

1 **The Impact of Glyphosate-Based Herbicides**
2 **and Their Components on *Daphnia Magna***

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4 Katherine Duan¹, Alexander Kish¹, Leanna Kish¹,

5 Peter Faletra¹, Kelly Salmon¹

6

7 ¹ New Hampshire Academy of Science, Lyme, New Hampshire, United
8 States of America

9 Corresponding author: Kelly.Salmon@nhacadsci.org

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40 Abstract

41 Recent studies suggest glyphosate-based herbicides (GBHs) are more harmful
42 to animals than suggested by the EPA and industry-funded studies. Both glyphosate
43 and the only known “other” ingredient in GBHs, polyethoxylated tallow amine (POEA),
44 have been implicated as safety hazards. In this study, we investigated the effects of the
45 commercial GBHs Roundup[®], Rodeo[®] and the two known GBH ingredients, POEA and
46 glyphosate, on the survival and heart rates of *Daphnia magna*. *D. magna* were exposed
47 to the retail herbicide mixture and the individual components dissolved in water to mimic
48 possible environmental exposure. When exposed to Roundup[®] and Rodeo[®], *D. magna*
49 survival and heart rates declined following a dose-response pattern. A commercial
50 formulation of Roundup[®] containing 98% unlisted ingredients had the greatest effect on
51 heart and survival rates, followed by two formulations of Rodeo with 4.62% unlisted
52 ingredients and 1.72% unlisted ingredients, respectively. The Rodeo[®] formulation with
53 1.72% unlisted ingredients had an equal concentration of glyphosate as the Roundup[®]
54 formulation, suggesting that the negative effects of GBHs are influenced by the unlisted
55 ingredients. Although differences in survival rates were not observed between controls
56 and glyphosate groups, groups exposed to glyphosate alone generally showed a
57 significant ($p < 0.05$) effect on *D. magna* heart rates. Heart rates following POEA
58 exposure were consistently and, in most cases, significantly ($p < 0.05$) lower than
59 controls. POEA caused a decrease in survival rate for all concentrations, but followed a
60 dose-response pattern only in the three highest concentrations. A Mock-GBH, made
61 with POEA and glyphosate, significantly ($p < 0.05$) lowered heart rates at some higher
62 concentrations, with no dose-response pattern. The Mock-GBH negatively affected

63 survival rates at approximately the same level as POEA alone. The heart rate data
64 suggest that there are undisclosed ingredients in Roundup® and Rodeo® other than
65 POEA and glyphosate that negatively affect *D. magna* since glyphosate and POEA
66 combined yielded less pronounced negative responses than the full GBH products.

67

68

69

70 **1 Introduction**

71 Although N-(Phosphonomethyl)glycine (glyphosate) was first synthesized in 1950, its
72 efficacy as an herbicide was not reported until the early 1970s (1,2). Glyphosate was
73 initially patented by Monsanto in 1971 (US 3799758) and, by the mid-1970s, marketed
74 with the trade name Roundup[®]. In this herbicide formulation, the “active ingredient” is
75 the isopropylamine salt of glyphosate while the “other ingredients” include the surfactant
76 polyethoxylated tallow amine (POEA) and other undisclosed chemicals (1,3).

77

78 Glyphosate is an amphoteric chemical that is often derived from the amino acid glycine
79 (4,5). It is a weak organic acid with a water solubility of 12g/L at 25°C (6). Glyphosate
80 and its salts are non-volatile, do not undergo photochemical degradation or hydrolysis at
81 pH values of 3, 6, or 9 between 5°C and 35°C, and are detectable in soil after
82 application for 1-151 days depending on soil types and environmental conditions (7–9).
83 Although it is often reported as a single chemical, to increase its water solubility, it is
84 produced for commercial use in a variety of salt forms including sodium, potassium,
85 ammonium, isopropylammonium, and trimesium salts (10,11).

86

87 Glyphosate’s herbicidal action has been attributed to inhibiting the synthesis of aromatic
88 amino acids through the shikemic acid pathway (12,13). This pathway is necessary for
89 viability in plants, bacteria, fungi, and archaea. A common rationale for glyphosate’s
90 safety was that it would be non-toxic to animals since the shikimic acid pathway is not
91 found in animals (12,13). When it was first released for use in agriculture in the early to

92 mid-1970s, glyphosate was hailed as “a once in a century herbicide” with the promise of
93 decreasing the world’s total use of herbicides (1,10,14). By the end of the 1970s,
94 glyphosate became the most commonly used herbicide in the world.

95

96 A number of developments helped glyphosate expand its dominance as the world’s
97 most common herbicide, including: 1) the use of glyphosate-based herbicides (GBHs) in
98 pre-emergent weed control in conjunction with no-till agricultural practices (2,15); 2) the
99 development of genetically modified crops, such as corn and soybeans, that were
100 resistant to Monsanto’s GBH Roundup® and first marketed as “Roundup Ready®” in
101 1996 (2); 3) the expiration of the Monsanto patent and the subsequent production by
102 other large agribusinesses such as *Dow AgroSciences*® and *Syngenta*® (3); and 4) the
103 use of GBHs as desiccants to speed crop harvesting (2,16). An analysis by Benbrook in
104 2016 found that in the 40 years between 1974 and 2014, about two-thirds of all
105 glyphosate applied in the US had been used in the last 10-year period (2).

106

107 The extensive amount of GBHs used in an expanding variety of agricultural practices
108 when growing crops both resistant and not resistant to glyphosate has led to residues of
109 glyphosate detected in human foods, beverages, cotton fabrics, and bandages as well
110 as in human urine and breast milk (17–24). Studies performed by non-agricultural
111 industry parties questioned the veracity of the safety conclusions of both governmental
112 and agricultural industry groups. As examples, although the US EPA states that
113 “glyphosate is no more than slightly toxic to birds and is practically nontoxic to fish,

114 aquatic invertebrates, and honeybees”, studies have shown a higher toxicity towards
115 amphibians and aquatic invertebrates (25–27). Glyphosate has been shown to change
116 the behavior and reproduction of earthworms (28) and negatively affect the growth of
117 algae and bacteria in aquatic systems (29). More recently, glyphosate has been shown
118 to affect the gut microbiota of honeybees and is suggested as a possible contributing
119 factor in colony collapse disorder (30,31). Glyphosate has been implicated in a variety
120 of toxicity risks in vertebrate animals, including cell signal disruption in rats (32),
121 endocrine disruption in human cell lines (33), and steroidogenesis disruption in the
122 mouse MA-10 Leydig tumor cell line (34).

123

124 In addition to glyphosate’s direct toxicity, the unlisted ingredients in GBHs (frequently
125 labeled “other ingredients”) have been shown to pose a risk to aquatic organisms
126 (29,35). The EPA policy of not including safety testing for unlisted ingredients in
127 pesticides has been called into question by studies of pesticide toxicity with and without
128 the active ingredient (11,36,37). As reported in a publication on the toxicity of pesticides’
129 unlisted ingredients in *Scientific Reports*, “These adjuvants are generally considered
130 by the EPA to be biologically inert; and therefore, their use is not monitored at the
131 federal level and they are exempt from residue tolerance for food use” (38).

132

133 The unlisted ingredients in GBHs are trade secrets. The most prominent known
134 component of the unlisted substances in Roundup® is POEA, which is a surfactant that
135 improves the penetration of glyphosate across the epidermis into plant tissues (39).

136 POEA and other surfactants are not typically added to commercial preparations sold for
137 use in aquatic environments, such as *Dow Agrosiences' Rodeo*[®] aquatic herbicide
138 (*Rodeo*), since surfactants are known to have harmful effects to aquatic organisms (40–
139 42). Correspondence with Dow technical services confirmed that there are no
140 surfactants present in *Rodeo*.

141

142 Since glyphosate is never applied as the sole ingredient in an herbicide, one must also
143 consider the “other ingredients” to properly assess the toxicity of the full GBH product
144 (10). To understand the safety, health, and environmental risks of GBHs, all their
145 ingredients should be assessed, as well as the active ingredient in all its various
146 chemical formulations.

147

148 A review of research regarding the effects of GBHs and their undisclosed ingredients on
149 *D. magna* by Chura et.al. revealed a wide variety of approaches for toxicity with
150 correspondingly varying conclusions (10). The effects have been monitored on
151 development, egg production, survival rate, and heart rate (10) with a variety of culture
152 waters, times monitored for survival, and sources of active and undisclosed ingredients.
153 The culture water varies widely and includes synthetic water (43), Aachener Daphnien
154 Medium (27), Elendt-M7 medium (40,41,44) , and “moderately hard synthetic
155 freshwater” (42). Survival assessments with EC50 or LC50 to the GBHs or glyphosate
156 are typically at 24 or 48 hours with 48 appearing to be the most common (27,42,45).
157 Tests regarding the toxicity of the GBHs' surfactants or residues have generally used an

158 EC50 or LC50 at 48 hours (40,42,45–47). The surfactants tested vary from those
159 supplied by Monsanto (45) to general surfactants (40) and the known surfactant in
160 Roundup®, POEA (42,48).

161

162 In this paper, we attempt to separate the effects of the active and undisclosed
163 ingredients that may be involved in GBH toxicity by comparing the full GBH product to
164 the known ingredients alone. We examined the effects on heart rate and survival over
165 more discrete and shorter time periods than previously used. Our approach sought to
166 mimic the natural environment and reduce stress during testing. Here, we report the
167 comparative effects of “*Roundup Ready-to-Use Weed and Grass Killer*®” (Roundup-
168 WGK), Rodeo, glyphosate, and POEA on the heart rates and survival rates of *Daphnia*
169 *magna*. Our research seeks to determine whether Roundup-WGK and Rodeo, including
170 their unlisted ingredients as well as their known components, glyphosate and POEA,
171 have any deleterious effects on *D. magna*. *D. magna* is a fresh water aquatic
172 invertebrate and a well-established model organism for toxicological studies (49,50).
173 Because it is a filter feeder, it is rapidly responsive to suspended or dissolved
174 substances, allowing for simple and efficient toxicological testing of chemicals (49,50).
175 *D. magna* is also transparent and its heart rate can be directly observed with a stereo
176 microscope (35). We hypothesized that the herbicide commercial formulations would be
177 the most toxic and would affect both the heart rates and survival more than the
178 individual components would by themselves.

179

180 **2 Materials and methods**

181 **2.1 Materials**

182 Adult *D. magna* were originally purchased from *Carolina Biological Supply* and grown in
183 our lab at 19-22°C in two 10-gallon glass tanks filled with non-chlorinated artesian well
184 water and fitted with aerators. Approximately 1/3rd of the water was removed every 10-
185 14 days and replaced with fresh non-chlorinated artesian well water previously allowed
186 to equilibrate to room temperature. *D. magna* were maintained on a diet of spirulina
187 (*Pure Planet*) and *Nannochloropsis* (*sp.*) unicellular green algae (*Carolina Biological*
188 *Supply* Item #142337). A Wolfe[®] stereomicroscope from *Carolina Biological Supply* was
189 fitted with a custom camera adapter to capture videos of the *D. magna* at a
190 magnification of 30x using an iPhone[®] 6 Plus (*Apple*[®]). The microscope was modified
191 with a cooling fan and LED illumination to control for temperature variation. Because of
192 *D. magna*'s sensitivity to temperature change, the temperature was maintained within
193 one degree of 21°C during testing. A Fluke[®] 62 Max+ infrared thermometer with a
194 resolution of 0.1°C was used to ensure that temperature was monitored accurately.

195
196 Four substances were tested: *Monsanto*'s Roundup-WGK containing 2% glyphosate
197 isopropylamine salt and 98% unlisted ingredients including POEA, *Dow Agrosiences*'
198 Rodeo containing 53.8% glyphosate isopropylamine salt and 46.2% unlisted
199 ingredients, pure glyphosate (*Sigma Aldrich*), and POEA (POE (15) tallow amine, *Chem*
200 *Service, Inc.*).

201

202 All test substances were diluted with water from the *D. magna* culture tanks to create
203 stock solutions. The choice of using tank water was based on our objective to minimize
204 stress on the *D. magna* by avoiding a sudden change in their water. Most ready-to-use
205 GBHs, including Roundup-WGK, contain 2% glyphosate salt and approximately 1%
206 POEA (39). Five stock solutions were created that contained the percentages of
207 glyphosate (2%) or POEA (1%) found in ready-to-use products. Since Rodeo's
208 recommended dilution results in a higher concentration of glyphosate, a sixth stock
209 solution was created for Rodeo according to the manufacturer's recommended dilution.
210 The six stock solutions are:

- 211 1) **Roundup-WGK**: The original undiluted Roundup-WGK herbicide stock
212 contained 2% glyphosate salt, an estimated 1% POEA, and 97% unlisted
213 ingredients.
- 214 2) **Rodeo-Recommended**: The Rodeo stock contained 5.38% glyphosate, no
215 surfactant or POEA, and 4.62% unlisted ingredients and was diluted from
216 *Dow Agrosciences' Rodeo* aquatic herbicide based on the manufacturer
217 recommendation.
- 218 3) **Rodeo-2%**: The Rodeo-2% stock contained 2% glyphosate salt, no surfactant
219 or POEA, and 1.7% unlisted ingredients and was diluted from *Dow*
220 *Agrosciences' Rodeo* aquatic herbicide. This solution was designed to match
221 the glyphosate content in Roundup-WGK solution.
- 222 4) **Glyphosate**: The glyphosate stock contained 2% pure glyphosate.
- 223 5) **POEA**: The POEA stock contained 1% POEA.
- 224 6) **Mock-GBH**: The Mock-GBH contained 2% pure glyphosate and 1% POEA.

225
 226 All stock solutions were diluted with water from the *D. magna* culture tanks to achieve
 227 concentrations ranging from 0.1% to 100% of the stock solution. A special 200% test
 228 concentration was created for both Rodeo-Recommended and Rodeo-2%. For
 229 comparison to other research using concentrations of glyphosate and/or POEA, we
 230 have provided a table with the concentrations (in mg/L) of each known ingredient in our
 231 dilution series (Table 1). Percent solutions were used since we wished to compare the
 232 effects of unlisted ingredients that have undeclared concentrations.

Stock Solution	Description	Ingredients	Concentration (mg/L)													
			0%	0.1%	0.5%	1%	3%	5%	7%	10%	25%	50%	75%	100%	200%	
Roundup-WGK	Mixture of Water and Ready-Use RoundUp (2% Glyphosate Isopropylamine Salt)	Glyphosate	0	11	55	109	327	545	763	1,090	2,725	5,451	8,176	9,761		
		POEA - inactive ingredient	0	11	56	112	335	558	782	1,117	2,792	5,584	8,376	10,000		
		Other/Unknown inactive ingredient	0	1,083	5,417	10,833	32,500	54,167	75,833	108,333	270,834	541,667	812,501	970,000		
Rodeo-Recommended	Mixture of Water and Commercial Concentrated Rodeo Solution (53.8% Glyphosate Isopropylamine Salt)	Glyphosate	0	32	159	318	953	1,589	2,224	3,177	7,943	15,886	23,829	31,773	63,545	
		POEA - inactive ingredient	0	0	0	0	0	0	0	0	0	0	0	0	0	
		Other/Unknown inactive ingredient	0	56	280	559	1,677	2,795	3,913	5,590	13,976	27,951	41,927	55,902	111,804	
Rodeo-2%	Mixture of Water and Commercial Concentrated Rodeo Solution (53.8% Glyphosate Isopropylamine Salt)	Glyphosate	0	12	59	118	354	591	827	1,181	2,953	5,906	8,859	11,811	23,623	
		POEA - inactive ingredient	0	0	0	0	0	0	0	0	0	0	0	0	0	
		Other/Unknown inactive ingredient	0	21	104	208	623	1,039	1,455	2,078	5,195	10,391	15,586	20,781	41,563	
Glyphosate	Mixture of Water and Pure Glyphosate (100%)	Glyphosate	0	20	100	200	600	1,000	1,400	2,000	5,000	10,000	15,000	20,000		
		POEA - inactive ingredient	0	0	0	0	0	0	0	0	0	0	0	0		
		Other/Unknown inactive ingredient	0	0	0	0	0	0	0	0	0	0	0	0		
POEA	Mixture of Water and Pure POEA (100%)	Glyphosate	0	0	0	0	0	0	0	0	0	0	0	0		
		POEA - inactive ingredient	0	10	48	97	290	483	676	966	2,415	4,830	7,245	9,660		
		Other/Unknown inactive ingredient	0	0	0	0	0	0	0	0	0	0	0	0		
Mock-GBH	Mixture of Water, Pure Glyphosate(100%) and Pure POEA (100%)	Glyphosate	0	20	100	200	600	1,000	1,400	2,000	5,000	10,000	15,000	20,000		
		POEA - inactive ingredient	0	10	48	97	290	483	676	966	2,415	4,830	7,245	9,660		
		Other/Unknown inactive ingredient	0	0	0	0	0	0	0	0	0	0	0	0		

233
 234 **Table 1. Concentration in mg/L of ingredients for each dilution of all stock solutions.** Table 1
 235 lists the mg/L of glyphosate, POEA, and “Other ingredients for the various percentage dilutions of
 236 the six stock solutions.

237 238 **2.2 *Daphnia magna* heart rate analysis methods**

239 Each experiment had a 0% control group containing water from *D. magna* culture tanks
 240 grown in our lab. Each *D. magna* was placed in an individual well of a 96-well microtiter
 241 plate filled with a given concentration of a test solution. Using our modified stereoscope,

242 *D. magna* heart rates were quantified by videotaping each *D. magna* individually for 10
243 seconds on an iPhone 240 fps slow-motion setting and then manually counting their
244 heart rates in the slow-motion video after the experiment was conducted. All heart rate
245 measurements and analyses are derived from beats per minute (BPM). For all
246 experiments, the heart rates of three *Daphnia magna* per condition were recorded.

247
248 For Roundup-WGK solutions, three experiments were conducted with narrowing
249 concentrations of Roundup-WGK tested. The first Roundup-WGK experiment tested the
250 concentrations 10%, 25%, 50%, 75%, and 100% and a video was captured of each *D.*
251 *magna* every minute for 14 minutes. To further assess the procedure's reliability and
252 examine dose responses in more narrow concentration ranges, two more experiments
253 were conducted. The second Roundup-WGK experiment tested the concentrations 1%,
254 3%, 5%, 7%, and 10%, and a video was captured of each *D. magna* every 3 minutes for
255 30 minutes. The third Roundup-WGK experiment tested the concentrations 0.1%, 0.5%,
256 1%, 5%, and 10%, and a video was captured of each *D. magna* every minute for 14
257 minutes.

258
259 For the test substances, Rodeo-Recommended, Rodeo-2%, glyphosate, POEA, and the
260 Mock-GBH solutions, the concentrations 0.1%, 0.5%, 1%, 5%, 10%, 25%, 50%, 75%,
261 and 100% were tested. An additional 200% concentration-was tested for Rodeo-
262 Recommended and Rodeo-2%. A video was captured of each *D. magna* every 5
263 minutes. For the concentrations 0.1%, 0.5%, 1% and 75% of Rodeo-Recommended

264 and Rodeo-2% solutions, the observation duration was 35 minutes, whereas all others
 265 were 45 minutes. Table 2 displays the concentrations tested for each test substance.

Heart Rate Analysis -- Concentrations Tested													
Stock Solution	0%	0.1%	0.5%	1%	3%	5%	7%	10%	25%	50%	75%	100%	200%
Roundup-WGK	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
Rodeo-Recommended	✓	✓	✓	✓		✓		✓	✓	✓	✓	✓	✓
Rodeo-2%	✓	✓	✓	✓		✓		✓	✓	✓	✓	✓	✓
Glyphosate	✓	✓	✓	✓		✓		✓	✓	✓	✓	✓	
POEA	✓	✓	✓	✓		✓		✓	✓	✓	✓	✓	
Mock-GBH	✓	✓	✓	✓		✓		✓	✓	✓	✓	✓	

266

267 **Table 2. Concentrations tested for heart rate analyses.** Table 2 shows the concentrations
 268 tested for Roundup-WGK, Rodeo-Recommended, Rodeo-2%, Glyphosate, POEA and Mock-GBH
 269 solutions. A check mark indicates that concentration was tested.

270

271 An expanded heart rate verification experiment was conducted to verify the results for
 272 Rodeo-Recommended, Rodeo-2%, and POEA. The concentrations 1%, 25%, and
 273 100% were tested for each stock solution and ten *D. magna* were individually observed
 274 for each concentration. A video was captured of each *D. magna* every 15 minutes for 60
 275 minutes.

276

277 Statistical analysis of the heart rate results was performed by applying a Kruskal-Wallis
 278 ANOVA test with Dunn's Multiple Comparison post-test using GraphPad Prism version
 279 8 for Mac (GraphPad Software, La Jolla, California, www.graphpad.com). Graphs were
 280 generated by plotting average heart rates with standard deviation using GraphPad
 281 Prism. Full statistical data is presented in Supporting Information (S1 Dataset), as is the

282 raw data for the initial heart rate experiments (S2 Dataset) and heart rate verification
283 experiments (S3 Dataset).

284

285 **2.3 *Daphnia magna* survival rate analysis methods**

286 To observe the effects of the test substances on *D. magna* survival, we used a larger
287 containment vessel with 15-25mls of test solution in 25mm x 150mm glass tissue
288 culture test tubes. Each experiment had a 0% control group. For each concentration,
289 the time of death was recorded starting from when the first *D. magna* was introduced
290 into a tube. Each tube was monitored continuously for eight hours. The ambient room
291 temperature was kept between 19 and 22°C.

292

293 Two experiments were conducted on the Roundup-WGK solution. The concentration
294 between 1% and 10% were tested. Each test concentration and control group contained
295 twelve *D. magna* with 3 tubes per group and four *D. magna* per tube; 25mls of each
296 concentration per test tube. Two experiments were conducted on Rodeo-
297 Recommended and Rodeo-2% solutions. One experiment was conducted on
298 glyphosate, POEA, and Mock-GBH solutions. For Rodeo-Recommended,
299 concentrations between 1% and 200% were tested. For Rodeo-2%, concentrations
300 between 1% and 75% were tested. For glyphosate, POEA and Mock-GBH,
301 concentrations between 1% and 100% were tested. Each test concentration and control
302 group contained 5-6 *D. magna* with 3 tubes per group and 1 to 2 *D. magna* per tube;
303 15mls of each concentration per test tube. Table 3 displays the concentrations tested
304 for each test substance.

Median Time Until Death Analysis -- Concentrations Tested														
Stock Solution	0%	1%	2%	3%	4%	5%	7.50%	10%	25%	30%	50%	75%	100%	200%
Roundup-WGK (12D, 2017-#1)	✓	✓	✓	✓	✓									
(12D, 2017-#3)	✓					✓	✓	✓						
Rodeo-Recom. (5D, 2017-02-04)	✓	✓				✓		✓					✓	✓
(6D, 2017-12-20)	✓								✓		✓	✓		
Rodeo-2% (5D, 2017-02-04)	✓	✓		✓		✓		✓		✓				
(6D, 2017-12-20)	✓								✓		✓	✓		
Glyphosate (6D, 2017-07-18)	✓	✓		✓				✓		✓				✓
POEA (6D, 2017-07-18)	✓	✓				✓		✓	✓		✓			✓
Mock-GBH (6D, 2017-07-18)	✓	✓				✓		✓	✓		✓	✓	✓	✓

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306

307

308

309

Table 3. Concentrations tested for survival rate analysis. Table 3 outlines the concentrations tested of Roundup-WGK, Rodeo-Recommended, Rodeo-2%, Glyphosate, POEA and Mock-GBH solutions for survival analysis. A check mark indicates that the concentration was tested.

310

Kaplan-Meier survival curves were generated in Microsoft Excel and median time until

311

death was determined manually for all concentrations. The survival rate raw data is

312

provided in the Supporting Information (S4 Dataset).

313

314

3 Results

315

3.1 *Daphnia magna* heart rate analysis

316

Heart rate experiments were performed with a 0% control. There is a wide range of

317

resting heart rates for control groups, ranging 126-546 BPM at a temperature of 21°C.

318

Supplemental Figure 1 (S1 Fig) shows the compiled average and median heart rates of

319

all the control groups in our heart rate experiments with each data point representing 25

320

D. magna (S1 Fig). Unlike test groups, control heart rates remained steady without

321

dramatic decreases or increases. The median and mean heart rates of *D. magna* in our

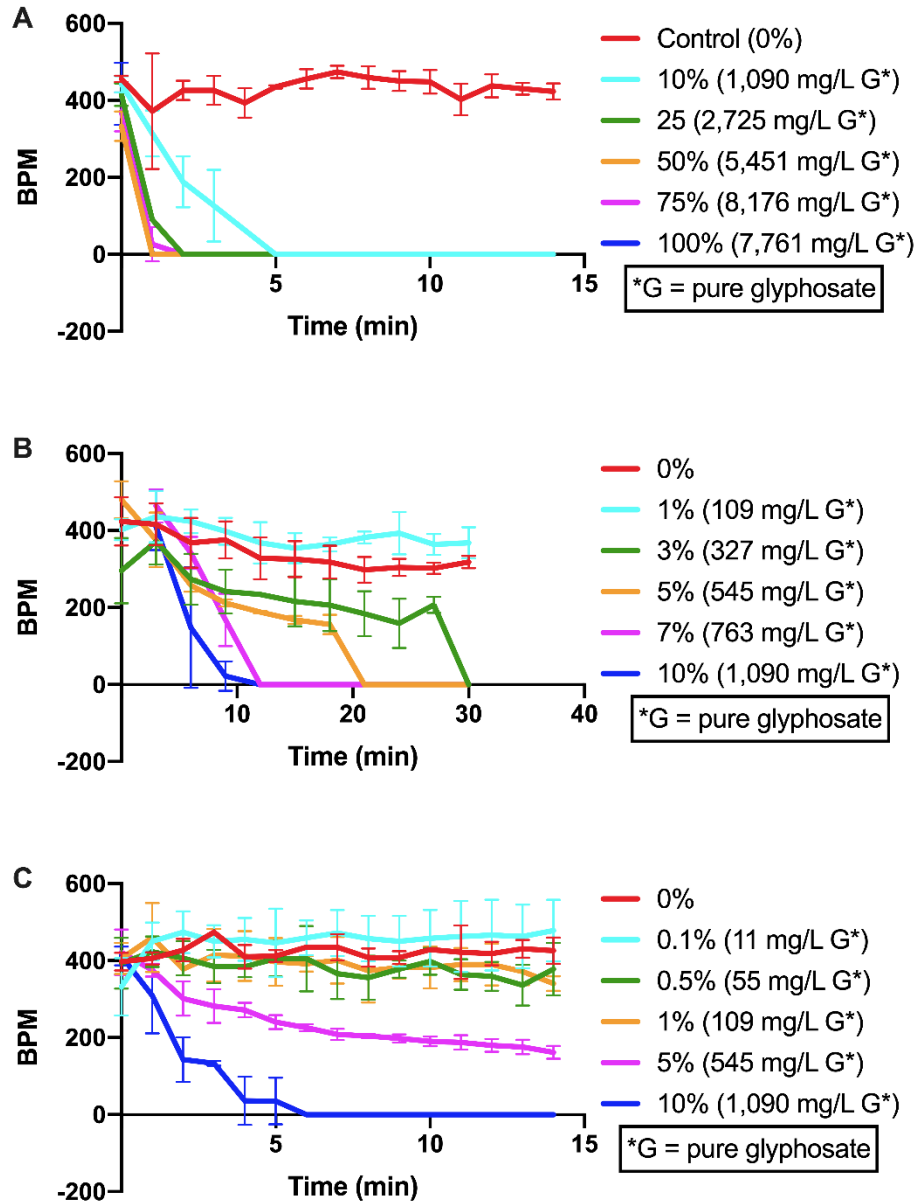
322 experiments were 369 and 357 BPM respectively. The consolidated control heart rate
323 raw data is provided in the Supporting Information (S5 Dataset).

324

325 **3.1.1 Roundup-WGK**

326 We first performed a study examining the effects of successively more narrow
327 concentration ranges of Roundup-WGK on heart rate. For the 10% to 100%
328 concentrations, *D. magna* heart rates dropped to 0 BPM within 8 minutes (Fig 1A) with
329 an average heart rate significantly lower ($p < 0.001$) than the control. To further explore
330 the effects of concentrations between 0% and 10%, we performed two more
331 experiments. In the second experiment, *D. magna* heart rates dropped to 0 BPM in less
332 than 30 minutes for the 3%, 5%, 7%, and 10% concentrations (Fig 1B). In the third
333 experiment, with lower concentrations, *D. magna* heart rates again dropped to 0 BPM in
334 less than 30 minutes for the 5% and 10% concentrations. For the 0.1%, 0.5%, and 1%
335 concentrations, *D. magna* heart rates remained within the normal range set by the
336 control (Fig 1C).

337



338

339 **Figure 1. Effects of Roundup-WGK solutions on heart rate.** Graphs represent the average BPM with
 340 standard deviation of the *D. magna* over exposure time in minutes for listed concentrations. Each data point
 341 represents 3 *D. magna*. The control (0% Roundup-WGK) is shown as a red line. **A)** The first experiment looked
 342 at a broad range of concentrations: 0%, 10%, 25%, 50%, 75%, and 100% Roundup-WGK (100% stock contains
 343 2% glyphosate, approximately 1% POEA). Heart rates show a precipitous decline for all concentrations tested,
 344 following a dose-response pattern. All test concentrations yielded an average heart rate significantly below that
 345 of the control ($p < 0.001$) **B)** The second experiment focused on the range of 0-10% with concentrations of 1%,
 346 3%, 5%, 7%, and 10% Roundup-WGK. Heart rates showed a clear dose response above 1% Roundup-WGK.
 347 The *D. magna* in the 7% and 10% concentrations had average heart rates significantly below that of the control
 348 group ($p < 0.05$) **C)** The last experiment tested lower concentrations of 0.1%, 0.5%, 1%, 5% and 10% Roundup-
 349 WGK. Heart rates showed a clear dose response above 1% Roundup-WGK, with 5% and 10% being
 350 significantly decreased compared to the control ($p < 0.05$). *D. magna* in the 0.1%, 0.5%, and 1% solutions had
 351 heart rates within the range set by the controls.

352

353 **3.1.2 Rodeo-Recommended and Rodeo-2%**

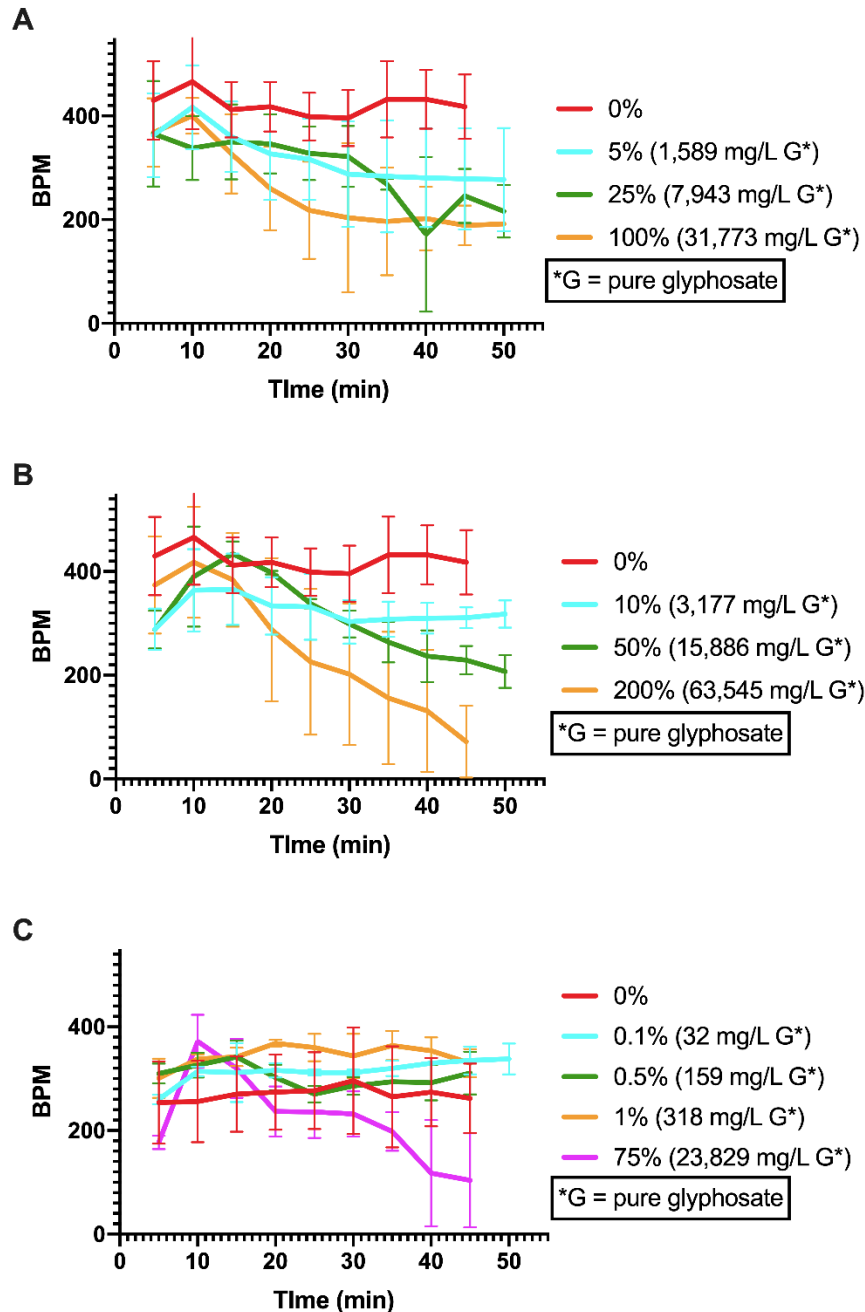
354 The 5% to 25% solutions reduced their heart rates to approximately 75% of the control
355 within 45 minutes (Fig 2A). The 75% to 100% concentrations of the Rodeo-
356 Recommended stock solution reduced the *D. magna*'s heart rate by at least 50% of the
357 control within 45 minutes (Figs 2A and 2C). The 200% solution reduced their heart rates
358 to approximately 20% of the control (Fig 2B). The 0.1% and 0.5% concentrations
359 remained within the normal range set by the control. No death was observed within the
360 45-minute observation period (Fig 2C).

361

362 Heart rates approximated a general dose-response pattern with higher concentrations
363 separating from the lower concentrations by the end of the 45-minute observation
364 period. Extensive crossover among test groups is observed at earlier time points.
365 Average heart rates in all concentrations $\geq 1\%$ Rodeo-Recommended varied significantly
366 from the control heart rates ($p < 0.05$) except the 75% group, which varied widely around
367 the control line, ending at less than 50% of the control (Figs 2A, 2B and 2C).

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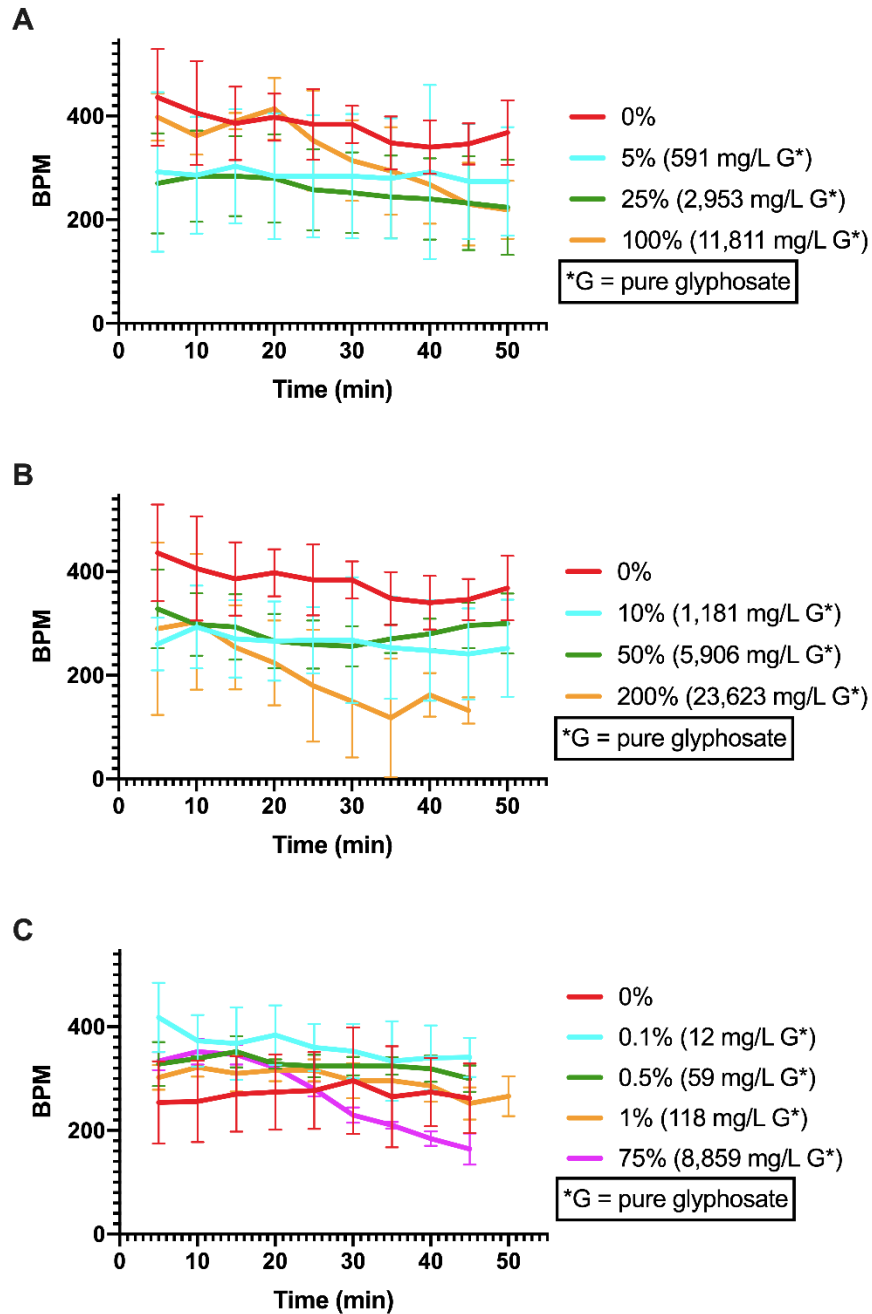
380

Fig 2. Effects of Rodeo-Recommended solutions on heart rate. This represents the average BPM with standard deviation of the *D. magna* over exposure time in minutes for listed concentrations of the Rodeo-Recommended stock (100% stock contains 5.38% glyphosate). The control (0%) is shown as a red line. Each data point represents 3 *D. magna*. **A)** Heart rates at the concentrations 0%, 5%, 25%, and 100%. **B)** Heart rates at the concentrations 0%, 10%, 50%, and 200%. Graphs **A** and **B** represent data from a single experiment, separated for easier viewing, and share the same control. **C)** Heart rates at the concentrations 0%, 0.1%, 0.5%, 1%, and 75%. There was extensive overlap for the lower concentrations. The higher concentrations of 75%, 100%, and 200% showed a clear decline by 30 minutes that continued out to 45 minutes. Average heart rates for all concentrations $\geq 1\%$ Rodeo-Recommended showed a significant difference from control except for the 75% test group ($p < 0.05$).

381

382 Heart rates ranged from 100% to 60% of the control for the 100% to 0.1%
383 concentrations within 45 minutes, approximating a general dose-response pattern. No
384 death was observed within 45 minutes (Figs 3A, 3B and 3C). For the Rodeo-2% stock,
385 the 200% concentration reduced the heart rates to approximately 50% of the control
386 within 45 minutes (Fig 3B).

387



388

389 **Fig 3. Effects of Rodeo-2% solutions on heart rate.** This represents the average BPM with standard
390 deviation of the *D. magna* over exposure time in minutes for listed concentrations of the Rodeo-2% stock
391 (100% stock contains 2% glyphosate). The control (0%) is shown as a red line. Each data point represents 3
392 *D. magna*. **A)** Heart rates in the concentrations 0%, 5%, 25%, and 100%. **B)** shows the concentrations 0%,
393 10%, 50%, and 200%. Graphs **A** and **B** represent data from a single experiment, separated for easier
394 viewing, and share the same control. Each graph contains a range of high and low concentrations for ease
395 of comparison. **C)** Heart rates in the concentrations 0%, 0.1%, 0.5%, 1%, and 75%. With the exception of
396 the 50%, there was a general trend for the higher concentrations of 25% to 200% to show decreasing heart
397 rates in a dose-response pattern. The 0.1%, 0.5%, 10%, 25%, 50%, and 200% Rodeo-2% group heart rates
398 varied significantly compared to the control ($p < 0.05$).

399

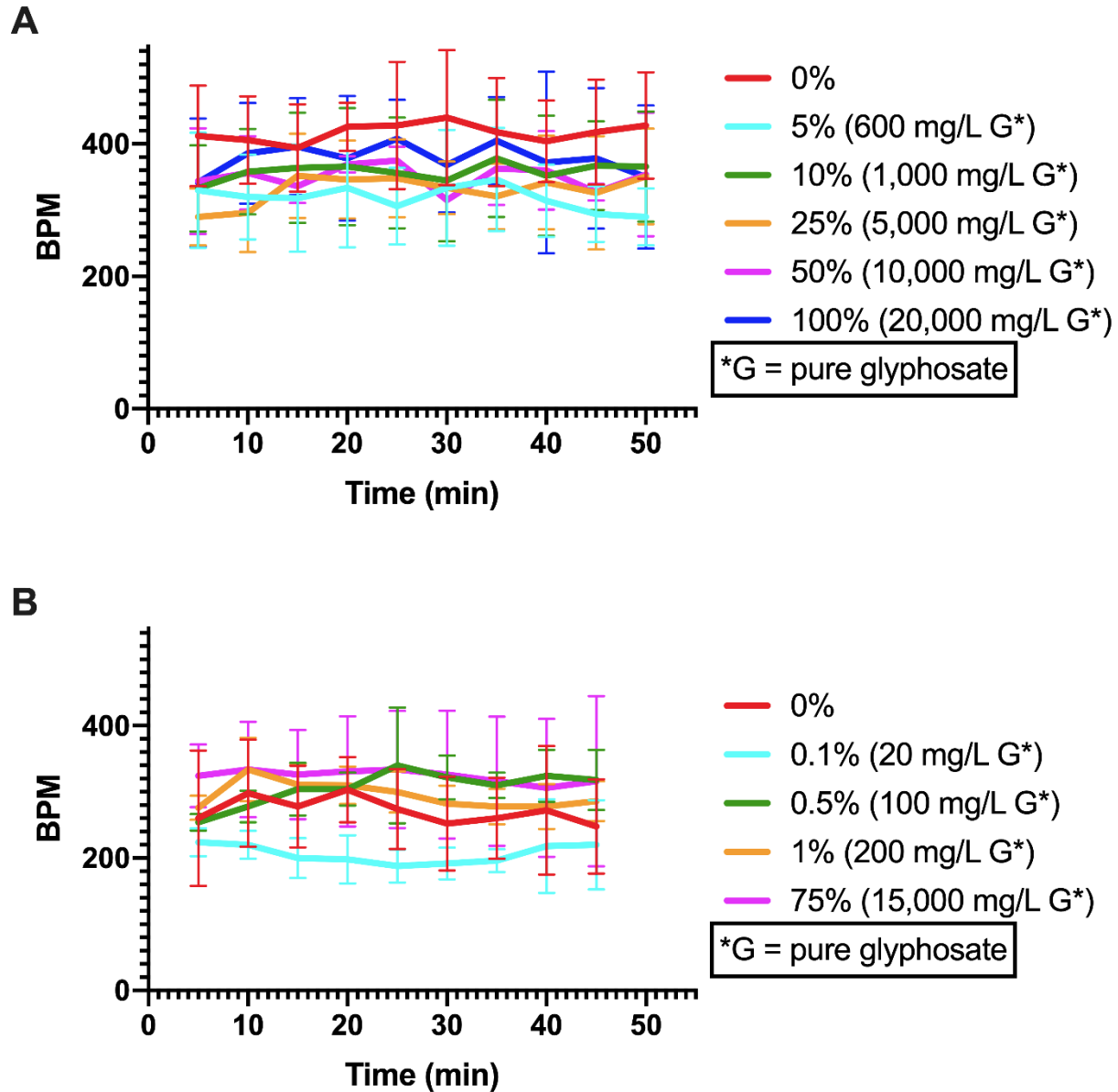
400 With the exception of the 50%, there was a general trend for the higher concentrations
401 of 25% to 200% to show decreasing heart rates in a dose-response pattern. Average
402 heart rates in the 0.1%, 0.5%, 10%, 25%, 50%, and 200% Rodeo-2% groups were
403 significantly different from control heart rates ($p < 0.05$) (Figs 3A, 3B and 3C).

404

405 **3.1.3 Glyphosate**

406 For most concentrations $> 1\%$ glyphosate, heart rates of *D. magna* varied from the
407 control group. However, the heart rates remained steady and did not show a dose-
408 response pattern relative to the control. Average heart rates in 5%, 10%, 25%, 50%,
409 and 75% glyphosate groups were significantly different from control heart rates ($p < 0.05$)
410 (Figs 4A and 4B).

411



412

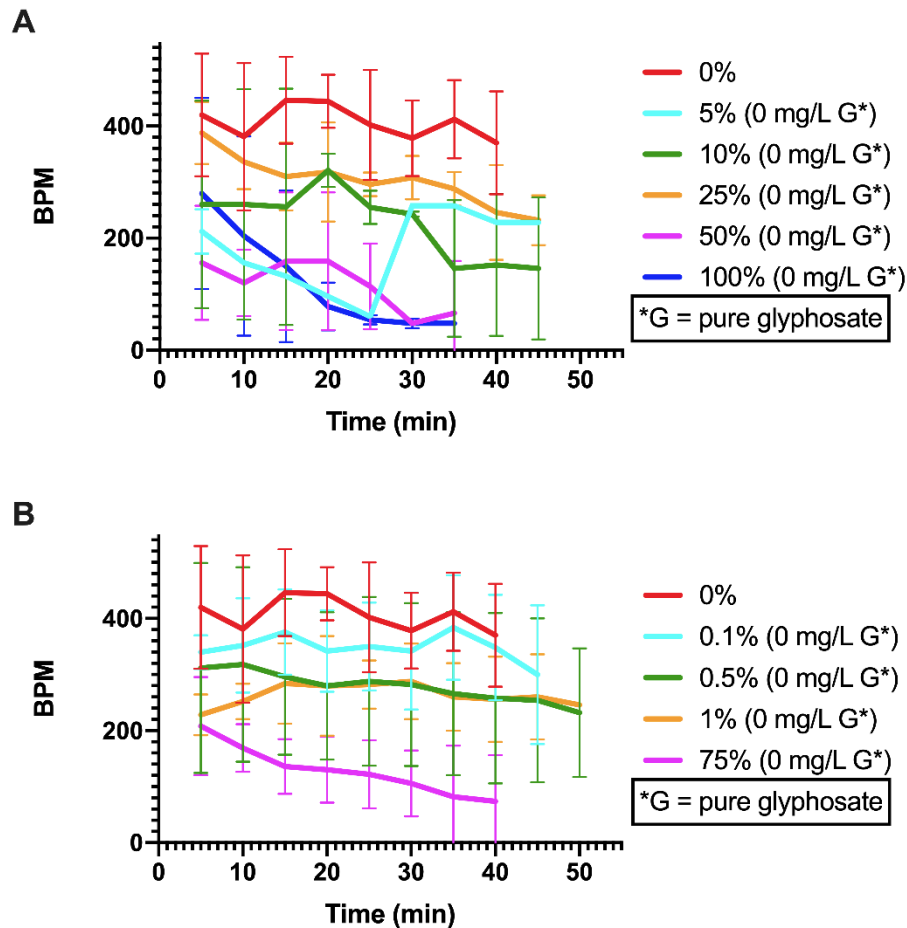
413 **Fig 4. Effects of glyphosate solutions on heart rate.** This represents the average BPM with standard
414 deviation of the *D. magna* over exposure time in minutes for listed concentrations of glyphosate (100% stock
415 contains 2% glyphosate). The control (0%) is shown as a red line. Each data point represents 3 *D. magna*.
416 Concentrations are divided into 2 graphs for clarity and to match the concentration groupings of the previous
417 experiments. All concentrations presented here share the same control. **A)** Heart rates in the concentrations
418 0%, 5%, 10%, 25%, 50%, and 100%. **B)** Heart rates in the concentrations 0%, 0.1%, 0.5%, 1%, and 75%.
419 For the tested concentrations, the heart rates remained steady and did not show a dose-response pattern.
420 The heart rates of *D. magna* in the 5%, 10%, 25%, 50%, and 75% glyphosate groups were significantly
421 different from those of the controls ($p < 0.05$).
422

423 **3.1.4 POEA**

424 In the POEA test groups, the *D. magna* heart rates ranged from approximately 15% to
425 100% of the control group, following no clear dose response (Figs 5A and 5B). The 5%
426 concentration showed steep jump in heart rate (Fig 5A). Average heart rates in all
427 solutions $\geq 1\%$ except for the 25% POEA solution were significantly lower than control
428 heart rates ($p < 0.05$). Though there is no clear dose-response, the *D. magna* in the three
429 highest concentrations of 50%, 75% and 100% had distinctly lower heart rates
430 ($p < 0.0001$) at less than 200 BPM (Figs 5A and 5B).

431

432



433

434 **Fig 5. Effects of POEA solutions on heart rate.** This represents the average BPM with standard deviation
435 of the *D. magna* over exposure time in minutes for listed concentrations of POEA (100% stock contains 1%
436 POEA and no glyphosate). The control (0%) is shown as a red line. Each data point represents 3 *D. magna*.
437 **A)** Heart rates in the concentrations 0%, 5%, 10%, 25%, 50%, and 100%. **B)** Heart rates in the
438 concentrations 0%, 0.1%, 0.5%, 1%, and 75%. Concentrations are divided into 2 graphs for clarity and to
439 match the concentration groupings of the previous experiments. All concentrations presented here share the
440 same control. Although all test concentrations had heart rates lower than controls, there was no clear dose-
441 response pattern. However, the 3 highest concentrations of POEA prompted the most drastic decreases in
442 heart rate ($p < 0.0001$). All concentrations $\geq 1\%$ of the POEA stock solution, except for the 25% solution,
443 produced heart rates significantly lower than the control group ($p < 0.05$). Missing data points were the result
444 of unreadable videos. This is generally revealed in Figs 5A and 5B where lines do not continue out to 45
445 minutes. For all missing data points, see highlighted data fields in S2 Dataset.

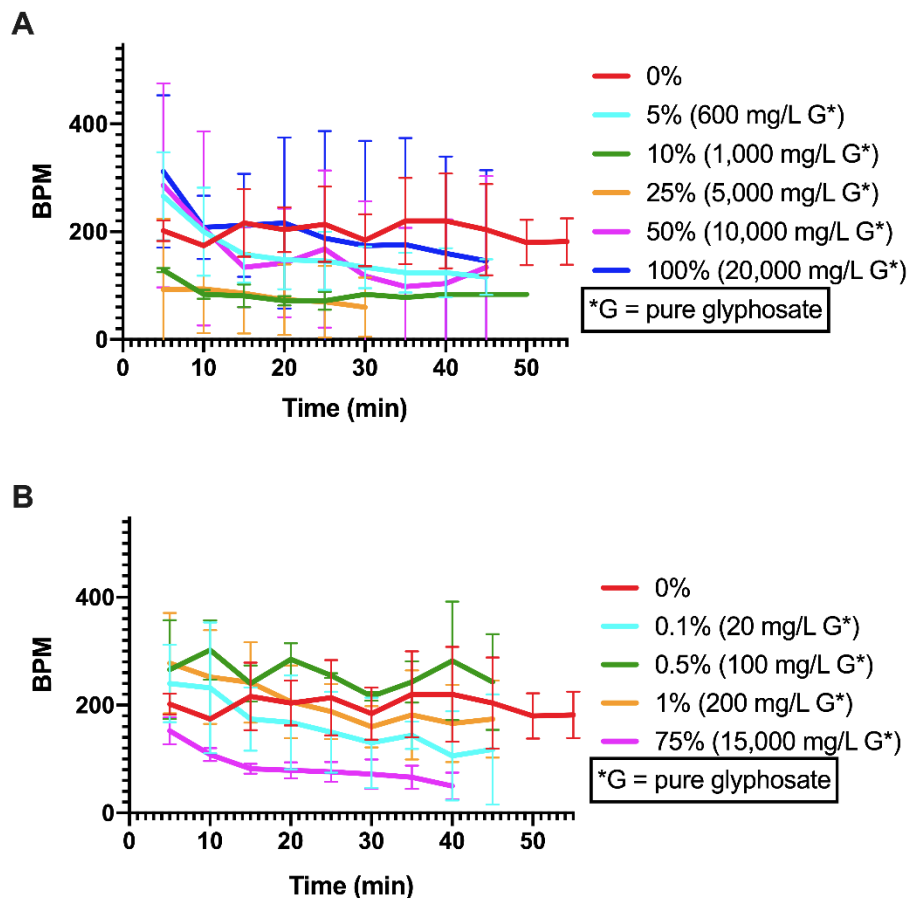
446

447 3.1.5 Mock-GBH (Glyphosate + POEA)

448 Heart rates following exposure to Mock-GBH concentrations decreased over time

449 compared to the control except for in the 0.5% concentration. The heart rates did not

450 follow a dose-response pattern and ranged from 150% to 25% of the control. Average
451 heart rates in the 10%, 25%, and 75% Mock-GBH groups were significantly lower than
452 control heart rates ($p < 0.05$) (Figs 6A and 6B).
453



454

455 **Fig 6. Effect of Mock-GBH (glyphosate + POEA) solutions on heart rate.** This represents the average
456 BPM with standard deviation of the *D. magna* over exposure time in minutes for listed concentrations of the
457 Mock-GBH (100% stock contains 1% POEA and 2% glyphosate). The control (0%) is shown as a red line.
458 Each data point represents 3 *D. magna*. **A)** Heart rates for the concentrations 0%, 5%, 10%, 25%, 50%, and
459 100%. **B)** Heart rates for the concentrations 0%, 0.1%, 0.5%, 1%, and 75%. Concentrations are divided into
460 2 graphs for clarity and to match the concentration groupings of the previous experiments. All concentrations
461 presented here share the same control. The control in this experiment had heart rates approximately 50%
462 slower than the heart rates of controls in other experiments. Heart rates did not follow a dose-response
463 pattern, although *D. magna* in the 10%, 25%, and 75% Mock-GBH concentrations had an average heart rate
464 significantly lower than that of the control. Missing data points were the result of unreadable videos. This is
465 generally revealed in Figs 6A and 6B where lines do not continue out to 45 minutes. For all missing data
466 points, see highlighted data fields in S2 Dataset.

467 All *D. magna* test and control groups displayed lower heart rates than in all other
468 experiments of this investigation. This is revealed by all concentrations settling at under
469 200 BPM, with exception of the 0.5% concentration (Figs 6A and 6B).

470

471

472 **3.1.6 Verification experiments for Rodeo-Recommended, Rodeo-2%, and** 473 **POEA**

474 The results for Rodeo-Recommended, Rodeo-2%, and POEA did not follow clear dose-
475 response patterns (Figs 2A,B,C, 3A,B,C and 5A,B). This led us to perform verification
476 experiments for the *D. magna* heart rates in Rodeo-Recommended, Rodeo-2%, and
477 POEA solutions. The concentrations 1%, 25%, and 100% were chosen and a larger test
478 group of 10 *D. magna* was used. *D. magna* heart rates were captured every 15 minutes
479 for one hour.

480

481 The 1% and 25% concentrations of both the Rodeo-Recommended and Rodeo-2%
482 stocks stayed within the normal range set by the control (S2 Figs A and B). Although the
483 100% concentrations of both Rodeo-Recommended and Rodeo-2% stock solutions
484 caused the *D. magna* heart rates to decrease to less than 50% of the control by 60
485 minutes (S2 Figs A and B), only the Rodeo-2% results at 100% concentration were
486 statistically significant from the control ($p < 0.05$) (S2 Fig B).

487

488 The heart rates for the 1% concentration of the POEA stock remained within the normal
489 range set by the control (S2 Fig C). The 25% and the 100% concentrations had lower

490 heart rates at less than 50% of the control for the entire 60-minute observation period
 491 (S2 Fig C). Unlike the 100% concentrations of Rodeo-Recommended and Rodeo-2%
 492 (S2 Figs A and B), the POEA concentrations did not cause any sharp changes in heart
 493 rates over time (S2 Fig C). POEA heart rates remained steady and level, but with
 494 significantly ($p < 0.05$) reduced BPM for the 25% and 100% concentrations. (S2 Fig. C).
 495

496 3.2 *Daphnia magna* survival rate analysis

497 All survival rate experiments were performed with a control concentration of 0%.
 498 Controls had 100% survival with no control *D. magna* dying within the observation
 499 period of 8 hours. Survival results are summarized in Table 4 using median time until
 500 death. Kaplan-Meier plots of survival over time are supplied in the Supporting
 501 Information (S3-8 Figs).

Stock Solution	Median Time Until Death (Hour : Minute : Second)													
	0%	1%	2%	3%	4%	5%	7.50%	10%	25%	30%	50%	75%	100%	200%
Roundup-WGK (12D, 2017-#1)	NA	NA	0:55:13	0:24:21	0:17:53									
(12D, 2017-#3)	NA					0:08:50	0:03:40	0:02:59						
Rodeo-Recom. (6D, 2017-02-04)	NA	NA				NA		NA					0:37:00	0:20:00
(6D, 2017-12-20)	NA								3:14:00		2:06:00	1:08:00		
Rodeo-2% (5D, 2017-02-04)	NA	NA		NA		NA		NA		NA				
(6D, 2017-12-20)	NA								6:32:00		2:40:00	2:19:00		
Glyphosate (6D, 2017-07-18)	NA	NA		NA				NA		NA			NA	
POEA (6D, 2017-07-18)	NA	5:18:00				NA		NA	3:49:00		2:52:00		2:16:00	
Mock-GBH (6D, 2017-07-18)	NA	NA				NA		2:30:00	NA		4:22:00	NA	7:23:00	

502
 503 **Table 4. Median time until death of *D. magna* exposed to Roundup, Rodeo, glyphosate, POEA, and Mock-GBH**
 504 **solutions.** Median time until death indicates the time at which half of the population died. “NA” indicates that fewer
 505 than half of the population died by the end of the experiment. A gray box indicates that the concentration was not
 506 tested. N=12 *D. magna* per concentration for Roundup-WGK; N=5 *D. magna* per concentration for 1%-10%, 100%
 507 and 200% of Rodeo-Recommended stock solution; and 1%-10% and 30% of Rodeo-2% stock solution; and N=6 *D.*
 508 *magna* per concentration for the rest of the stock solutions. For POEA and Mock GBH, all concentrations were tested
 509 on the same day. For Roundup, 1-4% were tested on one day and 5-10% tested on another. For Rodeo-
 510 Recommended and Rodeo-2%, the 25%, 50%, and 75% concentrations were tested on a separate day from the rest.
 511 All control *D. magna* survived for the entirety of the observation period of 8hrs. Kaplan-Meier plots of survival over
 512 time are supplied in the Supporting Information (S3-8 Figs).

513

514 **3.2.1 Roundup-WGK**

515 The 7.5% and 10% Roundup-WGK groups all *D. magna* died within 10 minutes (median
516 time until death of 3min 40sec and 2min 59sec respectively) (Table 4, S3 Fig). All of the
517 5% group died within 12 minutes (median time until death 8min 50sec). The 4% group
518 all died within 25min (median time until death 17min 53sec). The 3% group all died by
519 40 minutes (median time until death 24min 21sec) and the 2% group all died within 110
520 minutes (median time until death 55min 31sec) (Table 4, S3 Fig). In the 1% group, one
521 *D. magna* died within 5 minutes, the remaining *D. magna* did not die within the 8-hour
522 observation period.

523

524 **3.2.2 Rodeo-Recommended and Rodeo-2%**

525 All *D. magna* died within 2 hours in the 100% and 200% concentrations of the Rodeo-
526 Recommended stock (median time until death 37min and 20min, respectively) (Table 4,
527 S4 Fig). The 75% group died within 3 hours (median time until death 1hr 8min), the 50%
528 group died within 4 hours (median time until death 2hr 6min), and the 25% group died
529 within 6 hours (median time until death 3hr 14min). The 1%, 5%, and 10% groups did
530 not die within the 8-hour observation period (Table 4, S4 Fig.). Starting at 25%, death
531 rates show a precipitous decline with a clear dose-response pattern (S4 Fig).

532

533 For the Rodeo-2% stock, the 75% and the 50% groups all *D. magna* died within 4 and 5
534 hours, respectively (median time until death 2hr 19min and 2hr 40min, respectively)
535 (Table 4, S5 Fig). Half of the 25% group died within 8 hrs. The remaining groups of 10%

536 down to 1% concentrations showed no deaths within the 8-hour period (Table 4, S5
537 Fig). Survival rates followed a dose-response pattern starting at the 25% concentration
538 with the two highest concentrations showing a precipitous decline within 5 hours (S5
539 Fig).

540

541 **3.2.3 Glyphosate**

542 No deaths were observed in any of the glyphosate test groups (Table 4, S6 Fig).

543

544 **3.2.4 POEA**

545 The *D. magna* in the three highest concentrations of 100%, 50%, and 25% declined to
546 50% of control survival within the first 4 hours (median time until death 2hr 16min, 2hr
547 52 min, and 3hr 49 min, respectively). All of the *D. magna* died within 7 hours in the
548 100% concentration group. Half of the 1% group died within 6 hours (median time until
549 death 5hr 18min), but less than half of the 5% and 10% groups died within the 8-hour
550 observation period (Table 4, S7 Fig). Survival rates follow a dose-response pattern for
551 the three highest concentrations dropping to 50% survival within the first 4 hours. The
552 1%, 5%, and 10% concentrations did not follow a dose-response pattern (S7 Fig).

553

554 **3.2.5 Mock-GBH (POEA + Glyphosate)**

555 The Mock-GBH groups did not follow a dose-response pattern (Table 4, S8 Fig). All *D.*
556 *magna* in the 50% group died within 8 hours (median time until death 4hr 22min).
557 Approximately 67% of the *D. magna* died in the 10% group within 8 hours (median time
558 until death 2hr 30min) and about 50% died within 8 hours in the 100% group (median

559 time until death 7hr 23min). For all other groups of 1%, 5%, 25%, and 75%, about 33%
 560 died within 8 hours (Table 4, S8 Fig). Survival rates do not follow a dose-response
 561 pattern (S8 Fig).

562

563 4 Discussion

Stock Solution	Description	Ingredients	Percentage (V/V)	Concentration (g/L)	Impacts	
					Survival Rate Reduction	Heart Rate Reduction
Roundup-WGK	Mixture of Water and Ready-Use RoundUp (2% Glyphosate Isopropylamine Salt)	Glyphosate Isopropylamine Salt	2.00%	20.0	xxxx	xxxx
		Glyphosate	0.98%	9.8		
		POEA - inactive ingredient	1.00%	10.0		
		Other/Unknown inactive ingredient	97.00%	970.0		
Rodeo-Recommended	Mixture of Water and Commercial Concentrated Rodeo Solution (53.8% Glyphosate Isopropylamine Salt)	Glyphosate Isopropylamine Salt	5.38%	65.1	xxx	xxx
		Glyphosate	2.63%	31.8		
		POEA - inactive ingredient	0.00%	0.0		
		Other/Unknown inactive ingredient	4.62%	55.9		
Rodeo-2%	Mixture of Water and Commercial Concentrated Rodeo Solution (53.8% Glyphosate Isopropylamine Salt)	Glyphosate Isopropylamine Salt	2.00%	24.2	xx	xx
		Glyphosate	0.98%	11.8		
		POEA - inactive ingredient	0.00%	0.0		
		Other/Unknown inactive ingredient	1.7175%	20.8		
Glyphosate	Mixture of Water and Pure Glyphosate (100%)	Glyphosate	2.00%	20.0	None	None
		POEA - inactive ingredient	0.00%	0.0		
		Other/Unknown inactive ingredient	0.00%	0.0		
POEA	Mixture of Water and Pure POEA (100%)	Glyphosate	0.00%	0.0	x	x
		POEA - inactive ingredient	1.00%	9.7		
		Other/Unknown inactive ingredient	0.00%	0.0		
Mock-GBH	Mixture of Water, Pure Glyphosate(100%), and Pure POEA (100%)	Glyphosate	2.00%	20.0	x	x
		POEA - inactive ingredient	1.00%	9.7		
		Other/Unknown inactive ingredient	0.00%	0.0		

564

565 **Table 5. Summary of testing results.** A qualitative summary of the effects of Roundup-WGK, Rodeo-
 566 Recommended, Rodeo-2%, Glyphosate, POEA, and Mock-GBH solutions on *D. magna* survival rates and heart
 567 rates. The x, xx, xxx, xxxx approximate increasing severity of response seen for those stock solutions.

568

569 An important consideration in this study is the sensitivity of *D. magna* species' heart
 570 rates to variations in water temperature. For example, the heart rate of *D. magna pulex*
 571 increases by about 24 BPM per 1°C (51). Because water temperature was maintained
 572 at 21°C during all experiments, temperature had minimal effects on our results. The
 573 variation in our control heart rates is not uncommon and aligns with published data of
 574 normal adult *D. magna* heart rates at about 21°C (51,52). At this temperature, various
 575 publications have stated that *D. magna* heart rate ranges from approximately 180 to 350

576 BPM (50,52). Individual heart rates for *D. magna* species are also highly variable
577 among individuals and across conditions (51). We are unsure why the control and
578 experimental heart rates in the Mock-GBH experiment were unusually low compared to
579 our other experiments presented in this paper.

580

581 As summarized in Table 5, glyphosate alone had the least effect on *D. magna* heart
582 rates out of all test substances and showed no effect on survival rates (Fig 4, Table 4,
583 and S6 Fig). Although POEA lowered heart and survival rates (Fig 4, Table 4, and S7
584 Fig), the lack of a clear dose response makes definitive conclusions difficult. POEA and
585 glyphosate together in the Mock-GBH had approximately the same effect on heart rate
586 and survival rate as POEA alone (Figs 5-6, Table 4, and S7-8 Figs), reinforcing the
587 conclusion that glyphosate has a marginal effect on *D. magna* physiology at the
588 concentrations used in this study. For the glyphosate, POEA, and Mock-GBH solutions,
589 100% concentrations are closest to the concentrations of these chemicals (in mg/L) in
590 ready-to-use GBHs. Considering the unlisted ingredient concentration(s) are unknown,
591 we diluted the full products for dose response testing using percent rather than diluting
592 to a specific concentration in weight/volume of glyphosate or POEA. These results
593 emphasize the probable deleterious effects of unlisted ingredients in herbicides.

594

595 As an internal verification of our experiments, Rodeo-2% containing 2% glyphosate has
596 approximately half the concentration of glyphosate as is present in Rodeo-
597 Recommended containing 5.38% glyphosate. The unlisted ingredients in Rodeo are
598 also approximately halved. The decreased death rates for Rodeo-2% compared to

599 Rodeo-Recommended support this pattern (Table 4, S4-5 Figs). The median time until
600 death is approximately half in the Rodeo-2% compared to the Rodeo-Recommended
601 (Table 4).

602

603 Heart and survival rates largely decreased following exposure to the Mock-GBH
604 compared to controls (Fig 6, Table 4 and S8). However, the Roundup-WGK, Rodeo-
605 Recommended and Rodeo-2% solutions decreased heart and survival rates to a much
606 greater extent (Fig 1-3, Table 4, S3-5 Figs). This is despite the Mock-GBH containing
607 comparable levels of glyphosate to Roundup-WGK and Rodeo and, in the case of
608 Roundup-WGK, comparable levels of POEA.

609

610 These results suggest that there are other unlisted ingredients in addition to POEA in
611 both Roundup-WGK and Rodeo that have deleterious effects on *D. magna*. The results
612 of the Roundup-WGK and Rodeo-2% experiments suggest that the amount of unlisted
613 ingredients in a given stock solution is proportional to the negative effects of these
614 GBHs on *D. magna*. Although both stock solutions contain 2% glyphosate, Roundup-
615 WGK with 98% unlisted ingredients had a much greater effect on heart and survival
616 rates compared to Rodeo-2% with approximately 1.72% unlisted ingredients.

617

618 Our results agree with previous studies questioning the safety of the unlisted ingredients
619 in GBHs (27,29,37,38). This is supported by our results that show greater negative
620 impacts on heart rates and death rates of the full GBH products compared to our mock
621 GBH solution or glyphosate alone. The 5% solution of the Roundup-WGK killed all the

622 *D. magna* within 10 minutes, while the solution of 5% Mock-GBH caused a death rate of
623 50% within the 8-hour observation period (Table 4, S3 Fig, S8 Fig). Considering that the
624 5% solution of Mock-GBH contains more glyphosate by mass than the Roundup-WGK
625 because of different salt forms of glyphosate, one would have expected that the Mock
626 GBH would have been more harmful, based on the glyphosate alone. In comparing the
627 other concentrations and solutions, we found similar results that indicate more harmful
628 effects with the full GBH product compared to our solutions of the known ingredients.
629 Solutions of glyphosate alone did not cause any death in our experiments and caused
630 no clear effects on heart rates while higher concentrations of full GBH products
631 Roundup-WGK and Rodeo caused increasing death and decreasing heart rates.

632

633 These results challenges the veracity of the EPA-stated safety of herbicides since they
634 generally do not test and do not require reporting of the full ingredient makeup of
635 commercial herbicide preparations containing glyphosate (36). This also underscores
636 the limitations in the policy of not investigating the unlisted ingredients and total product
637 when determining the safety of GBHs and other herbicides by regulatory and licensing
638 agencies.

639

640 Challenges to furthering the research on the safety of GBHs as well as other
641 herbicides/pesticides include not only the identification of the ingredients, but also their
642 accessibility for testing. For example, in the case of glyphosate, although there are over
643 30 chemical vendors that sell over a dozen chemical variations, these vendors primarily
644 sell very large quantities (1000 liter minimum order) to manufacturers of herbicides

645 (6,10). In this investigation, we were unable to procure glyphosate formulations other
646 than pure glyphosate. For researchers to accurately assess safety concerns, we
647 propose three changes to the current GBH research environment:

648 A) Glyphosate formulations and POEA should be readily accessible in small
649 quantities suitable for laboratory testing.

650 B) All unlisted ingredients in GBHs should be disclosed by herbicide
651 manufacturers.

652 C) Glyphosate and POEA formulations should be disclosed by vendors to
653 allow standardization for testing.

654

655 We hope our investigation emphasizes the need for peer-reviewed research of
656 herbicide safety and the need to improve the transparency of product testing. This could
657 improve the public's confidence in government safety assessments.

658 Most importantly, we hope that further investigations using other organismal systems
659 will test the listed and unlisted ingredients, as well as the full GBH product, to reveal the
660 direct and indirect risks to human health and the environment as a result of their
661 continually increasing use.

662

663

664 **5 Acknowledgements**

665 We thank the *New Hampshire Academy of Science* for providing access to the
666 extensive equipment of their STEM Lab to conduct our research. We are grateful for the
667 guidance and support of Dr. Chery Whipple during the experimental phase and Elaine

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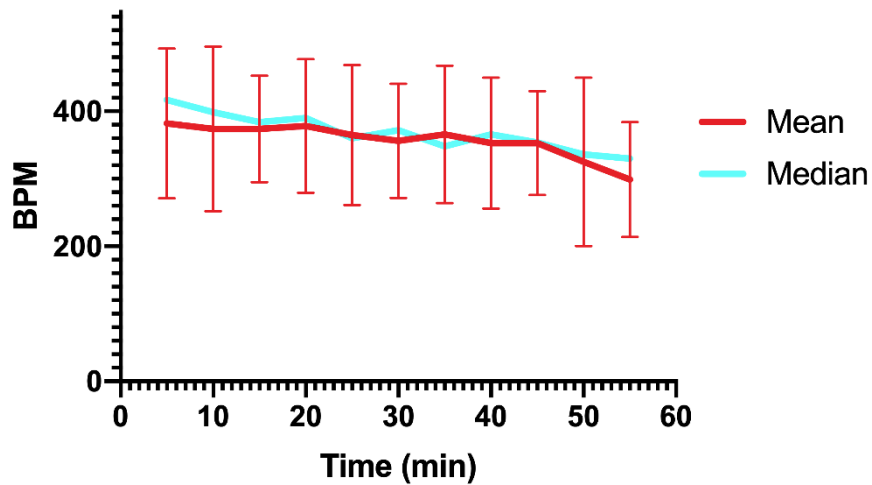
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801 7 Supporting information

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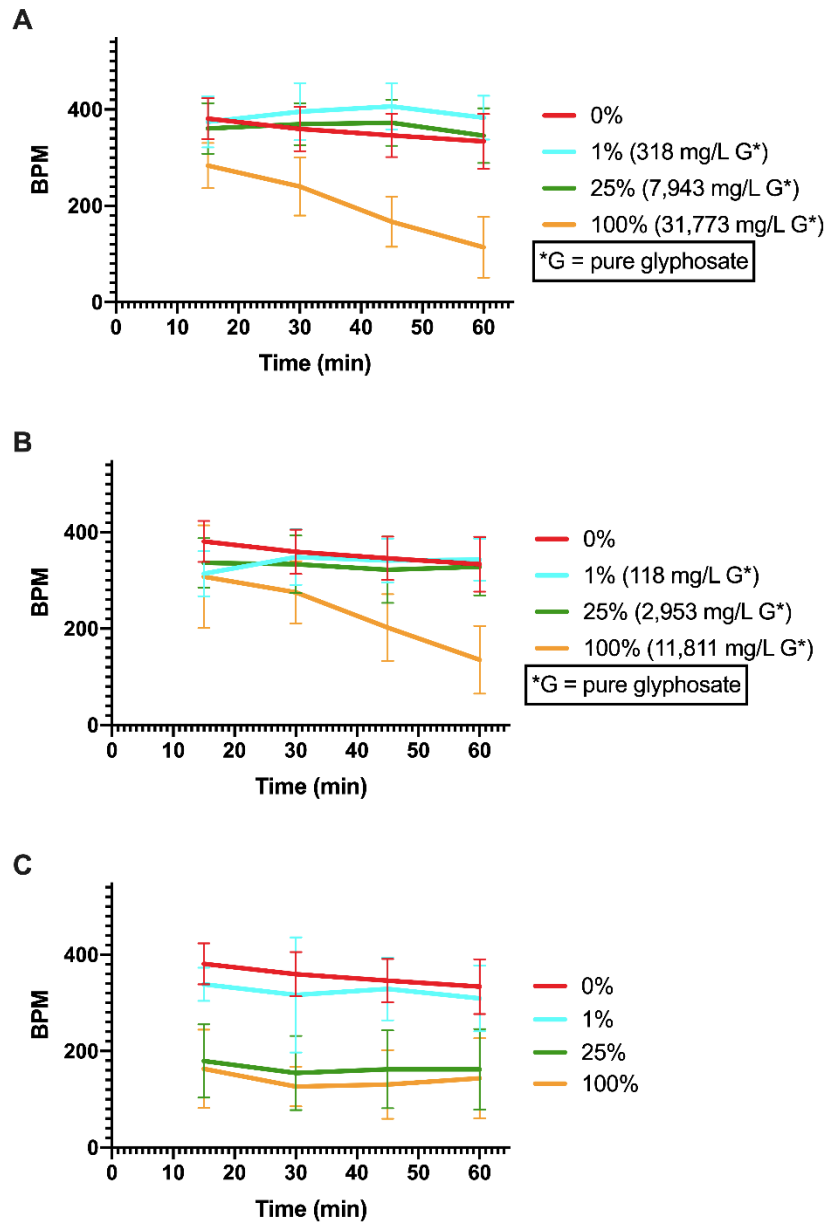
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805 **S1 Figure. Control heart rate verification.** This represents the average and median BPM of our control *D.*
806 *magna* with error bars representing standard deviations. Each data point represents 25 *D. magna*. The average
807 heart rate is shown with error bars representing standard deviation while the median heart rate is plotted without
808 error bars. The median and mean resting heart rate in our experiments were 369 and 357 BPM respectively.

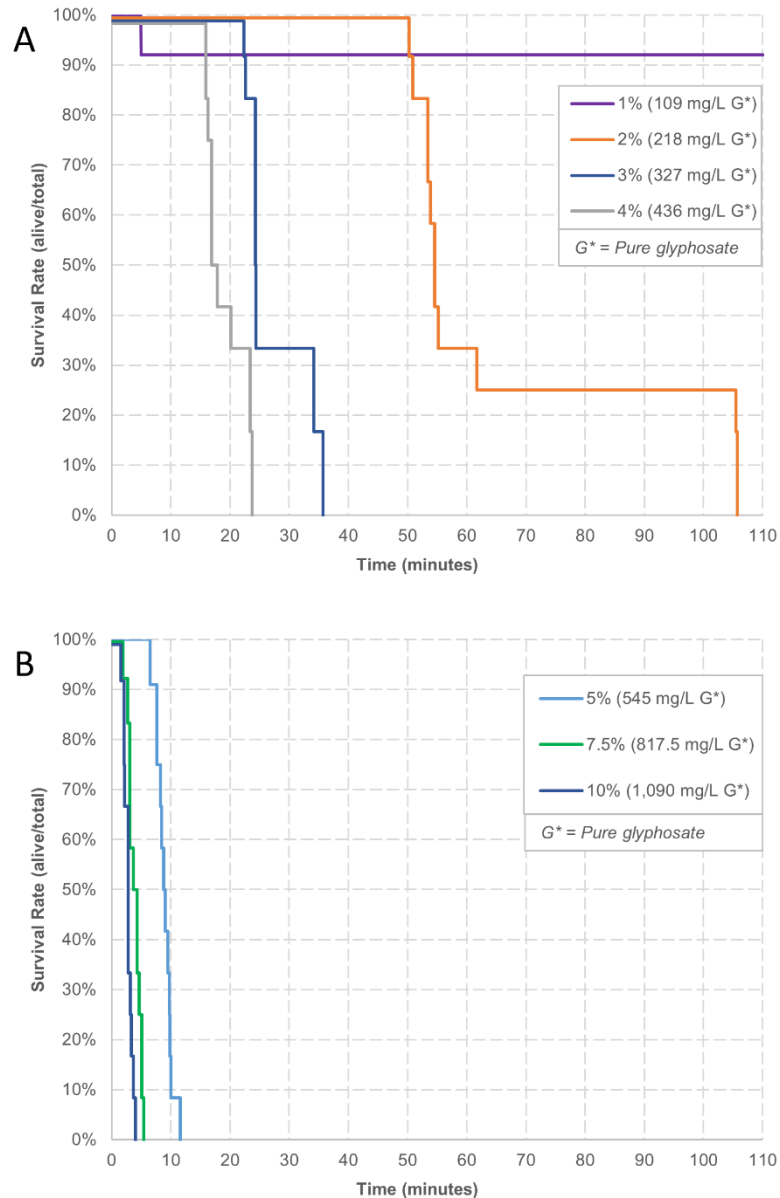
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812 **S2 Figure. Verification of Rodeo-Recommended, Rodeo-2%, and POEA heart rate experiments.** The
813 results of the verification experiments, showing the average BPM with standard deviation of *D. magna* over
814 exposure time in minutes. Each data point represents 10 *D. magna*. All experiments were performed at the
815 same time and share a control though they are plotted separately for clarity. **A)** Rodeo-Recommended
816 verification with concentrations of 1%, 25%, and 100% Rodeo-Recommended stock (100% stock contains
817 5.38% glyphosate). Both the 1% and 25% concentrations had little effect on the *Daphnia* while 100% caused
818 heart rates to steadily drop to under 50% of the control by 60 minutes. **B)** Rodeo-2% verification with
819 concentrations of 1%, 25%, and 100% Rodeo-2% stock (100% stock contains 2% glyphosate). Both the 1% and
820 25% concentrations had little effect on the *daphnia* while 100% caused heart rates to steadily drop to under
821 50% of the control by 60 minutes. This was a significant decrease compared to the control ($p < 0.05$) **C)** POEA
822 verification with concentrations of 1%, 25%, and 100% POEA stock (100% stock contains 1% POEA and no
823 glyphosate). The heart rates of *D. magna* exposed to the 25% and 100% concentrations had significantly lower
824 heart rates at less than 50% of control ($p < 0.05$).
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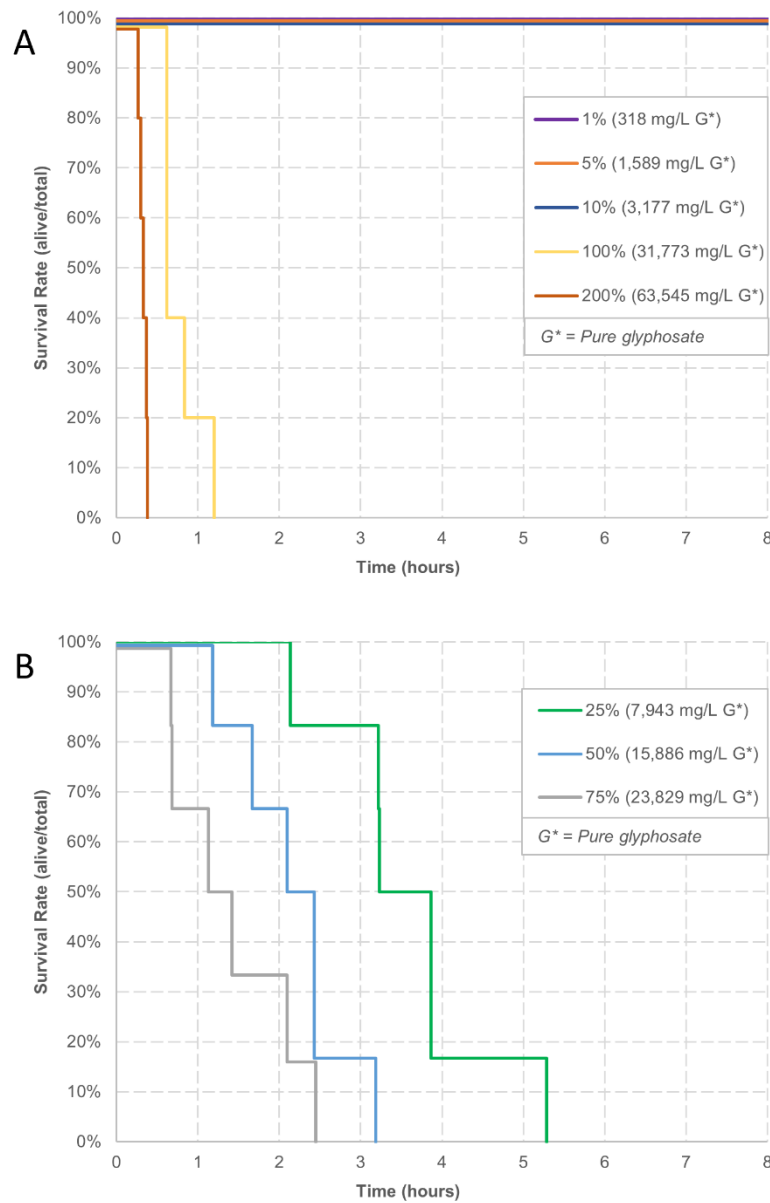
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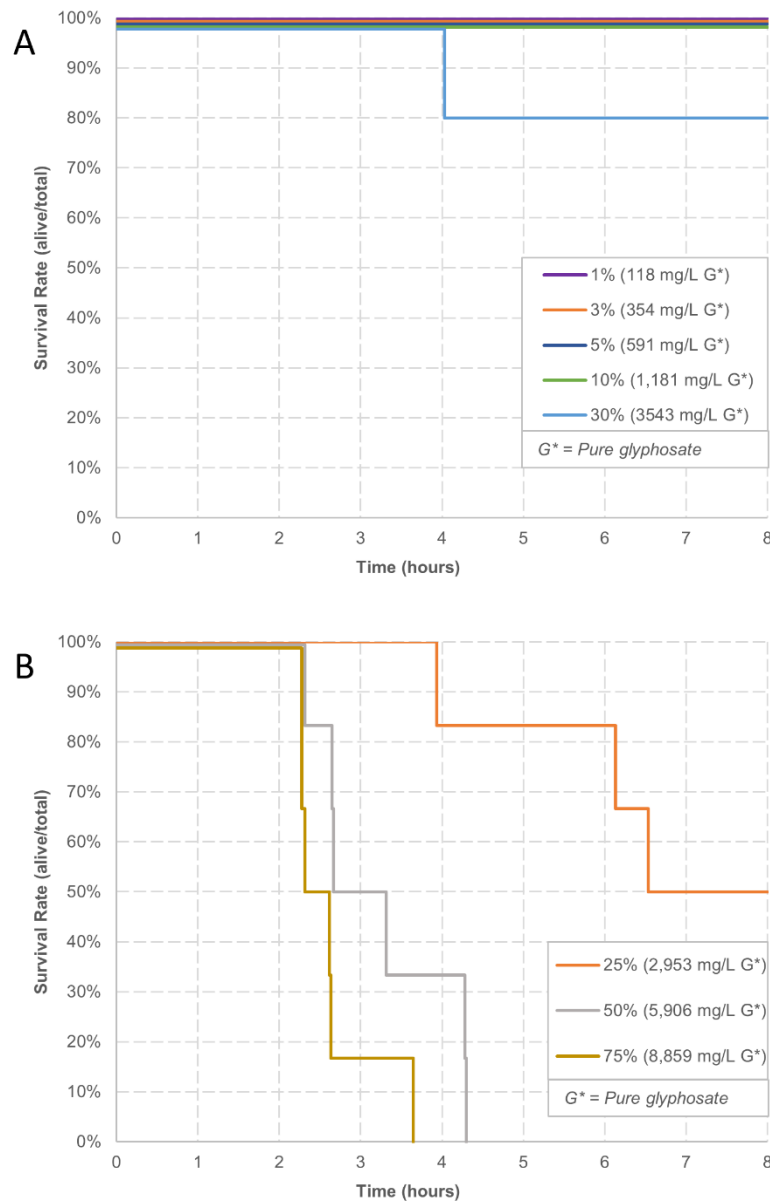
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S3 Figure. Roundup-WGK survival. This represents the survival rates of the *D. magna* exposed to Roundup-WGK dilutions over exposure time in minutes. **A)** Survival for concentrations 1%, 2%, 3%, 4% Roundup-WGK (100% stock contains 2% glyphosate). **B)** Survival in concentrations 5%, 7.5%, and 10% Roundup-WGK followed a clear dose-response pattern. Survival rates show a precipitous decline starting at 2% (A and B). Twelve *D. magna* were used for each concentration. Control group values, which had no deaths, are not shown to avoid obscuring test concentration data points. Observations were continuous over 8 hours and each death is shown by a vertical line drop at the time of death. This graph only displays the first 120 minutes of the 8-hour observational period to allow for discrimination among the groups. To avoid the colored lines representing different test groups obscuring each other, lines are slightly shifted to allow for clear visual recognition.



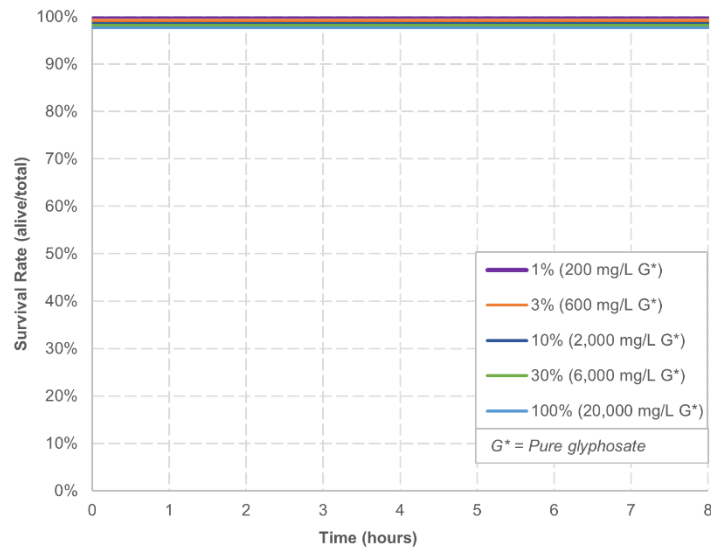
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S4 Figure. Rodeo-Recommended survival. This represents the survival rates of the *D. magna* in Rodeo-Recommended concentrations over exposure time in hours. **A)** Survival for concentrations 1%, 5%, 10%, 100%, and 200% Rodeo-Recommended stock (100% stock contains 5.38% glyphosate). **B)** Survival for concentrations 25%, 50%, and 75% Rodeo-Recommended stock, Five *D. magna* were used for each of concentrations 1%-10%, 100% and 200%; six *D. magna* were used for the remaining concentrations. Starting at 25%, death rates show a precipitous decline with a clear dose-response pattern. Control group values, which had no deaths, are not shown in graphs to avoid obscuring test concentration data points. Observations were continuous over 8 hours and each death is shown by a vertical line drop at the time of death. To avoid the colored lines representing different test groups obscuring each other, lines are slightly shifted to allow for clear visual recognition.



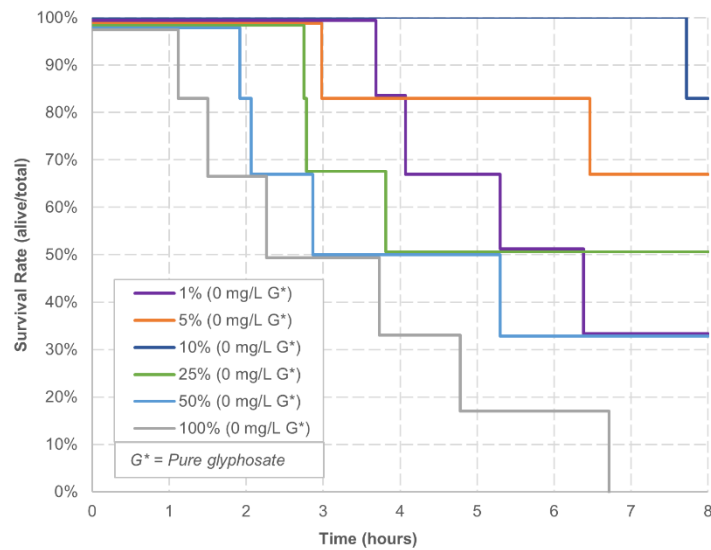
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S5 Figure. Rodeo-2% survival. This represents the survival rates of the *D. magna* over exposure time in minutes. **A)** Survival for concentrations 1%, 3%, 5%, 10%, and 30% Rodeo-2% stock (100% stock contains 2% glyphosate). **B)** Survival for concentrations 25%, 50%, and 75% Five *D. magna* were used for each of concentrations 1%-10% and 30%, six *D. magna* were used for the remaining concentrations. Survival rates followed a dose-response pattern starting at the 25% concentration with the two highest concentrations showing a precipitous decline within 5 hours. Control group values, which had no deaths, are not shown in graphs in order to avoid obscuring test concentration data points. Observations were continuous over 8 hours and each death is shown by a vertical line drop at the time of death. To avoid the colored lines representing different test groups obscuring each other, lines are slightly shifted to allow for clear visual recognition.



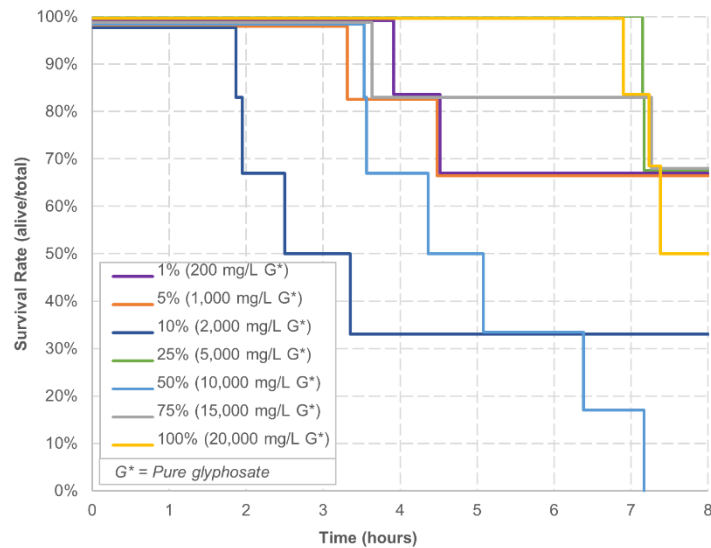
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S6 Figure. Glyphosate survival. This graph shows the survival rates of *D. magna* over an 8-hour exposure time for 1%, 3%, 10%, 30%, and 100% concentrations of the glyphosate stock solution. Six *D. magna* were used for each concentration. No *Daphnia* died during the observation times for any concentrations. Control group values, which had no deaths, are not shown in graphs to avoid obscuring test concentration data points. Observations were continuous over 8 hours. To avoid the colored lines representing different test groups obscuring each other, lines are slightly shifted to allow for clear visual recognition.



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S7 Figure. POEA survival. This represents the survival rates of the *D. magna* over exposure time in hours for concentrations 1%, 5%, 10%, 25%, 50%, and 100% POEA (100% stock contains 1% POEA and no glyphosate). Six *D. magna* were used for each concentration. Survival rates follow a dose-response pattern for the three highest concentrations dropping to 50% survival within the first 4 hours. The 1%, 5%, and 10% concentrations did not follow a dose-response pattern. Control group values, which had no deaths, are not shown in graphs to avoid obscuring test concentration data points. Observations were continuous over 8 hours and each death is shown by a vertical line drop at the time of death. To avoid the colored lines representing different test groups obscuring each other, lines are slightly shifted to allow for clear visual recognition.



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S8 Figure. Mock-GBH survival. This represents the survival rates of the *D. magna* over exposure time in hours for concentrations 1%, 5%, 10%, 25%, 50%, 75%, and 100% Mock-GBH (100% stock contains 1% POEA and 2% glyphosate). Five to six *D. magna* were used for each concentration. Survival rates do not follow a dose-response pattern. Control group values, which had no deaths, are not shown in graphs to avoid obscuring test concentration data points. Observations were continuous over 8 hours and each death is shown by a vertical line drop at the time of death. To avoid the colored lines representing different test groups obscuring each other, lines are slightly shifted to allow for clear visual recognition.

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S1 Dataset. Statistical results for heart rate experiments. Statistical analysis of the results was performed by applying a Kruskal-Wallis ANOVA test with Dunn's Multiple Comparison post-test using GraphPad Prism.

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S2 Dataset. Raw data for heart rate experiments.

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S3 Dataset. Raw data for heart rate verification experiments of Rodeo-Recommended, Rodeo-2% and POEA.

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S4 Dataset. Raw data for survival experiments.

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S5 Dataset. Consolidated control heart rate data.