

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



Ph.D. Thesis Summary

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### **Publications the Thesis is based on:**

- I. **Macasai 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. **Macasai 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 Complement Alternat Med*. 2018;3690683 doi: 10.1155/2018/3690683; **IF: 1.984; Q1**
- III. Datki Z, Olah Z, Hortobagyi T, **Macasai 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**
- IV. Datki Z, Olah Z, **Macasai L**, Pakaski M, Galik B, Mihaly G, Kalman J. Application of BisANS fluorescent dye for developing a novel protein assay. *PLOS One*. 2019;14:e0215863. doi: 10.1371/journal.pone.0215863; **IF: 2.776; Q1**

The cumulative impact factor of the publications  
directly related to Thesis: **15.354**

The cumulative impact factor of the whole scientific work: 22.827

### **Abbreviations**

**A $\beta$**  - beta-amyloid; **A $\beta$ 42** - beta-amyloid 1-42; **Au-A $\beta$ 42** - 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; **CRC** - cellular reduction capacity; **CR** - Congo red; **ECR** - extreme caloric restriction; **MCF** - mastax contraction frequency; **NAD<sup>+</sup>** - nicotinamide adenine dinucleotide, reduced form; **NADH** - nicotinamide adenine dinucleotide, reduced form; **NRA** - number of rotifers alive; **PI** - propidium iodide; **PMS** - phenazine methosulfate; **SEM** - scanning electron microscope; **XTT** - 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt.

## 1. Introduction

Aging can be defined as a natural and progressive loss of physiological integrity, leading to impaired function and increased vulnerability to diseases and death. The majority of adult-onset diseases that are direct consequences of aging concern a gradually increasing population. To slowing down the natural and basic processes of aging caused by extrinsic factors (e.g. diseases), it is necessary to have comprehensive knowledge. A model to study aging should, therefore, fulfil the following criteria: it must have measureable phenotypes of the process and the underlying mechanisms must be conserved. Ideally, the chosen organism should have a short lifespan and should be relatively easily to manipulate under laboratory conditions (e.g. small size and inexpensive to grow in large amount). *In vitro* human cell cultures provide useful information about the cellular processes of aging; however, we cannot speak of real lifespan. 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. Rotifer species are widely used in aging studies, especially in risk assessment of pharmaceuticals and in examining the roles of their metabolites in aquatic ecosystem. 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. The human aging studies are complicated in many reasons, including the ethical issues, environmental and social factors.

Bdelloid rotifers, as microinvertebrates, are commonly used models in ecotoxicology-, pharmacology- and aging-related studies. They are eutelic (approximately 950-1000 somatic cells) metazoans with distinct morphological characteristics (e.g. ciliated head structure, mastax and bilateral ovaries). Our reasons for choosing bdelloid rotifers for experiments based on their following biological advantages: (1) short lifespan; (2) characteristic micro-ethology; (3) clonal reproduction and direct development; (4) adaptive phenotypic plasticity; (5) no ethical concern.

Hormesis extends lifespan through the induction of mild redox stress. Nutrient deprivation can be a stressor per se due to normal caloric restriction and results in extended longevity. Amount of nutrition is associated with the redox state and

this restriction may cause cellular electron deprivation, impaired mitochondrial function and suppressed NADH availability. The ratio of  $\text{NAD}^+/\text{NADH}$  molecules reflects the cytoplasmic redox state and is a relevant marker of cellular metabolism. Treatment with electron carriers and acceptors increases the lifespan of numerous organisms by increasing the cellular  $\text{NAD}^+/\text{NADH}$  ratio. The XTT and PMS molecules are widely used in combination as electron acceptor and carrier, which indicates the cellular redox potential; moreover, they are suitable manipulators of the cellular redox state.

In the recent years the use of rotifers become emphasized as *in vivo* models for screening anti-aging, metabolic modulator or adverse effects of pharmaceuticals. Neurodegenerative disorders are predominantly initiated and intensified by the systemic enzyme resistance of toxic aggregated proteins and related pathological consequences. The application of different exogenous A $\beta$  isoforms are widely used models of Alzheimer's disease and earlier studies used various *in vitro* and *in vivo* systems to reveal their exact effects. The A $\beta$ s are crucial molecules in aging-associated dementias, representing a starting point in the development of these diseases. Their accumulation is one of the most essential processes during the course of cerebral A $\beta$ -related pathologies. There is an evident correlation between aged-related pathologies and protein aggregation. A single publication aimed at describing the comprehensive effects of A $\beta$  on bdelloid rotifers; however, they could not prove the neurotoxic effect of A $\beta$ 42 in these models. In another aspect of aging research, various studies and applications have confirmed that plant-originated adaptogens play important role in human health. Rotifer-based *in vivo* studies are suitable to investigate many compounds with their various effects. The characterization of plant extracts using rotifer model may provide promising candidates for subsequent testing in vertebrate animal models.

## 2. Aims

*The aims of the thesis were:*

- Investigation of aging and its modulating factors *in vivo*;
- Application of bdelloid rotifers in redox-based aging model with real lifespan monitoring, optimized to specific requirements of the experiments;

- Investigation and modulation of lifespan-influencing cellular redox processes;
- Revealing the optimal combination of chemical compounds and biological processes to achieve extremely increased lifespan related to our bdelloid rotifer, *Philodina acuticornis*;
- Interdisciplinary application of the adaptive capabilities of rotifers evoked by starvation-based *in vivo* electron deprivation for inducing exceptional catabolic processes of aggregated neurotoxins;
- Screening potential phenotypic plasticity-modulating agents derived from plant extracts in order to establish the species-specific biological limits.

### 3. Materials and methods

#### 3.1. 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 aquarist. Rotifer culturing, feeding and harvesting methods in brief: the specimens were **cultured** in a supervised and semi-sterile laboratory environment. The cultures 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 well-kept at room temperature and under a light/dark cycle of 12/12 hours. The steps of the rotifer manipulation and culturing was monitored under an inverted light microscopy. Standard feeding consisted of prepared *Saccharomyces cerevisiae* added to the flasks after every medium change. **Isolation** was a cooling and washing process utilizing the surface-binding ability of the healthy animals, then the individuals or groups were let to rest. We chose approximately 5-day-old rotifers after hatching (1-2 days before the beginning of the reproductive stage of the species) for the experiments. Two **application mode of microinvertebrates** were used in the experiments. The microdrop technique were applied to treat and observe **one-housed individual** rotifer in total isolation for longer time-periods. For this we used 24-well plates with one microdrop at the bottom of each well. The drop was covered with oil.

For screening the effect of compounds, **populations** of rotifers were isolated with the standard process and left to rest for 24 hours before treatment.

## **3.2. Preparation and treatment protocols**

### **3.2.1. Biological and chemical electron deprivation**

The treatment period comprises of two different phases of combined nutrient and chemical drug-based redox modulation. Before application, the impact of nutrient level on the longevity of *P. acuticornis* were examined. The applied nutrient (homogenized yeast) concentrations ( $\mu\text{g}/\text{mL}$ ) were: 0 (starved), 50 (ECR), 200 (normal caloric restriction) and 600 (standard feeding). The optimized and combined electron deprivation method was the following: (1) **Preselection phase**: relatively intense (72 hours) and short electron deprivation, where rotifer populations and middle-aged (15 days old) one-housed individuals were administered chemical (PMS, 5  $\mu\text{M}$ ; XTT and ascorbic acid both 1000  $\mu\text{M}$ ) compounds alone or combined under total food deprivation in darkness. (2) **Conditioning phase**: multistep washing process were followed by moderate and permanent (months; with the medium changed in every second day) treatment of the selected surviving individuals with 50  $\mu\text{M}$  XTT or ascorbic acid under standard feeding or ECR. 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).

### **3.2.2. Preparation of A $\beta$ 42 and treatment of rotifers**

The synthesis and characterization of relevant A $\beta$ 42 peptide was manifested as previously described by our cooperation partners with some minor modifications. The aggregation period was 3 hours or 3 days (24 °C, pH 3.5). To detect the exogenic A $\beta$ 42 in the rotifer's body (digestive system), the peptide was labelled with dyes according to the aggregation state and monitored under fluorescent (OLYMPUS) or light microscope. For the scanning electron microscope (SEM) detection the aggregated A $\beta$ 42 (1 mg/mL, for 3 days) solution was mixed for 2 hours with gold(III) chloride ( $\text{AuCl}_3 \times 2\text{H}_2\text{O}$ ; 2.8 mg/mL) solution in a 1:4 molar ratio, according to the number of Au-binding sites of monomeric A $\beta$ 42. The peptide amount of the pellet was determined by Qubit Protein Assay Kit. The 15 days old (middle-aged) harvested rotifers were starved for 2 days, then washed into 6-well plates, each well was treated with Au-A $\beta$ 42 complex in a dose of 100  $\mu\text{g}/\text{mL}$  for 1 day. 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% ethanol at  $-75\text{ }^{\circ}\text{C}$  (5 min), followed by a partial rehydration with 30% ethanol at  $24\text{ }^{\circ}\text{C}$  (30 min). After fixation with 1% paraformaldehyde (30 min) the wells were washed two times with distilled water. Collected rotifers were transferred to the center of a round glass coverslip to dry. Separately, the surface of dried rotifer samples was not covered with nano-gold.

### **3.2.3. Investigated plant-extracts**

Plant materials (*P. ginseng*, *W. frutescens*, *L. carthamoides*, and *R. rosea*) were collected and crude extracts were obtained. Purified compounds (Ginsenoside Rb1, withanolide A and B, withaferin A, rosavin, salidroside, tyrosol, cinnamyl alcohol, rhodiosin) were purchased. The 20-OH ecdysone and ajugasterone was isolated in the Department of Pharmacognosy, University of Szeged. The rotifers were treated with relevant compounds (final concentration was  $100\text{ }\mu\text{M}$  with 0.1% DMSO content) and were monitored daily. Rotifers were starved during the toxicity interval (72 hours); on the fourth day they were under ECR (one time food supplementation), which is enough for surviving, but cease the reproduction. The untreated control group was grown in standard medium, while the control group was grown in standard medium containing 0.1% DMSO. The condition of the treated specimens was compared to the control group.

### **3.2.4. Protein quantification of biological samples**

Microinvertebrates (*P. acuticornis*) and intact yeast (*S. cerevisiae*) cells served as biological samples for testing newly developed, total protein quantification method based on the fluorescence dye BisANS. The process of sample-homogenization contains several steps of centrifugation with freezing/thawing and ultrasonication.

## **3.3. Optical imaging- and detection assays**

### **3.3.1. Microscopy-based experiential monitoring (phenotypical markers)**

#### **3.3.1.1. Light microscopy**

The ‘**number of rotifers alive**’ (NRA) assay provides the mortality rate of rotifers after treatment at a representative date. The survival number of the control group in the specific experiments were regarded as 100%. The ‘**body size index**’ (BSI) is a special calibration curve to determine the age of

*P. acuticornis* rotifers, that were isolated from standard cultures. Determination of the relevant rotifer age was executed by the measured means of their daily BSI (%) = *maximal 'length x width' of body*. The mastax of rotifers is part of the digestive system with the specialized function to shred the food. The **'mastax contraction frequency'** (MCF; contraction/sec), served as a quantitative viability marker. The **'normalized mean lifespan'** (NML) presents the mean survival lifespan of rotifers under a period of treatment. The mean lifespan of the control (standard-fed) group were regarded as 100%. The **'bright light disturbance'** (BLD) were used to assess behavioral responsiveness with high sensitivity and resolution. We measured the reaction of the rotifer with two parameter: (1) 'bright light irritation' (BLI); (2) 'bright light avoidance' (BLA). We defined the index according to:  $BLD (\%) = (BLI/BLA) \times 100$ . The **reproduction capacity** assay were used to measure the viable egg production of rotifers in standard and/or restored conditions and the kinetics of reproduction. **Freezing tolerance** assay compares the tolerance and recovery of phenotypes exposed to subzero conditions (one week). The number of survivors was counted 24 hours after thawing. To **detect the exogenic A $\beta$ 42** with CR in the rotifer's body, the aggregated (3 days) peptide (100  $\mu$ g/mL) was labeled with CR (*in vitro*, 50  $\mu$ M, 1 hour) dye.

### 3.3.1.2. Fluorescence microscopy

The preselected and 1-day starved 5 days old (juvenile) rotifers were treated with unlabeled A $\beta$ 42 aggregates (100  $\mu$ g/mL; incubated 3 hours and 3 days) as 'food' source. After 12 days, well-contents were changed to dyed A $\beta$ 42 aggregated for 3 hours (*in vitro* marked; 10  $\mu$ M BisANS fluorescent dye for 30 min) or 3 days (*in vitro* marked; 50  $\mu$ M CR dye for 1 hour). Applying 5-hour treatment ('feeding'), we detected the optical signals in the digestive system of individuals by a fluorescence microscope. The representative digital photographs were taken of the localization of A $\beta$ 42 in the rotifers comparing untreated, standard fed and starved rotifers.

### 3.3.1.3. Scanning electron microscopy

The quality-based (e.g. morphological integrity) selected bodies were subjected to SEM (Zeiss), operating on 8-mm working distance at 10 kV and using a backscattered electron detector in various pressure mode at 30 Pa. The white balance of SEM photographs was normalized to the unspecific background.



### 3.3.2. Plate-reader-based optical detection (physiological markers)

#### 3.3.2.1. Absorbance (optical density).

To **validate the NADH dependency of PMS/XTT system** the reduction of XTT was measured both *in vitro* and *in vivo* with an absorbance plate-reader (Spectramax Plus) at 492/630 nm. In the *in vitro* experiments PMS (5  $\mu$ M), XTT (1000  $\mu$ M) and NADH (100  $\mu$ M) was administered alone or in their combination (incubated for 7 hours in dark; 24 °C, pH 7.5) in standard medium. The parameters of the *in vivo* investigations were the same, with different incubation time (24 hours) and without exogenous NADH. The readings were normalized to the background. For measuring the '**cellular reduction capacity**' (CRC) of rotifers, the EZ4U Cell Proliferation Assay was applied with modification to prevent toxicity. The absorbance was measured by an absorbance plate-reader set at 492/630 nm. The readings were normalized to the number of rotifers in each well. The percentages of the measures were calculated, 100% was defined as the ratio of absorbance and a matching number of animals in untreated control wells. The **intracellular NADH levels were quantified** (absorbance: 450 nm) with NADH Quantification Kit according to the protocol of the manufacturer. The reading were normalized exclusively to the survivals of preselection.

#### 3.3.2.2. Fluorescence (emission)

For the **BisANS-based protein quantification assay** the samples derived from *P. acuticornis* or yeast. They 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 (50  $\mu$ M; pH 6.5) in itself provided the unspecific background. The readings were carried out in a 96-well half-area plate using NOVOstar plate-reader at 405/520 nm. 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. Qubit Protein Assay Kit was used to validate our readings. To **measure the nucleic acid/protein ratio** of the normal- and super rotifers, PI (5  $\mu$ M) and BisANS (50  $\mu$ M) dyes were used. The measurements were run after a 10 min incubation period. The settings and the instrument were the same as described in the BisANS assay. The extinction/emission of PI was 530/620 nm. The readings were interrelated with rotifer number/well and this normalized fluorescence intensity was presented.

### 3.4. Statistics

Statistical analysis was performed with SPSS 23.0 software for Windows (SPSS Inc.) using one-way ANOVA with Bonferroni *post hoc* test or with GraphPad Prism 7.0b software (GraphPad Software Inc.) 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.

## 4. Results

### 4.1. Effects of combined biological and chemical redox modulations on *P. acuticornis*

Starved rotifers had the shortest lifespan (median = 13.5 days), whereas the group under normal caloric restriction had the longest (median = 48 days) one. During standard feeding, normal lifespan (median = 33.5 days) was observed. The longevity of rotifers under ECR was significantly shorter (median = 23 days) compared to the standard-fed counterpart.

The reduction of XTT was measured both *in vitro* (cell-free) and *in vivo* (middle-aged live rotifers). The reliability of the PMS- and XTT-containing system was tested (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. Without NADH, the reduction of XTT did not happen *in vitro*. The XTT alone was capable of forming formazan *in vivo*.

We preselected individuals by testing the electron deprivation tolerance, via treating with PMS, XTT and ascorbic acid and their combination under total food deprivation. As a parallel control for XTT, we used ascorbic acid. Intense chemical electron deprivation in the preselection phase caused significant toxicity under starvation. The PMS treatment caused significant decrease in all measured parameter of viability (live rotifer numbers, MCF and NADH level) compared to untreated control. Neither XTT nor ascorbic acid alone had any effect on viability markers compared to untreated control. 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 MCF. 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, MCF and intracellular NADH levels show positive correlation in their tendency.

The preselection phase-survived *P. acuticornis* rotifers were subjected to the next phase of experiments consisting of a permanent (for several months) and moderate (low-dose) chemical electron deprivation accompanied by standard feeding or ECR. The survival of the specimens was measured under various treatment conditions applying standard feeding or ECR. A pronounced longevity was observed in the corresponding XTT-treated group under standard feeding during second phase; however, the corresponding ascorbic acid-treated groups showed a slight superiority of ECR over standard feeding. Additionally, when ECR was combined with XTT treatment in preselected rotifers, it synergistically extended the lifespan to an extreme extent (up to 182 days) compared to the untreated-untreated control group. The XTT and ascorbic acid both significantly (though less remarkably) increased the lifespan of preconditioned rotifers compared to untreated-untreated controls and the respective treated-untreated controls, with no remarkable influence of diet.

The combination of starvation/PMS/XTT treatment during 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) rotifers 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. The BSI of super rotifers was higher than that of normal rotifers. Interestingly, the MCF of super rotifers was lower compared to normal ones, probably as a result of altered neuromuscular function due to increased size. The senescent 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. We assessed how super rotifers tolerate freezing conditions and found significantly more survivors in the super rotifer ( $5.0 \pm 0.52$  individuals) than in normal rotifer cultures ( $2.6 \pm 0.40$  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 ( $25 \pm 4.2$  days) until their natural death.

#### **4.2. Confirmation of eutely-status of enlarged entities**

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 rotifers and yeast, isolated with lysis buffer. Qubit assay was used to validate our BisANS type readings. No significant difference was discovered between the data measured by our assay and the Qubit. We measured simultaneously the protein and the nucleic acid amount of the normal- and super rotifers. The BisANS assay showed significant increase in the protein content of the super rotifers compared to the normal ones. With the PI-based assay we detected no difference between the content of the nucleic acid of normal- and super rotifers. These results indicate the enlargement of super rotifers was carried out via eutely.

#### **4.3. Beneficial effect of aggregated A $\beta$ 42 used as rotifer-food under caloric restriction**

We examined the effect of the supposed to be toxic A $\beta$ 42 on *P. acuticornis*. The treatment of the animals with A $\beta$ 42 resulted in a significantly longer mean lifespan ( $51 \pm 2.71$  days) than in the case of unfed ( $14 \pm 2.29$  days) and normally fed ( $32 \pm 2.72$  days) controls. We characterized the A $\beta$ 42-treated rotifers with experimental monitoring assays. The NML of groups treated with either 3 hours or 3 days aggregated A $\beta$ 42 significantly increased compared to unfed controls. The increase in the BSI (40%) and the BLD (60%) indicated phenotypical and physiological changes of the treated animals. The changes in the MCF (46%) and CRC (42%) suggested intensified energy level as represented by neuromuscular and cellular-redox activities. The A $\beta$ 42-treated one-housed rotifer individuals performed much better in the measured parameters than their unfed controls, and they do not considerably differ from the standard fed counterparts. These results suggest that A $\beta$ 42 is not toxic to *P. acuticornis* and it could be used by them as an exclusive dietary source to survive and develop in a hermetically-isolated environment.

We detected the presence of the exogenous aggregated (3 hours and 3 days) A $\beta$ 42 in the digestive system of the rotifers with  $\beta$ -sheet-specific fluorescent

(BisANS) and absorbent (CR) dyes after ‘feeding’ ad libitum. For monitoring the distribution of the potentially catabolized A $\beta$ 42 aggregates in the intact rotifers we applied Au-tagged A $\beta$ 42 aggregates and detected them with SEM. In the treated/fed animals, the signal of gold-metal could be found homogeneously everywhere in their body in contrast to the untreated ones. The only possible source of gold in the samples was the Au-A $\beta$ 42 complex taken up during the feeding process.

#### **4.4. Administration of various plant-extracts as nutrient, with phenotypic modulator properties**

Three viability parameters (NRA, MCF and BSI) were measured to assess effects of treatment with plant-extracts. The compounds rosavin, cinnamyl alcohol, ginsenosid Rb1, withanolide B, withanolide A and withaferin A caused a significant decrease in the NRA and in the MCF value. On the contrary, we observed a growth in BSI amongst the survivors, probably since they used these compounds as nutrient. A significant increase was observed in the BSI values of groups treated with *W. frutescens* and *R. rosea* crude extracts, parallel with a slight elevation in NRA and MCF. Exposure to the compound solidoside resulted in significantly decreased MCF with normal BSI in the survivors. In the 20-OH-ecdysone group we observed 40% decrease in rotifer number with less BSI and normal MCF values. From the 14 tested compounds, withaferin A proved to be the most toxic. 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.

### **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.

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* biological and chemical electron deprivation. The developed *in vivo* experimental setup was based on the data of the previous measurements and consisted of two main phases. The combination of PMS/XTT (under total food deprivation) preselection followed by XTT (under ECR) treatment phase 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 observed physiological changes in super rotifers are accompanied with slowed metabolic activity and cease of reproduction. To compare their viability markers, middle-aged normal rotifers and senescent super rotifers were followed-up for 10 days under standard feeding conditions without chemical treatment. Significant growth in BSI was accompanied by lower MCF compared to normal rotifers, probably as a result of altered neuromuscular function due to the increased body size. The senescent animals were capable of laying viable eggs (reflecting an effect on the reproduction phase); moreover, the number of offspring were significantly higher after the 10-day period compared to normal rotifers. To our knowledge, no similar long reproductive phase without dormant stage has ever been detected in bdelloid rotifers. The tolerance of freezing conditions and the recovery rate of the cultures were significantly higher in the super rotifer than in normal rotifer cultures. 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. For this investigation we applied our newly developed, BisANS-based protein quantification method. When we calculated the ratio of normal-BSI and super-BSI and the ratio of normal and super rotifers normalized fluorescence intensity, we found a distinct correlation between BSI 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 ( $25 \pm 4.2$  days) until their natural death.

During our work we discovered a yet unknown biological phenomenon concerning the effect of A $\beta$ 42 on *P. acuticornis*. Under total caloric restriction in isolated conditions we observed a novel catabolic activity in the rotifers. The one-housed and aggregated A $\beta$ 42-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 (NML) or maintain viability (BSI, BLD, MCF and CRC) showed the trophic effect of both aggregation state of A $\beta$ 42. The causality between the well-known negative effects of A $\beta$ 42 and their beneficial aspects on rotifers, is not evident, rather it can be viewed as contradictory. The administered exogenous A $\beta$ 42 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 homogeneously in their body. Since the treated *P. acuticornis* specimens maintained their health, function, and redox capacity, we presume that the neurodegeneration-related aggregates may serve as a required energy source for gluconeogenesis in our experimental conditions.

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. The treatment with 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 information related to biological limits fosters the correct application of our *micro-in vivo* model in large-scale drug screening.

The holistic aspects of the *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. Our future aims are to test various lifespan-modulating compounds (e.g. songorine) and investigate the molecular background of aging-modulation processes in ‘omics’ perspectives. Parallely with the extension of the natural rotifer-lifespan we revealed some special physiological parameters (e.g. attenuated metabolism) behind the longevity. The combination of the developed *in vivo* viability, phenotypical and systemic modulations allows us the interdisciplinary application of our model. Furthermore, by revealing evolutionary conserved biological processes in rotifers, it may lead to a possible human relevancy in the field of pharmacological research.

## 6. Conclusion

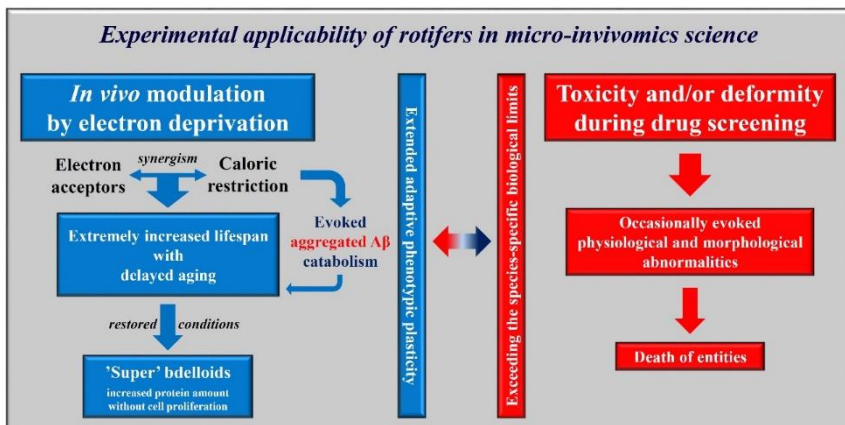
*The summary of our main findings is the following:*

- The bdelloid rotifer *P. acuticornis* is a suitable *in vivo* aging model according to the lifespan-measuring requirements stated in the academic literature;
- 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 ECR) on middle-aged rotifers resulted in exceedingly long, previously not documented lifespan in the survivors;
- 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;
- 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-phenomenon, we applied our newly developed BisANS-based protein quantification assay;
- The starvation-based *in vivo* electron deprivation resulted in adaptive phenotypic plasticity in the microinvertebrates. This property of the



bdelloids rotifers expressed also in the catabolism of the neurodegeneration-related A $\beta$ 42 peptide. This interdisciplinary phenomenon revealed the unique capability of these animals to use the neurotoxic aggregates as nutrients, which resulted also in extended lifespan. The investigation to unravel this unique ability could lend new scientific perspectives e.g. in the research of Alzheimer's disease;

- The *P. acuticornis* showed significant adaptability during the holistic electron deprivation. For test the limits of the phenotypic plasticity, we applied active agents and extracts derived plants occurring in their natural habitat. The appearance of physiological/morphological deformity of these animals were carefully monitored. This additional information contributed to the objective application of our rotifer-based aging model within the margin of error.
- The capabilities of *P. acuticornis* revealed during our work were presented the first time in the academic literature.



**Holistic and schematic summary of the thesis (A $\beta$  = beta-amyloid)**

## 7. Acknowledgement

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