

1 **Title: Gonadectomy and blood sampling procedures in small size teleost models**

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29 **Key words:** Gonadectomy, ovariectomy, orchidectomy, castration, medaka, zebrafish,
30 blood, steroids, fish, reproduction, plasticity, estradiol, 11-ketotestosterone

31

32 **SUMMARY:**

33 The article describes a quick protocol to gonadectomize and sample blood from small
34 teleost fish, using medaka (*Oryzias latipes*) as a model, to investigate the role of sex
35 steroids in animal physiology.

36

37 **Abstract:**

38 Sex steroids, produced by the gonads, play an essential role in the neuroendocrine
39 control of reproduction in all vertebrates by providing feedback to the brain and pituitary.
40 Sex steroids also play an important role in tissue plasticity by regulating cell proliferation
41 in several tissues including the brain and the pituitary. Therefore, investigating the role of
42 sex steroids and mechanisms by which they act is crucial to better understand both
43 feedback mechanism and tissue plasticity. Teleost fish, which possess a higher degree of
44 tissue plasticity and variations in reproduction strategies compared to mammals, appear
45 to be useful models to investigate these questions. The removal of the main source of sex
46 steroid production using gonadectomy together with blood sampling to measure steroid
47 levels, have been well-established and fairly feasible in bigger fish and are powerful
48 techniques to investigate the role and effects of sex steroids. However, small fish such as
49 zebrafish and medaka, which are particularly good model organisms considering the well-
50 developed genetic toolkit and the numerous protocols available to investigate their
51 biology and physiology, raise challenges for applying such protocols due to their small
52 size. Here, we demonstrate the step-by-step procedure of gonadectomy in both males
53 and females followed by blood sampling in a small sized teleost model, the Japanese
54 medaka (*Oryzias latipes*). The use of these procedures combined with the other
55 advantages of using these small teleost models will greatly improve our understanding of
56 feedback mechanisms in the neuroendocrine control of reproduction and tissue plasticity
57 provided by sex steroids in vertebrates.

58

59

60

61 **Introduction**

62 In vertebrates, sex steroids, which are mainly produced by the gonads, play
63 important roles in the regulation of the Brain-Pituitary-Gonadal (BPG) axis through

64 various feedback mechanisms (reviewed in ¹⁻⁵). In addition, sex steroids also affect the
65 proliferation and activity of neurons in the brain (reviewed in ⁶⁻⁸) and endocrine cells,
66 including gonadotropes, in the pituitary (reviewed in ^{9,10}), and thus serve crucial roles in
67 brain and pituitary plasticity.

68 Despite a relatively good knowledge in mammals, mechanism of BPG axis
69 regulation mediated by sex steroids is far from being understood in non-mammalian
70 species, leading to poor understanding of evolutionary conserved principles (reviewed in
71 ¹¹). Besides, there is still a limited number of studies documenting the role of sex steroids
72 on brain and pituitary plasticity, thus raising the need for further investigations of the
73 role and effects of sex steroids on diverse vertebrate species.

74 Among vertebrates, teleosts have become powerful model animals in addressing
75 numerous biological and physiological questions, including stress response (reviewed in
76 ^{12,13}), growth (reviewed in ^{14,15}), nutritional physiology (reviewed in ^{16,17}) and reproduction
77 (reviewed in ²). Teleosts, in which sex steroids are mostly represented by estradiol (E2) in
78 females and 11-ketotestosterone (11-KT) in males ^{18,19}, have thus long been reliable
79 experimental models for investigating the general principle of reproduction across
80 species. Also, teleosts possess unique characteristics in their hypothalamus and pituitary,
81 which are sometimes convenient for the elucidation of regulatory mechanisms. For
82 instance, they show direct pituitary innervation of hypophysiotropic neurons in the brain
83 instead of mediating the hypothalamic-hypophyseal portal system (reviewed in ^{20,21}) and
84 their gonadotropins (luteinizing and follicle stimulating hormones) are produced in two
85 separate cells in these animals (reviewed in ²²). Due to the high number of species (nearly
86 30 000 ²³) and their high diversity ²⁴, these animals offer interesting models to investigate
87 a wide range of biological questions (reviewed in ²⁵). Moreover, due to their amenability
88 to both laboratory and field experiments, teleosts offer many advantages compared to
89 other organisms. They are relatively inexpensive to purchase and maintain (reviewed in
90 ^{25,26}). In particular, small teleost models, such as zebrafish and medaka, are species with
91 very high fecundity and relatively short life cycle enabling rapid analysis of gene function
92 and disease mechanisms, thus providing even greater advantages in addressing a
93 plethora of biological and physiological questions, considering the numerous well-
94 developed protocols and genetic tools available for these species (reviewed in ^{27,28}).

95 In numerous studies, the removal of gonads (gonadectomy), the main source of
96 sex steroid production, has been used as a method for investigating many physiological
97 questions, including its impact in vertebrate reproductive physiology, including in
98 mammals²⁹⁻³¹, birds³² and amphibians³³. Meanwhile, blood collection is commonly
99 aimed for quantifying circulating hormone levels, including those of sex steroids³⁴⁻³⁷.
100 Together, these two techniques have shown their importance in a great number of
101 studies, including the investigation of feedback mechanisms and the effect of sex steroids
102 on BPG axis regulation³⁸⁻⁴⁰. In teleosts however, while these techniques are relatively
103 easy to perform in bigger species, such as European sea bass⁴¹, coral reef fish⁴², dogfish
104⁴³ and catfish^{44,45}, they raise challenges when applied in smaller fishes, such as zebrafish
105 and medaka. Therefore, a clear protocol demonstrating every step of gonadectomy and
106 blood sampling in small teleosts is of importance.

107 Here, we use Japanese medaka (*Oryzias latipes*) as a model, a small freshwater
108 fish native to East Asia, and similar to zebrafish in many aspects (size, genome sequenced,
109 molecular and genetic tools available). However, medaka has a smaller genome size than
110 that of zebrafish⁴⁶ and a genetic sex determination system allowing for investigation of
111 sexual differences before second sexual characters or gonads are well developed
112 (reviewed in⁴⁷).

113 This paper demonstrates gonadectomy and blood sampling in small teleost
114 models, a technique that takes only 8 minutes in total and that will complete the list of
115 detailed video protocols already existing for this species that included labeling of blood
116 vessels⁴⁸, patch-clamp on pituitary sections⁴⁹ and brain neurons⁵⁰, and primary cell
117 culture⁵¹. This technique will allow the research community to investigate and better
118 understand the roles of sex steroids in feedback mechanisms as well as brain and
119 pituitary plasticity in the future.

120

121 Protocol

122 All experimentations and animal handling were conducted in accordance with the
123 recommendations on the experimental animal welfare at Norwegian University of Life

124 Sciences. Experiments using gonadectomy were approved by the Norwegian Food Safety
125 Authority (FOTS ID 24305).

126 1. Instruments and solutions preparation

127 1.1. Prepare anesthesia stock solution (0.6% Tricaine):

128 1.1.1. Dilute 0.6 g of Tricaine in 100 ml of 10X Phosphate Buffer Saline (PBS).

129 1.1.2. Distribute 800 μ l of the tricaine stock solution into several 1.5 ml plastic tubes
130 and store at -20 °C until use.

131

132 1.2. Prepare recovery water (0.9% NaCl solution) by adding 18 g of NaCl into 2 L of
133 aquarium water. Store the solution at room temperature until use.

134

135 1.3. Prepare the incision tools by breaking a razor diagonally to get a sharp point
136 (**Figure 1A**).

137

138 1.4. Prepare blood anti-coagulant solution (0.05 U/ μ l of sodium heparin) by diluting 25
139 μ l of sodium heparin into 500 μ l of 1X PBS. Store the anti-coagulant solution at 4 °C
140 until use.

141

142 1.5. Prepare two glass needles from a 90 mm long glass capillary by pulling a glass
143 capillary with a needle puller (**Figure 1B**) following the instructions of the
144 manufacturer.

145

146 **NOTE:** The outer diameter of the glass needle is 1 mm, while the inner diameter is 0.6
147 mm.

148

149 1.6. Prepare a 1.5 ml plastic tube lid by cutting the lid and make a hole that fits with
150 the needle outer diameter (**Figure 1C**). To make the hole, heat one end of the 9-mm
151 glass capillary and stab the heated glass capillary through the lid. Alternatively, use a
152 needle to stab through the lid until the diameter of the hole fits with the 9-mm glass
153 capillary.

154

155 2. Gonadectomy procedure

156 2.1. Prepare 0.02% of anesthesia solution (MS-222) by diluting one tube of Tricaine
157 stock (0.6%) in 30 ml of recovery water.

158 **NOTE:** Depending on the size of the fish, additional Tricaine stock can be added, but final
159 concentration of 0.02 % usually works well.

160 In our experience, most cases that fish did not recover after surgery were due to over
161 exposure to high concentration of anesthesia, probably not due to the mistake in surgery.

162

163 2.2. Prepare dissection tools including one ultra-fine and two fine forceps (one with
164 relatively wide tip), small scissors, nylon thread and razor as described in **step 1.3**.

165

166 2.3. Anesthetize the fish by putting it into the 0.02% anesthesia solution, and make
167 sure that the fish is anesthetized enough to be operated.

168 **NOTE:** To ensure that the fish is fully anesthetized, the fish body can be pinched gently
169 using forceps. If the fish does not react, the gonadectomy can be started.

170

171 2.4. Place the anesthetized fish under a dissection microscope.

172

173 2.5. Ovariectomy in females

174 2.5.1. Remove oviposited eggs (eggs hanging outside the female body) if any, and
175 scrap the scales in the incision area (**Figure 2A**).

176

177 2.5.2. Incise gently the incision area between the ribs (**Figure 2A**) using the razor
178 blade, pinch gently the fish abdomen while taking out the ovary little by little
179 using fine forceps with wide tip.

180

181 2.5.3. Cut the end of the ovary using fine forceps and put aside the ovary (**Figure 2B**).

182 **NOTE:** It is important to take care not to break the ovarian sac as possible. In case of
183 breaking the ovarian sac, it is important to remove as completely as possible without
184 leaving even some non-ovulated eggs.

185

186 2.6. Orchidectomy in males

187 2.6.1. After making an incision with the razor blade, incise gently between the ribs
188 (Figure 2A) and open up the incision slowly using fine forceps.

189

190 2.6.2. Grab the testis gently using the fine forceps and take out the testes slowly.
191 Afterwards, cut the end of the testis to remove testis completely (Figure 2B).

192

193 **NOTE:** For male orchidectomy, all preparations are similar to in females until the incision
194 part. Incision area of males should be more dorsal side of the abdomen (Figure 2A).
195 When grabbing the testes, sometimes we obtain only the fat resembling the testes.
196 However, after restoring the fat, it is possible to try to find the testes again. (Figure 2B).

197

198 **NOTE:** For both males and females, it is important to minimize the incision size in the
199 abdomen to prevent excessive damage that can lead to mortality. Sometimes the
200 intestines may also appear through the incision along with the gonads, so make sure they
201 are properly returned inside the incision before closure. It is important to understand
202 where ovaries or testes are localized in medaka abdomen by dissection.

203

204 2.7. Suture the incision similarly in males and females (Figure 3).

205 2.7.1. Place the nylon thread beside the incision area, inject the right side of incision
206 part from inner body cavity using ultra-fine forceps to take the thread in with
207 the help of fine forceps (Figure 3;1-2).

208

209 2.7.2. Inject the left side of incision part from outer body cavity to take out the
210 thread (**Figure 3;3-4**).

211

212 2.7.3. Close the incision opening and make two knots and cut the excessive thread
213 (**Figure 3;4-6**).

214 2.7.4. Put the fish directly into the recovery water.

215 **NOTE:** The suture should be adequately tight, and the remaining thread on the fish
216 should be long enough to prevent the disattachment of the suture.

217

218 3. Blood sampling procedure

219 3.1. Prepare the tools including glass needle, silicone capillary, a plastic tube with a
220 hole, an empty 1.5 ml plastic tube, a 1.5 ml plastic tube containing 1X PBS, mini
221 centrifuge, and tape.

222

223 3.2. Anesthetize the fish using 0.02% MS-222 solution as described in **step 2.1**, and
224 place the fish under a dissection microscope in a vertical position (**Figure 4A**).

225 **NOTE:** It is highly recommended to place the fish on a bright surface to ease visualization
226 of the caudal puncture vein.

227

228 3.3. Install the blood drawer by attaching a glass needle to the silicone tubing (**Figure**
229 **4B**). Break the tip of the needle with forceps with wide tip, and coat the anti-
230 coagulant inside a needle by suctioning and blowing.

231 **NOTE:** Make sure that the opening of the needle tip is sufficiently large to allow drawing
232 the blood.

233

234 3.4. Direct the needle toward the peduncle area of the fish, aim at the caudal peduncle
235 vein (**Figure 5A**) and draw the blood using mouth until at least one fourth the total
236 volume of the needle is filled (**Figure 5B**).

237 **NOTE:** It is important to stop suctioning when removing the needle from fish body.

238

239 3.5. Release the needle and put a piece of tape on it. Place the lid with a hole on a
240 reservoir tube and put the needle inside the tube through the hole with the needle
241 tip on the outside (**Figure 5C**).

242

243 3.6. Spin down the blood to collect the blood in the tube

244

245 3.7. Proceed directly to downstream applications, or store the blood at -20 °C until
246 use.

247 **NOTE:** The blood analysis is dependent on what outcome is required. For sex steroid
248 analysis such as E2 or 11KT, 1 ul of collected blood is adequate for the analysis. The
249 blood can be diluted in 1X PBS and extract steroids with diethyl ether or
250 dichloromethane if necessary. In many previous studies, clot was removed, however, as
251 the volume of blood is so small that we can ignore the effects in most cases.

252

253

254 Representative Results

255 This protocol describes every step for performing gonadectomy and blood
256 sampling in small sized teleosts, using the Japanese medaka as a model. The survival rate
257 of the fish after ovariectomy (OVX) in females is 100% (10 out of 10 fish) while 94% (17
258 out of 18 fish) of the males survived after orchidectomy. Meanwhile, after blood sampling
259 procedure was performed, all (38 fish) fish survived.

260 Sham-operated females show oviposition (**Figure 6A**) and all the eggs are fertilized
261 and allow for embryonic development (**Figure 6B**). Sham operated males are also able to
262 fertilize eggs after only a couple of weeks. Similarly, partly-gonadectomized females
263 reared with partly-gonadectomized males also show oviposition and showed 100% of
264 fertilized eggs after 2 months. In contrast, no oviposition in females or fertilization by
265 males could be observed in fully gonadectomized fish, even after 4 months.

266 When performed correctly, the body shape of the fish slightly changes (Figure 7A).
267 If performed correctly, no piece of gonad should remain after the gonadectomy
268 procedure when dissected (Figure 7B).

269 Four weeks post-gonadectomy, the incision and suture completely disappeared
270 (**Figure 8**), and after 4 months, all gonadectomized fish still showed healthy phenotype,
271 and no gonadal tissue could be found.

272 E2 and 11-KT blood concentrations measured with ELISA following the
273 manufacturer's instructions revealed that E2 levels in OVX females ($0,36 \pm 0,2$ ng/ml) are
274 significantly lower than in sham-operated females ($4,15 \pm 0,5$ ng/ml) 24 hours after
275 surgery (**Figure 9A**). Likewise, 11-KT concentrations in orchidectomized males ($0,4 \pm 0,2$
276 ng/ml) are also significantly lower than in sham-operated males (10.38 ± 1.32 ng/ml) 24
277 hours after surgery (**Figure 9B**). There is no statistical difference in blood levels of E2 and
278 11KT in gonadectomized fish after 4 months compared to the levels of those after 24
279 hours (**Figure 9A-B**).

280 In contrast to fully OVX females, partly OVX fish, where only 1/3 to 1/2 of the
281 gonad was removed, showed no difference in E2 levels ($3,37 \pm 0,6$ ng/ml) compared to
282 sham-operated fish (**Figure 9A**). However, in males there is a difference observed
283 between 11KT levels of sham-operated fish and partly orchidectomized fish (8.37 ± 1.92
284 ng/ml) (**Figure 9B**). However, partly orchidectomized males, where only 1/3 to 1/2 of the
285 gonad was removed, showed significantly higher levels of 11KT compared to in fully
286 orchidectomized fish.

287

288 Discussion

289 As reported in previous literature, gonadectomy and blood sampling have long
290 been used in other model species to investigate questions related to the role of sex
291 steroids in regulation of the BPG axis. However, these techniques seem to be amenable
292 only for bigger animals. Considering the small size of the most used teleost models, we
293 hereby describe detailed protocols for gonadectomy and blood sampling that are feasible
294 for these small teleost models.

295 The fact that the survival rate of gonadectomized fish reached almost 100%
296 indicates that the gonadectomy procedure is feasible to be applied on small fish.
297 Similarly, the procedure of blood sampling does not affect the survivability of the fish as
298 shown by the 100% survival after undergoing this procedure. In addition, sham-operated
299 females reared together with sham-operated males show oviposition and 100% fertilized
300 eggs, indicating that the incision and suture procedure do not affect the reproduction of
301 the fish. In other words, they were healthy enough to spawn. Meanwhile, as shown in
302 **Figure 7**, the incision and suture mark on the fish completely disappeared 4 weeks post-
303 gonadectomy and the fish are still alive and look healthy 4 months after surgery,
304 indicating that the operation procedure is safe for the fish for long term purpose
305 gonadectomy and does not affect the life span of the fish. In addition, after 4 months no
306 gonads are observed. This is confirmed by the low levels of E2 and 11KT which are still
307 similar to that of those found in gonadectomized fish after 24 hours.

308 Partly gonadectomized fish showed comparable concentrations of sex steroids to
309 sham-operated fish, and as a consequence, resulted in oviposition in the females and
310 fertilization of eggs. These results suggest that the procedure of gonadectomy should be
311 performed with high precision, meaning that the ovary or testes should be completely
312 removed. Furthermore, since this procedure does not rely on Fish Anesthesia Delivery
313 System (FADS) as demonstrated in ⁵², the gonadectomy should be carried out as quickly
314 as possible to prevent mortality during surgery. Indeed, the use of FADS enables us to
315 maintain the rhythm of operation since this tool allows continuous anesthetic condition
316 to the fish despite being exposed to the air. Nonetheless, due to its lower feasibility in
317 smaller teleosts, the use of FADS cannot be performed with these sized fish. Many factors
318 can affect the success rate of the procedure, including anesthesia period, the wideness of
319 incision, the accuracy and tidiness of the suture and fish handling during the procedure.
320 Since the protocol relies so much on the quick and clean procedure, some training is
321 highly recommended until reaching high success rate, indicated by high survival rate of
322 the fish after gonadectomy as well as complete removal of the gonads (see the difference
323 of morphological and anatomical appearance of the fish before and after successful
324 gonadectomy in **Figure 7**). Another important point is that one should prepare healthy
325 fish by maintaining the fish optimally prior to performing the protocol.

326 With respect to blood sampling procedure, the steroid extraction is generally
327 performed using diethyl ether or dichloromethane. Meanwhile, the evaluation of sex
328 steroid concentrations is commonly carried out by using Enzyme-linked Immunosorbent
329 Assay (ELISA) kit, and there have been many ELISA kits commercially available for
330 different types of sex steroids. Due to the low amount of blood collected during blood
331 sampling, the assays performed to evaluate sex steroid concentrations in small teleosts is
332 not aimed for serum, but whole blood. This might influence the results obtained from the
333 assays. A previous study⁵³ suggested that the quantification of sex steroids using whole
334 blood can slightly differ from that of serum. Therefore, the comparison between plasma
335 and blood concentrations of sex steroids should be investigated for each assay in order to
336 determine whether measured concentrations from the assay should be re-calculated to
337 get comparable results as from serum.

338 As documented in previous studies with different animal models, the protocol
339 described here will allow us to investigate questions related to reproductive physiology
340 using small teleosts as model. In fact, these techniques have already contributed to
341 answer questions concerning the regulation of the BPG axis and its feedback
342 mechanisms, such as the involvement of *kiss1* (kisspeptin gene type 1) expressing
343 neurons in positive feedback loops⁵⁴, estrogen-mediated regulation of *kiss1* expressing
344 neurons in nucleus ventralis tuberis (NVT), and *kiss2* (kisspeptin gene type 2) expressing
345 neurons in preoptic area (POA)^{55,56}, the expression profile of *fshb* (follicle-stimulating
346 hormone beta sub-unit gene) in *esr2a* (estrogen receptor gene) knock out (KO) fish⁵⁷ as
347 well as the profile of circadian rhythm of E2 in female fish⁵³. Furthermore, since previous
348 studies demonstrated that sex steroids also affect the proliferation of gonadotropic cells
349 in the pituitary of teleosts^{58,59}, it would be intriguing to investigate the effects of sex
350 steroid clearance after gonadectomy on pituitary plasticity. Besides, due to the fact that
351 the protocol can also be applied for blood glucose measurements as demonstrated in
352 zebrafish⁶⁰ and medaka⁶¹, it may also be expanded to address research questions in
353 other fields of physiology.

354 Finally, the protocols described here are intended and optimized for adult
355 medaka, and the outcomes due to different size of fish and materials used during the
356 procedures may vary. Also, as medaka left and right ovaries/ testes are fused, which

357 might provide an important advantage for gonadectomy, this protocol might need few
358 small adaptations before to be used in other species where this is not the case such as in
359 zebrafish. Thus, an optimization according to the choice of laboratory equipment and fish
360 size should be taken into account before testing these protocols.

361

362 [Disclosures](#)

363 The authors have nothing to disclose.

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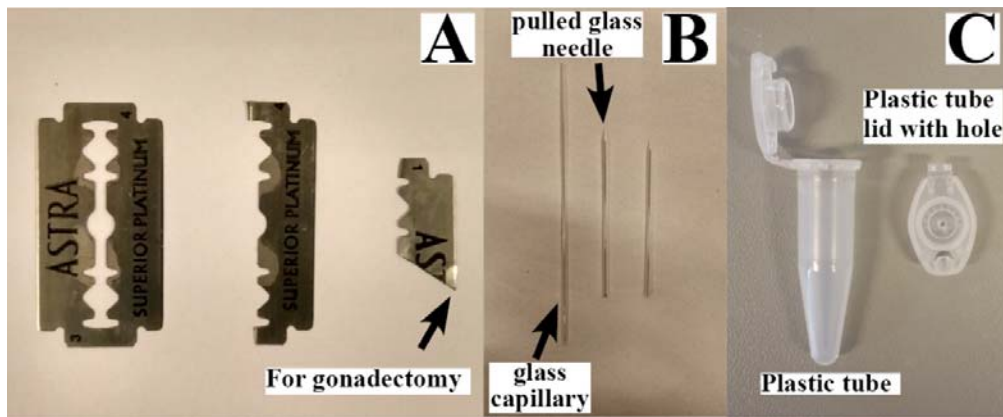


Fig 1. Razor blade for gonadectomy (A), glass needle for blood extraction (B), and plastic tube together with a lid with a hole for blood collection (C).

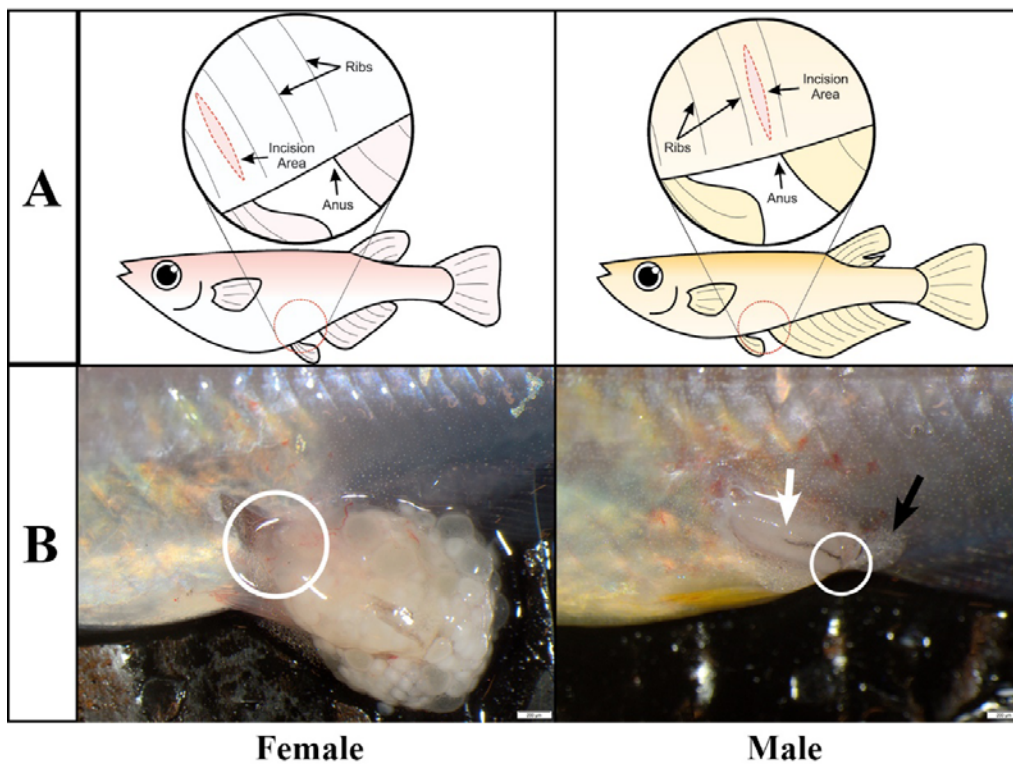


Fig 2. Location of the incision area. A) Drawing of the incision area located between the ribs in females (left panel) and males (right panel); B) gonad removal in females (left panel) and males (right panel), white circles showing the joint part, white arrow showing the testis and black arrow showing the fat.

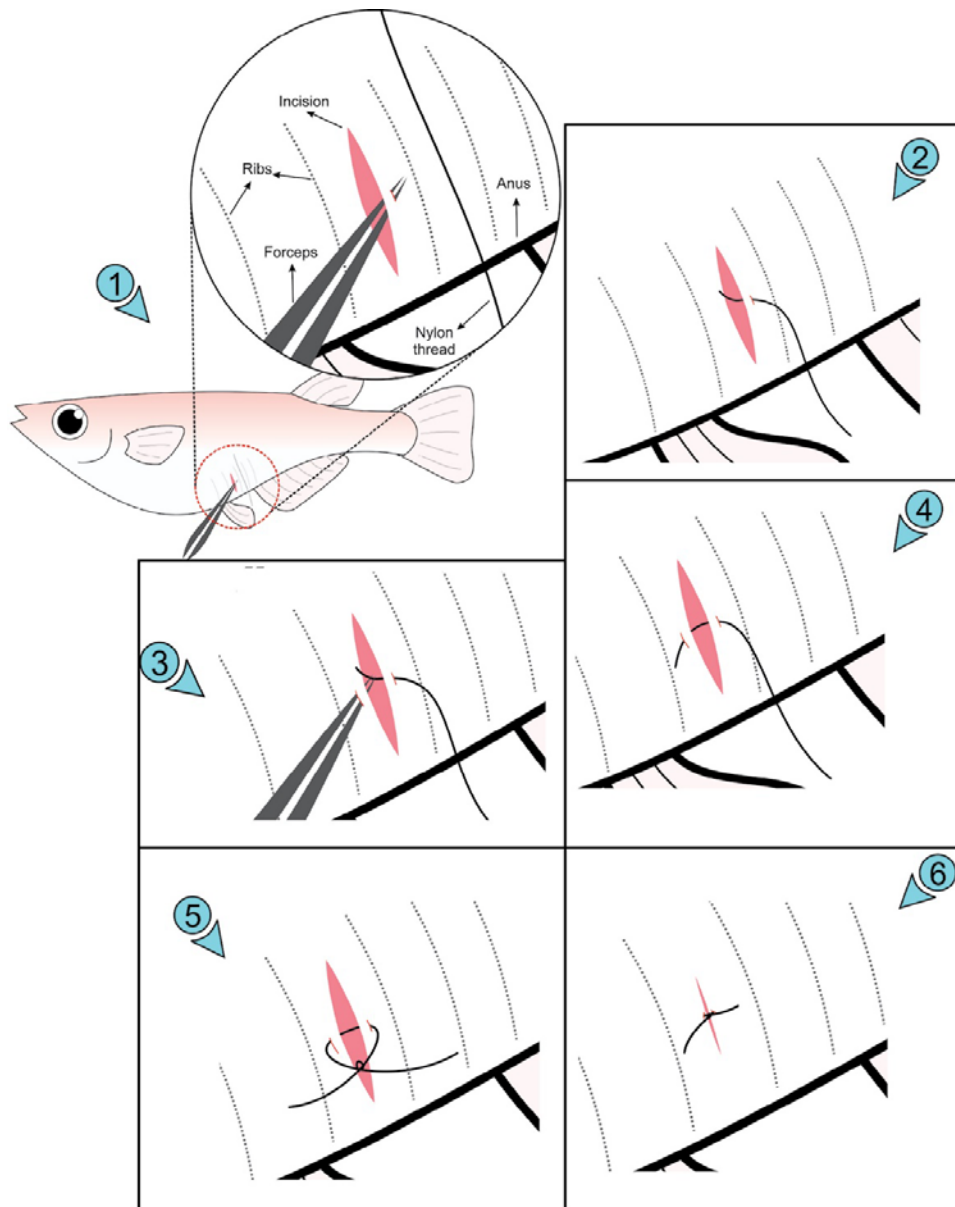


Figure 3. The procedure of suture. 1) a hole is made on the right side of the incision using fine forceps. 2) the nylon thread is passed through the skin using the hole made in 1. 3) a hole is made in the left side of the incision. 4) the nylon thread is passed through the hole made in 3. 5) an overhand knot is made twice to close the incision. 6) excess thread is cut.

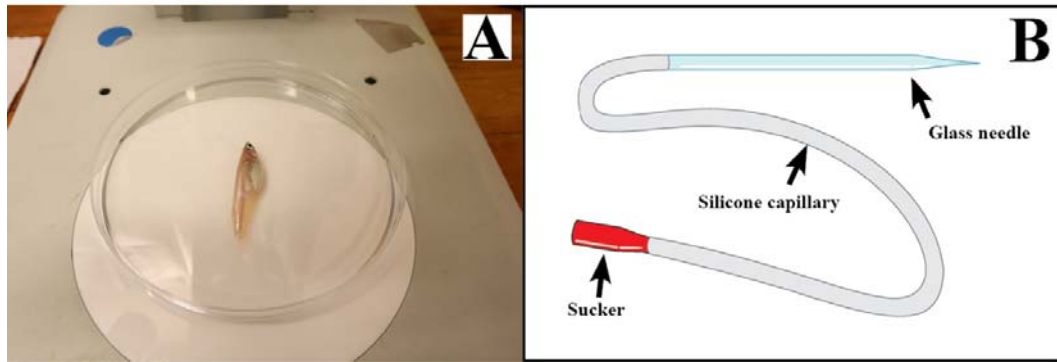


Fig 4. Fish position during blood sampling (A), the installation of glass needle with the silicone capillary (B).

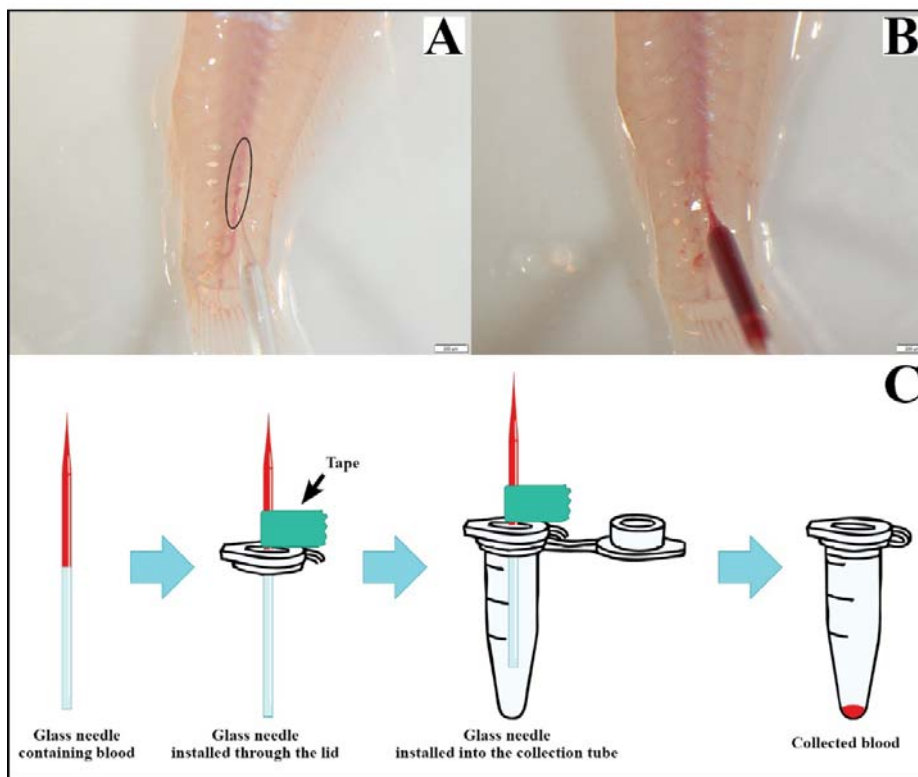


Fig 5. The suction area of blood sampling (A), drawn blood (B) and blood collection steps (C).

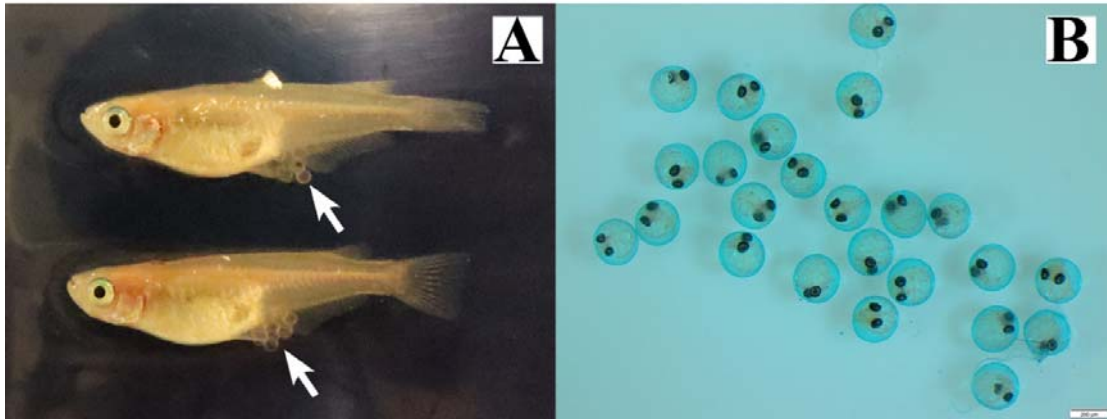


Fig 6. Sham-operated fish shows spawning. Oviposition of eggs (A) and fertilized eggs (B).

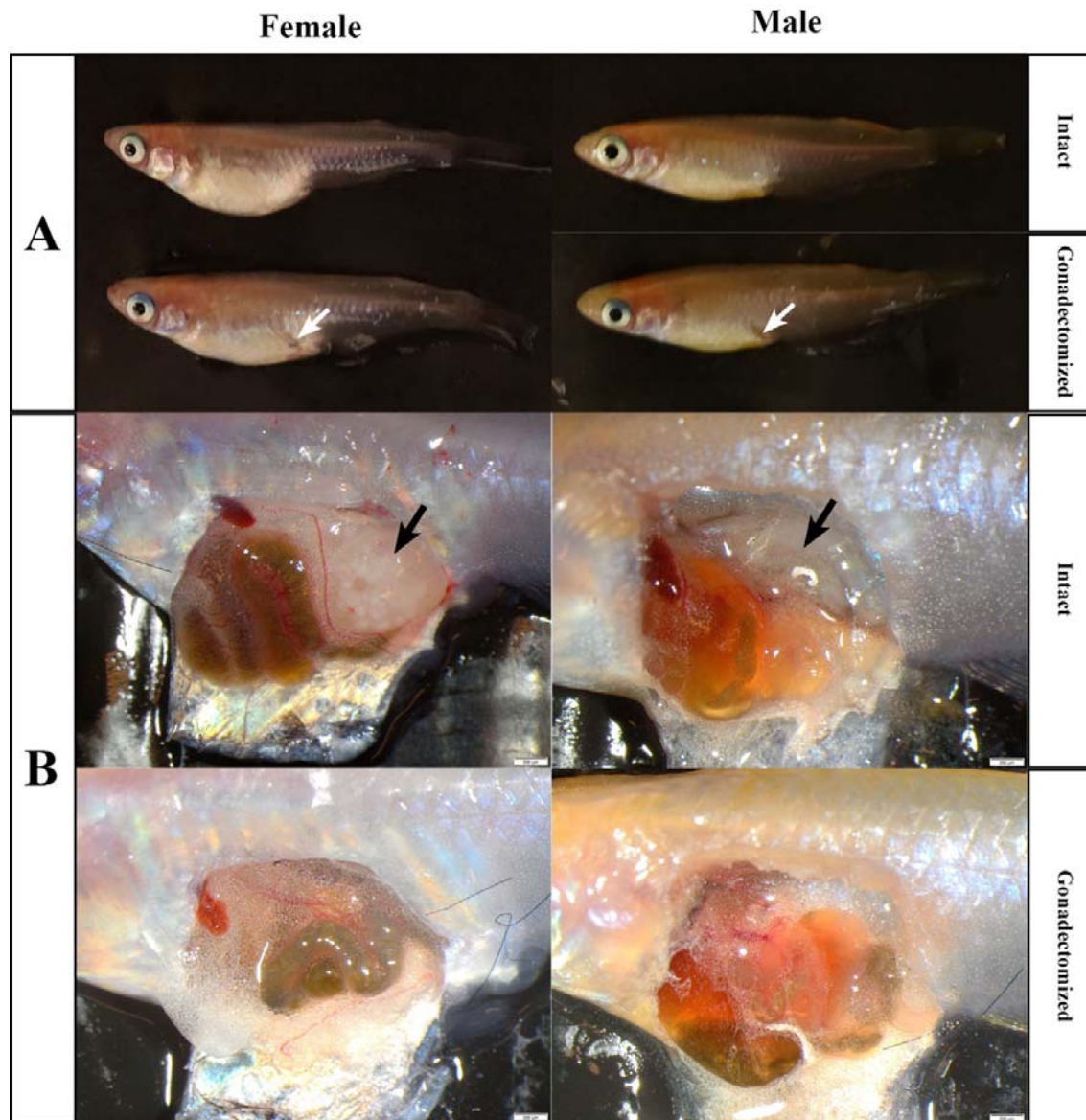


Fig 7. Morphological (A) and anatomical (B) appearance of intact and gonadectomized fish



Fig 8. The incision and suture after 4 weeks.

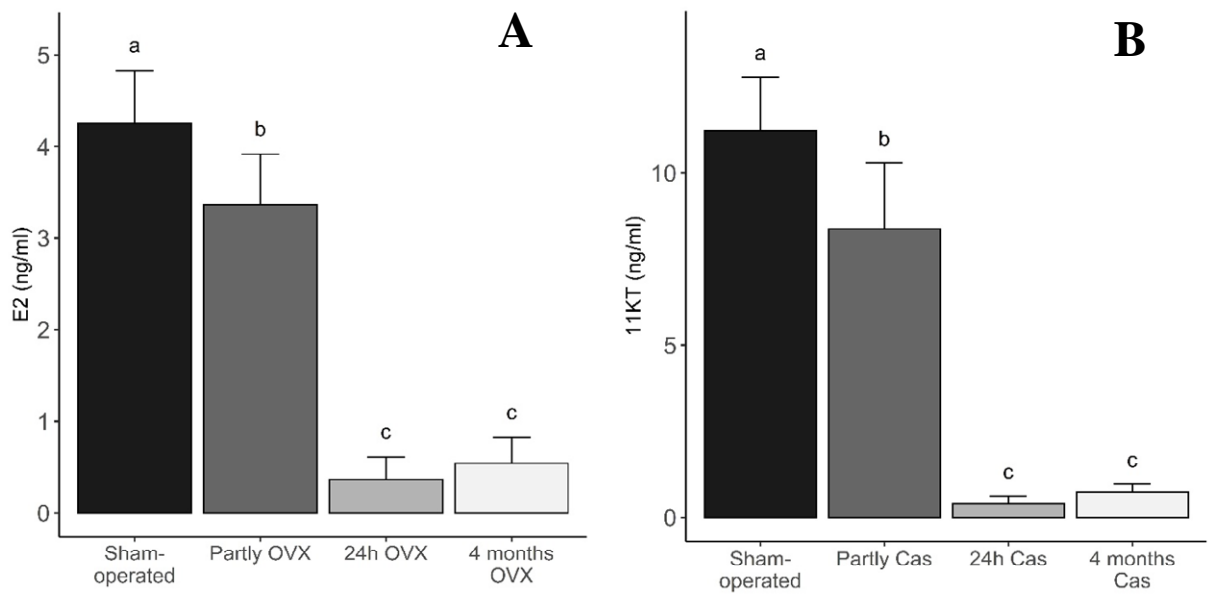


Fig 9. Blood levels of E2 in female (A) and 11KT in male (B) medaka, 24 hours after sham operation (control), partly gonadectomy or gonadectomy, and 4 months after gonadectomy (OVX, ovariectomy in females; Cas, castrated in males) (Data in the graph are provided as mean + SD; n = 5). Different letters (a-c) show statistically significant differences.