

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

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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; Knakiewicz 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 μ 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 (μ 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 μ l of planarian water. 20 μ 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

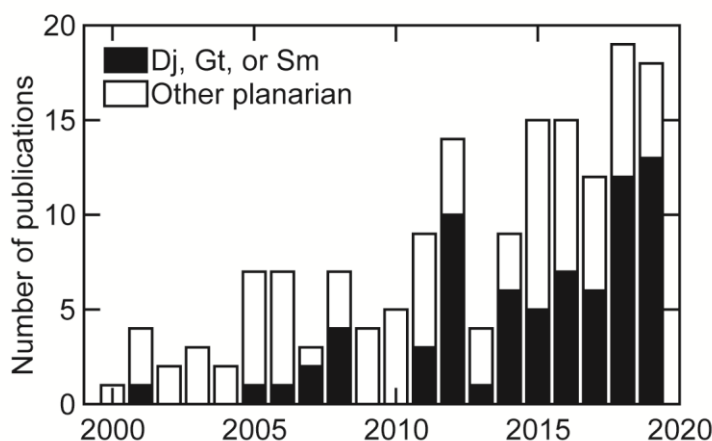
333

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

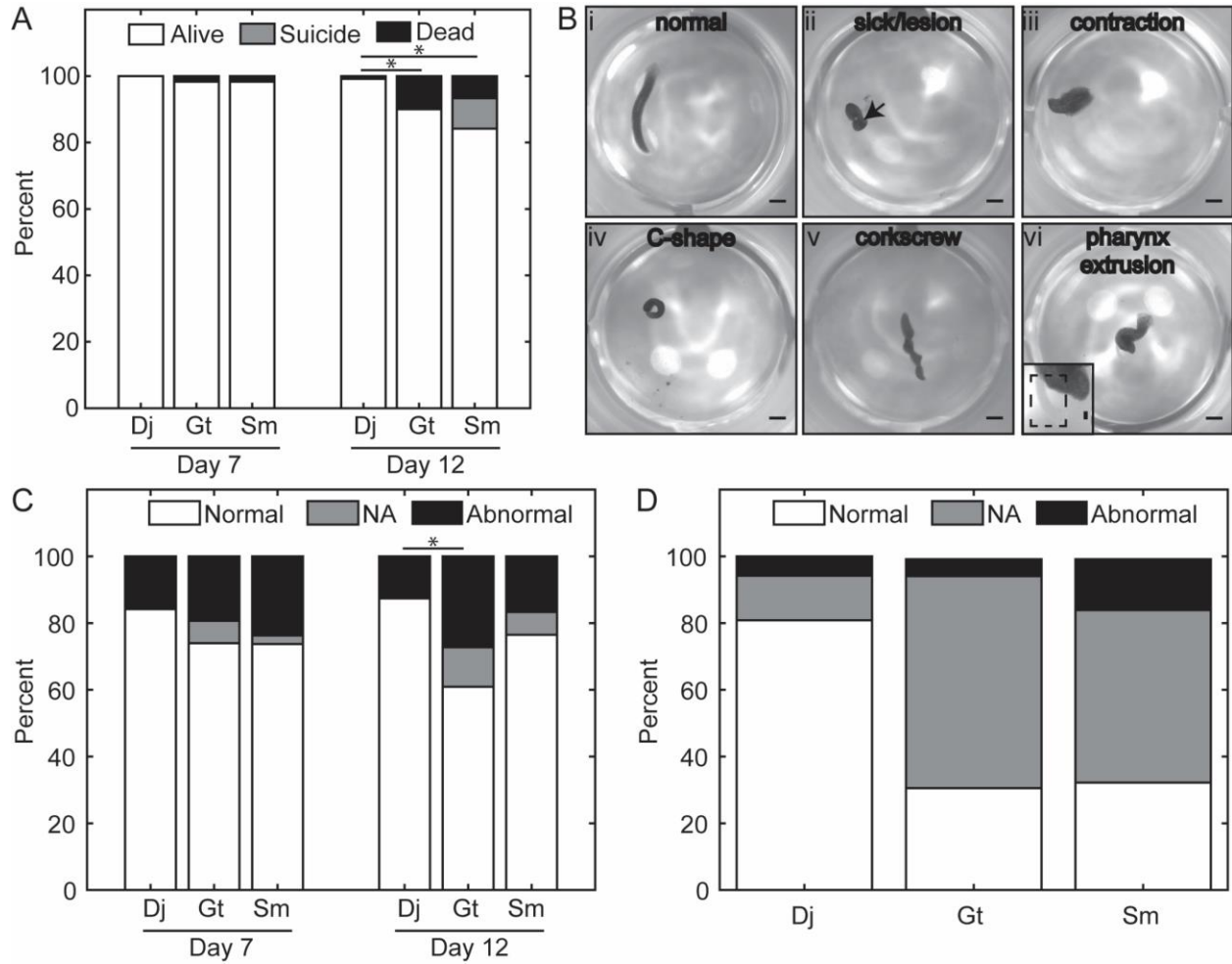
353 To directly compare the performance of these 3 popular species, we screened for potential
354 morphological and behavioral effects of 4 common solvents (DMSO, ethanol, methanol, ethyl
355 acetate) and 1 negative control (sorbitol) on regenerating *D. japonica*, *S. mediterranea*, and *G.*
356 *tigrina* 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

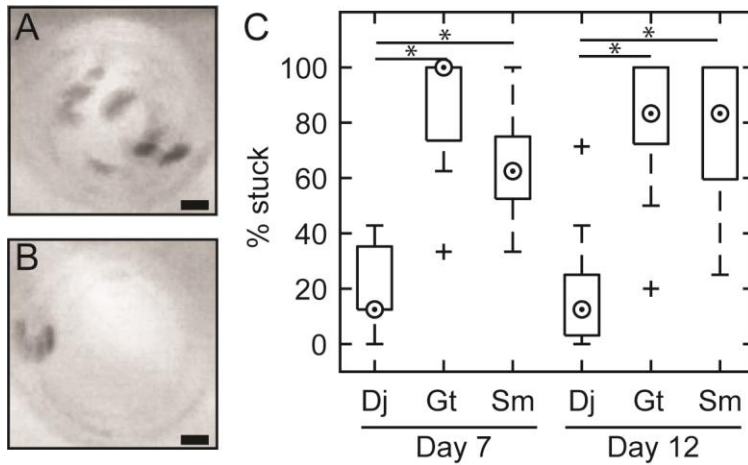
407 analyzable and were excluded from further analysis (Figure 2C). Eye regeneration was also
408 assessed through high resolution imaging of individual wells to discern whether the planarian has
409 regained both eyes (normal condition) or not (abnormal) (Zhang et al., 2019a). However, due to
410 the 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

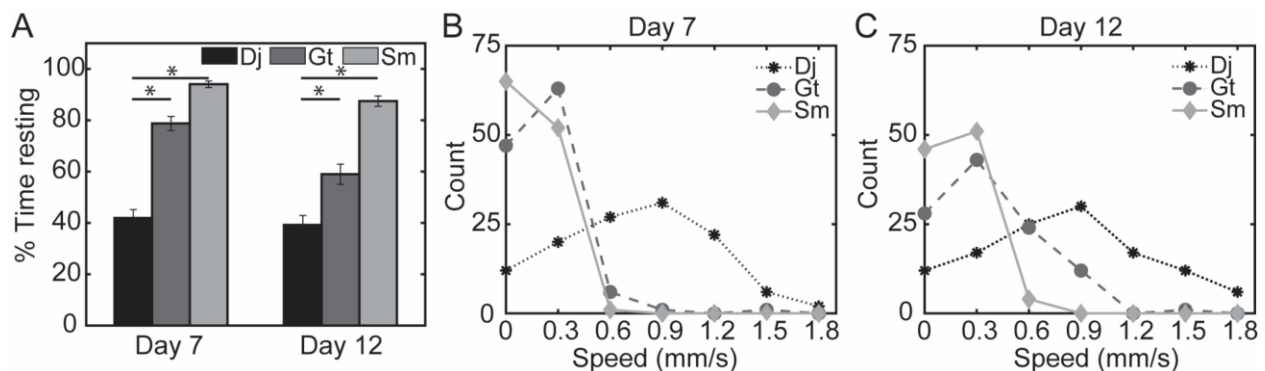
433 The shaking parameters were defined such that a reproducible majority of control *D.*
434 *japonica* planarians would be unstuck, allowing for identification of conditions that caused either
435 increased or decreased stickiness. We found that the other two species were significantly stickier
436 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: 1.1×10^{-5} , 2.6×10^{-5} [*S.*
438 *mediterranea* Day 7 and 12] and 4.9×10^{-6} , 1.5×10^{-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 5.8×10^{-31} , 2.1×10^{-23} [*S. mediterranea* Day 7 and 12] and 1.9×10^{-15} , 3.0×10^{-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: 2.0×10^{-28} , 6.2×10^{-23} [*S.*
452 *mediterranea* Day 7 and 12] and 3.5×10^{-22} , 3.5×10^{-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

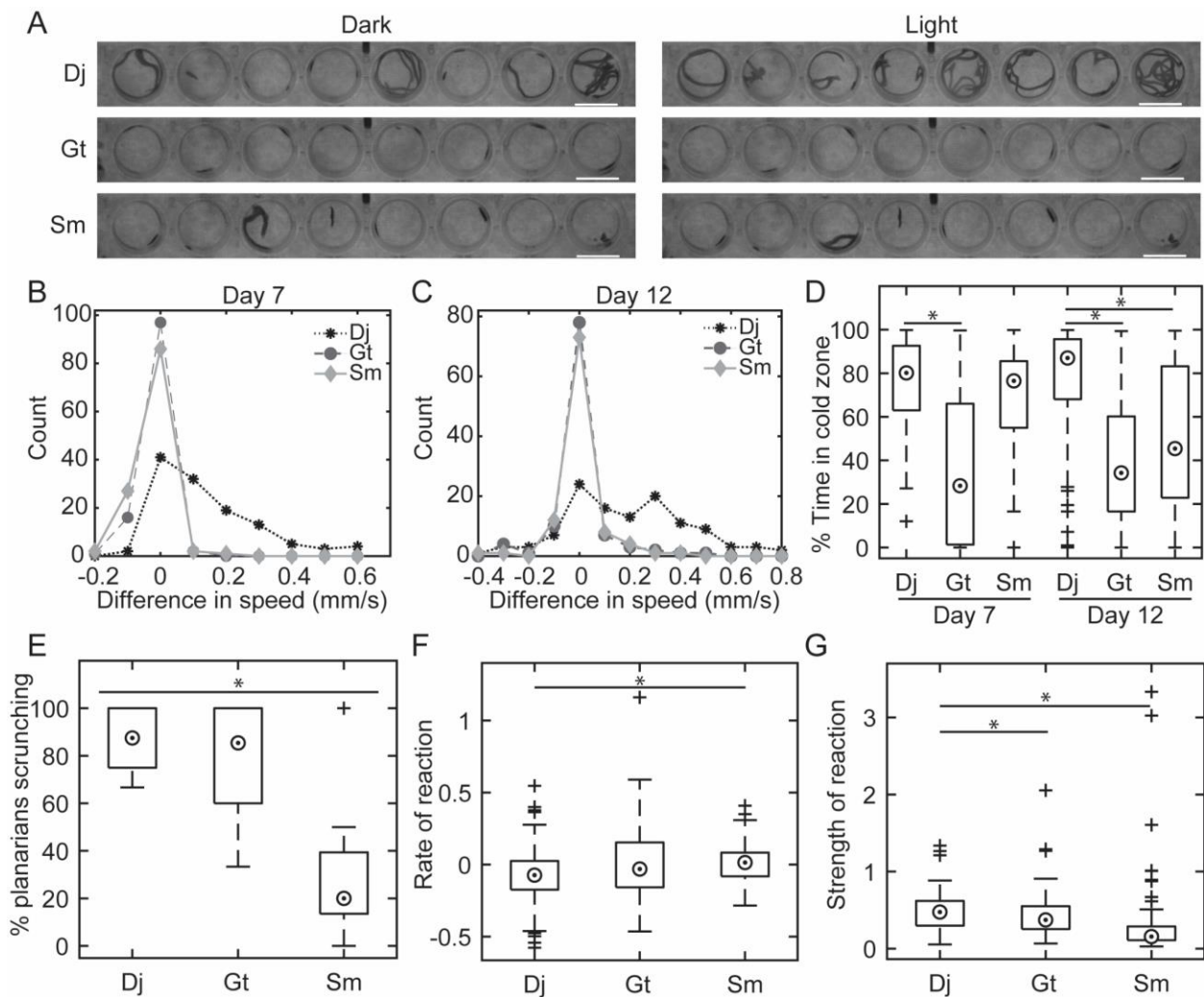
488 ***Lack of motility hinders analysis of stimulated behaviors in S. mediterranea and G. tigrina:***

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: 1.1×10^{-12}
536 [*G. tigrina*], 3.2×10^{-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 < 1.8×10^{-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 4.7×10^{-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 μ M	>100 μ M	>100 μ 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 thigmotaxis
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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716

717

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