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1 # **Embryonic development of the grass pufferfish (*Takifugu niphobles*): From egg to larvae.**

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20 ## Abstract

21 Tetraodontidae (pufferfish) family members carry the smallest genomes among
22 vertebrates, and these pocket-sized genomes have directly contributed to our
23 understanding of the structure and evolution of higher animals. The grass pufferfish
24 (*Takifugu niphobles*) could be considered a potential new model organism for
25 comparative genomics and development due to the potential access to embryos, and
26 availability of sequence data for two similar genomes: that of spotted green pufferfish
27 (*Tetraodon nigroviridis*) and Fugu (*Takifugu rubripes*). In this study, we provide the
28 first description of the normal embryonic development of *T. niphobles*, by drawing
29 comparisons with the closely related species cited above. Embryos were obtained by *in*
30 *vitro* fertilization of eggs, and subsequent development was monitored at a constant
31 temperature consistent with natural conditions. *T. niphobles* development was divided
32 into seven periods of embryogenesis: the zygote, cleavage, blastula, gastrula,
33 segmentation, pharyngula, and hatching periods; and stages subdividing these periods
34 are defined based on morphological characteristics. The developmental stage series
35 described in this study aims to provide the utilization of *T. niphobles* as an experimental
36 model organism for comparative developmental studies.

37

38 ## Keywords

39 Fugu; Oocyte; Staging; Embryogenesis; Embryo

40 # 1. Introduction

41 The grass pufferfish (*T. niphobles*) is a teleost fish with a wide distribution in the
42 Northwest Pacific Ocean. This species is one of around twenty four pufferfish species in
43 the genus *Takifugu*, and it presents interesting features to study in depth and preserve it:
44 *i*) it is placed on the IUCN Red List due to the reduced knowledge about the stage of its
45 current populations, making it a possible endangered species [1]; *ii*) other closely
46 related species (like *Takifugu rubripes*) are widely-kept by scientists as a model
47 organism for genomics [2,3]; and *iii*) some species of this genus are considered a
48 popular food in Japan.
49
50 The genome of the congeneric species *T. rubripes* (Fugu) has been sequenced and
51 assembled recently, the second vertebrate genome to be sequenced and the shortest
52 known genome of any vertebrate species [4]. In this respect, the pocket-sized genome of
53 Fugu should help to resolve contentious estimates of human gene number, where the
54 genome of Fugu has directly contributed to the annotation of protein-coding genes on
55 11 human chromosomes and has also helped unearth nearly 1,000 new human genes
56 [5,6]. In this regard, closely related species such as *T. niphobles* could be similarly
57 applied in this purpose due to its small and similar genome. One advantage of *T.*
58 *niphobles* over the other pufferfish species currently used for genomic studies is the
59 potential for the study of essential steps in development: staging series based on
60 morphological traits will provide in-depth knowledge of the developmental processes
61 governing teleost fish [7,8].
62
63 Staging by morphological criteria is an useful tool for generating developmental
64 comparisons between different species and, in this sense, to determine the underlying
65 mechanisms of evolutionary changes among them [9]. For Fugu (*T. rubripes*), a
66 developmental stage series has been published [10], but a standard and cost-effective
67 laboratory breeding protocol is not available. In contrast, *T. niphobles*, with a high
68 fertility rate during a wide spawning period (offering the availability of thousands of
69 eggs [11]), can be kept and maturated in laboratory conditions [12]. As a result, both
70 species have remained virtual models, mostly confined to genome sequence analyses. In
71 this study, we have set out to promote *T. niphobles* as a laboratory model for functional
72 and comparative genomic and developmental projects. We report the embryonic

73 development of *T. niphobles*, raised under laboratory conditions, describing the series of
74 embryonic stages and provide fundamental data to facilitate its use for future
75 developmental studies.
76

77 2. Materials and methods

78 2.1 Fish handling

79 *Takifugu niphobles* shows a singular spawning behavior at Arai Beach near Misaki
80 Marine Biological Station (MMBS, Japan). Large schools of fish (200-1000; [13])
81 arrive to the beach around the new or full moon at spring tide during the spawning
82 season, which occurs between May and July. Spawning takes place repeatedly from 2
83 hours before the sunset and includes a beach-spawning behavior, where the fish are
84 routinely found out of the water on the beach until the next wave. During this time,
85 males and females of *T. niphobles* were caught and moved to the MMBS seawater
86 facilities. Fish were kept in running seawater tanks at 18 °C and the trial was carried out
87 under the approval of the animal guidelines of the University of Tokyo on Animal Care.
88

89 2.2 Gamete collection and *in vitro* fertilization process

90 Genital area was cleaned with freshwater and thoroughly dried to avoid the
91 contamination of the samples with faeces, urine or seawater, and gentle abdomen
92 pressure was applied to obtain the gametes both in males and in females. Eggs from two
93 females were divided into batches of approximately 100 eggs and placed into 60 $\times$ 15
94 mm Petri dishes (x4) using a micropipette with the tip cut off to prevent compression of
95 the eggs. An aliquot of sperm from only one male (10^5 sperm/egg ratio) was put on to
96 the batches of eggs and 5 ml of seawater was then added in order to activate the sperm
97 and achieve fertilization success as described in Gallego et al. (2013) [14].
98

99 2.3 Embryo culture

100 The fertilized eggs were transferred into clean Petri dishes and were then incubated in
101 darkness at a controlled temperature of 20 °C (each Petri dish with approximately 50
102 eggs). Embryos were observed every 30 min using a Leica M165FC microscope to
103 check the embryogenic staging of pufferfish and detailed descriptions of each
104 development stage were performed. Images were taken with a camera (MicroPublisher

105 5.0; QImaging, Surrey, Canada). Dead eggs were removed during daily inspections, and
106 seawater was exchanged once a day.
107
108 **2.4 Presentation of the stage series**
109 To describe *T. niphobles* embryonic development in a standardized way,
110 embryogenesis was divided into periods following the scheme used for other model
111 organisms as zebrafish (*Danio rerio*, [7]) and medaka (*Oryzias latipes*, [8]): zygote,
112 cleavage, blastula, gastrula, segmentation, pharyngula, and hatching. Images of
113 individual embryos were cropped and arranged into figures using the Adobe Photoshop
114 CS3 (Adobe Systems).
115
116 **2.4 *In situ* hybridisation**
117 *T. niphobles* MyoD2 and Myogenin cDNA fragments were isolated by RT-PCR with 96
118 hpf total RNA. Primer sets were designed using *F. rubripes* genome information
119 available from Ensemble database [3]. Primers used and lengths of amplified products
120 are: MyoD2, 370bp with sp (5'-AGAAGGCCACCAGCACCTCCATCAC-3') and ap
121 (5'-. CAGCGGTGGGTAGAAGCTCTGGTCT-3'); Myogenin, 394 bp with sp (5'-
122 CCTACGACCAAGGCACCTAC-3') and ap (5'- TCAGTGTCCTGCTGGTTGAG-3').
123 Whole mount in situ hybridization was perfomed using digoxigenin-11-UTP labeled
124 antisense RNA probes as described [15].
125
126 *T. niphobles* MyoD2 partial cDNA seq.
127 AGAAGGCCACCAGCACCTCCATCACCACGTCCCCAGTGCAGAGGAGGAGCT
128 GGAGGAGGAGACGGTGGTGGAAGAGCACGTGAGAGCACCGGGGGGCCTCC
129 ACCAGGCCGGCCGATGCCTGCTCTGGGCCTGCAAAGCCTGTAAAAGGAAGA
130 CGACCCACGCGGACCGGCGGAAGGCGGCGACCATGCGGGAGCGGCGACGA
131 CTGAGCAAAGTCAACGACGCCTTTGAGACGCTAAAGCGCTGCACCGCCTCC
132 AACCCCAACCAGAGGCTCGCCAAGGTGGAGATCCTGCGCAACGCCATCAGC
133 TACATCGAGTCCCTGCAGGCCCTGCTGAGGACTTCGGGTCAAGACCAGAGC
134 TTCTACCCACCGCTG
135
136 *T. niphobles* Myogenin partial cDNA seq.
137 CCTACGACCAAGGCACCTACCAGGATAGGAACACCATGATGGGCTTGTGTG
138 GGAGTCTGTCCGGAGGTGTGGATGTTGGAGTGACAGGGACAGAGGACAAA

139 GCCTCTCCATCCAGCCTGTCACCTCACTCTGAGCCACACTGCCCGGGCCAGT
140 GCCTTCCCTGGGCCTGCAAGTTATGCAAGAGGAAGACGGTCACCATGGACC
141 GCCGGAGAGCGGCCACGCTGAGAGAGAAGAGGCGCCTGAAGAAGGTGAAC
142 GAGGCCTTCGACGCTTTGAAGAGGAGCACGTTGATGAACCCAAACCAGAGG
143 CTGCCCAAGGTGGAGATCCTCAGGAGCGCCATCCAGTACATCGAAAAGCTA
144 CAGGCCCTGGTGTCCTCCCTCAACCAGCAGGACACTGA
145

146 ## 3. Results

147 ***Zygote period***. The zygote period started from *in vitro* fertilisation until the onset of
148 cleavage period, when the embryonic polar cell mass transitioned from the 1-cell stage
149 to the 2-cell stage (Fig. 1A-B). Zygote period spanned 0-1.7 hpf for reaching the
150 cleavage.
151

152 ***Cleavage Period.*** During the cleavage period of *Takifugu niphobles* embryonic
153 development, a single cell (1st blastomere), formed at the animal pole by separation of
154 cytoplasm from the yolk, was divided (cleaved) into an increasing number of smaller
155 cells, decreasing in size with each division (Fig. 1C-H). This period took approximately
156 2.9 hours.
157

158 ***Blastula Period***. During the early blastula period from the 128-cell stage to dome stage
159 (Fig. 1I-M), the number of cells and the shape of the cell mound were used as criteria
160 for staging. *T. niphobles* embryos began this phase of development at 5.1 hpf when
161 100% of the embryos were consistently dividing into the blastula dome. This period
162 included the stages up to 20% epiboly, where the embryo forms multiple sheets of cells
163 through to gastrulation. During this period, the yolk pushed into the embryonic cells
164 (animal pole; Fig. M) as the embryo develops.
165

166 ***Gastrula period.*** During the gastrula period, the extent to which the blastoderm covers
167 the yolk cell and the form of blastoderm were used as criteria for staging. The gastrula
168 period from 40% epiboly to the tail bud stage proceeded during 14.1-25.5 hpf (Fig. 1N-
169 Q). We marked the beginning of the gastrula period when the majority of the embryos
170 in a given brood reached 40% epiboly (Fig. 1N). During the stages from 50 - 70%
171 epiboly the germ ring started to appear and soon after the embryonic shield developed

172 as a thickening at the germ ring poles (shield stage, Fig. 1O). Towards the end of
173 epiboly (90%) the margin of the blastoderm (germ ring) progressed around the yolk cell
174 (between 15.8 and 21.5 hpf) and the dorsal indentation occurred to mark the start of the
175 tail bud period (Fig. 1P-Q).
176
177 ***Segmentation period***. Segmentation refers to the division of territories and the
178 emergence of somitogenesis – this began soon after tail bud stage at approximately 32.9
179 hpf and when the embryo developed 3-somites the first indication of optic placode
180 formation begins (Fig. 1R). The number and division of somites is a universal indicator
181 of embryonic staging (Fig. 2), and in order to fully appreciate the staging during
182 somitogenesis, we conducted *in situ* hybridisation experiments to determine the precise
183 number of somites during development (6 and 8 somites; Fig. 2, *myoD* and *myogenin*,
184 respectively). Without any indication of gene expression the formation of somites is
185 relatively unclear in *T. niphobles*. *myoD* and *myogenin* (*myog*) are two Muscle
186 Regulatory Factors (MRFs); these genes encode related myogenic basic helix-loop-helix
187 (bHLH) transcription factors involved in myogenesis [16] and are associated with
188 establishing myogenic potential and delineating the process of somitogenesis [16,17].
189 During segmentation the tail began its extension and separation from the yolk
190 membrane (Fig. 1S). Within the latter stages of the segmentation period (approximately
191 the 18 to 21-somite stage) the first signs of pigmentation emerged with black
192 melanophores appear ahead (Fig. 1T) of the orange xanthophores that spread
193 concurrently in ventral regions of the embryo near to and covering the ventral boundary
194 between the embryo and the yolk.
195
196 ***Pharyngula period.*** Eye pigmentation emerged at the start of the pharyngula period,
197 with a weakly darkened retinal pigment equivalent to the Prim (primordial) -10 to Prim-
198 21 stages of development (in zebrafish). The pigmentation of the embryo by both the
199 xanthophores and melanophores spread during these pharyngula stages and covered the
200 dorsal regions of the exposed yolk, the ventral trunk of the embryo and began migration
201 to anterior and dorsal regions of the head image (Fig. 1U). During these migratory
202 periods of the pigment cells, the retinal pigment became darker (Fig. 1V).
203
204 ***Hatching period.*** The hatching period of *T. niphobles* was variable within a batch of
205 embryos, where this process can take from 24 h (in this trial) to several days [18].

206 Typical landmarks of this period of development were the formation of the jaw
207 cartilages, which defines the period by which the mouth develops. At these stages prior
208 to hatching the pigmentation in the retina began to transition from the dark/black to
209 reflective iridophore pigmentation. The mouth was clearly visible and began to
210 protrude beyond the limit of the eyes. Interestingly, the pigment of the ventral trunk and
211 the anterior head region became dominated by xanthophores, giving the embryo a
212 distinctive orange colouration (Fig. 1W). This colour pattern then appeared to permeate
213 throughout the body and on hatching the emerging *T. niphobles* hatchlings were orange
214 dotted with large dark melanocytes. The emerging *T. niphobles* fry were free-swimming
215 (Fig. 1X) and although still retaining a considerable yolk for several days after hatching,
216 they began to feed on zooplankton (rotifer) between 3 and 5 days after emergence from
217 the chorion.

218

219 ## 4. Discussion

220 In this study, we report the developmental stages of *T. niphobles* based on
221 morphological characteristics. This information is anticipated to allow the use of
222 pufferfish as a model for developmental studies [19], uncovering the morphological
223 diversification of this group of highly derived teleost fishes. Regarding the different
224 embryogenic stages during the egg development, cell division cycle from the 2-cell to
225 the 1k-cell stage lasted approximately 12 hours in *T. niphobles*. These intervals were
226 quite similar to the other closely related species, the green spotted pufferfish (*T.*
227 *nigroviridis,* [18]) and another model teleost species, as the medaka (Killifish; *O.*
228 *latipes;* [20]). However, the cleavage and blastula period of Fugu (*T. rubripes*)
229 embryogenesis was a little longer (about 16h) compared to *T. niphobles*; and
230 approximately a threefold shorter in zebrafish (*D. rerio*), probably due to the fast
231 embryo development in the model species par excellence.

232

233 The early embryonic development of *T. niphobles* to the start of the segmentation phase
234 of development was approximately 11 hours faster than *T. rubripes* [10] and 7 hours
235 shorter than *T. nigroviridis* [18]. This shows that even among closely related species
236 inhabiting a similar environment with equivalent standard temperatures for
237 development, there is a great degree of developmental heterochrony and potential
238 diversification. *T. niphobles* embryos at this stage are vastly more heavily pigmented

239 than the closely related *T. rubripes* embryos [10] suggesting the diversity in
240 development even during these later stages of embryogenesis. In contrast to *T.*
241 *niphobles*, *T. rubripes* appears almost clear of pigmentation in areas other than the trunk
242 (i.e. the head and majority of the body), although pigmentation only appears in the head
243 region of *T. rubripes* at the protruding mouth stages of development (188 h; [10]). In
244 comparison pigmentation in *T. nigroviridis* appears early, at 3 days and 5 hours (77 h;
245 [18]) and this obviously reflects the speed of development towards an earlier hatching
246 period in *Tetraodon*. It is clear that pigmentation becomes more pronounced in the
247 stages closest to the hatching period in all species. Pectoral fins have developed towards
248 the end of this protruding mouth stage of development (equivalent to the mid-high 'pec'
249 stages observed by Uji et al. (2011) [10]).
250
251 Hatching time of *Takifugu niphobles* was relatively similar to it closely related species
252 Fugu (*T. rubripes*): pufferfish embryos needed approximately 8 days until they start to
253 hatch while fugu embryos needed 6 days [10]. In contrast, another species of pufferfish
254 genus *Tetraodon* (*T. nigroviridis*) needed only a little more than 3 days for hatching
255 [18]. In this respect, embryo development period is widely variable in marine fish [21]:
256 from a few hours in some carangid fishes to several days (even weeks) in some species
257 of gadids, and this variation is directly related to the combined effects of body size,
258 temperature and life-history attributes [22].
259

260 In the case of pufferfish, long incubation times (about 8 days) are due to its peculiar
261 reproductive strategy, where pufferfish larvae must hatch within a narrow window
262 during the next high tides from the egg fertilization [11,23]. In this regard, this slow
263 developmental rate could enable analysis of gene expression patterns in greater detail,
264 as occur in other species like the model medaka fish (*O. latipes*) [20]. At the hatching
265 period, the time between the first and the last hatching larvae in *Takifugu niphobles*
266 showed a 24h interval, while in other species for example Fugu (*T. rubripes*) or
267 zebrafish (*D. rerio*) this period seems to be two-fold longer, about 48h. In this respect,
268 the hatching synchrony in *T. niphobles* could be due to intertidal reproductive
269 behaviour, where every larvae should hatch in close succession at the right time (high
270 tide) in order to reach the seawater [24].
271
272 On the other hand, regarding *Takifugu* as a model organism for future research, it is
273 important to keep in mind that *Takifugu niphobles* is better suited to experimentation
274 than its close relative Fugu, for its small adult size (up to 15 cm compared to 80 cm) and
275 its ability to survive in different salinity waters: seawater, brackish and freshwater.
276 However, one important caveat to the emergence of *T. niphobles* as a comparative lab-
277 based model for developmental biology is the fact that the fishes cannot breed easily in
278 captivity without hormone stimulation. The study of the embryonic stages in captivity is
279 therefore more accessible with proximity to beach breeding adults.
280
281 A great deal of research has been conducted on *T. niphobles* in several fields as sperm
282 physiology and quality, gamete storage, ecotoxicology, tooth evolution and
283 neuroscience [25–30]; and this study aims to provide a starting point for the
284 comprehensive description of *T. niphobles* development with the aim to enhance all
285 these research areas. The developmental stage series described in this study is one of the
286 essential steps toward the establishment of *T. niphobles* as an experimental model for
287 developmental biology.
288

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296

297 ## References

298 [1] Shao, K., Liu, M., Hardy, G., Leis, J.L., Matsuura, K. & Jing L. Takifugu
299 niphobles. The IUCN Red List of Threatened Species 2014: e.T21341A2775256e
300 2014.
301 [2] Yamanoue Y, Miya M, Matsuura K, Miyazawa S, Tsukamoto N, Doi H, et al.
302 Explosive speciation of Takifugu: another use of fugu as a model system for
303 evolutionary biology. Mol Biol Evol 2009;26:623–9.
304 doi:10.1093/molbev/msn283.
305 [3] Aparicio S, Chapman J, Stupka E, Putnam N, Chia J-M, Dehal P, et al. Whole-
306 genome shotgun assembly and analysis of the genome of Fugu rubripes. Science
307 2002;297:1301–10. doi:10.1126/science.1072104.
308 [4] Brenner S, Elgar G, Sanford R, Macrae A, Venkatesh B, Aparicio S, et al.
309 Characterization of the pufferfish (Fugu) genome as a compact model vertebrate
310 genome. Nature 1993;366:265–8. doi:10.1038/366265a0.
311 [5] Gilligan P, Brenner S, Venkatesh B. Fugu and human sequence comparison
312 identifies novel human genes and conserved non-coding sequences. Gene
313 2002;294:35–44. doi:10.1016/S0378-1119(02)00793-X.
314 [6] Venkatachalam K. The Molecular Paradigm of Human Complexity. Cloning
315 Transgenes 2013;3:1–2. doi:10.4172/2168-9849.1000e109.
316 [7] Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of
317 embryonic development of the zebrafish. Dev Dyn 1995;203:253–310.
318 [8] Iwamatsu T. Stages of normal development in the medaka Oryzias latipes. Mech
319 Dev 2004;121:605–18. doi:10.1016/j.mod.2004.03.012.
320 [9] Uji S, Suzuki T, Kurokawa T. Molecular cloning and expression of retinoic-acid
321 synthesizing enzyme raldh2 from Takifugu rubripes. Comp Biochem Physiol Part
322 D Genomics Proteomics 2006;1:133–8. doi:10.1016/j.cbd.2005.08.004.
323 [10] Uji S, Kurokawa T, Hashimoto H, Kasuya T, Suzuki T. Embryogenic staging of
324 fugu, Takifugu rubripes, and expression profiles of aldh1a2, aldh1a3 and
325 cyp26a1. Dev Growth Differ 2011;53:715–25. doi:10.1111/j.1440-
326 169X.2011.01281.x.
327 [11] Yamahira K. The role of intertidal egg deposition on survival of the puffer,
328 Takifugu niphobles (Jordan et Snyder), embryos. J Exp Mar Bio Ecol
329 1996;198:291–306. doi:10.1016/0022-0981(96)00002-0.

330 [12] Goo IB, Park I, Gil HW, Im JH. Stimulation of Spermiation by Human Chorionic
331 Gonadotropin and Carp Pituitary Extract in Grass Puffer , Takifugu niphobles.
332 Dev Reprod 2015;19:253–8.
333 [13] Motohashi E, Yoshihara T, Doi H, Ando H. Aggregating behavior of the grass
334 puffer, Takifugu niphobles, observed in aquarium during the spawning period.
335 Zoolog Sci 2010;27:559–64. doi:10.2108/zsj.27.559.
336 [14] Gallego V, Pérez L, Asturiano JF, Yoshida M. Relationship between
337 spermatozoa motility parameters, sperm/egg ratio, and fertilization and hatching
338 rates in pufferfish (Takifugu niphobles). Aquaculture 2013;416–417:238–43.
339 doi:10.1016/j.aquaculture.2013.08.035.
340 [15] Suda Y, Kurokawa D, Takeuchi M, Kajikawa E, Kuratani S, Amemiya C, et al.
341 Evolution of Otx paralogue usages in early patterning of the vertebrate head. Dev
342 Biol 2009;325:282–95. doi:10.1016/j.ydbio.2008.09.018.
343 [16] Weinberg ES, Allende ML, Kelly CS, Abdelhamid A, Murakami T, Andermann
344 P, et al. Developmental regulation of zebrafish MyoD in wild-type, no tail and
345 spadetail embryos. Development 1996;122:271–80.
346 [17] Galloway TF, Bardal T, Kvam SN, Dahle SW, Nesse G, Randøl M, et al. Somite
347 formation and expression of MyoD, myogenin and myosin in Atlantic halibut
348 (Hippoglossus hippoglossus L.) embryos incubated at different temperatures:
349 transient asymmetric expression of MyoD. J Exp Biol 2006;209:2432–41.
350 doi:10.1242/jeb.02269.
351 [18] Zaucker A, Bodur T, Roest Crollius H, Hadzhiev Y, Gehrig J, Loosli F, et al.
352 Description of Embryonic Development of Spotted Green Pufferfish (Tetraodon
353 nigroviridis). Zebrafish 2014;11:509–17. doi:10.1089/zeb.2014.0984.
354 [19] Tanaka M, Yu R, Kurokawa D. Anterior migration of lateral plate mesodermal
355 cells during embryogenesis of the pufferfish Takifugu niphobles: insight into the
356 rostral positioning of pelvic fins. J Anat 2015;227:81–8. doi:10.1111/joa.12324.
357 [20] Shima A, Mitani H. Medaka as a research organism: past, present and future.
358 Mech Dev 2004;121:599–604. doi:10.1016/j.mod.2004.03.011.
359 [21] Hirst A, López-Urrutia A. Effects of evolution on egg development time. Mar
360 Ecol Prog Ser 2006;326:29–35. doi:10.3354/meps326029.
361 [22] Pauly D, Pullin RS V. Hatching time in spherical, pelagic, marine fish eggs in
362 response to temperature and egg size. Environ Biol Fishes 1988;22:261–71.
363 doi:10.1007/BF00004892.

364 [23] Yamahira K. Hatching success affects the timing of spawning by the intertidally
365 spawning puffer Takifugu niphobles. Mar Ecol Prog Ser 1997.
366 [24] Yamahira K. Proximate factors influencing spawning site specificity of the puffer
367 fish Takifugu niphobles. Oceanogr Lit Rev 1997.
368 [25] Gallego V, Pérez L, Yoshida M, Asturiano JF. Study of pufferfish (Takifugu
369 niphobles) sperm: Development of methods for short-term storage, effects of
370 different activation media and role of intracellular changes in Ca2+ and K+ in the
371 initiation of sperm motility. Aquaculture 2013;414–415:82–91.
372 doi:10.1016/j.aquaculture.2013.07.046.
373 [26] Itoi S, Kozaki A, Komori K, Tsunashima T, Noguchi S, Kawane M, et al. Toxic
374 Takifugu pardalis eggs found in Takifugu niphobles gut: Implications for TTX
375 accumulation in the pufferfish. Toxicon 2015;108:141–6.
376 doi:10.1016/j.toxicon.2015.10.009.
377 [27] Fraser GJ, Britz R, Hall A, Johanson Z, Smith MM. Replacing the first-
378 generation dentition in pufferfish with a unique beak. Proc Natl Acad Sci U S A
379 2012;109:8179–84. doi:10.1073/pnas.1119635109.
380 [28] Motohashi E, Hamabata T, Ando H. Structure of neurohypophysial hormone
381 genes and changes in the levels of expression during spawning season in grass
382 puffer (Takifugu niphobles). Gen Comp Endocrinol 2008;155:456–63.
383 doi:10.1016/j.ygcen.2007.07.009.
384 [29] Amores A, Suzuki T, Yan Y-L, Pomeroy J, Singer A, Amemiya C, et al.
385 Developmental roles of pufferfish Hox clusters and genome evolution in ray-fin
386 fish. Genome Res 2004;14:1–10. doi:10.1101/gr.1717804.
387 [30] Gallego V, Pérez L, Asturiano JF, Yoshida M. Sperm motility parameters and
388 spermatozoa morphometric characterization in marine species: a study of
389 swimmer and sessile species. Theriogenology 2014;82:668–76.
390 doi:10.1016/j.theriogenology.2014.05.026.
391

392 ## Table legends

393 **Table 1.** Timing-stages of embryonic development of grass pufferfish (*T. niphobles*).

394

395 ## Figure legends

396 **Figure 1.** Developmental process of grass pufferfish (*T. niphobles*) embryo from zygote
397 to hatching. A) External appearance of egg. B) 1-cell stage. C) 2-cell stage. D) 4-cell
398 stage. E) 8-cell stage. F) 16-cell stage. G) 32-cell stage. H) 64-cell stage. I) 128-cell
399 stage. J) 256-cell stage. K) 512-cell stage. L) 1024-cell stage. M) 20% epiboly stage. N)
400 40% epiboly stage. O) 90% epiboly stage. P) Tail bud-1 stage. Q) Tail bud-2 stage. R)
401 3-somite stage. S) 14-somite stage. T) 21-somite stage. U) Prim-5 stage. V) Prim-21
402 stage. W) Hatching. X) Larvae. Scale bar = 200 μm. White arrow in S, T, V indicate the
403 position of the mouth opening. White arrow in W demarcates the pectoral fin, during
404 emergence from the chorion. Orientation of images show anterior to the left and
405 posterior to the right, dorsal is toward the top and ventral is toward the bottom of the
406 images.

407

408 **Figure 2. In situ Hybridisation of *MyoD2* and *Myogenin* (*myog*) during**
409 **somitogenesis in *T. niphobles***. **A**, **B**, *MyoD2* expression in developing somites at the 6-
410 somite stage embryo (A) dorsal view and (B) lateral view. **C**, **D**, *Myogenin* expression
411 demarcating the early bilateral somite blocks in the 8-somite stage *T. niphobles* embryo;
412 dorsal view (**C**) and lateral view (**D**).

413

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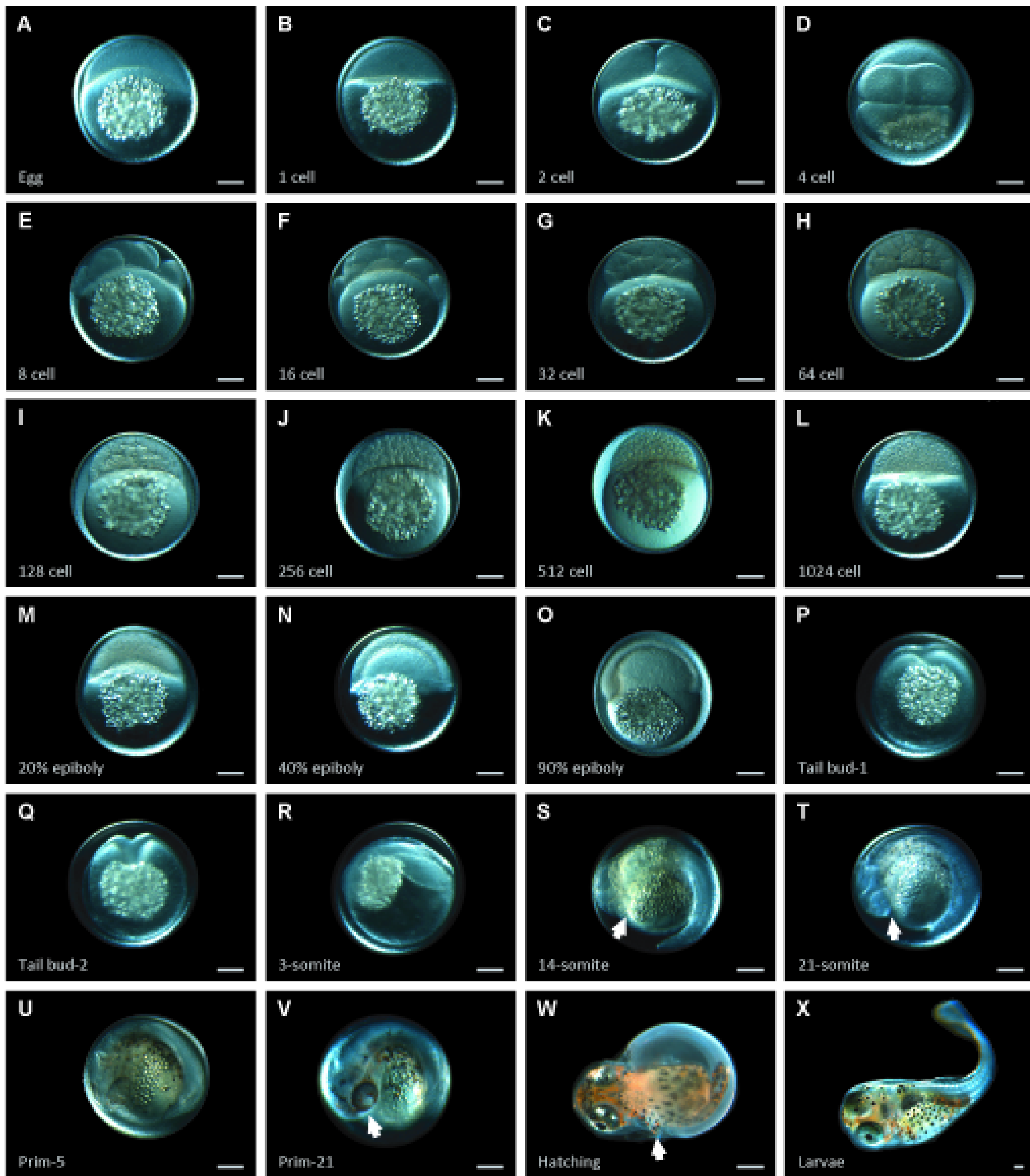
416 **Table 1**

Period	Stage	Time (h)
Zygote period	None	0.0
	1 cell	0.9
Cleavage period	2 cell	1.7
	4 cell	2.4
	8 cell	2.7
	16 cell	3.7
	32 cell	4.1
	64 cell	4.6
Blastula period	128 cell	5.1
	256 cell	5.8
	512 cell	6.5
	1024 cell	7.3
	Epiboly 20%	12.7
Gastrula period	Epiboly 40%	14.1
	Epiboly 90%	15.7
	Tail bud 1	23.7
	Tail bud 2	25.6
Segmentation period	3-somite	32.9
	14-somite	48.9
	21-somite	65.9
Pharyngula period	Prim-5	77.1
	Prim-24	108
	Fin stage	147
Hatching period	First eclosion	191
	Last eclosion	214

417
418

419 **Figure 1**



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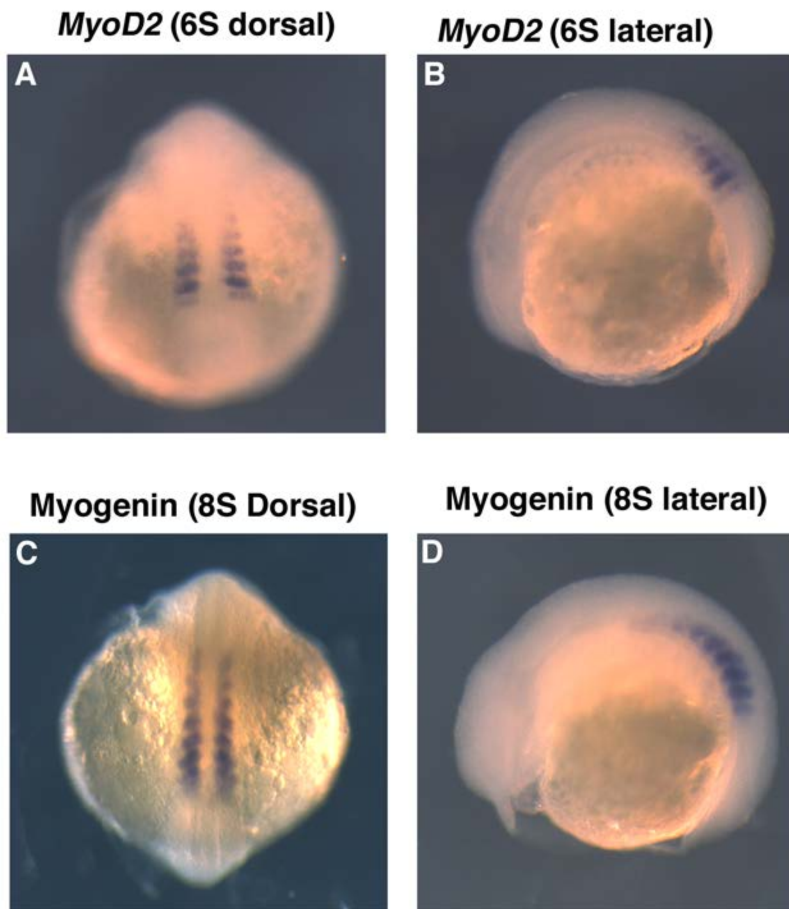
422 **Figure 2**



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