Sperm cryopreservation and in vitro fertilization techniques for the African turquoise killifish *Nothobranchius furzeri*

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1 **Sperm cryopreservation and *in vitro* fertilization techniques for the African**
2 **turquoise killifish *Nothobranchius furzeri***

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13 **Abstract**

14 Over the last decade, the African turquoise killifish, *Nothobranchius furzeri*, has
15 emerged as an important model system for the study of vertebrate biology and
16 ageing. *N. furzeri* propagation, however, can pose challenges due to the short
17 window of fertility, the efforts and space requirements involved in continuous
18 strain maintenance, and the risks of inbreeding. The current method for long
19 term strain preservation relies on arrest of embryos in diapause. Though useful,
20 it remains unclear how long embryos can survive in this state. To create an
21 alternative for long term maintenance, we developed a robust protocol to
22 cryopreserve and revive sperm for *in vitro* fertilization (IVF). We tested a variety
23 of extender and activator buffers for sperm IVF, as well as cryoprotectants to
24 achieve feasible long-term storage and fertilization conditions tailored to this
25 species. Our protocol enabled sperm to be preserved in a cryogenic condition for
26 months and to be revived an average of 40% viability upon thawing. Thawed
27 sperm were able to fertilize nearly the same number of eggs as natural
28 fertilization, with an average of ~25% and peaks of ~55% fertilization. This
29 technical advance will greatly facilitate the use of *N. furzeri* as a model organism.

30

31 **Abbreviations used:**

32 **HBSS:** Hank's Balanced Salt Solution

33 **BSMIS:** Buffered sperm motility-inhibiting solution

34 **FBS:** Fetal bovine serum

35 **DMSO:** Dimethyl sulfoxide

36 **DMF:** Dimethylformamide

37 **MetOH:** Methanol

38 **DMA:** Dimethylacetamide

39 **IVF:** *In vitro* fertilization

40

41 **Introduction**

42 Over the last few years, the African killifish *Nothobranchius furzeri* has
43 emerged as an important model system for the study of vertebrate ageing. *N.*
44 *furzeri*'s life cycle is characterized by a fast growth rate, reaching sexual maturity
45 by 4-5 weeks, and a maximum lifespan of 6.5-7 months in the wild type strain
46 GRZ-AD ^{1,2}, making this fish among the shortest-lived vertebrate species bred in
47 captivity and a unique platform for the rapid exploration of ageing and age-
48 associated diseases ³.

49 Rapid growth, maturation and ageing carry drawbacks, however, which
50 make maintenance in captivity of *N. furzeri* challenging. It leads to a swift
51 passing of generations, which makes it difficult to maintain a primary founder
52 genotype and prevent inbreeding ^{4,5}. Fertility is limited to the 5th-20th week and
53 maximal between the 6th -11th weeks ⁶⁻⁸ of life, giving a narrow breeding window,
54 whereas in other fish species the breeding window can correspond to years ⁹.
55 Importantly, the success of transposon-mediated transgenesis ¹⁰ and CRISPR-
56 mediated mutagenesis ¹¹ in *Nothobranchius* means that more genetically

57 engineered lines require continuous maintenance and space usage. Breeding to
58 preserve a line takes significant effort and carries the risk of accident or infection
59 that can result in strain loss. Furthermore, *N. furzeri* husbandry requires
60 considerable space, since they are often optimally grown when singly housed
61 because of fish-to-fish harassment and food competition ⁸.

62 Given these constraints, it is essential to develop protocols to maintain
63 stocks without constant breeding. Currently, the main way to maintain strains is
64 through diapause, a state of arrested development ². To date, it is difficult to
65 induce and release diapause in a controlled and synchronized manner from a
66 large pool of embryos ^{12,13}, though recently vitamin D has been shown to
67 regulate diapause in other killifish species ¹². Moreover, eggs in diapause need
68 periodic maintenance, since the medium and substrate must be checked,
69 cleaned and changed ⁸. As the number of strains and lines increase, this method
70 becomes untenable at larger scales.

71 To solve these problems, fish researchers rely on sperm cryopreservation
72 and *in vitro* fertilization techniques. Through cryopreservation, fish genetic pools
73 can be stored in sperm banks with minimum maintenance effort for years. Upon
74 revival, thawed sperm can usually fertilize eggs with a success rate from 10-80%
75 ¹⁴. Specific protocols have been established to preserve and activate the sperm
76 of various production and research species ¹⁵. To date, however, there is no
77 established protocol available for sperm cryopreservation and *in vitro* fertilization
78 for killifish species. Here we developed a feasible protocol for Killifish sperm
79 cryopreservation and *in vitro* fertilization. Our method will facilitate the husbandry
80 and the usefulness of *N. furzeri* as a model organism for research.

81 **Results**

82 **Extender and Activator**

83 The optimum conditions for spermatozoa cryopreservation and *in vitro*
84 fertilization can vary greatly for each fish species. For most freshwater fish,
85 sperm motility is initiated by hypotonic osmolalities ¹⁶ and/or by alteration of ion
86 concentration such as potassium or calcium ^{17,18}. Once activated, the sperm
87 exhibit a short period of motility (30 s to 5 min), depending on species ¹⁶.

88 Protocols from other fish species make use of an “extender” and an
89 “activator” solution. The extender is usually a high molality saline-buffered
90 solution mixed with the extracted sperm that keeps them stable and inactive, and
91 is comparable to the ion concentrations inside the gonad ^{14,19}. By contrast, the
92 activator is a low molality solution, which when added to the sperm-extender
93 solution, initiates sperm activity and movement. In some cases, the activator is
94 simply a dilution of the extender. In addition, specific concentrations of ions like
95 potassium, calcium and magnesium are required for proper sperm activation,
96 motility, directionality and endurance ^{14,20}.

97 For Zebrafish and Medaka there are several useful existing protocols.
98 These typically use various solutions, such as Hank's Balanced Salt Solution
99 (HBSS), Buffered sperm motility-inhibiting solution (BSMIS) or Fetal bovine
100 serum (FBS) as extender. A dilution of these solutions or the addition of another
101 composite salt, such as Instant Ocean or Iwamatsu solution, trigger activation
102 ^{15,21,22}. Our first goal therefore was to test sperm activation using these known
103 solutions. Sperm was collected from male fish between 9 and 11 weeks of age
104 through dissection and gonad isolation. Before dissection, fish were carefully

105 dried to prevent spontaneous sperm activation due to the presence of water.
106 Testes were placed into various solutions (extenders at various dilutions), in an
107 Eppendorf tube and quickly shaken for 5-10 seconds to release sperm into the
108 medium. We tested tank water, deionized water, BSMIS, FBS, HBSS and
109 Iwamatsu solution at different dilutions and molalities (Figure 1).

110

111

112 **Figure 1: Fresh sperm activation in different buffers.** (A) Motility tracing of different types of sperm movement that
 113 occur after activation, resulting in proper directional movements (left) or erratic inefficient vibrating movements
 114 (right). (B) Representative images of sperm movement in activated by 1:1 dilution of the indicated buffer conditions
 115 (C) Quantification of sperm activation in different buffers calculated as the average of number of active sperm
 116 divided by total sperm among biological replicates. Asterisks indicate solutions that are significantly more effective

117 than tank water in activating sperm based on a two way Anova post hoc Dunnett's test. . *p < 0.05, **p < 0.01, ***p
118 < 0.001. N = 3 biological replicates for each condition.

119

120 Immediately afterwards, the gonads were removed from the medium, 10 µl of the
121 mixture was put in a hemocytometer chamber and sperm movements were
122 video-recorded under a microscope. The videos were subsequently analyzed
123 with Imaris[®] and the percentage of directionally moving spermatozoa measured
124 (Figure 1A,B,C).

125 Tank water, the natural medium where fish biologically breed, was able to
126 activate ~40% of the sperm. Deionized water was able to trigger an initial
127 activation, which faded within seconds (data not shown), resulting immediately
128 afterwards in vibrating or immobile sperm. Stock solutions of BSMIS, FBS, HBSS
129 and Iwamatsu solutions kept the sperm inactive (Figure 1C, Extender part 1,
130 deionized water part 0), but upon dilution (Figure 1C, extender part 1, deionized
131 water parts >0) could activate sperm. Some BSMIS and FBS dilutions had
132 activation rates comparable to tank water, while BSMIS diluted with 1 part of
133 deionized water had significantly higher activation compared to tank water.
134 HBSS and Iwamatsu solutions were not significantly better than tank water as
135 activators at any dilution.

136 FBS is often used as an extender, which when diluted with other solutions
137 activates sperm^{21,23,24}. Aoki et al. used FBS, and then added 2 volumes of
138 Iwamatsu solution to promote directional activation in Medaka, the species
139 closest to *Nothobranchius* among model fish. We therefore tested FBS buffer as

extender, and added lwamatsu, as well as BSMIS and HBSS at different dilutions for activation (Figure 2A).

Figure 2: Fresh sperm activation using FBS as extender and different buffers for dilution. (A) Image tracking of sperm movements and quantification of directional sperm in different activating solutions, mixed in a ratio 1 to 3 with the FBS extender solution (1 volume of extender solution plus 2 volumes of activating solution at different dilutions). Ratios of extender, activator, and water for each mix are shown below each column. (B) Image tracking of sperm movements and quantification of directional sperm in different activating solutions, mixed in a ratio of 1 to 10 with the FBS extender solution (1 volume of extender solution plus 9 volumes of activating solution at different dilutions). Ratios of extender, activator, and water for each mix are shown below each column. Asterisks indicate solutions significantly more effective than tank water in activating sperm, based on a two way Anova post hoc Dunnett's test. *p < 0.05, **p < 0.01, ***p < 0.001. N=3 biological replicates for each condition.

Among the combinations, we observed the highest activation using FBS extender together with a dilution 1:4 of BSMIS (i.e. 1 part of FBS mixed with 0.5 parts of BSMIS and 1.5 parts of deionized water), reaching yields of >60%. In

155 contrast to *Medaka*, FBS mixed with Iwamatsu did not give appreciable
156 activation of *Nothobranchius* sperm. In another set of experiments, we
157 increased the dilution ratio between the extender (FBS) and the activators, from
158 1 plus 2 volumes respectively to 1 plus 9 volumes, respectively (Figure 2B).
159 However, the activation rates obtained in these experiments (Figure 2B) were
160 significantly worse than the previous test (Figure 2A), and most of the
161 spermatozoa were vibrating or not moving at all.

162 **Cryoprotection, Freezing and Thawing**

163 For sperm to survive freezing and kept long-term in a cryostatic condition,
164 the extender has to be supplemented with a cryoprotectant. These chemicals
165 surround sperm cells and prevent the formation of ice crystals that compromise
166 membrane integrity, thereby protecting them from cryodamage ²⁵. The most
167 commonly cryoprotectants used are DMSO, DMF, MetOH, glycerol, and DMA,
168 with concentrations usually set around 10% of the volume ^{15,26}. Since FBS and
169 BSMIS worked best as extenders (Figure 1C, 2A), we performed our cryostatic
170 experiments using these solutions. We mixed the selected extender with 10% of
171 DMSO, DMF, MetOH, DMA or glycerol. Sperm was released in pre-mixed
172 extender-cryoprotectant solutions (Figure 3C) and the mixture was incubated for
173 1h at 4°C (Figure 3D), allowing the cryoprotectants to be evenly absorbed by the
174 sperm cells. Samples were then aliquoted in 60ul volumes (Figure 3E), frozen in
175 dry ice (Figure 3F), and nitrogen gas phase in sequence (Figure 3G), and stored
176 in liquid nitrogen (Figure 3H).

Figure 3: Schematic of sperm collection and freezing procedure. (A) Male dissection. (B) Gonad extraction. (C) Gonads are spun in extender plus cryoprotectant solution. (D) Cryoprotectant incubation with sperm cells at 4°C. (E) Solution transfer in smaller aliquots to vials. (F) First freezing step setup with vials laying on the bottom of a glass beaker surrounded by dry ice in a closed styrofoam box. (G) Second freezing step setup with vials exposed to nitrogen vapor. (H) Long term storage of vials in liquid nitrogen. For a detailed explanation, see text.

After 24–48h freezing, the sperm were revived through rapid thawing in a 30°C water bath (see optimization below) and activated by the addition of 2 volumes of BSMIS (diluted 1:4 in the case of FBS extender and 1:2 in the case of BSMIS extender). Overall we found that sperm cryopreserved using 10% DMSO as cryoprotectant maintained the highest level of activation (Figure 4A), followed by methanol (in FBS extender only). DMF, Glycerol and DMA were ineffective at cryoprotection, resulting in immobile or inviable sperm upon thawing.

Figure 4: Cryoprotectants, cryopreservation and thawing efficiencies. (A) Effect of different cryoprotectants on sperm activation using BSMIS and FBS extender solutions. Y bar represents the portion of sperm able to reactivate upon thawing. Statistical analysis used is two way Anova with multiple comparison Tukey's post hoc correction. In BSMIS extender, DMSO performed significantly better compared to the other cryoprotectants. In FBS extender, DMSO and MetOH performed significantly better compared to the other cryoprotectants. (B) Effect of different concentrations of cryoprotectants in FBS extender. Asterisks indicate significance levels of two way Anova multiple comparison test with post hoc Dunnett's correction. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (C) Different freezing methods with different cooling rates applied to FBS extender plus 10% cryoprotectant. Asterisks indicate significance levels of two way Anova multiple comparison test with post hoc Sidak's correction. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (D) Different thawing methods (30°C water bath, 4°C refrigerator, RT, room temperature) with different thawing rates applied to FBS extender plus 10% DMSO. Asterisks indicate significance levels of one way Anova multiple comparison test with post hoc Tukey's correction. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. N = 3 biological replicates for each condition.

204 Several concentrations of cryoprotectants were further tested in combination with
205 FBS. Sperm cryopreserved in FBS with 10 to 20% DMSO maintained
206 comparably high activation ranging from 30-37% (Figure 4B), dropping slightly at
207 higher concentrations. Methanol was able to moderately protect sperm from
208 cryodamage at several concentrations, giving sperm activation of ca. 20%. DMA
209 or DMF gave less than 10% sperm revival at any concentration (Figure 4B).

210 Since >20% sperm survival and activation were observed in the samples with
211 DMSO and methanol at 10%, we used these concentrations of cryoprotectants
212 for further experiments.

213 The freezing procedure is also a crucial step for cryopreservation. Slow freezing
214 rates can produce large ice crystals that damage cellular ultrastructure, whereas
215 rapid freezing rates induce smaller intracellular ice crystals that are less likely to
216 trigger damage²⁷. We therefore used various setups to establish different rates
217 of freezing. We placed the Eppendorf tubes in 1) Mr. Frosty™ Freezing
218 Container (estimated -10°C per min), 2) a glass beaker surrounded by dry ice
219 (estimated -20°C per min), 3) direct contact with dry ice (estimated -50°C per
220 min), 5) a Dewar vessel partially filled with liquid nitrogen, where the Eppendorf
221 tubes were placed in a box without direct contact with liquid nitrogen but exposed
222 to nitrogen gas phase (estimated -100°C per min); and 6) direct liquid nitrogen
223 contact (estimated -200°C per min) (Figure 4C). Cooling rates were estimated
224 using a digital thermometer with a detection range -50°C / +110°C with the
225 probe inserted in the tube, measuring the temperature drop divided by the time
226 required to reach -50°C. 15 minutes after a temperature below -50°C was
227 reached, sperm vials from the various freezing setups were stored in liquid
228 nitrogen overnight. After one day, frozen sperm samples were revived using

BSMIS 1:4 and monitored for activation. Sperm vials frozen in a beaker surrounded by dry ice (-20°C per min) achieved the highest activation upon revival. Sperm cryoprotected with 10% DMSO had higher activation than those cryoprotected with 10% methanol (Figure 4C), similar to above. Therefore, we selected DMSO as the final cryoprotectant and a beaker surrounded by dry ice for our cryopreservation protocol.

To assess the effect of different thawing rates on the frozen sperm, we warmed the vials at 1) 30°C in a water bath, 2) room temperature or 3) 4°C in the refrigerator, to achieve rapid, medium, or slow thawing rates, respectively. We then revived sperm using BSMIS 1:4 and observed the sperm activation rate. We found that rapid thawing in a 30°C water bath achieved the highest survival and activation of sperm (Figure 4D).

241

Egg fertilization and survival

We next tested if the active sperm obtained with our protocol was able to fertilize eggs obtained from *N. furzeri* females. To perform this experiment in the most comparable way, we used the same males for natural and *in vitro* fertilization. We set up several aquarium tanks with 5 females and 2 males per tank and allowed them to breed naturally for 2 days. The age of the animals used for fertilization were typically 9-11 weeks of age. We then collected the eggs generated from the natural breeding and monitored their survival rate until mid somitogenesis. After the natural breeding, the same males were separated from the females, kept alone for 2 days, and then sacrificed for gonad extraction

252 (Figure 3A-B). The females were anesthetized, dried carefully (Figure 5A), and
253 their unfertilized eggs gently pushed out from their ovaries (Figure 5B).

255 **Figure 5: Schematic of sperm thawing and egg fertilization procedure. (A)** Female preparation. (B) Egg collection
256 through gentle abdominal squeezing. (C) Frozen sperm thawing in a water bath. (D-E) Eggs mixed with sperm-
257 extender solution. (F) Activating solution addition and (G) mix. (H) Fertilization. (I) Egg recovery in a petri dish. (J)
258 Fertilized eggs developing in a petri dish. For detailed explanation, refer to the protocol.

259 The sperm obtained from the male gonads was mixed with FBS or BSMIS buffer
260 containing 10% DMSO. Half of the sperm-extender-cryoprotectant mixture was
261 directly activated (with 1:2 or 1:4 BSMIS) and used for the fertilization of the eggs
262 (Figure 5D-J) while the other half was frozen and cryopreserved (Figure 3). A
263 subset of the sperm was also frozen in Cryovials with the same shape of the
264 1,5mL Eppendorf tubes.

265 After 2 weeks the frozen sperm were thawed, activated and used to fertilize
266 another pool of eggs derived from 10 week old females, following the same
267 procedure (Figure 5). Sperm concentrations used for fertilization were on
268 average 60,000/ul, a value estimated by counting the number of sperm in a 3x3
269 square field Burker chamber. Eggs fertilized in both ways were monitored until
270 the stage of mid-somitogenesis (Figure 6A) or later (Figure 6B). Survival rates
271 were scored (Figure 6F). This experiment was repeated again after 4 months
272 using aliquots from our collection. Yields of fertilization achieved were
273 comparable to those after 2 weeks freezing (Figure S1).

Figure 6: Fertilization rates and embryonic development. (A) Embryos fertilized with IVF using frozen sperm develop through diapause II/mid-somitogenesis stage and later (B), up to advanced developmental stage. (C) Adult fish derived from IVF embryos. (D) Fertilization efficiencies of natural breeding, IVF with fresh extracted sperm and IVF with sperm frozen for 2 weeks. None of the condition tested resulted in fertilization rates significantly different from natural fertilization when tested with one way Anova post hoc Tukey's test. N = 6,8,4,10,3 independent fertilization trials for each fertilization condition, respectively, in order of appearance in the graph. N = 1023,440,349,485,201 total eggs used for fertilization in the trials for each fertilization condition, respectively, in order of appearance in the graph. (E) Fertilization efficiencies of IVF using sperm frozen for 50 days in Cryovials or Eppendorf tubes. The egg pool used for fertilization derived from 10 weeks old females. 3 independent trials were performed using different females and sperm aliquots. N = 45 total eggs used for cryovials (fertilized with 3 different sperm aliquots) IVF and 52 total eggs for Eppendorfs (fertilized with 3 different sperm aliquots) tests. T-Test between the two groups results was not significant.

288 To approximate the fertility rate, we counted the total number of embryos that
289 developed until mid-somitogenesis (Figure 6A) over the total initial number of
290 eggs used for fertilization (Figure 6D). For the IVF experiments, several embryos
291 were followed through development to post-somitogenesis (Figure 6B), post-
292 hatching and up to adulthood (Figure 6C). The growth rates were normal,
293 reaching maturity in 5-6 weeks, and no developmental defects were detected
294 (n=20). These fish were fertile and able to produce viable embryos (n=70). We
295 found that under our conditions, rates of fertilization with frozen sperm was
296 approximately 25% on average and were only slightly below fresh IVF or natural
297 fertilization.

298 In a final experiment we used the sperm previously extracted and frozen in
299 Cryovials and Eppendorf tubes for 50 days to fertilize eggs of 10 weeks old
300 females. In this experiment we used FBS as extender and BSMIS diluted 1:4 in
301 deionized water as activator. Results were comparable between the two groups
302 (Figure 6E), suggesting that both containers are suitable for short-medium term
303 sperm storage. The long term storage (years) remains unexplored.

304 A detailed protocol for the entire procedure of egg in vitro fertilization is found in
305 the Supplemental Methods.

306 **Cortical reaction**

307 Interestingly, we noticed that upon contact with the aqueous medium,
308 eggs could spontaneously undergo the cortical reaction even in the absence of
309 sperm, as indicated by an increase in the distance (λ) of about $52.7 \pm 10.9 \mu\text{m}$
310 between the yolk and chorion membrane (Figure 7A, 7B).

Figure 7: Cortical reaction and cell division in fertilized and unfertilized eggs. (A) Oocytes that do not undergo the cortical display closely apposed chorion membrane and yolk (λ represent the average distance of $35.0 \pm 10.6 \mu\text{m}$. $N = 8$). (B) Embryo in which the cortical reaction has occurred properly where the yolk detaches from the chorion membrane (λ represent the average distance of $87.6 \pm 11.2 \mu\text{m}$. $N = 14$). (C) Embryo undergoing correct cell division at the 4 cell stage. (D) Pseudoembryo undergoing aberrant divisions, due to missing or incorrect fertilization. Cells are odd in number and of irregular size and shape during the earliest stages. (E) Average embryonic phenotypic distribution. $N = 4$ independent natural fertilizations with 470, 332, 101, 612 eggs collected, respectively. Embryos were screened after 6 hours from collection and phenotype was assessed by eye. The graph represents the average of the 4 different collections.

321

322 Under conditions of natural fertilization, a fraction (10% to 25%) of eggs
323 underwent spontaneous cortical reaction, while a smaller (1%-10%) fraction of
324 eggs remained blocked in the pre-cortical reaction stage (Figure 7E, yellow bar).
325 Eggs that underwent a spontaneous cortical reaction, which we term
326 pseudoembryos, displayed abnormal cell cleavages (compare Figure 7D and
327 7C), errant development and embryonic death in the first 10 days (Figure 7E,
328 orange bar). Eggs that did not carry out the cortical reaction were not fertilized,
329 but evidently not all eggs that carried out the cortical reaction were fertilized
330 either. Thus the cortical reaction itself does not necessarily indicate fertilization
331 success.

332

333 Discussion

334

335 In this study, we worked out conditions for sperm activation,
336 cryopreservation and *in vitro* fertilization in the species *N. furzeri*, aiming to
337 establish protocols to facilitate line maintenance and space usage of this fish.
338 Previous publications show how a decrease in osmolality together with a species
339 specific salt combination are required for fish sperm activation [13-14].
340 Concentrating on the most used buffers and testing them in several dilutions and
341 combination, we found that *N. furzeri* requires a specific protocol tailored for this
342 species.

343 After exploring several combinations, we narrowed down the viable
344 extender solutions to FBS and BSMIS and activators to BSMIS 1:4 and 1:2.
345 Cryoprotectants, 10% DMSO and MetOH best prevented cryodamage and
346 preserved sperm viability. Moderate freezing rates in a beaker using dry ice, and
347 rapid thawing at 30°C resulted in sperm that could be readily activated. Our IVF
348 method resulted in fertilization rates that were comparable between frozen
349 sperm, fresh sperm and natural fertilization, hovering around 20-30%.
350 Cryopreserved sperm was equally active at least 4 months later.

351 Nevertheless, there are several parameter that we did not explore and
352 possible limitations of our study. First and foremost, we do not know the maximal
353 length of time that sperm can remain well preserved under these conditions.
354 Based on other reports ²⁸, we anticipate preservation could last many years. We
355 used sperm densities of ca. 60,000/μl and male age of 9-11 weeks; we did not
356 systematically optimize these parameters. Another limitation of our study is that
357 males were sacrificed in order to retrieve sperm from the testes. Future
358 techniques could be developed to collect sufficient sperm from live animals.
359 Finally, sperm preservation only represents solving half the problem. In the
360 future, it would be extremely useful to develop techniques for egg preservation
361 as well.

362 Currently most *N. furzeri* lines are kept in embryonic diapause for long-
363 term maintenance. This still remains a very useful approach because it
364 preserves lines in the form of organism, not as gametes, and does not require
365 freezers or liquid nitrogen systems that can breakdown. However, it still remains
366 unclear how long embryos can be maintained in diapause, but it is likely less
367 time than cryopreservation of sperm. Maintaining embryos in diapause is more

368 time consuming and takes up greater space. Whether animals in embryonic
369 diapause can also be cryopreserved is also unknown. In any case, sperm
370 cryopreservation techniques and IVF can complement existing techniques of
371 diapause preservation.

372 One of the unexpected observations we made in the course of our studies
373 is that a considerable number of oocytes undergo the cortical reaction even in
374 the absence of sperm, with either IVF or natural fertilization. Such
375 “pseudoembryos” displayed abnormal development. Though the mechanism
376 underlying spontaneous cortical reaction remains unknown in this species, it is
377 thought to be related to the degree of egg maturation ^{29,30}. Whether conditions
378 can be improved to minimize such events to increase the fraction of fertilizable
379 oocytes is another parameter that deserves future attention.

380 **Conclusion**

381 In sum, we worked out conditions feasible for sperm cryopreservation and in vitro
382 fertilization in *Nothobranchius furzeri*. Our protocol provides useful techniques
383 that can enhance existing approaches for line maintenance and husbandry,
384 which should greatly facilitate the use of *Nothobranchius furzeri* as a model
385 organism.

386

387

388

389

390 **Materials and Methods**

391 **Fish husbandry and sample collection**

392 All adult fish used were *Nothobranchius furzeri* belonging to the GRZ strain and
393 were raised singularly in 2.8L tanks from the second week of life. Water
394 parameters were pH: 7-8; Kh: 3-5; T: 27 °C. 10% of the water in the system was
395 automatically replaced with clean water every day. Fish were raised in 12 hours
396 of light and 12 hours of darkness. Fish were fed with chironomus twice a day and
397 “Premium Artemia Coppins[®]” twice a day. Natural breeding events occurred in
398 8L tanks with 1 male and 3-5 females. To initiate breeding, one box (9cm x 9cm
399 x 4cm) half full of river sand ($\varnothing < 0,2\text{mm}$) was put on the bottom of the tank. The
400 boxes were put in the fish tanks for 2-3 hours during which the average number
401 of eggs laid by each female in the sand was between 5 -30. All the fish used for
402 the experiments were from 9-11 weeks old.

403 **Ethical approval**

404 All fish husbandry was performed in the fish facility of the Max Planck Institute for
405 Biology of Aging (Permit Nr. 576.1.36.6.G12/18 Be). The animal experiments
406 were approved by the Institutional Animal Welfare Body (RB.16.005) and
407 complied with national legislation and the European directive 2010/63/EU. The
408 study was overseen and reported to the appropriate authority, the Veterinarian
409 State Office Cologne.

410

411 **Gonad extraction and sperm collection**

412 Males were anesthetized with tricaine methanesulfonate (Sigma, 0.5 mg/ml) for
413 10+ minutes, until movement and breathing ceased. Fish were dried using tissue

414 paper and decapitated. The abdomen was cut open with scissors, the organs
415 removed and the testes flanking the swimming bladder were gently extracted
416 using a forceps. Gonads were placed in an Eppendorf tube containing 500 µl of
417 extender solution, held with forceps and spun back and forth for 1 min. After the
418 sperm was released into the solution, the gonads were removed from the
419 Eppendorf tube. On average around 30 million sperm (between 7,5 million and
420 100 million) were released. This value was estimated by counting the sperm cells
421 of several sperm-extender aliquots in a 3x3 square field of a Burkner chamber.
422 The initial total sperm value was calculated multiplying the number obtained by
423 the dilution factor.

424 **Fresh solutions trials (Figure 1)**

425 Each individual gonad was placed in 250µl of solution (such as tank water, DE
426 water, BSMIS, HBSS, FBS, Iwamatsu solution) at dilutions of (1:1, 1:2, 1:4, 1:8)
427 and the sperm was released inside by shaking for 5-10 seconds. 10µl of each
428 solution+sperm mix were placed in a hemocytometer chamber immediately after
429 the activation of sperm, visualized and recorded. Each trial combination was
430 repeated at least 3 times with gonads derived from different fish.

431 BSMIS: (75mM NaCl, 70mM KCl, 2mM CaCl₂, 1mM MgSO₄, 20mM Tris pH8.0)

432 HBSS: (Gibco™ #14025-092)

433 FBS: (Gibco® #10270-106)

434 **Extender solution sperm activation trials (Figure 2)**

435 One gonad was extracted and shaken in extender solutions as described above.

436 One volume (3µl) of sperm-extender mixture was mixed with two (6µl) or nine

437 volumes (27 μ l) of activator solutions and immediately transferred to the
438 hemocytometer chamber under the microscope for video recording. Each trial
439 combination was repeated at least 3 times with gonads derived from different fish
440 shaken in the extender.

441 **Imaging and video acquisition**

442 For sperm visualization, we used a Zeiss Imager Z1 microscope and recorded
443 videos with a Zeiss Axiocam 506 mono, using parameters of binning
444 3x3 ,resolution 2752x2208 pixels, and the ZEN 2.3 Pro software. Each video was
445 recorded for 8 seconds.

446 **Imaris conversion and sperm motility analysis.**

447 Videos were imported in Imaris 8.1 and frame time points rescaled by 10 times.
448 Black and white colors were inverted in order to visualize white dots over a black
449 background. The particle tracking function was set to identify all sperm with a
450 diameter of $\pm 2.9\mu$ m. Movements were tracked with the autoregressive motion
451 algorithm function, set with maximum distance of 25 μ m and a maximum gap
452 size of 5. Statistics relative to track displacement length were exported as excel
453 file and analyzed. As a cutoff, we set a displacement length of $>15\mu$ m to
454 distinguish directionally traveling sperm from non-active or vibrating sperm. In
455 case of general drift of the whole sample size we increased the cutoff to $>35\mu$ m
456 or $>50\mu$ m (in case of very strong drift).

457 **Cryoprotectant trials (Figure 4A,B)**

458 For each biological replicate the two testes were extracted from a male and were
459 shaken into 500 μ l of different extender solutions (FBS or BSMIS). In total we
460 used 3 biological replicates for each extender combination. Mixes were aliquoted

461 in smaller volumes (60µl) and different cryoprotectants (DMSO (Sigma), DMF
462 (Carl Roth), Methanol (Optima), DMA (Sigma) or glycerol ≥99.5% (Sigma) were
463 added in different concentrations in each Eppendorf and immediately mixed. 3µl
464 from each sample were taken and mixed with an activation solution to check the
465 sperm mobility, following the procedure previously described. The rest of each
466 mix was frozen following the cryopreservation procedure. Sperm survival was
467 checked after two weeks upon thawing (see below).

468 **Cryopreservation (Figure 5)**

469 Extender-sperm-cryoprotectant mixtures were incubated in 1.5ml Eppendorf
470 tubes for 1 hour at 4°C. The mixtures were distributed in Eppendorf tubes at a
471 volume of 60µl each, the tubes were closed and gently placed at the bottom of a
472 glass beaker sitting in a styrofoam box filled up to 10cm with dry ice. After 15
473 minutes, Eppendorf tubes were quickly moved into a rack in a nitrogen tank,
474 exposed to nitrogen vapors for 30 minutes. Samples were moved directly into
475 liquid nitrogen for long term storage.

476 **Freezing trials (Figure 4C)**

477 Both gonads from the same individual were placed in 500µl FBS solution and
478 swung for sperm release as previously described. 60µl of the resulting mix were
479 mixed half with 10% DMSO and half with 10% methanol in Eppendorf tubes (or
480 Cryovials, Sarstedt #72.692.005) and incubated for 1 hour at 4°C. Vials were
481 distributed to Mr. frosty container (-10°C per min), a beaker surrounded with dry
482 ice (-20°C per min), directly on dry ice (-50°C per min), directly in nitrogen gas
483 phase (-100°C per min), directly in nitrogen liquid (-200°C per min) phase for 30
484 minutes, until completely frozen. The samples were placed in the nitrogen liquid

485 phase after they reached a temperature of below -50°C. The thawing and
486 monitoring process were performed as described in the main text.

487 **Thawing and sperm revival (Figure 4 D)**

488 Frozen samples were thawed directly from liquid nitrogen into a 30°C warm
489 water bath until the ice completely melted. The procedure usually took 1 minute
490 or less. One volume (3µl) of thawed sperm mixtures was mixed with two volumes
491 (6µl) of BSMIS 1:4 and pipetted into a hemocytometer chamber to record sperm
492 movements.

493 ***In vitro* fertilization by thawed sperm (Figure 5,6)**

494 Females were anesthetized in a Tricaine methanesulfonate solution (0.5mg/ml)
495 and carefully dried of residual water to prevent spontaneous egg cortical reaction
496 or activation (Figure 5A). Placing the fish on an open hand, a gentle pressure
497 was applied with a finger on the abdomen, pushing gently from the middle
498 toward the anus (Figure 5B). Typically 5 to 35 eggs were expelled. Eggs were
499 collected using a forceps into an Eppendorf tube containing the extender-sperm
500 solution (Figure 5D). In the case of frozen sperm, the tube was thawed
501 immediately before fertilization in a 30°C water bath (Figure 5C). These steps
502 were best performed by two people, with one person thawing the sperm while
503 the other person expelled eggs from the female. Eggs were placed at the edge of
504 the tube and gently pushed to the bottom (Figure 5D). We used aliquots of
505 sperm-extender of 60 µl and no more than 35 eggs per aliquot. Once the eggs
506 were immersed, the tube was gently flicked for 10-20 seconds (Figure 5E),
507 allowing homogeneous distribution of sperm around the eggs. The activator
508 solution was then pipetted into the tube, letting drops slide over the tube's border

509 (Figure 5F) and then mixed with the extender by gently flicking the tube for 20-30
510 seconds (Figure 5G). The activated sperm was incubated with the eggs for 10
511 minutes with the tubes standing open on a bench at room temperature (Figure
512 5H). At this step, 10ul of the mixture was examined under the microscope to
513 evaluate sperm motility. To avoid damage due to DMSO toxicity, the embryos
514 were transferred after 10 minutes by pipette to a petri dish and methylene blue
515 buffered tank water (Figure 5I). The water was replaced twice and the petri dish
516 incubated at 28°C (Figure 5J).

517

518

519 **Embryo phenotype assessment and cortical reaction. (Figure 7)**

520 10 breeding tanks were set up with 1 male and 4 females each. The fish were
521 allowed to breed for 3-4 hours and then eggs were collected and cleaned of
522 empty shells or dead eggs and allowed to develop in an incubator at 28°C for 6
523 hours. Embryos were then carefully examined under a Leica M80
524 stereomicroscope to assess morphology, cortical reaction, cell shape and
525 number. Four independent breedings were performed with at least 2 days
526 between them. After 6 hours in the incubator, each breeding event yielded 470,
527 332, 101, 612 embryos, respectively. Data regarding the percentages of the various
528 phenotypes were averaged between the 4 collections to produce the graph.

529 **Graph production images acquisition and enhancement**

530 Raw data depicting track displacement length realized with Imaris were exported
531 as an excel chart. Data relative to all the experiments were pooled in a unique
532 excel file, containing 3 replicates for each individual trial or combination. Total

533 data were used to calculate averages, standard deviations and standard errors,
534 in case of mean of the means. Graphs were produced from these data using
535 excel.

536 Tracking images were acquired from videos using Imaris snapshot function.
537 Brightfield images were acquired using a Leica M80 microscope equipped with a
538 Leica MC170 HD camera. Images were enhanced in brightness, contrast and
539 saturation using GIMP to improve the visual quality. Quantifications were not
540 affected by imaging quality enhancing modifications.

541 Graphics and drawings were realized using Paint, GIMP and Power point.

542

543 **Data availability**

544 All data generated or analysed during this study are included in this
545 published article (and its Supplementary Information files).

546 The datasets generated during and/or analysed during the current study
547 are available from the corresponding author on reasonable request.

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628

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634 **Author contributions**

635 LD: Conceived and designed the analysis, collected and analyzed the data,

636 wrote the paper.

637 TKS: Conceived and designed the analysis, collected and analyzed the data,

638 revised the paper.

639 RR: Analyzed the data, revised the paper.

640 AA: Conceived and designed the analysis, wrote the paper.

641 **Additional information**

642 **Competing interests**

643 The author(s) declare no competing interests.

644 **Supporting information**

645 **Figure S1. Sperm resilience in FBS + 10% DMSO extender.** Fertilization efficiencies of IVF
646 with sperm frozen for 2 weeks or 4 months. N = 10 independent fertilization trials (485 total
647 eggs used) with sperm frozen for 2 weeks in FBS + 10% DMSO extender and N = 1
648 fertilization trial (52 total eggs used) with sperm frozen for 4 months in FBS + 10% DMSO
649 extender.

650 **S1 File. IVF protocol.** This is the protocol that we suggest and that uses FBS as extender,
651 DMSO 10% as cryoprotectant and BSMIS 1:4 as activator.

Figures

Figure 1

Fresh sperm activation in different buffers. (A) Motility tracing of different types of sperm movement that occur after activation, resulting in proper directional movements (left) or erratic inefficient vibrating movements (right). (B) Representative images of sperm movement in activated by 1:1 dilution of the

indicated buffer conditions (C) Quantification of sperm activation in different buffers calculated as the average of number of active sperm divided by total sperm among biological replicates. Asterisks indicate solutions that are significantly more effective than tank water in activating sperm based on a two way Anova post hoc Dunnett's test. . *p < 0.05, **p < 0.01, ***p < 0.001. N = 3 biological replicates for each condition.

Figure 2

Fresh sperm activation using FBS as extender and different buffers for dilution. (A) Image tracking of sperm movements and quantification of directional sperm in different activating solutions, mixed in a ratio 1 to 3 with the FBS extender solution (1 volume of extender solution plus 2 volumes of activating solution at different dilutions). Ratios of extender, activator, and water for each mix are shown below each column. (B) Image tracking of sperm movements and quantification of directional sperm in different activating solutions, mixed in a ratio of 1 to 10 with the FBS extender solution (1 volume of extender solution plus 9 volumes of activating solution at different dilutions). Ratios of extender, activator, and water for each mix are shown below each column. Asterisks indicate solutions significantly more effective than tank water in activating sperm, based on a two way Anova post hoc Dunnett's test. *p < 0.05, **p < 0.01, ***p < 0.001. N=3 biological replicates for each condition.

Figure 3

Schematic of sperm collection and freezing procedure. (A) Male dissection. (B) Gonad extraction. (C) Gonads are spun in extender plus cryoprotectant solution. (D) Cryoprotectant incubation with sperm cells at 4°C. (E) Solution transfer in smaller aliquots to vials. (F) First freezing step setup with vials laying on the bottom of a glass beaker surrounded by dry ice in a closed styrofoam box. (G) Second freezing step setup with vials exposed to nitrogen vapor. (H) Long term storage of vials in liquid nitrogen. For a detailed explanation, see text.

Figure 4

Cryoprotectants, cryopreservation and thawing efficiencies. (A) Effect of different cryoprotectants on sperm activation using BSMIS and FBS extender solutions. Y bar represents the portion of sperm able to reactivate upon thawing. Statistical analysis used is two way Anova with multiple comparison Tukey's post hoc correction. In BSMIS extender, DMSO performed significantly better compared to the other cryoprotectants. In FBS extender, DMSO and MetOH performed significantly better compared to the other cryoprotectants. (B) Effect of different concentrations of cryoprotectants in FBS extender. Asterisks indicate significance levels of two way Anova multiple comparison test with post hoc Dunnett's correction. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (C) Different freezing methods with different cooling rates applied to FBS extender plus 10% cryoprotectant. Asterisks indicate significance levels of two way Anova multiple comparison test with post hoc Sidak's correction. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (D) Different thawing methods (30°C water bath, 4°C refrigerator, RT, room temperature) with different thawing rates applied to FBS extender plus 10% DMSO. Asterisks indicate significance levels of one way Anova multiple comparison test with post hoc Tukey's correction. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. N = 3 biological replicates for each condition.

Figure 5

Schematic of sperm thawing and egg fertilization procedure. (A) Female preparation. (B) Egg collection through gentle abdominal squeezing. (C) Frozen sperm thawing in a water bath. (D-E) Eggs mixed with sperm-extender solution. (F) Activating solution addition and (G) mix. (H) Fertilization. (I) Egg recovery in a petri dish. (J) Fertilized eggs developing in a petri dish. For detailed explanation, refer to the protocol

Figure 6

Fertilization rates and embryonic development. (A) Embryos fertilized with IVF using frozen sperm develop through diapause II/mid-somitogenesis stage and later (B), up to advanced developmental stage. (C) Adult fish derived from IVF embryos. (D) Fertilization efficiencies of natural breeding, IVF with fresh extracted sperm and IVF with sperm frozen for 2 weeks. None of the condition tested resulted in fertilization rates significantly different from natural fertilization when tested with one way Anova post hoc Tukey's test. N = 6,8,4,10,3 independent fertilization trials for each fertilization condition, respectively, in order of appearance in the graph. N = 1023,440,349,485,201 total eggs used for fertilization in the trials for each fertilization condition, respectively, in order of appearance in the graph. (E) Fertilization efficiencies of IVF using sperm frozen for 50 days in Cryovials or Eppendorf tubes. The egg pool used for fertilization derived from 10 weeks old females. 3 independent trials were performed using different females and sperm aliquots. N = 45 total eggs used for cryovials (fertilized with 3 different sperm aliquots) IVF and 52 total eggs for Eppendorfs (fertilized with 3 different sperm aliquots) tests. T-Test between the two groups results was not significant.

Figure 7

Cortical reaction and cell division in fertilized and unfertilized eggs. (A) Oocytes that do not undergo the cortical display closely apposed chorion membrane and yolk (λ represent the average distance of $35.0 \pm 10.6 \mu\text{m}$. $N = 8$). (B) Embryo in which the cortical reaction has occurred properly where the yolk detaches from the chorion membrane (λ represent the average distance of $87.6 \pm 11.2 \mu\text{m}$. $N = 14$). (C) Embryo undergoing correct cell division at the 4 cell stage. (D) Pseudoembryo undergoing aberrant divisions, due to missing or incorrect fertilization. Cells are odd in number and of irregular size and shape during the earliest stages. (E) Average embryonic phenotypic distribution. $N = 4$ independent natural fertilizations

with 470, 332, 101, 612 eggs collected, respectively. Embryos were screened after 6 hours from collection and phenotype was assessed by eye. The graph represents the average of the 4 different collections.

Supplementary Files

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- [S1filelVFprotocol.pdf](#)