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3 # Cryopreservation of *Hydractinia symbiolongicarpus* sperm to support community-
4 based repository development for preservation of genetic resources

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21 # Abstract

22 *Hydractinia symbiolongicarpus* is an emerging model organism in which cutting-edge genomic
23 tools and resources are being developed for use in a growing number of research fields.
24 However, one limitation of this model system is the lack of long-term storage for genetic
25 resources. Our goal in this study was to establish a generalizable approach to sperm
26 cryopreservation that would support future repository development and could be applied to
27 many species according to available resources. Our approach was to: 1) Assess sperm
28 characteristics and standardize collection and processing; 2) Assess acute toxicity to
29 cryoprotectants, and 3) Evaluate and refine freezing conditions to permit post-thaw fertilization
30 and produce viable offspring. By following this approach, we found that *Hydractinia* sperm
31 incubated in 5% DMSO, equilibrated at 4°C for 20 min, and cooled at a rate of 20°C/min to -
32 80°C at a cell concentration of 10^8-10^9/mL in 0.25-mL aliquots were able to fertilize 150-300
33 eggs which yielded offspring that could metamorphose into juvenile polyps. In addition,
34 improvements were made for processing sperm using a customized 3-D printed collection
35 system. Other opportunities for improvement include optimizing the volumetric sperm-to-egg
36 ratio for fertilization. Establishing repository capabilities for the *Hydractinia* research
37 community will be essential for future development, maintenance, protection, and distribution
38 of genetic resources. More broadly, this application-based approach highlights the long-term
39 value of establishing repository-level resources that can be expanded to fit community needs.

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40 # Introduction

41 *Hydractinia symbiolongicarpus* is a colonial cnidarian and an established model for
42 evolutionary developmental biology, stem cell biology, regeneration, and allorecognition [1–3].
43 In recent years, efforts to improve *Hydractinia* as a model system have included generation of
44 robust laboratory strains for use by the research community, sequencing of these strains
45 through the *Hydractinia* Genome Project (https://research.nhgri.nih.gov/hydractinia/), and
46 establishment of methods to produce transgenic animals via the random integration of
47 exogenous DNA [4] or targeted integration via CRISPR/Cas9-mediated gene knock-in [5].

48 An increasing limitation to the expanded use of *Hydractinia* as a model is the lack of
49 long-term storage options for genetic resources. Over the years, laboratories have collected
50 and bred hundreds of genotypically distinct colonies, while simultaneously generating strains
51 bearing various transgenes. In all cases, these animals have had to be maintained as live
52 animals or they would be lost. While this is possible because *Hydractinia* colonies can be
53 maintained for decades under laboratory conditions, it is increasingly costly in terms of labor
54 and space. These costs are often minimized by reducing colonies to the smallest possible size,
55 and only expanding them via clonal reproduction when needed for experiments. However,
56 these colonies remain vulnerable to accidents, disease, and improper handling which can result
57 in death and permanent loss of genotypes important to previous and future research.

58 To address this limitation, we sought to evaluate the feasibility and potential utility of
59 cryopreservation as an archival storage method. As an immediate benefit, cryopreservation
60 would allow “backing-up” animals that are valuable genetic resources. And, as a long-term

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61 benefit beyond laboratory use, cryopreserved stocks would allow user groups from across the
62 research community to store and access samples on demand rather than requiring time and
63 resources to grow or collect new animals. While the ultimate goal would be cryopreservation of
64 germplasm and somatic tissues from all life stages, here we focused on *Hydractinia* sperm as
65 the most amenable to cryopreservation based on previous success in corals [6], and the
66 anemone *Nematostella* (Matt Gibson and Shane Merryman, personal communication).

67 Although much is known about *Hydractinia* embryonic development and the
68 differentiation of *Hydractinia* germ cells [7–9], much less is known about *Hydractinia*
69 germplasm after its release, beyond what is necessary for routine breeding. It is well
70 established that *Hydractinia* are dioecious and have gonozooids (reproductive polyps) that bear
71 multiple gonophores (gamete-filled structures) that release either sperm or eggs. Healthy
72 *Hydractinia* release gametes daily. Researchers typically allow male and female colonies to
73 spawn together in the same water or they collect eggs and sperm separately, then mix them
74 within 30 minutes. Anecdotal evidence suggests waiting longer than 30 minutes decreases the
75 quantity and quality of embryos.

76 After fertilization, each embryo develops into planula larva (1-4 d) before permanently
77 attaching to the surface and metamorphosizing into a juvenile primary polyp. The animal then
78 grows by extending structures called stolons across the surface, from which additional polyps
79 are produced to create a colony. Colonies become sexually mature within 1-2 months. Under
80 laboratory conditions the number of offspring that are male or female is consistent with a 1:1
81 sex ratio.

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82 Successful cryopreservation of sperm cells requires the balance of multiple parameters
83 [10]. These include the storage temperature and the time that elapses the time and storage
84 temperature that elapses between sperm collection and freezing, sperm concentration at the
85 time of freezing, choice and concentration of cryoprotectant, cooling method and rate, thawing
86 method and rate, and the conditions under which thawed sperm will be used for fertilization
87 [11]. Here we detail a systematic three-part approach to: 1) determine basic characteristics of
88 *Hydractinia* sperm and standardize collection and processing: 2) test the toxicity of commonly
89 used cryoprotectants, and 3) identify conditions that maximized the likelihood of cryopreserved
90 sperm samples being capable of fertilization after thawing.

91 Materials and methods

92 Ethics

93 Animal care is overseen by separate Institutional Animal Care and Use Committees at
94 the University of Pittsburgh and Louisiana State University. *Hydractinia symbiolongicarpus* is a
95 marine invertebrate lacking a central nervous system and is not regulated by specialized
96 guidelines. All animals used in this study were maintained in continuous culture as detailed
97 below.

98 Animal care and breeding

99 Experimental work was performed from February to April, 2019, at the Aquatic
100 Germplasm & Genetic Resources Center (AGGRC) in Baton Rouge with animals transported in
101 50-mL tubes by overnight shipping from University of Pittsburgh. Colonies were maintained and

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102 grown as previously described [5] and cultured for at least 2 weeks before use in experiments.
103 Briefly, colonies were established on 25 mm x 75 mm glass microscope slides and cultured in
104 38-L (10-gal) aquaria using artificial seawater (ASW) (Instant Ocean Reef Crystals, Spectrum
105 Brands, Blacksburg, VA) at between 29 and 31 ppt, held at 22-23°C, and maintained on an
106 8h:16h (light:dark) photoperiod. Adult colonies were fed 4-day-old *Artemia* nauplii on Monday,
107 Wednesday, and Friday. On Tuesday and Thursday, colonies were fed a suspension of pureed
108 oysters (fresh caught, shucked, pureed, aliquoted, flash frozen in liquid nitrogen, and stored at
109 -20°C).

110 In this study, we performed crosses between two half siblings, a male (colony 291-10)
111 and a female (colony 295-8) (Fig S1). Following first exposure to light, male and female colonies
112 were moved into separate bins filled with ASW and placed under supplemental lighting.
113 Gametes released approximately 1 hr after light exposure. Sperm were released in "clouds" or
114 "streams" from individual gonophores (**Error! Reference source not found.**A-C) and were
115 collected and pooled using a Pasteur pipette (**Error! Reference source not found.**D).

116 **Fig 1. Time lapse of sperm release.**
117 (A) Close-up view of Hydractinia polyps just prior to sperm release. Arrows
118 indicate polyp types. (B) Arrowhead points to sperm stream being released. (C)
119 Arrowheads point to sperm stream (polyps have retracted from B). (D) Top-down
120 view of slide with Hydractinia releasing sperm. Arrowheads point to multiple
121 streams of sperm released from the colony. (E) 1-d old larvae. (F) 2-d old larvae.

122 Eggs were collected by straining the water from the female bin with a 20-µm cell
123 strainer. For routine breeding and to serve as a positive control for fertilization, 20-30 clouds of

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124 sperm were collected from 10 male slides, transferred to a 50-mL conical tube, and brought to
125 a final volume of 15 mL with filtered sea water (FSW, artificial seawater filtered through 0.45
126 µm Polyethersulfone (PES) membrane Rapid-Flow Sterile Disposable Bottle Top Filters, Thermo
127 Scientific Nalgene, catalog #295-4545). To this were added 400-600 eggs harvested from 8-9
128 female slides. The final volume was brought to 30 mL with FSW and transferred to a 100-mm
129 polystyrene Petri dish. Within 1 hr, embryos began to cleave and developed into planulae by
130 the following day (**Error! Reference source not found.**E). On day 4 after fertilization, larvae (Fig
131 1F) were settled by exposure to 100 mM Cesium Chloride (CsCl diluted in FSW) for 4-5 hr until
132 ready for settlement, and were pipetted onto microscope slides and kept in the dark for 1-2 d
133 or until attachment and primary polyps formed.

134 Estimation of sperm concentration and motility

135 On six separate days, individual sperm clouds (cumulative N = 35) were collected in a 10-
136 µl volume and analyzed for motility within 20 min of collection. The sample was briefly
137 vortexed to form a uniform suspension, loaded onto a Makler® counting chamber (SEFI Medical
138 Instruments Ltd, Irvine Scientific, Santa Ana, CA, USA), and viewed with dark-field illumination
139 at 200-X magnification (Olympus CX41RF, Tokyo, Japan). Sperm were already motile when
140 observed and did not require activation. The sample concentration was counted twice
141 according to an established protocol [12] and the average used as the sperm concentration (at
142 10^6/mL). Motility was quantified using a computer-assisted sperm analysis (CASA) system
143 (CEROS model; Hamilton Thorne, Inc., Beverly, MA, USA). The settings used were based on a
144 previous study [13]. Briefly, motility and VCL (curvilinear velocity) were measured for 10 sec.

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145 Cell detection was set at a minimum of 25 pixels for contrast and 6 pixels for cell size. In each
146 measurement, 100 frames were captured at a rate of 60 frames/sec. Sperm with an average of
147 >20 µm/s measured path velocity (VAP) were counted by the program as being progressively
148 motile. GraphPad Prism (v8.2.0) was used to calculate correlations between sperm
149 characteristics (velocity, percent motile, and concentration).

150 Longevity and temperature sensitivity of sperm

151 To test the effects of time and temperature on sperm motility, approximately 30 sperm
152 clouds were collected using a 10-µL pipette, pooled and diluted to produce a concentration of 2
153 x 10^7 cells/mL, and then divided into two tubes. One tube was kept at room temperature (21-
154 23°C) and the other was kept in a 4°C refrigerator. Each treatment was evaluated hourly for
155 presence or absence of motility for 7 hr.

156 Fertility of sperm

157 To determine how long sperm could produce viable offspring when stored at 4°C, we
158 performed a time-series experiment using a single collection of sperm. Approximately 150
159 clouds of sperm were collected using a Pasteur pipette and stored in a 50-mL conical tube.
160 Concentration was determined as described above. On day 0, 3 mL of this sample (total of 2 x
161 10^7 sperm) were used to fertilize 200 eggs in a total volume of 30 mL FSW. The sperm sample
162 was stored at 4°C. On subsequent days, freshly collected eggs were fertilized with 3 mL (2 x 10^7
163 sperm) of sperm in 30 mL FSW. Offspring were followed until they metamorphosed into
164 juvenile polyps.

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165 ## Acute Toxicity of Cryoprotectants

166 Approximately 20 sperm clouds were collected using a 10-µL pipette, pooled, and
167 adjusted to a concentration of 1 x 10^7 sperm/mL using FSW. Three cryoprotectants, methanol
168 (Fisher Scientific, Waltham, MA) dimethyl sulfoxide (DMSO, Fisher Scientific, Waltham, MA),
169 and glycerol (Sigma-Aldrich, St. Louis, MO) were used. For each cryoprotectant, double strength
170 stocks of 10%, 20%, and 30% (v/v) were created using FSW. The sperm and double-strength
171 cryoprotectant were mixed in equal volumes (100 µL:100 µL) resulting in a final sperm
172 concentration of 5 x 10^6 sperm/mL and final cryoprotectant concentrations of 5%, 10%, or 15%.
173 Sperm were evaluated at 30 min after addition of cryoprotectant (30 min was chosen as a
174 practical total exposure time required for cryoprotectant equilibration and for packaging and
175 handling of the samples). Presence or absence of motility was used as an estimate for toxicity.

176 ## Standardized Sperm Collection (3-D printing)

177 Based on the difficulties and inefficiencies experienced during pilot experiments working
178 with *Hydractinia* sperm, we designed a custom sperm collection chamber with integrated slide
179 rack to collect and concentrate sperm for downstream applications (Fig 2) by use of free
180 computer-aided design (CAD) online software (Tinkercad, version 4.7, Autodesk, San Rafael,
181 CA). The design was exported as a stereolithography (STL) file and imported into a 3-D printer
182 slicer software (Simplifiy3D, version 4.0, Cincinnati, OH) to control the printing process (Table
183 S1). Collection chambers were printed in black PLA (ZYLtech Engineering, Spring, TX) filament
184 on a stock Prusa i3 MK3 3-D printer (Prusa Research, Czech Republic) (Table S2).

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185 **Fig 2. Sperm collection chamber.**

186 (A) CAD-rendering of the 3-D design. (B) Printed model with rack and box

187 separate. (C) Printed model with rack inserted. Object model deposited on

188 Thingiverse. https://www.thingiverse.com/thing:3661286

189

190 Freezing

191 To collect sperm for freezing, we placed nine slides of males in the 3-D printed sperm

192 collection chamber filled with ASW. An additional male was placed in a separate bin so that

193 sperm could be collected and used as a fertilization positive control. After sperm were released,

194 the slide rack was removed from the sperm collection chamber, the cloudy seawater poured

195 into two 50-mL conical tubes (~80 mL total) and spun for 20 min at 3,000 rpm (~1450-1500 x *g*)

196 which resulted in a visible white pellet. The supernatant was pipetted off and the pellets were

197 combined and resuspended in FSW to the appropriate concentrations (between 2 x 10^6 and 2 x

198 10^9 sperm/mL) and stored at 4°C until they were prepared for freezing (~3 hr).

199 To prepare for freezing, sperm were mixed with an equal volume of 10% DMSO or 10%

200 methanol in FSW (final concentrations of 5% cryoprotectant), drawn into 0.25-mL French straws

201 (IMV International, MN, USA), and held at 4°C in a controlled-rate freezer for the remaining

202 equilibration time (Minitube of America, IceCube 14M, SY-LAB). The total equilibration time,

203 from initial mixing with cryoprotectant to starting the freezing program, was set at 20 min.

204 Equilibrated samples were cooled to -80°C with one of three pre-programmed cooling rates:

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205 5°C/min, 20°C/min, or 30°C/min. Frozen samples were held at -80°C for at least 5 min before
206 transfer and storage in liquid nitrogen.

207 Thawing and use for fertilization

208 After 21-69 hr of storage, straws were removed from liquid nitrogen and immediately
209 plunged into room temperature (22°C) water for 8 sec. The straws were clipped and a 2-µL
210 sample was removed, diluted with 38 µL of FSW (1:20 dilution), and used for sperm
211 assessment. The remaining sample was held in a microfuge tube until fresh eggs were obtained
212 (15-30 min). After performing the fertilization positive control (routine breeding), 100-300 fresh
213 eggs were collected in 500 µL of FSW and added to the microfuge tube with the thawed sperm.
214 The mixed gametes were placed into a 100-mm Petri dish and ~50 mL FSW was added. An
215 estimate of the number of eggs used was obtained by counting in groups of ten. The resulting
216 fertilization was kept at room temperature and observed for 24 hr to determine how many
217 planulae had begun forming. The resulting offspring were observed until metamorphosis into
218 juvenile polyps.

219 Results

220 Sperm motility and viability

221 To assess sperm characteristics, we measured sperm from 35 clouds (each collected in
222 10 µl) using CASA. Mean velocity was 50.8 ± 26.2 µm/s, mean percent that were motile was 37
223 ± 22% and mean concentration was 9.37 ± 5.31 x 10^6/mL. Based on linear regression, velocity
224 and motility were correlated (R^2 = 0.2804, P = 0.0011) (Fig 3A), as were concentration and

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225 motility (R^2 = 0.2870, P = 0.0009) (Fig 3B). Velocity and concentration were not correlated (R^2 =
226 0.02365, P = 0.3778) (Fig 3C).

227 **Fig 3. Correlations among sperm velocity, motility, and concentration.**
228 Each point represents a sperm cloud (N = 35). (A) Distribution of sperm based on
229 velocity and the number motile. (B) Distribution of sperm based on
230 concentration and number motile. (C) Distribution of sperm comparing velocity
231 and concentration.

232 To determine the effect of temperature on sperm viability, we compared the motility of
233 freshly collected sperm held at room temperature (22°C) to that of sperm held in a 4°C
234 refrigerator. At room temperature, the number of motile sperm declined over 6 hr, such that by
235 7 hr only twitching was observed (tail movement without progressive motility). In contrast,
236 sperm kept at 4°C retained progressive motility 7 hr after collection, although the total number
237 of motile sperm and the velocity visibly decreased. By 23 hr, no sperm were motile, but
238 approximately 40%, assessed manually were still twitching. Thus, holding sperm at 4°C
239 prolonged motility.

240 The observation that sperm held at 4°C were still moving after 23 hr raised the question
241 of whether they could still fertilize eggs and, if so, whether sperm would remain viable after
242 longer storage times. To address this question, we collected ~150 sperm clouds and used the
243 sperm to fertilize freshly collected eggs over the following 6 d (Fig 4). We performed daily
244 routine breeding to serve as a positive control for egg fertilization; nearly all of the eggs (>95%)
245 were fertilized each day indicating that there was no appreciable differences in egg quality for

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246 fertilization. On day 0, we mixed 2 x 10^7 sperm (3 mL) with ~200 eggs, which resulted in ~150
247 embryos. Because ~50 eggs remained unfertilized, we interpreted this to indicate that the
248 defined sperm number in this sample (2 x 10^7) were capable of fertilizing ~150 eggs.

249 **Fig 4. Estimated sperm fertilization capacity over time.**
250 Each day, 2 x 10^7 sperm cells from the same collection aliquot were used to
251 fertilize the freshly collected eggs in 30 mL FSW. On Days 1 and 2, only ~100 eggs
252 were available for exposure to sperm. On the other days, a surplus of eggs were
253 collected for exposure.

254 On each subsequent day, we mixed the same amount of stored sperm with as many
255 eggs as we could collect and estimated the total number of fertilized and unfertilized embryos.
256 We found that 2 x 10^7 sperm consistently fertilized ~150 eggs after 3, 5, and 6 d at 4°C. On days
257 1 and 2, we were only able to collect ~95 eggs, nearly all of which were fertilized. These latter
258 data were consistent with the notion that 2 x 10^7 sperm could fertilize ~150 eggs. In these
259 experiments, all embryos developed and metamorphosed into normal juvenile colonies. These
260 results suggest that it is possible to store sperm for at least 6d at 4°C without an appreciable
261 drop in fertilization capability, thereby enabling shipment of sperm samples. This also
262 demonstrated that sperm motility is not necessarily a good predictor of fertilization success
263 when gametes are mixed under controlled conditions.

264 Determining cryoprotectant toxicity to sperm

265 We tested the acute toxicity of three common cryoprotectants (DMSO, methanol, and
266 glycerol). Sperm incubated with the three concentrations (5, 10 and 15%) of DMSO or

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267 methanol displayed comparable motility after 30 min. In contrast, sperm exposed to 15%
268 glycerol ceased moving immediately, while those exposed to 10% and 5% glycerol were non-
269 motile within 30 min.

270 To determine whether cryoprotectant-treated sperm would be able to fertilize eggs, we
271 exposed sperm to each cryoprotectant for 30 min and mixed 4.1 x 10^6 sperm with 40 freshly
272 collected eggs in a total volume of 50 mL. Sperm exposed to 10% or 15% of any cryoprotectant
273 were unable to fertilize eggs. In contrast, sperm treated with 5% of any cryoprotectant yielded
274 3-12 embryos (Fig 5). From this, we concluded that 5% DMSO or methanol would be suitable
275 cryoprotectants.

276 **Fig 5. Number of fertilized eggs using cryoprotectant-treated sperm.**
277 For each condition, 30-40 eggs were exposed to 4.1 x 10^6 sperm in a total
278 volume of 50 mL.

279 Identifying suitable freezing conditions

280 While many factors affect the quality of cryopreserved sperm, three key parameters
281 must be balanced: cryoprotectant concentration, sample concentration, and cooling rate. For
282 example, higher cryoprotectant concentrations can be more toxic, whereas lower
283 concentrations may not sufficiently protect the cells. Moreover, the toxicity of a given
284 concentration of cryoprotectant often decreases as the sample concentration increases [10].
285 The cooling rate must also be slow enough to allow cells to dehydrate sufficiently (to minimize
286 intracellular ice formation), but fast enough to freeze them before concentrations of
287 intracellular salts or pH (i.e., solution effects) or the cryoprotectant become damaging.

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288 To survey the effects of freezing rate on sperm in either 5% DMSO or 5% methanol, we
289 cooled sperm at 5°C/min and 30°C/min (Table 1, Experiment 1; Fig S2, S3). Samples were stored
290 in liquid nitrogen for at least 21 hr before they were thawed and evaluated. In all conditions,
291 the concentration of intact sperm in the thawed samples was reduced from 1 x 10^7 to 2 x 10^6 or
292 fewer, nearly ten-fold, likely due to cell rupture either during freezing or thawing. Overall,
293 between 5 x 10^4 and 1 x 10^5 fewer sperm were detected in the 30°C/min samples than in the
294 5°C/min samples suggesting that the faster rate did not allow sufficient osmotic egress and
295 intracellular ice was formed. We incubated aliquots of each thawed sample with 75 freshly
296 collected eggs. Despite the low numbers of sperm used (≤5 x 10^5), at least one egg was
297 fertilized in each condition. This indicated the presence of viable sperm and suggested that
298 increasing the effective sperm concentration would increase fertilization.

299 **Table 1. Overview of frozen samples and fertilization potential.**

	Experiment 1				**Experiment 2**
Cryoprotectant	5% DMSO	5% DMSO	5% Methanol	5% Methanol	5% DMSO
Initial sperm concentration (sperm/mL)	1 x 10^7	1 x 10^7	1 x 10^7	1 x 10^7	5 x 10^7
Cooling rate (°C/min)	5	30	5	30	20
Hours stored frozen	69	69	69	69	21
Thawed sperm concentration (sperm/mL)	2 x 10^6	1.5 x 10^6	2 x 10^6	1 x 10^6	5 x 10^7

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Total sperm mixed with 75 eggs	5×10^5	3.8×10^5	5×10^5	2.5×10^5	1.2×10^7
Number of embryos	2	2	2	1	10

300

301 We increased the volume and concentration of sperm collected by fabricating a sperm
302 collection chamber by 3-D printing that allowed incubation of as many as ten slides bearing
303 male colonies in <100 mL of water, thus eliminating the need to collect sperm with pipettes.
304 This enabled collection of 10^9 sperm per day (a 100-fold increase). We froze the sperm at a
305 concentration of 5×10^7/mL at a cooling rate of 20°C/min. When thawed, these sperm samples
306 remained at a concentration of 5×10^7 sperm/mL (Table 1, Experiment 2; Fig S4). Moreover, the
307 number of fertilized eggs increased to 10.

308 These results encouraged us to test whether we could further increase fertilization by
309 increasing the concentration of sperm samples. We froze sperm at five different concentrations
310 ranging from 10^7 to 10^9/mL (Fig 6) at the 20°C/min cooling rate (Fig S5) and stored them in
311 liquid nitrogen for 21 hr. The concentration of each sample post-thaw had the same count as
312 before freezing. When thawed, sperm frozen at 10^9/mL were able to fertilize 270-275 eggs.
313 Sperm (in descending order of concentration) at 5×10^8/mL fertilized 235-250 eggs; 1×10^8/mL
314 fertilized 150-250 eggs; 5×10^7/mL fertilized 26-150 eggs, and 1×10^7/mL fertilized 5-100 eggs.
315 All embryos developed into larvae and were able to metamorphose into a primary polyp with
316 no visual abnormalities. Thus, cooling sperm at a rate of 20°C/min and at concentrations in
317 excess of 1×10^7 showed best fertilization.

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318 **Fig 6. Fertilization comparing frozen sperm.**

319 Each thawed sperm sample was exposed to different number of eggs. In each
320 case, the number of eggs collected was manually estimated to be a surplus of
321 what each respective sperm sample could fertilize based on their concentration.

322 Discussion

323 Sperm motility and viability

324 The sperm motility and concentration-related phenomena reported herein provide
325 some insight of the basic characteristics of sperm clouds that have not been previously
326 observed. While the results from this feasibility study are promising, there are several
327 improvements and future experiments that can be pursued. There was a large standard
328 deviation (<50%) in motility and concentration between individually collected sperm streams.
329 Part of this variation reflects the imprecise manner traditionally used for collecting sperm as it
330 is released. These findings reinforce the need to standardize collection methods and sperm
331 concentrations. Future studies can also address other outstanding questions related to these
332 characteristics. For example, when and how are sperm activated? Does the sperm
333 concentration affect activation and motility? Determination of how these features could affect
334 cryopreservation, especially among different genotypes, would be useful in expanding and
335 making protocols more robust for *Hydractinia* and potentially other cnidarian models.

336 Concepts such as these have been studied quantitatively in aquatic species previously at
337 the commercial scale, for example in blue catfish, *Ictalurus furcatus*, (for hatchery production of

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338 hybrids) [14] by use of industrial engineering and simulation modeling approaches [15]. Those
339 studies were based on use of automated high-throughput processing [16] developed using
340 commercial dairy industry approaches [17] but are also relevant for processing at lower
341 throughput. The emphasis in such approaches is on the application level for repository
342 development, rather than on the research level for optimization of individual components (e.g.,
343 cooling rate or cryoprotectant choice) for protocol development.

344 Another application-level concept often overlooked in traditional research approaches is
345 the refrigerated storage of samples prior to freezing or use. Such storage enables shipping of
346 germplasm for processing elsewhere, and can avoid waste by identifying the usable working
347 lifetime of valuable material. We tested the retention of *Hydractinia* sperm fertility after
348 storage in FSW at 4°C and found that freshly collected sperm and sperm stored for 6 d could
349 fertilize comparable numbers of eggs. This result suggested that sperm could be stored in FSW
350 at 4°C even longer and still produce viable embryos. Identifying these basic storage conditions
351 is useful in cases when resources are not available to process on-site and samples must be
352 transported to another facility for processing and storage.

353 Future studies should compare fertility across a range of storage temperatures with
354 longer storage times when appropriate, and couple that with freezing experiments to evaluate
355 the effects of storage on cryopreservation survival. In addition, extender solutions (e.g. buffers)
356 can influence the quality and retention of fertility of sperm during storage [18–20]. Future
357 studies should also address the total fertilization window for eggs. While mixing gametes ≤30
358 mins post-release has been the community guideline for producing quality embryos, it has not

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359 been determined quantitatively and it is possible that storage at a cooler temperature may
360 extend fertility.

361 Determining cryoprotectant toxicity to sperm

362 The acute toxicity assay we performed was at a small scale, but yielded useful
363 information regarding potential cryoprotectants. We initially observed limited fertilization using
364 the treated sperm which demonstrated feasibility and a basis for improvement. In future
365 studies, the potential effects of cryoprotectant toxicity on sperm and egg should be evaluated
366 more clearly. If toxicity is affecting fertilization, sperm can be rinsed to reduce or eliminate the
367 cryoprotectant before exposing them to the eggs. The limited fertilization we observed also
368 emphasized the need to process sperm in concentrations that were relevant to those used for
369 breeding. This prompted the design of a custom 3-D printed collection chamber to improve
370 sperm collection, and enabled evaluation of cryopreservation conditions that resulted in
371 effective post-thaw fertilization rates. This improved collection method provides expanded
372 opportunities for standardized evaluation of cryoprotectants and concentrations, while bearing
373 in mind that such choices should be governed by overall utility at the process level rather than
374 optimizing singular factors (e.g., motility) at the individual step level. For example, a certain
375 cryoprotectant may yield a slightly lower motility value than other chemicals, but is cheaper,
376 less toxic to sperm cells, and allows more flexibility in timing and cooling rates. In research-
377 driven studies, the highest motility would be recommended; in application-driven work, the
378 cryoprotectant that increases efficiency and reliability would be recommended.

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379 Other benefits of placing a focus on application include that work in the present study
380 can be directly scaled up for use with hundreds of animals and multiple laboratories. Work
381 addressing repository development in previous studies, with blue catfish for example, can be
382 generalized to *Hydractinia* because the approaches used are the same, including the use of
383 French straws that can be filled, sealed, and labelled using automated equipment (e.g., the
384 Minitube Quattro system at the AGGRC can process 15,000 straws per hour). In addition,
385 cryopreservation in *Hydractinia* can be directly transferred from a central facility (such as the
386 AGGRC) to on-site work within an existing laboratory by use of high-throughput mobile
387 cryopreservation capabilities [21], or by establishment of full high-throughput cryopreservation
388 capabilities such as in creation of a central *Hydractinia* Stock Center (for economic analysis, see
389 [22]). Development of in-house cryopreservation capabilities within research laboratories will
390 be greatly strengthened by the recent developments in 3-D printing described above (e.g., [23])
391 including fabrication of probes for monitoring and storing temperature information [24], and
392 the potential for sharing of open-source design files for production of inexpensive, reproducible
393 freezing devices that can be integrated with strong quality control programs (e.g., [25,26]).

394 Identifying suitable freezing conditions

395 While there are no other *Hydractinia* cryopreservation protocols to directly compare
396 our results to, there are protocols that have been developed for sperm from various coral
397 species which can serve as an indirect comparison for some of the key parameters. One
398 protocol in particular has been instrumental in banking the germplasm of 31 coral species from
399 around the world [6,27,28] and additional protocols have been developed in two coral species

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400 [29,30]. Briefly, we can compare our method with the cryoprotectant, container, and cooling
401 methods of these studies. Similar to two of the studies [28,30], DMSO was used as the
402 cryoprotectant but at higher final concentrations (≥10%), and in the other [29] 20% methanol
403 was used with 0.9 M sucrose as an extender. These cryoprotectant concentrations are higher
404 than what our trials suggested would be suitable for *Hydractinia* sperm, however there are two
405 major differences that may explain this discrepancy and proffer improvements to this study.
406 The *in vitro* fertilization in this study used a volumetric sperm to egg ratio of 50 mL with
407 ~10^5/mL sperm to 30-40 eggs, which was considerably more dilute in comparison to each coral
408 study in which the ratios used to determine post-cryoprotectant fertility were 5 mL with 10^6/mL
409 sperm to 30-50 eggs [28], 1 mL with 10^5/mL sperm to 20 eggs [29], or 4 mL with 1.5 x 10^7/mL
410 sperm to 50 eggs [30]. In future acute toxicity assays, optimizing the volumetric sperm to egg
411 ratio (in our case, reducing the volume and increasing the sperm concentration) would improve
412 the assessment of acute toxicity before moving onto freezing. Previous studies with eastern
413 oyster *Crassostrea gigas* have shown that much of the variation in sperm cryopreservation
414 response is procedural rather than biological (e.g., "male-to-male variation"), and control of
415 sperm concentration is necessary for reproducible results [31].

416 One of the coral protocols cryopreserved 1-mL samples in 2-mL cryovials [28], whereas
417 the other two studies [29,30] cryopreserved samples in 0.25-mL French straws. French straws
418 offer several advantages over traditional cryovials. French straws require less storage space and
419 can be easily processed manually in the case of a few samples, or more efficiently in high-
420 throughput with automated filling, labeling, and sealing for hundreds to thousands of samples.
421 In addition, samples can generally be cooled in French straws at a faster rate than in cryovials,

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422 in large part due to the higher surface-area-to-volume ratio of straws (which can also decrease
423 variability during freezing). In cryovials, there is potentially more variation across the sample
424 volume as material on the periphery could freeze more rapidly than that closer to the center.
425 Also, vials typically have thicker walls with greater insulative potential slowing heat removal
426 from the sample.

427 With regard to cooling rates, there are several differences which make these studies
428 difficult to compare. First, the equilibration temperature and time used were slightly different
429 [28,30] or not explicitly quantified [29]. Second, the ending temperatures used to calculate the
430 freezing curve were different where one study used -80°C [28] but the other two used the
431 coldest achievable temperature between -110°C and -130°C. Theoretically, the ending
432 temperature should not affect the rate calculation if the freezing rate is constant, but unless the
433 temperature is monitored while the samples are being frozen, fluctuations are difficult to
434 account for. Although the different procedures make studies difficult to compare, it is critical
435 that all details surrounding the freezing process be documented for quality samples and
436 reproducible results [11]. For this reason, only two of the studies can be referenced for
437 reproducibility and generally compared in relation to their cooling rate [28,30]. Both studies
438 used an equilibration temperature between 24-29°C and equilibration time of 15 [30] or 20 min
439 [28] where in this study the equilibration temperature was 4°C for 20 min. The selection of 4°C
440 as the equilibration temperature in our study was in part due to the usage of a controlled-rate
441 freezer.

442 One obvious difference among the present study and the three published coral studies
443 is that each used suspension at defined heights above liquid nitrogen to freeze samples. This

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444 method is difficult to standardize, and is less precise than using a controlled-rate freezer.
445 However, it has significant advantages including affordability, availability, and portability. In
446 future studies, a comparison of samples frozen at comparable nominal rates by various
447 methods should be done to enable harmonization of results and reporting, providing multiple
448 options for freezing that could be selected based on the needs of the user. Other factors that
449 could be investigated in future work include whether offspring produced from cryopreserved
450 sperm will mature into full adults and whether the male-to-female ratio is affected.

451 Approaches to repository development for aquatic species

452 Recent advances in consumer-level technology provide opportunities to custom-design
453 open-source options for hardware and other tools necessary to assist repository development
454 beyond that provided by adaptation of traditional livestock practices. Customizing the design
455 of the 3-D printed collection chamber greatly increased the efficiency and success in identifying
456 suitable freezing conditions. To collect a useful number of sperm, the standard collection
457 method via Pasteur pipette or micropipette is labor intensive, and poses logistical problems in
458 the case of multiple collectors. Given our previous approach, collecting all sperm would be
459 possible but would require filtering all the water from the bin (~2 L) or having access to a large
460 capacity centrifuge. Thus, by customizing a chamber to minimize the collection volume (<100
461 mL) and maximize the total yield of sperm (as many as ten slides bearing *Hydractinia*), we were
462 able to directly improve and standardize processing efficiency.

463 In addition, custom design of devices is also possible for freezing activities. The
464 polylactic acid (PLA) used for 3-D printing does not become brittle or stiff as do other plastics

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465 when exposed to cryogenic temperatures (such as liquid nitrogen) [32] making 3-D printed
466 objects safe and useful for such applications. Various devices can typically be fabricated at low
467 cost (e.g., $10 or less for material costs) using consumer-level printers ($250 or less) that offer
468 high resolution, flexibility, and short learning curves. There are large internet-driven user
469 communities for these printers and thousands of videos (such as on YouTube) are available for
470 printer set up, training, and troubleshooting. In addition, design files can be shared on a
471 number of sites (e.g., Thingiverse, and Github) for others to print and customize. In this way,
472 devices used in cryopreservation and repository development can be developed, shared, and
473 standardized within research communities, greatly reducing costs of cryopreservation, and
474 making reliable methods widely available. Systems such as this must be accompanied by quality
475 control and quality assurance programs, however, to ensure that samples meet minimum
476 thresholds for repository use [25,26].)

477 Overall, the success in the present study of using a generalizable approach for
478 *Hydractinia* sperm provides further evidence that cryopreservation protocols are not
479 necessarily species-specific. For example, a single generalized protocol was applied to more
480 than 20 species within the genus *Xiphophorus* and two other species in the genus *Poecilia* to
481 enable repository development to safeguard the genetic resources of these valuable biomedical
482 model species [12]. Overall, more research is needed for aquatic species in general to
483 quantitatively assess factors important to practical repository operation with cryopreserved
484 sperm (e.g., [14]), and standardization of procedures and reporting is necessary to enable
485 meaningful comparisons across studies [11]. The present study offers evidence that substantial
486 repository-level benefits can be realized by generalizing cryopreservation at the application

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487 level, rather than trying to optimize new protocols on a species-by-species basis, and restricting
488 this work to the traditional (reductionist) research level [11].

489 Conclusions

490 This feasibility study showed that it is possible and a worthwhile endeavor to pursue
491 *Hydractinia* sperm cryopreservation as a long-term storage option for genetic resources.
492 Specifically, we demonstrated that sperm cooled at 20°C per min in 5% DMSO at a
493 concentration of 10^8-10^9/ml in 0.25-mL French straws were able to fertilize 150-300 eggs, which
494 developed into juvenile colonies. In our experience, a population of 150 juvenile colonies
495 typically contains sufficient numbers to establish a strain for propagation via asexual
496 reproduction (i.e., they will grow into healthy adults with the genotype of interest) or breeding
497 to produce subsequent generations. With some additional work it should be possible to reliably
498 freeze and re-derive specific genotypes. This would greatly enhance the utility of *Hydractinia* as
499 a model system for cnidarian genetics. In addition, we expect this work could also provide a
500 guide to researchers seeking to develop cryopreservation approaches in other cnidarian
501 species.

502 While this study has direct implications for the *Hydractinia* community, there are several
503 considerations that can be discussed with regard to communities that work on other cnidarian
504 models. Lack of long-term storage options has been one of the limitations to nearly all cnidarian
505 research. Cryopreservation has not been pursued either due to the lack of resources to achieve
506 and maintain frozen samples, or the lack of necessity as many cnidarian models can be cultured
507 relatively simply and the animals can regenerate. A notable exception to this are

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508 cryopreservation efforts for conservation in coral species and their symbionts due to
509 importance of corals to reef biodiversity, and the overall decline in health and prevalence of
510 corals globally over the past several decades [33–36].

511 Another emerging problem for popular research models is the rapid proliferation of new
512 lines and mutants that would require maintenance as live animals which is expensive and risky
513 without cryopreservation. With these common limitations in mind, cnidarian communities need
514 to come together and agree on a consistent and foundational approach towards
515 cryopreservation of all cnidarian models for the ultimate purpose of repository development
516 and establishment of repository networks. By having this long-term goal in mind, we can more
517 systematically work towards developing, protecting, maintaining, distributing, and utilizing an
518 expanding pool of cnidarian genetic resources.

519 A centralized repository or stock center is a necessity for well-developed research
520 organisms and part of the success with these has been due to collaboration among laboratories
521 and the sharing of tools, systems, and resources throughout the communities. For example,
522 mouse resources are largely centralized with The Jackson Laboratory (https://www.jax.org/),
523 zebrafish databases and lines are found in the Zebrafish International Resources Center (ZIRC,
524 University of Oregon), *Drosophila* utilizes the Bloomington *Drosophila* Stock Center (BDSC,
525 Indiana University Bloomington), *Caenorhabditis elegans* and other worm-related models
526 localize their resources in WormBase (wormbase.org/), and *Xenopus* related resources are
527 found in Xenbase (https://www.xenbase.org). Having a wealth of such resources and
528 information available to these communities makes these model systems much more useful and

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529 available to investigators, whereas model systems that require development of basic tools can
530 be more challenging on many levels.

531 Future studies should establish a standardized approach for the storage, shipment, and
532 use of frozen *Hydractinia* samples that can be made available throughout the research
533 community. Current models for this would include development of repositories or a repository
534 system, and the potential incorporation of these entities into a community-based stock center.
535 An existing model for such organization exists in ZIRC which maintains more than 43,000
536 research lines of zebrafish as frozen sperm (https://zebrafish.org/). In addition, to assist
537 standardization of protocols and approaches, it may be useful to establish community-level
538 mechanisms to design and share inexpensive devices that can be used to support users across a
539 wide range of experience and skill levels in culture, spawning, and cryopreservation of
540 *Hydractinia*. Lastly, cryopreservation and repository development should be expanded to
541 include additional germplasm and somatic cell types.

542

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546

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641 # Supporting Information Captions

642 **Figure S1. Pedigree of the colonies used to generate germplasm and offspring.** Field-collected
643 colonies are denoted with black symbols. Colony 291-10 is the offspring of two colonies
644 collected from Lighthouse Point, New Haven, CT in 2014. Colony 295-8 is the offspring of a field
645 collected colony and a laboratory strain, 235-33. The pedigree of colony 235-33 can be
646 recreated by concatenating previously published pedigrees (shaded area) (Cadavid et al. 2004;
647 Powell et al. 2007). Colony AP100-88 is from the mapping population in Powell et al. (2007).
648 Colony 431-44 is from the mapping population in Cadavid et al. (2004).
649

650 **Figure S2. Experiment 1 cooling curve (5°C/min).**
651 The first trial included a slow cooling rate of 5°C/min with a sperm concentration of $1x10^7$/mL.
652 The green line represents the programmed temperature profile, red is the chamber
653 temperature, and blue is the sample temperature. Samples were held at -80°C for at least 5 min
654 before plunging into liquid nitrogen.
655

656 **Figure S3. Experiment 1 cooling curve (30°C/min).**
657 The first trial included a fast freezing rate of 30°C/min with a sperm concentration of $1x10^7$/mL.
658 The green line represents the programmed temperature profile, red is the chamber
659 temperature, and blue is the sample temperature. Samples were held at -80°C for at least 5
660 minutes before plunging into liquid nitrogen.
661

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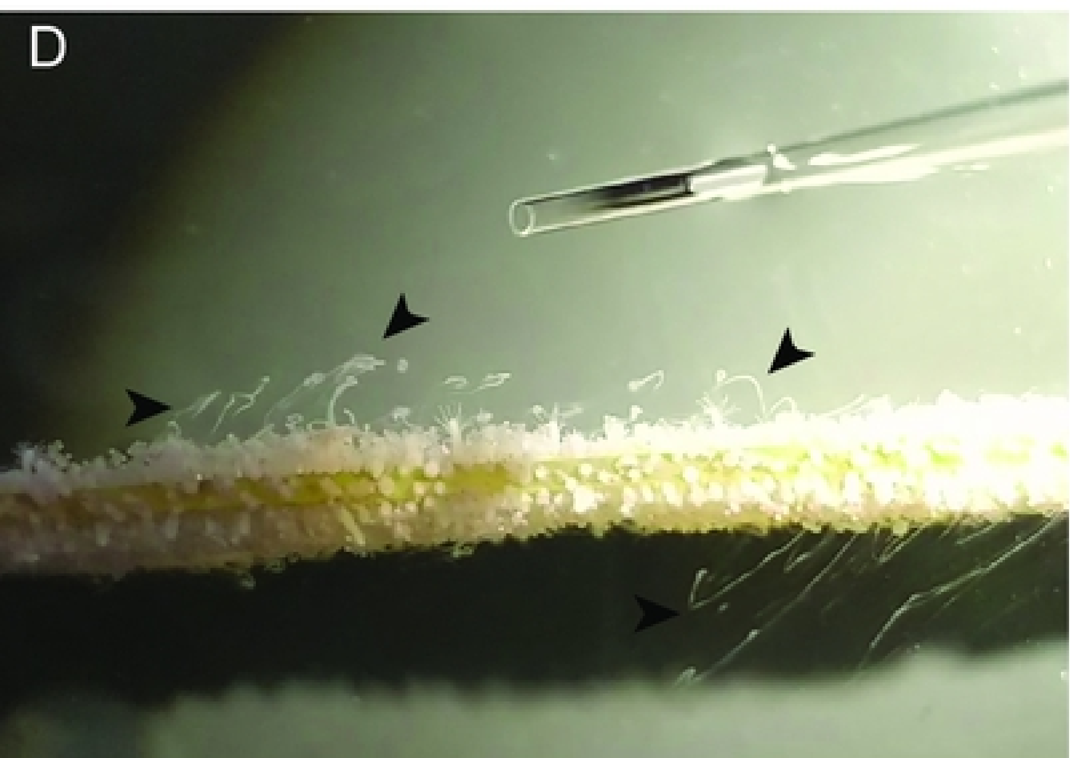
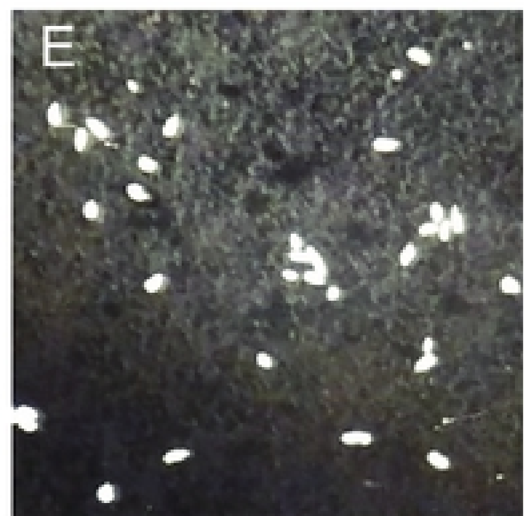
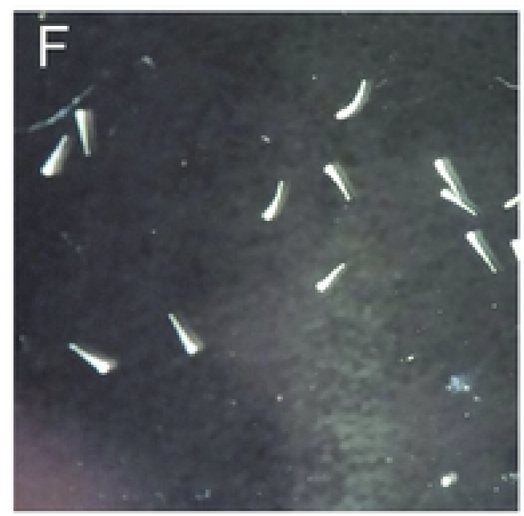
662 **Figure S4. Experiment 2 cooling curve (20°C/min).** The second trial adjusted the freezing rate
663 to a moderate rate of 20°C/min with a more concentrated sperm sample (5x10^7/mL). The green
664 line represents the programmed temperature profile, red is the chamber temperature, and
665 blue is the sample temperature. Samples were held at -80°C for at least 5 minutes before
666 plunging into liquid nitrogen.
667
668 **Figure S5. Cooling curve of various sperm concentrations at (20°C/min).** Each sperm
669 concentration (10^7-10^9/mL) was frozen at the same time at 20°C/min. The green line represents
670 the programmed temperature profile, red is the chamber temperature, and blue is the sample
671 temperature. Samples were held at -80°C for at least 5 min before plunging into liquid nitrogen.
672
673 **Table S1. Slicer software settings used to 3-D print collection chamber.**
674
675 **Table S2. Printer hardware features.**







Key

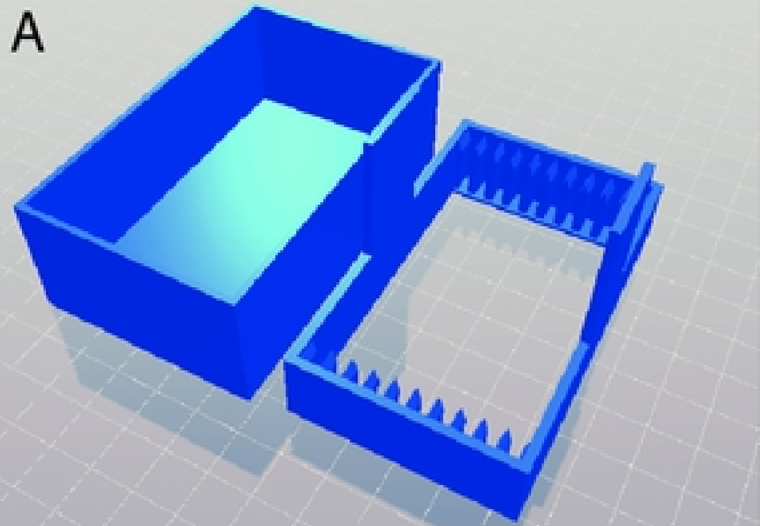
→ Gastrozooids

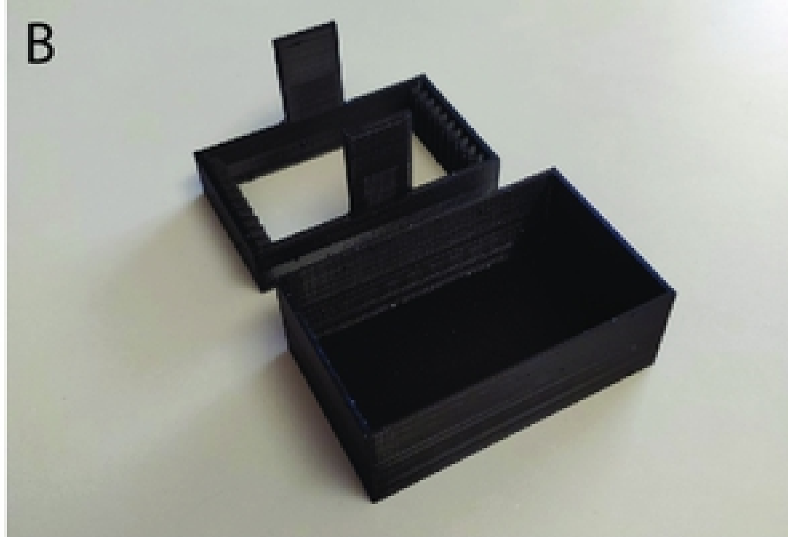
- - ➤ Gonozooids

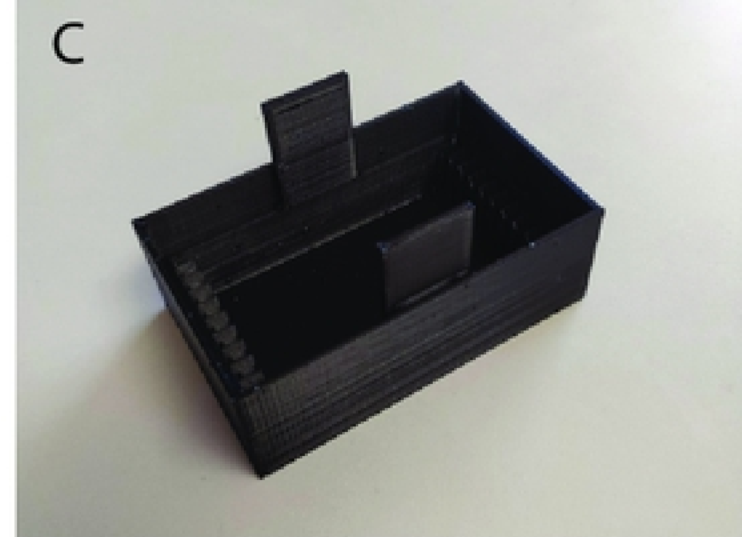
❯ Gonophore

➤ Sperm stream

Fig1







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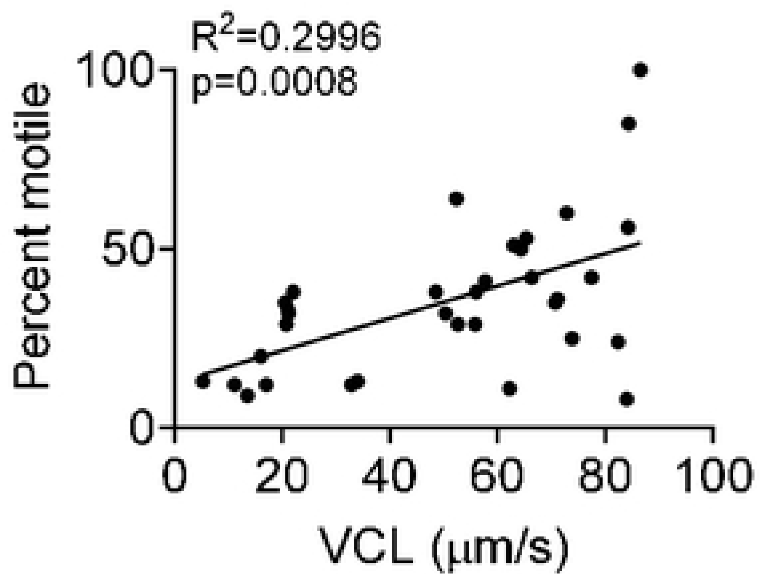

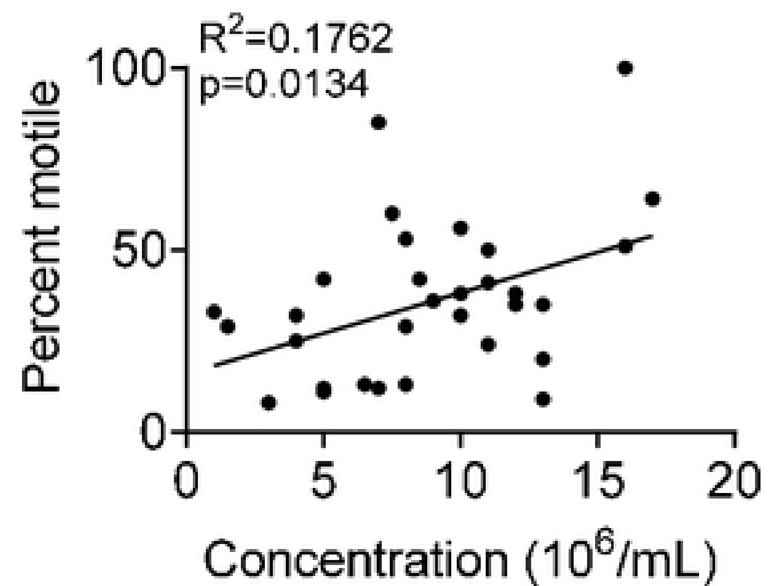

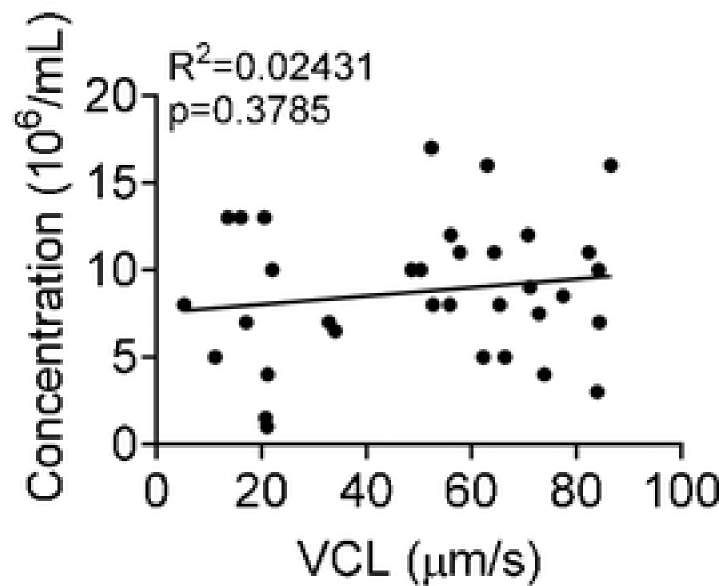

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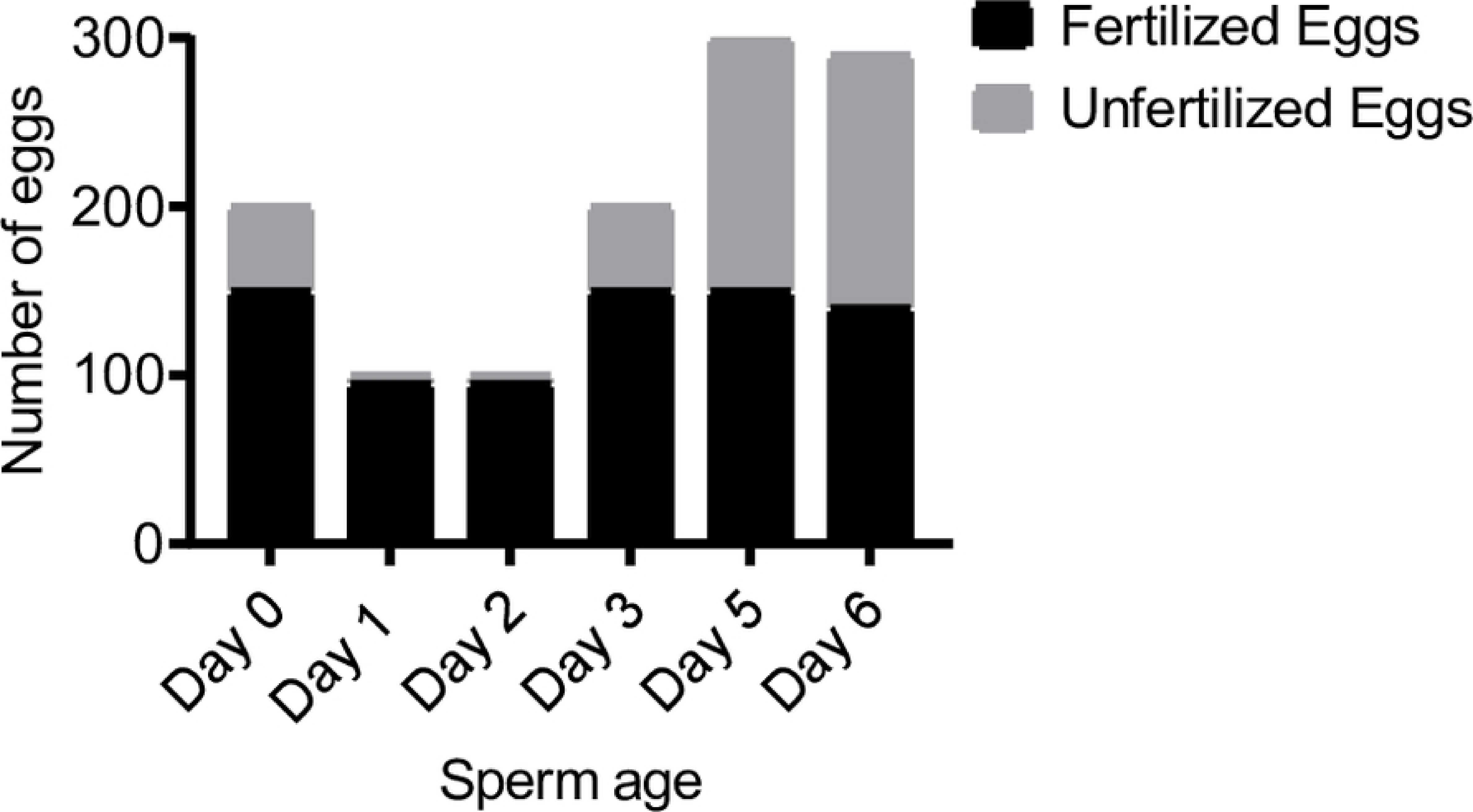

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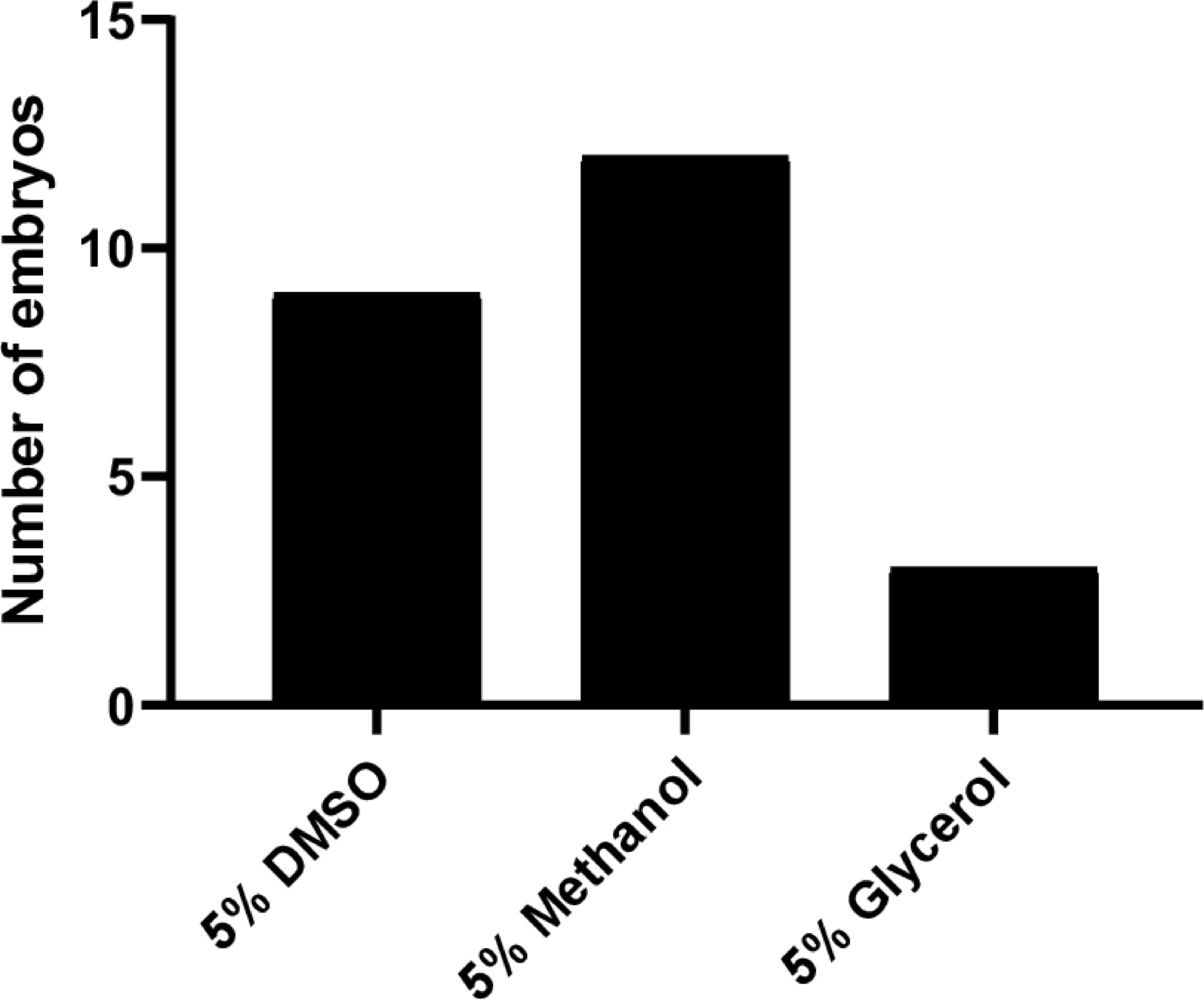

Fig5

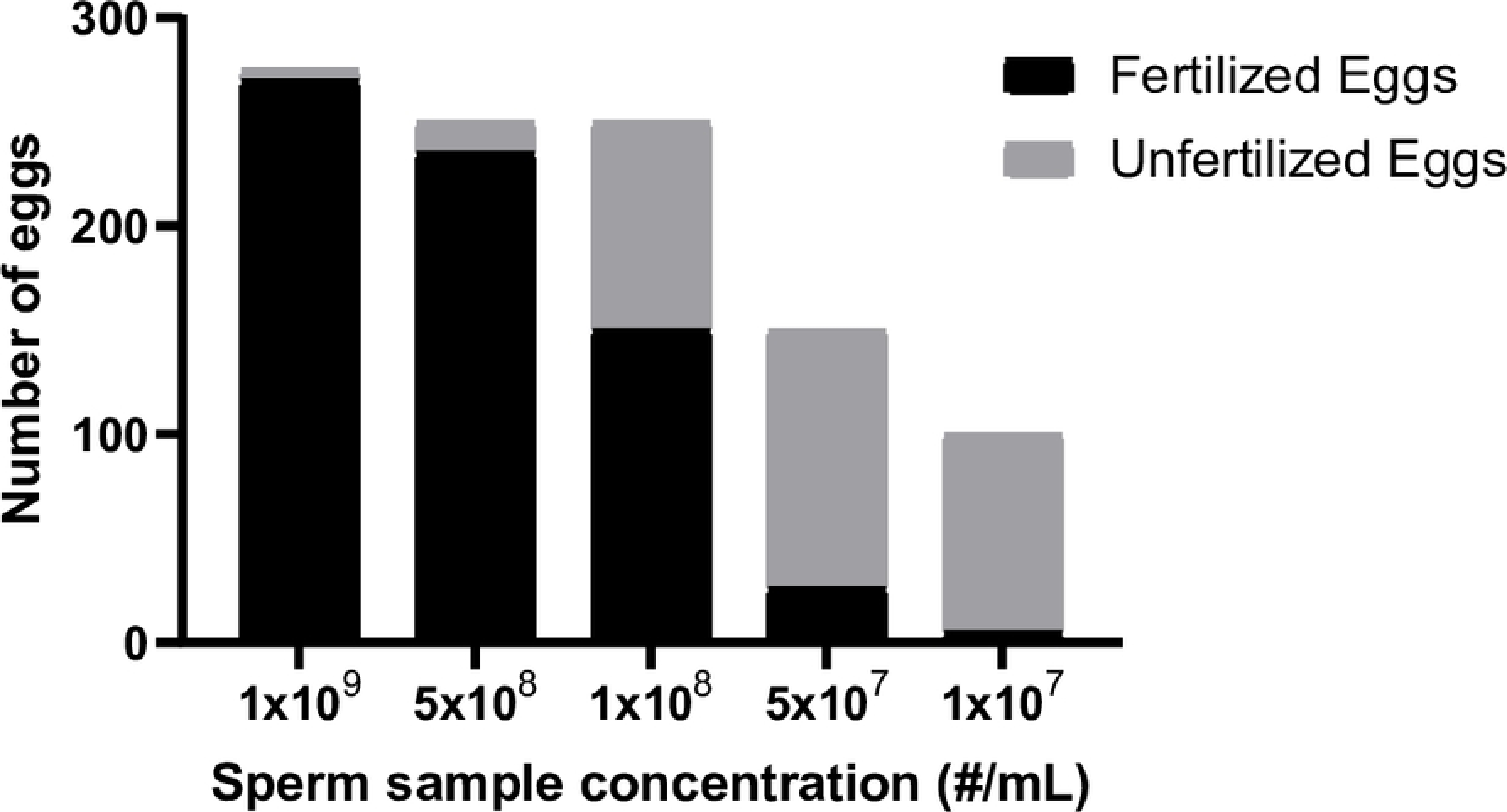

Fig6