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Gonadectomy and blood sampling procedures in the small size teleost model Japanese medaka (Oryzias latipes)

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TITLE:

Gonadectomy and blood sampling procedures in the small size teleost model Japanese medaka (*Oryzias latipes*)

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KEYWORDS:

Gonadectomy, ovariectomy, orchidectomy, castration, medaka, blood, steroids, fish, reproduction, plasticity, estradiol, 11-ketotestosterone

SUMMARY:

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

ABSTRACT:

Sex steroids, produced by the gonads, play an essential role in brain and pituitary tissue plasticity and in the neuroendocrine control of reproduction in all vertebrates by providing feedback to the brain and pituitary. Teleost fishes possess a higher degree of tissue plasticity and variation in reproduction strategies compared to mammals and appear to be useful models to investigate the role of sex steroids and the mechanisms by which they act. The removal of the main source of sex steroid production using gonadectomy together with blood sampling to measure steroid levels has been well-established and fairly feasible in bigger fish and is a powerful technique to investigate the role and effects of sex steroids. However, these techniques raise challenges when implemented in small size teleost

models. Here, we describe the step-by-step procedures of gonadectomy in both males and female Japanese medaka followed by blood sampling. These protocols are shown to be highly feasible in medaka indicated by a high survival rate, safety for the life span and phenotype of the fish, and reproducibility in terms of sex steroid clearance. The use of these procedures combined with the other advantages of using this small teleost model will greatly improve the understanding of feedback mechanisms in the neuroendocrine control of reproduction and tissue plasticity provided by sex steroids in vertebrates.

INTRODUCTION

In vertebrates, sex steroids, which are mainly produced by the gonads, play important roles in the regulation of the Brain-Pituitary-Gonadal (BPG) axis through various feedback mechanisms[1-5]. In addition, sex steroids affect the proliferation and activity of neurons in the brain[6-8] and endocrine cells, including gonadotropes, in the pituitary[9,10], and thus serve crucial roles in brain and pituitary plasticity. Despite relatively good knowledge in mammals, the mechanism of BPG axis regulation mediated by sex steroids is far from understood in non-mammalian species, leading to poor understanding of evolutionary conserved principles[11]. There is still a limited number of studies documenting the role of sex steroids on brain and pituitary plasticity, thus raising the need for further investigations of the role and effects of sex steroids on diverse vertebrate species.

Among vertebrates, teleosts have become powerful model animals in addressing numerous biological and physiological questions, including stress response[12,13], growth[14,15], nutritional physiology[16,17] and reproduction[2]. Teleosts, in which sex steroids are mostly represented by estradiol (E2) in females and 11-ketotestosterone (11-KT) in males[18,19], have thus long been reliable experimental models for investigating the general principle of reproduction across species. Teleosts show uniqueness in their hypothalamic-pituitary connection[20,21] and distinct gonadotrope cells[22], which are sometimes convenient for the elucidation of regulatory mechanisms. Moreover, due to their amenability to both laboratory and field experiments, teleosts offer many advantages compared to other organisms. They are relatively inexpensive to purchase and maintain[23,24]. In particular, small teleost models such as zebrafish (*Danio rerio*) and the Japanese medaka (*Oryzias latipes*), are species with very high fecundity and a relatively short life cycle enabling rapid analysis of gene function and disease mechanisms[23], thus providing even greater advantages in addressing a plethora of biological and physiological questions, considering the numerous well-developed protocols and genetic toolkit available for this species[25].

In numerous studies, the removal of gonads (gonadectomy) along with blood sampling techniques have been used as a method for investigating many physiological questions, including its impact in vertebrate reproductive physiology in mammals[26-28], birds[29] and amphibians[30]. Although the gonadectomy effect on reproductive physiology can be alternatively mimicked by sex steroid antagonists, such as tamoxifen and clomiphene, the effect of the drugs appears to be inconsistent due to bimodal effects[31,32]. Chronic exposure to a sex steroid antagonist may lead to ovarian enlargement[33,34], which may disable observation of its effects for long-term purposes due to an unhealthy phenotype. In addition, it is impossible to perform a recovery experiment after sex steroid antagonist treatment, to warrant the specific effect of certain sex steroids. Along with those aforementioned points, other trade-offs of sex steroid antagonist use have been

extensively reviewed[31,32]. Therefore, gonadectomy still appears today as a powerful technique to investigate the role of sex steroids.

While gonadectomy and blood sampling techniques are relatively easy to perform in bigger species, such as European sea bass (*Dicentrarchus labrax*)[35], bluehead wrasse (*Thalassoma bifasciatum*)[36], dogfish (*Scyliorhinus canicula*)[37] and catfish (*Heteropneustes fossilis* and *Clarias bathracus*)[38,39], they raise challenges when applied in small fish as medaka. For instance, the use of Fish Anesthesia Delivery System (FADS)[40] is less feasible and appears to be prone to excessive physical damage for small fish. In addition, a gonadectomy procedure that is commonly used for bigger fish[40] is not suitable for small fish that requires high precision to avoid excessive damage. Finally, blood sampling is challenging due to the limited access to blood vessels and the small amount of blood in those animals. Therefore, a clear protocol demonstrating every step of gonadectomy and blood sampling in a small teleost is of importance.

This paper demonstrates the step-by-step procedures of gonadectomy followed by blood sampling in Japanese medaka, a small freshwater fish native to East Asia. Japanese medaka have a sequenced genome, several molecular and genetic tools available[25], and a genetic sex determination system allowing for investigation of sexual differences before secondary sexual characters or gonads are well developed[41]. Interestingly, Japanese medaka possess fused gonads contrary to many other teleost species[42]. These two techniques combined take only 8 minutes in total and will complete the list of video protocols already existing for this species that included labeling of blood vessels[43], patch-clamp on pituitary sections[44] and brain neurons[45], and primary cell culture[46]. These techniques will allow the research community to investigate and better understand the roles of sex steroids in feedback mechanisms as well as brain and pituitary plasticity in the future.

PROTOCOL

All experimentations and animal handling were conducted in accordance with the recommendations on the experimental animal welfare at Norwegian University of Life Sciences. Experiments using gonadectomy were approved by the Norwegian Food Safety Authority (FOTS ID 24305).

NOTE: The experiments were performed using adult male and female (6-7 months old, weight ca. 0.35 g, length ca. 2.7 cm) Japanese medaka. The sex was determined by distinguishing the secondary sexual characteristics, such as the size and shape of dorsal and anal fin, as described in[42,47].

1. Instruments and solutions preparation

1.1. Prepare anesthetic stock solution (0.6% Tricaine).

1.1.1. Dilute 0.6 g of Tricaine (MS-222) in 100 mL of 10x Phosphate Buffer Saline (PBS).

1.1.2. Distribute 1 mL of the Tricaine stock solution into several 1.5 mL plastic tubes and store at -20 °C until use.

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

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

1.4. Prepare blood anti-coagulant solution (0.05 U/µL of sodium heparin) by diluting 25 µL of sodium heparin into 500 µL of 1x PBS. Store the anti-coagulant solution at 4 °C until use.

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

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

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

2. Gonadectomy procedure

2.1. Prepare 0.02% of anesthetic solution by diluting one tube of Tricaine stock (0.6%) in 30 mL of recovery water.

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

2.3. Anesthetize the fish by putting it into the 0.02% anesthetic solution for 30-60 seconds.

NOTE: The duration of the anesthesia depends on the size and weight of the fish, and must be adapted. To ensure that the fish is fully anesthetized, the fish body can be pinched gently using forceps. If the fish does not react, the gonadectomy can be started.

2.4. Take out the fish from the anesthetic solution and place the fish horizontally on its side, out-of-water under a dissection microscope.

2.5. Ovariectomy in females

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

2.5.2. Gently make an incision about 2-2.5 mm long between the ribs, between the pelvic and anal fins (**Figure 2A**), using the razor blade. Then, pinch gently the fish abdomen while taking out the ovaries little by little using fine forceps with wide tip.

2.5.3. Cut the end of the ovaries using fine forceps and place the ovaries aside (**Figure 2B**).

NOTE: Take care not to break the ovarian sac if possible. If the ovarian sac is broken, remove any gonad traces as completely as possible without leaving any non-ovulated eggs.

2.6. Orchidectomy in males

2.6.1. Gently make an incision between the ribs above the anus (**Figure 2A**), and open up the incision slowly using fine forceps.

2.6.2. Gently grab the testes using the fine forceps and slowly take out the testes. Afterwards, cut the end of the testes to completely remove the testes (**Figure 2B**). For male orchidectomy, all preparations are similar to in females until the incision part. When grabbing the testes, sometimes the fat resembling the testes is obtained. However, after restoring the fat, it is possible to try to find the testes again (**Figure 2B**).

NOTE: For both males and females, it is important to minimize the incision size in the abdomen to prevent excessive damage that can lead to mortality. Sometimes the intestines may also appear through the incision along with the gonads, so make sure they are properly returned inside the incision before closure. Prior knowledge on ovaries and testes location in medaka abdomen is essential.

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

2.7.1. Place the nylon thread beside the incision area, and stab the skin from the right side of the incision through inner body cavity using ultra-fine forceps to take the thread in with fine forceps (**Figure 3; 1-2**).

2.7.2. Stab the skin from the left side of the incision through outer body cavity to take out the thread (**Figure 3; 3-4**).

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

NOTE: The suture must be adequately tight, and the remaining thread on the fish must be long enough to prevent loosening of the suture. The whole procedure from anesthesia until suturing commonly takes up to 6 minutes. Longer times may increase mortality rate.

2.7.4. Put the fish in the recovery water and leave them for at least 24 hours before transferring them to the aquarium system.

NOTE: Gonadectomized fish usually show normal behavior after 1-2 hours in the recovery water. Therefore, depending on the experiment purpose, one can sample the fish after this time interval.

3. Blood sampling procedure

3.1. Prepare the tools: a glass needle, a silicone capillary, a plastic tube with a hole, an empty 1.5 mL plastic tube, a minicentrifuge and tape.

3.2. Anesthetize the fish using 0.02% anesthetic solution as described in step 2.1 and place the fish under a dissection microscope in a vertical position (**Figure 4A**). Place the fish on a bright surface to ease visualization of the caudal puncture vein.

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

NOTE: The use of a sucker and a silicone capillary with at least 50 cm length are recommended for safety measures to avoid any direct contact of the blood when suctioning. In addition, make sure that the opening of the needle tip is sufficiently large to allow drawing the blood.

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

NOTE: It is important to stop suctioning before removing the needle from fish body.

3.5. Release the needle and put a piece of tape at the proximity of the sharp side of the needle. Place the lid with a hole on a collection tube and put the needle inside the tube through the hole with the needle tip on the outside (**Figure 5C**).

3.6. Put the fish in the recovery water and leave them for at least 24 hours before transferring them to the aquarium system.

NOTE: To perform a second blood sampling from the same fish, sample the blood one week after the first blood sampling.

3.7. Flash spin down the collected blood for 1-2 seconds at 1,000 x g and room temperature to collect the blood in the tube.

3.8. Proceed directly to downstream applications or store the blood at -20 °C until use.

NOTE: Refer to the previous study for sex steroid extraction from the whole blood[48].

REPRESENTATIVE RESULTS

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

Sham-operated females show oviposition (**Figure 6A**) and all the eggs were fertilized and allowed for embryonic development (**Figure 6B**). Sham operated males were also able to fertilize eggs after only 1-2 weeks. Two out of six partly-gonadectomized females reared with partly-gonadectomized males also show oviposition and showed 100% of fertilized eggs after 2 months. In contrast, no oviposition in females or fertilization by males was observed in fully gonadectomized fish, even after 4 months.

When performed correctly, the body shape of the fish slightly changes (**Figure 7A**), and no piece of gonad should remain after the gonadectomy procedure when dissected (**Figure 7B**). Meanwhile, 4 weeks post-gonadectomy, the incision and suture completely disappeared (**Figure 8**), and after 4 months, all gonadectomized fish still showed healthy phenotype, and no gonadal tissue was found.

E2 blood concentrations in females (**Table 1**), measured with ELISA following the manufacturer's instructions, revealed that the E2 level in OVX fish is significantly lower than in sham-operated fish 24 hours after surgery ($p < 0.00001$). After 4 months, the E2 level in OVX fish is also significantly lower than in sham-operated fish ($p < 0.00001$) and shows no significant difference compared to that in 24h post-OVX fish ($p > 0.05$). Finally, partly OVX fish, where only 1/3 to 1/2 of the gonad was removed, show significantly lower E2 levels than sham-operated fish ($p = 0.0437$) and significantly higher E2 levels than fully OVX fish ($p < 0.00001$) (**Figure 9A**).

Similarly in males (**Table 1**), the 11-KT concentration in orchidectomized fish is significantly lower than in sham-operated fish 24 hours after surgery ($p < 0.00001$). The level of 11-KT in orchidectomized fish after 4 months is also significantly lower than in sham-operated fish ($p < 0.00001$) and shows no difference compared to 24 h post-orchidectomized fish ($p > 0.05$). Finally, partly orchidectomized fish show significantly lower levels of 11-KT than sham-operated fish ($p = 0.0428$) and significantly higher levels of 11-KT than fully orchidectomized fish ($p < 0.00001$) (**Figure 9B**).

Figure legends:

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

Figure 2. Location of the incision area. A) Drawing of the incision area located between the ribs, between the pelvic and anal fins 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.

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

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

Figure 6. Sham-operated fish shows oviposition of eggs pointed by white arrow (A) and fertilized eggs (B).

Figure 7. Morphological (A) and anatomical (B) appearance of intact and gonadectomized fish. White arrows (top panels) show the surgery mark on gonadectomized fish. Black arrows (bottom panels) show gonads in intact fish.

Figure 8. Surgery marks in male and female fish after 4 weeks.

Figure 9. Blood levels of E2 in female (A) and 11-KT in male (B) Japanese medaka, 24 hours after sham operation (control), partly gonadectomy or gonadectomy, and 4 months after gonadectomy (OVX, ovariectomy in females; Cas, orchidectomy in males). The statistical analyses were performed using One Way ANOVA followed by Tukey *Post Hoc* test. Different letters (a-c) show significant differences (p-value < 0.05). Data in the graph are provided as mean ± SD, n = 5.

Table 1. E2 and 11-KT levels (ng/mL) in females and males of sham-operated and gonadectomized and partly gonadectomized fish.

DISCUSSION

As reported in previous literature, gonadectomy and blood sampling have long been used in other model species to investigate questions related to the role of sex steroids in regulation of the BPG axis. However, these techniques seem to be amenable only for bigger animals. Considering the small size of the commonly used teleost model, Japanese medaka, we provide a detailed protocol for gonadectomy and blood sampling that is feasible for this species.

The fact that the survival rate of gonadectomized fish reached almost 100% indicates that the gonadectomy procedure is feasible to be applied on medaka. Similarly, the procedure of blood sampling does not affect the survivability of the fish as shown by the 100% survival after undergoing this procedure. In addition, sham-operated females reared together with sham-operated males show oviposition and 100% fertilized eggs, indicating that the incision and suture procedure do not affect the reproduction of the fish. In other words, they were healthy enough to spawn. Furthermore, partly gonadectomized fish showed comparable concentrations of sex steroids to sham-operated fish, and oviposition in some females as well as fertilization of eggs by males were observed in those partly gonadectomized fish. These results suggest that the procedure of gonadectomy should be performed with high precision, meaning that the ovaries or testes should be completely removed.

As shown in **Figure 7**, the incision and suture mark on the fish completely disappeared 4 weeks post-gonadectomy, and the fish are still alive and look healthy 4 months after surgery. These indicate that the operation procedure is safe for the fish for long term

purpose gonadectomy and does not affect the life span of the fish. In addition, after 4 months, no gonads are observed. This is confirmed by the low levels of E2 and 11-KT that are still similar to those found in gonadectomized fish after 24 hours.

The levels of E2 and 11-KT in gonadectomized fish are significantly lower than sham-operated fish, already after 24 hours post-surgery and remain lower in fish sampled 4 months after gonadectomy. The significantly lower sex steroid levels in gonadectomized fish compared to control have been observed in previous studies in dogfish[37], catfish[39] and medaka[48]. These consistent evidences suggest that the gonadectomy procedure described in the protocol is a reliable technique to clear circulating sex steroids.

Since this procedure does not rely on FADS as demonstrated in[40], the gonadectomy should be carried out as quickly as possible to prevent mortality during surgery. Indeed, the use of FADS enables to maintain the rhythm of operation since this tool allows continuous anesthetic condition to the fish despite being exposed to the air. Nonetheless, due to its lower feasibility in the small teleost as medaka, the use of FADS cannot be performed with that fish size. In addition, unlike the previous gonadectomy protocol in bigger fish that enables wide incision to reach the gonad, the protocol described in this manuscript does not allow wide incision to avoid excessive damage to the small fish. Therefore, one should be very careful when trying to access the gonad using forceps to prevent damage in other tissues inside the fish body cavity.

The protocol relies on a quick and clean procedure. Training is thus highly recommended until reaching a high success rate, indicated by a high survival rate of the fish after gonadectomy as well as the complete removal of the gonads (see the difference of morphological and anatomical appearance of the fish before and after successful gonadectomy in **Figure 7**). In fact, many factors can affect the success rate of the procedure, including the anesthesia period, the wideness of incision, the accuracy and tidiness of the suture and fish handling during the procedure. Another important point is that one should prepare healthy fish by maintaining the fish optimally prior to performing the protocol.

With respect to blood sampling procedure, the previous studies have attempted to sample the blood from medaka[48] and zebrafish[49-51], but the procedure does not allow repeated blood sampling in the same fish since the blood is taken after euthanizing the fish. Repeated blood sampling has been demonstrated using zebrafish in another study[52], but we report this type of protocol for the first time in medaka.

The evaluation of sex steroid concentrations is commonly carried out using an enzyme-linked immunosorbent assay (ELISA) kit, and there have been many ELISA kits commercially available for different types of sex steroids. Due to the low amount of blood collected during blood sampling, the downstream assays are intended for the whole blood. Previous studies have shown that there is a difference in the measured level of circulating steroid levels extracted from whole blood and plasma[53,54]. Therefore, the difference in the sex steroid levels from whole blood and plasma needs to be validated prior to performing the real experiment using the protocol.

As documented in previous studies with different animal models, the protocol described here will allow to investigate questions related to reproductive physiology using a small size teleost as a model. In fact, these techniques have already contributed to answer questions concerning the regulation of the BPG axis and its feedback mechanisms, such as the involvement of *kiss1* (kisspeptin gene type 1) expressing neurons in positive feedback loops[55], estrogen-mediated regulation of *kiss1* expressing neurons in nucleus ventralis tuberis (NVT), and *kiss2* (kisspeptin gene type 2) expressing neurons in preoptic area (POA)[56,57], the possible involvement of estrogen receptor β1 (Esr2a) in down-regulating *fsh* expression level in female Japanese medaka[58] as well as the profile of the circadian rhythm of E2 in female fish[48]. Furthermore, since previous studies demonstrated that sex steroids also affect the proliferation of gonadotrope cells in the pituitary of teleosts[59,60], it is intriguing to investigate the effects of sex steroid clearance after gonadectomy on pituitary plasticity.

The blood sampling technique not only can be used for sex steroid analysis, but also for other blood content analysis, including blood glucose levels. Indeed, the protocol can also be applied for blood glucose measurements as demonstrated in zebrafish[52] and medaka[61]. Therefore, this technique may be expanded to address research questions in other fields of physiology.

Finally, the protocols described here are intended and optimized for adult Japanese medaka, and the outcomes due to different size of fish and materials used during the procedures may vary. Also, as medaka left and right ovaries/ testes are fused, which might provide an important advantage for gonadectomy, this protocol might need several adaptations before being used in other species where this is not the case such as in zebrafish. Thus, an optimization according to the choice of laboratory equipment and fish size should be taken into account before testing these protocols.

Disclosures

The authors have nothing to disclose.

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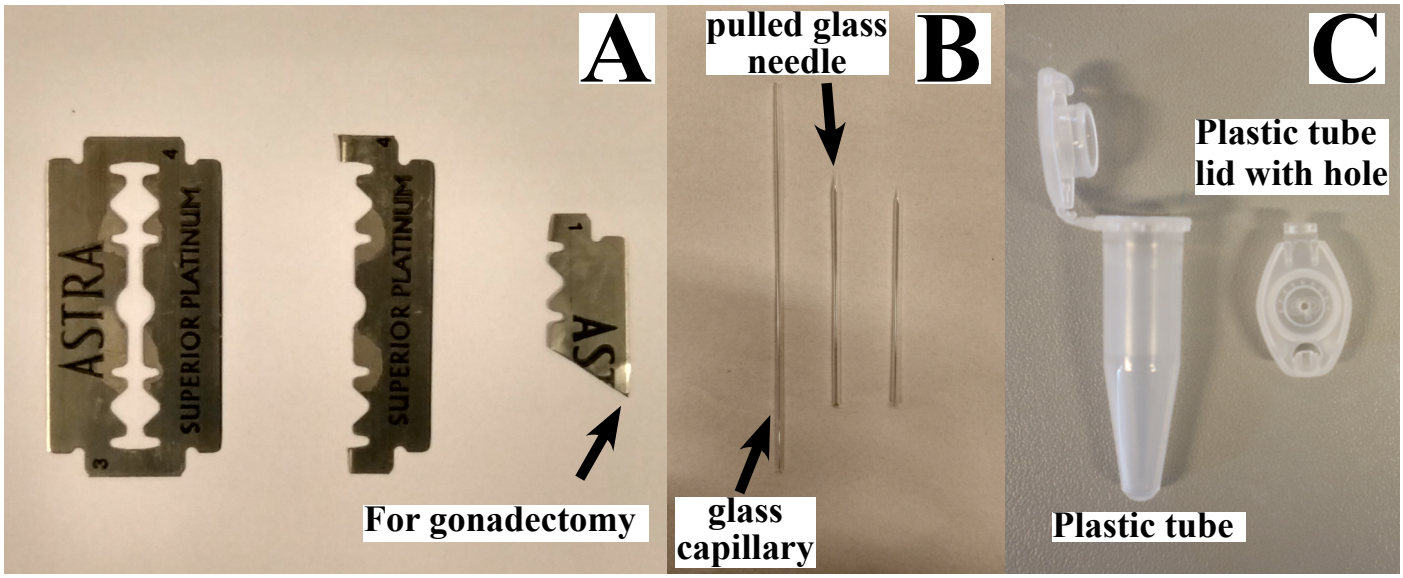
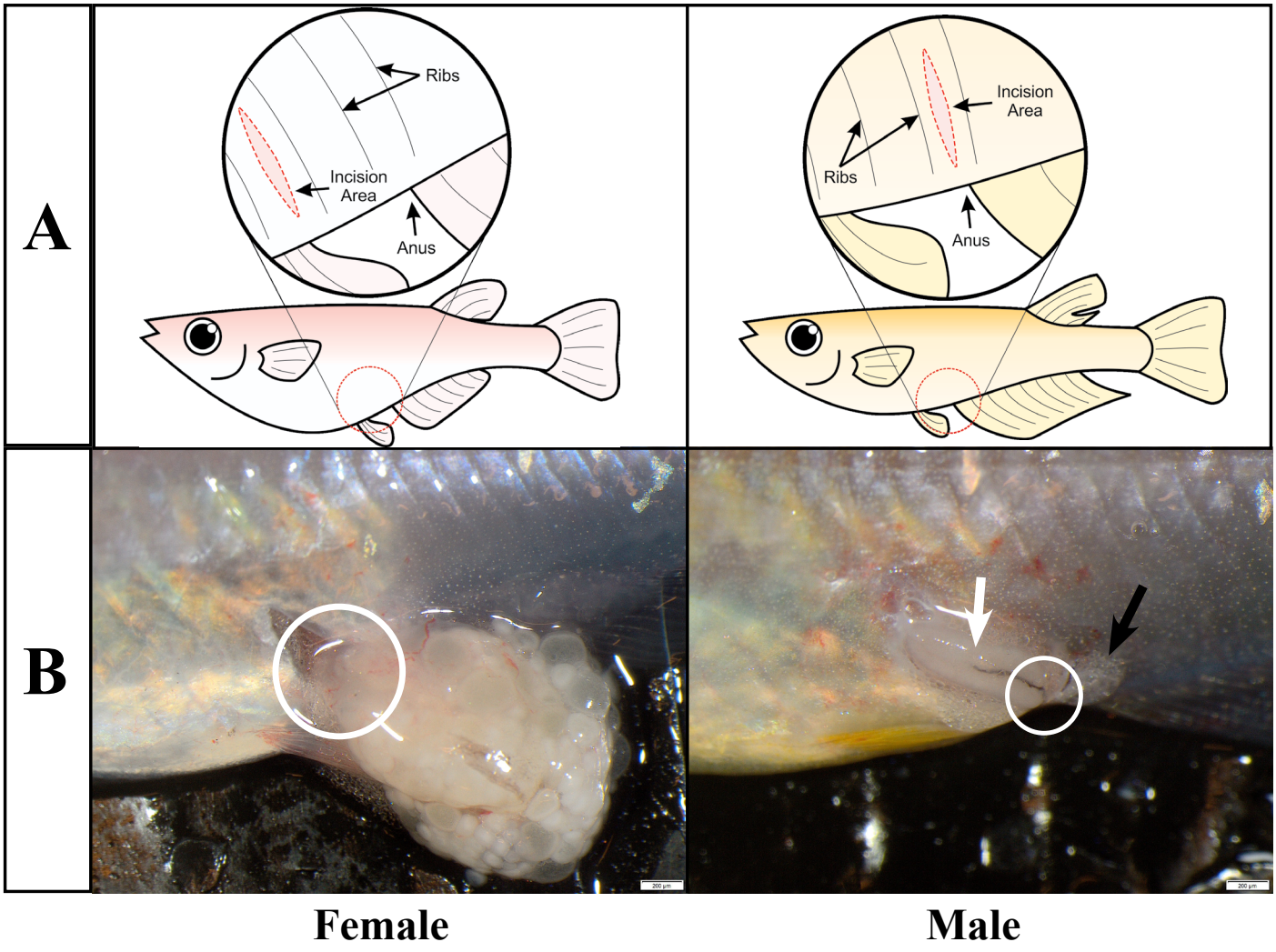


FIGURE 1



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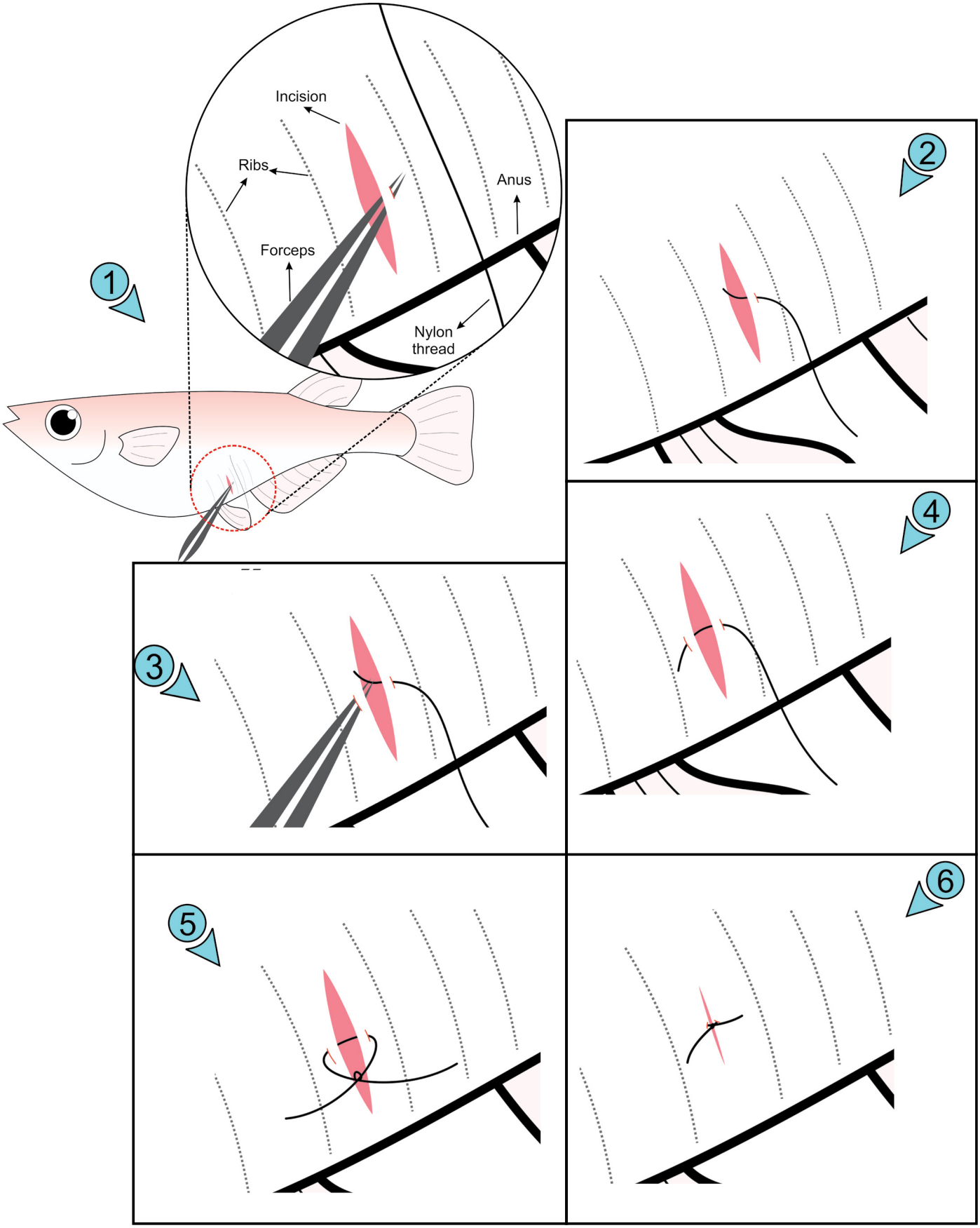

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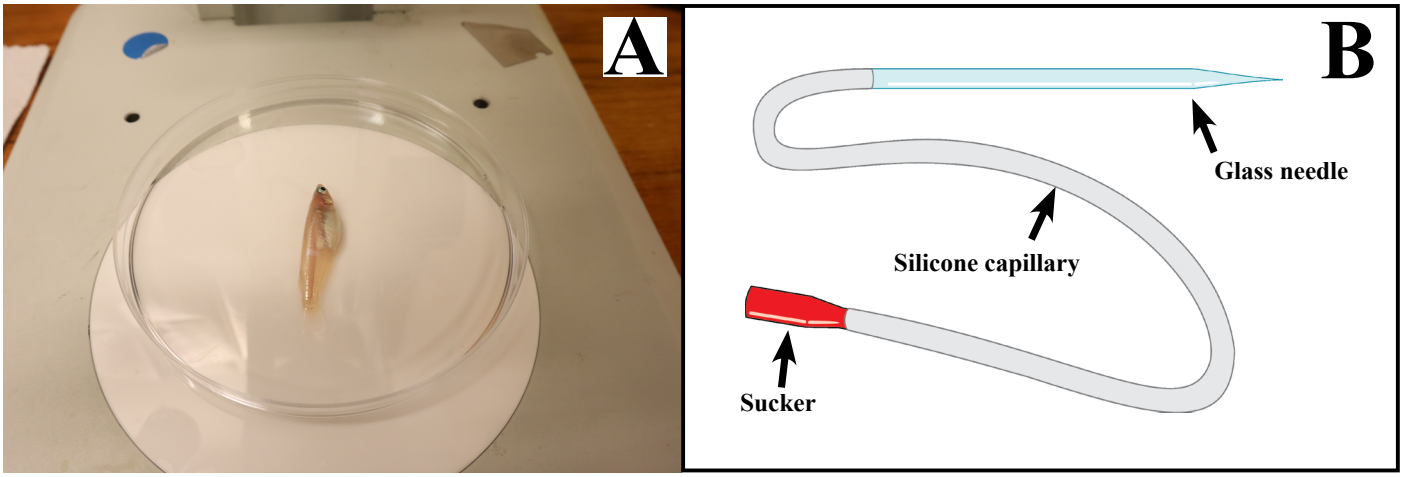

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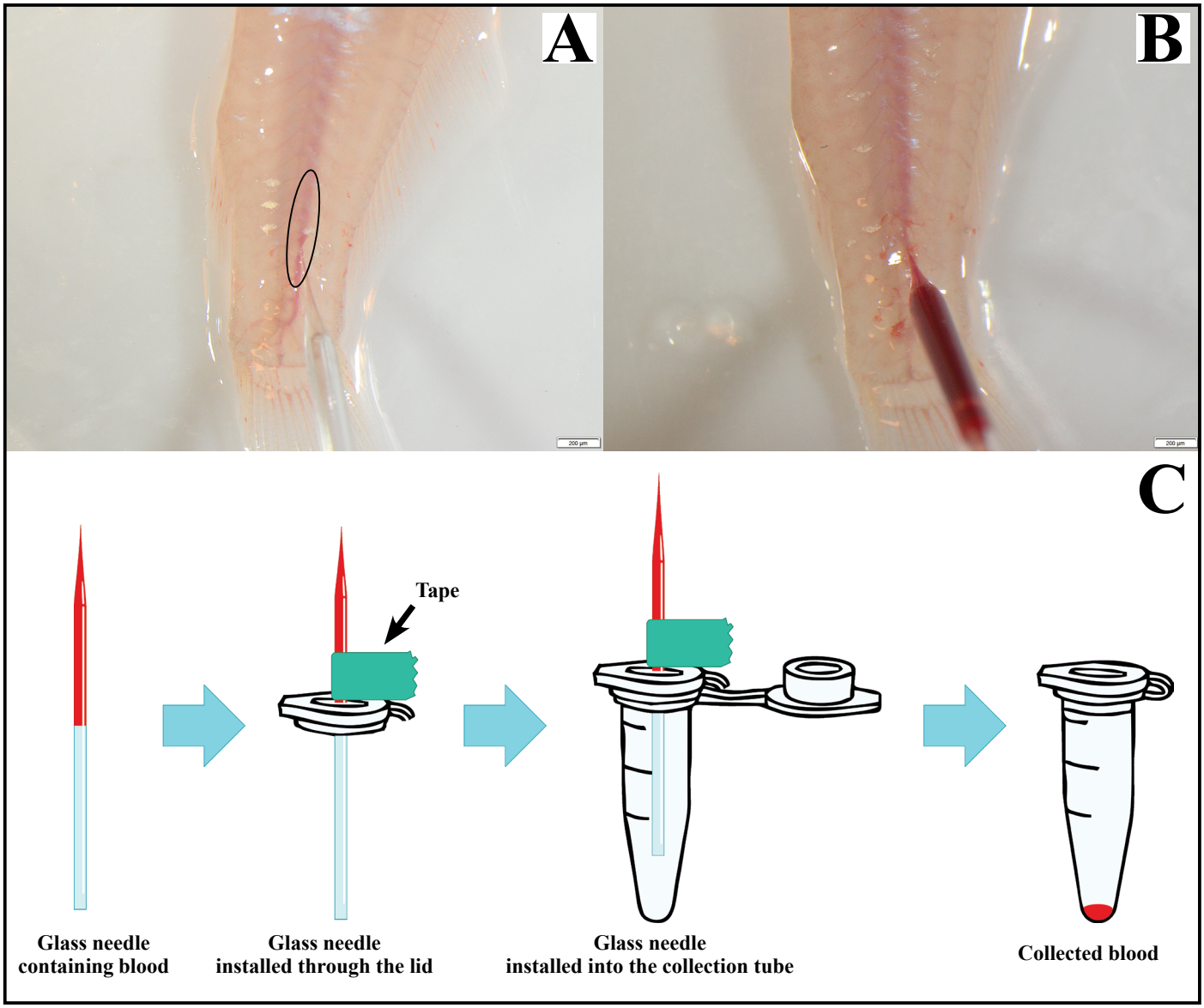

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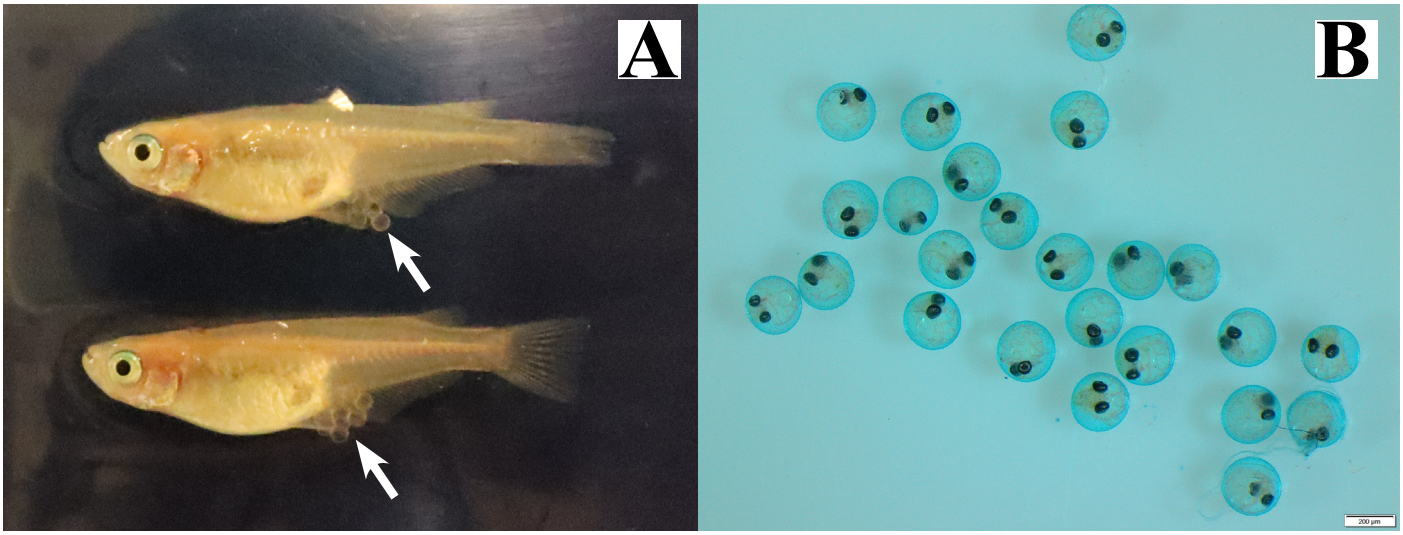

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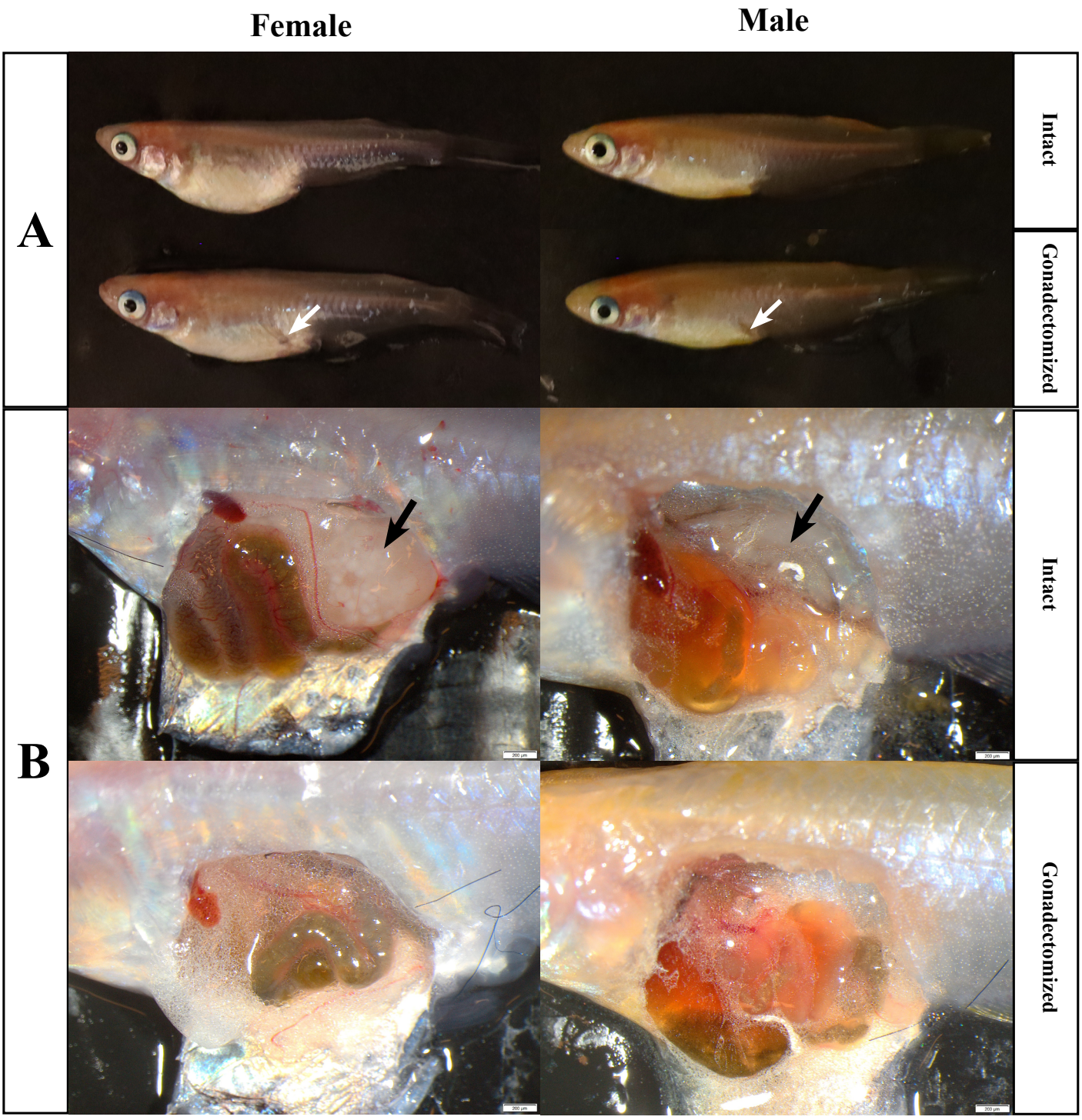

FIGURE 7

FIGURE 8

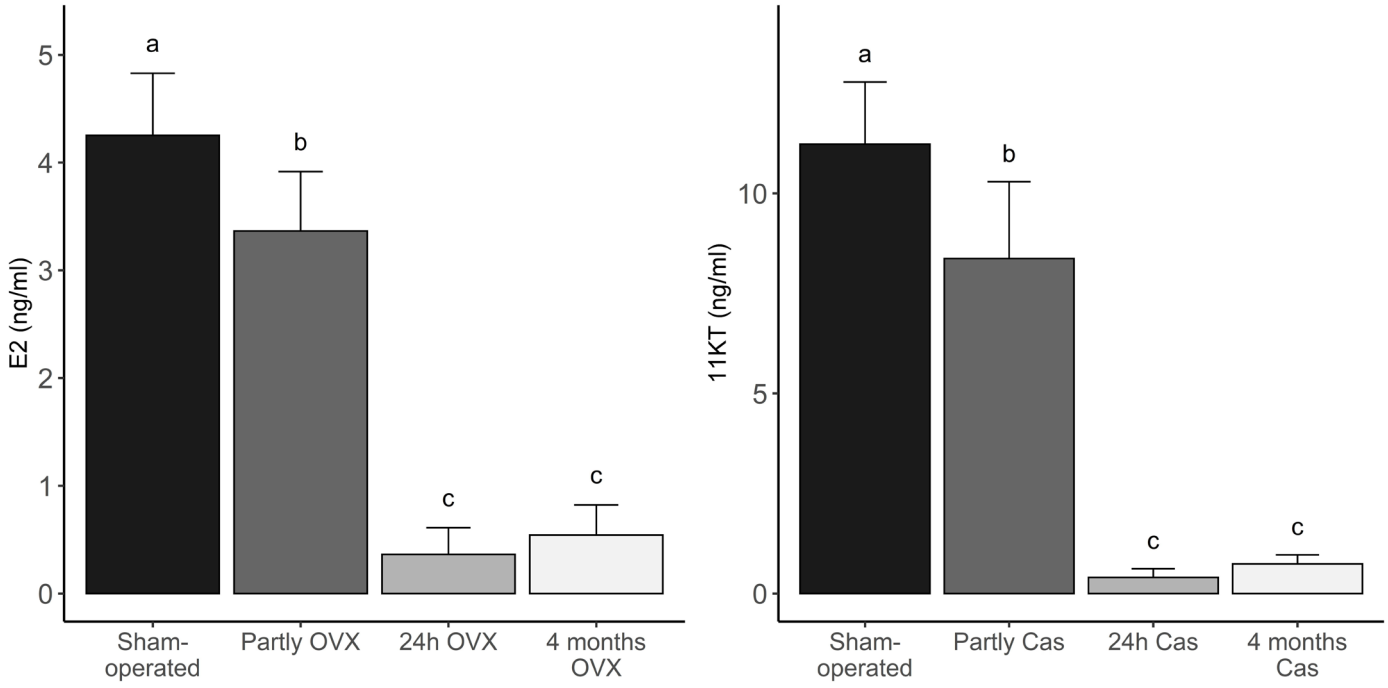

FIGURE 9

Table 1

	E2 levels (Females)	11-KT levels (Males)
Sham-operated	4.15 ± 0.5 (n = 5)	10.38 ± 1.32 (n = 5)
Partly-gonadectomized	3.37 ± 0.6 (n = 5)	8.37 ± 1.92 (n = 5)
24h post-gonadectomy	0.36 ± 0.2 (n = 5)	0.4 ± 0.2 (n = 5)
4 months post-gonadectomy	0.54 ± 0.28 (n = 5)	0.74 ± 0.22 (n = 5)

Table of Materials

Click here to access/download;Table of Materials;List of tools and materials_V28102020.xlsx

No.	Name	Referance Number
1	Glass capilary	GD1
2	Heparin sodium salt	H4784-1G
3	Needle puller	P97
4	Nylon thread	N45VL
5	Plastic tube	T9661
6	Razor blade	-
7	Silicone capillary	a16090800ux0403
8	Tricaine	WXBC9102V

Source

Glass Capillary with Filament GD-1; Narishige
Sigma-aldrich
Flaming/Brown Micropipette puller Model P-97; Sutter Instrument
Polyamide suture, 0.2 metric; Crownjun
Eppendorf Safe-lock microcentifuge tube 1.5 ml, Sigma-aldrich
Astra Superior Platinum Double Edge Razor Blades Green, salonwholesale.com
Uxcell Silicone Tube 1 mm ID x 2 mm OD, amazon.com
Aldrich chemistry

We thank the editor and both reviewers for their time and comments which have helped to improve the manuscript quality.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.
2. It is enough to cite the reference, there is no need to say “reviewed in #”. For example, line 64: “mechanisms1–5.” Instead of “mechanisms (reviewed in 1–5).”
3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

These (1-4) have been checked and fixed.

5. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have made sure that all steps are correctly described.

6. Note after 2.1: Besides the final concentration of 0.02% that you have recommended, please specify the concentration that should not be exceeded to avoid death of the fish.

We realized that the way we presented the adaptation of the anesthetic treatment from fish to fish was unclear. In fact, to adjust the anesthesia protocol to the fish, we only changed the duration of the anesthesia treatment. Therefore, for clarity we have changed the statement and do not discuss the concentration of MS222 but only the duration of the anesthetic. We also suggest in the note the way we can make sure that the fish is asleep before to start the surgery (gently pinching the fish using the forceps). This part has now been rewritten in the manuscript.

7. 3.6: Please specify centrifuge speed in centrifugal force (x g), duration, and temperature.

This information has now been provided in the manuscript.

8. Note after 3.7: If steroid extraction is not the focus of this protocol, please cite a reference in case the readers wish to replicate the protocol along with steroid extraction.

We have updated the information of this section in the manuscript.

9. Lines 279-281: As you state that there is a difference in 11KT levels of sham-operated fish and partly orchidectomized fish, please mention the levels of both types of fish.

For more clarity, we have removed all the values in the text and provide a table (Table 1) that now resumes all the measured concentrations. It makes easier comparison between groups.

10. Please refer to instructions for authors: include a section “Figure and Table Legends” right after Representative Results and before Discussion and move the legends here.
This has been fixed.

11. As we are a methods journal, please add limitations of the technique to the Discussion with citations.
We have accommodated this point by discussing some limitations of the method in the Discussion part.

12. Please sort the Materials Table alphabetically by the name of the material.
The Materials Table has been adjusted accordingly.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The methods described within the manuscript seem logical and concise. Having worked with small-bodied teleosts I understand the modifications required for smaller tissue weights and blood volumes well! The authors do a good job of outlining their proposed methodology but have omitted a few pieces of information that I feel would increase the applicability of their methods to people who are not as experienced dealing with small bodied teleosts as they themselves are. These concerns are outlined below. In addition, there are some poorly defined linkages within the manuscript that would benefit from expansion or removal from the manuscript (also outlined in the next section). Overall, once these comments are addressed I feel this methods manuscript will be a valuable tool to researchers.

Major Concerns:

Introduction:

Further justification of the methodology is required. Why is a gonadectomy required? Can't specific sex hormone or gonadotroph antagonists be used to elicit the same effects? What is the benefit of gonad removal?

We acknowledge that the use of sex steroid antagonist may exert similar effect to gonadectomy. However, sex steroid antagonists not only can provide antagonist effect but also agonist effect on reproductive physiology (Clark and Markaverich, 1981; Mourits et al., 2001). In addition, it is impossible to perform recovery experiment after using the sex steroid antagonist, to warrant the specific effect of certain sex steroids. Therefore, gonadectomy remains one powerful technique to investigate the physiological role of sex steroids. According to your recommendation on this point, we have added some other information on this concern.

Why are there constant references to the similarity between zebrafish and medaka? This methodology is developed for medaka and there is no proof provided it would work identically for zebrafish. The relationship between these species, and whether the procedure would work for zebrafish, needs to be addressed. If there is no data on the efficacy of this procedure on zebrafish, it is not advisable to include them in the introduction or discussion.
The experimental unit that we use in our protocol is Japanese medaka. However, we think that with few adjustments, this protocol could be adapted to zebrafish and thus should be of high interest for the zebrafish community. Through the manuscript, we did not argue that this method can be directly applied to zebrafish, but we argue that both medaka and zebrafish are teleost models who share several features, such as the small size and limited amount of blood. We have specified the title, removed zebrafish from the abstract and keywords but we have kept few words about the similarities between zebrafish and medaka in the introduction, and about the possibility to adapt this protocol to other species, including zebrafish, in the discussion.

Repetitive statements, such as Lines 95-96, hamper the impact of the introduction. Authors should ensure these statements are edited out for clarity and conciseness.
We have addressed reviewer's comment on this part by excluding redundancy in the paragraph.

Methods:
Is there a way to determine fish sex before the initial incision? This information needs to be added, as the procedure varies based on sex.
Even if the sex of adult Japanese medaka can be known by genotyping as medaka has a sex determination system, it can also be easily distinguished by their secondary sexual characteristics, i.e. the size and shape for the dorsal and anal fin (Kenji Murata et al., 2019; Wittbrodt et al., 2002). We have adopted reviewer's suggestion above and added the information in the protocol before the section 1.

What are the settings for blood centrifugation? These need to be included. Does this not cause separation of plasma from the rest of the blood volume? Why or why not?
In our experience, flash spin down (1-2 seconds) does not cause separation of plasma and the blood. As recommended by Thermo Fisher Scientific protocol regarding plasma and serum separation from the blood, ten-minute centrifugation is required to separate plasma from the blood cells. (Please refer to https://www.thermofisher.com/no/en/home/references/protocols/cell-and-tissue-analysis/elisa-protocol/elisa-sample-preparation-protocols/plasma-and-serum-preparation.html). We have added the information in section 3.7. of the Protocol based on the reviewer's recommendation.

Can the gonadectomy and blood collection protocols be combined? Would a fish survive both procedures happening during the same anesthetic event? Clarification on the length of recovery time (if any) before the next procedure is needed. How often can blood be collected? Is it a single, lethal, sampling?
We think that it is not possible to combine the two procedures during the same anesthetic event since it will be deleterious to the fish. During gonadectomy, the fish loses some amount of blood, thus taking even a small amount during the procedure might be dangerous for the animal. In

addition, the whole procedure of gonadectomy takes approximately 6 minutes, while adding 2-3 more minutes for blood sampling may cause mortality. However, in our experience, it is possible to do blood sampling at least 4 hours after gonadectomy.
Regarding the recovery time after the procedure, the fish start to behave normally already after 1-2 hours, however we never tried to do blood sampling at this time point. In previous studies, they usually let the fish recovering at least 1-4 days after gonadectomy before sampling the blood (Kanda et al., 2008; Kanda et al., 2012; Kayo et al., 2020; Kayo et al., 2019; Mitani et al., 2010). According to your concern, we have added the information in the protocol section 2.7.4.
Concerning the frequency of blood sampling, it is possible to re-sample the blood twice from the same fish with one-week interval, according to our experience. However, we never tried to do more than that since we think that it may give more damage to the fish, and it is not included In our application approved by the Norwegian Food Safety Authority. We also have added the information regarding possible subsequent blood sampling in the manuscript section 3.6.

Discussion:
Is the paragraph from Line 232-334 necessary? The methodology is not extended to quantifying hormones from the blood. And it is not something the authors tested. Nor does the paragraph reference any study that has.
In our case, we have measured sex steroids directly from the blood (not plasma) as described in Kayo et al,. 2020). We now cite this reference at the end of the protocol. Since previous studies have reported that there is a difference in steroid levels from whole blood and plasma (Holtkamp et al., 1975; Taves et al., 2010), we would like to remind the future user that this difference needs to be validated in the assay. For medaka, we have checked and confirmed that by diluting the blood, the ELISA is not affected by blood cell content and are currently writing an original research article focusing on the sex steroids ELISA on blood where we discuss more in detail on this point. But in the Jove manuscript, the focus is placed on the gonadectomy and blood sampling and not hormone measurements. Hormone measurement is just a way to demonstrate that the gonadectomy and blood sampling techniques are working. Following this, we still think that it is important to briefly mention this aspect in the discussion section.

Line 343: A knockout fish is not a fish that has had its gonads removed through surgery. This example should not be included.
We apologize for the wording mistake. What we want to point out here is that the use of gonadectomy in this study suggests the possible involvement of Esr2a in down regulating *fsh* expression level in medaka. We hope that by rephrasing this part, we satisfy your concern here.

The authors state that additional research questions, such as blood glucose measurements, could be address using this technique. But there is zero discussion on the relationship between gonads and glucose in teleosts. This needs to be addressed, or the statement removed.
We are sorry for giving information that seems irrelevant due to mistake in paragraphing. What we would like to raise in this matter is the fact that our blood sampling technique not only can be used for sex steroid analysis, but the other blood contents including glucose levels can also be analyzed. We hope after re-paragraphing that part, we fulfill your concern.

Minor Concerns:

Species scientific names are not introduced in the proper placement (for example line 48 requires danio rerio, while line 53 is NOT the first time Japanese medaka was used and therefore doesn't require Oryzias latipes). There are other areas in the text this occurs, and the authors should ensure this is fixed.
11-ketotestosterone is misspelled in the text. Please include the 'e' at the end of the word wherever it appears.
Line 111: Secondary not second
We have adjusted the three preceding points according to reviewer's suggestions.

Line 247-248: Unclear sentence. Small volumes of blood don't clot? Please reword or clarify.
We apologize for the wording mistake. We removed the sentence which does not provide any information.

Line 259: Be specific. How many weeks elapse between sham operation and successful fertilization?
Line 289: Insert 'commonly' before 'used'
Line 358: Disclosures misspelled
We have modified those three points above based on reviewer's comments.

Figure 9: The stats on this figure do not match the description in the text. When comparing E2 levels between sham-operated and partly OVX fish, the text states there is NO difference but the figure shows a statistical difference. This needs to be addressed. How were stats completed on this? What were the p-values? Which post-hoc test was used?
We apologize for the wording mistake in the Representative Result section. We have fixed it according to reviewer's concerns and have included the details regarding the statistics in the figure legend.

Reviewer #2:
Comments:
Abstract
The abstract is clear and comprehensible, however, it is overly general with the bulk of the synthesis on the feedback mechanisms in the neuroendocrine control of reproduction and tissue plasticity provided by sex steroids (which was not investigated or included in the title of the study). Please note, while it is necessary to appreciate the importance of the study in understanding reproductive physiology, this can be captured in a topic sentence such that bulk of the summary (abstract) is allowed to deal with key steps in the procedure described.
We hope that after re-paragraphing the abstract section, it satisfies the reviewer's concerns.

Introduction
Among others, it is very important at this point to note that it is not enough to only state that general techniques (including even recently described ones in principles) of gonadectomy raise challenges when applied to smaller fish (lines 102, 103 & 104), some of the challenges should be mentioned, and with some described as appropriate.

We have modified the paragraph and added the information according to reviewer's recommendations.

Lines 64-94: reduce your use of "reviewed in", you can use only reference number.
This concern has been fixed in the manuscript.

Methodology
1. The age, size and weight of the fish should be mentioned; this is the Standard procedure and for ease of reproducibility (as some of the chemicals used in this procedure are stated with their concentration in relation to the size of the fish, e.g. mg of chemical/kg of the fish).
We have added the information required by the reviewer.

2. The anesthesia protocol applied should be stated for clarity: out-of-water/ continuous delivery protocol. The induction and maintenance doses of anesthetics should be stated if continuous delivery was applied.
We have added the information required by the reviewer in the manuscript section 2.4.

3. The exact duration of the operation in your study should be clearly stated.
We have added the information required by the reviewer.

4. Line 135: is there any reason for the preference of razor blade to standard scalpel?
We prefer to use razor blade because scalpel blade is thicker than razor. In our experience, using thicker blade put more damage to the fish compared to razor blade.

5. Lines 166/167: the induction dose and the duration of anesthesia (just before surgery) are more appropriate here or can be added to the subjective reaction of the fish upon 'gently' pinching with forceps.
We have adopted reviewer's suggestion and reorganize point 2.1. and 2.3. along with the notes.

6. Line 175: mention should be clearly made of the incision site in relation to the pelvic and anal fins (and not „incision area between the ribs").
We agree with reviewer's concern on this point. Since it is also important to incise between the ribs to avoid mortality, we have adjusted this information to be more precise.

7. Line 207: the use of inject as in "Inject the left side of incision part from outer body cavity…" is not clear, consider rephrasing.
We have modified the above point according to reviewer's recommendations.

8. Line 212: postoperative care (recovery water) is for how long?
Before transferring to the aquarium system, the gonadectomized fish needs to be stay in the recovery water for at least 24 hours. The information is now provided in the manuscript in section 2.7.4 of the protocol.

9. Line 221: MS-222 should be used in parenthesis with Tricaine in line 127.
We have modified the above point according to reviewer's recommendations.

10. Line 216: How this method is different from previously described method was not stated.
We have added this information according to reviewer's comment on this point in the Discussion section.

11. Line 223: Please, let us know if there is any safety consideration since drawing of blood sample is done with mouth.
It is true that suction with mouth may cause direct contact with the blood. We can prevent this possible accident by using a sucker and silicon capillary with at least 50 cm long. That way, we assume that the few microliters of blood available in such small fish will not be able to reach the mouth. Therefore, we have added information regarding this safety consideration in the text.

12. Lines 270/271/272/278: the use of comma in the plasma hormone level (e.g., 0,36 ± 0,2 ng/ml) not clear.
For the clarity, we now provide a table (Table 1) that summarizes all measured concentrations.

13. Line 274: replace "statistical‟ with"significant‟ in the statement: "There is no statistical difference in blood levels of E2 and 11KT…" as statistic can assume any value which in most cases are different in the numerical sense of it.
The information above has been fixed based on reviewer's comments.

14. Line 274: it is more appropriate to compare females with females (E2) separately from males with males (11k), across different durations of the experiment, and not E2 with
11k as reported here.
We have adopted reviewer's comment on this point and adjusted accordingly.

15. Lines 277-282: comparison of the plasma hormone level will be more appropriate with values before and after gonadectomy (the plasma hormone level before gonadectomy in the operated fish with respect to the control cannot be assumed). Alternatively, you may clearly state the number of individual gonadectomised fish that showed this variation in the plasma level of the stated hormones.
Although it is not impossible to combine gonadectomy and repeated blood sampling within the same individual, we realize that it is quite tricky to perform those with a short time interval as it might give a lot of damages to the fish. Therefore, according to reviewer's suggestion, we have provided more detailed information concerning the number of gonadectomized fish in a table (Table 1) as the alternative and have rephrased our statement.

16. The statistical method used should be clearly stated.
We have added this information in the figure legend according to reviewer's suggestion.

Discussion
1. Lines 331-334: the comparison between plasma and blood concentrations of sex steroids was not investigated and cannot be included here (not even by way of recommendation).
Since previous studies have reported that there is a difference in steroid levels from whole blood and plasma (Holtkamp et al., 1975; Taves et al., 2010), we would like to remind the future user that

this difference needs to be first validated in the assay. For medaka, we have checked and confirmed that by diluting the blood, the ELISA is not affected by blood cell content and are currently writing an original research article focusing on the sex steroids ELISA on blood where we discuss more in detail on this point. But in the Jove manuscript, the focus is placed on the gonadectomy and blood sampling and not hormone measurements. Hormone measurement is just a way to demonstrate that the gonadectomy and blood sampling techniques are working. Following this, we still think that it is important to briefly mention this aspect in the discussion section.

2. It will be better to discuss your results alongside previous studies on gonadectomy and blood sampling in medaka.

Jove is a journal that focuses on the method, the results are just here as proof that the method is working. We can gonadectomize medaka and collect blood for sex steroid measurement. However, we agree with the reviewer that comparing with previous study will further demonstrate the reproducibility of the technique. Therefore, we have added several information concerning the relevancy of our result with previous studies.

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