Specially regulated electron deprivation extremely prolongs lifespan and delays aging in middle-aged rotifers

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Publications

Publications the Thesis is based on:

I. **Macsai L**, Olah Z, Bush AI, Galik B, Onody R, Kalman J, Datki Z. Redox modulating factors impact longevity regulation in rotifers. J Gerontol A Biol Sci Med Sci. 2018;74:811-814. doi: 10.1093/gerona/gly193; **IF: 4.711; Q1**

II. **Macsai L**, Datki Z, Csupor D, Horvath A, Zomborszki ZP. Biological activities of four adaptogenic plant extracts and their active substances on a rotifer model. Evid-Based Comp Alt. 2018;3690683. doi: 10.1155/2018/3690683; **IF: 1.984; Q1**

III. Datki Z, Olah Z, Hortobagyi T, **Macsai L**, Zsuga K, Fulop L, Bozso Zs, Galik B, Acs, E, Foldi A, Szarvas A, Kalman J. Exceptional *in vivo* catabolism of neurodegeneration-related aggregates. Acta Neuropathol Commun. 2018;6:6. doi: 10.1186/s40478-018-0507-3; **IF: 5.883; Q1**

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II. Olah Z, Bush AI, Aleksza D, Galik B, Ivitz E, **Macsai L**, Janka Z, Karman Z, Kalman J, Datki Z. Novel *in vivo* experimental viability assays with high sensitivity and throughput capacity using a bdelloid rotifer. Ecotoxicol Environ Saf. 2017;144:115-122. doi: 10.1016/j.ecoenv.2017.06.005; **IF: 3.974; Q1**

III. Chuluunbaatar B, Beni Z, Dekany M, Kovacs B, Sarkozy A, Datki Z, **Macsai L,** Kalman J, Hohmann J, Vanyolos A. Triterpenes from the mushroom *Hypholoma lateritium*: Isolation, structure determination and investigation in bdelloid rotifer sssays. Molecules. 2019;24:301. doi: 10.3390/molecules24020301; **IF: 3.060; Q1**

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Abbreviations

AA → ascorbic acid

Aβ → beta-amyloid

Aβ42 → beta-amyloid 1-42

Au-Aβ42 → gold-tagged beta-amyloid-1-42

BisANS → 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid dipotassium salt

BLA → bright light avoidance

BLD → bright light disturbance

BLI → bright light irritation

BSI → body size index

C → control (containing 0.1% DMSO)

CR → Congo red

CRC → cellular reduction capacity

DMSO → dimethyl sulfoxide

DW → distilled water

ECR → extreme caloric restriction

EDTA → ethylenediaminetetraacetic acid

EtOH → ethanol

MCF → mastax contraction frequency

MTT → 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAD^+ → nicotinamide adenine dinucleotide, oxidized form

NADH → nicotinamide adenine dinucleotide, reduced form

NaOH → sodium hydroxide

NFI → normalized fluorescence intensity

NML → normalized mean lifespan

NR → normal rotifer

NRA → number of rotifers alive

PI → propidium iodide

PMS → phenazine methosulfate

SDS → sodium dodecyl sulfate

SEM → scanning electron microscope

SR → super rotifer

XTT → 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt

UC → untreated control (agent-free)

1. Introduction

1.1. Aging, longevity and their models, focusing on rotifers

„Aging can be fun if you lay back and enjoy it."
— Clint Eastwood

For humanity and life sciences, age and aging were always a matter of perspective. However, the processes of aging can be defined as a natural and progressive loss of physiological integrity, leading to impaired function and increased vulnerability to diseases and death [López-Otin et al., 2013]. Most of the adult-onset diseases that are direct consequences of aging concern a gradually increasing population [Valenzano et al., 2017]. The knowledge of delaying or even preventing the negative aspects of old age could decrease the chances of most relevant diseases and may increase longevity [Seals et al., 2016]. To slowing down the natural and basic processes of aging, the exacerbated progression caused by extrinsic factors (e.g. diseases), it is necessary to have comprehensive knowledge.

Although aging is a widespread phenomenon in biology, it is far from universal. A model to study aging should, therefore, fulfill the following criteria: it must have measurable phenotypes of the process and the underlying mechanisms must be phylogenetically conserved. Ideally, the chosen organism should have a short lifespan and should be relatively easy to manipulate under laboratory conditions (e.g. small size and inexpensive to grow in large amount). Further ethical consideration should be given to the work with sentient higher organisms [Lees et al., 2016]**.** To understand the diversity of aging models, we conclude the advantages and potential disadvantages of the relevant animals briefly. *In vitro* human cell cultures provide useful information about the cellular processes of aging, [Mitchell et al., 2015]; however, we cannot speak of real lifespan. (**Figure 1**).

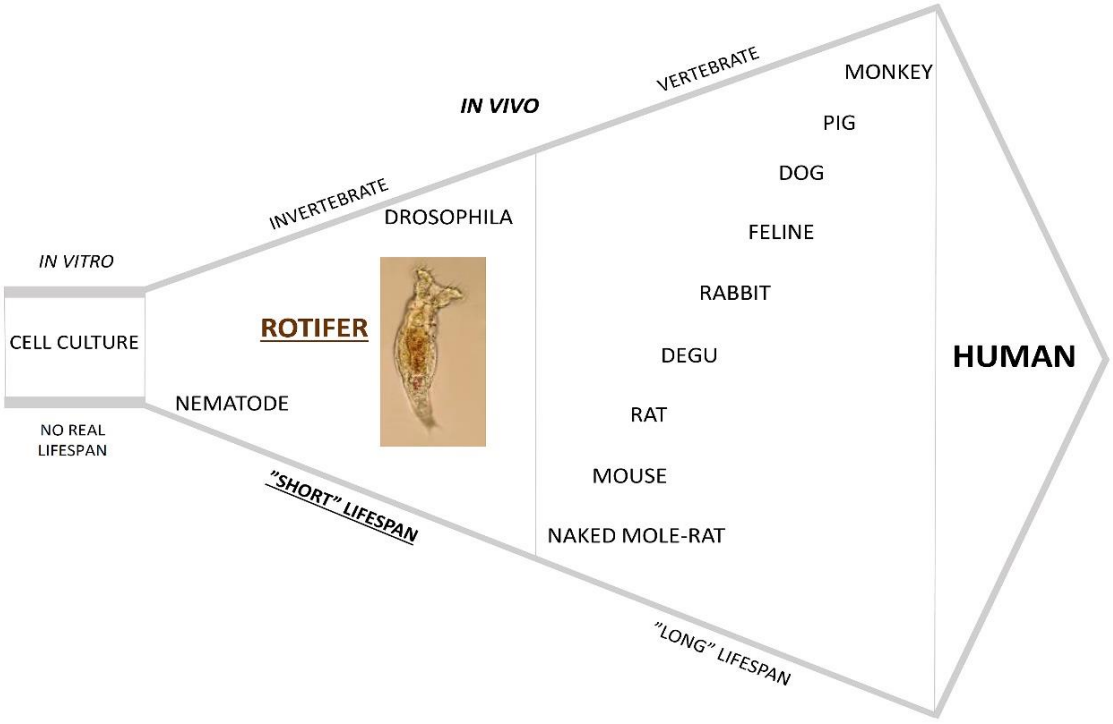

Figure 1. ***Biological models in aging studies***

Invertebrates (e.g. *Caenorhabditis elegans* and *Drosophila melanogaster*) were the first proposed genetic models of aging amongst the *in vivo* systems with precisely determinable lifespan [Partridge and Gems, 2002]. Investigation of genetic and environmental manipulations to alter the lifespan of *C. elegans* has shown remarkable insights related to some genes with very marked impacts on longevity [Tissenbaum, 2015]. The fruit flies together with nematodes have the advantage of the fully sequenced and annotated genome, as well the tools developed for genetic manipulation, coupled with well-defined and accepted phenotypic markers for analysis [Lees et al., 2016]. The potential disadvantages of these species are the lower penetration of the treatment agents through the cuticle (nematodes) and the difficulty in measuring phenotypes of aging beside lifespan (fruit flies) [Gravato-Nobre et al., 2005; Lees et al., 2016]. Rotifer species are widely used in aging studies [Enesco, 1993; Snare et al., 2013; Snell, 2014; Snell et al., 2015], in risk assessment of pharmaceuticals and in examining the roles of their metabolites in aquatic ecosystem [Dahms et al., 2011].

For the precise modeling of multi-level molecular and physiological alterations related to aging, vertebrate models could have numerous advantages such as the closer similarity in molecular regulatory pathways (e.g. nutrient-sensing) and the age-associated pathologies

[López-Otin et al., 2013; Lees et al., 2016; Chaudhari and Rizvi, 2019]. There is a great variety of well-known model species from those with short lifespan (e.g. mice, rat) to the extremely long-lived entities (e.g. naked mole-rat, primates) [Triplett et al., 2015; Colman, 2018]. There are practical- (e.g. rodents are nocturnal) and ethical concerns (particularly about the non-human primates), and their lifespan is counted in years (up to even 30 years or more). The human aging studies are even more complicated for many reasons, including the ethical issues, environmental and social factors [Mitchell et al., 2015].

Although various biological systems are established, a newly developed model or an unorthodox application of an old one is always welcomed due to it may give a novel insight into an unknown process of aging. Among the scientific models based on microscopic metazoan species (e.g. rotifers) have a long history in speciation, phylogenetic- and ecological research [Dahms et al., 2011]. Phylum Rotifera ("wheel-bearer") comprises approximately 2200 microscopic-sized aquatic invertebrate species [Segers, 2007] occurring almost any permanent or intermittent fresh- and saltwater. They are characterized by their unique ciliated corona [Pechenik, 2005] and by the muscular pharynx called mastax [Wallace et al., 2006]. They have a bilaterally symmetrical body, usually divided to head, trunk, and foot more or less clearly by transversal folds. With the exception of few colored species, they are transparent animals with complete internal organs: digestive-, reproductive- and excretory systems, bilobed brain and photosensory apparatus, sensory and tactile receptors [Clément et al., 1980; Hochberg and Litvaitis, 2000; Snell, 2014]. Rotifer species display the trait of eutely, the invariance of adult somatic cell number [Clément and Wurdak, 1991].

Three separate classes are recognized: owing to the diversity in habitat, morphology, and most importantly in reproduction. *Seisonidea* species are obligate sexual epibionts. Monogonant rotifers are free-living specimens with peculiar facultative cyclical parthenogenesis [Wallace et al., 2006]. Bdelloid specimens occur in any moist or wet habitat, surviving desiccation through dormancy; furthermore, they are known as "evolutionary scandals" due to their asexual reproduction, in which the horizontal gene transfer may provide genetic variability [Smith, 1986; Ricci and Fontaneto, 2009; Fontaneto and de Smet 2015.].

1.2. Advantages of bdelloid rotifers in scientific research

Bdelloid rotifers, as microinvertebrates, are commonly used models in ecotoxicology-, pharmacology- and aging-related studies. They are eutelic multicellular (approximately 950-

1000 somatic cells) animals with distinct morphological characteristics [Marotta et al., 2012]. They possess ganglia, muscles, digestive-, nervous- and secretory systems, moreover photosensitive, and tactile organs [Clément et al., 1980; Hochberg and Litvaitis, 2000]. Rotifers have most of the same or further advantages in culturing, anatomy, physiology and behavior compared to *C. elegans* [Birky, 2004]. They have more than 10% of human-analog genes with fundamental vertebrate homology. These genes can be found neither in nematodes nor in fruit flies. [Dahms et al., 2011] Standardized laboratory assays have been developed using them as target or model organisms [Preston and Snell, 2001; Snell, 2014]. They have been used to study the effects of reproduction, maternal age, and dietary modification on lifespan [Verdone-Smith and Enesco, 1982; Kirk, 2001; Snare et al., 2013]. The maintenance of cultures is cost-effective.

Our reasons for choosing bdelloid rotifers for experiments based on their biological advantages are the followings:

1. **Short lifespan**. The natural mean longevity of bdelloid rotifers is between 20-30 days, and sometimes it shows significant dependency on the environmental or laboratory conditions.
2. **Characteristic micro-ethology**. Our aquatic bdelloid species are capable of swimming and anchoring themselves to various surfaces. The head carries the peculiar rotatory apparatus, which is responsible for the movement (swimming) and the sessile feeding by its cilia. Rotifers have sensory structures, including photoreceptors [Fontaneto and de Smet, 2015]. We noticed that the animals reacted to the intensely illuminated zones by evading them quickly. Based on this phenomenon a novel assay system was developed to monitor the behavioral responsiveness with high resolution and sensitivity. By this method, we could quantify the sensory-motor system response (i.e. reflex) and investigate the impact after exposure to different compounds at sublethal concentrations [Olah et al., 2017].
3. **Clonal reproduction and direct development**. According to careful estimation, bdelloid males have been extinct for more than 30 million years [Gladyshev and Arkhipova, 2010]. Bdelloids lay diploid eggs formed by mitosis, resulting in clonal lines [Fontaneto and de Smet, 2015]. According to the academic literature, there is no documented evidence of meiosis having a fundamental role in the bdelloid class [Birky, 2010]. In the same culturing conditions, these lines can be maintained

continuously, providing reproducibility and comparability between the experiments in an extended time interval. Unlike some invertebrate species and the sessile rotifers, there is no metamorphosis during development [Snell, 2014]. The young specimens hatched from eggs are identical to the adults in every aspect besides the reproductive activity and size, which was the base of our 'body size index' (BSI) calibration curve [Olah et al., 2017].

4. **Adaptive phenotypic plasticity**. Bdelloids have a unique ability to enter dormancy in response to environmental and/or predatory stress, in the form of desiccation. In this phase, they can survive long periods and can recover after weeks or even years with no changes in their natural active lifespan [Ricci and Fontaneto, 2009; Wilson and Sherman, 2010]. Their insensitivity to physical stress and sensitivity to chemical agents allows wide-scaled experimental possibilities in the fields of pharmacology- and aging research. Rotifers adapt successfully to the various types and amounts of nutrients that exist in their natural habitat [Ricci, 1984]. The natural degradation of organic materials is a process that results in different aggregates and precipitates, which represent potential nutrients for many microinvertebrates, e.g. for rotifers [Wallace and Snell, 2010]. Catabolic processes of all these available organic mass resources are a unique property of some rotifers [Castro et al., 2005]. Their capability to use the most unconventional food sources are useful for studying the effect of these compounds as nutriments on rotifer lifespan and aging. To determine the changes related to the lifespan, we defined various morphological viability markers: (i) normal anatomy and the active locomotion (motility) of the body; (ii) general movement within the body; (iii) naturally red eyes. We considered a rotifer specimen dead when the following features were all discernible: lack of motility when touched by the tip of the micro-pipette, the loss of red color of the eyes, loss of the telescopic reflex, and the appearance of fragmentation inside the body [Olah et al., 2017].
5. **No ethical concern.** Bdelloids are microinvertebrates; therefore, according to the current ethical regulations, no specific ethical permission is needed for the experiments.

1.3. Electron deprivation and lifespan

Different factors of environmental stress can induce homeostasis disruption in biological systems, which is reducing the survival of relevant living organisms; however, applying mild

stress *in vitro* or *in vivo* could result in adaptive response of cells and/or animals [Calabrese and Baldwin, 2002; Lajqi et al., 2019]. Hormesis extends lifespan through the induction of mild redox stress [Cypser and Johnson, 2002], which improves energy metabolism, activates antioxidant mechanisms, and represses reactive oxygen species accumulation [Hyun et al., 2006a; Rea et al., 2007; Weithoff, 2007; Szalárdy et al., 2015]. Nutrient deprivation can be a stressor *per se* due to normal caloric restriction and results in extended longevity [Sinclair, 2005; Gribble et al., 2014]. Amount of nutrition is associated with the redox state [Hyun et al., 2006a], and this restriction may cause cellular electron deprivation, impaired mitochondrial function, and suppressed NADH (nicotinamide adenine dinucleotide, reduced form) availability [Chandrasekaran et al., 2017]. The ratio of NAD^+ (nicotinamide adenine dinucleotide, oxidized form) and NADH molecules reflects the cytoplasmic redox state and is a relevant marker of cellular metabolism. Cell viability assays use different tetrazolium salts (e.g. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT; 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt, XTT) based on metabolic activity of cells (using endogenic NADH as substrate) from microbial to mammalian origin [Berridge et al., 2005]. Rotifers could be also an *in vivo* source of NADH for redox-based assays. Treatment with electron carriers and acceptors increases the lifespan of numerous organisms by increasing the cellular NAD^+/NADH ratio [Lin et al., 2004; Marcu et al., 2014]. According to Lin and her colleagues [2004], this ratio can serve a critical regulatory function, in their case to determine the lifespan of yeast cells [Lin et al., 2004]. The XTT (as electron acceptor) and PMS (phenazine methosulfate, as electron carrier) molecules are suitable manipulators of the cellular redox state [Berridge et al., 2005]. XTT has a positively charged quaternary tetrazole ring core containing four nitrogen atoms. It possesses a redox potential that is lower than the transmembrane potential, therefore promoting the transfer of intracellular electrons to the extracellular space [Paull et al., 1988; Scudiero et al., 1988]. The sulfonate groups provide a net negative charge, promoting extracellular exclusion and water solubility [Berridge et al., 2005]. The XTT reduction depends on the NADH level of the actively respiring cells forming a non-toxic, aqueous formazan [Comley and Turner, 1990]. Both PMS and XTT are widely used in combination as electron carrier and acceptor, respectively, in a colorimetric viability assay of diaphorase-catalyzed redox-cycling [Halaka et al., 1982], which indicates the cellular redox potential [Comley and Turner, 1990; Berridge et al., 2005].

1.4. Application of rotifers in pharmacology

In recent years the use of rotifers became emphasized as *in vivo* models for screening anti-aging [Snell et al., 2016, 2018], metabolic modulator [Datki et al., 2019] or adverse effects of pharmaceuticals [Park et al., 2018]. Screening of existing and approved by the United States Food and Drug Administration drugs with rotifers could list potential compounds with anti-aging effects [Snell et al., 2016].

The application of different exogenous beta-amyloid (Aβ) isoforms are widely used protocols and models of Alzheimer's disease (AD) that using simultaneously various *in vitro* and *in vivo* systems to reveal their exact effects [Datki et al., 2018]. Neurodegenerative disorders are predominantly initiated and intensified by the systemic enzyme resistance of toxic aggregated proteins and related pathological consequences. They could be regarded as phenotypes secondary to the progressive functional impairment of proteomes [Wyss-Coray, 2016; Makin, 2016; Wildburger et al., 2017]. Physiological and molecular basis of aging may be described as an extended accumulation accompanied by an attenuated clearance and degradation of misfolded peptides and proteins in the brain [Mawuenyega et al., 2010; Tomita, 2017]. The intramolecularly localized β-sheet conformation is strictly resistant to enzymatic degradation [Morell et al., 2008; Wang et al., 2008]. The Aβs are crucial molecules in aging-associated dementias, representing a starting point in the development of these diseases. There is an evident correlation between age-related pathologies and protein aggregation. Numerous studies were performed on human neuroblastoma cells [Datki et al., 2003; Poeggeler et al., 2005], invertebrates, rodents, and primates [Harkany et al., 2000; Kong et al., 2016, Sharma et al., 2017]; however, only one article aimed at describing the comprehensive effects of Aβ on a bdelloid rotifer, named *Philodina acuticornis* [Poeggeler et al., 2005]. This unique study by Poeggeler et al. [2005] reported the treatment of rotifers with beta-amyloid 1-42 (Aβ42) in order to test the efficacy of the LPBNAH (N-[4-(octa-O-acetyllactobionamidomethylene)benzylidene]-N-[1,1-dimethyl-2-(N-octanoyl)amido]-ethylamine N-oxide) antioxidant molecule against the supposed toxicity of the aggregates. In their final *in vivo* studies with rotifers the authors applied doxorubicin instead of Aβ42. This antitumor antibiotic compound gave more consistent results in the specimens. They could not prove the neurotoxic effect of Aβ42 in this model.

In another aspect of aging research, various studies and applications have confirmed that plant-originated adaptogens play an important role in human health. However, their clinical application and/or use in healthcare remains in the preliminary stage [Liao et al., 2018]. The comprehensive chemical and/or biological investigations have resulted in the study of their active components; moreover, the further possible applications of these plants [Zomborszki

et al., 2016]. The beneficial or toxic effects of these adaptogenic plants (e.g. *Panax ginseng*, *Withania frutescens*, *Leuzea carthamoides*, and *Rhodiola rosea*) have not yet been examined on rotifer models. Rotifer-based *in vivo* studies are suitable to investigate many compounds with their various effects. These animals have high sensitivity for measuring the pharmacological activities and toxicity of relevant drugs. The characterization of plant extracts using rotifer-based model may provide promising candidates for subsequent testing in vertebrate animal models.

2. Specific aims

The aims of the thesis were:

A. Investigation of aging and its modulating factors *in vivo*;
B. Application of bdelloid rotifers in redox-based aging model with real lifespan monitoring, optimized to specific requirements of the experiments;
C. Investigation and modulation of lifespan-influencing cellular redox processes;
D. Revealing the optimal combination of chemical compounds and biological processes to achieve extremely increased lifespan related to our bdelloid rotifer, *P. acuticornis*;
E. Interdisciplinary application of the adaptive capabilities of rotifers evoked by starvation-based *in vivo* electron deprivation for inducing exceptional catabolic processes of aggregated neurotoxins;
F. Screening potential phenotypic plasticity-modulating agents derived from plant extracts to establish the species-specific biological limits.

3. Material and methods

3.1. Materials

The cultures of rotifers were kept in standard cell culturing flasks (cat. no.: 83.3910.302, Sarstedt AG and Co., Germany). The anion and cation content of the distilled water (DW; Millipore type, ultra-pure, demineralized; prepared in our laboratory) based standard medium (mg/L): Ca^{2+} 31.05; Mg^{2+} 17.6; Na^{+} 0.9; K^{+} 0.25; Fe^{2+} 0.001; HCO_3^{-} 153.097; SO_4^{-} 3; Cl^{-} 0.8; F^{-} 0.02; H_2SiO_3 3.3.

For the one-housed individual setup, high purity paraffin oil (cat. no.: 18512-1L; Sigma-Aldrich) and SAGE tissue culture oil (cat. no.: ART-4008; Origio, Denmark) were applied.

The one-housed individual and population-based experiments were carried out either in 6-, 24-, 96- or 384-well plastic cell culture plates (cat. no.: CLS3526, CLS3516, CLS3695 and CLS3640 respectively; Costar, Corning Inc., USA).

For the two treatment phases, the PMS (cat. no.: P9625), the XTT (cat. no.: X4626) and the ascorbic acid (cat. no.: A1300000) were obtained from Sigma-Aldrich (USA). NADH (cat. no.: 10107735001, Sigma-Aldrich) was used to confirm the XTT reduction *in vitro*. For measuring the intracellular NADH levels we applied NADH Quantification Kit (cat. no.: K337-100, BioVision, USA).

The Aβ42 was synthesized and prepared in the Department of Medical Chemistry, University of Szeged (Hungary) on an Fmoc-Ala-Wang resin (cat. no.: 47644, Sigma-Aldrich) using Nα-Fmoc-protected amino acids with a CEM Liberty microwave peptide synthesizer (Matthews, NC, USA). For *in vivo* and *in vitro* investigations of the Aβ42 aggregates, we applied 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid dipotassium salt (BisANS; cat. no.: D4162) and Congo red (CR; cat. no.: C6277) dyes obtained from Sigma-Aldrich. For measuring the 'cellular reduction capacity', EZ4U Cell Proliferation Assay was used (cat. no.: BI-5000; Biomedica Hungary). To observe the gold-tagged beta-amyloid 1-42 (Au-Aβ42) in *P. acuticornis* rotifers with scanning electron microscope (SEM), we applied gold(III) chloride ($AuCl_3$ x $2H_2O$; cat. no.: 01216, Reanal, Hungary) and prepared Aβ42 aggregates (3 days). The preparation of rotifers required ethanol (EtOH; cat no.: 1.59010; Sigma-Aldrich) and paraformaldehyde (cat. no.: 1.04005; Sigma-Aldrich). The specimens were placed on the center of a round glass coverslip (diameter: 12 mm, thickness: 0.15 mm; 89167-106, VWR International, Houston, USA).

In protein-quantification assay the contents (in mM) of the basic medium are: NaCl (cat. no.: S7653) 115, KCl (cat. no.: P9333) 3, HEPES (cat. no.: H3375) 25, D-glucose (cat. no.:

G7021) 10, pH 6.5; ingredients were obtained from Sigma-Aldrich. The lysis buffer was basic medium supplemented with ethylenediaminetetraacetic acid (EDTA; cat. no.: E6758), sodium dodecyl sulfate (SDS; cat. no.: L3771) and leupeptin hydrochloride (cat. no.: L9783) pepstatin A; cat no.: P5318). For comparison of assays the Qubit Protein Assay Kit (cat. no.: Q33212, Thermo Fisher, USA) was applied. For the nucleic acid measurement the propidium iodide (PI; cat. no.: 81845; Sigma-Aldrich) was used.

Plants were purchased from the local market (*P. ginseng*) or collected from culture (*W. frutescens, L. carthamoides,* and *R. rosea*). Ginsenoside Rb1 (cat. no.: G0777) was purchased from HWI Analytik Gmbh (Tübingen, Germany). Withanolide A (cat. no.: 80556), withanolide B (cat. no.: 80557), and withaferin A (cat. no.: 89824) were purchased from Phytolab (Vestenbergsgreuth, Germany). Rosavin (cat. no.: SML0336), salidroside (cat. no.: 05410590), tyrosol (cat. no.: 188255), and cinnamyl alcohol (cat. no.: W229407) were purchased from Sigma-Aldrich. Rhodiosin (cat. no.: FR65344) was purchased from Carbosynth (Compton-Burkshire, UK). The 20-OH-ecdysone and ajugasterone were isolated in the Department of Pharmacognosy, University of Szeged, Hungary. The extracts stock solutions were prepared with aqueous form of the dimethyl sulfoxide (DMSO; cat. no.: D8418, Sigma-Aldrich).

3.2. General *in vivo* setups applied on bdelloid rotifers

Our work was performed on invertebrate bdelloid rotifer species, named *P. acuticornis*; therefore, according to the current ethical regulations, no specific ethical permission was needed. *P. acuticornis* was obtained from Hungarian aquavaristique. Our experiments were carried out in accordance with globally accepted norms: Animals (Scientific Procedures) Act, 1986, associated guidelines, EU Directive 2010/63/EU for animal experiments, and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Animal studies comply with the ARRIVE guidelines. The culturing (e.g. in standard medium), harvesting and monitoring methods of *P. acuticornis* have been reported in detail in our prior publication [Olah et al., 2017].

3.2.1. Culturing

Rotifer-related methods applied in our project were developed based on previous literature [Ricci, 1984] and have been described in our earlier publication [Olah et al., 2017]. In brief: The specimens were grown in a supervised and semi-sterile laboratory environment. The cultures of *P. acuticornis* were kept in cell culturing flasks in 15 mL standard medium (pH

7.5), which was changed every two days. New cultures were started from previous ones by transferring one *P. acuticornis* specimen in standard medium. The flasks were kept at room temperature (24 ± 1 °C) and under a light/dark cycle of 12/12 hours and were monitored daily under an inverted light microscope (Leitz Labovert FS, with 32 x to 1000 x magnification). The steps of rotifer manipulation techniques were implemented in a strictly precise way. Standard feeding consisted of 0.35 mL of prepared *Saccharomyces cerevisiae* stock solution (7.5 g of dried yeast in 300 mL standard medium) added to the culture after every medium change. The final concentration of yeast in the medium was 600 μg/mL.

3.2.2. Isolation

To start harvesting rotifers, the medium was poured off and the flasks were washed twice with standard medium, which was also removed either in Petri dish or in well plates without feeding. The dishes or plates were then left for 30 minutes to let healthy animals attach to the bottom; therefore, the surface was washed twice with DW and once with the standard medium. We chose approximately 5-day-old rotifers after hatching (determined by 'BSI' calibration curve; length 220 ± 10 μm and width 60 ± 5 μm). The ontogenetic phase of these animals are 1-2 days before the beginning of the reproductive (mature) stage. For all investigations, the isolated colonies were left in Petri dish and/or well-plates without feeding for 24 hours. After a medium change, the viable specimen with an empty digestive system were selected for treatment. Every step was monitored with light microscopy.

3.2.3. Application mode of microinvertebrates

3.2.3.1. One-housed individual

We developed a new microdrop culturing technique [Olah et al., 2017] to treat and observe one individual rotifer at a time. This method was adapted from human *in vitro* fertilization procedures [Nagy et al., 2012]. For this application, we used 24-well plates with artificially treated (with amide and carboxyl side chains) surface for tissue culture. To the bottom of each well of the plate a 30 μL microdrop of standard medium (pH 7.5) was added and was then covered with a 0.64 mL mixture (1:1) of the high purity paraffin oil and SAGE type tissue culture oil. These oils were standardly used in human *in vitro* fertilization as a protective barrier for embryonic cell cultures [Nagy et al., 2012] allowing normal gas diffusion between the microdrop and the environment, preventing evaporation and hypoxia. The harvested animals and the oil-covered water-drop setup were rested for 24 hours, separately. Next day the individuals were isolated from the Petri dish and they were placed

in microdrop by pipetting via 20 μL standard medium. The end volume of a microdrop with a one-housed specimen was 55 μL that contain the 5 μL stock solution of the respective drug. Oil thickness was 1 mm on the top of each drop and these plates were kept at room temperature during the experiments. The wells were observed two times per day until the animal was considered dead inside the drop.

3.2.3.2. Population

For assessing the effect of compounds, colonies of *P. acuticornis* rotifers were isolated with the standard process and let to rest for 24 hours before treatment.

3.3. Preparation and treatment protocols

3.3.1. Biological and chemical electron deprivation

The treatment period (**Figure 2**) comprises of two different phases of combined nutrient and chemical drug-based redox modulation. Before application, the impact of nutrient level (**Figure 3**) on the longevity of *P. acuticornis* were examined (n = 24, one-housed rotifer per dose of nutrient indicated). The applied nutrient (homogenized yeast) concentrations (μg/mL) were: 0 (starved), 50 (extreme caloric restriction; ECR), 200 (normal caloric restriction), and 600 (standard feeding). The optimized (**Figure 4**) and combined electron deprivation method was the following: (**1**) *Preselection phase*: related to rotifer populations (n = 35, well; 100±5 individual/well) and middle-aged (15 days old) one-housed animals (one specimen/well) were administered PMS (5 μM) combined with XTT (1000 μM) under total food deprivation for 72 hours in darkness. We applied a parallel control for XTT (**Figure 5**), the ascorbic acid (1000 μM), a well-known antioxidant [Szalárdy et al., 2015]; (**2**) *Conditioning phase*: in the first step, the dead animals and the remnants of the treatment agents were eliminated from the wells by washing. The selected surviving individuals were treated permanently (with the medium changed in every second day) with 50 μM XTT or ascorbic acid (corresponding to their respective preselection treatment; n = 90) under standard feeding (600 μg/mL) or ECR (50 μg/mL) (**Figure 6**). The respective treated-untreated controls underwent chemical/dietary preselection, but received no chemical treatment in the second phase or received no treatment at all (untreated-untreated).

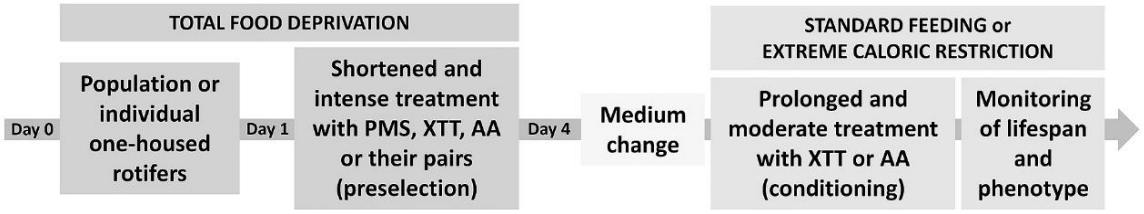

Figure 2. ***The schematic work-flow of the redox modulating protocol.*** *Abbreviations: AA = ascorbic acid; PMS = phenazine methosulfate; XTT = 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt.*

3.3.2. Preparation of Aβ42 and treatment of rotifers

The synthesis and characterization of relevant Aβ42 peptide was manifested as previously described by Bozso et al. [2010] with some minor modifications: the concentrations of the stock solutions were 1 mg/mL (DW); the aggregation period was 3 hours or 3 days (24 °C, pH 3.5) and the neutralization (to pH 7.5) was performed with sodium hydroxide (NaOH, 1 N; cat. no.: S8045, Sigma-Aldrich) [Kalweit et al., 2015]; the final concentrations were 100 µg/mL. Before treatment (by micropipette) of entities with Aβ42, the stock solutions were ultrasonicated (Emmi-40 HC, EMAG AG, Mörfelden-Walldorf, Germany) for 10 min at 45 kHz for sterilization and homogenization.

The samples of Aβ42 (1 mg/mL) and $AuCl_3$ (2.8 mg/mL) for the SEM detection were prepared with DW. The aggregated Aβ42 (for 3 days) solution was mixed for 2 h with the $AuCl_3$ solution in a 1:4 molar ratio, according to the number of Au-binding sites of monomeric Aβ42 (three histidine residues and one methionine). To remove excess Au ions, we used two rounds of centrifugation (25,000 x g for 10 min at 24 °C) with supernatant substitution; furthermore, the NaOH (1 N) was used to adjust the pH to 7.5. The peptide amount of the pellet was determined by Qubit Protein Assay Kit. The 15 days old (middle-aged) harvested rotifers were starved (complete food deprivation) for 2 days. After washing the animals into 6-well plates (containing 10^4 entities per well), each well was treated with Au-Aβ42 complex in a dose of 100 µg/mL for 1 day. The plates were decanted and washed two times with standard medium and after incubation (6 hours; the time to empty the digestive tracts) the wells were washed again. These treated populations were fixed and dehydrated with 96% EtOH at –75 °C (5 min), followed by a partial rehydration with 30% EtOH at 24 °C (30 min). After fixation with 1% paraformaldehyde (30 min) the wells were washed two times with DW. Collected rotifers were transferred to the center of a round glass

coverslip (VWR International) to dry. Separately, the surface of dried rotifer samples was not covered with nano-gold. The structural integrity of the specimen's bodies was monitored and recorded with digital photography, applying a Nikon D5500 camera.

3.3.3. Investigated plant-extracts

Extraction of investigated materials (1 g) were carried out with 10 mL of extraction solvent (50% of ethanol for *P. ginseng*, *W. frutescens*, *L. carthamoides* and 70% for *R. rosea*) and were ultrasonicated for at 24 °C (10 min). By solvents evaporation the extracts were dried. The investigated compounds were: Ginsenoside Rb1, withanolide A and B, withaferin A, rosavin, salidroside, tyrosol, cinnamyl alcohol, rhodiosin, 20-OH ecdysone and ajugasterone.

For *in vivo* screening (**Figure 12**) the stock solutions (1 mM) of plant extracts were prepared with 1% aqueous DMSO. In these experiments, the stock solutions were added to standard medium where the final concentration was 100 μM with 0.1% DMSO content. After 24 hours of the normal isolation process, the rotifers were treated in a 384-well plate (n = 16/well/compounds) and were monitored daily. The untreated control (UC) group was grown in standard medium, while the control (C) group was kept in standard medium containing 0.1% DMSO. The rotifers were starved (without feeding) during the toxicity interval (72 hours); however, from the fourth day began a period with ECR (food supplementation), which is enough for surviving, but cease the reproduction. The condition of the treated specimens was compared to the C group.

3.3.4. Protein quantification of biological samples

Microinvertebrates served as biological samples for testing new, total protein quantification method using the fluorescence dye BisANS. The source of protein for the BisANS assay was the *P. acuticornis* and yeast samples. The isolation process was the following: the live animals or the intact yeast cells were centrifuged at 3000 x g, at 24 °C (10 min). The supernatants were decanted and the pellets were resuspended in lysis buffer ($2x10^5$ animals/mL or 10^6 yeast cells/mL). The prepared samples were frozen to -75 °C (60 min). After thawing, they were ultrasonicated (10 min). The isolates were centrifuged at 1500 x g (5 min) to eliminate the cell debris or the exoskeletons from the homogenates.

3.4. Optical imaging- and detection assays

3.4.1. Microscopy-based experiential monitoring (phenotypical markers)

3.4.1.1. Light microscopy

The experiments were monitored by Leitz Labovert FS microscope, and the representative photographs were taken by Nikon DSLR camera applied to the microscope in different magnification.

3.4.1.1.1. Number of rotifers alive

This assay provides the mortality rate of rotifers after treatment at a representative date. The experimental setup was the following: n = 35, well with 100 ± 5 rotifer/well (**Figure 5**); n = 90, one-housed individual per dose indicated (**Figure 6**); n = 16, well with one population/well (**Figure 12**). The survival number of the control group in the specific experiments was regarded as 100%.

3.4.1.1.2. Body size index

In our previous studies [Olah et al. 2017; in supplement], we created a special calibration curve to determine the age of *P. acuticornis* rotifers, that were isolated from standard cultures. Determination of the rotifer age was calculated by the measured means of their daily BSI (%) = *maximal 'length* x *width' of body* (μm; n = 300 animals). The dimensions of hatched offspring are 95 x 25 μm (6%), while the maximal dimensions of senescent individuals are 350 x 110 μm (100%). Three phases of life can be detected concerning these rotifers: **1**) juvenile (1-6 days), a pre-reproductive phase without egg production; **2**) mature reproductive (7-27 days), a reproductive phase with egg production; **3**) senescent (≥ 28 days), post-reproductive phase without egg production. The experimental case numbers were the followings: n = 35 (**Figure 7**), n = 50 (**Figure 10**), n = 15 (**Figure 12**), one-housed individuals in all cases.

3.4.1.1.3. Mastax contraction frequency

The mastax of rotifers is part of the digestive system with a powerful muscular wall. This pharynx-type organ contains tiny, calcified, jaw-like structures named trophi. The periodic opening and closing function of the mastax specialized to shred food. To evaluate and standardize the viability of one-housed rotifers in the experiments, we developed and presented in our previous work [Olah et al., 2017] the 'mastax contraction frequency' (contraction/sec), as a quantitative viability marker. The experimental lineups were the following: n = 35 (**Figure 5**); n = 35 (**Figure 7**); n = 24 (**Figure 10**); n = 24 (**Figure 12**) one-housed individuals in all cases.

3.4.1.1.4. Normalized mean lifespan

This assay measures the mean survival lifespan of rotifers under a period of treatment. The experimental case number was the following during the Aβ42 screening (100 μg/mL): n = 30, one-housed individual (**Figure 10**). The mean lifespan of the control (standard-fed) group was regarded as 100%.

3.4.1.1.5. Bright light disturbance

The animals reacted to intensely illuminated zones by evading them immediately. For measuring the sensory-motor reflex the microdrop setup was placed under a Hund Wetzlar H500 microscope (ocular: PK 20 x /8/; objective: HW-A 10/0.25, 160/-) with the light source illumination set to 20 lux in relation to one well of the plate. We identified the active specimen (i.e. a surface-attached animal exhibiting mastax chewing and beating of the coronal cilia) and we adjusted the well so that the animal was in the middle of a 0.5 mm^2 illuminated field created by narrowing and masking the light source of the microscope with the aperture mechanism. After 30 sec, if the specimen was still in place and moving coronal cilia, we increased the illumination to 40,000 lux and measured the reaction of the rotifer. Two reactions (markers) were recorded: (**1**) 'bright light irritation' (BLI), which is the total period that the animal is crawling or contracted, recapitulating the unfed-state phenotype where cilia are not used for motility. When the treated rotifer remains in a cilia-motile (fed-state) form, it is considered insensitive to the intense light stimulation; (**2**) 'bright light avoidance' (BLA), which is the period spent in the illuminated area (time to leave the designated platform). We defined the 'bright light disturbance' index: $BLD\ (\%) = (BLI/BLA) \times 100$. As a health index, the 'bright light disturbance' approaches 100% when it reflects the maximum sensitivity and reaction to light of rotifers. The rotifers had a maximum time interval (5 min) to leave the illuminated area in the 'bright light disturbance' test. The treated animals were considered to be unable to escape and assigned a 'bright light disturbance' of 0. The experimental setup was the following: n = 20, individual (**Figure 10**).

3.4.1.1.6. Reproduction capacity

The viable egg production of rotifers in standard and/or restored conditions (n = 35-35 for both groups, one-housed individuals) and the kinetics of reproduction (number of descendants; n = 10 flasks, started from 5-5 rotifer of both phenotype) was measured on Day 10 (**Figure 7**).

3.4.1.1.7. Freezing tolerance

This assay compares the tolerance and recovery of phenotypes exposed to freezing conditions. Ten-ten flasks (2000 ± 100 individuals; medium fully-decanted) of differently treated and conditioned rotifers were frozen for a day at –75° C. The number of survivors (**Figure 7**) was counted 24 hours after thawing (using 20 ml medium per flasks at room temperature).

3.4.1.1.8. Congo red labeling of exogenic Aβ42

For the detection of exogenic Aβ42 in the rotifers body (primary in the digestive system), the aggregated (3 days) peptide (100 μg/mL) was labeled with CR (50 μM; 1 hour) dye. The methods described by Klunk et al. [1999] and Datki et al. [2004] were used for these experiments. The CR stock solution (10 μL; 0.5 mM) was added to every aggregate-containing tube (490 μL; 100 μg/mL) and the relevant mixtures were incubated at 24 °C (20 min) and shaken every 5 min (at 50 rpm for 10 sec). The centrifugation was executed at 25,000 x g for 15 min. The supernatant was removed and the pellet was resuspended in standard medium (0.5 mL).

3.4.1.2. Fluorescence microscopy

The 5 days old (juvenile) animals were identified based on their 'body size index' as read from a calibration curve (size per age) previously described [Olah et al., 2017]. For the treatments in 96-well plate of the preselected and 1-day starved rotifers, we applied unlabeled Aβ42 aggregates (100 μg/mL; incubated 3 hours and 3 days) as 'food' source. After 12 days the well-contents were changed to dyed Aβ42, aggregated for 3 hours (*in vitro* marked; 10 μM BisANS fluorescent dye for 30 min) or 3 days (*in vitro* marked; 50 μM CR dye for 1 hour). Applying 5 hours treatment ('feeding'), we detected the optical signals (magnification was 200x) in the digestive system (stomach and intestine) of individuals by an inverted fluorescence microscope (Olympus IX71, OLYMPUS). The representative digital photographs demonstrate the localization of Aβ42 in the rotifers compared to untreated, standard fed (600 μg/mL yeast) and unfed (starved) controls (**Figure 11**). Unfed individuals marked with these two dye molecules (control background without Aβ42 treatment) had no signal (image not shown).

3.4.1.3. Scanning electron microscopy

The quality-based (e.g. morphological integrity) selected bodies were subjected to SEM (Zeiss EVO MA 10, Carl Zeiss). This protocol is a modified version of work of Guerrero-Jiménez et al. [2013]. The carrier coverslip of samples was fixed onto a stub by a double-sided carbon tape. The high-resolution structure of the rotifers was observed and documented with the SEM (**Figure 11**), operating on 8-mm working distance at 10 kV and using a backscattered electron detector in various pressure modes at 30 Pa. The white balance of SEM photographs was normalized to the unspecific background.

3.4.2. Plate-reader-based optical detection (physiological markers)

3.4.2.1. Absorbance (optical density)

3.4.2.1.1. Validation of NADH dependency of PMS/XTT system

The reduction of XTT was measured (**Figure 4**) both *in vitro* (cell-free; n = 5 well) and *in vivo* (middle-aged live rotifers; n = 24, well; 100 ± 5 rotifer/well). In the *in vitro* experiments PMS (5 μM), XTT (1000 μM) and NADH (100 μM) were administered alone or in their combination (incubated for 7 hours in darkness, 24 °C, pH 7.5; working volume: 200 μL/well) in standard medium. Experiments were run in triplicates. Parameters of *in vivo* investigations were the same as those of the *in vitro* ones, except for the following: rotifers were treated for 24 hours, and no exogenous NADH was used. The optical density was measured *in vitro* and *in vivo* with an absorbance plate-reader (Spectramax Plus 384, Molecular Science) in a 96-well plate at 492 nm with 630 nm as a background reference. The readings were normalized to the background.

3.4.2.1.2. Cellular reduction capacity of rotifers

To measure the redox activity of multicellular specimens, the EZ4U Cell Proliferation Assay (non-radioactive cytotoxicity-, cell proliferation- and reduction capacity assay using XTT solution) was applied. This assay is widely used on cell cultures and tissues [Berridge et al., 2005], but to our knowledge, there is no data about applying it to intact invertebrates. We modified the standard protocol, given by the manufacturer, to measure the 'cellular reduction capacity' of rotifers (n = 24, well with normalized absorbance to rotifer number; **Figure 10**). To preventing toxicity, 20 x diluted XTT solution (475 μL standard medium with 25 μL XTT kit-stock solution per well) was used. The plates were incubated for 24 hours without direct light at 24 °C. The supernatant of the all well was then transferred to a 96-well plate. The absorbance was measured by an absorbance plate-reader (Spectramax Plus 384) set at 491/630 nm. The readings were normalized to the number of rotifers in each well. The

percentages of the results were calculated, 100% was defined as the ratio of absorbance (OD) and a matching number of animals in untreated control wells.

3.4.2.1.3. Endogenic NADH quantification

The intracellular NADH levels (n = 10 well/group indicated) were measured (absorbance: 450 nm) with NADH Quantification Kit, according to the protocol of the manufacturer. The readings were normalized exclusively to the number of survivals of preselection (**Figure 5**).

3.4.2.2. Fluorescence (emission)

3.4.2.2.1. BisANS-based protein quantification assay

The samples derived from bdelloid rotifer (*P. acuticornis*) or yeast (*S. cerevisiae*) were isolated in lysis buffer, where the basic medium was supplemented with the chelator EDTA, the detergent SDS and inhibitors (leupeptin hydrochloride and pepstatin A). In the protein assays the lysis buffer was used as a blank and the BisANS fluorescent dye (working concentration 50 μM; pH 6.5) in itself provided the unspecific background. In every case 0.5 μL of protein samples were added to the wells, where the working volume was 50 μL/well (1:100 dilution). The readings were carried out in a 96-well half-area plate (n = 12, well; **Figure 8**) using NOVOstar plate-reader (BMG Labtech). The extinction/emission was set to 405/520 nm and the number of flash per well and cycle was 30. Before the first cycle, orbital shaking was applied, the shaking time was 3 secs and the plate-rounds per minute were 600. The gain adjustment was set to both lysis buffer and BisANS background. The buffer readings were normalized to the dye background, and they were averaged with the dye readings. Commercially available Qubit Protein Assay Kit was used to validate our readings, following the protocol given by the manufacturer.

3.4.2.2.2. Nucleic acid/protein ratio detection assay

To measure the amount of nucleic acids in normal- and super rotifers, PI (1 mM stock solution) was used (**Figure 9**). In all cases 50 μL rotifer sample (100 ± 10 specimen on 13 mm^2), 70 μL BisANS (50 μM final concentration) and 0.6 μL PI (5 μM final concentration) were added to each well of 96-well plates. The measurements were run after a 10-min incubation period. The settings and the instrument were the same as described in the 3.4.2.2.1. section. The extinction/emission of PI was 530/620 nm. The readings were interrelated with rotifer number/well, and this normalized fluorescence intensity was presented.

3.5. Statistics

Statistical analysis was performed with SPSS 23.0 software for Windows (SPSS Inc., IL, USA) using one-way ANOVA with Bonferroni *post hoc* test or with GraphPad Prism 7.0b software (GraphPad Software Inc., CA, USA) that was used for the illustration and statistical analysis. Kaplan-Meier curves (log-rank; Mantel-Cox) were applied to present the survival of different treated groups. Data was presented in mean ± standard error of the mean (S.E.M), the levels of significance were $p^{*,\#} < 0.05$; $p^{**,\#\#} < 0.01$; $p^{***,\#\#\#} < 0.001$; $p^{****,\#\#\#\#} < 0.0001$.

4. Results

4.1. Survival of rotifers under different dietary conditions

We examined the impact of nutrient level on the longevity of *P. acuticornis* (**Figure 3**). Starved rotifers had the shortest lifespan (median = 13.5 days, log-rank $p < 0.0001$), whereas the group under normal caloric restriction had the longest (median = 48 days, log-rank $p < 0.0001$) one. During standard feeding, normal lifespan (median = 33.5 days, log-rank $p < 0.0001$) was observed. The longevity of rotifers under ECR was significantly shorter (median = 23 days, log-rank $p < 0.0013$) compared to the standard-fed counterpart.

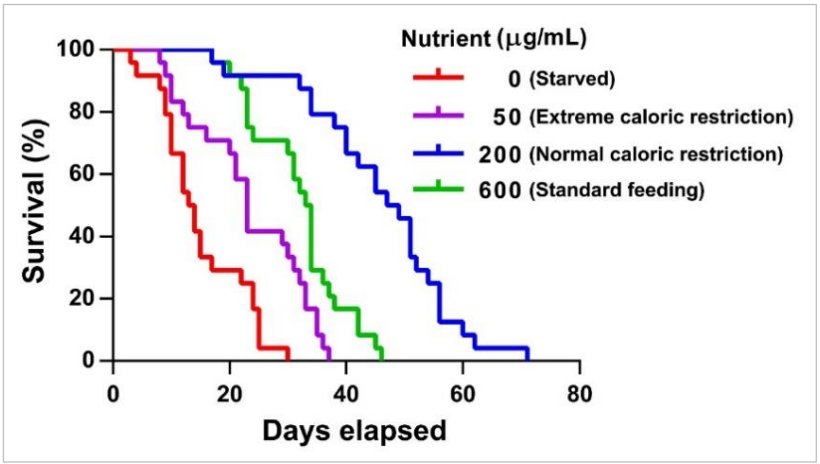

Figure 3. ***Survival of rotifers under various dietary conditions.*** *The lifespan was the shortest under starvation (0 µg/mL), and the longest under normal caloric restriction (200 µg/mL). Standard feeding (600 µg/mL) resulted in normal lifespan. The longevity of rotifers under extreme caloric restriction (50 µg/mL) was significantly shorter compared to the standard-fed control. Kaplan-Meier survival curves were presented (n = 24, one-housed rotifer per dose of nutrient indicated). The Mantel-Cox log-rank test was used to assess significance.*

4.2. Redox properties of XTT *in vitro* and *in vivo*

The reduction of XTT was measured both *in vitro* (cell-free) and *in vivo* (middle-aged live rotifers) and was presented on **Figure 4**. To confirm the reliability of the PMS- and XTT-containing *in vivo* system, we tested this combination (cell-free) in the presence of NADH. The extra- and intracellular NADH is the reducing (electron) source for extracellular XTT reduction. The PMS, as an intermediate electron carrier markedly promotes this reaction (**Figure 4**). Without NADH, the reduction of XTT did not happen *in vitro* (not shown). The XTT alone was capable of forming formazan *in vivo* after 24 hours incubation.

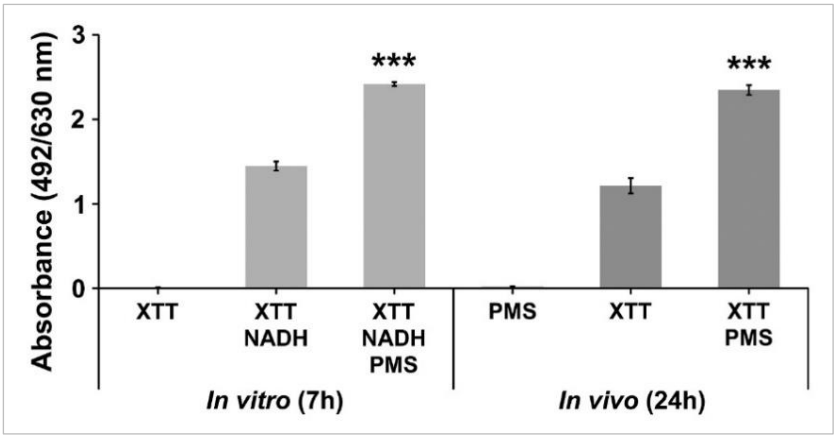

Figure 4. ***Validation of XTT reduction in vitro and in vivo.*** *The reaction was measured in vitro in the presence of XTT (1000 µM), NADH (100 µM) and PMS (5 µM) after 7 hours incubation. The XTT reduction capacity of rotifers was recorded in vivo after 24 hours incubation. This absorbent dye alone was capable of forming formazan in vivo. The error bars present S.E.M. One-way ANOVA with Bonferroni post hoc test was used for statistical analysis, the level of significance was p*** < 0.001; in vitro: n = 5 (well) in triplicate; in vivo: n = 24 (well; 100±5 rotifer/well) in triplicate. (*, significant difference from the parallel groups within the respective category). Abbreviations: NADH = nicotinamide adenine dinucleotide, reduced form; PMS = phenazine methosulfate; XTT = 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt.*

4.3. Intense redox state modulation as a preselection mode of rotifers

Our *in vivo* viability experiments consisted of two main treatment phases (**Figure 2**) using middle-aged rotifers. First, we preselected individuals by testing the electron deprivation tolerance via treating with PMS, XTT, ascorbic acid and their combinations (PMS/XTT and PMS/ascorbic acid) under total food deprivation for 72 hours (**Figure 5**). The antioxidant drug ascorbic acid [Szalárdy et al., 2015] was used as a parallel control for XTT. The PMS treatment caused significant decrease in all measured parameter of viability (live rotifer numbers, ‘mastax contraction frequency’ and NADH level) compared to untreated control (UC) since the reduced form of PMS can alone generate reactive oxygen species in a dose-dependent manner [Gabriel et al., 2015]. The intense chemical electron deprivation caused significant toxicity under starvation in the preselection phase. Neither XTT nor ascorbic acid alone had any effect on viability markers compared to UC. The combinations of PMS/XTT or PMS/ascorbic acid resulted in significantly lower decreases in the ‘number of live rotifers’ and intracellular NADH levels compared to PMS treated group. The same decrease occurred to ‘mastax contraction frequency’, a validated index of rotifer viability. We presume that the protective role of XTT and ascorbic acid (via facilitation of NADH metabolism) might be secondary to an irreversible capturing of electrons from PMS. The data of live rotifers,

'mastax contraction frequency' and intracellular NADH levels show positive correlation in their tendency (**Figure 5**).

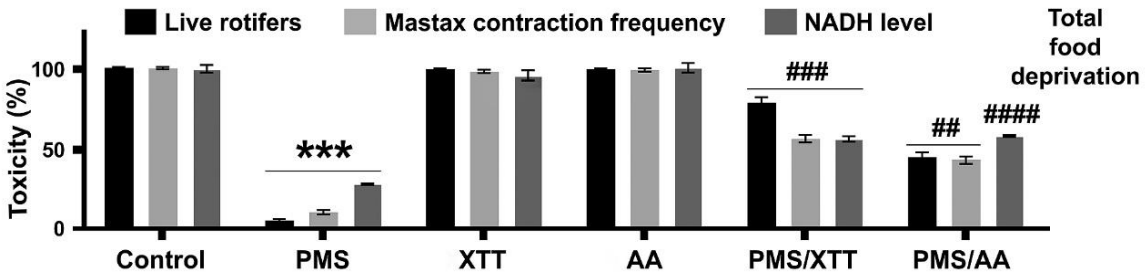

Figure 5. ***The effect of preselection on the survival and phenotype of P. acuticornis.*** *The columns present the percentage values of live rotifers (n = 35, well; 100 ± 5 rotifer/well), mastax contraction frequency (n = 35, one-housed individual) and the cellular NADH level (n = 10 well/group indicated). The treatment concentrations were the following: 5 μM (PMS), 1000 μM (XTT or ascorbic acid). The error bars present S.E.M. One-way ANOVA with Bonferroni post hoc test was used for statistical analysis, the levels of significance were* $p^{\#\#} < 0.01$, $p^{***,\#\#\#} < 0.001$ *and* $p^{\#\#\#\#} < 0.0001$ *(*, significant difference from untreated, XTT and AA controls;* $^{\#}$*, significant difference from the group receiving PMS alone). Abbreviations: AA = ascorbic acid; NADH = nicotinamide adenine dinucleotide, reduced form; PMS = phenazine methosulfate; XTT = 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt.*

4.4. Prolonged and moderate redox conditioning of preselected survivals

The preselection phase-survived *P. acuticornis* rotifers were subjected to the next phase (**Figure 2**) of experiments. The survival of rotifers was measured under various treatment conditions applying standard feeding (**Figure 6A**) or ECR (**Figure 6B**). This period was associated with a permanent (for several months) and moderate (low-dose) chemical electron deprivation accompanied by standard feeding or ECR. Extreme low nutrition alone is capable of triggering electron deprivation [Weithoff, 2007; Gribble et al., 2014]. A pronounced longevity was observed in the corresponding XTT-treated group (PMS/XTT/total food deprivation-preconditioned) under standard feeding (**Figure 6A**) during second phase; however, in the corresponding ascorbic acid-treated groups (PMS/ascorbic acid/total food deprivation-preconditioned) showed a slight superiority of ECR over standard feeding. Additionally, when ECR was combined with XTT treatment in preselected (PMS/XTT/total food deprivation-preconditioned) rotifers, it synergistically extended the lifespan to an extreme extent (up to 182 days) compared to the untreated-untreated control group. (**Figure 6B**) The XTT and ascorbic acid both significantly (though

less remarkably) increased the lifespan of preconditioned rotifers compared to the untreated-untreated controls (**Figure 6A** and **B**) and the respective treated-untreated controls (not shown), with no remarkable influence of diet.

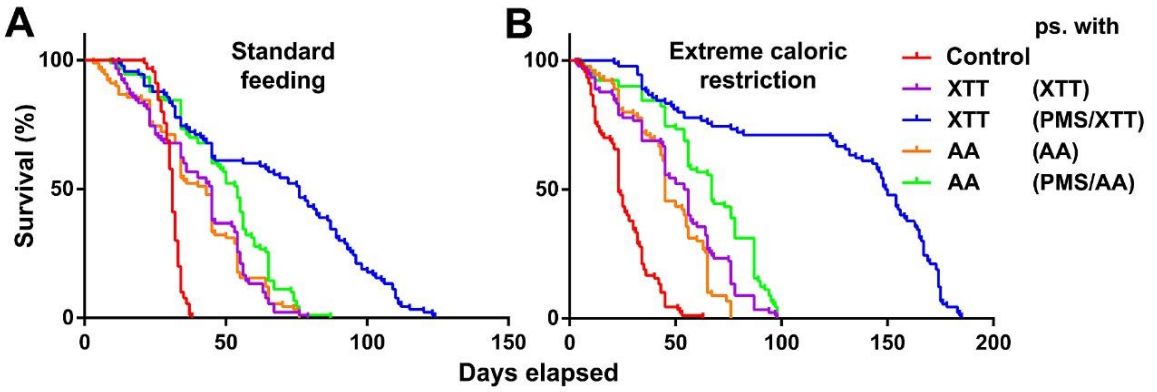

Figure 6. ***The effect of conditioning on the survival of rotifers****. Treatment of preselected surviving specimens with moderate chemical electron deprivation (XTT or AA; 50 µM per dose indicated) under standard feeding (600 µg/mL) (**A**) or under ECR (50 µg/mL) (**B**) significantly extended their lifespan. Kaplan-Meier survival curves are presented (n = 90, one-housed individual per dose of agent indicated). Abbreviations: AA = ascorbic acid; PMS = phenazine methosulfate; ECR = extreme caloric restriction; XTT = 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt.*

4.5. Characterization of electron-deprived and refed 'super rotifers'

The combination of PMS/XTT (under total food deprivation) preselection followed by XTT (under ECR) made a synergistic positive impact on lifespan and phenotype of preselection survivors resulting 'super rotifers', a nickname given by us reflecting their collection of unique properties. Middle-aged normal (15 days old) and redox-manipulated senescent (110 days old) animals were followed-up for 10 days under standard feeding conditions without any chemical treatment to compare their phenotype and viability markers (**Figure 7**). There were significant differences in the size (**Figure 7A**) and physiology (**Figure 7B**). First, the 'body size index' of super rotifers was higher than that of normal rotifers. Interestingly, the 'mastax contraction frequency' of super rotifers was lower compared to normal rotifers, probably as a result of altered neuromuscular function due to increased size. More surprisingly, these senescent (110-day-old) animals were capable of laying viable eggs (reflecting an effect on the reproduction phase); moreover, the numbers of descendants were significantly higher after the 10-day period than in normal rotifers. Furthermore, we assessed

how super rotifers tolerate freezing conditions (one week), monitoring the recovery rate of cultures for 24 hours after being thawed. We found significantly more survivors in the super rotifer (5.0 ± 0.52 individuals) than in normal rotifer cultures (2.6 ± 0.40 individuals). After reintroduction of standard feeding and thus cessation of permanent conditioning (with ECR and low-dose XTT treatment) at 110 days of age, the 'remaining lifetime' of super rotifers was restored near to their original expected longevity (25 ± 4.2 days) until their natural death.

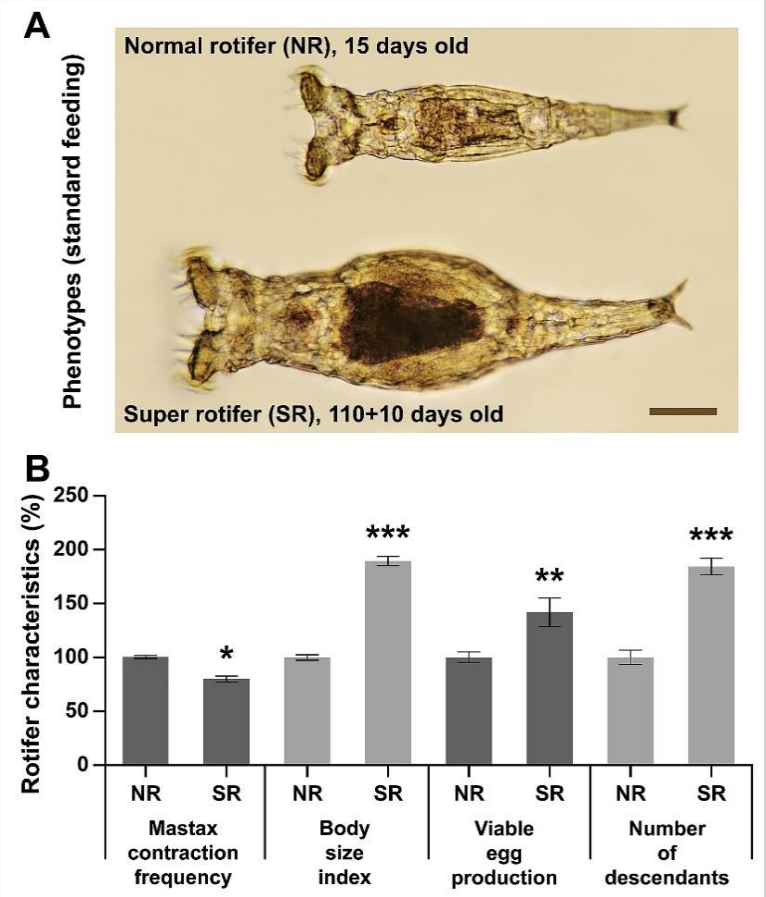

Figure 7. ***Characterization and comparison of middle-aged NR and senescent SR.*** *The representative illustration of the difference in body size between NR (middle-aged, 15 days old) and SR (senescent, 110 days old) is provided (**A**; scale bar: 50 µm) after a 10-day period of standard feeding without any chemical treatment. The viability markers of SR compared to NR specimens (mastax contraction frequency, body size index, viable egg production: n = 35-35 for all features in both groups, one-housed individual; the number of descendants in the population: n = 10-10, flasks; started from 5-5 NR or SR animals per each) significantly changed (**B**). The error bars present S.E.M. One-way ANOVA with Bonferroni post hoc test was used for statistical analysis, the levels of significance were p* < 0.05; p** < 0.01, and p*** < 0.001. (*, significant difference from NR within the respective category). Abbreviations: NR = normal rotifer; SR = super rotifer.*

4.6. Confirmation of eutely status of enlarged entities

4.6.1. A novel protein quantification assay based on BisANS fluorescent dye

To determine the optimal applicability of our BisANS assay, we tested the compatibility of the dye with interfering agents on complex biological samples such as a *P. acuticornis*

rotifers and yeast. Measured protein samples were isolated with lysis buffer and their protein contents were determined both with Qubit- and novel BisANS-assay. The Qubit-based measurements were used to validate our BisANS type readings. No significant difference was discovered between the data measured by our assay and the Qubit (**Figure 8**).

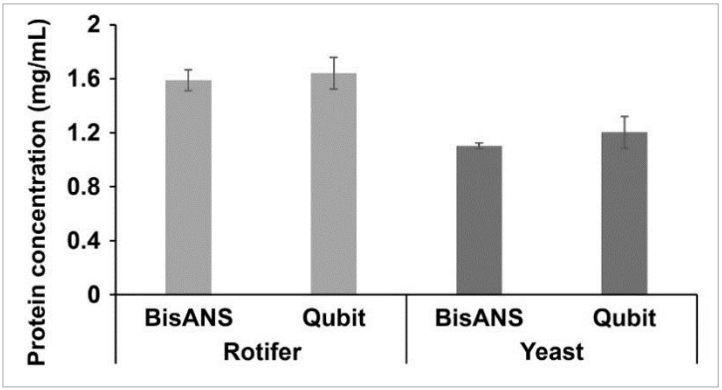

Figure 8. ***Validation and application of BisANS assay on protein samples isolated in lysis buffer.*** *Isolated protein samples from rotifers or yeast ($2x10^5$ animals/mL or 10^6 yeast cells/mL) were paralelly measured by BisANS assay (n = 12, well) and with Qubit assay (n = 12, PCR tubes). The means are presented by the columns and the error bars show the S.E.M. For statistical analysis, one-way ANOVA was used followed by the Bonferroni post hoc test. Abbreviation: BisANS = 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid dipotassium salt.*

4.6.2. Comparison of protein and nucleic acid amounts between normal- and super rotifer

We measured the protein and the nucleic acid amount of normal- and super rotifers simultaneously. The BisANS assay showed an increase in the normalized fluorescence intensity in the super rotifers compared to the controls. With the PI-based assay we detected no difference between the content of the nucleic acid of normal- and super rotifers (**Figure 9**) suggesting the eutely. The protein content of super rotifers was significantly higher than normal rotifers. The nucleic acid amounts did not differ. These results indicate the enlargement of super rotifers was carried out via eutely.

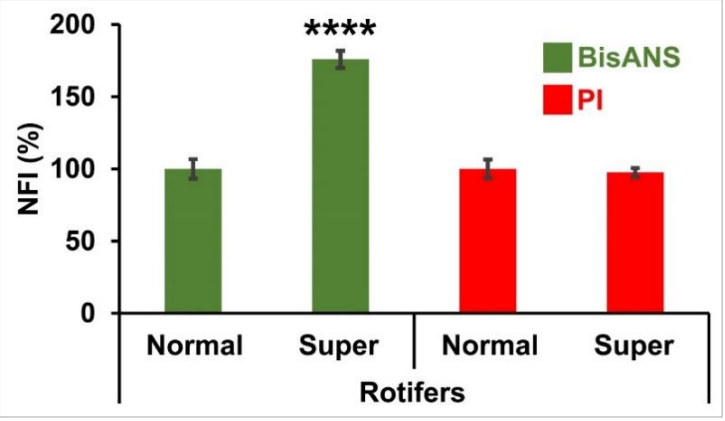

Figure 9. ***Detection of protein and nucleic acid quantity of normal and super rotifers.*** *Application of BisANS assay (50 µM) simultaneously with PI (5 µM) on rotifer samples (n = 6, well; 100 specimens on 13 mm^2). The normalized means are presented by the columns and the error bars show the S.E.M. One-way ANOVA with post hoc Bonferroni test was used for the statistical analysis. Level*

*of significance is p**** ≤ 0.0001. Abbreviations: BisANS = 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid dipotassium salt; NFI = normalized fluorescence intensity (normalized to the rotifer number/well); PI = propidium iodide.*

4.7. Beneficial effect of aggregated Aβ42 used as rotifer-food under caloric restriction

To understand the phenomenon related to less 'consistent results' with Aβ42 toxicity in bdelloid rotifers published by Poeggeler et al. [2005], we revealed the effect of oligomer/fibril-aggregates on one-housed *P. acuticornis*.

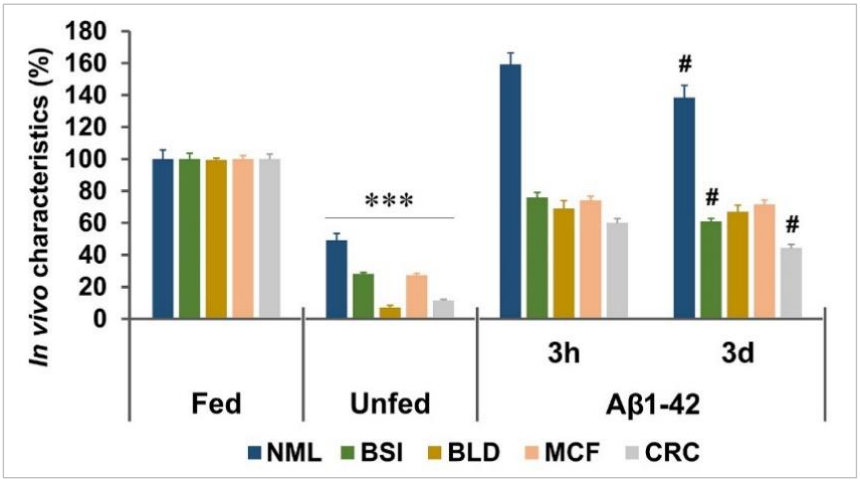

Figure 10. ***Viability assays of P. acuticornis treated with aggregated Aβ42****. The NML (n =30, one-housed individual) of the Aβ42-treated (100 µg/mL) animals was significantly longer than that of their standard fed and untreated starved controls, with a significant difference between the 3 hours- and 3 days-aggregated Aβ42 types. Besides NML, further viability markers were measured: n = 50 (BSI), 20 (BLD), and 24 (MCF) one-housed individuals per group; n = 24, well with normalized absorbance to rotifer number (CRC). The Aβ42-treated rotifers performed significantly better than their untreated starved controls. In BSI and CRC were significant differences between the subgroups treated with two differently aggregated Aβ42 forms. For comparative statistical analysis, the one-way ANOVA was used followed by the Bonferroni post hoc test. A p ≤ 0.05 was regarded as statistically significant, with the different levels of significance indicated as follows: p# ≤ 0.05 and p*** ≤ 0.001 (*, significant difference from the fed and Aβ42-treated groups; #, significant difference from the group treated with the 3 h aggregated form). Abbreviations: Aβ42 = beta-amyloid 1-42; 3d = Aβ42 aggregated for 3 days; 3 h = Aβ42 aggregated for 3 hours; BLD = bright light disturbance; BSI = body size index; CRC = cellular reduction capacity; MCF = mastax contraction frequency; NML = normalized mean lifespan.*

We examined the effect of Aβ42, which supposed to be toxic to *P. acuticornis* (**Figure 10**). Surprisingly; however, Aβ42-treatment of the rotifers resulted in significantly longer mean lifespan (51 ± 2.71 days) compared to the case of starved (14 ± 2.29 days) and standard fed (32 ± 2.72 days) control groups. To characterize the Aβ42-treated *P. acuticornis* specimens,

we applied further experiential monitoring assays previously published in Olah et al. [2017]. The results presented on **Figure 10** verified the fact that this bdelloid can use the Aβ42 as a nutrient in an isolated environment without the presence of other organic material. The 'normalized mean lifespan' of groups treated with either 3 hours or 3 days aggregated Aβ42 significantly increased compared to starved controls. The 'body size index' and the 'bright light disturbance' represented both physiological and/or phenotypical changes of the treated animals. These characteristics were increased by 40% and 60% compared to untreated starved controls, respectively. The 'mastax contraction frequency' and the 'cellular reduction capacity' suggested elevated energy levels as indicated by neuromuscular and cellular-redox activities. The markers were increased by 46% and 42% in comparison to unfed entities, correspondingly. The Aβ42-treated one-housed rotifers performed significantly better in the measured parameters than their starved controls, and they do not considerably differ from the standard fed counterparts. These results suggest that Aβ42 is not toxic to *P. acuticornis*. The aggregated neurotoxic peptide could be used by them as a sole nutrient source to survive and develop in a hermetically-isolated environment.

For the localization and demonstration of the existence of Aβ42 aggregates in the body of *P. acuticornis*, we used β-sheet-specific fluorescent (BisANS) and absorbent (CR) dyes. Animals in the representative photographs (**Figure 11**) are shown in proportional sizes of the animals and display strong differences between the groups (fed, **11A**; starved, **11B**; Aβ42-fed/treated, with 3 hours /**11C**/ or 3 days /**11D**/ aggregates). BisANS dye is specific both to Aβ42 oligomers (3 hours aggregated**; Figure 11C**) and fibrils in contrast to CR dye which labels only the protofibrils and fibrils (3 days aggregated; **Figure 11D**). These figures present the exogenous Aβ42 localization in the digestive system (above is the stomach and below is the intestine) of the rotifers after 'feeding' *ad libitum*.

For monitoring gold (Au)-tagged Aβ42 aggregates in the intact rotifers (distribution of metal-peptide complex in the body), we applied scanning electron microscopy (SEM) for detection (**Figure 11E** and **F**). After fixating and drying the Au-Aβ42-treated (fed) and untreated specimens, we detected the possible distribution of the potentially catabolized peptide intermediers. In the treated/fed animals, the signal of gold-metal could be found homogeneously everywhere in their body (**Figure 11F)** in contrast to the untreated ones **(Figure 11E**). The only source of gold in the samples was the Au-Aβ42 particles taken up during the feeding processes, as no aspecific gold coating was applied. We recorded representative photographs without exact quantification of the gold signal.

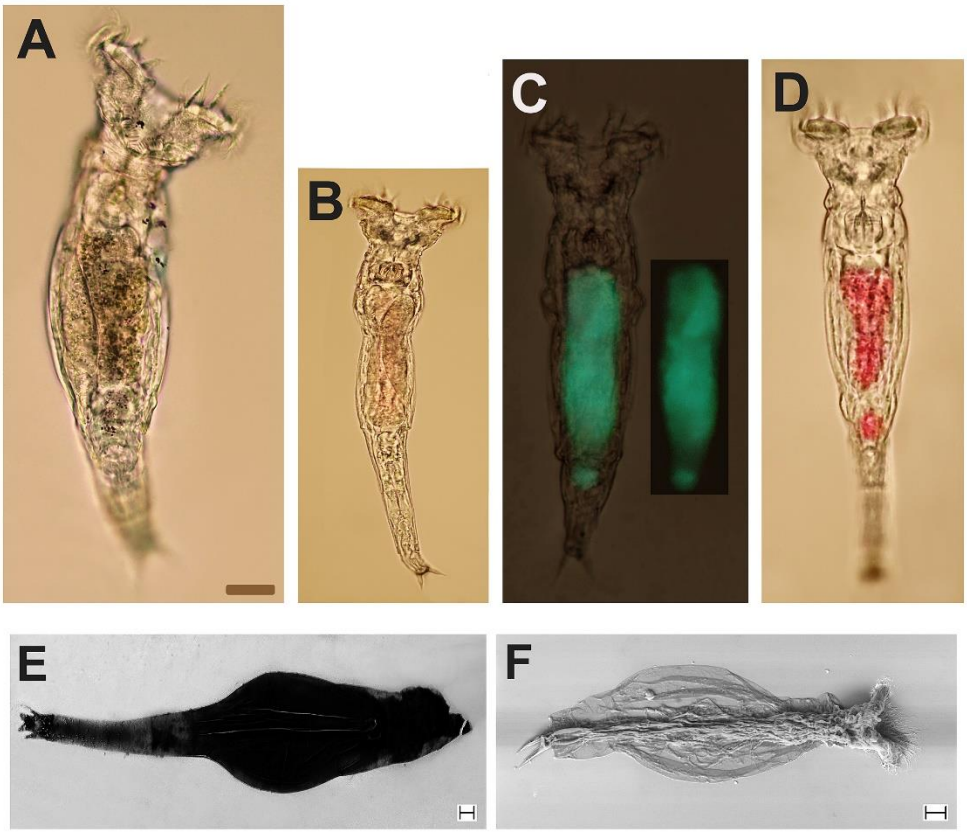

Figure 11. ***Localization of aggregated Aβ42 in P. acuticornis.*** *Juvenile rotifers were sorted in different groups after 24 hours starvation: fed (**A**; 12 days standard feeding, 600 µg/mL), starved (**B**; 12 days) and Aβ42-treated (12 days; 100 µg/mL). The Aβ42 in stomach and intestine (digestive system) of rotifers were monitored by fluorescent dye, named BisANS (**C**; green color in the representative photograph; 3 h = the Aβ42 was aggregated for 3 h; 10 µM) and absorbent CR (**D**; red color in the representative photograph; 3d = the Aβ42 was aggregated for 3 days; 50 µM). The scale bars in the proportional representative photographs represent 20 µm. Untreated specimens labeled with BisANS and CR dyes showed no signal (not shown). The possible in vivo distribution of the exogenous Au-Aβ42 complex (100 µg/mL) was investigated with SEM, detected in the body of P. acuticornis (three animal/glass coverslip): (**E**) rotifer without Au-Aβ42 treatment. (**F**) Au-Aβ42-treated (fed) rotifer. Homogenous signals can be detected in Au-Aβ42-treated entities. Scale bar represents 10 µm. Abbreviation: Aβ = beta-amyloid 1-42; Au-Aβ42 = gold-tagged beta-amyloid 1-42; BisANS = 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid dipotassium salt; CR = Congo red; SEM = scanning electron microscope.*

4.8. Administration of various plant-extracts as nutrient, with phenotypic modulator properties

Three viability parameters (‘number of rotifers alive’, ‘mastax contraction frequency’ and ‘body size index’) were measured to assess the effects of plant-extracts on *P. acuticornis*

(**Figure 12**) in the presence of 0.1% DMSO. This concentration of the mentioned solvent had no significant impact on rotifers' viability. The following compounds: rosavin, cinnamyl alcohol, ginsenosid Rb1, withanolide B, withanolide A, and withaferin A caused a significant decrease in the 'number of rotifers alive' and in the 'mastax contraction frequency' value. On the contrary, we observed a growth in 'body size index' value amongst the survivors, probably since they used these compounds as nutrients. A significant increase was observed in the 'body size index' values of the groups treated with *W. frutescens* and *R. rosea* crude extracts, parallel with a slight elevation in 'number of rotifers alive' and 'mastax contraction frequency'. The nomenclature of the 'number of rotifers alive' is equivalent with 'toxicity and survival lifespan' data, described previously in our publication [Macsai et al. 2018]. One treatment resulted in unique changes: exposure to compound salidroside resulted in significantly decreased 'mastax contraction frequency' with normal 'body size index' in the survivor specimens. In the 20-OH-ecdysone-treated specimens we observed 40% decrease in the number of survival with less 'body size index' and normal 'mastax contraction frequency' values. From the 14 tested compounds, withaferin A proved to be the most toxic. This sensitive action and well-reproducible model might be a useful tool in the characterization of bioactivities of plants and compounds, moreover, in the identification of key components with viability effects.

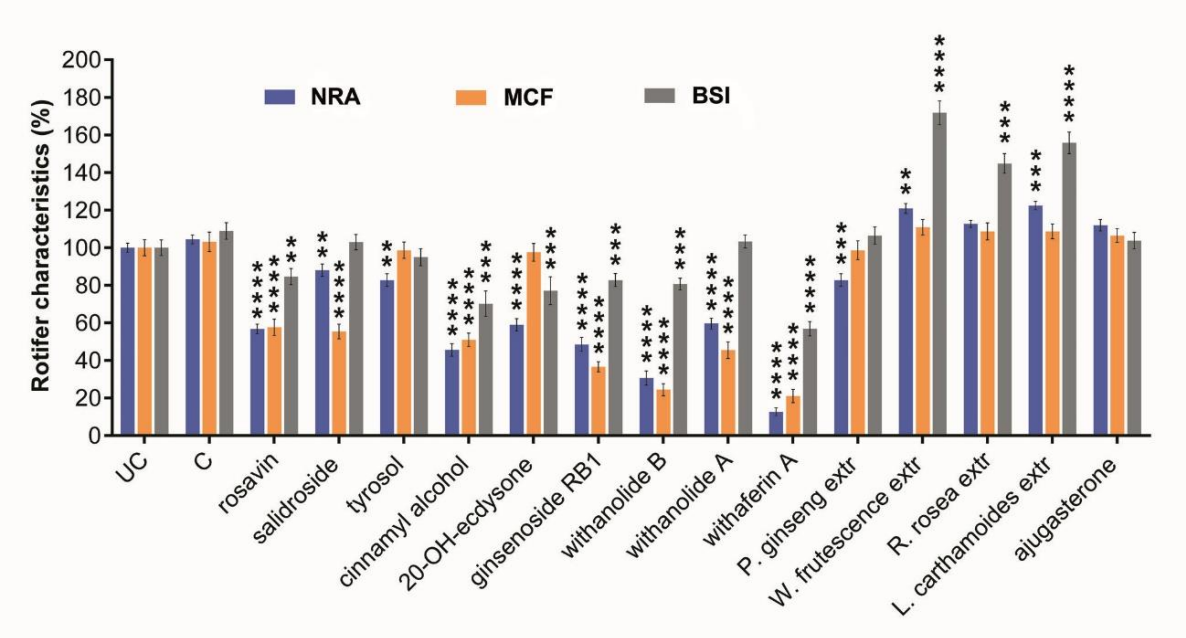

Figure 12. ***Viability values of treated P. acuticornis.*** *Changes in the viability markers (NRA, n = 16, well; BSI, n = 15, MCF, n = 24, one-housed rotifer in both cases) of the specimens were measured after 6 days' treatment and compared to the C group. The final concentration for the compounds*

*was 100 µM, with 0.1% DMSO content. Values are the mean ± S.E.M. One-way ANOVA with post hoc Bonferroni test was used for the statistical analysis. Different levels of significance were indicated as follows: p** ≤ 0.01, p*** ≤ 0.001, and p**** ≤ 0.0001. Abbreviations: C = control with 0.1% DMSO; BSI = body size index; MCF = mastax contraction frequency; NRA = number of rotifers alive; UC = untreated control.*

4.9. The 20-OH-ecdyson-induced abnormalities, as a model of surpassing phenotypic plasticity in rotifers

Similarly with previous plant-related experiments the viability of the rotifers was monitored for six days, with no feeding for the first three days, to investigate the impact of plant extracts on the specimens. In earlier experiments, we observed a special reproduction (egg production) in the presence of standard nutrient and 20-OH-ecdysone; however, the mothers could not lay down the eggs and eventually deceased after 4-5 days. Surprisingly, the eggs hatched inside the mother's body, more precisely in the germovitellarium, and the viable young rotifer left the host's body (**Figure 13**). The lifespan of the offspring was extremely short, only one or two days (not shown).

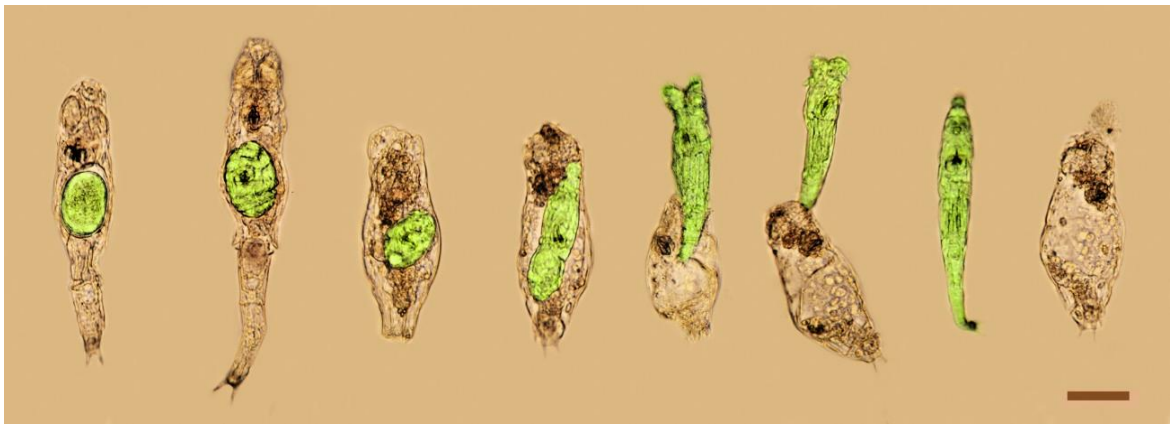

Figure 13. ***Effect of 20-OH-ecdysone on P. acuticornis****. The figure presents the egg (green, digitally painted) growing and eventually hatching inside the mother's body (dark brown color). Due to the 20-OH-ecdysone treatment, the mother specimens were unable to lay the eggs and eventually deceased. The viable egg hatched, and the young rotifer (green, digitally painted) left the body. The picture was colored for the better presentation. Scale bar: 50 µm.*

5. Discussion

Our special *micro-in vivo* model system, characterized in detail, will make it possible to measure numerous conditions independently and simultaneously, providing a reliable and highly replicable investigation in connection with lifespan, aging and plasticity. Our results may give further explanation to the mechanism of aging and the causality relations between redox system and extended lifespan. Moreover, our rotifer-based experiments might not only provide new theories to understand the holistic platform of aging-related physiology, but they may as well help to clarify the biochemical background of longevity, one of the most fascinating phenomenon in biology.

Highlights

- The bdelloid rotifer *P. acuticornis* is validated *in vivo* aging model;
- Chemical and biological electron deprivation prolonged lifespan;
- Characteristics of long-lived old rotifers outperform that of the normal ones;
- Long-term redox influence enhanced the reproductive ability of senescent rotifers;
- Electron deprivation markedly extends lifespan without typical signs of aging;
- The *P. acuticornis* can catabolize and use the neurotoxic Aβ42 aggregates as nutriments;
- The aggregated protein catabolism *in vivo* is a novel phenomenon in current biology;
- Our rotifer model seems to be appropriate for the comprehensive testing of adaptogenic plants and their constituents related to the biological limits of the phenotypic plasticity and deformity.

5.1. Extremely increased lifespan with delayed aging in the framework of extended adaptive phenotypic plasticity

In academic literature some research groups have published the positive impact of caloric restriction on lifespan [Verdone-Smith and Enesco, 1982; Weithoff, 2007] but in our experiments a special *in vivo* electron modulation was applied in order to achieve extremely prolonged life. We aimed to develop an aging model by targeting electrons, the smallest representatives of the cellular redox system via modulating the redox processes in the bdelloid rotifer *P. acuticornis* with *in vivo* chemical and biological electron deprivation (**Figure 14**).

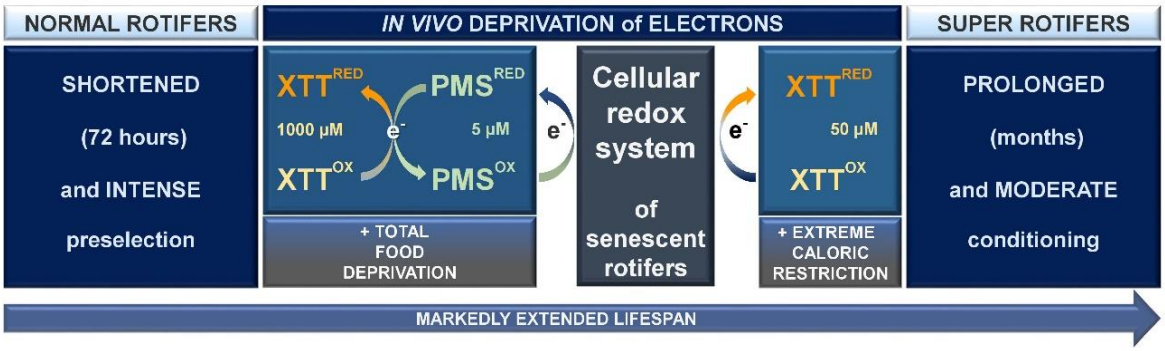

Figure 14. ***Relation of redox modulating system with extended lifespan of super rotifers.*** *Abbreviations: OX = oxidized form; RED = reduced form; PMS = phenazine methosulfate; XTT = 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt.*

In the *P. acuticornis* specimens the electron source was their plasma membrane redox system. This apparatus supposedly attenuates the aging-associated oxidative stress by providing NAD^+ for ATP biological oxidation-related ATP [Hyun et al., 2006b]. Intracellular NADH is the reducing source for extracellular XTT reduction (applied as chemical modulator of specimens), providing electrons via the plasma membrane redox system [McCluskey et al., 2005]. The PMS, as an intermediate electron carrier, significantly promotes the reductive reactions. The low redox potential of PMS results electron deprivation in living systems [Kettisen et al., 2015]. The reliability of the PMS- and XTT-applied system was confirmed by testing this combination pair (cell-free) in the presence of NADH. The developed *in vivo* experimental setup was based on the data of the previous measurements and consisted of two main phases (**Figure 14**). The combination of PMS/XTT (under total food deprivation) preselection followed by XTT (under ECR) treatment phase, this setup made a synergistic positive impact on lifespan and phenotype of preselection survivors resulting so-called super rotifers, with unique properties. The phenomenon of this markedly prolonged lifespan (up to 182 days), related to *P. acuticornis*, has not been previously published. The documented maximal lifespan was approximately 75 days, with mean 43 days [Ricci and Perletti, 2006]. The observed physiological changes in super rotifers are accompanied by slowed metabolic activity and cease of reproduction [Marotta et al., 2012]. To compare the viability markers, middle-aged (15 days old) normal rotifers and senescent (110 days old) super rotifers were followed-up for 10 days under standard feeding condition without chemical treatment (**Figure 7**). Significant growth in body size (detected under light-microscope and measured by 'body size index') was accompanied by lower 'mastax contraction frequency' compared to normal rotifers, probably as a result of altered

neuromuscular function due to the increased body size. More surprisingly, these senescent (110 days old) animals were capable of laying viable eggs (reflecting an effect on the reproduction phase); moreover, the number of offspring was significantly higher after the 10-day period compared to normal rotifers (**Figure 7B**). To our knowledge, no similar long reproductive phase without dormant stage has ever been detected in bdelloid rotifers. Furthermore, the tolerance of freezing conditions and the recovery rate of the cultures were significantly higher in the super rotifer than in normal rotifer cultures. According to the academic literature, it is a common knowledge that bdelloid rotifers are eutelic animals, with fix cell number and without cell proliferation [Clément and Wurdak, 1991]. We measured the nucleic acid and protein quantity in normal rotifers and super rotifers to confirm that the size change is the result of elevated protein amount (**Figure 9**) instead of changing cell numbers. For this investigation we applied our newly developed, BisANS-based protein quantification method. This versatile fluorescent dye can be utilized in various forms of qualitative (localization) and quantitative (concentration) protein detection in complex biological samples (rotifers and/or yeast). The BisANS showed no exclusionary sensitivity to the presence of interfering agents (chelator, detergent, inhibitors). When we calculated the ratio of normal-BSI and super-BSI and the ratio of normal and super rotifer's normalized fluorescence intensity, we found a distinct correlation between 'body size index' and protein quantity. These results are complementary data to the confirmation of the lack of cell division in our model specimens.

Extended lifespan and delayed aging accompanied by increased viability remained in the framework of the *P. acuticornis*-specific adaptive phenotypic plasticity. After reintroduction of standard feeding and cessation of permanent conditioning (combined ECR and low-dose XTT treatment) at 110 days of age, the 'remaining lifetime' of super rotifers was restored close to their original expected longevity (25 ± 4.2 days) until their natural death.

Neurodegenerative diseases are predominately initiated and/or intensified by the systemic enzyme resistance of toxic aggregated peptides and related pathological consequences. During our work we discovered a yet unknown biological phenomenon. Under total caloric restriction in isolated conditions (oil-covered microdrop), we observed the special catabolic activity in the *P. acuticornis* specimens. These microinvertebrate specimens are capable of using even neurotoxic aggregates as exclusive energy and organic material sources, therefore prolonging their lifespan. The one-housed and aggregated Aβ42-treated individuals have significantly longer lifespan and improved viability markers compared to their untreated controls under starvation in a completely isolated environment. This ability to increase

survival (‘normalized mean lifespan’) and maintain viability (indicating by ‘body size index’, ‘bright light disturbance’, ‘mastax contraction frequency’ and ‘cellular reduction capacity’) showed the trophic effect of both aggregation state of Aβ42. The causality between the well-known negative effects of Aβ42 and their beneficial aspects on rotifers, is not evident, rather it can be viewed as contradictory! Available literature does not describe any other animal species with a similar ability. The administered exogenous Aβ42 aggregates were first localized and presented within the digestive system of the rotifers (applying BisANS or CR), providing evidence of being consumed. In parallel investigations the remnants of gold-tagged aggregate were detected further with SEM homogenously in their body (**Figure 11**). Previously published article by our research team reflect to the effect of further neurotoxic aggregates, e.g. beta-amyloid isoforms, alpha-synuclein, and prion (normal cellular- and pathogenic forms) on various microscopic species (rotifers and non-rotifers) [Datki et al., 2018]. The catabolic capability was not limited to Aβ42; however, only the bdelloid rotifers showed this unique phenomenon. For the other species, these aggregates were either toxic or indifferent [Datki et al., 2018]. Since the treated *P. acuticornis* specimens maintained their health, function, and redox capacity, we presume that these neurodegeneration-related aggregates may serve as a required energy source for gluconeogenesis in our experimental conditions. In their natural habitat, bdelloid rotifers had to adapt continuously to extreme fluctuations in nutrient availability [Castro et al., 2005]. Furthermore, their basic sustenance includes particulate organic detritus, algae, protozoa and dead bacteria, which offer a considerable variation of natural aggregates [Wallace and Snell, 2010]. Therefore, the capability of these individuals to metabolize nearly every type of aggregated proteins and peptides might be an evolutionary strategy to adapt and survive. Phylogenetically (evolutionarily) conserved biological characteristics (e.g. glycolysis; mitochondrial alterations; redox regulations; NAD/NADH ratio; glutamatergic and serotonergic system; autophagy; receptor agonism or antagonism) of rotifers and other microinvertebrates have good mammalian and/or human relevancy.

5.2. Physiological and morphological abnormalities beyond the species-specific biological limits

Adaptogenic plants have been widely used for human medicine; however, their exact mechanism of and the full spectrum of the active constituents have not yet been revealed. Rotifers are adequate models for the complex description and characterization of the biological effects of different requested homo- and/or heterogeneous samples (such as

molecules, ions, drug candidates, analogs, derivatives, dyes, plant extracts, natural waters, cosmetics). It was documented in several planktonic rotifer species that they respond with morphological changes (e.g. development of spines) to exogenic (environmental) hormones [Gilbert, 2011]. This response can provide effective post-encounter defense against predators, a form of adaptive phenotypical change to the stimuli of their environment. During our work, we had investigated this extended phenotypic plasticity specifically in *P. acuticornis*. To understand the limitation of the species' adaptability, we tested active agents derived from plants natural to the species' environment. While the crude extracts seemed to be nontoxic to the rotifers, the pure substances of the plants influenced the viability of the animals negatively. One compound (20-OH-ecdysone) resulted in abnormalities in the reproduction process beyond the physiological tolerance, leading to the specimens and the offspring's death. Further investigation of active agents and extracts on rotifers would give us a thorough understanding of our model's adaptive capability. Moreover, the species-specific informations related to biological limits fosters the correct application of our *micro-in vivo* model in large-scale drug screening.

5.3. Perspectives

Nowadays, molecular investigations are dominant parts of biological research. Despite of this, the renaissance of supramolecular and experiential biology is actual together with the application of their numerous advantages. The holistic aspects of these *invivomics* systems are inevitable in all types of studies; however, the *micro-in vivo* is one of the quintessence of fundamental research and high throughput screening.

The developed redox modulating system resulted in an exceedingly extended lifespan in our microinvertebrate specimens; furthermore, this setup also tested the capacity of the species-related adaptive phenotypic plasticity. Parallelly with the extension of the natural rotifer-lifespan we revealed some special physiological parameters (e.g. attenuated metabolism) behind the longevity. The experiments on *P. acuticornis* revealed novel phenotypical and physiological changes. Our future aims are the testing of various lifespan-modulating compounds (e.g. songorine) and expanding their molecular background in 'omics' perspectives. The combination of the developed *in vivo* viability, phenotypical and systemic modulations allows us the interdisciplinary application of our model.

According to the academic literature, several phylogenetically conserved biological processes (e.g. nutrient-sensing pathways, NAD/NADH ratio) are shared by microinvertebrate and mammalian model species [Vaiserman et al., 2016; Verdin, 2016]. By

revealing evolutionary conserved biological processes in rotifers, it may lead to a possible human relevancy in the field of pharmacological research. The various neurotoxic aggregates (including Aβ42) tested by in our laboratory [Datki et al., 2018] share mutual features, with their accumulation and aggregation facilitating neurodegeneration. The universal capability of bdelloid rotifers to catabolize the well-known neurotoxic peptide aggregates suggested that the hypothetic metabolic pathway might contain degrading enzyme(s) and their possible cofactors or anti-aggregation compounds. Investigation of *in vivo* aggregate-degradation has emerged from an interdisciplinary area of protein chemistry with a relevant field in neuropharmacology [Langer et al., 2011]. The understanding of these special catabolic capabilities of bdelloid species, including *P. acuticornis,* on neurotoxic aggregates in hermetically isolated environment without any additional energy source accessible may provide the basis of a new therapeutic approach (identification of relevant molecules and metabolic pathways) in neurodegeneration-related proteinopathies.

6. Conclusion

The general aim of our work was to investigate aging and its modulating factors in a microinvertebrate animal that is a novel redox-based *in vivo* model. Our preferred species was a bdelloid rotifer, named *P. acuticornis*. We adjusted the biological (starvation or extreme caloric restriction) and chemical (drug-based electron deprivation) modulations and their optimal time-phases. Applying this complex system, we tested redox-modulating agents, neurotoxic aggregates and adaptogenic compounds on rotifers, monitored their lifespan and viability. The phenotypic plasticity-related limitations of the rotifers were investigated in a separate conception.

The summary of our main findings were the followings:

A. The bdelloid rotifer *P. acuticornis* is a suitable *in vivo* aging model according to the lifespan-measuring requirements stated in the academic literature;
B. The biological- and chemical-based electron deprivation significantly increased the lifespan of the rotifer species. The combination of intense, 72-hour long preselection (PMS/XTT electron carrier-acceptor, under starvation) and moderate, long-term conditioning (XTT with extreme caloric restriction) on middle-aged rotifers resulted in exceedingly long, previously not documented lifespan in the survivors;
C. The synergistic effect of the applied biological and chemical treatments caused extremely increased lifespan, furthermore triggered specific phenotypic changes, e.g. enlarged body size and heightened reproductive capacity;
D. Our experiments confirmed that the growth in the specimens possessing constant cell number was caused by the increased cellular protein amount without cell division. To prove this eutely-related phenomenon, we applied our newly developed BisANS-based protein quantification assay;
E. The starvation-based *in vivo* electron deprivation resulted in adaptive phenotypic plasticity in the microinvertebrates. This property of the bdelloids rotifers also expressed in the catabolism of the neurodegeneration-related Aβ42 peptide. This interdisciplinary phenomenon revealed the unique capability of these animals to use the neurotoxic aggregates as nutrients, which also resulted extended lifespan. The investigation to unravel this unique ability could provide/give new scientific perspectives e.g. in the research of Alzheimer's disease;

F. The *P. acuticornis* showed significant adaptability during the holistic electron deprivation. To test the limits of phenotypic plasticity, we applied active agents and extracts derived plants occurring in their natural habitat. The appearance of physiological/morphological deformities of these animals was carefully monitored. This additional information contributed to the objective application of our rotifer-based aging model within the margin of error.

G. The capabilities of *P. acuticornis* revealed during our work were presented the first time in the academic literature.

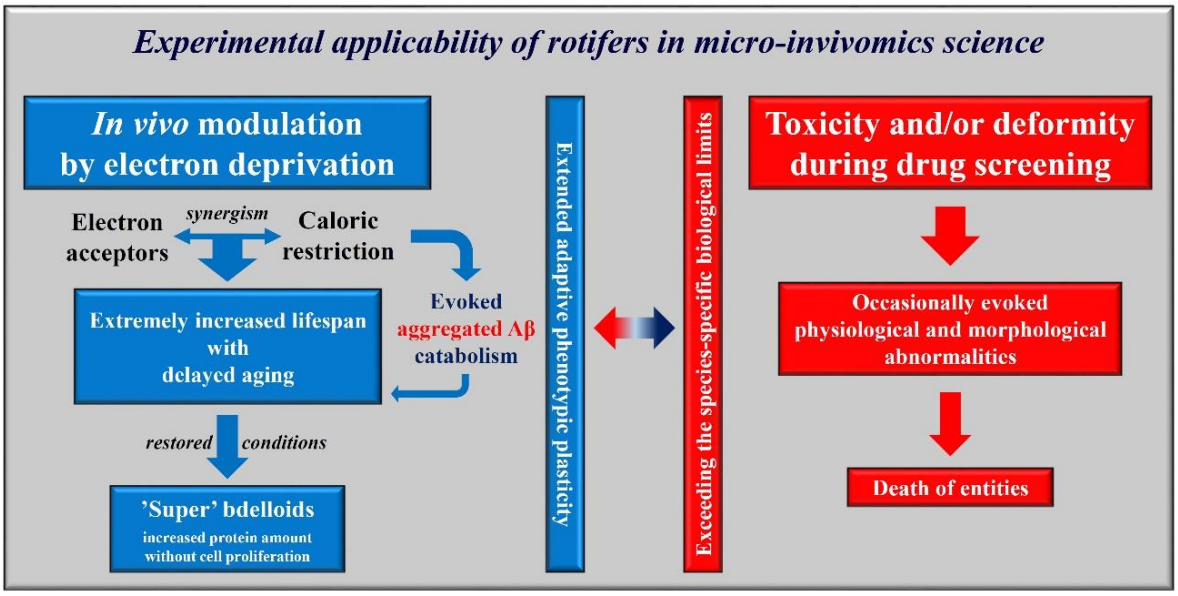

Figure 15. ***Holistic and schematic summary of the thesis*** *(Aβ = beta-amyloid)*

Our developed lifespan- and phenotype-modifying system (**Figure 15**) can be a reliable experimental model to provide new insights into the processes of senescence. We declare that the cellular NADH regulation phylogenetically is an important part of the complex mechanism of aging and longevity. The monitored physiological changes are accompanied by slowed metabolic activity and the cease of reproduction. Markedly extended lifespan, together with the delayed aging, is a unique combination of rotifer-phenotypes, which is to our knowledge unprecedented in the academic literature concerning any multicellular species. Furthermore, we revealed the available connections between starvation-induced electron deprivation and exceptional capability of rotifers to catabolize neurotoxic aggregates.

Intention to deeper understanding the connections between ***redox processes***, ***exceptional catabolism***, ***phenotype plasticity*** and ***longevity*** is at the same time recent and eternal in entity-based *in vivo* **aging** research.

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9. Appendix