Novel *in vivo* experimental viability assays with high sensitivity and throughput capacity using a bdelloid rotifer

Zita Olah[1], Ashley I. Bush[2], David Aleksza[3], Bence Galik[4], Eszter Ivitz[1], Lilla Macsai[1], Zoltan Janka[1], Zoltan Karman[1], Janos Kalman[1], Zsolt Datki[1]

[1] Department of Psychiatry, University of Szeged, Szeged, Hungary

[2] Oxidation Biology Unit, Florey Institute of Neuroscience and Mental Health, University of Melbourne, Parkville, Victoria, Australia

[3] Institute of Plant Biology, Biological Research Centre, Szeged, Hungary

[4] Bioinformatics & Scientific Computing, Vienna Biocenter Core Facilities, Vienna, Austria

Corresponding author:

Zsolt Datki,

Department of Psychiatry, University of Szeged, Szeged, Hungary, 6725 Szeged, Kalvaria sgt. 57, Hungary

e-mail: datkizsolt@gmail.com; datki.zsolt@med.u-szeged.hu

Abstract

Rotifers have been used in biological research as well-characterized models of aging. Their multi-organ characters and their sensitivity for chemicals and environmental changes make them useful as *in vivo* toxicological and lifespan models. Our aim was to create a bdelloid rotifer model to use in high-throughput viability and non-invasive assays. In order to identify our species *Philodina acuticornis odiosa* (PA), 18S rDNA-based phylogenetic analysis was carried out and their species-specific morphological markers identified. To execute the rotifer-based experiments, we developed an oil-covered water-drop methodology adapted from human *in vitro* fertilization techniques. This enables toxicological observations of individual one-housed rotifers in a closed and controllable micro-environment for up to several weeks. Hydrogen peroxide (H_2O_2) and sodium azide (NaN_3) exposures were used as well-understood toxins. The *toxicity and survival lifespan* (TSL), the *bright light disturbance* (BLD) the *mastax contraction frequency* (MCF) and *the cellular reduction capacity* (CRC), indices were recorded. These newly developed assays were used to test the effects of lethal and sublethal doses of the toxins. The results showed the expected dose-dependent decrease in indices. These four different assays can either be used independently or as an integrated system for studying rotifers. These new indices render the PA invertebrate rotifer model a quantitative system for measuring viability, toxicity and lifespan (with TSL), systemic reaction capacity (with BLD), organic functionality (with MCF) and reductive capability of rotifers (with CRC), *in vivo*. This novel multi-level system is a reliable, sensitive and replicable screening tool with potential application in pharmaceutical science.

Bdelloid; rotifer; pharmacological model; high throughput assay; oxidative stress; 18S rDNA; mastax; culturing; Philodina acuticornis odiosa; oil-drop method.

Chemical compounds studied in the article

Agarose (PubChem CID: 11966311)

Borate (PubChem CID: 26574)

EDTA (PubChem CID: 6049)

Ethanol (PubChem CID: 702)

Hydrogen peroxide (PubChem CID: 784)

Sodium azide (PubChem CID: 33557)

Sodium chloride (PubChem CID: 5234)

TRIS (PubChem CID: 13286)

XTT (PubChem CID: 497813)

Abbreviations

BLA - bright light avoidance; BLD - bright light disturbance; BLI - bright light irritation; CA - Cladophora aegagropila; CE - Caenorhabditis elegans; CRC – cellular reduction capacity; DW - distilled water; gDNA - genomic DNA; LCA - least common ancestor; MCF - mastax contraction frequency; ML - maximum likelihood; MSA - multiple sequence alignment; NJ - Neighbour-Joining; PA - Philodina acuticornis odiosa; bp – base pair; PM - Philodina megalotrocha; rDNA - ribosomal DNA; ROS – reactive oxygen species; TBE - Tris-Borate-EDTA buffer; TBS - Tris-buffered saline; TSL – toxicity and survival lifespan; XTT - (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide)

1. Introduction

Pharmaceutical and toxicological researches are aided by *in vivo* assays using small model organisms. Such models ideally should be multi-cellular and should meet the basic requirements of *in vitro* culturing methods. The ideal experimental methods should be non-invasive, sensitive and report on complex systems. The nematode *Caenorhabditis elegans* (CE) has been useful as a model organism for such studies despite being distant from mammals phylogenetically, with several vertebrate genes having no homolog (Snell, 2014).

Rotifers (phylum *Rotifera*) have similar advantages in culturing, anatomy, physiology and behavior similar to CE (Birky, 2004). They have the additional advantage that more than 10% of their genes have vertebrate homologs that can be found neither in nematodes nor in *Drosophila melanogaster* (Dahms et al., 2011). Rotifers are inexpensive to maintain, easy to handle, and have a relatively short lifespan; they are multicellular transparent animals with individual organs and nervous system (Snell et al., 1991). The bdelloids possess about 950 - 1000 somatic cells and have several well-described anatomic characteristics, such as ciliated head structure, bilateral ovaries, jaw-like mastax, ganglia, muscles, digestive- and secretory systems, photosensitive- and tactile organs (Marotta et al., 2012). They are resistant to harsh conditions (e.g. UV light, temperature, toxins) in their diapause stage. Only bdelloid rotifers have an uncommon degenerate tetraploid genome consisting of numerous genes acquired by horizontal gene transfer (Hagen et al., 2009). The reproduction of bdelloid rotifers is also obligatorily asexual, and males have been extinct for more than 30 million years. So, meiosis has never been documented in the bdelloid class (Gladyshev and Arkhipova, 2010).

Without any treatment or feeding, the bdelloid rotifers survive for approximately 15 days and are used as subjects of ecotoxicological research (Preston and Snell, 2011). Here, we validate novel assays to characterize *Philodina acuticornis odiosa* (PA) in terms of survival, health

and behavior, named *toxicity and survival lifespan* (TSL), *bright light disturbance* (BLD), *mastax contraction frequency* (MCF) and *cellular reduction capacity* (CRC). The TSL provides mortality rate (without feeding). The BLD index measures photostimulus-triggered reflexes, individual ethology and behavioral changes (Eakin and Westfall, 1965; Snell, 2014). Bdelloids have specialized photosensitive organs ("primitive eyes"), and avoid bright light, providing information about the neurological reflexes of the subjects. CE is also sensitive to light, but its sensation is based on photochemical-triggered reactive oxygen species (ROS) production, which could be confounded by other factors of metabolism (Bhatla et al., 2015). The MCF index assays the chewing organ function, providing information about the resilience of individuals and the need for nutrition. Assaying grinder movement is difficult in CE, but, in contrast, MCF is facile for PA. CE can react to stress by entering dormancy, which can be mistaken for death (Trojanowski and Raizen, 2016). The MCF will only reach zero when the PA is dead. The CRC gives information about the degree of reduction capacity and oxidative stress triggered by treatment. These four *in vivo* screening assays give continuous and quantitative indices.

To validate the newly developed assays we tested responses to well-known toxins. Hydrogen peroxide (H_2O_2) is a biocide that can cause massive cellular damage. It removes electrons from the susceptible chemical groups, oxidizing them and overwhelming the antioxidant defense system (Russel, 2003). At low concentrations, the oxidation caused by H_2O_2 can be rescued. H_2O_2 is readily permeable through cell membranes, interacting with intracellular components. The physiological response to H_2O_2 is always similar (Yang et al., 2013). The molecule affects multiple targets, such as peroxidation or disruption of membranes, oxidation of scavengers and thiol-groups, enzymatic inhibition, oxidation of nucleosides, impairment of energy production, causing inhibition of protein synthesis (Imlay, 2003; Poeggeler et al., 2005).

113 The use of sodium azide (NaN_3) as an alternative insecticide, herbicide, nematocide,
114 bactericide and fungicide has spread in the last few decades and its vasodilator effects are also
115 well-established (Bennett et al., 1996). NaN_3 as a cytochrome oxidase inhibitor is able to
116 inhibit the mitochondrial complex IV, therefore, it causes chemical hypoxia and lowers
117 energy production (Ye et al., 2016). It can partially inhibit the electron transfer chain in a
118 dose-dependent manner, leading to elevated ROS, mitochondrial membrane potential
119 reduction and the ultra-structural changes in mitochondria (Morales-Cruz et al., 2014).

120 Here, we report the development and validation of novel *in vivo* experimental monitoring
121 assays for the rotifer PA and providing reliable readout indices in four domains. The tests
122 compile a non-invasive (TSL, BLD, MCF), invasive (CRC) and high-throughput screening
123 index of the impact of different chemicals and environments on individual animals.

2. Materials and methods

2.1. Animal and plant

Among the bdelloid rotifers studied in our laboratory PA were considered suited for our methodological innovation because of their numerous advantages: short lifecycle, parthenogenic reproduction capability, discrete individual viability, ethological/behavioral markers and controllable culturing (Ricci, 1984).

The PA was obtained from Hungarian aquavaristique together with *Cladophora aegagropila* (CA; alternative names: *Aegogropila linnaei and/or Cladophora sauteri*) alga that is part of the environmental matrix of rotifers for living and reproduction (Hanyuda et al., 2002). This alga is found in an approximately 5 to 10 cm diameter spherical form in its natural habitat, but does not serve as a food source for animals, which feed off organic detritus or micro-organisms. In our experimental set-up, pasteurized *Saccharomyces cerevisiae* (yeast) homogenate was introduced as food (see in section 2.2.). The filamentous algae were necessary for the optimal culturing to form 3D-matrix, increasing the specific surface area.

2.2. Culturing rotifers

Rotifer culturing methods were developed based on previous literature (Ricci, 1984). The animals were cultured in a supervised and semi-sterile environment. These cultures contain Jana mineral water (origin: Croatia; distributed by Fonyódi Ásványvíz Kft., Hungary), half diluted with distilled water (Millipore type ultra-pure, demineralized DW), a mixture nominated ‘standard medium’ (pH=7.5). The amount of diluted cations and anions in 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. Every step of the rotifer manipulation was monitored by microscope. Clear cultures of PA were kept in standardized cell culturing flasks (cat. no.: 83.3910.302, Sarstedt AG & Co., Germany) in 15 mL standard medium. The flasks contained

CA alga fibers and sterile cotton wool buds in 1:1 ratio (mixed matrix) for structural stabilization. The thickness of algae/cotton wool matrix was ≈1 cm, thus significantly increasing the surface area for rotifer attachment, and so increasing the density of the culture from ≈8500 rotifers (2D; without matrix) up to ≈90,000 animals (3D; with matrix) per flask (after one month of culture, starting from one individual).

The CA alga balls were kept in standard medium, which was changed every three days. During dissection and isolation, a small part of the ball was removed using forceps and placed into a plastic petri dish (cat. no.: 430165, Corning Inc., USA). The resulting CA fragments, dispersed further into strings were washed once with 10% ethanol for 8 min and twice with standard medium for 30 min. The prepared alga strings were incubated for two days in standard medium and their quality checked daily by microscopy.

A further component of the culturing matrix was sterile cotton wool, which was dispersed with forceps into a petri dish and washed with DW thoroughly. Cotton wool fragments were then soaked in 96% ethanol for 30 minutes and then washed multiple times in DW. After two days the prepared pieces of cotton wool were put in a cell culture flask mixed with CA alga (1:1 volume ratio) in the medium and rotifers were then added. New cultures were started from previous ones by transferring one PA rotifer with standard medium. The flasks were kept at 25 °C and under a light/dark cycle of 12:12 hours and were examined every 24 hours under an inverted transparent light microscope (Leitz Labovert FS, magnification range from 32 x to 1000 x; Germany).

The culture medium was changed every two days. First, the culturing flasks were shaken carefully several times, then the old medium was decanted. As the next step, the mixed matrix was washed with abundant DW after which we supplied the standard medium again to the

flask and checked the clarity of the medium under microscope. If the medium was visibly cloudy the procedure was repeated.

The animals were fed with 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 0.6 mg/mL. The yeast stock solution was pasteurized at 65 °C for three hours in a water bath (Grant Sub 6; Grant Instruments Ltd., UK), then left at room temperature overnight, after which it was filtered (15 µm pore size) and divided into 2 mL aliquots. These were pasteurized again and stored at -75 °C until further use.

2.3. Harvesting rotifers

To commence harvesting, the medium was first changed, without feeding. The flasks were placed at -75 °C for 3 minutes for rapid cooling (up to 4 °C) in order to dissect attached rotifers from the alga/wool fibrils on ice. The medium was poured off, and the flasks were washed with standard medium twice, which was also removed in petri dish. The dish was then left for 30 minutes to let healthy animals attach to the bottom, then the surface was washed twice with DW and once with the standard medium (25 °C). We chose rotifers approximately 5-day-old after hatching (determined by body size; length 220±10 µm and width 60±5 µm), 1-2 days before the beginning of the reproductive stage of these animals. The calibration curve to determine the age of individual *Philodina acuticornis* rotifers isolated from standard medium can be found in Suppl. fig. 1.

For genetic studies the rotifers were used instantly after harvesting. For studies using the microdrop technique, the isolated colony was left for 24 hours in the petri dish without feeding, then, after a medium change, individual specimens were selected for treatment. This

was to select viable rotifers and to empty their digestive system to avoid any contamination in the microdrop setup. Every step was monitored by light microscopy.

2.4. Characterization of rotifers

2.4.1. Phenotypic analysis

Rotifer cultures used in our experiments were started with parthenogenesis from one unique animal to preserve genetic homogeneity. The live and healthy specimens were studied under light microscope using both photo and video analysis (Nikon D5500, Nikon Corp., Japan). When needed, we fixed the animals by adding cooled (-75 °C) ethanol (96%) when they adopted an elongated shape after decanting the medium from petri dishes. After fixation we used decreasing concentration steps of ethanol and then DW to rehydrate the sample with 10 minutes between each step. The bodies prepared this way were placed on a microscope slide with pipetting between 0.3 mm thick spacers and were covered with a glass slip. The animals were photographed under the microscope by taking multiple pictures at 5 µm sections (30-40 photo-layers/animal), which were then combined into one superimposed picture (created by Photoshop CC) to achieve better detail. Characterization was performed by examining the size of specimen (whole body or body parts), shape, special morphologic markers (e.g. characteristics of trophi, foot, corona, eyes, antennae) and different types of movement (swimming, crawling, the use of corona, the pulsation of mastax, body contractions, etc.). Animals were typified based on the works of K. Kertész (1894), L. Varga (1966) about the endemic species of microscopic animals in Hungary, and the publications of Ricci and Melone (2000).

215 2.4.2. Phylogenetic analysis

216 2.4.2.1. DNA isolation

217 The rotifers were placed from the culture into a 25 mL flask and after attachment they were
218 washed twice with DW. The density was approximately 100 animals/cm^2. The isolated
219 rotifers were recovered from the flask with 10 mL 50% ethanol and were concentrated in a
220 two-step centrifugation. First, the 10 mL recovered solution was centrifuged at 6000 x g for
221 20 minutes and then the pellet was suspended in 1 mL and centrifuged at 12,000 x g for 5
222 minutes. We tried various isolation methods in order to maximize the quality and recovery of
223 genomic DNA (gDNA; data not shown). Finally, we selected the Power Soil gDNA isolation
224 kit (cat. no.: 12888-100; MO BIO Laboratories, INC., Qiagen Company), following the
225 protocol given by the manufacturer. The quality and the size of the sample was checked on
226 1% agarose gel (cat. no.: A9539; Sigma-Aldrich, Co., USA), 65 V, 45 min, in TBS. The
227 concentration of the sample was 3.3 ng/µL, determined by Qubit HS DNA kit (cat. no.:
228 Q32854; Thermo Fisher Scientific).

229 2.4.2.2. PCR reaction

230 The PCR reaction contained 6 ng of gDNA sample, 10 µL DT Green PCR MM (cat. no.:
231 K1081; Thermo Fisher Scientific) 0.5 µL each of 10 µM forward and reverse primers, 7 µL
232 nuclease-free water (detailed PCR protocol steps are included in Suppl file 1). The primers
233 were bdelloid-specific and were selected based on the literature (Robeson et al., 2009). The
234 sequences of the primers are the following: Bdel_forward (5'-
235 CGGCTCATTACATCAGCTATAACTT-3'); Bdel_reverse1 (5'-
236 GATCCTTCCGCAGGTTCACC-3'); Bdel_reverse2 (5'-
237 GACTCAACACGGGAAACCTCACC-3'); and Bdel_reverse3 (5'-
238 CTAAGGGCATCACAGACC-3'). We checked the primers using the available PA sequence

239 from the SILVA database (www.arb-silva.de) and we found that the mix of Bdel_forward and
240 Bdel_reverse1 amplified a 1714 base pair (bp) fragment and the mix of Bdel_forward and
241 Bdel_reverse3 amplified 1360 bp fragment *in silico*. The extracted fragments were sent to
242 Sanger sequencing, using the latest versions of Life Technologies Genetic Analyzer Capillary
243 Electrophoresis Systems (3500 Series Genetic Analyzer).

244 2.4.2.3. Sequence analysis

245 The amplicons were sequenced from both ends (5' and 3'), which were then used to create a
246 consensus sequence. Primers and low quality (<20 quality value) 5' and 3' ends were
247 trimmed, and the sequences were edited by hand using DNA Chromatogram Explorer (DNA
248 Chromatogram Explorer Lite, Version 4.0, Heracle BioSoft). DNA Baser Sequence
249 Assembler (DNA Baser Sequence Assembler v4, Heracle BioSoft) was used to create contigs
250 from the forward and reverse reads of the sequenced PCR products (Jeanmougin et al., 1998).
251 Thus, we could increase the quality, reduce mismatches and obtain the longest possible read.

252 2.4.2.4. Classification

253 Following the previously described quality control, SILVA aligner was used to search and
254 classify our sequences against the SILVA SSU 128 Ref NR database (Quast et al., 2013)
255 using the least common ancestor (LCA) method with 95% minimal identity to query
256 sequence. Reference sequences were rejected under 70% identity against the query to only
257 write sequences with an alignment identity larger than the specified. With this step our
258 species were determined based on its 18S rDNA sequence. The alignment and classification
259 results were saved in FASTA format with metadata and upload into NCBI GenBank.

260 2.4.2.5. Phylogenetic analysis

261 Our model organism PA was previously identified based on phenotypical and morphological
262 features (Ricci and Melone, 2000; Varga, 1966). The next step was to identify the

evolutionary placement using the sequenced rDNA from our model organism. However, there are dozens of species in Rotifera, but only a subset of 18S rDNA sequences are available in the SILVA database for this clade. All Rotifera sequences were downloaded and used for further analyses, listed by accessions in the Table. First, a classical taxonomic tree was drawn with PhyloT (PhyloT, biobyte solutions GmbH, http://phylot.biobyte.de/index.html, database version 2017.1) using scientific names of all species listed in the Table. Then the first 18S rDNA based phylogenetic tree (Fig. 1.) was built using the most popular distance-based Neighbor-Joining method in MEGA7 (Gascuel and Steel, 2006). The detailed analytic method (Kumar et al., 2016) and statistical analysis of the results (Efron et al., 1996; Ronquist et al., 2012) are described in Suppl. file.

2.5. Microdrop technique for the treatment of rotifers

We devised a novel microdrop culturing technique in order to treat and observe one individual PA rotifer at a time, based on human *in vitro* fertilization procedures (Nagy et al., 2012). For this we used 24-well plastic cell culture plates (cat. no.: 3526, Corning Inc., USA). At the bottom of each well of the plate a 30 μL microdrop of standard medium (pH=7.5) was placed by pipetting and was then covered with 640 μL of a mixture (1:1) of the high purity paraffin oil (Cat. no.: 18512-1L; Sigma-Aldrich Co., USA) and SAGE tissue culture oil (Cat. no.: ART-4008; Origio, Denmark) used in human *in vitro* fertilization as a protective barrier for embryonic cell cultures (Nagy et al., 2012). These are mineral oils that facilitate normal gas diffusion between the microdrop and the environment while preventing evaporation and hypoxia in the microdrop.

After preparation, the oil covered water-drop setup was left to rest for one day without animals (Fig. 2A). The rotifers were harvested, isolated and selected and then placed in a microdrop by pipetting with 20 μL standard medium. Treatment was administered one day

after the transfection of animals and for each dose the treating substance was injected into the microdrop in 5 µL of liquid containing H_2O_2 (cat. no.: 95321; Sigma-Aldrich, Co., USA) or NaN_3 (cat. no.: S2002; Sigma-Aldrich, Co., USA) in 11 x concentration. Thus, the end volume of a microdrop with a one-housed animal was 55 µL. The oil thickness was 1 mm on top of each drop. The plates were kept at 25 °C throughout the experiment. After the treatment the wells were observed twice a day until the animal was considered dead inside the drop.

2.6. Experimental monitoring assays

The viability of rotifers was assessed with four different assays (Fig. 2B) utilizing video recordings with a Nikon D5500 DSLR camera. The treatment agents (H_2O_2 and NaN_3) were used in decreased concentration in the individual assays. Overlapping doses enabled the response of the various tests to be cross-validated over their dynamic range. The H_2O_2 and NaN_3 doses applied in TSL (µM): 70; 100; 200; 400; 500; 800; 1000; 2000; in BLD: 5; 10; 30; 50; 70; 100; 200; 400; in MCF: 0.1; 0.5; 1; 5; 10; 30; 50; 70; in CRC: 0.1; 1; 10; 50; 100; 400; 800; 2000.

2.6.1. Toxicity and survival lifespan (TSL) assay

The impact of test compounds on the lifespan of unfed PA rotifers was assessed. We defined different morphological viability markers: (i) normal anatomy (Fig. 3A) and the active locomotion (motility) of the body, (ii) general movement within the body and (iii) naturally red eyes (Fig. 3B/1 and B/2, marked with stars). A rotifer was considered dead when the following features were all apparent: the absence of motility when touched by the end of the micro-pipette, the loss of red color of the eyes, loss of the telescopic reflex, and the appearance of fragmentation or amorphous granules in the soma (Poeggeler et al., 2005).

2.6.2. Bright light disturbance (BLD) assay

311 Based on our observations of PA rotifers we devised a novel method to assess behavioral
312 responsiveness with high sensitivity and resolution. We noticed that the animals reacted to
313 intensely illuminated zones by evading them promptly. We could quantify the sensory-motor
314 system response (i.e. reflex) and monitor the impact after exposure to different compounds at
315 sublethal concentrations.

316 The microdrop setup was placed under a Hund Wetzlar H500 microscope (ocular: PK 20 x
317 /8/; objective: HW-A 10/0.25, 160/-) with the light source illumination set to 20 lux in
318 relation to one well of the plate. We identified the active specimen (i.e. a surface-attached
319 animal exhibiting mastax chewing and beating of the coronal cilia) and we adjusted the well
320 so that the animal was in the middle of a 0.5 mm^2 illuminated area created by narrowing and
321 masking the light source of the microscope with the aperture mechanism. After 30 sec, if the
322 specimen was still in place and moving coronal cilia, we increased the illumination to 40,000
323 lux and measured the reaction of the rotifer. Two reactions (parameters) were recorded: (i)
324 *bright light irritation* (BLI), which is the total time that the rotifer is contracted or crawling,
325 recapitulating the unfed-state phenotype where cilia are not used for motility. When the
326 treated animal remains in a cilia-motile (fed-state) form it is considered insensitive to the
327 bright light stimulation; (ii) *bright light avoidance* (BLA), which is the time spent in
328 illuminated area (time to leave the designated area). We defined the BLD index, according to:
329 $BLD\ (\%) = (BLI/BLA) \times 100$. As a health index, the BLD approaches 100% when it reflects
330 the maximum sensitivity and reaction to light of rotifers. The rotifers had a maximum of five
331 minutes to leave the illuminated area in the BLD test, whereupon the animals were considered
332 to be unable to escape and assigned a BLD of 0.

333 2.6.3. Mastax contraction frequency (MCF) assay

The mastax (pharynx) is part of the digestive system, has a powerful muscular wall and contains tiny, calcified, jaw-like structures named trophi. The function of the mastax is to shred the food by periodic opening and closing (Fig. 3C/1 and C/2; marked with star). To evaluate and standardize the viability of one-housed rotifers in our experiments, we developed the MCF (contraction/sec) as a quantitative viability marker.

2.6.4. Cellular reduction capacity (CRC) assay

The EZ4U Cell Proliferation Assay (non-radioactive cell proliferation, cytotoxicity and reduction capacity assay with XTT solution) was used (cat. no.: BI-5000; Biomedica Hungary). This assay is widely used on cell cultures and tissues (Berridge et al., 2005), but to our knowledge there are no data about applying it to intact invertebrates. We modified the standard protocol, given by the manufacturer, in order to measure the cellular reductive capacity of rotifers. After standard harvesting, approximately 300 rotifers per well were assayed a 24 well-plate. To avoid toxicity, 20 x diluted XTT solution was used (475 µL standard medium with 25 µL XTT kit-stock solution per well). The plates were incubated for 24 hours without direct light at room temperature (25 °C). The supernatant of each well was then transferred to a 96 well-plate. The absorbance was measured by a microplate-reader (Spectramax 384, Molecular Science, Hungary) set at 491 nm with 630 nm as a reference. The readings were normalized to the number of animals/well. The percentages of the measures were calculated, the 100% was the ratio of absorbance (OD) and matching number of animals in untreated control wells.

2.7. Statistics

Data are presented as means±S.E.M. Statistical evaluation and curve fitting were performed with Prism 7.0b (GraphPad Software) and with SPSS 23.0 software for Windows.

3. Results

3.1. Sequencing

After quality control and assembly of rotifer DNA samples, compared to the PCR products (1714 and 1340 bp), we achieved 1265 and 1624 bp query sequences (NCBI GenBank accession numbers: SUB2522809 and KY829026). As a result of SILVA aligner analysis, sequences were classified as *Philodina acuticornis odiosa* (PA) with 98% (for the 1265 bp long read) and showed 97% (for the 1624 bp long read) similarity to the reference. Because the DNA was isolated and amplified from an isolated population, SNPs could have biased the similarity scores. Indeed, the longer sequence showed lower similarity, therefore, phylogenetic analysis was performed.

3.2. Phylogenetic analysis

Based on the morphological features and the SILVA aligner analysis, query species were identified as PA (Suppl. fig. 2). The classical phylogenetic position of PA was determined by NCBI taxonomy. Other phylogenetic trees constructed by modern methods can depict the relationship between clades and taxonomic groups. This supported separation of the two main orders (*Monogononta, Bdelloidea*) by 18S-based phylogenetic analysis (Fig. 1 and Suppl. fig. 2). The two different phylogenetic tree calculating methods gave similar results, validating each other. However, there are a few differences compared to the classical one. These differences are caused by the relatively small evolutionary distances between the sequences. For example, in *Bdelloidea* clades none of the trees showed the previously drawn classical taxonomy. Notably, our PA in the phylograms stands much closer to *Philodina megalotrocha* than to the reference PA/U41281.

3.3. Validation of the *in vivo* toxicity assays

To validate our novel screening system, we exposed the PA to two toxic agents, hydrogen peroxide (H_2O_2) and sodium azide (NaN_3) with well-known modes of action. Unchallenged

PA had a median TSL of 15 days, and both toxins suppressed TSL in a significantly dose-dependent manner ($p<0.0001$ for both agents, logrank test for trend, Fig. 4A-C), with lifespan halving induced by concentrations of H_2O_2=163 µM, NaN_3=399 µM (iterated from the survival curves, Fig. 4C). The lowest concentrations tested to significantly impair TSL were H_2O_2 70 µM (median survival 11 days, log-rank $p<0.0001$), NaN_3 70 µM (median survival 13.5 days, log-rank $p=0.0341$).

PA was then challenged with various doses of H_2O_2 and NaN_3 over a 3-day exposure to test viability and behavioral effects using the BLD. To exhibit the response features of this assay, we employed concentrations of H_2O_2 and NaN_3 at an order of magnitude lower than the TSL tests. We found that the tested toxins induced significant dose-dependent inhibition of BLD (Fig. 4D, IC50s H_2O_2=51.9 µM, NaN_3=83.4 µM). The lowest concentrations tested to significantly impair BLD were H_2O_2 10 µM (-14.2%, one-way ANOVA with Dunnett's multiple comparison test, $p<0.0001$), NaN_3 10 µM (-16.9%, one-way ANOVA with Dunnett's multiple comparison test, $p<0.0001$).

We examined the effects of H_2O_2 and NaN_3 in the *mastax contraction frequency* (MCF) test and since this test is potentially a more sensitive index, we focused on the effects of toxin concentrations an order of magnitude lower than used in the BLD tests. After three days of exposure, the toxins at ≥10 µM significantly inhibited MCF (Fig. 4E, IC50s H_2O_2>70 µM, NaN_3=46.4 µM). The lowest concentrations tested to significantly impair MCF were H_2O_2 10 µM (-0.6 sec^{-1}, one-way ANOVA with Dunnett's multiple comparison test, $p<0.0430$), NaN_3 30 µM (-1.83 sec^{-1}, one-way ANOVA with Dunnett's multiple comparison test, $p<0.0001$). Curiously, H_2O_2 1 µM significantly but modestly promoted MCF by 0.7 sec^{-1} (one-way ANOVA with Dunnett's multiple comparison test, $p=0.0113$). This may indicate that low concentrations of H_2O_2, which is known to be a cellular signaling molecule at concentrations of this order (Veal et al., 2007) may act to stimulate rotifer activity. However, the CRC

response at this dose of H_2O_2 for the same time interval (3 days) indicated oxidative stress. So the increase in the MCF in response to H_2O_2 at 1 µM we hypothesize is a compensatory response to oxidative stress e.g. a reflex to increase nutriture.

Finally, the *cellular reduction capacity* (CRC) measurements were performed after three days of treatment. H_2O_2 and NaN_3 induced significant dose-dependent inhibition of CRC, in line with our expectations (Fig. 4F, IC50s H_2O_2=32.9 µM, NaN_3=91.2 µM). The lowest concentrations tested to significantly impair CRC were H_2O_2 1 µM (-30.3%, one-way ANOVA with Dunnett's multiple comparison test, $p<0.0001$), NaN_3 1 µM (-13.5%, one-way ANOVA with Dunnett's multiple comparison test, $p<0.0001$).

By pooling the data across the various treatments and doses, it was possible to appraise the ability of individual tests to predict lifespan and other viability indices. Both BLD and CRC strongly and significantly predicted lifespan (Fig. 5A, B). Similarly, we could also validate the impairment indices by examining their ability to predict each other. The BLD index was strongly and significantly associated with MCF (Fig. 5C) and CRC (Fig. 5D), and the MCF index was significantly associated with the CRC (Fig. 5E). While the TSL only shows the live/dead status of the rotifers, the other three tests also quantitatively assess aspects of the viability of individuals upon harmful, but sublethal toxin exposure. In toxicological experiments, our assays are suitable for detecting lethal and sublethal doses.

4. Discussion

Over the last 30 years the interest in experimental research related to rotifers has increased. Their use as a model in marine ecotoxicological and environmental studies provides reliable information about acute and chronic deleterious effects of aquatic pollution (Breitholtz et al., 2006). They also provide important data about specialization (Gómez et al., 2002), evolutionary ecology (Snell et al., 2006), the evolution of sex, population dynamics

(Yoshinaga et al., 2003) and ecotoxicity (Kaneko et al., 2005). Their main advantages in toxicological research derive from their small size, relatively short lifespan, high population density, rapid population growth rates, availability of culture methods; predominantly parthenogenic reproduction and sensitivity to a vast number of toxic agents (Hagiwara et al., 1997). Despite these advantages, standardized behavioral toxicity tests with invertebrates as model organisms are scarce in pharmaceutical research.

We have developed straightforward and multifaceted methods that support quantifiable *in vivo* toxicological testing on rotifers. While there have been previous assays developed for the lifespan and survival of rotifers (Kaneko et al., 2016), these have been on the basis of assaying large groups of aggregated animals. A caveat with this approach is that the dead animals may serve as food sources for the living ones. The novel aspect of our approach lies in measuring the individual lifespan of isolated rotifers, as well as combining this information with a matrix of readouts about the animals' well-being, functioning and redox state. The oil-covered water-drop methodology, adapted from human *in vitro* fertilization, enables toxicological measurements on a single, isolated animal (one-housed rotifer) under controlled conditions in a microenvironment. Our model was confirmed to be *Philodina acuticornis odiosa* (PA). The sequence similarity showed the lower limit of 18S-based classification using the currently available sequences of SILVA database. The similarity level could be influenced by the unknown strain-specific SNP variability (Gribble et al., 2014). The phylogenetic analysis also validated our theory that our species diverge from the available PA/U41281 sequences.

We validated four new high-throughput *in vivo* toxicological screening indices using two widely-used toxic agents with well-known mechanisms of action, hydrogen peroxide (H_2O_2) and sodium azide (NaN_3), which can now serve as standards for future eco- or pharmaco-toxicological surveys using the PA model.

457 The *toxicity and survival lifespan* (TSL) index is a life-conditional marker of animals'
458 existence. It showed a dose-dependent relationship of median lifespan to dose of toxin. The
459 criteria for survival were sufficiently stringent for reproducible data to be achieved and for
460 predictive validity to be achieved for the two indices (BLD and CRC) where overlapping
461 toxin concentration data were available.

462 The *bright light disturbance* (BLD) index is able to detect effects of sublethal doses of toxic
463 compounds based on a complex behavioral reflex of PA. Bdelloid rotifers have a specialized
464 photosensitivity deriving from their primitive eyes, and are able to sense light directly. The
465 rotifers show active escaping behavior in reaction to bright light, therefore the test gives
466 complex information on a systemic level (Imlay, 2003). A common invertebrate model is the
467 *Caenorhabditis elegans* (CE), which is also sensitive to light but detects light based on a
468 metabolic reaction: light-induced reactive oxygen species (ROS) production (Bhatla et al.,
469 2015). Using the bdelloid rotifer PA, the validation of BLD showed that sublethal doses of
470 H_2O_2 and NaN_3 also attenuated TSL.

471 The *mastax contraction frequency* (MCF) index provides information about the muscular
472 condition of the animals. The measurement is methodologically similar to the CE pharyngeal
473 pumping rate measurements (Bhatla et al., 2015). Both of their organs have a role in feeding
474 (moving food into the intestine), pump throughout the lifetime of an individual, and react to
475 modulators (Lazakovitch et al., 2008). The MCF was found to be the most sensitive index, as
476 it was attenuated by both toxins at low concentrations, but correlated with the results provided
477 by the BLD and CRC indices.

478 The *cellular reduction capacity* (CRC) is an adaptation of a widely used assay to quantify the
479 reductive capacity of the rotifer tissue. MCF and BDL assays also predicted changes in this
480 assay.

In summary, our new *in vivo* screening system contains four different methods that enable assay of various parameters at different sensitivity levels with high lifespan predictive value, independently and/or simultaneously, providing a reliable and highly replicable screening method for pharmaceutical and toxicological science.

Conflict of interest

Prof. Bush is a shareholder in Prana Biotechnology Ltd, Cogstate Ltd, Brighton Biotech LLC, Collaborative Medicinal Development Pty Ltd and Mesoblast Ltd. He is a paid consultant for Collaborative Medicinal Development Pty Ltd.

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Appendix: Supplementary files

Suppl. file

Suppl. fig. 1

Suppl. fig. 2

Suppl. fig. legends

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Figure legends

Figure 1. Evolutionary relationships of taxa obtained from MEGA7 analysis

The evolutionary history was inferred by using the Neighbour-Joining method. The numbers next to the nodes represent the number of times certain species were grouped together in the analysis. The numbers under the branches represent the statistics of percentile of evolutionary similarity. Our *Philodina acuticornis odiosa* (PA) and *Philodina megalotrocha* (PM) are related; PM is the closest evolutionary relative of our PA species, which stands closer than the reference PA/U41281. The analysis involved 26 nucleotide sequences. The evolutionary distances were computed using the p-distance method (Efron et al., 1996) and are shown under the branches and in the units of the number of base differences per site. To validate and confirm our first phylogenetic tree, a second tree was created using a recent version of maximum likelihood character-based probabilistic analysis in MrBayes (data not shown).

Figure 2. Workflow schematic of our *in vivo* experimental monitoring systems

A. Experimental setup of the rotifer treatment and monitoring. On Day 1, animals are harvested and separated from the culture and simultaneously microdrops are prepared in a 24 well-plate. On Day 2, the rotifers are transferred to microdrops (one rotifer to one microdrop per well) and they are placed under oil to accommodate to the new environment. On Day 3, the one-housed rotifers are treated with molecules of interest. From Day 4, the recording of monitoring assays commences to provide information on dose- and time-kinetic effects on *toxicity and survival lifespan* (TSL), *bright light disturbance* (BLD), *mastax contraction frequency* (MCF) *and cellular reduction capacity* (CRC). **B**. Ranges of H_2O_2 and NaN_3 (in µM) used in the TSL, BLD, MCF and CRC assays indicating the range of sensitivity of each assay, as well as the overlapping doses studied (used in the analyses in Fig. 5).

Figure 3. Anatomic characteristics of *Philodina acuticornis odiosa* (PA) used in viability assays

655 Micrographs of the characteristics of the PA used in the screening system. **A**. A whole rotifer
656 displaying corona, head, body and feet (scale bar: 50 µm). The transparency of the body
657 enables observations of the movement of the internal organs as a viability marker. **B/1.** The
658 anterior part of rotifer showing the head, corona and eyes (scale bar: 15 µm). **B/2**.
659 Enlargement of the primitive red eyes (marked with stars; scale bar: 5 µm): specialized
660 photosensitive organs that facilitate the bright light disturbance response. **C.** The mastax
661 (marked with stars) is the muscle pharynx of rotifers, and its periodic closing (C/1) and
662 opening (C/2) are sensitive to toxin exposure, providing information about organ functionality
663 (scale bar: 15 µm).

664 **Figure 4. Experimental screening assays with different sensitivities**

665 In each experiment the one-housed rotifers were treated with different doses of H_2O_2 and
666 NaN_3 as indicated, in accordance with the sensitivity of selected assay method. **A, B.** Kaplan-
667 Meier survival curves in the TSL assay of rotifers (n=30 per dose of toxin indicated). **C.**
668 Median survival in the TSL assays of rotifers exposed to a range of toxin concentrations. **D.**
669 BLD assay measured the ethological/behavioral reaction of rotifers to irritation with bright
670 light. The rotifers (n=30 individual one-housed rotifers) were challenged with various doses
671 of toxins, as shown. The means of normalized BLD±S.E.M. are shown. **E.** MCF
672 measurements assessed the mastax activity after three days treatment. The one-housed rotifers
673 (n=30 individual one-housed rotifers) were treated as indicated. The means of
674 MCF/sec±S.E.M. are shown. **F.** CRC, a cellular reduction capacity indicator, was measured
675 after three days treatment. The means of normalized CRC±S.E.M. (n=24 replicates at each
676 dose) are shown. Curve fitting was modelled with **C, E,** the [Inhibitor] vs. response algorithm,
677 and **D, F,** the [Inhibitor] vs. normalized response -- Variable slope algorithm (Prism 7.0).

678 **Figure 5. Predictive validity of the indices**

679 The different measurements of survival and viability were correlated with the other outcomes
680 where ≥4 overlapping toxin concentrations had been tested for both H_2O_2 and NaN_3 (see Fig.
681 2B). All available indices were significantly correlated. TSL results were significantly
682 predicted by **A,** BLD and **B,** CRC. CRC results were significantly predicted by **C,** BLD and
683 **D,** MCF. CRC was significantly predicted by **E,** MCF. The correlational analysis was
684 performed by Prism 7.0. Means and S.E.M., with 95% CI of the regression lines, are shown.
685

686 **Table legends**

687 **List of SILVA accession numbers by major groups used in phylogenetic analyses**

688 *Philodina* IDs are highlighted with bold italic.

Author/s:
Olah, Z; Bush, AI; Aleksza, D; Galik, B; Ivitz, E; Macsai, L; Janka, Z; Karman, Z; Kalman, J; Datki, Z

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