriction mapping of the L4440 vector

The plasmid used to express dsRNA for the RNAi experiment was subjected to restriction mapping. The electrophoretic analysis of the restricted L4440 plasmid with different restriction enzymes is shown in figure 19 A. The first well was loaded with a marker as a reference. The second well was loaded with L4440 vector, cut with SacI and XhoI which showed a pair of bands at 2700 bp and a further band at 130 bp. The third well was loaded with L4440 plasmid digested with HindIII and XhoI. This lane shows a relatively large band of 2800 bp and the second band that is expected to be 10 bp was not visible on the agarose gel.

3.5.2. Restriction mapping of the vector PCR 2.1 topo and pcDNA containing *Philo*-cDNA insert

The cDNA corresponding to the coding region of *Philo-lea, Philo-sod* and *Philo-cat* genes was amplified. The PCR product was then cloned into PCR 2.1 Topo vector (cDNA-*sod*) and pcDNA vector (cDNA-*lea* and cDNA-*cat*). In order to subclone each cDNA insert into a L4440 vector, the cDNA-*lea* and cDNA-*cat* were excised from the plasmid vector pcDNA using HindIII/XhoI and the cDNA-*sod* was excised from the plasmid vector PCR 2.1vector using SacI/XhoI. Each cut insert was gel-purified and subcloned into the corresponding sites of plasmid L4440. It was necessary to first control that the restriction site corresponding to the restriction enzyme was not present in the gene of interest. As is shown in figure 19 B the restriction of *lea* and *cat* plasmid resulted in a pair of 2 bands of approximatively 4700 bp and 690 bp and of 4800 bp and 700 bp respectively. The restriction of the *sod* plasmid resulted to a pair of two bands of

approximatively 3300 bp and 480 bp. In order to control that the insert of cDNA-*lea*, cDNA-*sod* and cDNA-*cat* were successfully subcloned into the L4440 vector, RNAi L4440 containing cDNA-*lea*, cDNA-*sod* and cDNA-*cat* insert were amplified using both forward and reverse T7 primer. Empty L4440 vector without insert served as control. Figure 20 shows the electrophoretic pattern of each amplified PCR product. The amplification of the empty vector resulted in the approximatively 240 bp band. Agarose electrophoresis of the amplified L4440(cDNA-*lea*), L4440(cDNA-*sod*) and L4440(cDNA-*cat*) plasmid revealed bands of about 960 bp, 900 bp and 580 bp respectively which correspond to the insert lengthsof the cDNA-*lea*, cDNA-*sod* and cDNA-*cat* after subtracting the sequence length between T7 forward and reverse primer. This shows that the subcloning into the RNAi vector was successfull.

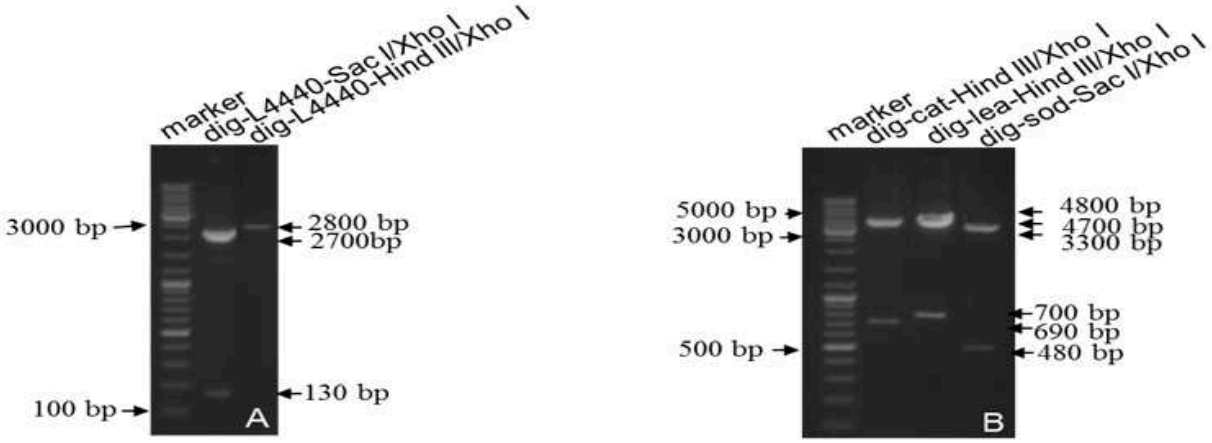

Figure 19: Electrophoretic analysis of the digested L4440, P 2.1 Topo and pcDNA vector (A) Restriction gel of L4440. From left to right, the first lane contains the marker; the second and the third contain the RNAi L4440 plasmid cut with SacI/XhoI and HindIII/XhoI respectively. (B) Lane 2 and 3 show digestion of pcDNA/*cat*-vector and pcDNA/*lea*-vector each with HindIII/XhoI and the third lane shows the PCR 2.1/SOD-plasmid cut with SacI/XhoI

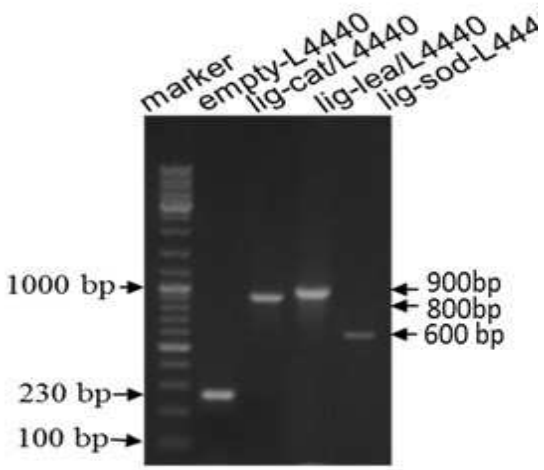

Figure 20: Electrophoretic analysis of the amplified PCR product of empty L4440 (lane 2), L4440 (cDNA-*cat*) plasmid (lane 3), L4440 (cDNA-*lea*) plasmid. (Lane 4) and L4440 (cDNA-*sod*) plasmid (Lane 5) using T7 forward and reverse L4440 vector primer.Lane 1 represents the marker

3.6. Percentages of bacterial cells after RNAi by feeding

The number of bacterial cells before and after rotifer feeding was determined with a Neubauer counting chamber. Figure 23 shows the percentages (raw data needed) of RNAi bacterial cells remaining after 3 days of feeding. Approximately 18 %, 19 %, 30 % and 22 % of the empty treated, *cat-, lea- and sod*-dsRNA bacterial cells respectively remained in culture whereas 25 % and 33 % of the *sod/cat*- and *lea/sod/cat*-dsRNA treated rotifers were still present in the culture.

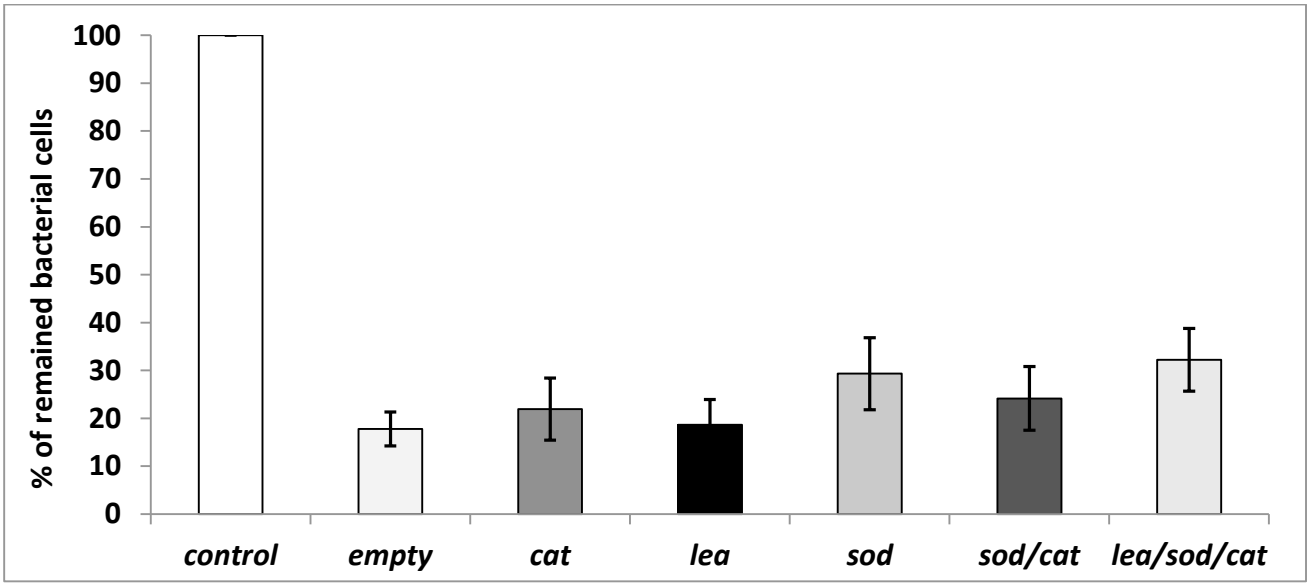

Figure 23: Percentages of RNAi bacteria H115 remaining in the culture of rotifers after feeding. The mean values were calculated from 5 independent experiments. Standard deviation bars are shown.

3.7. RNAi efficiency analysis

Following 24 h of rotifers dehydration at 88 % relative humidity, the expression of *Philo-lea*, *Philo-sod* and *Philo-cat* genes was compared in control and desiccated dsRNA-treated rotifers by real-time PCR. The DNA agarose gel in figure 21 shows the relative gene expression of each control and dsRNA-treated form of *lea, sod* and *cat* in relation to *actin*. Exposure of rotifers to homologous dsRNA by feeding resulted in a reduction of mRNA transcript levels for the target genes. Figure 22 shows the quantification of the expression level of *Philo-lea*, *Philo-sod* and *Philo-cat* genes in control, *cat*-, *lea*- and

sod-dsRNA treated rotifers after desiccation. A reduction in mRNA transcript levels occurred for the target genes in the dsRNA-treated rotifers. For the *Philo-lea* and *Philo-sod* genes, the reduction in transcript level was 80 % and 85 % respectively compared to the controls, while for the *Philo-cat*, the mRNA transcript level was reduced by 70 % compared to the control.

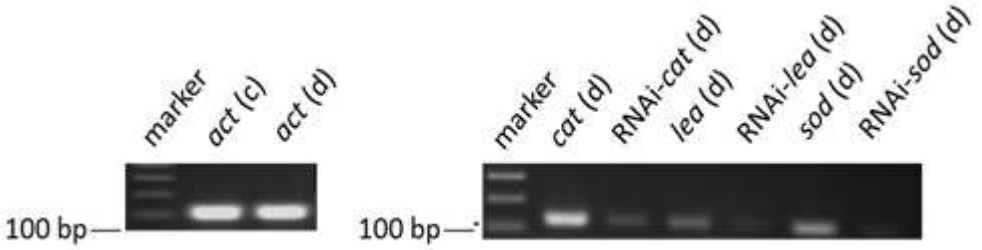

Figure 21: Electrophoretic analysis of the amplified RT-PCR product of the *Philo-lea, Philo-sod* and *Philo-cat* Knockdown efficiency assay. Picture (A) and (B) show images of the qPCR assay loaded on an agarose gel. (A) Lane 2 and 3: Transcript levels for the control and treated *Philo-actin* genes, lane1: marker. (B) Lane 1: marker; lane 2 and 3: transcript levels for the control and treated *Philo-cat* genes in rotifers fed on *E. coli* expressing *cat*-dsRNA; lane 4 and lane 5 represent transcript levels for the control and treated *Philo-lea* genes in rotifers fed on *E. coli* expressing *lea*-dsRNA; lane 6 and lane 7 represent transcript levels for the control and treated *Philo-sod* genes in rotifers fed on *E. coli* expressing *sod*-dsRNA.

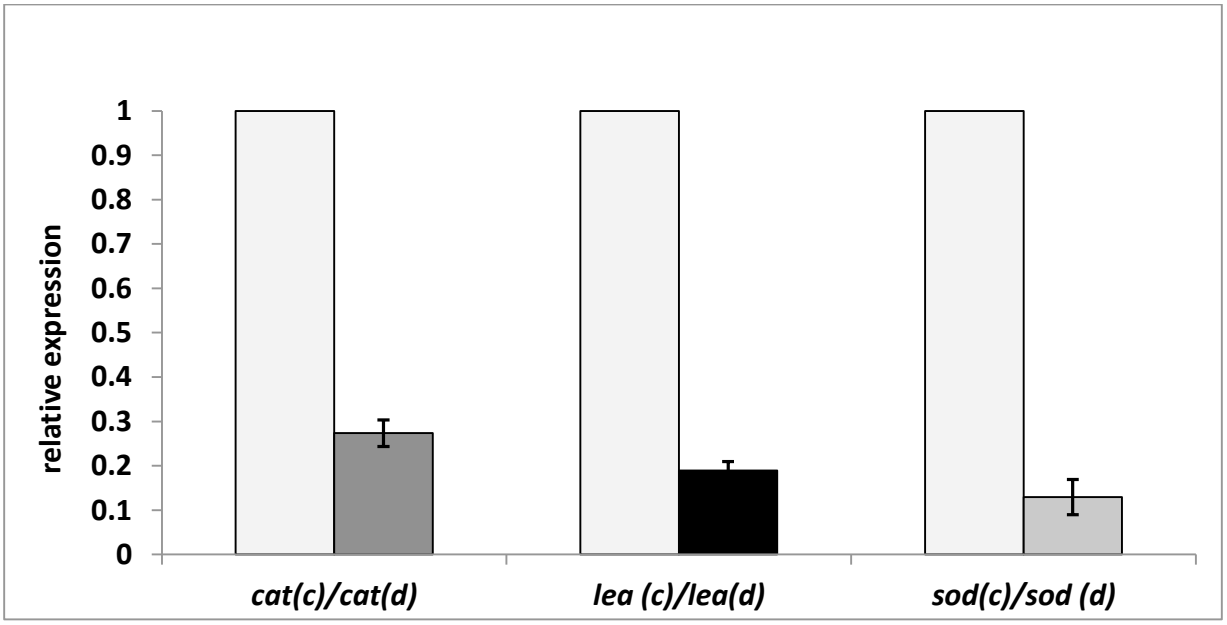

Figure 22: Expression levels of *Philo-lea, Philo-sod* and *Philo-cat* genes in control and dsRNA-treated rotifers following exposure to desiccation. The expression of each gene was tested in triplicate in each of five biologically independent experiments. Standard deviation bars are shown.

3.8. Effect of dsRNA treatment on the population dynamic of rotifers

Treatment of rotifers either with *cat-, lea-, sod-, sod/cat-* or *lea/sod/cat*-dsRNA differently influenced the increase of rotifers population. The Population of rotifers that were fed with bacterial cells carrying the empty vector (control) increased from 6000 to 48500 rotifers after 72 h. Comparing to control, treatment of rotifers with *cat, lea* and *sod*-dsRNA reduced the population of 60 %, 76 % and 59 % respectively. After *sod/cat*- and *lea/sod/cat*-dsRNA treatment, the population was dramatically decreased of 41 % and 35 % respectively (figure 23).

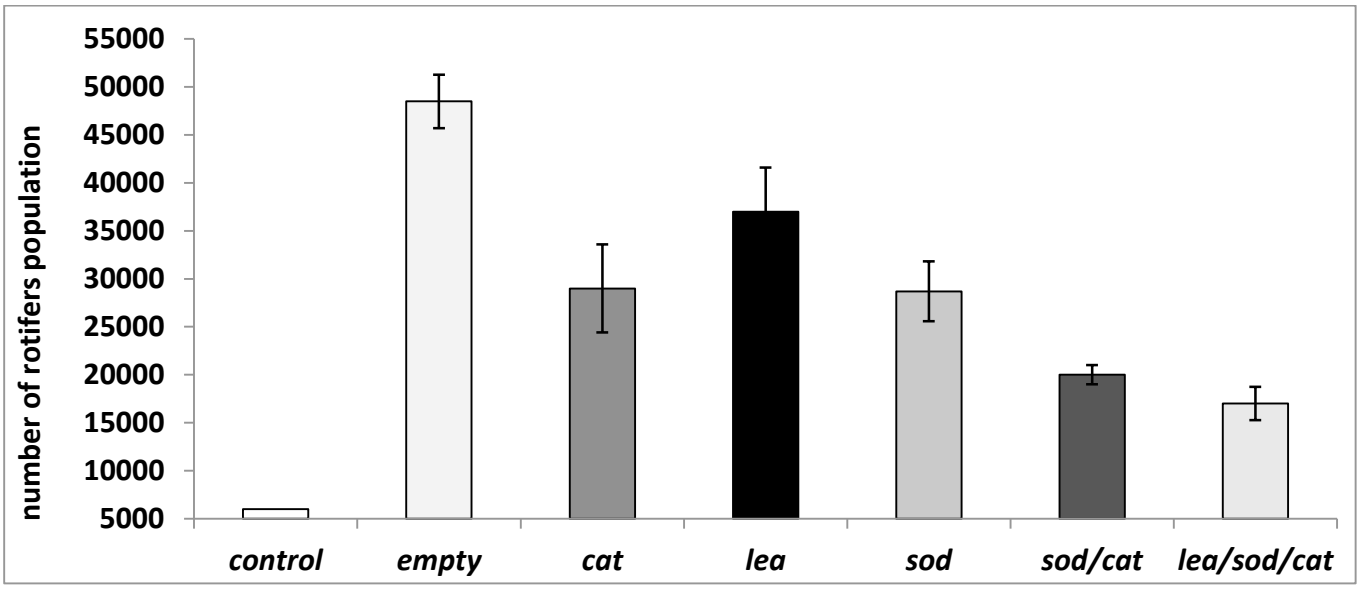

Figure 23: Change in rotifers populations after dsRNA treatment. Population of rotifers was determined after 72 h of dsRNA treatment. The mean values were calculated from three independent experiments. Standard deviation bars are shown.

3.9. Effect of dsRNA pretreatment on the survival of rotifers

In order to investigate the role of the LEA, SOD and catalase, the effect of RNAi-mediated silencing of *Philo-lea, Philo-sod* and *Philo-cat* was analyzed. Rotifers were fed with transformed H115 *E. coli* strain with either an empty L4440 vector or a L4440 vector containing an insert of *lea, sod-* or *cat*-dsRNA. As is shown in figure 24, *cat-, lea-*

and *sod*-dsRNA treatment resulted in a significant decrease of rotifer survival following desiccation. Only 67 %, 50 %, 43 % and of the *cat-, lea*- and *sod*-dsRNA treated rotifers survived after desiccation, whereas 96 % of the empty treated rotifers were still alive. Feeding *sod* and *cat* together reduced the survival rate to 15 % and feeding *lea*, *sod* and *cat* together reduced the recovery to 4 %. It appears that feeding two or three genes considerably reduces the rate of recovery of rotifers after desiccation.

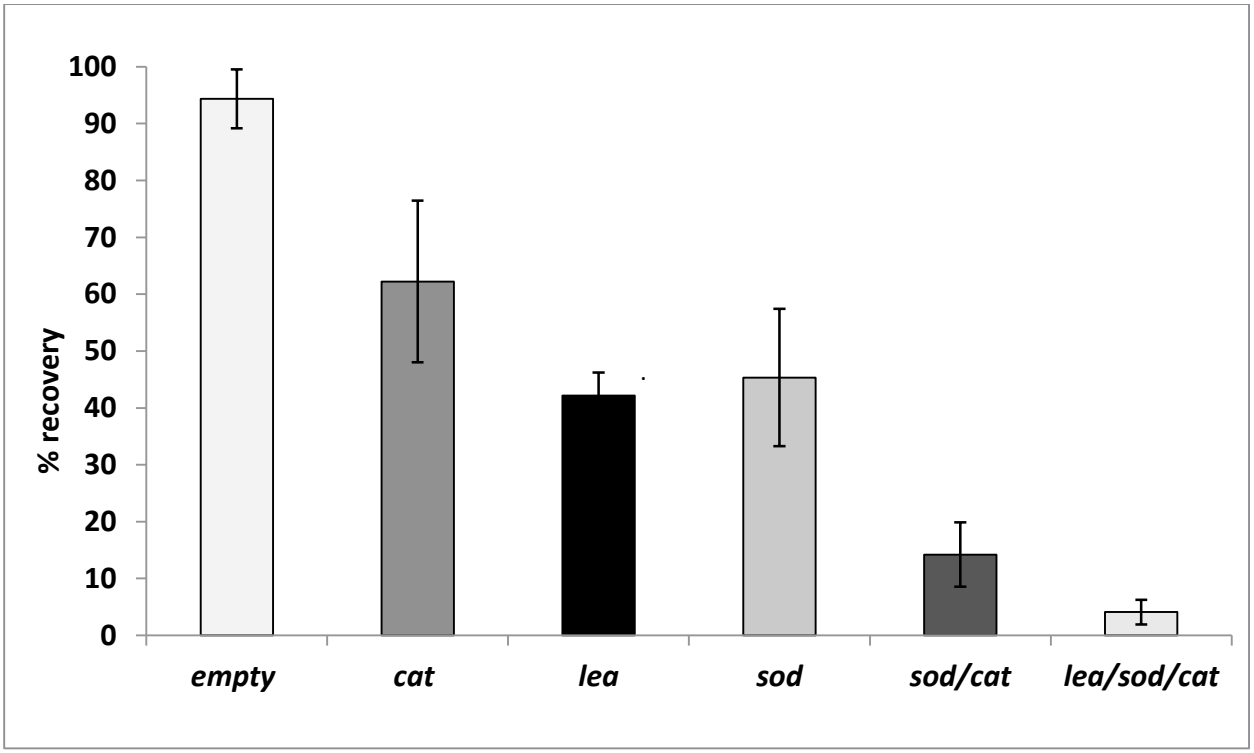

Figure 24: Sensitivity of rotifers to desiccation after dsRNA-*Philo-cat,-lea, -sod, -sod/cat* and *lea/sod/ca*-dsRNA treatment. The survival rate was estimated after 24 h of rehydration. The mean values were calculated from five independent experiments. Standard deviation bars are shown.

The reduced survival rates of rotifers under desiccation stress, following *Philo-cat, Philo-lea, Philo-sod, Philo-sod/cat* and *Philo-lea/sod/cat* silencing suggested that LEA, SOD and catalase are essential factors to tolerate the dehydration stress.

3.10. Morphological changes during desiccation

From the beginning of desiccation up to 4h empty treated, *lea*-, *cat*- and *sod*-dsRNA treated rotifers maintained the same appearance and shape and no striking morphological modification was noticed where by 26 % and 35 % of dsRNA-*sod/cat* and dsRNA-*sod/cat/lea* treated rotifers respectively were already contracted. After 6 to 8 h, 37 % to 100 % of the rotifers with a contracted tun shape were visible in each rotifer group. At the end of the dry period (10 h), all rotifers (100 %) were contracted into the tun shape typical of the dry rotifer, with roundish extremities and smooth body surface (figure 25).

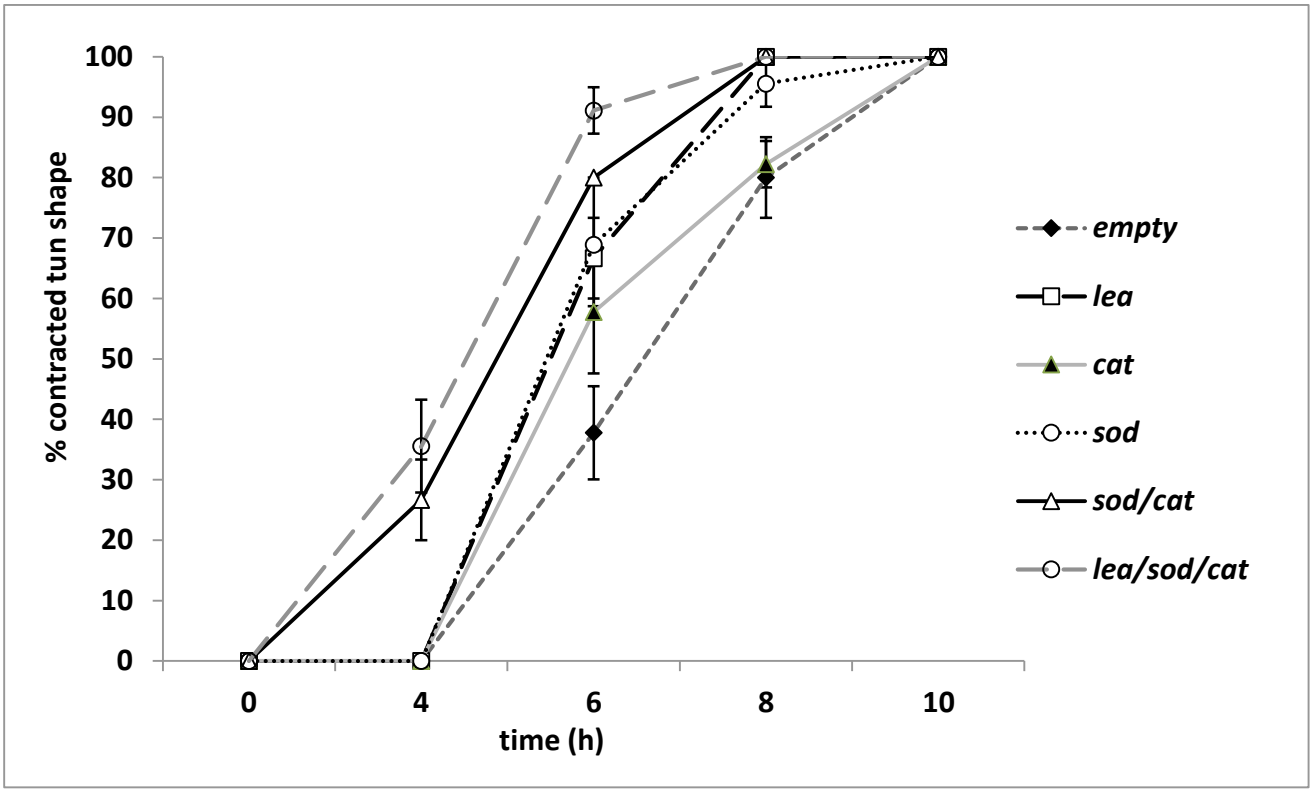

Figure: 25: Percentages of contracted tun shape rotifers during desiccation and following dsRNApretreatment. The mean values were calculated from five independent experiments. Standard deviation bars are shown.

3.11. Effects of desiccation on the accumulation of ROS

The accumulation of ROS level in rotifers was detected after exposure to desiccation (figure 26). The time dependent accumulation of ROS within desiccated rotifers was measured following exposure to desiccation at 88 % RH and for 0 h (control), 4 h, 14 h and 24 h using H_2DCF-DA. As is shown in figure 26 the ROS level clearly increased with time in both rotifers groups. The ROS accumulation in control rotifers was always less than in desiccated rotifers.

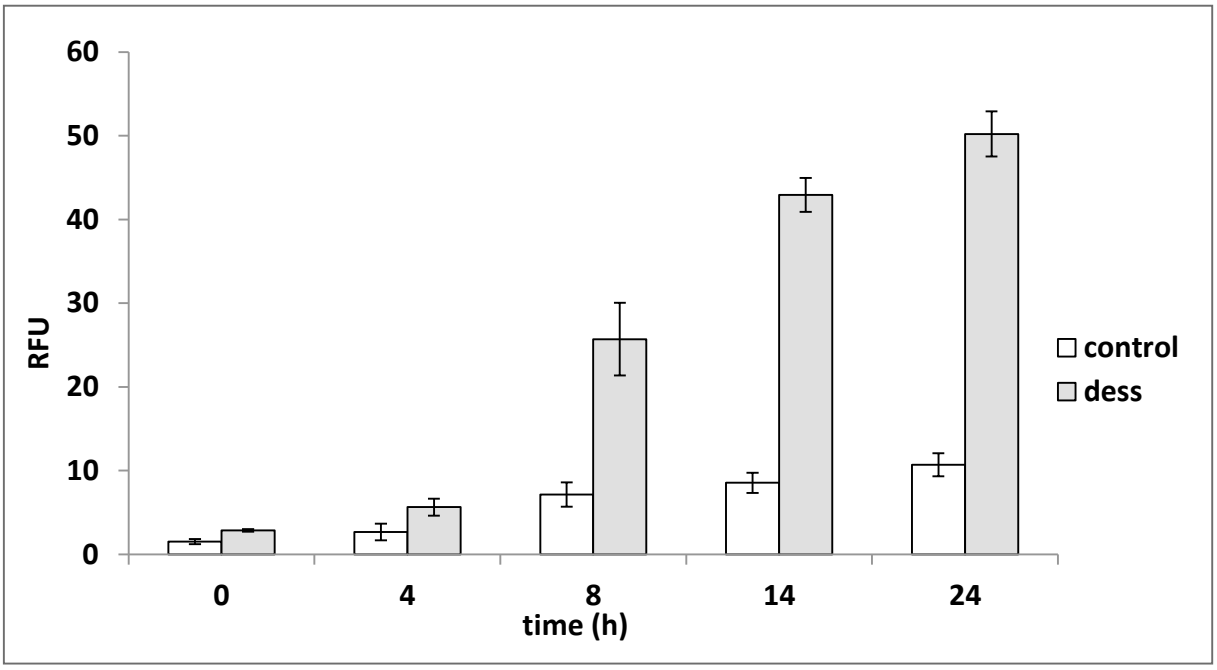

Figure 26: Measurement of ROS level in rotifers before and after exposure to desiccation at 88 % and for 0 h (control), 4 h, 8 h, 14 h and 24 h. Results are presented as mean values of relative fluorescence units (RFU) of four independent experiments. Standard deviation bars are shown.

3.12. Effect of desiccation on the ROS level after dsRNA pretreatment

The treatment with *sod*- and *cat*-dsRNA increased the ROS amount in rotifers with time compared to control, whereby the increase of ROS in *sod*-dsRNA treated rotifers was stronger. Control rotifers were fed with bacteria carrying the empty L4440 vector. The

increase of the ROS level with time in control and *lea*-dsRNA treated rotifers wassimilar. In a separate experiment the ROS level using RNAi by feeding for *sod/cat* and for *lea/sod/cat* was determined simultaneously. In both cases the ROS level increased significantly compared to feeding a single gene alone (figure 26). The intracellular ROS level in *sod/cat*-and *lea/sod/cat*-dsRNA treated rotifers were almost the same.

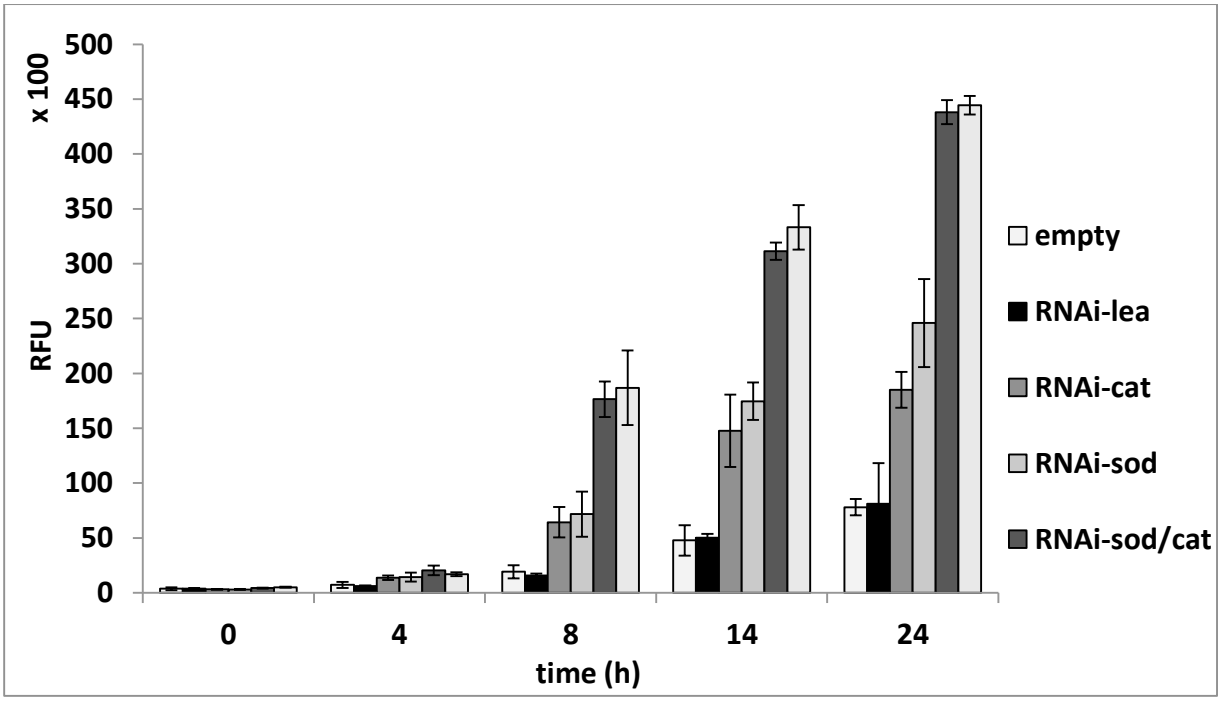

Figure 27: Measurement of ROS level in rotifers after dsRNA pretreatment following desiccation at 88 % relative humidity for 0 h (control), 4 h, 8 h, 14 h and 24 h. Results are presented as mean values of relative fluorescence units (RFU) of four independent experiments.Standard deviation bars are shown.

Taken together, these results confirm that SOD and catalase can help to scavenge free radicals generated by desiccation resulting in an increasing survival rate of rotifers as is shown in figure 24. LEA showed no influence on the ROS level, although figure 24 demonstrates that LEA was able to increase the survival rate of rotifers after dehydration

3.13. Determination of abasic sites in genomic DNA isolated from control and desiccated rotifers

For the AP site determination a calibration curve was made with the ARP DNA standard solution supplied by the manufacturer. After treatment of genomic DNA with ARP, the number of AP sites in extracted genomic DNA from control and treated rotifers was determined by measuring the enzymatic activity of streptavidin-conjugated peroxidase (HRP) and calculated using a standard curve generated by ARP standard DNA solutions. Figure 28 shows the electrophoretics analysis of DNA extracted from both, control and desiccated rotifers. The extracted genomic DNA from control rotifers showed about 2.5 AP sites per 10^5 kb (figure 30). The number of AP sites in the extracted DNA from desiccated rotifers increased to about 15 AP sitesper 10^5 kb. These results give evidence for the damage of DNA during dehydration stress.

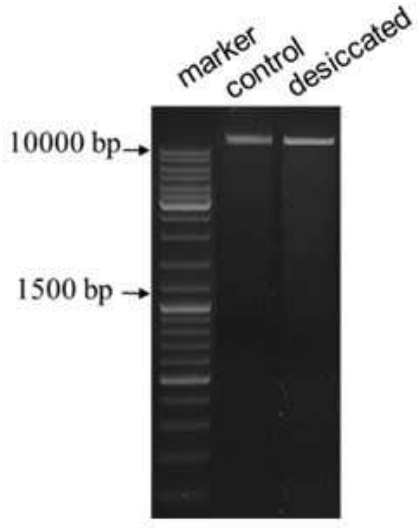

Figure 28: Electrophoretic analysis of genomic DNA isolated from control and desiccated rotifers. DNA was extracted from control and desiccated rotifers after 24 h of dehydration at 88 % relative humidity. Control rotifers were maintained hydrated. DNA from each rotifers group was analyzed on a 1 % agarose gel. Lane 1: marker; lane 2: DNA extracted from control rotifers; lane 3: DNA extracted from desiccated rotifers

3.14. Determination of AP sites in genomic DNA after dsRNA pretreatment

Genomic DNA was extracted from dsRNA treated rotifers and the number of AP sites was then determined by measuring the enzymatic activity of streptavidin-peroxidase (HRP) and calculated, using a standard curve generated by ARP standard DNA solutions. Figure 29 shows the electrophoretic analysis of each treated genomic DNA.

Control DNA was extracted from rotifers fed with bacteria carrying an empty vector. A considerable amount of DNA fragmentation was apparent in dsRNA treated rotifers compared to control who showed little fragmentation. The AP site detection is shown in figure 30. DsRNA treatment of rotifers resulted in an increase of the AP sites after desiccation. The number of AP sites in control rotifers was about 17 AP per 10^5 kb. The AP sites were highly increased in *lea-, cat-* and sod-dsRNA treated rotifers to about 84, 89 and 77 AP sites per 10^5 kb respectively.

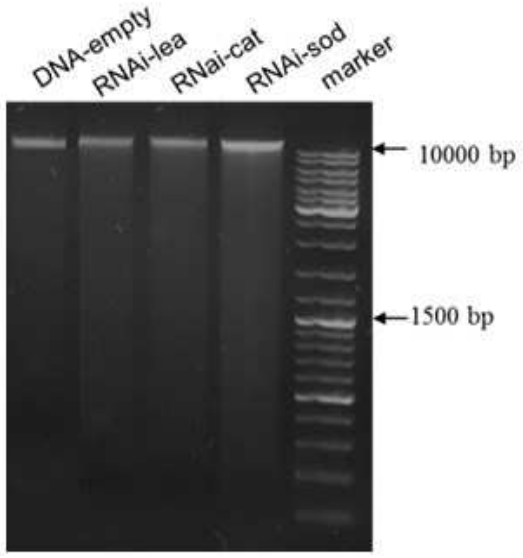

Figure 29: Electrophoretic analysis of genomic DNA after dsRNA pretreatment. Lane 1: DNA extracted from control rotifers; lane 2, 3 and 4: DNA extracted from desiccated rotifers after *lea-*, *cat-* and *sod*-dsRNA treatment respectively; lane 5: marker

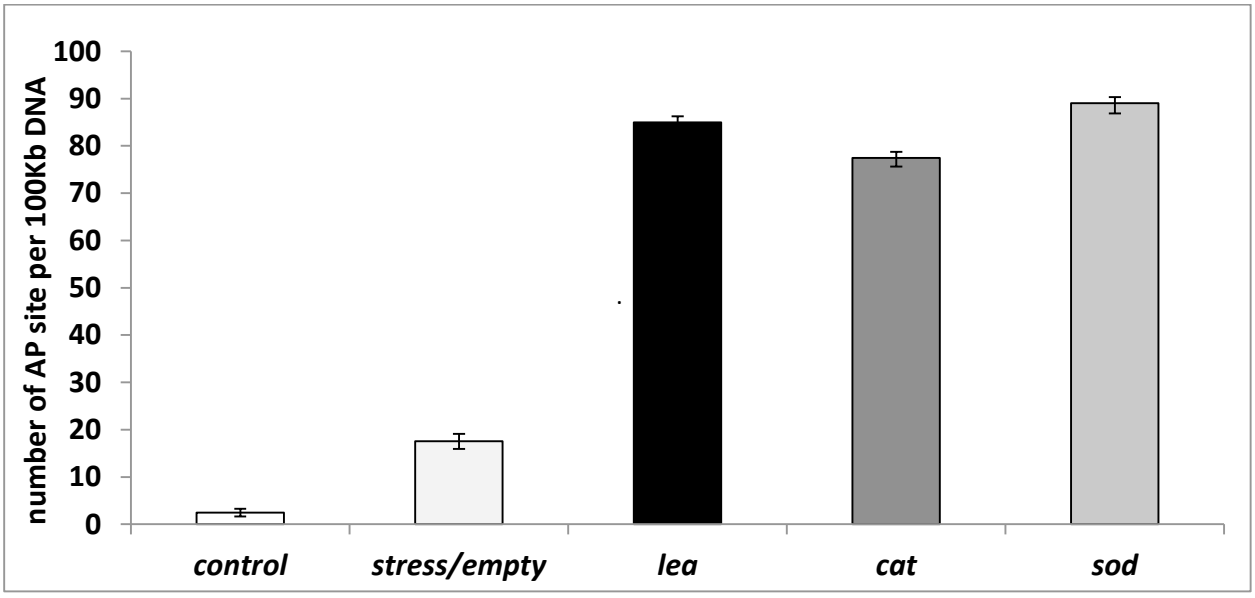

Figure 30: DNA damage analysis in rotifers. (A) Colorimetric measurement of AP sites in DNA from control and desiccated rotifers at 88 % RH and for 24 h. (B) Colorimetric measurement of AP sites in DNA extracted from *lea-, sod*-and *cat*-dsRNA treated rotifers after desiccation. The assay was done in triplicate and the data presented here are an average of three individual experiments with standard deviation bars.

Taken together, these results indicate that LEA, SOD and catalase can reduce the DNA damage in rotifers during dehydration.

4. Discussion

4.1. Survival after desiccation

Generally, all bdelloid rotifers are able to tolerate desiccation. Most species inhabit freshwater ponds, lakes, temporary pools, brackish waters, sewage and the interstices of soil in an environment that dries up more or less periodically (e.g soil, mosses and lichens) (Ricci, 1998). The presented desiccation experiments with *Philodina roseola* showedthat this species appears not to be able to survive very harsh anhydrobiosis at least under the experimental conditions provided in this study. The best survival rate (approx. 100 %) was obtained at 88 % relative humidity for 24 h hours and after a rehydration time of 24 h. Exposure of *Philodina roseola* to a very low RH (24 %) for 3 days led to a high decrease of rotifer survival. Thus the recovery capacity of anhydrobiotic rotifers may be affected by the conditions during the anhydrobiotic period, e.g. duration of the dry period and relative humidity, as shown previously (Ricci & Pagani, 1997, Ricci & Caprioli, 1998).

Studies on the effect of drying on rotifer recovery indicated that longer dryness and low relative humidity are unfavourable conditions for viability of anhydrobiotic animals, while high relative humidity (approx. 97 %) gave high recovery after 24 h of desiccation (Ricci & Pagani, 1997; Ricci & Caprioli, 1998). Generally, these findings suggest an accumulation of several damages during anhydrobiosis that may limit the survival rate of anhydrobiotes (Ricci & Caprioli, 1998).

4.2. Factors involved in desiccation tolerance

The present study was undertaken with the aim to identify the significance of factors influencing the capacity to withstand desiccation in *Philodina roseola.* To enter anhydrobiosis successfully, bdelloids undergo a series of morphological and biochemical changes. During dehydration rotifers, (and other anhydrobionts such as

tardigrades) contract into a “tun” leading to a reduction of the body surface and in tardigrades certain layers of the cuticle even simultaneously form a permeability barrier (Wright, 2001; Ricci *et al*., 2003). For *Philodina roseola* the results of the present work indicate a contraction in tun shape after 6 h to 10 h of desiccation at 88 % relative humidity. The reduction of their body surface slows down evaporation, which appears to be necessary to allow the animals to prepare for anhydrobiosis, e.g. to pack organs, cells and organelles (Higa & Womersley, 1993; Ricci *et al*., 2003). During that time it is also conceivable that rotifers synthesize substances to protect biological structures. Many molecules are thought to be involved in the phenomenon of anhydrobiosis such as trehalose (Wolkers *et al*., 2002; Goyal *et al*., 2005), heat shock proteins (Clegg *et al*., 1999) and LEA proteins (Browne *et al*., 2002; Tunnacliffe *et al*., 2005; Kikawada *et al.,* 2006).

Various studies have been undertaken to understand the cellular events leading to water deficit-induced gene expression, signal transduction and the regulation of gene expression. The first step in the regulation of the water deficit response is the recognition of the stres triggering a cellular signal transduction pathway. In this way, a physical stress can lead a biochemical response. Following cellular perception of water loss, a signaling mechanism must be activated to induce specific genes. Examples of a signal operating during drought stress is the transcription factor Leucine Zipper (bZIP) (Shinozaki & Yamaguchi-Shinozaki, 1997) that regulates the expression of *Arabidopsis lea* gene (Em) (Bensmihen *et al*., 2002). The forkhead transcription factors (FOXO) play a role in oxidative stress by upregulating expression of antioxidant genes (Storz, 2011). FOXO regulates antioxidant enzymes manganese superoxide dismutase (MnSOD) and catalase in the African clawed frogs, *Xenopus laevis,* during dehydration (Malik & Storey, 2011).

Regarding bdelloid rotifers, *sod* (superoxide dismutase), *cat* (catalase) and Ar-*lea*1A (group 3 late embryogenesis abundant) genes have been identified to be

overexpressed in *Adineta ricciae* after exposure at 100 % RH for 24 h (Boschetti *et al.,* 2010). The present work on *Philodina roseola* using similar experimental conditions and a substracted cDNA library confirms these results. The genes group 3 *lea, sod* and *cat* found in the present study suggest a similar response in both species.

The function of the genes *lea*, *sod* and *cat* was tested on its ability to protect *Philodina roseola* by reduction of the ROS level and the DNA damage during desiccation and by influencing the time used for contraction in a tun shape during desiccation. Potential protection mechanisms of *lea*, *sod* and *cat* in rotatoria were analyzed for the first time with RNA interference experiments by feeding *Philodina roseola* with RNAi bacteria H115, which allows gene expression to be selectively suppressed and thus a determination of gene function (Khvorova *et al*., 2003). Intake of RNAi was shown in the rotifers *Branchionus plicatilis* and *Brachionus manjavacas* after electroporation with dsRNA (Snell *et al*., 2010; Shearer *et al*., 2007), whereas other studies demonstrated that RNAi could be immitted in *Caenorhabditis elegans* and *Paramecium sp*. by feeding them with *Escherichia coli* expressing target-gene dsRNA. Here the number of ingested bacterial cells is crucial for a successful knockdown. In the culture of *Philodina roseola* the amount of remaining bacteria showed that an important quantity of bacteria harboring the RNAi vector L4440 with an insert of *lea, sod* and *cat* respectively was consumed by the rotifers after RNAi feeding. Suppression of mRNA transcripts was confirmed with quantitative PCR, which showed a considerable inhibition of gene expression in the range of 75 %, 80 % and 85 %.

To investigate the effect of dsRNA treatment of rotifers on the reproduction, rotifers were ingested in culture with bacterial cells carrying *cat-, lea-, -sod, sod/cat-* or *lea/sod/cat*-dsRNA. It has been reported that the rotifer *Brachionus plicalitis* can eat and digest bacteria (Watanabe *et al*., 1992). Ingestion with bacterial cells producing dsRNA inhibited the reproduction rate of rotifers within 72 h. After *cat, lea*, and *sod*-dsRNA treatment, population declined comparing to control. The lowest reproduction of rotifers

occurred after *sod/cat*-dsRNA and *lea/sod/cat*-dsRNA. The reduction of the expression of *sod, lea and cat* seems to affect the reproduction of rotifers. These genes are likely to be crucial for rotifers reproduction.

4.2.1. Late embryogenesis abundant (LEA)

Blast analyses of the isolated *Philo*-LEA indicate that it is closest to group 3 LEA of *Adinetaricciae*. Most LEA proteins from animal species show resemblance to group 3 LEA proteins, except for one LEA protein of group 1 reported in Artemia cysts (Sharon *et al*., 2009). LEA motifs were searched using BLASTP. The search analysis revealed that the *Philo*-LEA protein show an apparent higher resemblance to group 3 LEA proteins. The amino acid sequence show 6 matches, with repeats of the 11mer motifs (TAQAAKEKAGE) typical of group 3 LEA proteins and display 40 - 50 % identical amino acid residues. *Philo*-LEA shows three repeat motifs with a length of eight to nine amino acids and of the form KANEALKA, GEAYENIAP and VDGAAFVK. Each motif occurs 2 to 3 times in the amino sequence of LEA and all three motifs are present in the bdelloid rotifer *Adineta ricciae* and *Adineta vaga* as well. The amino acid composition of LEA also points out a hydrophilic character of the protein suggesting that this protein is highly hydrated in aqueous solutions. LEA proteins are considered to be intrinsically unstructured proteins, forming random coiled proteins in solution (Garraya-Arroyo *et al*, 2000; Battaglia *et al*., 2008; Tunnacliffe *et al.,* 2005). In this regard, LEA might act as a hydration buffer during the dehydration process, slowing the rate of water loss and maintaining a minimal level of bound water within the cell even at low relative humidities. It is striking that LEA proteins may be found in all cell compartments of rotifers (Tunnacliffe & Wise, 2007), among others in the nucleus, which has been also found in plants despite the absence of a nucleus localization signal (Battaglia *et al*., 2008). A translocation of LEA in *Philodina roseola* under desiccation stress is therefore possible that the subcellular localisation of LEA proteins may be closely related with

important functions under stress conditions. The mentioned repeat motifs among bdelloid rotifers, the hydrophilic character of LEA proteins and its probable omnipresence in rotifers underline the importance of this protein for the organism.

In context with desiccation an overexpression of *lea* or the induction of its expression has been observed among a variety of species. In the nematode *Aphelenchus avenae* similiar *lea* genes were isolated as an up-regulated transcript (10-fold overexpression) after exposure to 90 % RH for 24 h (Browne *et al.,* 2002) and in the nematode *Steinernema feltiae* (IS-6) induction of high levels of LEA proteins has been associated with water stress after desiccation at 97 % RH for 3 days (Solomon *et al*., 2000). A high concentration of LEA proteins in consequence of water stress has also been monitored in different plants, e.g. in the moss *Ceratodon purpureus* as well as in *Craterostigma pumilum* and *C. plantagineum* (Scrophulariaceae) (Hoekstra *et al.,* 2001; Solomon *et al*., 2000). Consequently, LEA proteins seem to play a fundamental role in the protection of cell structures, like cell membranes (Hoekstra *et al*., 2001). This protective role of LEA proteins against desiccation stress has been also supported by transgenic expression studies, e.g. in transgenic rice overexpressing LEA, which improved its tolerance to dehydration (Xu *et al*., 1996).

The assumption of LEA as a protective protein against dehydration damage has been reinforced in this study. Silencing of *Philo-lea* transcription resulted in the decrease of the survival rate compared to control rotifers after desiccation. Similar results were achieved for the bacterium *Deinococcus radiodurans*, inactivation of *lea* genes resulted in a significant reduction of desiccation tolerance (Battista *et al.,* 2001). Possible factors for dehydration damages are ROS (reactive oxygen species) and DNA-lesions. The formation of reactive ROS in anhydrobionts has been proven to be a consequence of desiccation and may penetrate membrane systems, proteins and nucleic acids (Oliver *et al*., 2001; França *et al*., 2007; Cruz de Carvalho, 2008). Therefore, ROS scavenging mechanims in anhydrobiotic organsims would be useful. Regarding *Philodina roseola*

an increase of the ROS level was observed in desiccated specimens. The *lea* knockout mutants were unaffected by ROS after desiccation, suggesting that other protective molecules must be able to compensate the absence of LEA during desiccation stress. Still an important effect of LEA on DNA-damages could be demonstrated. Investigation of DNA integrity in *Philodina roseola* was monitored in *lea*-dsRNA treated specimens. Exposure of *lea*-dsRNA treated rotifers to desiccation caused extensive DNA damage. Since LEA had no effect on ROS accumulation during anhydrobiosis, it must enable the survival of rotifers by DNA protection probably by binding to DNA. Structural analysis of LEA showed the presence of DNA binding domains, which may also indicate a nuclear localization (Rajesh & Manickam, 2006). Another important effect of LEA during anhydrobiosis may be a delay in contraction of the rotifers. The percentage of contracted *lea*-dsRNA treated rotifers was constantly higher than of empty treated rotifers, which showed the best recovery rate, needed more time to contract into a tun shape. *Lea* appears to enable rotifers to slowly contract to a tun shape, which seems to be necessary for a correct body contraction and for entering and performing anhydrobiosis (Ricci, 1998).

4.2.2. Superoxide dismutase and catalase

Aerobic organisms utilize antioxidant enzymes, including catalase and superoxide dismutase (SOD) for protection against ROS formed as by-products of aerobic metabolism (Newton *et al*., 1995, Huynh *et al*., 2003). In the present study for the first time the function of the antioxidant enzymes SOD and catalase has been investigated in bdelloid rotifers during desiccation. An up-regulation of these detoxifying enzymes suggests that also *Philodina roseola* uses both to efficiently scavenge ROS under desiccation stress.

It is known that the activity of antioxidant enzymes, such as ascorbate peroxidase (APX) and glutathione reductase, increases in response to drought, e.g. in *Helianthus annuus*

and in grass seedlings of *Sorghum bicolor* (Zhang & Kirkham, 1996; Manivannan *et al.*, 2007) just as well the activity of SOD and catalase. In the asian rice *Oryza sativa* (Guo *et al*., 2006) or in leaves of canola (Tohidi- Moghaddam *et al.,* 2009) the activity was found to be higher under drought stress, whereas Hsieh *et al* (2002) demonstrated that transgenic tomato plants resistant to water deficiency overexpress the catalase gene.

An increased SOD concentration also enforces the production of hydrogen peroxide (H_2O_2), which is normally detoxified by catalase or GPx (Avissar *et al*., 1996). Hence, a decreased catalase activity leads to the accumulation of H_2O_2 in tumor cells, which causes DNA damage and/or cell death (Islam *et al.,* 1997, Sandstrom *et al.,* 1993, Yabuki *et al.,* 1999). Silencing of *Philo-sod, Philo-cat* and *Philo-sod/cat* transcription in rotifers *Philodina roseola* resulted in a decrease of the survival rate. It appears that tolerance to dehydration of rotifers is also dependent on catalase and SOD and may be correlated with enzymatic defense (Stajner *et al*., 1995). Similar results were observed in *lea/sod/cat*-dsRNA treated rotifers indicating that tolerance to dehydration of rotifers is also dependent on LEA.

The influence of SOD and catalase on the survival rate has also been observed for other species. The maximum life span of *Drosophila melanogaster* was significantly increased by the transgenic overexpression of both SOD and catalase (Orr & Sohal, 1994). Schriner *et al*. (2005) have significantly extended life span of transgenic mice expressing a catalase localized in the mitochondria. JagTap and Bhargava (1995), stated that the activity of SOD increased in drought-tolerant cultivars of maize. Further, Fu and Huang (2001) reported that the ability for adaptation to drought stress depended on the capability to detoxify superoxide radical by antioxidant enzymes in *Poa pratensis* (Kentucky bluegrass) and tall fescue (*Festuca arundinacea*).

To further investigate the role of catalase in protecting rotifers during dehydration the cell redox status was analyzed in *lea-, sod-,* and *cat*-dsRNA treated rotifers. The level of ROS caused by water stress was higher in *lea*-, sod-, *cat*-a nd *sod/cat*-dsRNA treated

rotifers than in the control strain implying that SOD and catalase favor resistance to dehydration by controlling reactive oxygen species (ROS) production, probably through removal of O_2- and H_2O_2. After *lea/sod/cat*-dsRNA treatment the accumulation of ROS in rotifers following desiccation was similar to the ROS level in *sod/cat*-dsRNA treated rotifers confirming that LEA has no effect on the ROS level in rotifers during anhydrobiosis.

After silencing of *sod* and *cat*, DNA damage was visible in dsRNA treated rotifers, which is an indication that the protective antioxidant mechanisms of SOD and catalase are likely to be responsible for the resistance of *Philodina roseola* during dehydration through scavenging of ROS and leading to a reduction of the oxidative DNA damage. *Cat*- and *sod*-dsRNA treated rotifers were more rapidly contracted to a tun shape than empty treated rotifers. S*od/cat*- and *lea/sod/cat* treated rotifers that showed the worst recovery rates were faster contracted into a tun than *lea*-, *sod*- and *cat*-dsRNA treated rotifers. Retraction of head and foot into the trunk is the usual response of bdelloids to perturbations (Ricci & Melone, 1984; Melone & Ricci, 1995; Ricci *et al*., 2001). The limited time noticed in dsRNA-treated rotifers until the occurance of tun shape formation seems to be due to the inhibition of gene expression of *lea*, *sod* and *cat*, which was an additional stress.

5. Summary

Anhydrobiosis is a special form of cryptobiosis, which enables various organisms to tolerate desiccation. In this state, however, they may suffer various and sometimes irreversible damages such as membrane peroxidation and protein aggregation. To weaken or even prevent harmful consequences of desiccation protective and repair mechanisms have been developed.

The bdelloid rotifer *Philodina roseola* is able to survive phases of dryness, which occur in its habitat, i.e. mosses that dry up more or less periodically. Hence, rotifers permanently adapt morphologically and physiologically to their current environment. Entering anhydrobiosis rotifers reduce their body surface and form a tun. After 6 h to 10 h and at a relative humidity of 88 % the rotifers contracted into a tun. After exposition of *Philodina roseola* to a relative humidity of 88 % for 24 h approx. 100 % of the specimen survived. Therefore these conditions were used for further experiments as longer desiccation periods (48 h to 72 h) and a lower relative humidity (44 % to 23 %) reduced the capacity of *Philodina roseola* to recover (60 % to 12 %).

In a cDNA subtractive library of *Philodina roseola* in which desiccation induced transcipts were enriched cDNAs of *lea*, *sod* and late embryogenesis abundant, superoxide dismutase and catalase have been successfully identified. The analysis of the rate of transcription of these genes was determined by real time PCR and showed a fourty fold overexpression of *lea*, and seven fold overexpression of *sod* and a four fold overexpression of *cat* compared to the non desiccated control rotifers. To examine the possible essential significance of the proteins LEA, SOD and catalase for the survival of *Philodina roseola*, the suppression of these genes via RNA interference was carried out experimentally. The vector L4440 was used as carrier for the RNA intereference and was transformed into *E. coli* H115. 80 % of

the bacteria harbouring the empty L4440-vector, *lea*-, *cat*- and *sod*-dsRNA respectively were ingested by *Philodina roseola* and only 70 % of bacteria treated with *sod/cat*- and *lea/sod/cat*-dsRNA respectively were ingested.This treatment with dsRNA led to a down regulation of the expression of the genes *lea, cat* and *sod* in all of the combinations mentioned above. In the present work *sod/cat* and *lea/sod/cat* respectively were knocked down simultaneously for the first time. The expression of the single genes was reduced by approx. 80 %.

Treatment of rotifers with *cat-, lea*- and *sod*-dsRNA resulted in a decrease of population of 60 %, 76 % and 59 % respectively compared with control. The reproduction rate of rotifers was considerably decreased of 41 % and 35 % respectively after treatment with *sod/cat*- and *lea/sod/cat*-dsRNA.

The recovery rate after desiccation considerably decreased to 67 % (*cat-dsRNA*), 50 % (*sod*-dsRNA) and to 43 % (*lea*-dsRNA) respectively, whereas 96 % of empty treated rotifers survived. The simultaneous knockdown of *sod/cat* and *lea/sod/cat* reduced the recovery rate even more to 4 % (*lea/sod/cat*) and 14 % (*sod/cat*) respectively.

An Exposure to desiccation for 24 h resulted in a 4 fold increase of the ROS level in non dsRNA treated rotifers in comparison to control rotifers in a humid environment. After *sod*-dsRNA and *cat*-dsRNA treatment a 12 fold increase of ROS was observed, whereas a simultaneous treatment with *sod/cat* and *lea/sod/cat* resulted in a 28 times higher ROS level. Surprisingly the knockdown of *lea* showed no effect on the ROS concentration.

A desiccation for 24 h also has shown to have impact on DNA integrity. In dsRNA untreated rotifers as well as in rotifers harbouring an empty vector the amount of DNA-lesions increased about 4 times compared to rotifers under humid conditions. After knockdown of the single genes the rate of DNA-lesions arose about 20 times.

The present study shows for the first time a protective effect of SOD and catalase by reducing the ROS level and DNA damages. LEA was shown as not previously described to prevent DNA damage during desiccation.

6. Zusammenfassung

Anhydrobiose ist eine spezielle Form der Kryptobiose. Sie ermöglicht einem Organismus, extreme Austrocknung zu überleben, kann aber dennoch zu verschiedenen, irreversiblen Schäden führen, wie z.B. einer oxidativen Membrandegradation und Proteinaggregation. Viele Organismen haben Schutz- und Reparaturmechanismen entwickelt, um potentiell schädliche Folgen einer Austrocknung abzuschwächen oder sogar zu verhindern.

Philodina roseola zählt zu den bdelloiden Rädertierchen und ist zur Anhydrobiose fähig, um wiederkehrende Trockenperioden in seinem Habitat zu überdauern. Zu diesem zählen Moose, die während ihres Lebenszyklus immer wieder beinahe vollständig austrocknen, so dass Radertierchen zu einer ständigen morphologischen und physiologischen Anpassung an ihre Umgebung gezwungen sind. Zu Beginn einer Anhydrobiose nehmen Rädertierchen die Gestalt eines Tönnchens an, wodurch ihre Körperoberfläche beträchtlich verkleinert wird. Bei einer relativen Luftfeuchtigkeit von 88 % benötigten sie dafür 6 - 10 h. Nach 24 h Trocknung bei gleicher relativer Luftfeuchtigkeit von 88 % überlebten fast alle Tiere. Demzufolge wurden diese Bedingungen für nachfolgende Experimente gewählt, da bereits eine längerfristiges Aussetzen (48 h bis 72 h) der Rädertierchen bei niedriger relativer Luftfeuchtigkeit (44 % bis 23 %) zu einer schlechteren Erholungsfähigkeit der Tiere führte (60 % bis 12 %).

In einer Subtraktions-cDNA-Bibliothek von *Philodina roseola*, in der durch den Trocknungsprozess induzierte Transkripte angereichert waren, gelang die Identifizierung von late embryogenesis abundant, Superoxide Dismutase und Katalase cDNAs. Die Analyse der Transkriptionsrate der drei Gene mittels real time PCR in getrockneten Tieren zeigte eine vierzigfache Erhöhung für *lea*, eine siebenfache für *sod* und eine vierfache für *cat* gegenüber nicht getrockneten Kontrolltieren.

Eine Unterdrückung der Expression von *lea, sod* und *cat* anhand von RNA-Interferenz sollte Aufschluss über eine mögliche essentielle Bedeutung dieser Proteine für das Überleben von *Philodina roseola* geben. Träger der RNA-Interferenz für *lea, sod* bzw. *cat* war der Vektor L4440, der in *E. coli* H115 transformiert wurde. Die verfütterten Bakterien wurden zu 80 % in *Philodina*-Kulturen gefressen, die jeweils entweder Leervektor, *lea*-dsRNA, *cat*-dsRNA bzw. *sod*-dsRNA aufgenommen haben. Im Gegensatz dazu wurden in Ansätzen mit dsRNA-*sod/cat* bzw. *lea/sod/cat* behandelten Rädertierchen nur 70 % der Bakterien gefressen.

Die Behandlung der Rotatorien mit *cat-, lea-* und *sod*-dsRNA resultierte in einem erniedrigten Populationswachstum und sank auf 60 %, 76 % bzw. 59 % im Vergleich zur Kontrolle. Die Populationsgröße der Rotatorien nach Behandlung mit *sod/cat*- und *lea/sod/cat*-dsRNA fiel sogar auf 41 % bzw. 35 % im Vergleich zur Kontrolle.
Die dsRNA-Behandlung führte in allen Fällen zu einer unterschiedlichen Runterregulation der Expression der drei Gene. In der vorliegenden Arbeit wurde zum ersten Mal die Expression von *sod/cat* bzw. *lea/sod/cat* gleichzeitig unterbunden. Der einzelne ‚knockdown' der drei Gene senkte die Expression um jeweils ca. 80 %.

Auch die Überlebensrate von dsRNA-behandelten Rädertierchen nach Rehydrierung nahm deutlich ab und zwar auf 67 % (*cat*-dsRNA), 50 % (*sod*-dsRNA) bzw. 43 % (*lea*-dsRNA) während mit Leervektor behandelte Rotatorien zu 96 % überlebten. Der gleichzeitige ‚knockdown' von *lea/sod/cat* bzw. *sod/cat* senkte die Überlebensrate sogar auf nur 4 % bzw. 14 %.

In dsRNA-unbehandelten Tieren führte die 24stündige Trocknung zu einem vierfachen Anstieg der intrazellulären Menge an reaktiven Sauerstoffspezies (ROS) im Vergleich zu Tieren in feuchter Umgebung. In *sod*- und *cat*-dsRNA behandelten Rädertierchen wurde eine zwölffach höhere ROS-Konzentration gemessen und in dsRNA-*lea/sod/cat* bzw. dsRNA-*sod/cat* behandelten Rädertierchen stieg die ROS-Menge sogar um das

28-fache. Der ‚knockdown' der *lea*-Expression zeigt überraschend keinen Einfluß auf die ROS-Menge.

Die 24stündige Trocknung hat auch Auswirkungen auf die DNA-Integrität. In dsRNA-unbehandelten Tieren sowie mit Leervektor behandelten Tiere zeigte sich jeweils eine vierfache Zunahme der DNA-Schäden im Vergleich zu Tieren in feuchter Umgebung. Behandlung mit jeweils *sod*-dsRNA, *lea*-dsRNA bzw. *cat*-dsRNA führte zu einer 20-fachen ROS-Erhöhung nach Trocknung.

Die vorliegende Arbeit zeigt zum ersten Mal, dass die Überexpression der Proteine SOD und Katalase während des Trocknungsprozesses von Rotatorien einen sehr bedeutungsvollen schützenden Effekt im Hinblick auf ROS- und DNA-Schäden bewirkt. LEA verhindert offenbar ebenfalls DNA-Schäden während der Trocknung, was zuvor noch nicht nachgewiesen wurde.

7. Literature

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9. Erklärung

Die hier vorgelegte Dissertation habe ich eigenständig und ohne unerlaubte Hilfe angefertigt. Die Dissertation wurde in vorgelegter oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, den 01.06.2012

Marthe Christiane Gombitang Nkwonkam