

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

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18 **Abstract**

19 Rotifers have been used in biological research as well-characterized models of aging. Their  
20 multi-organ characters and their sensitivity for chemicals and environmental changes make  
21 them useful as *in vivo* toxicological and lifespan models. Our aim was to create a bdelloid  
22 rotifer model to use in high-throughput viability and non-invasive assays. In order to identify  
23 our species *Philodina acuticornis odiosa* (PA), 18S rDNA-based phylogenetic analysis was  
24 carried out and their species-specific morphological markers identified. To execute the rotifer-  
25 based experiments, we developed an oil-covered water-drop methodology adapted from  
26 human *in vitro* fertilization techniques. This enables toxicological observations of individual  
27 one-housed rotifers in a closed and controllable micro-environment for up to several weeks.  
28 Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and sodium azide (NaN<sub>3</sub>) exposures were used as well-understood  
29 toxins. The *toxicity and survival lifespan* (TSL), the *bright light disturbance* (BLD) the  
30 *mastax contraction frequency* (MCF) and *the cellular reduction capacity* (CRC), indices were  
31 recorded. These newly developed assays were used to test the effects of lethal and sublethal  
32 doses of the toxins. The results showed the expected dose-dependent decrease in indices.  
33 These four different assays can either be used independently or as an integrated system for  
34 studying rotifers. These new indices render the PA invertebrate rotifer model a quantitative  
35 system for measuring viability, toxicity and lifespan (with TSL), systemic reaction capacity  
36 (with BLD), organic functionality (with MCF) and reductive capability of rotifers (with  
37 CRC), *in vivo*. This novel multi-level system is a reliable, sensitive and replicable screening  
38 tool with potential application in pharmaceutical science.

39

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

42 **Chemical compounds studied in the article**

43

44 Agarose (PubChem CID: 11966311)

45 Borate (PubChem CID: 26574)

46 EDTA (PubChem CID: 6049)

47 Ethanol (PubChem CID: 702)

48 Hydrogen peroxide (PubChem CID: 784)

49 Sodium azide (PubChem CID: 33557)

50 Sodium chloride (PubChem CID: 5234)

51 TRIS (PubChem CID: 13286)

52 XTT (PubChem CID: 497813)

53 **Abbreviations**

54

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

63

## 64 1. Introduction

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

71 Rotifers (phylum *Rotifera*) have similar advantages in culturing, anatomy, physiology and  
72 behavior similar to CE (Birky, 2004). They have the additional advantage that more than 10%  
73 of their genes have vertebrate homologs that can be found neither in nematodes nor in  
74 *Drosophila melanogaster* (Dahms et al., 2011). Rotifers are inexpensive to maintain, easy to  
75 handle, and have a relatively short lifespan; they are multicellular transparent animals with  
76 individual organs and nervous system (Snell et al., 1991). The bdelloids possess about 950 -  
77 1000 somatic cells and have several well-described anatomic characteristics, such as ciliated  
78 head structure, bilateral ovaries, jaw-like mastax, ganglia, muscles, digestive- and secretory  
79 systems, photosensitive- and tactile organs (Marotta et al., 2012). They are resistant to harsh  
80 conditions (e.g. UV light, temperature, toxins) in their diapause stage. Only bdelloid rotifers  
81 have an uncommon degenerate tetraploid genome consisting of numerous genes acquired by  
82 horizontal gene transfer (Hagen et al., 2009). The reproduction of bdelloid rotifers is also  
83 obligatorily asexual, and males have been extinct for more than 30 million years. So, meiosis  
84 has never been documented in the bdelloid class (Gladyshev and Arkhipova, 2010).

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

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

103 To validate the newly developed assays we tested responses to well-known toxins. Hydrogen  
104 peroxide (H<sub>2</sub>O<sub>2</sub>) is a biocide that can cause massive cellular damage. It removes electrons  
105 from the susceptible chemical groups, oxidizing them and overwhelming the antioxidant  
106 defense system (Russel, 2003). At low concentrations, the oxidation caused by H<sub>2</sub>O<sub>2</sub> can be  
107 rescued. H<sub>2</sub>O<sub>2</sub> is readily permeable through cell membranes, interacting with intracellular  
108 components. The physiological response to H<sub>2</sub>O<sub>2</sub> is always similar (Yang et al., 2013). The  
109 molecule affects multiple targets, such as peroxidation or disruption of membranes, oxidation  
110 of scavengers and thiol-groups, enzymatic inhibition, oxidation of nucleosides, impairment of  
111 energy production, causing inhibition of protein synthesis (Imlay, 2003; Poeggeler et al.,  
112 2005).

113 The use of sodium azide ( $\text{NaN}_3$ ) 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).  $\text{NaN}_3$  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.

124 **2. Materials and methods**

125 **2.1. Animal and plant**

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

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

138 **2.2. Culturing rotifers**

139 Rotifer culturing methods were developed based on previous literature (Ricci, 1984). The  
140 animals were cultured in a supervised and semi-sterile environment. These cultures contain  
141 Jana mineral water (origin: Croatia; distributed by Fonyódi Ásványvíz Kft., Hungary), half  
142 diluted with distilled water (Millipore type ultra-pure, demineralized DW), a mixture  
143 nominated 'standard medium' (pH=7.5). The amount of diluted cations and anions in standard  
144 medium (mg/L): Ca<sup>2+</sup> 31.05; Mg<sup>2+</sup> 17.6; Na<sup>+</sup> 0.9; K<sup>+</sup> 0.25; Fe<sup>2+</sup> 0.001; HCO<sub>3</sub><sup>-</sup> 153.097; SO<sub>4</sub><sup>-</sup> 3;  
145 Cl<sup>-</sup> 0.8; F<sup>-</sup> 0.02; H<sub>2</sub>SiO<sub>3</sub> 3.3. Every step of the rotifer manipulation was monitored by  
146 microscope. Clear cultures of PA were kept in standardized cell culturing flasks (cat. no.:  
147 83.3910.302, Sarstedt AG & Co., Germany) in 15 mL standard medium. The flasks contained



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

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

159 A further component of the culturing matrix was sterile cotton wool, which was dispersed  
160 with forceps into a petri dish and washed with DW thoroughly. Cotton wool fragments were  
161 then soaked in 96% ethanol for 30 minutes and then washed multiple times in DW. After two  
162 days the prepared pieces of cotton wool were put in a cell culture flask mixed with CA alga  
163 (1:1 volume ratio) in the medium and rotifers were then added. New cultures were started  
164 from previous ones by transferring one PA rotifer with standard medium. The flasks were  
165 kept at 25 °C and under a light/dark cycle of 12:12 hours and were examined every 24 hours  
166 under an inverted transparent light microscope (Leitz Labovert FS, magnification range from  
167 32 x to 1000 x; Germany).

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

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

173 The animals were fed with 0.35 mL of prepared *Saccharomyces cerevisiae* stock solution (7.5  
174 g of dried yeast in 300 mL standard medium) added to the culture after every medium change.  
175 The final concentration of yeast in the medium was 0.6 mg/mL. The yeast stock solution was  
176 pasteurized at 65 °C for three hours in a water bath (Grant Sub 6; Grant Instruments Ltd.,  
177 UK), then left at room temperature overnight, after which it was filtered (15 µm pore size)  
178 and divided into 2 mL aliquots. These were pasteurized again and stored at -75 °C until  
179 further use.

### 180 **2.3. Harvesting rotifers**

181 To commence harvesting, the medium was first changed, without feeding. The flasks were  
182 placed at -75 °C for 3 minutes for rapid cooling (up to 4 °C) in order to dissect attached  
183 rotifers from the alga/wool fibrils on ice. The medium was poured off, and the flasks were  
184 washed with standard medium twice, which was also removed in petri dish. The dish was then  
185 left for 30 minutes to let healthy animals attach to the bottom, then the surface was washed  
186 twice with DW and once with the standard medium (25 °C). We chose rotifers approximately  
187 5-day-old after hatching (determined by body size; length  $220\pm 10$  µm and width  $60\pm 5$  µm), 1-  
188 2 days before the beginning of the reproductive stage of these animals. The calibration curve  
189 to determine the age of individual *Philodina acuticornis* rotifers isolated from standard  
190 medium can be found in Suppl. fig. 1.

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

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

## 196 **2.4. Characterization of rotifers**

### 197 2.4.1. Phenotypic analysis

198 Rotifer cultures used in our experiments were started with parthenogenesis from one unique  
199 animal to preserve genetic homogeneity. The live and healthy specimens were studied under  
200 light microscope using both photo and video analysis (Nikon D5500, Nikon Corp., Japan).  
201 When needed, we fixed the animals by adding cooled (-75 °C) ethanol (96%) when they  
202 adopted an elongated shape after decanting the medium from petri dishes. After fixation we  
203 used decreasing concentration steps of ethanol and then DW to rehydrate the sample with 10  
204 minutes between each step. The bodies prepared this way were placed on a microscope slide  
205 with pipetting between 0.3 mm thick spacers and were covered with a glass slip. The animals  
206 were photographed under the microscope by taking multiple pictures at 5 µm sections (30-40  
207 photo-layers/animal), which were then combined into one superimposed picture (created by  
208 Photoshop CC) to achieve better detail. Characterization was performed by examining the  
209 size of specimen (whole body or body parts), shape, special morphologic markers (e.g.  
210 characteristics of trophi, foot, corona, eyes, antennae) and different types of movement  
211 (swimming, crawling, the use of corona, the pulsation of mastax, body contractions, etc.).  
212 Animals were typified based on the works of K. Kertész (1894), L. Varga (1966) about the  
213 endemic species of microscopic animals in Hungary, and the publications of Ricci and  
214 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<sup>2</sup>. 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 GATCCTTCCGCAGGTTTACC-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](http://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

263 evolutionary placement using the sequenced rDNA from our model organism. However, there  
264 are dozens of species in Rotifera, but only a subset of 18S rDNA sequences are available in  
265 the SILVA database for this clade. All Rotifera sequences were downloaded and used for  
266 further analyses, listed by accessions in the Table. First, a classical taxonomic tree was drawn  
267 with PhyloT (PhyloT, biobyte solutions GmbH, <http://phylot.biobyte.de/index.html>, database  
268 version 2017.1) using scientific names of all species listed in the Table. Then the first 18S  
269 rDNA based phylogenetic tree (Fig. 1.) was built using the most popular distance-based  
270 Neighbor-Joining method in MEGA7 (Gascuel and Steel, 2006). The detailed analytic method  
271 (Kumar et al., 2016) and statistical analysis of the results (Efron et al., 1996; Ronquist et al.,  
272 2012) are described in Suppl. file.

## 273 **2.5. Microdrop technique for the treatment of rotifers**

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

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

287 after the transfection of animals and for each dose the treating substance was injected into the  
288 microdrop in 5  $\mu$ L of liquid containing H<sub>2</sub>O<sub>2</sub> (cat. no.: 95321; Sigma-Aldrich, Co., USA) or  
289 NaN<sub>3</sub> (cat. no.: S2002; Sigma-Aldrich, Co., USA) in 11 x concentration. Thus, the end  
290 volume of a microdrop with a one-housed animal was 55  $\mu$ L. The oil thickness was 1 mm on  
291 top of each drop. The plates were kept at 25 °C throughout the experiment. After the  
292 treatment the wells were observed twice a day until the animal was considered dead inside the  
293 drop.

## 294 **2.6. Experimental monitoring assays**

295 The viability of rotifers was assessed with four different assays (Fig. 2B) utilizing video  
296 recordings with a Nikon D5500 DSLR camera. The treatment agents (H<sub>2</sub>O<sub>2</sub> and NaN<sub>3</sub>) were  
297 used in decreased concentration in the individual assays. Overlapping doses enabled the  
298 response of the various tests to be cross-validated over their dynamic range. The H<sub>2</sub>O<sub>2</sub> and  
299 NaN<sub>3</sub> doses applied in TSL ( $\mu$ M): 70; 100; 200; 400; 500; 800; 1000; 2000; in BLD: 5; 10;  
300 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;  
301 400; 800; 2000.

### 302 2.6.1. Toxicity and survival lifespan (TSL) assay

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

### 310 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<sup>2</sup> 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



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

#### 339 2.6.4. Cellular reduction capacity (CRC) assay

340 The EZ4U Cell Proliferation Assay (non-radioactive cell proliferation, cytotoxicity and  
341 reduction capacity assay with XTT solution) was used (cat. no.: BI-5000; Biomedica  
342 Hungary). This assay is widely used on cell cultures and tissues (Berridge et al., 2005), but to  
343 our knowledge there are no data about applying it to intact invertebrates. We modified the  
344 standard protocol, given by the manufacturer, in order to measure the cellular reductive  
345 capacity of rotifers. After standard harvesting, approximately 300 rotifers per well were  
346 assayed a 24 well-plate. To avoid toxicity, 20 x diluted XTT solution was used (475  $\mu$ L  
347 standard medium with 25  $\mu$ L XTT kit-stock solution per well). The plates were incubated for  
348 24 hours without direct light at room temperature (25 °C). The supernatant of each well was  
349 then transferred to a 96 well-plate. The absorbance was measured by a microplate-reader  
350 (Spectramax 384, Molecular Science, Hungary) set at 491 nm with 630 nm as a reference.  
351 The readings were normalized to the number of animals/well. The percentages of the  
352 measures were calculated, the 100% was the ratio of absorbance (OD) and matching number  
353 of animals in untreated control wells.

354

#### 355 **2.7. Statistics**

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

### 358 **3. Results**

#### 359 **3.1. Sequencing**

360 After quality control and assembly of rotifer DNA samples, compared to the PCR products  
361 (1714 and 1340 bp), we achieved 1265 and 1624 bp query sequences (NCBI GenBank  
362 accession numbers: SUB2522809 and KY829026). As a result of SILVA aligner analysis,  
363 sequences were classified as *Philodina acuticornis odiosa* (PA) with 98% (for the 1265 bp  
364 long read) and showed 97% (for the 1624 bp long read) similarity to the reference. Because  
365 the DNA was isolated and amplified from an isolated population, SNPs could have biased the  
366 similarity scores. Indeed, the longer sequence showed lower similarity, therefore,  
367 phylogenetic analysis was performed.

#### 368 **3.2. Phylogenetic analysis**

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

#### 380 **3.3. Validation of the *in vivo* toxicity assays**

381 To validate our novel screening system, we exposed the PA to two toxic agents, hydrogen  
382 peroxide (H<sub>2</sub>O<sub>2</sub>) and sodium azide (NaN<sub>3</sub>) with well-known modes of action. Unchallenged

383 PA had a median TSL of 15 days, and both toxins suppressed TSL in a significantly dose-  
384 dependent manner ( $p < 0.0001$  for both agents, logrank test for trend, Fig. 4A-C), with lifespan  
385 halving induced by concentrations of  $\text{H}_2\text{O}_2 = 163 \mu\text{M}$ ,  $\text{NaN}_3 = 399 \mu\text{M}$  (iterated from the  
386 survival curves, Fig. 4C). The lowest concentrations tested to significantly impair TSL were  
387  $\text{H}_2\text{O}_2$   $70 \mu\text{M}$  (median survival 11 days, log-rank  $p < 0.0001$ ),  $\text{NaN}_3$   $70 \mu\text{M}$  (median survival  
388 13.5 days, log-rank  $p = 0.0341$ ).

389 PA was then challenged with various doses of  $\text{H}_2\text{O}_2$  and  $\text{NaN}_3$  over a 3-day exposure to test  
390 viability and behavioral effects using the BLD. To exhibit the response features of this assay,  
391 we employed concentrations of  $\text{H}_2\text{O}_2$  and  $\text{NaN}_3$  at an order of magnitude lower than the TSL  
392 tests. We found that the tested toxins induced significant dose-dependent inhibition of BLD  
393 (Fig. 4D,  $\text{IC}_{50}$ s  $\text{H}_2\text{O}_2 = 51.9 \mu\text{M}$ ,  $\text{NaN}_3 = 83.4 \mu\text{M}$ ). The lowest concentrations tested to  
394 significantly impair BLD were  $\text{H}_2\text{O}_2$   $10 \mu\text{M}$  (-14.2%, one-way ANOVA with Dunnett's  
395 multiple comparison test,  $p < 0.0001$ ),  $\text{NaN}_3$   $10 \mu\text{M}$  (-16.9%, one-way ANOVA with Dunnett's  
396 multiple comparison test,  $p < 0.0001$ ).

397 We examined the effects of  $\text{H}_2\text{O}_2$  and  $\text{NaN}_3$  in the *mastax contraction frequency* (MCF) test  
398 and since this test is potentially a more sensitive index, we focused on the effects of toxin  
399 concentrations an order of magnitude lower than used in the BLD tests. After three days of  
400 exposure, the toxins at  $\geq 10 \mu\text{M}$  significantly inhibited MCF (Fig. 4E,  $\text{IC}_{50}$ s  $\text{H}_2\text{O}_2 > 70 \mu\text{M}$ ,  
401  $\text{NaN}_3 = 46.4 \mu\text{M}$ ). The lowest concentrations tested to significantly impair MCF were  $\text{H}_2\text{O}_2$   $10$   
402  $\mu\text{M}$  ( $-0.6 \text{ sec}^{-1}$ , one-way ANOVA with Dunnett's multiple comparison test,  $p < 0.0430$ ),  $\text{NaN}_3$   
403  $30 \mu\text{M}$  ( $-1.83 \text{ sec}^{-1}$ , one-way ANOVA with Dunnett's multiple comparison test,  $p < 0.0001$ ).  
404 Curiously,  $\text{H}_2\text{O}_2$   $1 \mu\text{M}$  significantly but modestly promoted MCF by  $0.7 \text{ sec}^{-1}$  (one-way  
405 ANOVA with Dunnett's multiple comparison test,  $p = 0.0113$ ). This may indicate that low  
406 concentrations of  $\text{H}_2\text{O}_2$ , which is known to be a cellular signaling molecule at concentrations  
407 of this order (Veal et al., 2007) may act to stimulate rotifer activity. However, the CRC

408 response at this dose of H<sub>2</sub>O<sub>2</sub> for the same time interval (3 days) indicated oxidative stress. So  
409 the increase in the MCF in response to H<sub>2</sub>O<sub>2</sub> at 1 μM we hypothesize is a compensatory  
410 response to oxidative stress e.g. a reflex to increase nutriture.

411 Finally, the *cellular reduction capacity* (CRC) measurements were performed after three days  
412 of treatment. H<sub>2</sub>O<sub>2</sub> and NaN<sub>3</sub> induced significant dose-dependent inhibition of CRC, in line  
413 with our expectations (Fig. 4F, IC<sub>50</sub>s H<sub>2</sub>O<sub>2</sub>=32.9 μM, NaN<sub>3</sub>=91.2 μM). The lowest  
414 concentrations tested to significantly impair CRC were H<sub>2</sub>O<sub>2</sub> 1 μM (-30.3%, one-way  
415 ANOVA with Dunnett's multiple comparison test, p<0.0001), NaN<sub>3</sub> 1 μM (-13.5%, one-way  
416 ANOVA with Dunnett's multiple comparison test, p<0.0001).

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

#### 426 **4. Discussion**

427 Over the last 30 years the interest in experimental research related to rotifers has increased.  
428 Their use as a model in marine ecotoxicological and environmental studies provides reliable  
429 information about acute and chronic deleterious effects of aquatic pollution (Breitholtz et al.,  
430 2006). They also provide important data about specialization (Gómez et al., 2002),  
431 evolutionary ecology (Snell et al., 2006), the evolution of sex, population dynamics

432 (Yoshinaga et al., 2003) and ecotoxicity (Kaneko et al., 2005). Their main advantages in  
433 toxicological research derive from their small size, relatively short lifespan, high population  
434 density, rapid population growth rates, availability of culture methods; predominantly  
435 parthenogenic reproduction and sensitivity to a vast number of toxic agents (Hagiwara et al.,  
436 1997). Despite these advantages, standardized behavioral toxicity tests with invertebrates as  
437 model organisms are scarce in pharmaceutical research.

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

453 We validated four new high-throughput *in vivo* toxicological screening indices using two  
454 widely-used toxic agents with well-known mechanisms of action, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)  
455 and sodium azide (NaN<sub>3</sub>), which can now serve as standards for future eco- or pharmaco-  
456 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<sub>2</sub>O<sub>2</sub> and NaN<sub>3</sub> 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.

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

485

#### 486 **Conflict of interest**

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

490

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498

#### 499 **Appendix: Supplementary files**

500       Suppl. file

501       Suppl. fig. 1

502       Suppl. fig. 2

503       Suppl. fig. legends

504

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630 **Figure legends**

631 **Figure 1. Evolutionary relationships of taxa obtained from MEGA7 analysis**

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

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

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

653 **Figure 3. Anatomic characteristics of *Philodina acuticornis odiosa* (PA) used in viability**  
654 **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  $\mu\text{m}$ ). 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  $\mu\text{m}$ ). **B/2.**  
659 Enlargement of the primitive red eyes (marked with stars; scale bar: 5  $\mu\text{m}$ ): 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  $\mu\text{m}$ ).

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

665 In each experiment the one-housed rotifers were treated with different doses of  $\text{H}_2\text{O}_2$  and  
666  $\text{NaN}_3$  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  $\text{BLD} \pm \text{S.E.M.}$  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  $\text{MCF/sec} \pm \text{S.E.M.}$  are shown. **F.** CRC, a cellular reduction capacity indicator, was measured  
675 after three days treatment. The means of normalized  $\text{CRC} \pm \text{S.E.M.}$  (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  $\geq 4$  overlapping toxin concentrations had been tested for both H<sub>2</sub>O<sub>2</sub> and NaN<sub>3</sub> (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.