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Abstract

Previously, we examined whether aromatase inhibitor (AI) treatment induces a sex change in adult female zebrafish. A 5-month AI treatment regime resulted in the retraction of the ovaries and testis formation. Eight weeks after changing the diet to AI-free food, a large number of normal sperm were obtained. Artificial fertilization using sperm from the sex-changed females was successful. These results demonstrated that sex plasticity remains in the mature ovaries of zebrafish. However, more than 7 months of treatment were necessary; thus, pairing was unsuccessful.

In this study, we tried to induce sex change via the injection of an AI to shorten the time course of sex change. When the AI solution was directly injected into the abdomen of zebrafish, retraction of the ovary was induced within 2 months. The natural mating of sex-changed females with normal females was successful at 3 months. Although the fertilization rate was low, juveniles resulting from these matings developed normally. We succeeded in establishing a method for inducing sex changes in adult zebrafish within 3 months. The procedure will support the study of how sexual plasticity persists in adult zebrafish following sex differentiation and the identification of undifferentiated stem cells.

Introduction

Female-to-male sex change is associated with a decrease in estrogen levels [1]. Aromatase inhibitors (AIs) have been developed to reduce estrogen synthesis as pharmaceuticals for the treatment of cancers [2]. Among third-generation AIs, exemestane, an oral steroidal-type aromatase inhibitor, is very effective in the treatment of metastatic breast cancer [3]. Exemestane inhibits aromatase with a chemical structure resembling that of the natural substrate androstenedione.

Studies have shown that sex change can be induced in many types of fish by aromatase inhibitor (AI) treatment during sex differentiation. In Japanese flounder (*Paralichthys olivaceus*) and tilapia (*Oreochromis niloticus*), brief treatment with an AI during sex differentiation causes a type of sex reversal in which genetic females develop into phenotypically normal males [4, 5]. Furthermore, successful sex change in sex-differentiated fish has been achieved in Medaka, tilapia and zebrafish by long-term treatment with AIs [6-8].

The sex differentiation of zebrafish is known to involve juvenile hermaphroditism. Initially, undifferentiated ovary-like gonads are formed during gonadal development in all juvenile zebrafish, regardless of genotypic sex [9]. In genotypically male zebrafish, oocytes disappear from the gonad by apoptosis, and spermatocytes develop concomitant with testicular differentiation [10, 11]. In contrast, oocytes in the female ovaries continue to grow to maturation. In zebrafish, the gonadal masculinization of juvenile genetic females can be induced by the dietary administration of an AI (fadrozole)[12].

In a previous study, we examined whether AI (fadrozole) treatment induces a sex change in adult female zebrafish [7]. Our results support the hypothesis that sexual plasticity persists in adult zebrafish following sex differentiation, indicating that undifferentiated stem cells are maintained in adult fish that do not undergo a sex change under natural conditions. Female-to-male sex change in adult fish can be categorized as secondary sex reversal (SSR) [13]. It has been proposed that the sex change induced by AI after sex differentiation is a form of SSR rather than primary sex reversal (PSR), in which treatment with an AI is initiated before sex differentiation. In female-to-male SSR, testis formation starts on the ventral side of the ovary. Our previous work in zebrafish showed that newly synthesized testes formed separately on the ventral side of the ovaries after ovarian degeneration [7]. The results suggested that undifferentiated germ stem cells that remained alongside the ovaries developed into testes under these conditions [14].

In this study, we tried to shorten the time period required for sex change by the injection of an AI (exemestane). We succeeded in shortening the total time from 7 to 3 months. Furthermore, we successfully paired sex-changed females with normal females. The method established in this study might provide a good model for purposes such as the analysis of changes in sex behavior or the identification of remaining undifferentiated germ stem cells in adult zebrafish.

Materials and Methods

Animals

To monitor the changes in the ovaries of living fish, the *TG (β-actin:EGFP)*;roy (*β-roy*) strain was used as previously described [7]. The TG strain is highly transparent, and its oocytes are easily observed by fluorescence imaging in living fish. The original *TG (β-actin:EGFP)* transgenic line was established by Hsiao et al.[15, 16]. We crossed the roy and TG strains to establish a strain enabling the direct observation of oocytes in living fish [14]. The TG zebrafish were bred and maintained at 28.5°C under a 14-h light/10-h dark cycle [17]. All zebrafish experiments were carried out with approval from the Institutional Ethics Committee of Shizuoka University, Japan (approved No. 2019F-5); the guidelines set by this committee for the use of animals were strictly followed.

Reagents

17,20β-DHP and tricaine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Aromasin was obtained from Pfizer Inc. (Tokyo, Japan).

AI preparation and treatments

Aromasin tablets containing 25 mg of exemestane reagent were crushed and dissolved in zebrafish Ringer's solution at a final concentration of 3 mg/ml (corresponding to 10 mM), followed by filtration through a 0.22 μm filter. The exemestane solution was separated into 100 μl aliquots and stored at -30°C. A 35 μl volume of exemestane solution was injected into the abdomen of female zebrafish by using a fine needle (29G syringe from TERUMO) following anaesthetization with 0.5% tricaine at three-day intervals (Fig. 1A). The amount of exemestane injected (0.1 mg/fish) was determined based on the results of pilot experiments. The sexually mature females used for the treatments were selected after checking for maturity, as confirmed by the ability to ovulate, using a technique for the induction of ovulation [18]. The experimental fish were housed in separate small aquaria with air pumps.

Ovarian morphology was monitored by fluorescence microscopy observations at each injection interval during the treatment period following anaesthetization with 0.5% tricaine. The ovaries were photographed under a binocular microscope under both brightfield and fluorescent lighting conditions every month.

Fertility check

The exemestane-injected individuals were allowed to mate with normal females. Each mating pair was housed in a pairing tank with stainless steel net on the bottom to separate the spawned eggs from fishes from the evening until the morning of the next day.

Results

Morphological changes in ovaries

To allow us to monitor the changes in the ovaries of living fishes during the injection of exemestane, we used ovarian fluorescent and transparent transgenic zebrafish lines as described previously [7]. The transparent *roy* strain of zebrafish is deficient in the production of

iridescent color by iridophores, and the viscera and reproductive organs are therefore visible from the outside of the fish body. In transgenic *β-roy*, GFP is expressed predominantly in oocytes; thus, the morphology of the ovaries can be monitored by fluorescence. This fluorescence is relatively strong in small oocytes at stages 1 and 2. This property is well suited for the monitoring of ovarian retraction. The solution of exemestane was injected into the upper edge of the ovaries by passing the needle between the skin and ovary (Fig. 1A).

The ovaries of the exemestane-injected fish gradually decreased in size. Although there was a difference in the pace of reduction between individuals, ovary size was reduced by the injection of exemestane. Almost all the ovaries were reduced to an invisible size within 2 months. Oscillatory changes in size during treatment with food intake were not observed in a previous study [7]. Three months after the initiation of the injections, the ovaries had retracted completely, and the fluorescence-expressing oocytes had disappeared in all fish (100%, n=9) (Fig. 1B). The injection of exemestane was stopped at 3 months to induce sperm formation, which was blocked by loss of estradiol under the effect of AI [7]. Then, we cultured the zebrafish without any treatment. The methods for female to male sex-change in adult zebrafish previously reported and established in this study were compared (Table.1). The morphology of sex-changed females was compared with that of normal females and males (Fig. 2). Breeding tubercle in pectoral fin of male fish was found to start development (Fig. 2B) [19]. Typical characteristics of females including the existence of the genital papilla and large anal fins remained in the sex-changed females. However, the color of the anal fin changed to yellow, as observed in males (Fig. 2). Additionally, the tissues of the sex-changed females were subjected to histological examination (Fig. 3). In the newly formed testis, cysts filled with spermatozoa-like cells were observed in the exemestane-injected fish. Remaining retracted oocytes were observed during absorption beside the newly formed testis (Fig. 3A arrowheads). We also analyzed changes in ureter and gonadal ducts. In the case of female,

ureter and oviduct are independent (Supplementary Figure. 1). In contrast, ureter and sperm duct fuse and form a common sperm duct before open as urogenital pore. In exemestane-injected fish, oviduct and newly formed sperm duct fused but did not fuse with ureter (Fig. 3B, Supplementary Figure. 2).

Finally, mating experiments were conducted using the sex-changed fish to examine whether functional testes had developed in these fish. Sex-changed females showed mating behavior with nontransgenic normal females, and fertilized eggs could be obtained. Although the fertilization rate was low (average 15.2 ± 4.7 %, n=3)(Supplementary Table. 1), embryos with GFP fluorescence developed from the fertilized eggs (Fig. 4). This result proved that the embryos were produced from the sex-changed females. All the two-month-old juveniles developed fluorescent ovaries, further confirming that the juveniles were produced from sex-changed females. This result indicates that the sperm from the sex-changed females were capable of fertilization. ~~Contrast to previous study, only low percentages (10-20%) but male juveniles were developed in this study.~~

Discussion

In a previous study, we succeeded in inducing female-to-male sex change in adult zebrafish by the administration of an AI through food intake. Five months of the treatment of zebrafish with AI-containing food induced ovarian retraction and testis formation.

The results suggested that the capacity for sex change remained in the adult fish of species that do not undergo sex change in nature. Specifically, we suggest that undifferentiated germ and/or somatic stem cells remain in the body of zebrafish. We suggest that these cells are located outside of the ovaries near the cloaca [14]. Our results demonstrated that undifferentiated germ stem cells persist in adult fish, similar to the results obtained in tilapia [6]. We also investigated whether the sex-changed females would engage in

reproductive behavior with normal females by trying to pair sex-changed and normal females. Although we used females that were ready to spawn, carrying ovulated eggs, the sex-changed females did not show any behavior directed toward normal females in a previous study. The results suggested that the sex change of the fish was restricted to the gonadal tissues and did not cause changes in the brain, resulting in the failure to show any mating behavior. However, a long period of 7 months was required for complete sex change starting with 3-month-old fish; thus, the experimental fish were 1 year old at the time of the pairing experiment. Additionally, the sex-changed fish seemed to exhibit side effects of AI treatment and damage. It is possible that age and side effects in the sex-changed fish resulted in the absence of pairing behavior.

To address the possibility of changes in sex behavior, we performed this experiment in fish in which sex change was completed over a short period that therefore remained young in this study. As a result, the mating trial succeeded. We obtained embryos by mating sex-changed females and normal females. The results demonstrated that the females underwent a sex change not only in the gonads but also in the brain. However, the rate of fertilization was low. When *in vitro* fertilization was conducted with sperm from the sex-changed fish, the success rate was not different from that of normal males as previously reported [7]. The changes in duct formation were not completed in female-to-male sex-changed fish (Fig 2B). Thus we hypothesized that sperm could not travel smoothly through incomplete ducts. We observed that a change in the duct structure from female (three ducts) to male (two ducts) required more than 3 months, similar to that required for the disappearance of the genital papilla. The genital papilla and the large size of the anal fin remained in sex-changed females in this study (Fig. 2). Thus, it is thought that the female-to-male reconstruction of the body structure requires more than 3 months.

In all the females tested, AI injection caused ovarian retraction, followed by the development of testes. Cyst structures in the newly formed testis filled with spermatids were observed in sections of these organs. The testes appeared to have developed from an area near the cloaca rather than the ovarian tissue [7]. Indeed, we found fluorescent tissue that might contain undifferentiated germ cells close to the cloaca in this strain [14]. It is thought that a small number of undifferentiated germ stem cells, which serve as the source of spermatogenic cells in sex-changed fish, may remain in this area. Future detailed analyses of the retraction of the ovary and formation of the testis should lead to the identification of stem cells. In contrast, evidence of the presence of undifferentiated stem cells in adult ovaries has been reported. Adult zebrafish can be sex reversed to fully functional males following the depletion of most of their germ cells [20]. Fertile sperm were produced from the transplanted germ cells via the transplantation of female germ cells into a male [21]. However, our previous study suggested that testes developed from a different cell than the ovaries [7].

Our next target is to address that undifferentiated germ stem cells in outside the ovary of adult zebrafish are present or not. The rapid induction method established in this study will accelerate this research.

Acknowledgments

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Disclosure Statement

No competing financial interest exists.

221 **Table.1 Comparison of two ways to induce sex change in zebrafish.**

Method	Aromatase inhibitor	Frequency of delivery	Duration of treatment	Outcomes of sex changes	Sperm Fertility	Mating
Food intake (ref. 7)	fadrozole	everyday (twice per day)	> 5 months	retraction of ovary: ≅3 months formation of testis: ≅5 months	+ > 7 months	-
Injection (this study)	exemestane (Aromasin tablet)	3 day intervals	3 months	retraction of ovary: ≅2 month formation of testis: ≅3 months	+ > 3 months	+

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References

1. Bhandari RK, Komuro H, Nakamura S, Higa M, Nakamura M: Gonadal restructuring and correlative steroid hormone profiles during natural sex change in protogynous honeycomb grouper (Epinephelus merra). Zoolog Sci. 2003;20:1399-404.
2. Steele RE, Mellor LB, Sawyer WK, Wasvary JM, Browne LJ: *In vitro* and *in vivo* studies demonstrating potent and selective estrogen inhibition with the nonsteroidal aromatase inhibitor CGS 16949A. Steroids. 1987;50:147-61.
3. Dank M: The role of aromasin in the hormonal therapy of breast cancer. Pathol Oncol Res. 2002;8:87-92.
4. Kitano T, Takamune K, Nagahama Y, Abe SI: Aromatase inhibitor and 17alpha-methyltestosterone cause sex-reversal from genetical females to phenotypic males and suppression of P450 aromatase gene expression in Japanese flounder (*Paralichthys olivaceus*). Mol Reprod Dev. 2000;56:1-5.
5. Kwon JY, Haghpanah V, Kogson-Hurtado LM, McAndrew BJ, Penman DJ: Masculinization of genetic female nile tilapia (*Oreochromis niloticus*) by dietary administration of an aromatase inhibitor during sexual differentiation. J Exp Zool. 2000;287:46-53.
6. Paul-Prasanth B, Bhandari RK, Kobayashi T, Horiguchi R, Kobayashi Y, Nakamoto M, et al.: Estrogen oversees the maintenance of the female genetic program in terminally differentiated gonochorists. Sci Rep. 2013;3:2862.
7. Takatsu K, Miyaoku K, Roy SR, Murono Y, Sago T, Itagaki H, et al.: Induction of female-to-male sex change in adult zebrafish by aromatase inhibitor treatment. Sci Rep. 2013;3:3400.
8. Sun LN, Jiang XL, Xie QP, Yuan J, Huang BF, Tao WJ, et al.: Transdifferentiation of Differentiated Ovary into Functional Testis by Long-Term Treatment of Aromatase Inhibitor in Nile Tilapia. Endocrinology. 2014;155:1476-88.
9. Orban L, Sreenivasan R, Olsson PE: Long and winding roads: testis differentiation in zebrafish. Mol Cell Endocrinol. 2009;312:35-41.
10. Takahashi H: Juvenile hermaphroditism in the zebrafish, *Brachydanio rerio*. Bull Fac Fish Hokkaido Univ. 1977;28:57-65.
11. Uchida D, Yamashita M, Kitano T, Iguchi T: Oocyte apoptosis during the transition from ovary-like tissue to testes during sex differentiation of juvenile zebrafish. J Exp Biol. 2002;205:711-8.
12. Uchida D, Yamashita M, Kitano T, Iguchi T: An aromatase inhibitor or high water temperature induce oocyte apoptosis and depletion of P450 aromatase activity in the gonads of genetic female zebrafish during sex-reversal. Comp Biochem Physiol A Mol Integr Physiol. 2004;137:11-20.
13. Li MH, Sun LN, Wang DS: Roles of estrogens in fish sexual plasticity and sex differentiation. General and Comparative Endocrinology. 2019;277:9-16.
14. Akhter A, Kumagai RI, Roy SR, Ii S, Tokumoto M, Hossain B, et al.: Generation of Transparent Zebrafish with Fluorescent Ovaries: A Living Visible Model for Reproductive Biology. Zebrafish. 2016;13:155-60.
15. Hsiao CD, Tsai HJ: Transgenic zebrafish with fluorescent germ cell: a useful tool to visualize germ cell proliferation and juvenile hermaphroditism *in vivo*. Dev Biol. 2003;262:313-23.

16. Tokumoto T, Ishikawa K, Furusawa T, Ii S, Hachisuka K, Tokumoto M, et al.: Sonophotocatalysis of endocrine-disrupting chemicals. Mar Environ Res. 2008;66:372-7.
17. Westerfield M: "The Zebrafish Book: a Guide for the laboratory Use of Zebrafish (*Danio rerio*).": Univ. of Oregon Press, Eugene, OR.; 1995.
18. Tokumoto T, Yamaguchi T, Ii S, Tokumoto M: In vivo induction of oocyte maturation and ovulation in zebrafish. PLoS One. 2011;6:e25206.
19. McMillan SC, Geraudie J, Akimenko MA: Pectoral fin breeding tubercle clusters: a method to determine zebrafish sex. Zebrafish. 2015;12:121-3.
20. Dranow DB, Tucker RP, Draper BW: Germ cells are required to maintain a stable sexual phenotype in adult zebrafish. Dev Biol. 2013;376:43-50.
21. Wong TT, Saito T, Crodian J, Collodi P: Zebrafish germline chimeras produced by transplantation of ovarian germ cells into sterile host larvae. Biol Reprod. 2011;84:1190-7.

Figure legends

FIG. 1. The effect of the injection of exemestane on sexually mature female zebrafish. (A) Injection of exemestane solution into experimental fish. The circle indicates the position of the injection of the needle. The dotted line indicates the position of the needle between the skin and ovary. The dotted circle indicates the position of the solution dispensed. (B) The morphology of the ovaries during treatment was monitored by the observation of fluorescence at 1-month intervals for 3 months. Photographs of whole fish and ovarian tissues under bright field microscopy (B. field) and a GFP filter (GFP) are shown. The white scale bars indicate 1 cm.

FIG. 2. Secondary sexual characteristics of exemestane-injected females. (A) Photographs of whole fish (upper panels), enlarged images of pectoral fin (B), the genital papilla (C) and anal fin (D) of a normal female, an exemestane-injected female and a normal male are indicated. Breeding tubercle clusters found in an exemestane-injected female and a normal male are indicated by white line (B). Extended genital papillae in normal females and exemestane-injected females are indicated by arrowheads (C). The black scale bars indicate 1

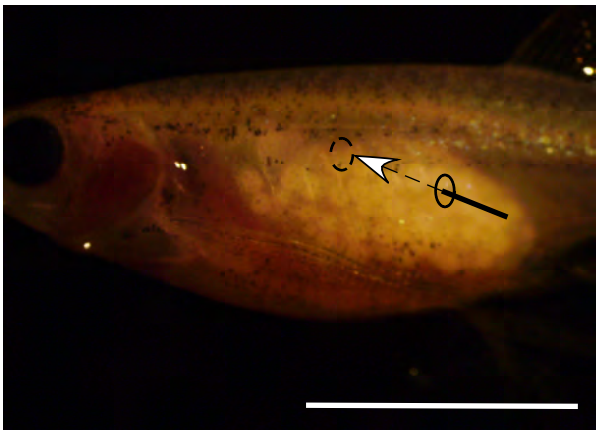
cm. The white scale bars in panel B indicate 50 μm. The white scale bars in C and D indicate 200 μm.

FIG. 3. Histological analysis of exemestane-injected females. (A) Testes formed in exemestane-injected females. A photograph of a section of an exemestane-injected female is shown. In the enlarged photograph on the left side, retracted and degraded oocytes are indicated by arrowheads. On both sides of the testis, cysts filled with spermatozoa (Sp) are shown. The scale bars indicate 10 μm. (B) Changes in ducts in sex-changed females. Photographs of sections around urogenital papilla of a normal female, an exemestane-injected female and a normal male are indicated. Ureter and gonadal ducts (oviduct in female and sperm duct in male) are indicated by black arrow and white arrow, respectively. The scale bars indicate 200 μm.

FIG. 4. Fluorescent juveniles developed from the pairing of sex-changed females and normal females.

(A) Photographs of fluorescent embryos at 1 day post fertilization (1 dpf) and 1 day post hatching (1 dph) are shown in GFP filter views. The scale bars indicate 1 mm. (B) Photographs of 2-month-old juveniles (2 months) are shown in bright-field (B. field) and GFP filter views. The scale bars indicate 1 cm.

A



B

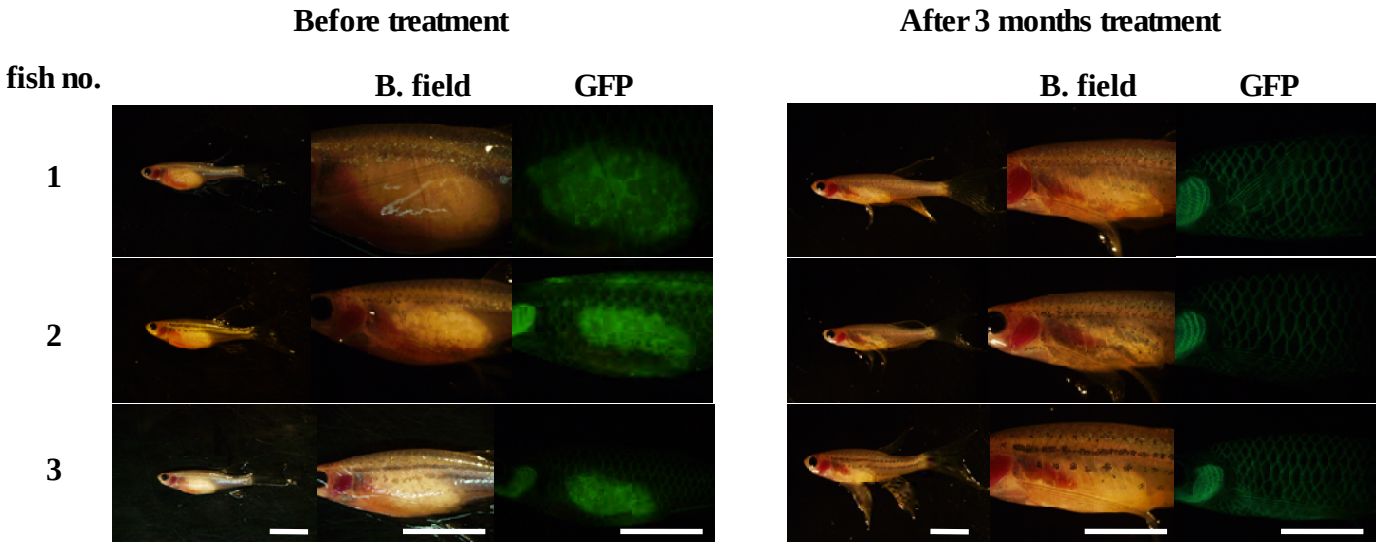

Before treatment
After 3 months treatment
fish no.
B. field
GFP
B. field
GFP
1
2
3

Figure 1.

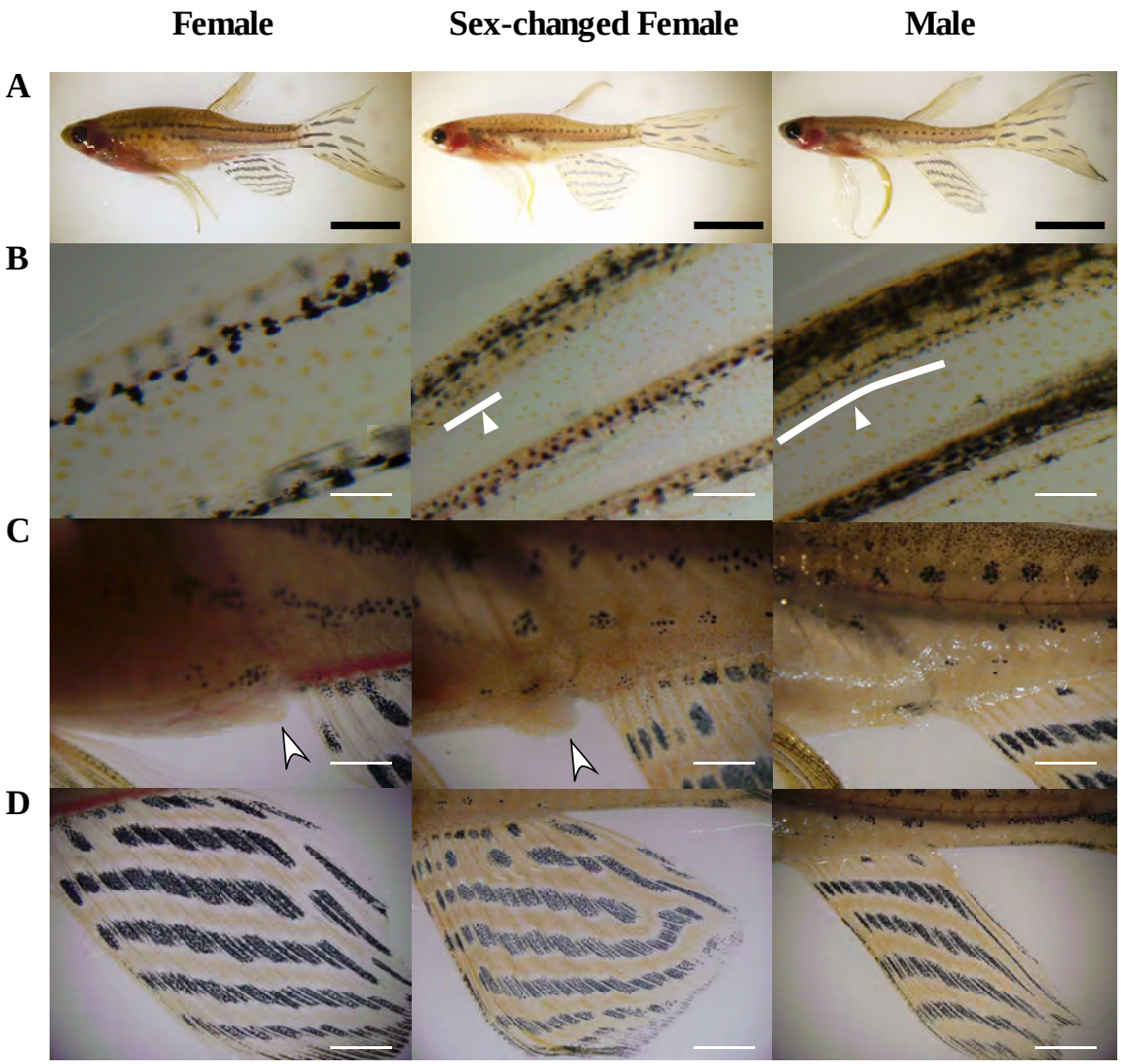


Figure 2.

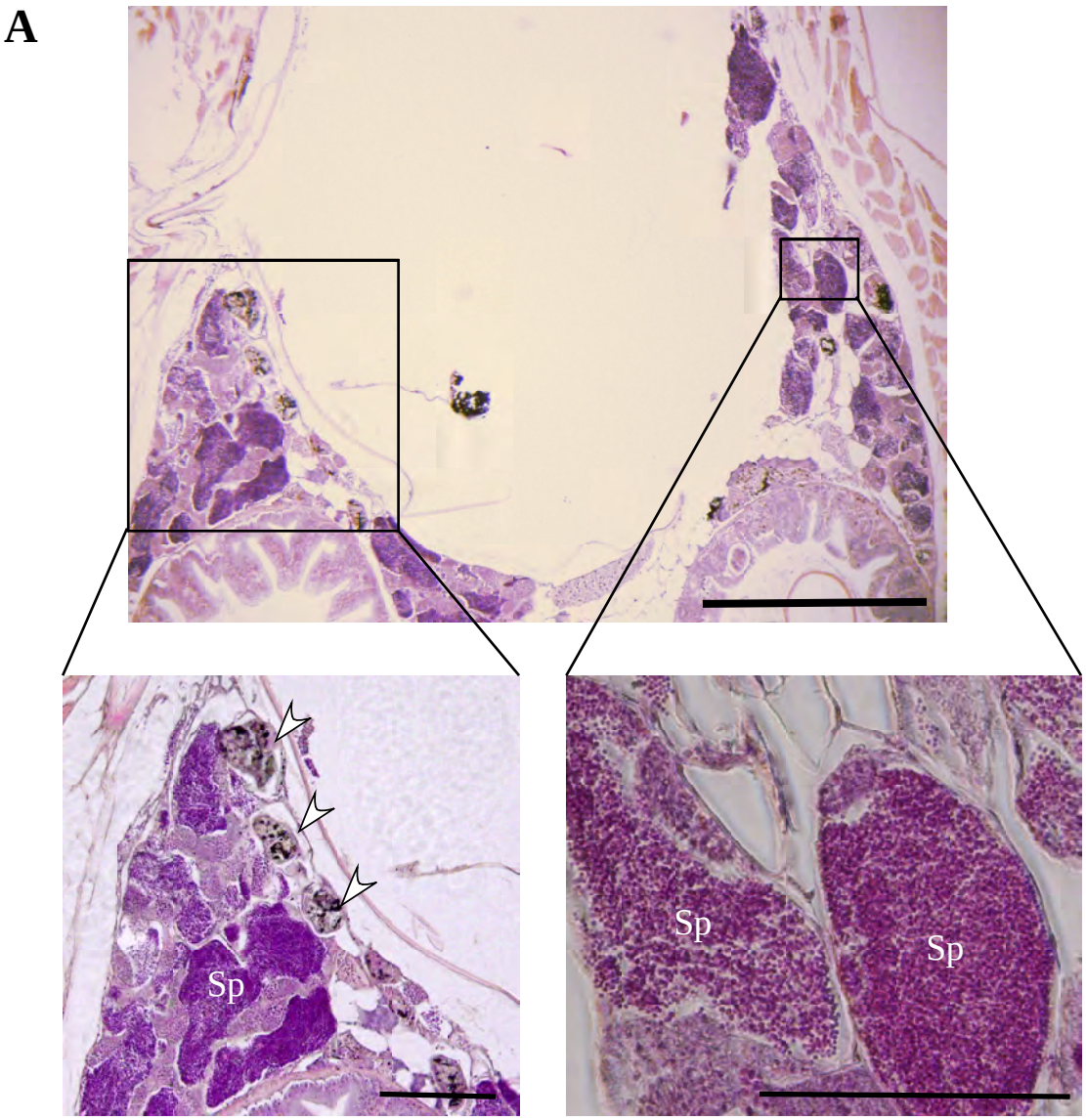


Figure 3.

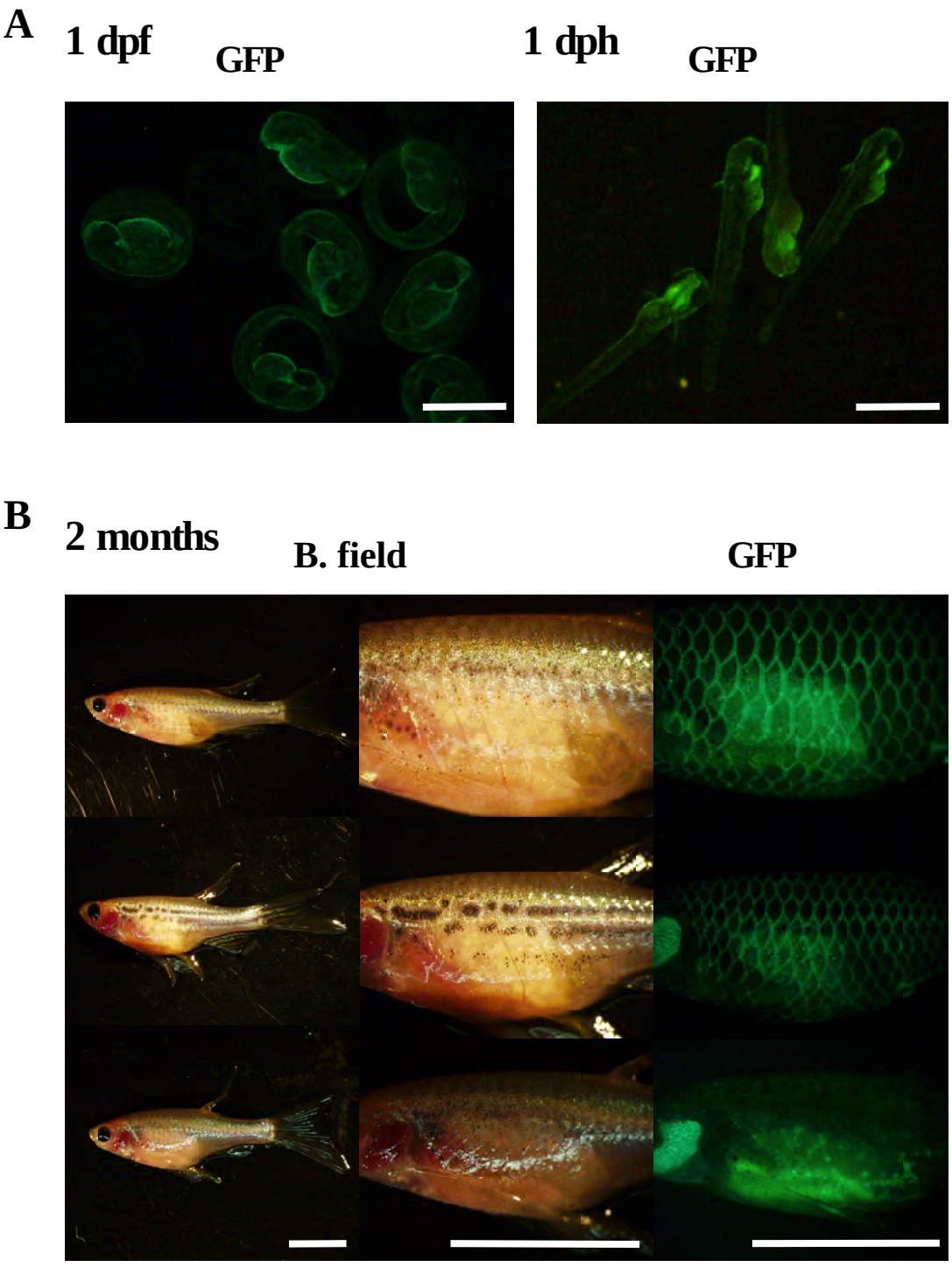


Figure 4.

Supplementary Table. 1 Results of mating of sex changed fish with nontransgenic normal female.

Sex changed fish	Paring[1)]	Total eggs	Unfertilized eggs[2)]	Dead embryos[3)]	Hatched embryos (HE)	% of HE
1	1	0	0	0	0	0
	2	192	152	173	19	10
	3	137	109	121	16	12
	4	0	0	0	0	0
	5	151	117	130	21	14
	6	205	144	178	27	13
	7	98	79	87	11	11
	8	0	0	0	0	0
	9	118	73	89	29	24
	6/9 = 66.7 %[4)]					Ave 14.0 %[5)]
2	1	162	135	144	18	12
	2	0	0	0	0	0
	3	203	168	181	22	25
	4	240	161	197	43	18
	5	178	128	149	29	16
	6	133	106	117	16	12
	7	183	121	152	31	13
	8	0	0	0	0	0
	9	0	0	0	0	0
	6/9 = 66.7 %					Ave. 16.0 %
3	1	0	0	0	0	0
	2	0	0	0	0	0
	3	0	0	0	0	0
	4	187	152	170	17	10
	5	110	76	90	20	19
	6	0	0	0	0	0
	7	65	41	52	13	20
	8	247	184	212	35	14
	9	0	0	0	0	0
	4/9 = 44.4 %					Ave. 15.8 %
						Ave of 3 fishes 15.2 ± 4.7 %

[1)] For three sex changed fishes, 9 trails of paring were conducted.

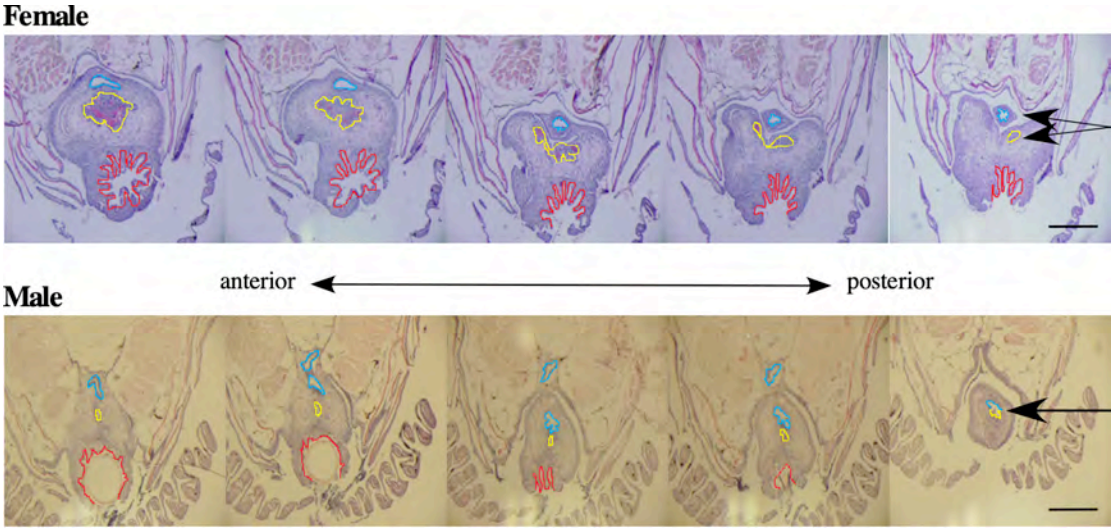
[2)] Number of unfertilized eggs (number of dead eggs until next morning of paring).

[3)] Number of dead embryo until hatch.

[4)] Successful rate of paring.

[5)] Average of hatching rate among successful paring.

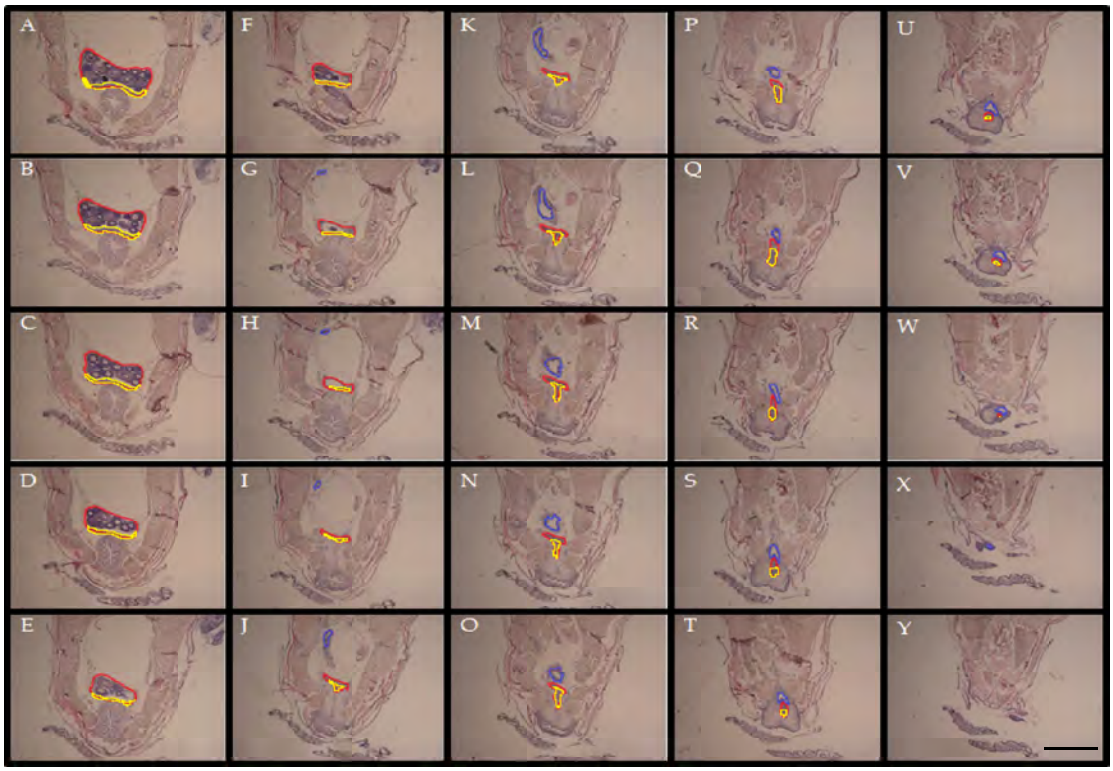
Supplementary Figure 1



Supplementary Figure 1. Duct structure of male and female zebrafish.

Sections of around urogenital papilla of female and male zebrafish. Ureter is indicated by cyan. Gonadal ducts (oviduct in female and sperm duct in male) are indicated in yellow. Intestine is indicated in red. Ureter and oviduct are separate until open as urinary pore and oviducal pore in the case of female (two arrows). Ureter and sperm duct are connect to form common sperm duct (arrow) and open as urogenital pore in male. The scale bars indicate 200 μm.

Supplementary Figure 2



Supplementary Figure 2. Structure of ducts in female during sex change. Sections of around urogenital papilla of female during sex change treatment were prepared serially from anterior to posterior (A to Y). Ureter is indicated by cyan. Ovary and oviduct are indicated in red. Newly formed testis and sperm duct are indicated in yellow. Ureter, oviduct and sperm duct are open separately. The scale bar indicates 1mm.