

1 **A chromosome-level genome of black rockfish, *Sebastes***  
2 ***schlegelii*, provides insights into the evolution of live birth**

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## 34 **Abstract**

35 Black rockfish (*Sebastes schlegelii*) is a teleost species where eggs are fertilized  
36 internally and retained in the maternal reproductive system, where they undergo  
37 development until live birth (termed viviparity). In the present study, we report a  
38 chromosome-level black rockfish genome assembly. High-throughput transcriptome  
39 analysis (RNA-seq and ATAC-seq), coupled with *in situ* hybridization (ISH) and  
40 immunofluorescence, identify several candidate genes for maternal preparation, sperm  
41 storage and release, and hatching. We propose that zona pellucida (ZP) genes retain  
42 sperm at the oocyte envelope, while genes in two distinct astacin metalloproteinase  
43 subfamilies serve to release sperm from the ZP and free the embryo from chorion at  
44 pre-hatching stage. Finally, we present a model of black rockfish reproduction, and  
45 propose that the rockfish ovarian wall has a similar function to uterus of mammals.  
46 Taken together, these genomic data reveal unprecedented insights into the evolution of  
47 an unusual teleost life history strategy, and provide a sound foundation for studying  
48 viviparity in non-mammalian vertebrates and an invaluable resource for rockfish  
49 ecology and evolution research.

50

51 **Keywords:** *rockfish genome, viviparity, evolution, ATAC-seq, zona pellucida, astacin*  
52 *metalloproteinase*

## 53 **Introduction**

54 Viviparity – the process of internal fertilization of an egg, development in a parental  
55 reproductive system (usually maternal), and live birth – has evolved independently in  
56 diverse vertebrate groups<sup>1,2</sup>. It is rare in teleosts, ray-finned fishes, where only 500 out  
57 of 30,000 species employ this life-history strategy while the remaining species are egg-  
58 laying (oviparous)<sup>3</sup>. Viviparity has been reported in five teleost orders (Lophiiformes,  
59 Beloniformes, Cyprinodontiformes, Scorpaeniformes, and Perciformes)<sup>4,5</sup>. Previous  
60 studies in fish focused on species of the Poeciliidae family within Cyprinodontiformes<sup>6-</sup>  
61 <sup>8</sup>. Recent genomic analyses on platyfish (*Xiphophorus maculatus*) and swordtail  
62 (*Xiphophorus hellerii*), viviparous species in the Poeciliidae family, revealed positive  
63 selection of protein-coding genes associated with reproductive features<sup>2,9</sup>. Exploring  
64 genetic mechanisms in orders other than Cyprinodontiformes promises to further  
65 improve our understanding of viviparity.

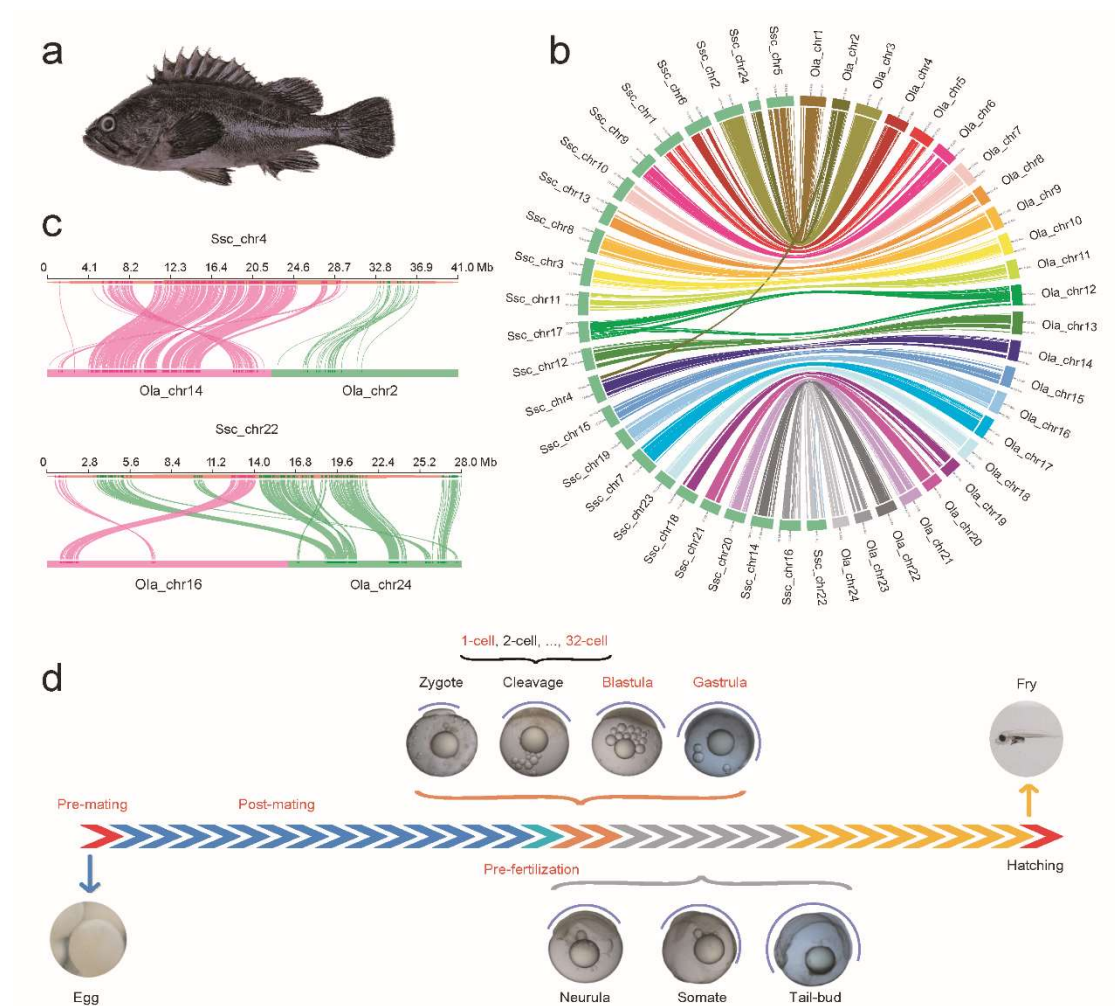
66 Rockfish (order Scorpaeniformes) include both viviparous and oviparous species.  
67 Black rockfish (*Sebastes schlegelii*; hereafter denoted ‘rockfish’) (Fig. 1a) has evolved  
68 viviparity. Previous reports on its reproduction process<sup>10-12</sup> lay an extensive  
69 understanding of viviparity. Yet the associated genetic mechanism remains unexplored.  
70 We report here a first chromosome-level whole-genome assembly for rockfish and the  
71 dissection of rockfish reproduction – from mating to hatching – by integrating RNA-  
72 seq and ATAC-seq data, *in situ* hybridization (ISH), and immunofluorescence. From  
73 this dataset, we were able to identify crucial genes and gene families related to  
74 viviparity, especially in the stages sperm storage, pre-fertilization and hatching –  
75 providing an unprecedented genome-wide view of an unusual reproductive mode in  
76 teleost fishes.

77

## 78 **Results**

### 79 **Rockfish genome assembly and annotation**

80 A critical first step in our effort to understand rockfish reproduction is the generation  
81 of an underlying high-quality genome assembly. We assembled the genome of a male  
82 rockfish ( $2n=48$ ) by combining 57.3 Gb ( $\sim 66\times$ , genome estimation 868Mb based on *k*-  
83 mer analysis; Fig. S1, Tables S1) long PacBio reads and 114.6 Gb ( $\sim 132\times$ ) short  
84 BGISEQ-500 reads (Methods, Tables S2). The genome assembly was 811 Mb, with a  
85 contig N50 size of 3.85 Mb (Table S3). We anchored  $\sim 99.86\%$  of the assembled  
86 sequences onto 24 chromosomes using Hi-C (high-through chromosome conformation  
87 capture) data (Fig. S2, Table S4). Finally, we identified a 35.4% repeat content (Fig.  
88 S3, Table S5) and 24,094 protein-coding genes in the genome (Table S6). The structure  
89 of rockfish genes is similar to proximal species (Fig. S4, Table S7) and 99.71% genes  
90 could be annotated by a least one public database (Fig. S5, Table S8). To evaluate  
91 genome assembly quality, we first mapped short reads back to the final assembly,  
92 revealing a 98.13% mapping rate (Table S9). Using BUSCO (Benchmarking Universal  
93 Single-Copy Orthologues)<sup>13</sup>, we estimated the coverage of core vertebrate genes to be  
94 93.9% and 94.4% in the assembly and gene set, respectively (Table S10). Furthermore,  
95 we found a good collinearity between rockfish and medaka genomes (Fig. 1b), with the  
96 exception of rockfish chromosomes 4 and 22. Each of them aligned to two medaka  
97 chromosomes (Fig. 1c), indicative of chromosome fusions. These assessments reflect  
98 the high quality of our rockfish genome assembly. Based on 1761 single-copy orthologs,  
99 we constructed a phylogenetic tree of rockfish and 15 other fish species. The tree  
100 suggests that rockfish (order Scorpaeniformes; a viviparous species with female  
101 parental care) and three-spined stickleback (order Gasterosteiformes; an egg-laying  
102 species with male parental care) diverged from a common ancestor approximately 84.9  
103 Mya (Fig. S6), which corresponds to the Cretaceous period.



104

105

106 **Fig. 1 Synteny analysis and overview of rockfish reproduction** (a) Adult black

107 rockfish (*Sebastes schlegelii*). (b) Synteny of rockfish and medaka genomes. (c)

108 Recombination of chromosomes could only be observed in chr4 and chr22 of

109 rockfish. (d) Schematic representation of rockfish reproduction and transcriptome

110 sampling in the present study. Reproduction includes mating, pre-fertilization (six

111 months; indicated in blue), embryo and early larvae development (hours; indicated in

112 orange). Includes zygote, cleavage stage embryo, blastula, and gastrula),

113 organogenesis (days; indicated in grey. Includes neurula, somite, and tail-bud),

114 gestation (more than a month; indicated in yellow), and hatching. Purple marks the

115 embryo proper. We sampled seven time points, highlighted in red font.

116

## 117 **Identification of gene expression correlating with maternal** 118 **preparation**

119 Black rockfish reproduction spans about eight months (Fig. 1d). Copulation occurs in  
120 November and December, while fertilization occurs approximately six months later, in  
121 April. The sperm storage stage is crucial, allowing maternal preparation for embryo  
122 development and hatching. In viviparous teleost fishes the ovary acts as both the source  
123 of eggs and the site in which eggs and embryos develop. After fertilization, the embryos  
124 develop in the ovary until hatching. Organogenesis completes within one day and is  
125 followed by about 50 days of gestation, when the offspring need to receive nutrition  
126 from the mother<sup>14</sup>.

127 The transcriptional program from mating to birth is highly stage- and cell/tissue-  
128 specific. We generated the transcriptomes of 21 adult tissues (Table S11) and carried  
129 out weighted gene co-expression network analysis (WGCNA)<sup>15</sup> to identify genes  
130 expressed in concert in particular tissue(s) (termed modules) (Fig. 2a). Of 28 modules,  
131 two significantly correlated ( $P < 0.01$ ) with a single sample type: TM08 with the oocyte  
132 and TM07 with the ovarian wall. Moreover, the expression of the genes within these  
133 modules were high in their correlating tissue. We further looked into functions of the  
134 genes in the two modules. Genes in TM07 are associated with processes likely  
135 important for maternal preparation for embryo implantation (Table S12). These include  
136 cell adhesion (collagen), blood vessel formation (*sox7*, *nln*, *vash1*, and *angpt2b*),  
137 response to blood vessel expansion and contraction (*ednrb*), guanylate cyclase activity  
138 (*gucy1a2*, *gucy2f*), NO-sGc-cGMP biosynthesis (*gucy1a2*), and extracellular calcium-  
139 sensing (*casr*). Other interesting genes in TM07 include a homolog to the oxygen-  
140 binding protein neuroglobin (*ngb*)<sup>16,17</sup>, and genes associated with trophoblast invasion  
141 into the maternal decidua (*htra3*)<sup>18</sup> and smooth muscle development (*coll2a1b* and  
142 *trpc4a*)<sup>19</sup>. These genes are related to early-stage embryo development in mammals;  
143 especially the NO/cGMP signalling pathway which plays an essential role in

144 insemination, pregnancy, and birth<sup>20-23</sup>. These data suggest that maternal preparation of  
145 the ovarian wall is critical for rockfish viviparity.

146 We next obtained the transcriptomes of the pre-mating, post-mating, and pre-  
147 fertilization ovary; as well as the later 1-cell, 32-cell, blastula, and gastrula stage  
148 embryos (Table S13, Fig. 1d). In total, we sequenced 21 biological samples and carried  
149 out WGCNA (Fig. 2b). We identified more co-expressed genes in the pre-fertilization  
150 ovary (2,765 genes in SM16 and SM19) and gastrula embryos (4,998 genes in SM05  
151 and SM31) compared to the post-mating ovary (141 genes in SM13) and the 32-cell  
152 (611 genes in SM28) and blastula embryos (343 genes in SM38). We did not detect any  
153 co-expressed modules in the pre-mating ovary and 1-cell embryo, indicating that these  
154 are relatively transcriptionally ‘dormant’ periods, whereas the pre-fertilization ovary  
155 and the gastrula embryos are more ‘active’. Furthermore, we identified a module (3,128  
156 genes in SM20) with a large number of genes co-expressed by the ovary before  
157 fertilization (pre-mating, post-mating, and pre-fertilization). These genes were  
158 significantly enriched for several gene families ( $P$ -value < 0.05, Table 1, Table S14),  
159 including the zona pellucida (ZP) domain, prefoldin subunit, and DEAD/DEAH box  
160 helicase domain families. Zona pellucida, a component of the envelop surrounding fish  
161 eggs, is important for oogenesis, ovulation, fertilization, and embryogenesis. ZP genes  
162 constitute a species-restricted barrier for sperm at fertilization, act as a post-fertilization  
163 block to prevent polyspermy after gamete fusion, and contribute a hardened structure  
164 which protects the developing embryos until hatching<sup>24</sup>. Prefoldin subunit 1 (*pfid-1*)  
165 mutated animals with maternally contributed PFD-1 develop to the L4 larval stage and  
166 present with gonadogenesis defects which include aberrant distal tip cell migration<sup>25</sup>.  
167 Previous studies on DEAD-box proteins in model organisms have revealed their  
168 functions in the maintenance of gametogenesis<sup>26</sup> and vascular endothelial growth<sup>27</sup>.  
169 DEAD-box proteins have an indispensable role in mammalian placental formation,  
170 which connect the developing embryo to the uterine wall and enables the delivery of

171 oxygen and nutrients to the fetus and the return of metabolic wastes from the fetus to  
172 maternal circulation<sup>28</sup>.

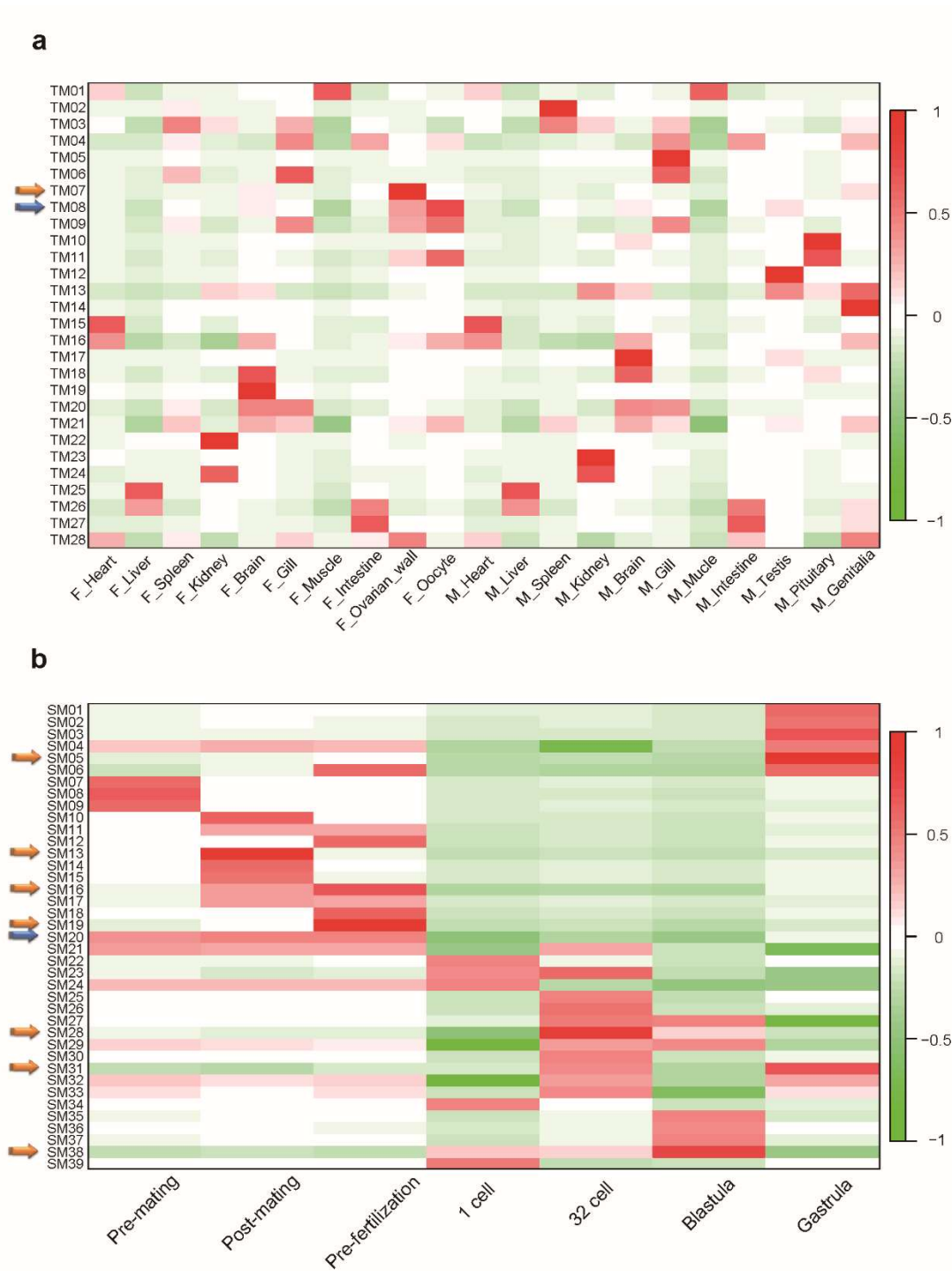
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174 **Table 1 Protein family enrichment analysis of genes co-expressed by the ovary**  
175 **before fertilization.** Enrichment analysis of genes in WGNCA module SM20 was  
176 carried out by searches against the STRING database online<sup>29</sup>.

Pfam ID	Description	Gene number	<i>P</i> -value
PF00100	Zona pellucida-like domain	16	0.010
PF02996	Prefoldin subunit	4	0.041
PF00270	DEAD/DEAH box helicase	14	0.049

177





178

179 **Fig. 2 Identification of genes co-expressed in rockfish** (a) WGCNA co-expression

180 modules were constructed by comparing (a) 21 tissues (b) seven reproduction time

181 points. The *x*-axis shows sampled tissues in (a) and (b), with the prefix F\_ for female

182 and M\_ for male samples, the *y*-axis WGCNA modules. For each module, the

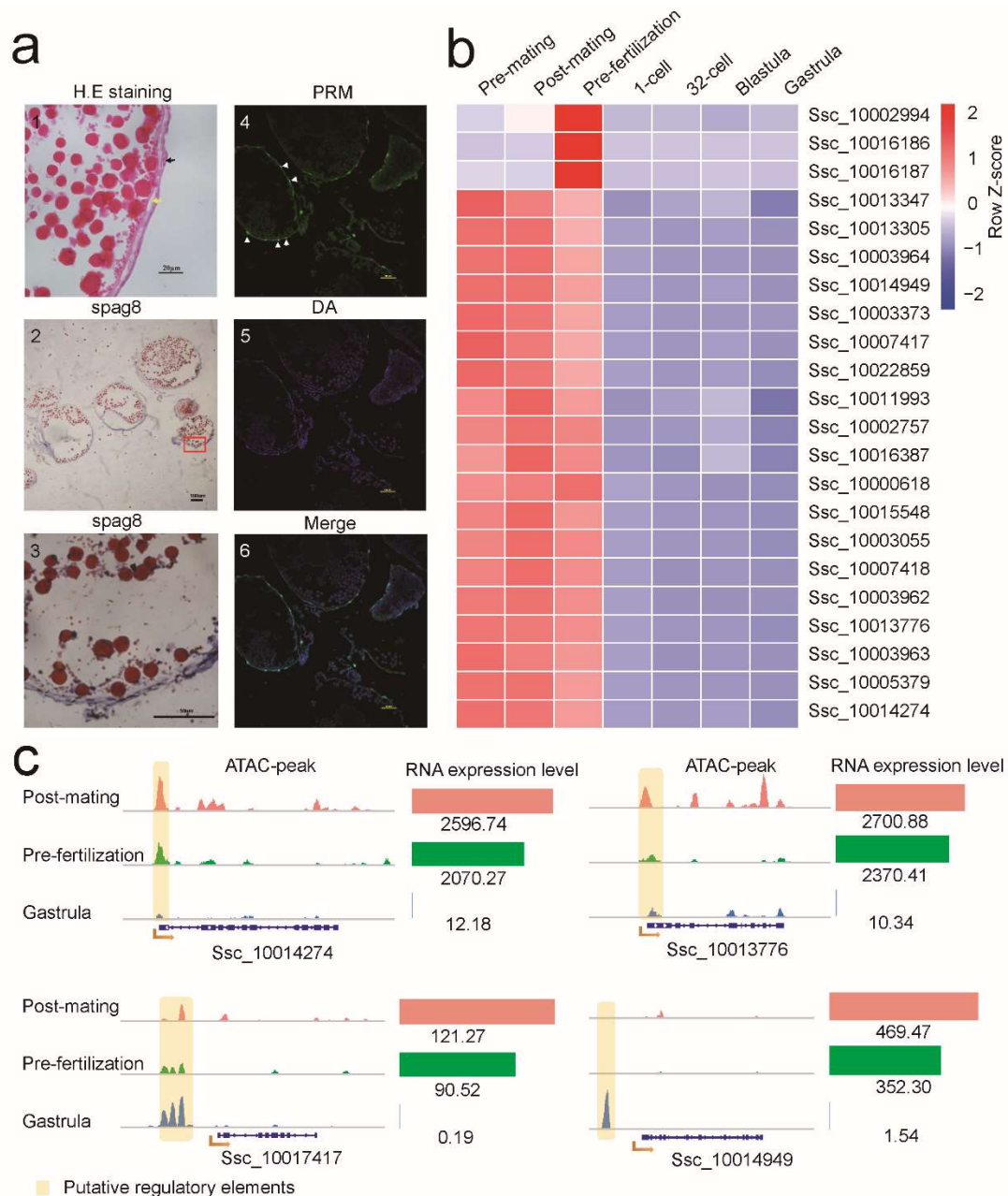
183 correlation value is indicated by a heat map ranging from -1 to 1. In total, 28 and 39

184 modules were identified in (a) and (b), respectively. Modules significantly correlated  
185 with a single tissue or stage are indicated by orange arrows; modules correlated with  
186 multiple tissues or stages by blue arrows.

187

## 188 **Zona pellucida protein gene family in rockfish**

189 To further dissect the role of ZP protein genes during viviparity, we annotated 22 ZP  
190 genes, aided by manual curation (Fig. S7, Table S15, S16), and performed *in situ*  
191 hybridization (ISH) and immunofluorescence (IF) to locate the position of sperm cells  
192 during post-mating and pre-fertilization. Sperm associated antigen 8 (*spag8*) ISH  
193 revealed strong mRNA staining of oocyte epithelium (Fig. 3a: 1-3). Furthermore,  
194 fluorescent staining of protamine 2 protein (PRM2) localized to the oocyte epithelium  
195 (Fig. 3a: 4-6). This indicates that sperm cells are in proximity of ZP proteins after  
196 mating. RNA-seq analysis showed that 19 out of 22 ZP genes are highly expressed  
197 before fertilization, while their expression after fertilization is relatively low (Fig. 3b,  
198 Table S17). We hypothesize that ZP proteins retain sperm after mating and that sperm  
199 is released for fertilization upon ZP protein degradation. To strengthen our hypothesis  
200 we performed chromatin accessibility profiling (ATAC-seq) during post-mating, pre-  
201 fertilization, and gastrula (Fig. S8). Consistently, a distinct difference in open  
202 chromatin regions adjacent to 12 ZP genes could be observed between maternal  
203 preparation (post-mating and pre-fertilization) and the gastrula stage (Fig. 3c, Fig. S9).



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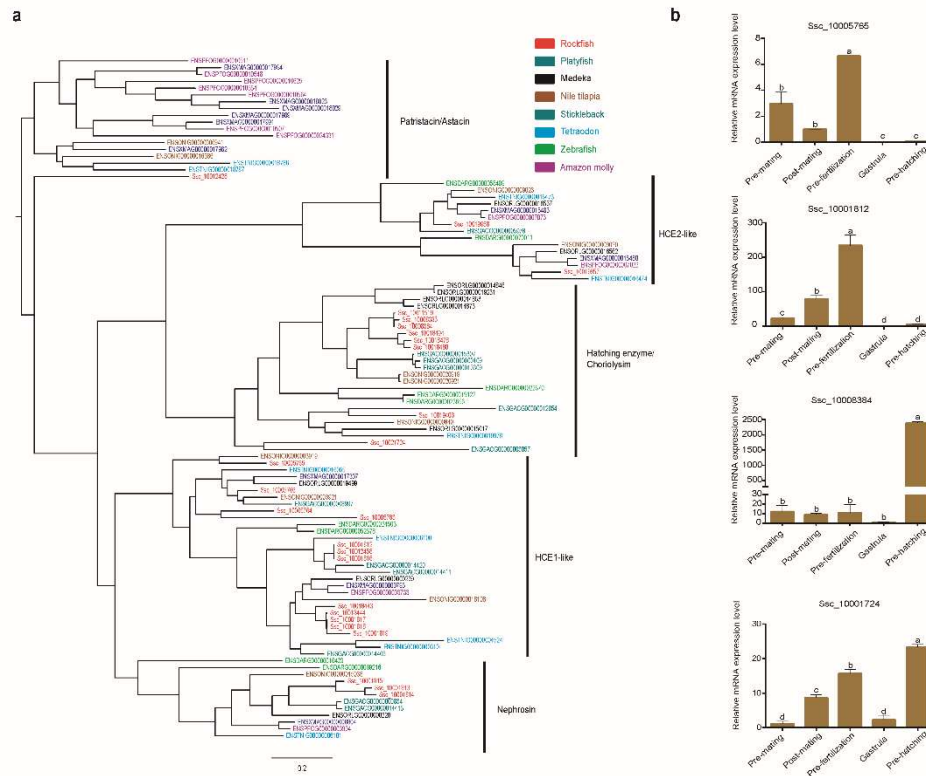
205 **Fig. 3 Zona pellucida protein gene family in rockfish** (a) Location of sperm and ZP  
 206 on the surface of eggs. 1: Paraffin sections of an egg. H.E. staining. Scale bar=10 $\mu$ m.  
 207 A number of spermatozoa surrounding the egg (black arrow) and the ZP region are  
 208 indicated (yellow arrow); 2, 3: Expression of the spermatozoa marker *spag8* (DIG-  
 209 labelled RNA) on the surface of eggs (blue region); 4, 5: Expression of the spermatozoa  
 210 marker PRM2 (fluorescent antibody) on eggs. 6: Merged pictures. (b) Expression  
 211 pattern of 22 ZP genes at seven time points. The log ratio expression is indicated in a  
 212 heat map (-2 to 2; red: high, blue: low). (c) Signals of accessible chromatin and

213 associated RNA expression levels of selected ZP genes from (b) in the post-mating and  
214 pre-fertilization ovary and gastrula stage of embryonic development. ATAC result of  
215 each gene is shown on the left, with peaks indicating accessible chromatin regions; gene  
216 expression levels (in TPM) on the right.

217

## 218 **Astacin metalloproteinase family in rockfish**

219 After fertilization, polymerized and cross-linked ZP proteins are digested by hatching  
220 enzymes<sup>30</sup>, a subfamily of astacin metalloproteinases which lyse the chorion  
221 surrounding the egg, leading to hatching of embryos<sup>31</sup>. Astacin metalloproteinase can  
222 be classified into five subfamilies: hatching enzyme, HCE1-like, HCE2-like,  
223 patristacin/astacin, and nephrosin (Fig. 4a). We identified 26 astacin family genes, with  
224 two expanded subfamilies of hatching enzyme and HCE1-like in rockfish (Table S18,  
225 S19). These are distinct to the patristacin/astacin subfamily expansion in seahorse  
226 which play a role in brood pouch development and/or hatching of embryos within the  
227 brood pouch prior to parturition<sup>32</sup>. To examine the expression patterns of rockfish  
228 astacin genes we performed qRT-PCR of five time-points: pre-mating, post-mating,  
229 pre-fertilization, gastrula, and pre-hatching. Two of twelve HCE1-like genes  
230 (Ssc\_10005765 and Ssc\_10001812) were highly expressed pre-fertilization, while two  
231 of the eight hatching enzyme genes (Ssc\_10008384 and Ssc\_10021724) were highly  
232 expressed pre-hatching (Fig. 4b). Thus, we propose that astacin family members play  
233 distinct roles in rockfish viviparity. HCE1-like proteins play a role in releasing  
234 spermatozoa from the zona pellucida at the pre-fertilization stage, while hatching  
235 enzymes are responsible for freeing the embryo from the chorion at the pre-hatching  
236 stage.



237

238 **Fig. 4 Assessment of the astacin metalloproteinase family** (a) Phylogenetic tree of  
 239 astacin family genes in eight fish species. In rockfish eight genes were found in the  
 240 hatching enzyme subfamily and 12 genes in the HCE1-like subfamily (b) the expression  
 241 profiles of four hatching enzyme genes in rockfish, quantified by qRT-PCR. Columns  
 242 with different letters (a–b) represents significantly different expression ( $P < 0.05$ , one-  
 243 way ANOVA). Data are mean±s.e.m. of three biological replicates.

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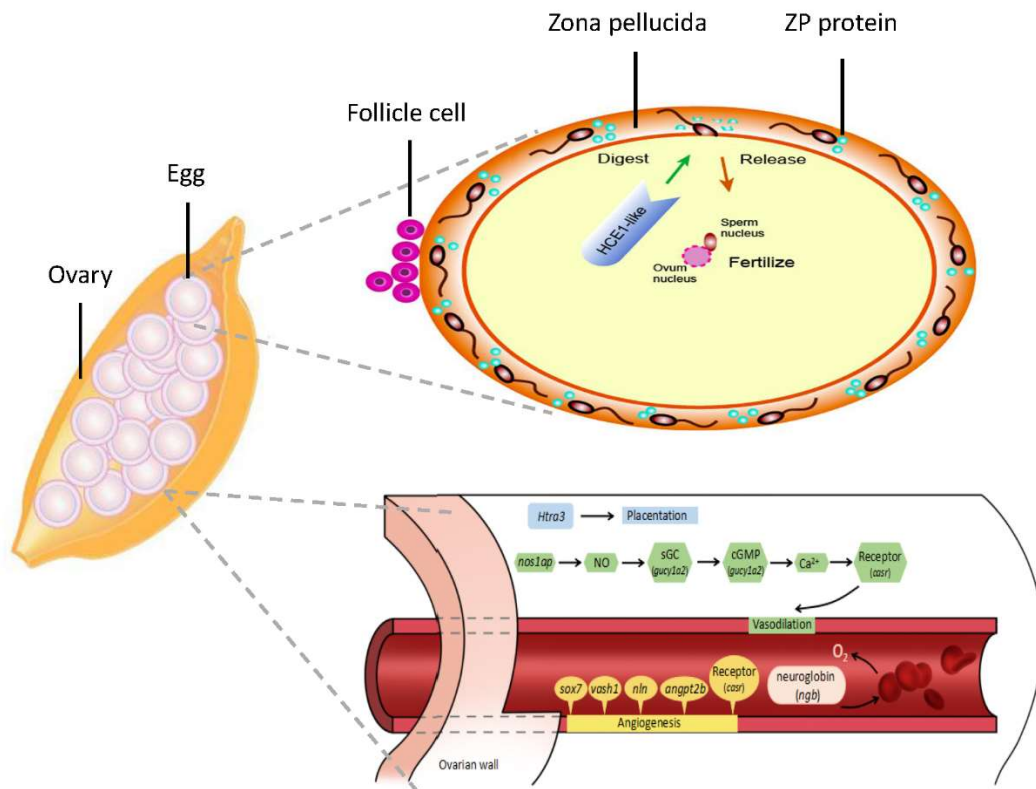
## 245 Discussion

246 In this study, we present a high-quality genome assembly of the rockfish *Sebastes*  
 247 *schlegelii*, a viviparous fish in the teleost order Scorpaeniformes. We generated  
 248 transcriptomes of different tissues and different developmental time points, to reveal  
 249 gene expression patterns related to viviparity. The reproduction of viviparous rockfish  
 250 is well-established, however the genetic changes associated with the long period

251 between copulation and fertilization (six months, including maternal preparation and  
252 sperm storage), embryo development, and hatching has hitherto remained unknown.  
253 We found that ZP proteins and HCE-1 like proteins likely play important roles during  
254 sperm storage and release, respectively (Fig. 5). We propose that ZP proteins retain and  
255 stabilize sperm in lamellae epithelium prior to fertilization – a six-month period in  
256 rockfish. In contrast, HCE1-like proteins digest the zona pellucida and contribute to  
257 sperm release. In the maternal preparation stage, the preparation for ‘embryo  
258 implantation’, we found expression of genes related to cell adhesion, trophoblast  
259 invasion, calcium-sensing receptors, the NO-sGc-cGMP signaling pathway, and blood  
260 vessel function. Significant differences in the internal circulation system of fish  
261 embryos has previously been reported<sup>33</sup>, believed to due to the high demand for oxygen  
262 associated with parental care. In rockfish we not only observed high expression of  
263 vasodilatation and angiogenesis-related genes, but also genes associated with the  
264 growth of blood vessels in the ovarian wall during reproduction. For example, a  
265 homolog of the oxygen-binding neuroglobin was highly expressed by the ovarian wall.  
266 In mammals, the function of the uterus is to transfer oxygen and nutrients to the growing  
267 embryo. The chemical messenger nitric oxide (NO) is a key regulator of fetoplacental  
268 circulation in mammals<sup>23</sup> and also induces oocyte maturation in the zebrafish via the  
269 NO-sGc-cGMP pathway<sup>20</sup>. In the rockfish ovarian wall we observed high expression  
270 of nitric oxide synthase 1 adaptor protein (*nos1ap*; also known as *CAPON*), a regulator  
271 of NO bioavailability<sup>34</sup>. Rockfish embryos not only receive parental care, but also  
272 parental nutrition<sup>14</sup>. We provide evidence, at the transcriptome level, for maternal  
273 preparation for embryo implantation and the formation of an interface between mother  
274 and offspring (embryo). We hypothesize that the ovarian wall of the rockfish functions  
275 similar to the mammalian uterus.

276 In conclusion, we have generated and analysed a high-quality genomic data set for  
277 black rockfish to generate a model of its reproduction – the first such model of fish  
278 viviparity. This model, along with the candidate genes identified in our study, will

279 greatly facilitate future studies on the evolution of fish, as well as vertebrate life history  
280 strategies.  
281



282

283 **Fig. 5 Proposed model of viviparous reproduction in rockfish.** Sperm is retained in  
284 the zona pellucida (ZP) region of eggs and released for fertilization upon degradation  
285 of ZP proteins. The rockfish ovarian wall is analogous to the mammalian uterus.

286

287

## 288 **Methods**

### 289 **Ethics statement**

290 This study was approved by the Animal Care and Use Committee of the Centre for  
291 Applied Aquatic Genomics at the Ocean University of China.

292

### 293 **Samples**

294 Black rockfish (*Sebastes schlegelii*) were obtained from the Zhucha Island (Qingdao,  
295 Shandong, China). A three-year-old male adult (weight 665g) was used for genome  
296 sequencing. Samples used for RNA- and ATAC-sequencing were collected from  
297 November 2017 to March 2018. Twelve healthy three-year-old fish (six males and six  
298 females) were randomly selected for sampling of heart, liver, spleen, kidney, pituitary,  
299 brain, intestine, gill, muscle, genitalia (testis or ovary), ovarian wall, and oocytes in  
300 November 2017. The ovary of the female at different developmental stages (pre-mating,  
301 post-mating, and pre-fertilization) were collected on November 2017, February 2018,  
302 and March 2018, respectively. Embryos (20-30 each) at various developmental stages  
303 including 1-cell, 32-cell, blastula, gastrula and pre-hatching were collected.

304

### 305 **Sequencing, assembly and evaluation of rockfish genome**

306 The black rockfish genome was assembled using CANU v1.2 software<sup>35</sup> with the  
307 parameters ‘MhapSensitivity=high corMinCoverage=0 minReadLength=500  
308 genomeSize=868m errorRate=0.04’ in three steps: correct, trim, and assemble. Though  
309 the assembly had been corrected by CANU, a strict error-correcting procedure was  
310 performed: firstly, the draft genome was corrected using PacBio long reads, secondly,



311 the assembly was further corrected using Pilon v1.22<sup>36</sup> and 98.1G (114.6X) WGS short  
312 reads.

313 To generate a chromosomal-level assembly of the genome, we took advantage of  
314 sequencing data from a Hi-C library<sup>37</sup>. We performed quality control of Hi-C raw data  
315 using HiC-Pro (v2.8.0)<sup>38</sup>. We used bowtie2 (v2.2.5)<sup>39</sup> to compare raw reads to the draft  
316 assembled genome, filtering out low-quality reads to build raw inter/intra-chromosomal  
317 contact maps. Our final data set was 41.75 Gb (48.1×), accounting for 54.59% of the  
318 total Hi-C sequencing data. We next used Juicer (v1.5)<sup>40</sup>, an open-source tool to analyse  
319 Hi-C datasets, and a 3D *de novo* assembly (3d-dna, v170123) pipeline to scaffold  
320 spotted rockfish genome to chromosomes. We further conducted whole-genome  
321 alignment between the rockfish genome and the published medaka genome using  
322 LASTZ (v1.10)<sup>41</sup> to compare consistency between these two genomes.

323

## 324 **Completeness assessment of genome assemblies**

325 To evaluate the consistence and integrity of genome assembly, short-insert library  
326 reads were used to map with the assembled genome using BWA<sup>42</sup> to generate mapping  
327 ratio statistics. BUSCO (Benchmarking Universal Single-Copy Orthologues)<sup>13</sup>  
328 provides quantitative measures for the assessment of genome assembly completeness,  
329 based on evolutionarily-informed expectations of gene content from near-universal  
330 single-copy orthologs.

331

## 332 **Repeat and gene annotation**

333 We constructed a transposable element (TE) library of rockfish genome using a  
334 combination of homology-based and *de novo* approaches. For the *ab-initio* method, we  
335 used RepeatModeler and LTR\_FINDER<sup>43</sup> to build the rockfish custom repeat database.

336 In homology-based method, we used the Repbase database<sup>44</sup> to identify repeats with  
337 RepeatMasker and RepeatProteinMask (<http://www.repeatmasker.org>).  
338 The annotation strategy of *Rockfish* protein-coding genes was to integrate *de novo*  
339 prediction and evidence based including homology and transcriptome data. Protein  
340 sequences of zebrafish (*Danio rerio*), fugu (*Takifugu rubripes*), spotted green pufferfish  
341 (*Tetraodon nigroviridis*), stickleback (*Gasterosteus aculeatus*), large yellow croaker  
342 (*Larimichthys crocea*), tongue sole (*Cynoglossus semilaevis*), tilapia (*Oreochromis*  
343 *niloticus*), medaka (*Oryzias latipes*), and Amazon molly (*Poecilia formosa*) were  
344 downloaded from Ensembl (<http://ensemblgenomes.org/>) or NCBI  
345 (<https://www.ncbi.nlm.nih.gov/genome/>). These sequences were aligned to the rockfish  
346 genome with tBLASTn ( $E\text{-value} \leq 10^{-5}$ ) and GeneWise<sup>45</sup> was used to generate gene  
347 structure based on the alignment. The transcriptome data (including 11 tissues in  
348 Method Fish section) were assembled by Trinity<sup>46</sup> and mapped to rockfish genome by  
349 BLAT<sup>47</sup>. The *de novo* prediction of rockfish was carried out with Augustus<sup>48</sup>. All  
350 evidences of gene model were integrated with GLEAN<sup>49</sup>.

## 351 **RNA-seq and transcriptome data processing**

352 In total, 71 RNA-seq libraries, 58 of which from 21 tissues, and 29 from seven different  
353 developmental stages (pre-mating, post-mating, pre-fertilization, 1-cell, 32-cell,  
354 blastula, gastrula) with three biological replicates. These libraries were sequenced 100  
355 bp at each end using BGI-seq 500 platform. The transcriptomic data were mapped to  
356 the rockfish genome and the expression level of genes was calculated using Salmon<sup>50</sup>  
357 with default parameters. The genes of interest were subsequently visualized by  
358 Heatmap package. In order to investigate the regulatory network, we used the gene  
359 co-expression network constructed by the method of Weighted Gene Co-Expression  
360 Network Analysis (WGCNA)<sup>15</sup>.

361

## 362 **ATAC experiment and data processing**

363 We treated the samples at pre-, post-mating and gastrula stage and build the ATAC-seq  
364 libraries with a protocol modified from previous report<sup>51</sup>. All ATAC-seq libraries were  
365 sequenced using BGI-SEQ500. After filtering low quality data, duplication and  
366 removing chondriosome DNA, the clean reads were aligned to the genomes using  
367 Bowtie2 (version 2.2.2)<sup>52</sup>. All the ATAC-seq peaks were called by MACS<sup>53</sup> with the  
368 parameters ‘nolambda –nomodel’.

369

## 370 **Prediction and clustering of ZP and Astacin** 371 **metalloproteinase**

372 The previously reported ZP and astacin metalloproteinase in zebrafish and medaka<sup>32,54</sup>  
373 were manually verified, and subsequently used as baits to predict the target proteins in  
374 rockfish. The candidates were filtered with domain information obtained with  
375 SMART<sup>55</sup> and confirmed by blast against NR database. Furthermore, multiple sequence  
376 alignment of ZP and astacin metalloproteinase was respectively carried out using  
377 MUSCLE<sup>56</sup>, with both predicted and reported proteins in rockfish, medaka, zebrafish,  
378 tilapia, Amazon molly, platyfish, tetraodon and stickleback<sup>32,54</sup>. Phylogenetic trees  
379 were subsequently constructed based on the alignment results with Fasttree<sup>57</sup> by  
380 Maximum Likelihood.

381

## 382 **RNA isolation, cDNA synthesis and quantitative real-time** 383 **RT-PCR**

384 Total RNA was extracted using TRIzol (Invitrogen, Carlsbad CA, USA) and  
385 complementary DNA (cDNA) was synthesized using the Reverse Transcriptase M-

386 MLV Kit (TaKaRa). All quantitative real-time RT-PCR experiments were performed  
387 in triplicate on a Light-Cycler Roche 480 instrument (Roche Applied Science,  
388 Mannheim, Germany), using primers against hatching enzyme genes and the  
389 housekeeping gene ribosomal protein L17 (*rpl17*) (primers shown in **Table S20**). The  
390 relative expression of each hatching enzyme gene was calculated using the comparative  
391  $2^{-\Delta\Delta Ct}$  method<sup>58</sup>. The results were expressed as mean  $\pm$  standard error of the mean  
392 (s.e.m.). Data was evaluated using a one-way ANOVA.

393

### 394 ***In situ* hybridization**

395 *In situ* hybridization of ovaries were conducted as described<sup>32</sup>. Antisense mRNA probes  
396 of *spag8*, a marker of spermatozoa, were synthesized using a DIG RNA Labelling Mix  
397 (Indianapolis, IN, USA). A pair of primers (*Ssc-spag8-Fw/Rv*) was used for probe  
398 synthesis (**Table S20**). The results were photographed by AZ100 (Nikon, Tokyo,  
399 Japan).

400

### 401 **Immunofluorescence**

402 Ovaries of fishes were removed, cleaned of excess fat, fixed in 4% formaldehyde  
403 solution overnight at 4 °C, and dehydrated to 100% for histological analysis. Ovaries  
404 were serially-sectioned at 7  $\mu$ m on a RM2016 Paraffin slicer onto glass slides and  
405 washed in 1x Phosphate-Buffered Saline (PBS) containing 0.1% Triton-X  
406 (CalBiochem). Tissue sections were incubated in blocking buffer (3% Goat Serum;  
407 Sigma), 1% Bovine Serum Albumin (Sigma), and 0.5% Tween-20 (Fisher Scientific)  
408 in 1X PBS], and incubated with rabbit primary antibodies against the spermatozoa  
409 marker protamine 2 protein (PRM2; Uscn Life Science Inc., PAH307Hu01). A  
410 secondary FITC AffiniPure Goat Anti-Rabbit IgG antibody was used to enable green

411 fluorescence detection. Sections were stained with DAPI to visualize nuclei and  
412 analysed on a laser scanning confocal microscope.

413

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420

## 421 **Author contributions**

422 Yan He, Guangyi Fan, Quanqi Zhang, Huanming Yang, Jian Wang and Jie Qi  
423 conceived the study; Yan He, Yue Chang, Lisui Bao, Mengjun Yu, He Zhang and  
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427 Wang performed the experiments. Haiyang Yu, Xubo Wang, Jie Cheng, Xuangang  
428 Wang, Zhigang Wang, Yaolei Zhang, Jiao Guo, Yang Liu and Kaiqiang Liu prepared  
429 the material; Yan He, Yue Chang, Lisui Bao, Mengjun Yu, Guangyi Fan and Jie Qi  
430 drafted the manuscript. Xin Liu, Guangyi Fan, Inge Seim, Yue Chang, Mengjun Yu,  
431 Yan He, Quanqi Zhang, Simon Ming-Yuen Lee, Xun Xu and Jie Qi contributed to the  
432 final manuscript editing.

433

## 434 **Data Accession**

435 The project has been deposited at CNSA(CNGB Nucleotide Sequence Archive) under  
436 the accession ID CNP0000222. The assembled genome can be obtained by assembly  
437 ID CNA0000824.

438

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