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### ***Dugesia japonica* Is The Best Suited Of Three Planarian Species For High-Throughput Toxicology Screening**

D. Ireland

Veronica Bochenek , '22

Daniel Chaiken , '20

*See next page for additional authors*

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**Authors**

D. Ireland; Veronica Bochenek , '22; Daniel Chaiken , '20; C. Rabeler; Sumi Onoe , '21; Ameet Soni; and Eva-Maria S. Collins

1 *Dugesia japonica* is the best suited of three planarian species for high-throughput  
2 toxicology screening

3 Danielle Ireland<sup>a</sup>, Veronica Bochenek<sup>a</sup>, Daniel Chaiken<sup>b</sup>, Christina Rabeler<sup>a</sup>, Sumi Onoe<sup>b</sup>, Ameet  
4 Soni<sup>b</sup>, and Eva-Maria S. Collins<sup>a,c\*</sup>

5  
6 <sup>a</sup> Department of Biology, Swarthmore College, Swarthmore, Pennsylvania, United States of  
7 America

8 <sup>b</sup> Department of Computer Science, Swarthmore College, Swarthmore, Pennsylvania, United  
9 States of America

10 <sup>c</sup> Department of Physics, University of California San Diego, La Jolla, California, United States of  
11 America

12

13

14

15

16 \* Corresponding author

17 Email: [ecollin3@swarthmore.edu](mailto:ecollin3@swarthmore.edu) (E-MSO)

18 Address: Martin Hall 202, 500 College Avenue, Swarthmore College, Swarthmore, PA 19081

19 Phone number: 610-690-5380

20

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22

## 23 **Abstract**

24 High-throughput screening (HTS) using new approach methods is revolutionizing  
25 toxicology. Asexual freshwater planarians are a promising invertebrate model for neurotoxicity  
26 HTS because their diverse behaviors can be used as quantitative readouts of neuronal function.  
27 Currently, three planarian species are commonly used in toxicology research: *Dugesia japonica*,  
28 *Schmidtea mediterranea*, and *Girardia tigrina*. However, only *D. japonica* has been demonstrated  
29 to be suitable for HTS. Here, we assess the two other species for HTS suitability by direct  
30 comparison with *D. japonica*. Through quantitative assessments of morphology and multiple  
31 behaviors, we assayed the effects of 4 common solvents (DMSO, ethanol, methanol, ethyl acetate)  
32 and a negative control (sorbitol) on neurodevelopment. Each chemical was screened blind at 5  
33 concentrations at two time points over a twelve-day period. We obtained two main results: First,  
34 *G. tigrina* and *S. mediterranea* planarians showed significantly reduced movement compared to  
35 *D. japonica* under HTS conditions, due to decreased health over time and lack of movement under  
36 red lighting, respectively. This made it difficult to obtain meaningful readouts from these species.  
37 Second, we observed species differences in sensitivity to the solvents, suggesting that care must  
38 be taken when extrapolating chemical effects across planarian species. Overall, our data show that  
39 *D. japonica* is best suited for behavioral HTS given the limitations of the other species.  
40 Standardizing which planarian species is used in neurotoxicity screening will facilitate data  
41 comparisons across research groups and accelerate the application of this promising invertebrate  
42 system for first-tier chemical HTS, helping streamline toxicology testing.

43

## 44 **Keywords**

45 Planarian, high-throughput screening, invertebrate, developmental neurotoxicity, solvents

46

## 47 **Introduction**

48 Toxicology is currently undergoing a paradigm shift, focusing considerable effort on  
49 replacing, reducing, and refining (3Rs) vertebrate animal testing. This change has been driven by  
50 the high cost, low throughput, and questionable relevance of traditional mammalian guideline tests  
51 used for regulatory decisions. This is especially true for assessing developmental neurotoxicity  
52 (DNT) (Tsuji and Crofton, 2012). New approach methods which are amenable to economical high-  
53 throughput screening (HTS), including *in silico* modeling, *in vitro* models, and invertebrate  
54 systems, promise to fill the gap, alone or as part of a test battery (Fritsche et al., 2018; Lein et al.,  
55 2005; Thomas et al., 2019). A recent directive from the Environmental Protection Agency (EPA)  
56 details a plan to stop all funding of mammalian testing by 2035 (Wheeler, 2019). This directive  
57 reinforces the agency's previous commitment to reduce vertebrate testing for chemicals regulated  
58 under the Toxic Substances Control Act through integration of new approach methods into  
59 regulatory decisions (US EPA, 2018). To achieve this challenging goal, an increased effort is  
60 necessary to validate these new approach methods to ensure sensitivity, robustness, and relevance,  
61 and standardize best testing practices (Bal-Price et al., 2018; Crofton et al., 2011). Common test  
62 standards for a particular model system are essential for meaningful direct comparisons of data  
63 across laboratories and ultimately will build the basis for the development of the necessary  
64 regulatory guidelines.

65

66 We have developed the asexual freshwater planarian *Dugesia japonica* as a promising new  
67 invertebrate model for high-throughput neurotoxicity and DNT screening (Hagstrom et al., 2016,  
68 2015; Zhang et al., 2019a, 2019b). We have shown that it possesses comparable sensitivity to more

69 established new approach methods and is predictive of mammalian DNT (Hagstrom et al., 2019,  
70 2015; Zhang et al., 2019a, 2019b). The key advantage of the planarian system is its sufficiently  
71 complex behavioral repertoire which enables distinct behaviors to be used as a multifaceted  
72 quantitative readout of neuronal function (Hagstrom et al., 2019; Zhang et al., 2019a, 2019b). The  
73 planarian nervous system is of medium size (~10,000 neurons), possessing >95% gene homology  
74 and sharing most of the same neurotransmitters and neuronal cell types as the mammalian brain  
75 (Buttarelli et al., 2008; Mineta et al., 2003; Ross et al., 2017). Thus, the planarian system allows  
76 for mechanistic insights into how different cells and pathways control specific behaviors (Birkholz  
77 and Beane, 2017; Currie and Pearson, 2013; Inoue et al., 2015, 2014; Nishimura et al., 2010, 2008;  
78 Pearce et al., 2017; Sabry et al., 2019; Zhang et al., 2019b). Because planarians are simultaneously  
79 amenable to high-throughput screening (HTS), they are a promising alternative neurotoxicology  
80 model. We and others have recently reviewed the benefits and limitations of planarians for  
81 toxicology, particularly neurotoxicity and DNT (Hagstrom et al., 2016; Wu and Li, 2018; Zhang  
82 et al., 2019a).

83  
84 Our previous work demonstrated the potential of *D. japonica* as an invertebrate model for  
85 neurotoxicity and DNT studies and demonstrated the reliability and robustness of our screening  
86 methodology (Hagstrom et al., 2015; Zhang et al., 2019a, 2019b). However, since other research  
87 groups have used other planarian species and other, generally low-throughput and small scale,  
88 screening methods, it is difficult to compare results or standardize testing conditions (Hagstrom et  
89 al., 2016; Wu and Li, 2018; Zhang et al., 2019a). The two most common planarian species that  
90 have been used in toxicology studies besides *D. japonica* are *Girardia tigrina*, formerly *Dugesia*  
91 *tigrina*, (Byrne, 2018; Córdova López et al., 2019; Knakievicz and Ferreira, 2008; Moustakas et

92 al., 2015; Ramakrishnan and DeSaer, 2011) and *Schmidtea mediterranea* (Lowe et al., 2015;  
93 Plusquin et al., 2012; Poirier et al., 2017; Stevens et al., 2014; Tran et al., 2019). Of these three, *S.*  
94 *mediterranea* is the most popular planarian species for molecular studies because its annotated  
95 genome is readily available (Grohme et al., 2018; Robb et al., 2008; Rozanski et al., 2019), whereas  
96 only a draft genome exists for *D. japonica* (An et al., 2018). Transcriptomes are available for all  
97 three species (Rozanski et al., 2019; Wheeler et al., 2015). Genomic studies have been hindered in  
98 *D. japonica* and *G. tigrina* because of the larger size ( $2n=16$ , compared to  $2n=8$  in *S.*  
99 *mediterranea*), mixoploidy, and abundance of repetitive, transposable elements in the genomes of  
100 these species (An et al., 2018; Benazzi, 1993; Garcia-Fernandez et al., 1995; Hoshino et al., 1991;  
101 Wheeler et al., 2015). Comparatively, *G. tigrina* is the least well characterized, but is commercially  
102 available and has thus found widespread use across research laboratories and schools. *G. tigrina*  
103 has been largely utilized for its characteristic head morphology (auricles), which facilitates scoring  
104 of morphological head abnormalities and regeneration defects (Córdova López et al., 2019;  
105 Knakievicz and Ferreira, 2008).

106

107 We have previously found that there are significant differences in terms of growth and  
108 reproductive strategies in the laboratory among these three species (Carter et al., 2015). Most  
109 relevant in respect to HTS suitability are our findings that *G. tigrina* and *S. mediterranea* are more  
110 sensitive to water conditions than *D. japonica* (Carter et al., 2015), which could be problematic  
111 when these species are stored in small volumes for extended periods of time, such as during HTS  
112 in multi-well plates.

113

114 In the context of toxicology screens, only *D. japonica* has so far been tested and  
115 demonstrated to be a suitable HTS system (Zhang et al., 2019a, 2019b), because the same  
116 repertoire of behaviors which can be observed in low-throughput experiments are reproducible in  
117 a HTS setting (a sealed 48-well plate, with 1 planarian per 200  $\mu$ l of solution per well) (Hagstrom  
118 et al., 2015; Zhang et al., 2019a) and specimen can be recovered from the HTS setup without  
119 obvious long-term negative health effects.

120  
121 Thus, we aim to evaluate two criteria: 1) which species is the best suited for HTS conditions  
122 and 2) how sensitive the different species are to solvents commonly used in toxicology. To directly  
123 compare the suitability of these three planarian species for HTS, we utilized our custom robotic  
124 screening platform because it was demonstrated to be reliable and robust (Zhang et al., 2019b). On  
125 this automated platform, chemicals are screened in a 48-well plate, testing 5 concentrations along  
126 with a solvent control for n=8 planarians (1/well) per condition and experiment. Planarian  
127 morphology and behaviors are assayed and quantified at Days 7 and 12 of  
128 neurodevelopment/exposure (Zhang et al., 2019a). Regeneration occurs on similar time scales for  
129 the three species, allowing comparisons to be made using the same time points.

130 The use of solvents is often necessary for chemical testing, particularly for aqueous  
131 solutions; thus, it is important to assess the potential toxicity of relevant solvent concentrations to  
132 ensure this does not interfere with assessment of test chemicals. Therefore, we assayed 4 common  
133 solvents (dimethyl sulfoxide (DMSO), ethanol, methanol, ethyl acetate) and a negative control  
134 (sorbitol) at concentrations previously determined to be sublethal in *D. japonica* (Hagstrom et al.,  
135 2015; Zhang et al., 2019a).

136



137           Unexpectedly, we found that under these HTS test conditions, *S. mediterranea* and *G.*  
138 *tigrina* exhibited limited motility, hindering our ability to evaluate meaningful morphological and  
139 behavioral defects in these species. In addition, these species tended to be more sensitive to solvent  
140 toxicity than *D. japonica*. For example, significant lethality was observed in methanol in *S.*  
141 *mediterranea* and *G. tigrina*, but only behavioral defects were found in *D. japonica* at the same  
142 concentrations. Together, our data show that *D. japonica* performs the best under the experimental  
143 constraints required for HTS and thus is the species of choice for planarian HTS.

144

## 145 **Material and Methods**

### 146 ***Specimen:***

147           Asexual *D. japonica*, *G. tigrina*, and *S. mediterranea* freshwater planarians were cultivated  
148 using standard protocols. *D. japonica* and *S. mediterranea* planarians were from established lab  
149 cultures. *G. tigrina* planarians were purchased from Ward's Science (Rochester, NY, USA) and  
150 thus is it unknown how long this population has been reared under laboratory conditions. *S.*  
151 *mediterranea* were kept in 1X Montjuïc salts (Cebrià and Newmark, 2005). *D. japonica* and *G.*  
152 *tigrina* were stored in dilute (0.5 g/L) Instant Ocean Salts (IO) (Spectrum Brands, Blacksburg, VA,  
153 USA). For simplicity, “planarian water” will refer to the respective water used for each species.  
154 Planarians were stored in tupperware containers at 20°C in a temperature-controlled Panasonic  
155 incubator in the dark when not used for experiments. The animals were fed organic chicken or beef  
156 liver 1-2 times per week and cleaned twice per week (Dunkel et al., 2011). Liver was purchased  
157 frozen from a local farm, thawed, cut into small pieces and aliquoted. Aliquots were stored at -20  
158 °C for up to 6 months before use. For experiments, we randomly selected similarly sized, intact

159 planarians that were starved 5-7 days prior to experiment onset. On Day 1, selected specimens  
160 were amputated pre-pharyngeally via an ethanol-sterilized razor blade.

161

### 162 ***Chemical Preparation:***

163 Table 1 summarizes the details on the chemicals and concentrations that were used. The  
164 highest tested concentration of each solvent was chosen to be sublethal to *D. japonica*, as  
165 determined from previous experiments (Hagstrom et al., 2015) and by preliminary testing. Stocks  
166 of all chemicals were prepared in IO water at 10x of the highest tested concentration. Experimental  
167 concentrations were made using 2-fold serial dilutions in IO water. D-sorbitol (D-glucitol) served  
168 as a negative control (Zhang et al., 2018) and was prepared using serial half-log dilutions in IO  
169 water. All dilutions were made and used fresh on Day 1 of the assay.

170

171 **Table 1.** Tested solvents and their experimental concentrations.

172

Chemical Name	CAS#	Supplier	Purity (%)	Tested Concentrations
Ethanol	64-17-5	Greenfield Global	(ACS): 99.98%, (USP): 99.99%.	1, 0.5, 0.1, 0.05, 0.01 (% v/v)
Methanol	67-56-1	Sigma Aldrich	99.9	3.2, 1.6, 0.8, 0.4, 0.2 (% v/v)
DMSO	67-68-5	Sigma Aldrich	99.9	1, 0.5, 0.1, 0.05, 0.01 (% v/v)
Ethyl Acetate	141-78-6	Sigma Aldrich	99.8	0.04, 0.02, 0.01, 0.005, 0.0025 (% v/v)
D-sorbitol	50-70-4	Sigma Aldrich	99	100, 31.6, 10, 3, 1 ( $\mu$ M)

173

174 ***Exposure set-up:***

175 For every chemical concentration and planarian species, 3 technical replicates of n=8 (n=24  
176 in total) developing/regenerating planarians were assayed in independent screening plates, using  
177 independent chemical preparations. Screening plates were prepared as described in (Zhang et al.,  
178 2019a). In brief, on Day 1 of the screen, planarians were decapitated, and their tails were randomly  
179 placed in separate wells of a 48-well plate (Genesee Scientific, San Diego, CA) (1 worm/well)  
180 containing 180  $\mu$ l of planarian water. 20  $\mu$ l of 10x stocks of the respective chemicals or the vehicle  
181 control were added to the screening plates within 3 hours following amputation. For each chemical  
182 and replicate, 1 screening plate was prepared such that the 5 test concentrations and 1 vehicle  
183 control (planarian water) were contained within the plate (one condition per row). The  
184 concentration pattern in each plate was shifted down 2 rows in each replicate to control for edge  
185 effects (Zhang et al., 2019a). Plates were sealed with ThermalSeal RTS sealing film (Excel  
186 Scientific, Victorville, CA) and stored in the dark at room temperature for the duration of screening  
187 (12 days). The plates were moved to the screening platform only when screened on Day 7 and Day  
188 12. Chemical solutions were not replaced over the course of the screening period.

189

#### 190 ***Planarian motility experiments:***

191 To test why *G. tigrina* and *S. mediterranea* planarians showed limited motility under the  
192 HTS screening conditions, we set up 48-well plates as described above using regenerating or intact  
193 planarians of each species. For regenerating planarian tests, we first screened the initial intact  
194 worms in a 48-well plate within 30 min of plate setup. The planarians were then amputated as  
195 described above, allowed to regenerate in petri dishes, and screened in 48-well plates again on  
196 Days 7 and 12. For intact planarian tests, the intact planarians were first confirmed to show normal  
197 locomotion under white light conditions in a petri dish. The planarians were then loaded into 48-

198 well plates, which were sealed as described above. The 48-well plates were screened within 30  
199 min of plate setup and again on Day 7 and Day 12. In addition, we tested the behavior of *S.*  
200 *mediterranea* planarians under different lighting conditions. Specifically, we compared their  
201 locomotion and thermotaxis behavior under red light conditions, as used in our HTS setup, with  
202 those under white light conditions, by laterally adding white light illumination to the assay station.  
203 For *G. tigrina* low throughput thermotaxis experiments, we used a custom peltier to assay 6 wells  
204 of a 6-well plate simultaneously (3 planarians/well). Ambient red lighting from an  
205 electroluminescence strip was used. Wells were filled with 3 ml IO water/well. Three wells  
206 contained *D. japonica* planarians (as experimental controls for the gradient) and 3 wells *G. tigrina*.  
207 Plate loading was rotated between triplicate experiments to account for any variability in gradient  
208 strength across the peltier. Plates were recorded for 2 min without and then 4 min with the gradient  
209 on.

210

### 211 ***Screening platform:***

212 Our custom screening platform consists of a commercial robotic microplate handler  
213 (Hudson Robotics, Springfield Township, NJ), two custom-built imaging systems, and multiple  
214 assay stations as described in detail in (Zhang et al., 2019a). The imaging systems, assay stations,  
215 and plate handler were controlled by a computer. Image analysis was performed using custom  
216 MATLAB or Python scripts. In addition to the assays performed in (Zhang et al., 2019a), we  
217 have expanded the platform in the following ways (described in detail below): 1) modification of  
218 the phototaxis assay to increase the resting period before the blue light stimulus, 2) modification  
219 of the scrunching assay to capture differences in the timing of reaction, and 3) addition of an

220 automated “stickiness” assay. Moreover, analysis of the morphology/regeneration assay was  
221 expanded to also detect body shape changes.

222  
223 First, the timing of the phototaxis assay was modified to increase the resting time in the red  
224 light (dark cycle) to 2 minutes before a 1 min blue light stimulation (light cycle), though only the  
225 activity in the last minute in the dark cycle was analyzed. The increased time in the dark cycle  
226 allowed the planarians to acclimate and settle before the blue light stimulus. The phototactic  
227 response was quantified by calculating the difference of the average speed in the blue light cycle  
228 to that in the preceding 1 min of the dark cycle (Zhang et al., 2019a). Dead planarians were  
229 discarded from the analysis.

230  
231 Second, the scrunching assay was modified to allow for a dynamic analysis of noxious heat  
232 sensing as we previously found that some chemicals interfere with the rate of reaction to noxious  
233 heat (Hagstrom et al., 2018). Thus, the rate of heating of the peltier was modified to allow for a  
234 more gradual ramping up in temperature. In addition to the binary scoring of scrunching, two new  
235 endpoints were added to this assay to evaluate 1) the rate of reaction and 2) the strength of reaction  
236 to the noxious heat. Similar to (Hagstrom et al., 2018), the center of mass (COM) of each planarian  
237 was tracked over the course of the experiment and the displacement (scaled by body length) of  
238 each worm across 6 second intervals was calculated in MATLAB. The mean displacement for  
239 every 30 second bin was then calculated. We previously found that under similar, low-throughput  
240 conditions, wild-type *D. japonica* exposed to noxious heat exhibit frequent turns and decreased  
241 movement followed by eventual paralysis (Hagstrom et al., 2018). Thus, the assay was separated  
242 into phases: 1) the initial dynamic reaction and 2) the persistent decreased movement once the

243 reaction stabilized (Supplementary Figure 1). During the initial reaction phase, the mean  
244 displacement of control *D. japonica* planarians generally decreases over time. Therefore, the rate  
245 of reaction was quantified as the slope of mean displacement for the first 2.5 minutes of the assay,  
246 which is typically negative for control *D. japonica*. Of note, quantification of this endpoint  
247 required that the planarian moved within at least three (of the five total) 30 second intervals in the  
248 first 2.5 minutes of the assay. During the second half of the assay, *D. japonica* planarians tend to  
249 become mostly immobile but may still move their heads or wiggle in place, resulting in small  
250 displacements. Therefore, the strength of the reaction was quantified as the mean of mean  
251 displacement during minutes 3-5 of the assay (Supplementary Figure 1).

252

253 Third, we introduced a new assay, which we named the “stickiness assay” since it  
254 quantifies the worm’s tendency to stick/adhere to the substrate. This new assay is a high-  
255 throughput implementation of a previous low-throughput endpoint which we have shown is  
256 correlated with mucus production (Hagstrom et al., 2018; Malinowski et al., 2017). A microplate  
257 orbital shaker (Big Bear Automation, Santa Clara, CA) was used to shake the screening plates and  
258 thus create controlled water flow within each well to unstick the planarians from the bottom of the  
259 plate well. Different rotation speeds for regenerating planarians at Day 7 and 12 were chosen based  
260 on preliminary testing to achieve a reproducible majority fraction of wild-type *D. japonica*  
261 planarians to unstick. This intermediate unsticking capacity was chosen to be able to detect both  
262 an increase or decrease in planarian “stickiness”. Day 7 was observed as the relatively stickiest  
263 time-point, potentially due to locally increased secretion of mucus because the worms are less  
264 motile during regeneration. At Day 7, the plates were shaken for 3 seconds at 1017 revolutions per  
265 minute (rpm), whereas at Day 12, the plates were shaken for 3 seconds at 665 rpm. The plate was

266 imaged from above by a USB3 camera (FLIR Systems Inc., Wilsonville, OR) mounted on a ring  
267 stand and imaged at 8 frames per second (fps).

268

269 Each worm was scored as either “unstuck” (defined as being displaced by the water flow  
270 and floating in the well) or “stuck” (defined as worms which did not float during the whole plate  
271 shaking session) using a custom script written in Python using functions from the Scikit-Image  
272 (Van Der Walt et al., 2014) library. The script analyzes a series of 50 frames of a plate, with  
273 approximately the first 30 frames showing the plate shaking and applies four major steps. First,  
274 the plate is cropped, registered, and segmented into 48 wells for each frame. Li thresholding (Li  
275 and Tam, 1998) is used to segment the plate from the background. For each well, candidate worms  
276 are identified across each shaking frame by removing the lightest 80 percent of pixels in each well  
277 (since the worms are the darkest object) and using three successive rounds of Otsu segmentation  
278 (Otsu, 1979). A morphological opening is applied to join close objects and a morphological closing  
279 is applied to remove small objects. The area and COM of each detected object is calculated. For  
280 the first frame of the shaking well, the largest object is identified as the worm; for each following  
281 frame, the nearest segmented object is the worm. Third, the total movement of the worm is  
282 measured by summing the distance between the weighted COM of the identified worm in each  
283 frame. Weights are determined by the ratio of the areas of the identified worms in adjacent frames,  
284 accounting for uncertainty by down-weighting movements where the frames have disagreements  
285 about the size of the worm. Fourth, stickiness is classified based on the tracked movements. For  
286 wells where a worm is detected, the well is marked as “stuck” when there is a mean of fewer than  
287 five units of movement of COM per frame or marked as “unstuck” when there are greater than five  
288 units of movement per frame. Wells for which a worm is never detected are marked as uncertain.

289 In wells with multiple worms detected, as a result of the planarians undergoing fission during the  
290 screen duration, the stickiness of the larger worm is detected. Parameters for this script were  
291 determined using an independent test set of images of *D. japonica* planarians.

292  
293 As a quality control check of this new methodology, we quantified the accuracy of the  
294 automated analysis by comparing to manual scoring (Supplementary Figure 2). Worms which were  
295 dead, were not visible by eye, or which were flagged as “unsure” by the automated analysis were  
296 excluded from the accuracy calculations. For *D. japonica* planarians, the automated analysis had  
297 an average accuracy of 84 and 88 % for Day 7 and 12, respectively. The automated analysis had  
298 slightly reduced accuracy for *S. mediterranea* and *G. tigrina*, ranging from 68-77% for the two  
299 days, due to an underestimation of stickiness. Thus, while there is room for improvement, the  
300 automated analysis works reasonably well for classifying stickiness in *D. japonica*.

301  
302 Additionally, in the morphology assay, different body shapes were classified for each alive  
303 planarian, including normal body shape, general sickness (lesions, loss of pigment, head  
304 regression), contraction, curled up or C-shape, corkscrew-like, and pharynx extrusion. Of note,  
305 one planarian could be classified as having multiple body shapes, for example, C-shape and  
306 pharynx extrusion.

307  
308 All assays were performed in the following order, whereby the notation in brackets  
309 indicates on which day(s) the assay was performed: phototaxis (D7/D12), unstimulated locomotion  
310 (D7/D12), stickiness (D7/D12), lethality/fission/morphology (D7/D12), eye regeneration (D7),  
311 thermotaxis (D7/D12), and scrunching (D12). Any data analysis which had to be cross-checked



312 manually was performed blinded by a single investigator, who was not given the chemical identity  
313 of the plates.

314

### 315 *Statistical Analysis:*

316 Statistical testing was performed on compiled data from the triplicate runs. For all  
317 endpoints, comparisons were made between the test population and the internal set of controls for  
318 that chemical. For lethality, eye regeneration, body shape morphology, stickiness, and scrunching  
319 endpoints, a one-tailed Fisher's exact test was used. For thermotaxis, phototaxis, noxious heat  
320 sensing, and unstimulated behavioral endpoints, Tukey's interquartile test was first used to remove  
321 any outliers, with at most 5% of the data removed. A non-parametric one-tailed Mann Whitney U-  
322 test was used to determine significant effects in thermotaxis. For unstimulated behavior endpoints  
323 (speed and fraction of time resting) and noxious heat endpoints (rate and strength of reaction),  
324 Lilliefors test was first used to test the normality of the samples. Thus, we performed either a  
325 parametric two-tailed t-test or a nonparametric two-tailed Mann-Whitney U-test depending on  
326 whether the sample distributions were normal or not, respectively.

327 Statistical significance was determined as instances where the p-value was less than 0.05.  
328 When a single plate in the triplicates was responsible for designating a "hit," the triplicate was  
329 considered inconsistent and excluded as a hit. The lowest observed effect level was determined as  
330 the lowest concentration designated as a statistically significant hit. All data are available upon  
331 request.

332

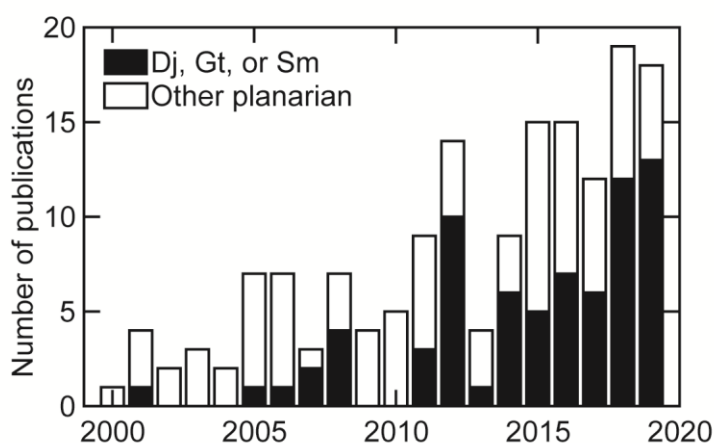
333

## 334 **Results**

### 335 *Need for standardization of planarian species used in toxicological studies*

336 The use of freshwater planarians in toxicological studies has been increasing in recent years  
337 from only a handful of papers published annually prior to 2000 to approximately 20 papers  
338 published annually in recent years (Wu and Li, 2018). Three planarian species (*D. japonica*, *S.*  
339 *mediterranea* and *G. tigrina*) have emerged as the most popular planarian models used for  
340 toxicological studies (Figure 1), because they are widely available and have published genomes  
341 (Grohme et al., 2018; Robb et al., 2008; Rozanski et al., 2019) or transcriptomes (Rozanski et al.,  
342 2019; Wheeler et al., 2015). In addition, stereotypical behaviors in these species have been  
343 characterized and employed as readouts for neuronal function, albeit to differing extents and with  
344 differing levels of throughput (Supplementary Table 1).

345



346

### 347 **Figure 1. Number of journal articles reporting toxicological effects on planarian species**

348 **over time.** Literature search was conducted using PubMed with the following keywords:

349 (((planarian OR flatworm) NOT marine NOT parasitic NOT Schistosoma) AND (toxic) NOT  
350 review) and (((Dugesia japonica) OR (Schmidtea mediterranea) OR ((Girardia OR Dugesia  
351 tigrina)) AND (toxic) NOT review).

352

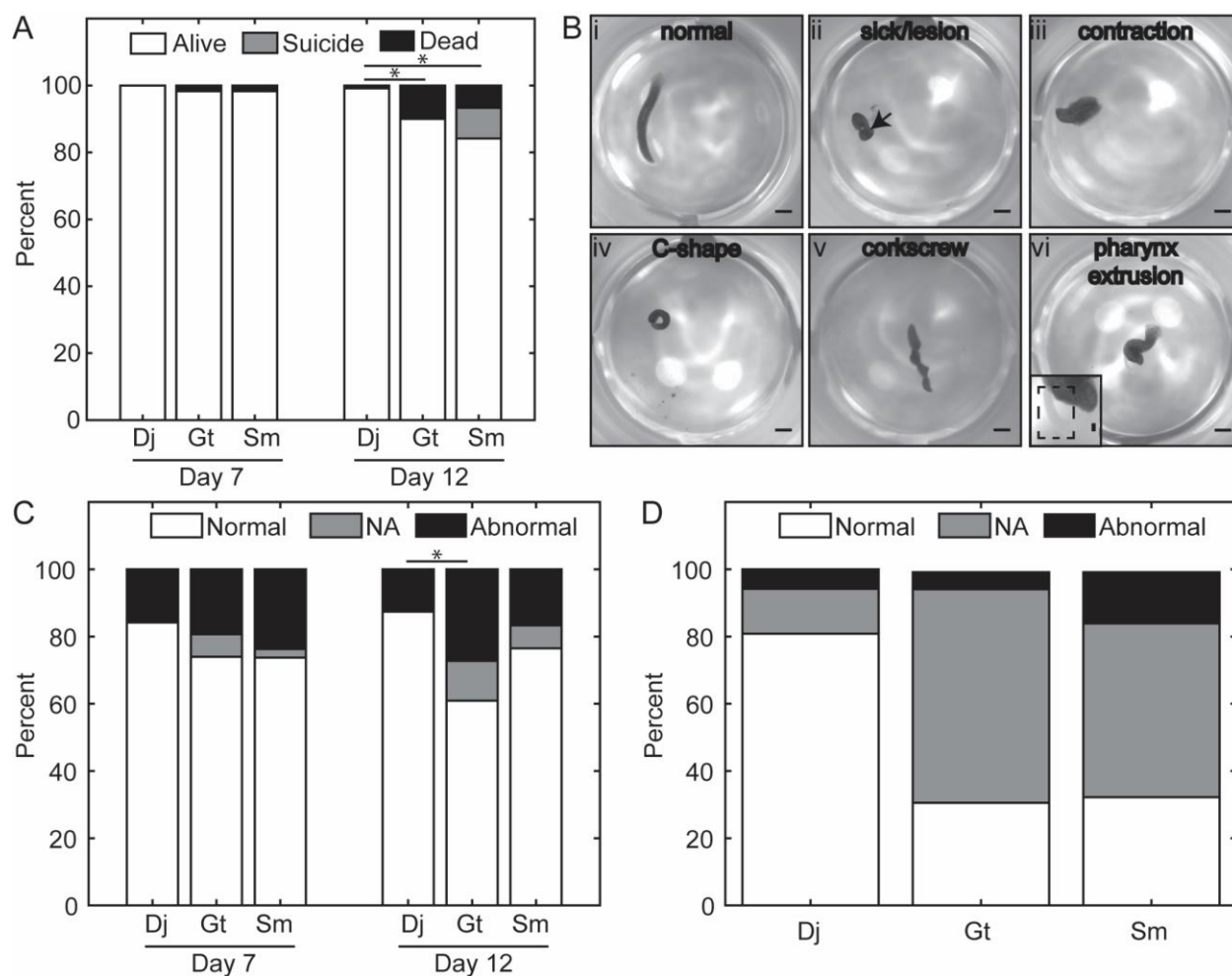
353 To directly compare the performance of these 3 popular species, we screened for potential  
354 morphological and behavioral effects of 4 common solvents (DMSO, ethanol, methanol, ethyl  
355 acetate) and 1 negative control (sorbitol) on regenerating *D. japonica*, *S. mediterranea*, and *G.*  
356 *tigrina* planarians using a robotic screening platform (Zhang et al., 2019a). We evaluated 1) the  
357 suitability of each planarian species for HTS by analyzing their performance in automated assays  
358 and 2) the sensitivity of each species to solvents commonly used in toxicology. For simplicity, we  
359 first report on the overall performance of each species, using data from control populations in the  
360 different morphological and behavioral assays, as this performance directly impacts our ability to  
361 assess chemical sensitivity in the different species.

362

### 363 ***Lethality, body shape, and eye regeneration***

364 As in previous screens (Zhang et al., 2019a, 2019b), control *D. japonica* exhibited very  
365 little background lethality. In contrast, significant lethality was observed in both *S. mediterranea*  
366 and *G. tigrina* control populations at Day 12, with approximately 14% and 7% lethality,  
367 respectively, (p-values: 0.015 [*S. mediterranea*] and 0.0027 [*G. tigrina*] compared to *D. japonica*  
368 using Fisher's exact test) (Figure 2A). Death can also occur by "suicide" wherein planarians leave  
369 the water and subsequently dry out (Zhang et al., 2019a). We excluded suicides from our lethality  
370 statistics because the mechanism causing death is different. A significant number of suicides (9%)  
371 were observed in *S. mediterranea* control planarians but were not observed in the control  
372 populations of the other two species.

373



374  
 375 **Figure 2. Lethality and morphology are compromised in *S. mediterranea* and *G. tigrina***  
 376 **controls.** A) Percentage of control planarians which were alive, dead, or committed "suicide" for  
 377 each species on Days 7 and 12. n=120. \* indicates statistical significance with p-values < 0.05 as  
 378 determined by a Fisher's exact test. B) Examples of normal and abnormal body shapes. i) Normal  
 379 planarian, ii) sick, iii) contracted, iv) curled or C-shape, v) corkscrew, vi) pharynx extrusion.  
 380 Arrow points to a lesion. Inset shows pharynx protruding outside the planarian body. Scale: 1 mm.  
 381 C) Percentage of alive control planarians demonstrating abnormal or normal body shapes in each  
 382 species at Days 7 and 12. NA indicates the planarians could not be analyzed. \* indicates statistical  
 383 significance with p-values < 0.05 as determined by a Fisher's exact test. D) Percentage of alive

384 control planarians in each species which showed normal (2 eyes) or abnormal (0 or 1 eye) eye  
385 regeneration at Day 7. NA indicates the planarians could not be analyzed.

386  
387 Planarians can exhibit a variety of abnormal morphologies and body shapes, including  
388 signs of general sickness (e.g. lesions, loss of pigment, or head regression), contraction, being  
389 curled up or C-shape, corkscrew-like, and displaying pharynx extrusion (Figure 2B). Some body  
390 shapes have been associated with disturbances to specific neurotransmitter systems (Buttarelli et  
391 al., 2008; Passarelli et al., 1999), making body shape a potentially sensitive readout for  
392 neurotoxicity. In all three species, some abnormal body shapes were observed in control  
393 populations (Figure 2C). Generally, more abnormalities were observed at Day 7 than Day 12. At  
394 both Day 7 and Day 12, the most prominent abnormal body shape in all species was contraction.  
395 At Day 7, approximately 16% of *D. japonica* controls exhibited some abnormal body shape,  
396 whereas in *S. mediterranea* and *G. tigrina* abnormal body shapes were found in 24% and 19% of  
397 controls, respectively, though these differences were not statistically significant. Only control *G.*  
398 *tigrina* showed greater abnormal body shapes at Day 12 than at Day 7, which was significantly  
399 greater than *D. japonica* at Day 12 (p-value < 0.001).

400  
401 Analysis of lethality and body shape in *S. mediterranea* and *G. tigrina* was hindered by the  
402 fact that many of these planarians were sitting on or at the well edge for the entire morphology  
403 assay. For many of these, assessments of lethality and body shape had to be manually cross-  
404 checked by observing the animals in the other assays, which is not a viable approach for HTS.  
405 Particularly for body shape, even manual cross-checks were insufficient to determine the  
406 morphology of some planarians. In these cases, the planarians were scored as “NA” for not

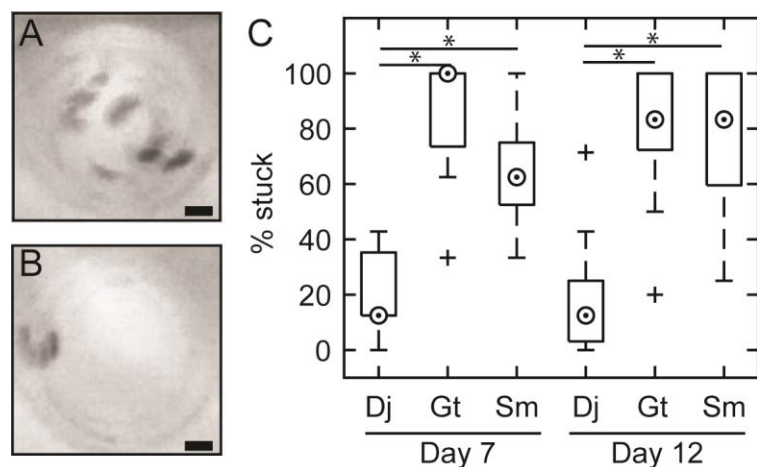
407 analyzable and were excluded from further analysis (Figure 2C). Eye regeneration was also  
408 assessed through high resolution imaging of individual wells to discern whether the planarian has  
409 regained both eyes (normal condition) or not (abnormal) (Zhang et al., 2019a). However, due to  
410 the limited visibility of many of the *S. mediterranea* and *G. tigrina* planarians in this assay, it was  
411 impossible to assess eye regeneration status in many of the control worms (Figure 2D). This led to  
412 very low sample sizes (Supplementary Table 2), decreasing the statistical power of this assay for  
413 these species.

414

415 ***Stickiness:***

416 Normal planarian locomotion relies on cilia beating in a layer of secreted mucus (Martin,  
417 1978; Rompolas et al., 2010). We previously found that increased mucus production is correlated  
418 with increased “stickiness” of the worm, which can be assessed by evaluating how easily the  
419 planarian is dislodged from its substrate, and that certain chemicals can increase planarian  
420 stickiness (Hagstrom et al., 2018; Malinowski et al., 2017). We have automated this originally  
421 low-throughput assay (Hagstrom et al., 2018; Malinowski et al., 2017), which now relies on  
422 shaking of the screening plate to create controlled water flow with the potential to unstick the  
423 planarian from the bottom of the well (Figure 3A-B).

424



425

426 **Figure 3. Overview of stickiness assay.** (A-B) Minimum intensity projections of the shaking  
427 phase of the stickiness assay showing an (A) unstuck or (B) stuck planarian. Scale bars: 2 mm.

428 C) Boxplot of the percent control planarians stuck in each replicate plate (n=8 per data point,  
429 n=15 data points per condition) as determined using manual analysis. Medians are shown as a  
430 dot in a circle, outliers are shown as crosses. \* indicates statistical significance with p-values <  
431 0.05 using the Mann-Whitney U-Test.

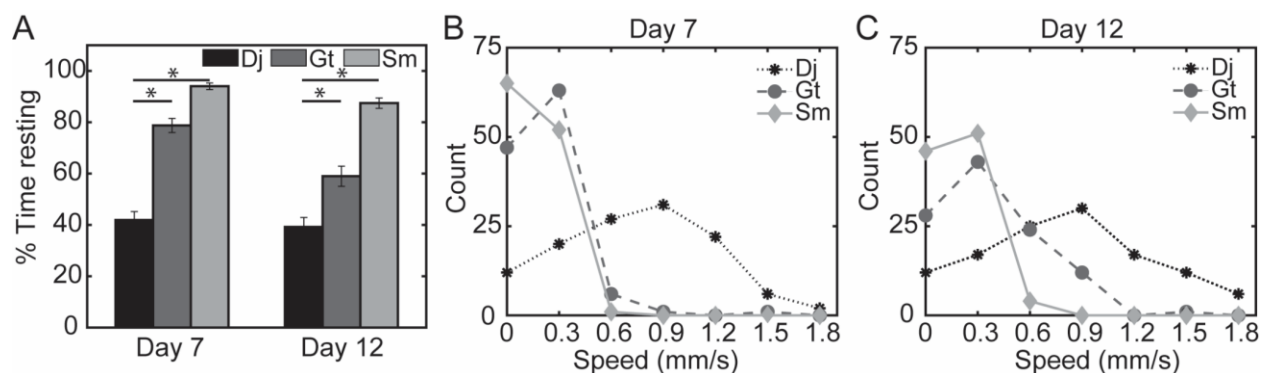
432

433 The shaking parameters were defined such that a reproducible majority of control *D.*  
434 *japonica* planarians would be unstuck, allowing for identification of conditions that caused either  
435 increased or decreased stickiness. We found that the other two species were significantly stickier  
436 than *D. japonica*, as the majority of controls in both *S. mediterranea* and *G. tigrina* were still stuck  
437 after shaking, and exhibited larger plate-to-plate variability (p-values:  $1.1 \times 10^{-5}$ ,  $2.6 \times 10^{-5}$  [*S.*  
438 *mediterranea* Day 7 and 12] and  $4.9 \times 10^{-6}$ ,  $1.5 \times 10^{-5}$  [*G. tigrina* Day 7 and 12] compared to *D.*  
439 *japonica* using Fisher's exact test) (Figure 3C).

440

441 *S. mediterranea* and *G. tigrina* show decreased motility under HTS conditions:

442 Next, we assayed the planarians' unstimulated locomotion by quantifying speed and the  
443 fraction of time resting. Unexpectedly, we found that both *S. mediterranea* and *G. tigrina* controls  
444 had significantly decreased motility evidenced by the large fraction of time spent resting (p-values:  
445  $5.8 \times 10^{-31}$ ,  $2.1 \times 10^{-23}$  [*S. mediterranea* Day 7 and 12] and  $1.9 \times 10^{-15}$ ,  $3.0 \times 10^{-4}$  [*G. tigrina* Day 7  
446 and 12] compared to *D. japonica* using a two-tailed student's t-test), (Figure 4A). Because gliding  
447 speed is only calculated for animals that glide for at least 10 continuous frames (out of 900 total),  
448 this large amount of resting greatly reduced the sample size for this endpoint (Supplementary Table  
449 2). This effect was more pronounced in *S. mediterranea* and in Day 7 for both *S. mediterranea* and  
450 *G. tigrina*. Moreover, even when the control planarians of these two species did glide, the speed  
451 was significantly less than seen in *D. japonica* (Figure 4B-C) (p-values:  $2.0 \times 10^{-28}$ ,  $6.2 \times 10^{-23}$  [*S.*  
452 *mediterranea* Day 7 and 12] and  $3.5 \times 10^{-22}$ ,  $3.5 \times 10^{-12}$  [*G. tigrina* Day 7 and 12]). In *S.*  
453 *mediterranea*, control gliding speeds were marginally greater than the resting speed cutoff of 0.3  
454 mm/s. This value is extremely reduced compared to published *S. mediterranea* mean speeds of  
455 1.62 mm/s (Talbot and Schötz, 2011) emphasizing the extreme lack of movement seen in these  
456 worms under these conditions.  
457



458  
459 **Figure 4. *S. mediterranea* and *G. tigrina* barely move during the locomotion assay. A)** Average  
460 percent time spent resting in controls of different species on Days 7 and 12 during the unstimulated



461 behavior assay. Error bars indicate  $\pm$  SE. \* indicates statistical significance with p-values  $< 0.05$   
462 as determined by a student's t-test. B-C) Distribution of speeds of controls from different species  
463 at B) Day 7 or C) Day 12. For visualization, planarians which were resting for the entire assay  
464 were set to speeds of 0 mm/s.

465  
466 To understand the reason for this difference in motility, we performed tests on intact *S.*  
467 *mediterranea*. Despite moving normally in a petri dish (Supplementary Figure 3A), these intact  
468 planarians exhibited reduced motility in the screening platform even when placed in 48-well plates  
469 within a few hours of petri dish testing. This motility defect was rescued if the planarians were  
470 imaged under bright white light (Supplementary Figure 3B), suggesting that the reason for *S.*  
471 *mediterranea* not to move in our assays on the screening platform was because the red lighting  
472 used for imaging is a wavelength that *S. mediterranea* are insensitive to (Paskin et al., 2014).

473  
474 We also performed additional tests on *G. tigrina* planarians to investigate why their  
475 movement was reduced compared to *D. japonica*. Based on our previous data on population  
476 growth (Carter et al., 2015), we hypothesized that the small volume confinement of the 48-well  
477 plate may cause general health issues and increased immobility. To test this, we analyzed the  
478 performance of *G. tigrina* regenerating tails which were not stored in the 48-well plates but instead  
479 allowed to regenerate in petri dishes until screening. These planarians had no motility defects,  
480 whereas intact *G. tigrina* stored in sealed 48-well plates for 12 days displayed increased lethality  
481 and resting (Supplementary Figure 4). Together, these data suggest that *G. tigrina* move normally  
482 under the imaging conditions of the platform and that this population did not have general health  
483 issues but that the long-term storage conditions necessary for HTS (small volumes, sealed plate)

484 are detrimental to *G. tigrina* health, leading to increased immobility in our screen. *S. mediterranea*  
485 motility was not significantly changed regardless of developmental condition (regenerating vs  
486 intact) or storage conditions (Supplementary Figure 5).

487

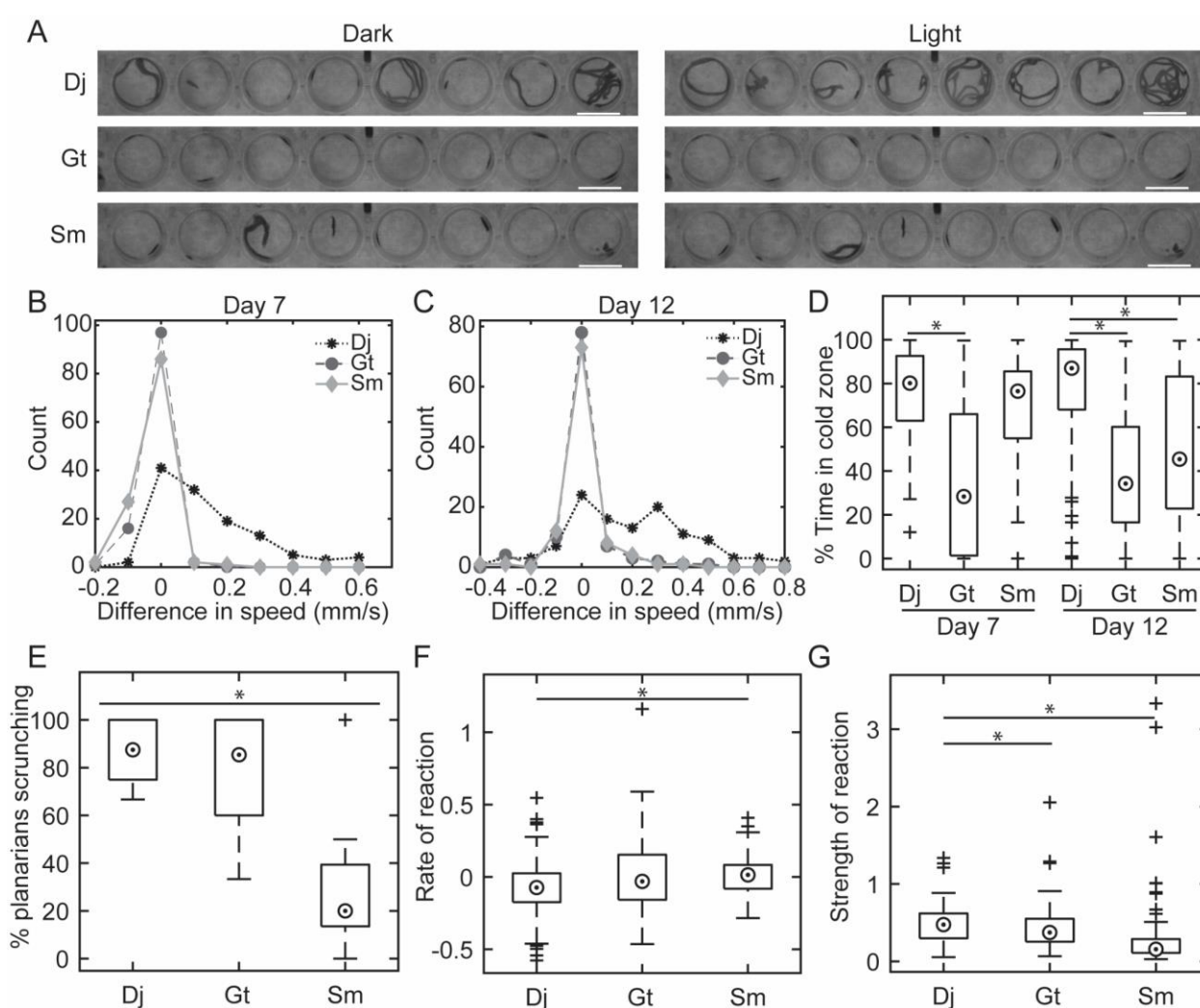
488 ***Lack of motility hinders analysis of stimulated behaviors in S. mediterranea and G. tigrina:***

489 A major advantage of the planarian system is their complex repertoire of stereotypical  
490 behaviors in response to various stimuli, including light, temperature gradients, and noxious heat  
491 (Cochet-Escartin et al., 2015; Inoue et al., 2014, 2004; Paskin et al., 2014). We have found that  
492 our methodology for automated assessment of these behaviors in *D. japonica* is robust and  
493 sensitive to detect neuronal defects induced by neurotoxicants (Hagstrom et al., 2019; Zhang et  
494 al., 2019a, 2019b). Several of these behaviors (phototaxis and scrunching) have previously been  
495 evaluated in *S. mediterranea* and *G. tigrina* (Supplementary Table 1), albeit using low-throughput  
496 assays. Therefore, we evaluated whether these species were also capable of exhibiting robust  
497 stimulated behaviors using our automated methodology. Even though we found that *S.*  
498 *mediterranea* and *G. tigrina* showed decreased motility during unstimulated locomotion, it was  
499 still possible that the various stimuli could induce movement.

500

501 Planarians are negatively phototactic. Multiple planarian species have been shown to be  
502 most sensitive to blue light while being insensitive to red light (Davidson et al., 2011; Marriott,  
503 1958; Paskin et al., 2014; Zhang et al., 2019a). Therefore, we exposed the planarians to 2 minutes  
504 of red light (dark cycle) followed by 1 minute of blue light (light cycle) and quantified the reaction  
505 as the difference in speeds between the light and dark cycles. Under these conditions, control *D.*  
506 *japonica* exhibited a robust increase in movement and speed during the light cycle, with an average

507 speed difference of approximately 0.2 mm/s (Figure 5A-C). In contrast, *S. mediterranea* and *G.*  
 508 *tigrina* control planarians exhibited much weaker reactions to the light, with average speed  
 509 differences of only approximately 0.01-0.03 mm/s. These attenuated reactions were mainly a result  
 510 of the immobility seen in these species as many of the planarians barely moved throughout the  
 511 assay, regardless of the presence of the light (Figure 5A). As a result, the sensitivity of this assay  
 512 in these species was limited.  
 513



514  
 515 **Figure 5. *S. mediterranea* and *G. tigrina* have attenuated performance in the various**  
 516 **stimulated behavior assays.** A) Minimum intensity projections showing the tracks of 8 control

517 planarians in each species during the last minute of the dark cycle (Dark) or during the 1 minute  
518 blue light cycle (Light) in the phototaxis assay. Notice *D. japonica* planarians move more during  
519 the light period while the other two species barely move in either lighting. Scale bars: 10 mm. B-  
520 C) Distribution of the difference of average speed in the light and dark cycles during the phototaxis  
521 assay for control planarians of the different species at B) Day 7 or C) Day 12. The lower bin edge  
522 is plotted. D) Boxplot of the time spent in the cold zone during thermotaxis for controls of each  
523 species at Days 7 and 12. E) Boxplot of the percentage of control planarians scrunching in each  
524 replicate plate (n=8 per data point, n=15 data points per condition). F) Boxplot of the rate of  
525 reaction to noxious heat of controls for each species. G) Boxplot of the strength of reaction to  
526 noxious heat of controls for each species. For all boxplots, medians are shown as a dot in a circle,  
527 outliers are shown as crosses. For D-G, \* indicates statistical significance with p-values < 0.05 as  
528 determined by a Mann-Whitney U-test.

529

530         Next, we evaluated how the different species performed in respect to thermotaxis. In this  
531 assay, a uniform temperature gradient is established within each well using a custom peltier setup,  
532 such that a cold region is established in each well, taking up an approximately 120 degree sector  
533 of the well (Zhang et al., 2019a). Control *D. japonica* prefer colder temperatures and thus spend  
534 the majority of time in the cold region (Figure 5D). In contrast, *G. tigrina* and *S. mediterranea* had  
535 significantly less robust preferences for the cold zone, especially at Day 12 (p-values:  $1.1 \times 10^{-12}$   
536 [*G. tigrina*],  $3.2 \times 10^{-6}$  [*S. mediterranea*] compared to *D. japonica* using a Mann-Whitney U-Test)  
537 (Figure 5D). First, because this assay only uses data from moving worms, the sample size was  
538 greatly diminished in these two species due to the general immobility mentioned previously  
539 (Supplementary Table 2). Second, when the *S. mediterranea* and *G. tigrina* planarians did move,

540 they spent less time in the cold region than *D. japonica* and had greater intraspecies variability  
541 (Figure 5D). Interestingly, *G. tigrina* reactions were similar to what would be expected from  
542 random motion across the well given that the cold sector is approximately 30% of the well area,  
543 suggesting these planarians do not react to the temperature gradient. *G. tigrina* planarians did not  
544 exhibit thermotaxis even when allowed to regenerate in petri dishes, and thus moved normally  
545 (Supplementary Figure 6A). Because we could neither induce thermotaxis in *G. tigrina* in the  
546 automated assay nor found any literature demonstrating thermotaxis in this species, we tested  
547 whether intact *G. tigrina* planarians could sense temperature gradients under low-throughput  
548 conditions using 6-well plates. In agreement with the HTS data, intact *G. tigrina* did not display  
549 thermotaxis under these conditions, while simultaneously assayed *D. japonica* planarians did.  
550 (Supplementary Figure 6B). These data suggest that *G. tigrina* do not exhibit thermotaxis in the  
551 same temperature ranges as the other two species.

552  
553         Some of the *S. mediterranea* control planarians appeared to be successfully exhibiting  
554 thermotaxis and spent a majority of time in the cold region. However, the variability in this species  
555 was substantial and appears to be caused by their lack of motility. For example, *S. mediterranea*  
556 planarians that were resting near the cold zone showed successful thermotaxis, since they were  
557 able to sense the temperature gradient and move enough to enter the cold zone. To increase  
558 motility, we also tested thermotaxis under bright white light. Imaging under these conditions was  
559 sufficient to stimulate *S. mediterranea* to move (Supplementary Figure 3B) but failed to induce  
560 successful thermotaxis in *S. mediterranea* and *D. japonica* controls (Supplementary Figure 7).  
561 This suggests the addition of a light stimulus (bright white light) masked the behavioral response

562 to the temperature gradient, in agreement with previous reports that when presented  
563 simultaneously, light is a stronger stimulus than temperature for *D. japonica* (Inoue et al., 2015).

564

565 Lastly, we evaluated the planarians' ability to react to noxious heat. We have previously  
566 demonstrated that scrunching, a musculature-driven planarian escape gait that is conserved across  
567 species (Cochet-Escartin et al., 2015), can be induced by noxious heat (Cochet-Escartin et al.,  
568 2015; Sabry et al., 2019) and is a sensitive readout of neuronal function (Zhang et al., 2019a,  
569 2019b). Scrunching was induced in approximately 88% of *D. japonica* control planarians under  
570 our experimental conditions; however, scrunching was much less prominent in the other two  
571 species (Figure 5E), with *S. mediterranea* showing a significantly lower scrunching induction rate  
572 compared to *D. japonica* (p-value <  $1.8 \times 10^{-4}$  using a Mann-Whitney U-Test ).

573

574 In addition to binary classification of whether a planarian scrunched, we captured the  
575 dynamics of the noxious heat response by quantifying: 1) the rate at which the planarians  
576 responded to the heat and 2) the strength of their final reaction. (Supplementary Figure 1 and  
577 Materials and Methods). *S. mediterranea* and *G. tigrina* controls had weaker rates of reaction to  
578 the noxious heat compared to *D. japonica*, with a significant difference for *S. mediterranea*  
579 compared to *D. japonica* (p-value < 0.008; Mann-Whitney U-test) (Figure 5F). In *S. mediterranea*  
580 and *G. tigrina*, the median rate of reaction was approximately 0 (Figure 5F). This indicates little  
581 change in displacement, which results from the general lack of motility observed in these species  
582 (i.e. since these planarians were already not moving, a decrease in motion could not be assessed).  
583 Moreover, the lack of motility in these species greatly decreased the sample size for this endpoint  
584 (Supplementary Table 2). *G. tigrina* and *S. mediterranea* control planarians also showed

585 significantly decreased “strength of reaction” scores compared to *D. japonica* (p-values: 0.03 [*G.*  
586 *tigrina*] and  $4.7 \times 10^{-14}$  [*S. mediterranea*]; Mann-Whitney U-test) (Figure 5G). These lower scores  
587 indicate these planarians were moving less than *D. japonica* during the second phase of the noxious  
588 heat assay, though this may be a result of their general lack of movement.

589

590 In summary, our data show that using our high-throughput methodology, motility and  
591 health issues in *S. mediterranea* and *G. tigrina* planarians greatly hindered our ability to assess the  
592 effects of chemical substances on morphology or behavior as even control animals demonstrated  
593 poor performance and high levels of variability.

594

#### 595 ***Toxicity of common solvents***

596 The second aim of this study was to evaluate the effect of 4 common solvents in  
597 pharmacology and toxicology (DMSO, ethanol, methanol, ethyl acetate) on the three planarian  
598 species. Sorbitol served as a negative control. However, the lack of motility of *S. mediterranea*  
599 and *G. tigrina* planarians impaired our ability to evaluate solvent toxicity for certain endpoints due  
600 to data scarcity, as explained above. This effect was the greatest with *S. mediterranea*, resulting in  
601 many endpoints which could not be adequately evaluated (marked as “indeterminate” in  
602 Supplementary Figure 8). The only endpoint we could accurately use to compare solvent toxicity  
603 across all three species was lethality (Table 2). Of note, since we were interested in studying  
604 behavioral phenotypes in the absence of overt toxicity, the test concentrations had been chosen to  
605 not cause significant lethality in *D. japonica*.

606

607 **Table 2. Lowest observed effect level for Day 12 lethality in each species.** If lethality was not  
608 observed, the concentration is listed as > X, where X is the maximum tested concentration.

Solvent	<i>D. japonica</i>	<i>G. tigrina</i>	<i>S. mediterranea</i>
DMSO	>1%	1%	>1%
Ethanol	>1%	0.05%	>1%
Methanol	>3.2%	1.6%	3.2%
Ethyl acetate	>0.04%	>0.04%	>0.04%
Sorbitol	>100 $\mu$ M	>100 $\mu$ M	>100 $\mu$ M

609  
610 Overall, *D. japonica* planarians were less sensitive to the lethal effects of these solvents  
611 than the other two species. For methanol, lethality was observed in *G. tigrina* and *S. mediterranea*  
612 but not *D. japonica* at the concentrations tested (maximum 3.2%). *G. tigrina* showed the greatest  
613 sensitivity as lethality was observed in three of the tested solvents (DMSO, ethanol and methanol).  
614 The observed species differences in sensitivity highlight that care needs to be taken when  
615 extrapolating findings of chemical exposure between planarian species.

616

## 617 **Discussion and Conclusions**

618 While existing studies have provided useful insight into how certain chemicals affect  
619 different aspects of planarian biology, the range of species and techniques used has made it difficult  
620 to compare results across different planarian studies to harmonize findings and contextualize how  
621 results in planarians relate to other species, especially humans.

622

623 Planarians and other new approach methods should have sufficiently high throughput to  
624 provide a robust, efficient alternative to existing testing methodologies. To this end, the use of  
625 multi-well plates and fully automated screening methodology is indispensable. Multi-well plates  
626 allow for the use of small testing volumes and the ability to test multiple conditions  
627 simultaneously, thus reducing chemical usage and experimental variability, respectively. We have



628 found that, for *D. japonica*, 48-well plates provide a balance between throughput, maintaining  
629 planarian health long-term, and being able to robustly induce and quantify various behaviors.  
630 Moreover, in our testing paradigm, exposures are static with the plate sealed throughout exposure  
631 to reduce agitation to the planarians, reduce the amount of chemical required, and prevent changes  
632 in chemical concentration due to volatility/evaporation. Fully automated screening methodology  
633 is critical to obtain robust, unbiased results with sufficiently high throughput. Thus, we have  
634 focused our efforts on creating automated methodologies, in both the engineering of the screening  
635 platform and in the associated image and data analysis, such as for stickiness presented here. To  
636 ensure the necessary accuracy and robustness, all new automated analyses are manually cross-  
637 checked before full implementation, allowing us to refine the analysis as necessary.

638  
639 Thus far, *D. japonica* is the only planarian species that has been successfully employed in  
640 large-scale automated screening (Zhang et al., 2019a, 2019b). Here, we have directly compared  
641 the performance of the 3 most commonly used freshwater planarian species in toxicology (*D.*  
642 *japonica*, *S. mediterranea*, and *G. tigrina*) under HTS conditions and evaluated their sensitivity to  
643 4 common solvents (DMSO, ethanol, methanol and ethyl acetate). We found that *S. mediterranea*  
644 and *G. tigrina* are ill-suited for HTS because they do not display robust behaviors under the  
645 necessary experimental conditions (Figures 4-5, Supplementary Figures 3-4).

646  
647 The reasons why the two species are not amenable to automated screening in 48-well plates  
648 differ between the two species. *S. mediterranea* exhibited limited locomotion when imaged with  
649 red light, but could be rescued using bright white light illumination (Supplementary Figure 3).  
650 This lack of motility prevented us from robustly evaluating locomotion or stimulated behaviors in

651 the automated testing platform. Red lighting conditions are necessary to properly evaluate non-  
652 phototaxis behaviors, because the planarians' response to light overrides other stimuli (Inoue et  
653 al., 2015) (Supplementary Figure 7). This lack of motility caused a major data loss for unstimulated  
654 locomotion and thermotaxis. In addition, *S. mediterranea* did not display a robust phototaxis  
655 response; it is unclear why, given that both *S. mediterranea* and *D. japonica* exhibit similar  
656 behaviors when exposed to a light gradient, though these behaviors often rely on moving  
657 planarians (Inoue et al., 2004; Paskin et al., 2014). Behavioral responses may also differ between  
658 exposure to a light gradient versus to a global light stimulus, as used here. While scrunching is one  
659 of the most sensitive readouts for assaying neurotoxicological effects in *D. japonica* (Zhang et al.,  
660 2019b, 2019a), we have been unable to robustly induce scrunching in *S. mediterranea* using a  
661 noxious heat bath here and in our previous work (Sabry et al., 2019). Together, these data suggest  
662 that *S. mediterranea* planarians are not well suited to multi-endpoint behavioral HTS. However,  
663 this species would be suitable to HTS assaying lethality, morphology and unstimulated behavior,  
664 if imaged using white light.

665  
666 In contrast, *G. tigrina* moved normally under red light conditions when tested immediately  
667 after plate setup or if allowed to regenerate in petri dishes, but were negatively impacted by the  
668 confinement and small water volumes in the 48-well plates. Thus, while general health issues were  
669 not found in this species under normal laboratory conditions, their health declined over the 12 days  
670 of confinement, causing them to stop moving and/or die (Supplementary Figure 4), greatly limiting  
671 the number of planarians that could be analyzed (Supplementary Table 1). The observed health  
672 issues in *G. tigrina* in the small test volumes are perhaps not surprising since we have previously  
673 shown that *G. tigrina* are more sensitive to environmental conditions than *D. japonica* (Carter et

674 al., 2015). Since *G. tigrina* planarians exhibit health problems during long-term storage in 48-well  
675 plates, this species is not suited for HTS of sub-chronic/chronic effects relying on small volume  
676 testing, independent of the details of the testing paradigm. Moreover, the lack of a thymotaxis  
677 response in *G. tigrina* without health or motility issues (Supplementary Figure 6) suggests this  
678 species may not have the same breadth of behaviors as *D. japonica*.

679  
680 We have recently shown that both sensitivity and behavioral phenotypes to the  
681 pharmacological and toxicological effects of certain drugs can differ among *D. japonica* and *S.*  
682 *mediterranea* planarians (Sabry et al., 2019). Similarly, we have shown here that the 3 planarian  
683 species exhibit differential sensitivity to 4 common solvents. *G. tigrina* showed the greatest  
684 sensitivity to the tested solvents, though it is possible this sensitivity was a result of the general  
685 decline in health observed in this species under long-term confinement in 48-well plates. These  
686 species differences highlight that not all planarian research should be unified under a singular  
687 planarian model and that care needs to be taken when extrapolating from one planarian species to  
688 another. Moreover, the lower sensitivity of *D. japonica* planarians to these solvents suggests that  
689 higher solvent concentrations can be used in this species compared to the other two without fear  
690 of toxicological effects, further supporting our conclusion that this species is the best suited for  
691 toxicological research.

692  
693 To be used in a regulatory context, new approach methods such as HTS in freshwater  
694 planarians must meet several “readiness criteria”, which evaluate the models’ technical  
695 capabilities, robustness, and relevancy to human health (Bal-Price et al., 2018; Crofton et al.,  
696 2011). A large aspect of this validation effort is to ensure results are reproducible across different

697 laboratories. This necessitates that methods are transparent and standardized across different  
698 research groups. Our data here emphasize this need for method harmonization among planarian  
699 toxicological research as different species and different testing conditions produced significantly  
700 different effects. Our data show that, of the 3 most common planarian species used, only *D.*  
701 *japonica* is suitable for practical HTS conditions. We have also previously shown that data  
702 obtained with this species and our testing methodology is robust and relevant to mammalian  
703 outcomes (Hagstrom et al., 2019; Zhang et al., 2019b, 2019a). By standardizing testing methods,  
704 including the species used, the planarian toxicological community can work together towards  
705 validation of this promising invertebrate model.

706

707

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