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Research Article

First Data on Sexual Dimorphic Growth of Cultured Pacific Bluefin Tuna, *Thunnus orientalis* (Temminck et Schlegel), and Its Sex Manipulation by Treatment with an Aromatase Inhibitor

Takao Hayashida [1,2] Kentaro Higuchi [3] Kogen Okita,[1] Yukinori Kazeto,[4] Masaomi Hamasaki,[5] Sota Yoshikawa,[5] Toshinori Takashi,[1] and Koichiro Gen[1]

[1]*Nagasaki Field Station, Fisheries Technology Institute, Japan Fisheries Research and Education Agency, 1551-8 Taira-machi, Nagasaki 851-2213, Japan*
[2]*Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato-ku, Tokyo 108-8477, Japan*
[3]*Headquarters, Japan Fisheries Research and Education Agency, 1-1-25 Shin-urashima, Kanagawa-ku, Kanagawa 221-8529, Japan*
[4]*Minamiizu Field Station, Fisheries Technology Institute, Japan Fisheries Research and Education Agency, 183-2 Minamiizu, Kamo, Shizuoka 415-0156, Japan*
[5]*Nagasaki Prefectural Institute of Fisheries, 1551-4 Taira-machi, Nagasaki 851-2213, Japan*

Correspondence should be addressed to Takao Hayashida; hayashida_takao43@fra.go.jp

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Tuna (genus *Thunnus*), particularly Pacific bluefin tuna (*T. orientalis*; PBT), are commercially important fish in the aquaculture industry worldwide. The objective of this study was to investigate sexual dimorphism in the growth performance of aquaculture-produced PBT and develop techniques for its sex manipulation, for the first time in tuna. A comparison of the body size between sexes revealed that male-cultured PBTs were larger than females at harvest. We also confirmed that *cyp19a1a* (encoding a gonadal aromatase) expression increased specifically in the genotypic female PBT gonads during sex differentiation. This suggested that aromatase plays an important role in ovarian differentiation and that the suppression of aromatase activity may effectively induce masculinization in genotypic females. Therefore, we administered letrozole—an aromatase inhibitor (AI)—into sexually undifferentiated PBT through the oral route. AI administration resulted in a 100% sex reversal of genotypic females into phenotypic males at the molecular level. Our results provide the basis for future studies on the establishment of mono-sex male production technology in PBT, which would help improve the productivity of closed-cycle PBT aquaculture. Furthermore, this study offers important insights into the understanding of the sex-wise growth of tuna species in aquacultural settings, and developing sex manipulation techniques.

1. Introduction

Tuna (genus *Thunnus*) are commercially important fish in the global aquaculture industry [1]. Pacific bluefin tuna (*T. orientalis*; hereafter referred to as PBT) specifically, due to its high market value, is one of the most important species for aquaculture worldwide [2]. Currently, several research institutes and companies in Japan have achieved closed-cycle production of this species [2, 3] and have made hatchery-produced tuna seed available for practical tuna aquaculture [4, 5]. However, most bluefin tuna aquaculture practices rely on wild-caught juveniles for seed stocks, owing to the low productivity of closed-cycle aquaculture [6, 7]. There are numerous issues associated with the use of wild-

caught juveniles for aquaculture, including the unstable supply and the negative impacts on wild stock management [2]. Because PBT are endangered, due to overfishing of wild stocks, the fishing of this species is currently controlled based on an assessment by the Western and Central Pacific Fisheries Commission (WCPFC). The promotion of closed-cycle aquaculture is necessary for species conservation and sustainable development of the tuna farming industry [8]; however, this requires the development of technology that improves its productivity.

Sexual dimorphism in growth performance is common in fishes, with some species exhibiting superior female growth and others exhibiting superior male growth [9, 10]. For example, the male tilapia (*Oreochromis niloticus*) grows to a larger size than females [11], whereas the female barfin flounder (*Verasper moseri*) grows to a larger size than males [12]. Due to these patterns of sexually dimorphic growth, mono-sex production is a desirable technology for the aquaculture industry, as it can help improve productivity by shortening production times and reducing production costs [10]. In addition, mono-sex stocks can also have other beneficial effects in aquaculture, such as preventing unwanted reproduction, reducing aggressive interactions, and improving the dress-out percentage and body coloration at harvest [10, 13, 14].

To the best of our knowledge, body size measurement data of cultured tuna at harvest for each sex have only been obtained in the study by Sawada et al. [8], and detailed data regarding the sex-wise growth performance of tuna species in aquacultural settings are limited. Previous studies reported that in wild stocks of PBT, males tend to be larger than females [15, 16]. A similar tendency has also been reported in several tuna species, including the Atlantic bluefin tuna (*T. thynnus*) [17], southern bluefin tuna (*T. maccoyii*) [18], bigeye tuna (*T. obesus*) [19], and albacore (*T. alalunga*) [20]. These findings potentially suggest that in tuna species, males also exhibit higher growth performance compared to females in aquacultural settings. Long periods of time (approximately, more than 3 years) and huge economic costs are required to rear PBT until harvest size [8, 21]. If male-cultured PBT exhibit higher growth performance than females, the production of mono-sex male stocks seems to act as a prominent tool in significantly improving the productivity of closed-cycle PBT aquaculture by shortening production times and reducing production costs.

Sex steroid hormones play a critical role in fish sex differentiation [22]. Therefore, sex manipulation in fish can be achieved via exogenous administration of sex steroids, such as 17β-estradiol (estrogen) and 17α-methyltestosterone (androgen) [10, 23–25]. Apart from sex steroids, aromatase inhibitors (AIs)—such as fadrozole—are commonly used to induce masculinization in fish [10]. Androgens are converted to estrogens through a reaction catalyzed by cytochrome P450 aromatase, the product of the *cyp19a1a* gene [24]. During the critical period of molecular sex differentiation in fish, aromatase and endogenous estrogens are expressed and synthesized specifically in the female gonads and act as inducers of ovarian differentiation [23, 24]. Aromatase inhibitors work by irreversibly deactivating the aromatase enzyme and suppressing estrogen synthesis in female gonads, resulting in the sex reversal of genotypic females into phenotypic males [10].

Fish are highly sensitive to the effects of exogenous sex steroids or AI during the labile period, when the gonads are undifferentiated [22]. Therefore, the administration of exogenous sex steroids or AI should be initiated prior to gonadal sex differentiation. Moreover, carefully selecting the method of administration is important for the development of mono-sex production technology, as sex manipulation efficiency, cost-effectivity, and usability depends on the method [10]. Sex steroids or AI can be administered through several routes, including direct injection of the agent into the muscle or body cavity of the fish, direct immersion of the fish into culture water containing the agent, or oral delivery via incorporation into the feed [26]. Administering exogenous sex steroids or AI via dietary supplementation is one of the most common approaches for sex manipulation in aquaculture due to its simplicity of administration and applicability at a commercial scale [10]. As such, this method has been proven to be an effective strategy for producing 100% mono-sex stocks [10, 27–29].

The objective of this study was to investigate sexual dimorphism in the growth performance of PBT in aquacultural settings and develop a technique for its sex manipulation, for the first time in tuna species. First, we compared the body sizes of aquaculture-produced PBTs at harvest between sexes. In addition, as a first step toward establishing mono-sex male PBT production technology, we examined the sex-dependent expression pattern of *cyp19a1a* during gonadal sex differentiation. Subsequently, based on the result of the expression analysis, we orally administered AI into juvenile PBT during the labile period, when the gonads differentiate [30]. We used letrozole, a third-generation nonsteroidal AI with high specificity and potency to aromatase than other AIs [31]—since successful and effective masculinization by letrozole treatment has been reported in several marine fish species including dusky grouper (*Epinephelus marginatus*) [32], blue drum (*Nibea mitsukurii*), and yellow drum (*N. albiflora*) [33]. Our results provide the basis for future studies on the development and establishment of technologies for mono-sex male production in PBT, which will contribute to the further development of closed-cycle PBT aquaculture and meet conservation objectives for wild PBT stocks. Moreover, this study offers important insights into the understanding of sex-wise growth of tuna species in aquacultural settings, and developing techniques for its sex manipulation.

2. Materials and Methods

2.1. Ethical Approval. All experiments were performed in accordance with the Guidelines for the care and use of Live Fish of the Fisheries Technology Institute (FTI), Japan Fisheries Research and Education Agency (FRA), and were approved by the Institutional Animal Care and Use Committee of FTI.

2.2. Sampling of Cultured PBT at Harvest. In February and August 2021, we sampled 4-year-old PBTs that had been commercially cultured from wild-caught seedlings in the coastal area of Takashima (Nagasaki, Japan). Wild-caught 1-year-old juveniles were transferred to the offshore net cage and reared until 4 years old under natural water temperature and photoperiod conditions. Fish were mainly fed defrosted chub mackerel (*Scomber japonicus*) until harvest. The fishes in the offshore net cage were caught and sacrificed through electroshock. Immediately after harvesting, the gills and guts of the fish were removed, placed on ice, and transported to an onshore facility. The fork length (FL, cm) and body weight (BW, kg) of each fish were measured. The individuals were sexed by visual observation of the dissected gonads, and the gonad weight (GW, g) was measured. The gonads were then fixed with Bouin's solution, stored in 70% ethanol at 4°C, and processed for histological analysis. Individual gonads were classified into different groups based on their maturity status after histological observation. Briefly, female gonads were categorized into the perinucleolar (oocyte at perinucleolus stage), oil droplet (oocyte at oil droplet stage), early vitellogenic (oocyte at primary and/or secondary yolk globule stages), and late vitellogenic (oocyte at tertiary yolk globule and/or migratory stages) according to the most advanced type of oocytes found in the female. Male gonads were categorized into the early (spermatogonia and spermatocytes dominant), mid (with spermatogonia, spermatocytes, spermatids, and sperm present), and late (sperm becoming dominant) spermatogenesis stages. The condition factor (CF) and gonad index (GI) were calculated as follows:

$$\begin{aligned} \mathrm{CF} &= \frac{\mathrm{BW}}{\mathrm{FL}^3} \times 10^5, \\ \mathrm{GI} &= \frac{\mathrm{GW}}{\mathrm{FL}^3} \times 10^4. \end{aligned} \tag{1}$$

2.3. Measurement of cyp19a1a Expression during Gonadal Sex Differentiation in PBT. The PBT gonads were obtained as described in our previous study [30] and stored in RNAlater (Ambion, Austin, TX, USA) at −30 °C until RNA extraction. For each genotypic sex, total RNA was extracted from the gonads of PBT at morphologically undifferentiated (41 days posthatching, dph), differentiating (57 dph), and differentiated (83 dph) stages. The RNA was used to measure the expression levels of the *cyp19a1a* gene through quantitative real-time PCR (qPCR) analysis.

2.4. AI Treatment. PBT juveniles reared from fertilized eggs at Nagasaki Prefectural Institute of Fisheries (Nagasaki, Japan) were transferred to Nagasaki Field Station, FTI, FRA (Nagasaki, Japan), and used for AI treatment experiments. In trial 1 (2019), a total of 180 and 80 fish at 31 dph were grouped into AI-treated (experimental) and nontreated (control) groups, respectively. The TL and BW of PBT juveniles at 31 dph in trial 1 were 3.20 ± 0.04 cm and 0.35 ± 0.01 g, respectively ($n = 25$ fish, 17 genotypic females, and 8 genotypic males; mean ± standard error of the mean, SEM). In trial 2 (2021), a total of 600 and 11,200 fish at 35 dph were grouped into AI-treated (experimental) and control groups, respectively. The TL and BW of PBT juveniles at 35 dph in trial 2 were 4.50 ± 0.06 cm and 0.88 ± 0.06 g, respectively ($n = 40$ fish, 25 genotypic females, and 15 genotypic males; mean ± SEM). The genotypic sex of each fish was identified using male-specific DNA markers [34].

PBT juveniles were reared in 7- and 38-ton indoor tanks with natural seawater during the experiments. Both groups were fed an artificial diet of Magokoro (Nisshin Feed, Tokyo, Japan) and Ambrosia (FEED ONE, Kanagawa, Japan) to apparent satiety 3–5 times per day. Letrozole (Tokyo Chemical Industry, Tokyo, Japan)—a nonsteroidal AI—was administered at a dose of 100 mg/kg feed [32, 35] until the end of the experiment (59 dph in trial 1 and 70 dph in trial 2). The photoperiod was fixed at 24 h of light in both trials. The natural seawater was sterilized by exposure to UV before being poured into the rearing tanks. The water temperature, depending on the natural seawater, was in the range of 23.2–27.5°C in trial 1 and 22.4–26.2°C in trial 2 during the experimental period. At the end of the experiment, the fish were sampled after being anesthetized with 2-phenoxyethanol. Blood samples were collected using heparinized syringes and kept on ice until centrifugation at 1,500 ×g for 15 min at 4°C. Following this, the plasma was stored at −30°C until the measurement of sex steroid levels. The TL (cm) and BW (g) of each sampled fish were measured. The dissected gonads were fixed with Bouin's solution, stored in 70% ethanol at 4°C, and processed for histological analysis. The phenotypic sex of individuals was determined by the presence of ovarian characteristics (an ovarian cavity with ovarian lamellae) and testicular features (the efferent duct appears as a slit in the stromal tissue with a triangular shape), as reported previously [30]. The blood vessels in gonads appeared to be larger in the ovaries than in the testes during gonadal sex differentiation in PBT [30]. Therefore, the cross-sectional area of the blood vessels in the gonads was measured from digital images using the image analysis software, ImageJ (National Institutes of Health, Bethesda, MD) [30]. The dissected gonads were also placed in RNAlater (Ambion), stored at −30°C, and processed for reverse transcription PCR (RT-PCR) with sex-specific gene primers. The fin or muscle samples of each fish were stored in 300 μl TNES-urea buffer (6 M urea, 10 mM Tris-HCl, pH 7.5, 125 mM NaCl, 10 mM EDTA, and 1% SDS) at room temperature according to Asahida et al. [36]; and processed for PCR-based genotypic sex identification.

2.5. Histology. The fixed gonads were dehydrated and embedded using standard paraffin embedding methods. The gonads were then cut into 5 μm-thick sections using a microtome (HistoCore BIOCUT, Leica Biosystems, Wetzlar, Germany) and stained with hematoxylin and eosin. The stained sections were imaged and photographed using a light microscope (BX-43, Olympus, Tokyo, Japan) equipped with a digital camera (DP-70, Olympus).

2.6. Total RNA Extraction. Total RNA was extracted using ISOGEN (Nippon Gene, Toyama, Japan) and then treated with TURBO DNase (Ambion) according to the manufacturer's instructions. A 1 μl aliquot of the total RNA was used for quantification with a NanoDrop spectrometer (ND-1000, Thermo Scientific, Rockford, IL).

2.7. qPCR. The quantified RNA samples were used for qPCR with a one-step RT-PCR system using the One Step TB Green PrimeScript PLUS RT-PCR Kit (Takara Bio, Shiga, Japan). The reactions were run on a Light Cycler 480 instrument (Roche Diagnostics, Mannheim, Germany) in 96-well plates (Roche Diagnostics) using the following cycling conditions: 42°C for 5 min, 95°C for 10 s, followed by 40 cycles of 95°C for 5 s and 60°C for 20 s. The reaction volumes (20 μl) contained 10 ng of the total RNA template, 10 μl of 2× One Step TB Green RT-PCR Buffer 4, 1.2 μl of TaKaRa Ex *Taq* Hs Mix, 0.4 μl of PrimeScript PLUS RTase Mix, and 0.4 μM each of forward and reverse *cyp19a1a*-specific primers (Table 1). The specific primers were designed using CLC Main Workbench 8.0.1 (QIAGEN GmbH, Hilden, Germany). As the standard for quantification, we used a plasmid containing a partial cDNA sequence of a target gene. The standard sets of seven points ranged from 1×10^8 to 1×10^2 copies and were prepared by 10 × serial dilutions. Technical duplicates were performed for all experimental samples and standards. The intraassay coefficient of variation (CV) was determined using repeated measurements ($n = 8$) of the standard samples and was calculated as 0.25%.

2.8. RT-PCR. Total RNA (200 ng) was reverse-transcribed using PrimeScript Reverse Transcriptase (TaKaRa Bio) according to the manufacturer's instructions. For RT-PCR analysis, we used ovarian (*cyp19a1a*) and testicular (*cyp11b* and *dmrt1*) marker genes and an internal control gene (*b-actin*). The sequences of the *cyp11b* and *dmrt1* genes were predicted from available genomes [34, 38], and cDNA fragments of *cyp11b* and *dmrt1* amplified using Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions were cloned into the pGEM T-Easy Vector (Promega, Madison, WI, USA) and sequenced. The following PCR conditions were used for RT-PCR analysis: 35 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 1 min. The reaction volumes (10 μl) contained 10 ng of the cDNA template, 0.25 U of Takara Ex *Taq* DNA polymerase (Takara Bio), 1 μl of 10× Ex *Taq* buffer (Takara Bio), 0.2 mM of dNTPs, and 1 μM each of the forward and reverse primers (Table 1). Specific primers were designed using the CLC Main Workbench 8.0.1 (QIAGEN GmbH). The PCR products were electrophoresed and visualized on a 2% agarose gel containing ethidium bromide.

2.9. Sex Steroid Measurement. Sex steroid measurements were performed as described by Higuchi et al. [39]. Briefly, steroids were extracted from 100 μl of plasma with dimethyl ether, which were then evaporated. The dried extracts were reconstituted in an assay buffer (ELISA buffer, Cayman Chemical, Ann Arbor, MI, USA). The plasma levels of E2 and 11-KT were analyzed with an enzyme-linked immunosorbent assay (ELISA) using the commercially available Estradiol ELISA kit and Testosterone 11-Keto-EIA kit, respectively (Cayman Chemical). The intraassay CV was determined by repeated measurements ($n = 7$) of standard plasma samples from the same plate. For the E2 and 11-KT assays, the intraassay CV was 13.31% and 10.05%, respectively.

2.10. Genotypic Sex Identification. Genomic DNA was extracted from fin or muscle tissues stored in TNES-urea buffer using the standard phenol-chloroform protocol, as described by Asahida et al. [36]. After DNA extraction, the genotypic sex of each fish was identified using a PCR-based sex identification method based on male-specific DNA markers, as described by Suda et al. [34]. The following PCR conditions were used: 35 cycles of denaturation at 94°C for 30 s, annealing at 62°C (male-specific primers) or 60°C (*b-actin*) for 30 s, and extension at 72°C for 30 s. The reaction volumes (10 μl) contained 10 ng of the DNA template, 0.25 U of Takara Ex *Taq* DNA polymerase (Takara Bio), 1 μl of 10× Ex *Taq* buffer (Takara Bio), 0.2 mM of dNTPs, and 1 μM each of the forward and reverse primers (Table 1). The PCR products were analyzed by electrophoresis.

2.11. Statistical Analysis. Data are presented as the mean ± SEM. For our analyses, we used the FL, BW, BW including GW, CF, and GI of harvested PBTs, and the expression levels of *cyp19a1a* in the gonads during sex differentiation. We tested for differences in these characters at each sampling period between the sexes using Welch's t-test ($P < 0.05$). We also performed a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test to identify trends in *cyp19a1a* expression levels during gonadal sex differentiation in each genotypic sex. Significant differences in the TL, BW, gonadal blood vessel area, and plasma sex steroid levels of PBTs in the AI treatment experiment were tested by a two-way ANOVA followed by Tukey's multiple comparison test. Statistical analyses were performed using Prism 7.0 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Sexual Dimorphism in Growth Performance of Cultured PBT at 4 Years of Age. The FL, BW, BW including GW, CF, GI, and maturity status of cultured PBTs of both sexes at 4 years of age are listed in Table 2.

In February 2021 ($n = 50$ females and 47 males), there were no significant differences in the FL, BW, BW including GW, and CF between the sexes ($P > 0.05$). The GI was significantly higher in females than in males ($P < 0.05$). During this period, the ovaries of all females were in the previtellogenic stage (perinucleolar and oil droplet stages). Similarly, all males had testes at the early- and mid-spermatogenesis stages.

Table 1: List of primers.

Purpose	Target	Primer (5′–3′)		GenBank accession no	Reference
Genotypic sex identification	Male-specific region	Forward	TGCACCTGTAACTCACTAACCG		[34]
		Reverse	CCTTTTCCTGGCCTCTTTACAT		
	actb	Forward	AGCTGCCTGACGGACAGGTCATCA		[37]
		Reverse	TCGTACTCCTGCTTGCTGATCCA		
qPCR	*cyp19a1a*	Forward	CTGTTGTAGGTGACAGACAG	AB610668.1	
		Reverse	CACGACGCATGGTGAAGTC		
RT-PCR	*actb*	Forward	AGCTGCCTGACGGACAGGTCATCA		[37]
		Reverse	TCGTACTCCTGCTTGCTGATCCA		
	cyp19a1a	Forward	TTTCTGTCTGGGTGTGGG	AB610668.1	
		Reverse	TGGGAGAGGTTGTTGGTTT		
	dmrt1	Forward	AGTCATGGCGGCTCAGGTC	LC727626	
		Reverse	GGTGAAGTTTGGCGTCTCG		
	cyp11b	Forward	TAGCGGCCACGTGCTCTACG	LC727625	
		Reverse	GAGGAAGAGCTGCATCTC		

Table 2: Fork length, body weight, body weight including gonad weight, condition factor, gonad index, and maturity status of cultured Pacific bluefin tuna at 4 years of age for each sex.

Sampling period	Sex*	No. of sampled fish	Fork length (cm)**	Body weight (kg)**	Body weight including gonad weight (kg)**	Condition factor**	Gonad index**	*Gonadal developmental stage****						
								PN (%)	Od (%)	EVG (%)	LVG (%)	ESG (%)	MSG (%)	LSG (%)
February 2021	Female	50	144.64 ± 0.68^{a}	51.34 ± 0.70^{a}	51.50 ± 0.66^{a}	1.69 ± 0.01^{a}	0.75 ± 0.02^{a}	42 (84.0)	8 (16.0)	0	0	—	—	—
	Male	47	144.78 ± 0.81^{a}	51.35 ± 0.79^{a}	51.40 ± 0.79^{a}	1.68 ± 0.01^{a}	0.15 ± 0.00^{b}	—	—	—	—	35 (74.5)	12 (25.5)	0
August 2021	Female	24	155.58 ± 1.00^{A}	63.54 ± 1.69^{A}	64.95 ± 1.77^{A}	1.68 ± 0.02^{A}	3.82 ± 0.66^{A}	3 (12.5)	1 (4.2)	3 (12.5)	17 (70.8)	—	—	—
	Male	40	158.80 ± 1.15^{B}	68.69 ± 1.83^{B}	69.36 ± 1.82^{A}	1.70 ± 0.02^{A}	1.76 ± 0.22^{B}	—	—	—	—	0	15 (37.5)	25 (62.5)

*Sex of individuals was identified by visual observation of gonads. **Data are presented as the mean ± standard error of the mean (SEM). Different letters indicate a significant difference between sexes at the same sampling period ($P < 0.05$, Welch's *t*-test). The body weight of individuals was recorded after being gilled and gutted. Condition factor = body weight (kg)/fork length $(cm)^3 \times 10^5$. Gonad index = gonad weight (g)/ fork length $(cm)^3 \times 10^4$. ***Maturity status of gonads is classified into perinucleolus (PN), oil droplet (OD), early vitellogenic (EVG), and late vitellogenic (LVG) stages according to the most advanced type of oocytes found in female, and into early (ESG), mid (MSG), and late spermatogenesis (LSG) stages in the male by histological observation of gonads [39].

In August 2021 ($n = 24$ females and 40 males), the FL and BW values of males were significantly higher than those of females ($P < 0.05$). The BW including GW of males tended to be larger than that of females, although there was no significant difference observed ($P > 0.05$). There was no significant difference in the CF between the sexes ($P > 0.05$). The GI was significantly higher in females than in males ($P < 0.05$). During this period, approximately, 70% of females and 60% of males had reached sexual maturation (late vitellogenic stage in females and late spermatogenesis stage in males).

3.2. Expression of cyp19a1a during Gonadal Sex Differentiation in PBT. The expression levels of *cyp19a1a* during gonadal sex differentiation in PBT are illustrated in Figure 1 ($n = 3$ fish). At the morphologically undifferentiated stage (41 dph), the expression level of *cyp19a1a* was significantly higher in genotypic females than in genotypic males ($P < 0.05$). In genotypic females, the expression levels of *cyp19a1a* increased significantly from the undifferentiated stage to the differentiating stage (57 dph) ($P < 0.05$) and remained high until the differentiated stage (83 dph). In contrast, although the expression levels of *cyp19a1a* increased significantly from the undifferentiated stage to the differentiating stage ($P < 0.05$) in genotypic males, it remained low during gonadal sex differentiation. In fact, the expression level of *cyp19a1a* was significantly higher in genotypic females than in genotypic males at both the differentiating (57 dph) and differentiated (83 dph) stages ($P < 0.05$).

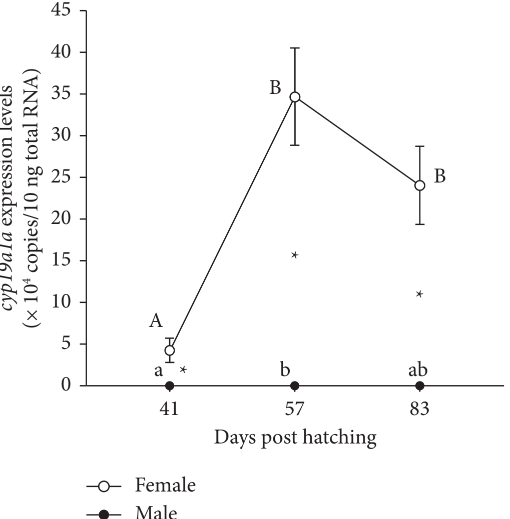


Figure 1: Expression levels of *cyp19a1a* during gonadal sex differentiation in Pacific bluefin tuna. Total RNAs extracted from Pacific bluefin tuna gonads for each genotypic sex at morphologically undifferentiated (41 days post-hatching, dph), differentiating (57 dph), and differentiated (83 dph) stages [30] were subjected to quantitative real-time PCR. Open and closed circles indicate genotypic female and male, respectively. Bars represent the mean ± standard error of the mean (SEM) ($n = 3$ fish). Different letters indicate significant differences ($P < 0.05$, one-way ANOVA followed by Tukey's multiple comparison test). Asterisks indicate significant differences between sexes at each sampling point ($P < 0.05$, Welch's t-test).

3.3. Effects of AI Treatment on Survival, Growth, and Gonadal Development in PBT. The survival, growth, and gonadal development of PBTs of both sexes in the AI-treated and nontreated (control) groups are summarized in Table 3. The survival rate tended to be higher in the AI-treated group than in the control group in both trials. In trial 2, there were no significant differences in TL and BW between the groups ($P > 0.05$). In both trials, the gonadal blood vessel areas of genotypic females in the AI-treated group tended to be smaller (Figure 2(c)) than those of genotypic females in the control group (Figure 2(a)). The genotypic males in the AI-treated (Figure 2(d)) and control (Figure 2(b)) groups also showed similar differences in gonadal blood vessel area. In trial 2, the gonadal blood vessel area of the genotypic female in the AI-treated group was significantly smaller than that of the genotypic female in the control group, as with of genotypic male in both AI-treated and control groups ($P < 0.05$) ($n = 4$ fish). In both trials, all individual gonads in the control group had differentiated into ovaries or testes depending on the genotypic sex of the individual (Figures 2(a) and 2(b)). In contrast, the gonads of genotypic males (Figure 2(d)), as well as those of genotypic females (Figure 2(c)) in the AI-treated group showed testicular characteristics (the efferent duct appears as a slit in the stromal tissue with a triangular shape).

3.4. Gene Expression Pattern of Sex-Reversed PBT Gonads. Figure 3 shows the expression patterns of sex-specific marker genes in the sex-reversed gonads of genotypic females in the AI-treated group ($n = 9$ fish). In the control group, the ovarian marker gene (*cyp19a1a*) and the testicular marker genes (*cyp11b* and *dmrt1*) were expressed specifically in the gonads of genotypic females and males, respectively. In the AI-treated group, *cyp19a1a* expression was not detected in the gonads of either sex (genotypic females or genotypic males). In contrast, *cyp11b* and *dmrt1* were expressed in genotypic males, as well as in the sex-reversed gonads of genotypic females.

3.5. Effects of AI Treatment on Plasma Levels of E2 and 11-KT in PBT. Figure 4 shows the plasma levels of E2 and 11-KT for each genotypic sex in the AI treated and control groups in trial 2 ($n = 8$ fish). Plasma E2 levels tended to be lower in the AI-treated group than in the control group, and it was significantly lower in genotypic males in the AI-treated group than in genotypic females in the control group ($P < 0.05$). In both groups, although there were no significant differences ($P > 0.05$), plasma E2 levels tended to be lower in genotypic males than in genotypic females. Plasma 11-KT levels in the AI-treated group were significantly higher than those in the control group ($P < 0.05$). In addition, plasma 11-KT levels of genotypic males were significantly higher than those of genotypic females in both AI-treated and control groups ($P < 0.05$).

Table 3: Effects of treatment with an aromatase inhibitor (AI) on the survival, growth, and gonadal development of the Pacific bluefin tuna.

Trial (year)	Sampling period (dph[†])	Treatment	Rearing tank (ton)	Survival rate (%)	Genotype[‡]	No. of sampled fish	Total length[$] (cm)	Body weight[$] (g)	Histological area of gonadal blood vessels[$] ($\mu m^2$)	*Phenotype*[¶]	
										Female (%)	Male (%)
1 (2019)	59	Control	7	12.00	Female	7	12.31 ± 0.52	21.99 ± 2.61	1159.84 ± 324.00	7 (100)	0
					Male	3	12.09 ± 1.14	22.78 ± 5.33	178.88 ± 63.61	0	3 (100)
		AI	38	23.00	Female	18	13.26 ± 0.23	27.69 ± 1.67	370.26 ± 106.87	0	18 (100)
					Male	2	14.78	37.26	281.25	0	2 (100)
2 (2021)	70	Control	38	2.67	Female	15	14.30 ± 0.18^{a}	29.55 ± 1.50^{a}	2758.27 ± 638.48^{a}	15 (100)	0
					Male	15	14.37 ± 0.15^{a}	29.85 ± 1.00^{a}	1117.95 ± 306.77^{b}	0	15 (100)
		AI	7	12.83	Female	40	14.06 ± 0.15^{a}	28.10 ± 1.11^{a}	928.12 ± 190.58^{b}	0	40 (100)
					Male	37	14.04 ± 0.18^{a}	27.15 ± 1.23^{a}	613.84 ± 110.08^{b}	0	37 (100)

[†]dph: days post hatching. [‡]The genotypic sex of each fish was identified using male-specific DNA markers [34]. [$]Data are presented as mean ± standard error of the mean. The data of AI-treated genotypic males in trial 1 are presented as mean values ($n = 2$ fish). Two-way ANOVA followed by Tukey's multiple comparison test was performed to determine statistical differences in total length, body weight, and histological area of gonadal blood vessels among groups in trial 2. Different letters indicate significant differences ($P < 0.05$). [¶]The phenotypic sex of each fish was identified through the histological observation of gonads [30].

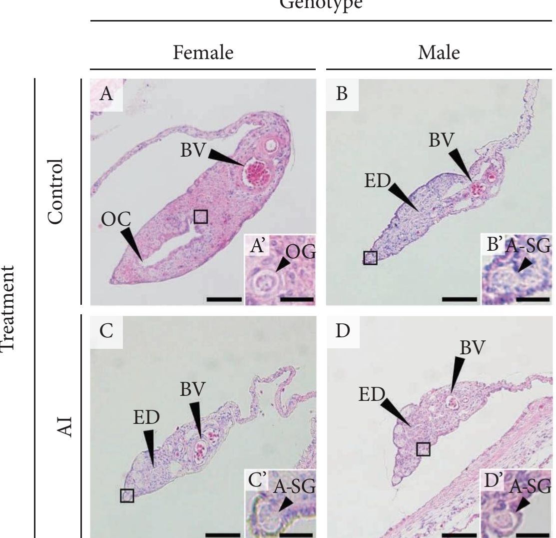


Figure 2: Histological images of the gonads of Pacific bluefin tuna in aromatase inhibitor (AI)-treated and nontreated (control) groups. Images are shown for each genotypic sex at 70 days post hatching. (a and b) All individual gonads in the control group have differentiated into ovaries or testes depending on the genotypic sex of the individual. (c and d) The gonads of genotypic males, as well as those of genotypic females in the AI-treated group show testicular characteristics: smaller gonadal blood vessels, and the efferent duct appears as a slit in the stromal tissue with a triangular shape. Transverse sections of the gonads are stained with hematoxylin and eosin. The genotypic sex of individuals was identified using male-specific DNA markers. The insets show a higher magnification of the area where the germ cells are located. Arrowheads indicate the following: OG, oogonia, A-SG, type-A spermatogonia, OC, ovarian cavity, BV, blood vessels, and ED, efferent duct. Bars = 50 μm (A–D) and 10 μm (A′–D′).

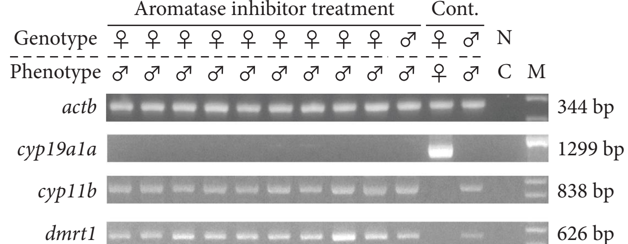


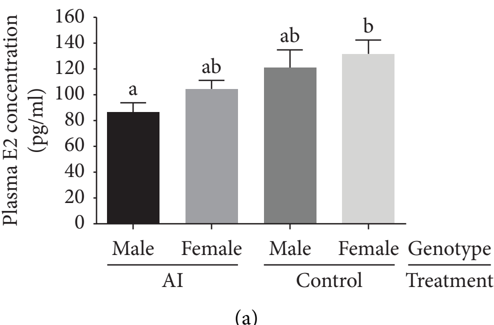
Figure 3: RT-PCR results for the expression levels of the ovarian marker gene, *cyp19a1a*, and testicular marker genes, *cyp11b* and *dmrt1*, in the gonads of Pacific bluefin tuna of each genotypic sex in the aromatase inhibitor (AI)-treated and nontreated (control) groups. *β-actin* (*actb*) has been used as an internal control. The genotypic and phenotypic sex of each fish was identified by a PCR-based sex identification method using male-specific DNA markers and by histological observation, respectively. NC: nontemplate control and M: molecular marker.

4. Discussion

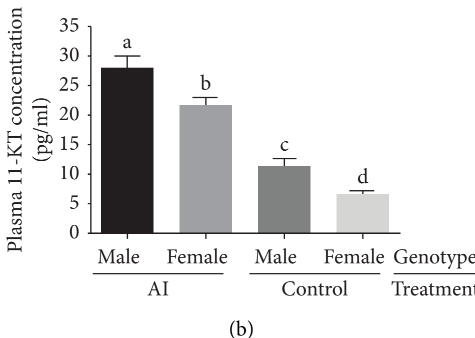
Sexual dimorphism in growth performance has been confirmed in various aquaculture target species. For this, sex manipulation in fish to produce mono-sex stocks is an important area of aquaculture research [10]. The present study provides the first data on the sexually dimorphic growth of a tuna species in aquacultural settings, and its sex manipulation.

We found that the FL and BW of cultured PBTs at 4 years of age were significantly larger in males than in females during the reproductive season (August 2021). Cultured PBTs, which are reared from wild-caught seedlings, generally reach sexual maturation and stable spawning at 4 years of age [2, 40, 41]. In this study, almost all PBTs sampled during the reproductive season had reached sexual maturation. Furthermore, we did not observe any differences in body size between sexes before the fish had reached sexual maturation at 4 years of age (February 2021). Our results suggest that male-cultured PBTs exhibit higher growth performance than females at maturity. The most common reason for the higher growth performance of males compared to females at maturity is that after sexual maturation, females allocate more energy for gonadal maturation and less energy for growth compared to males [42]. Previous studies on wild stocks have reported that several species of tuna—including the Atlantic bluefin tuna [17], southern bluefin tuna [18], bigeye tuna [19], albacore [20], and PBT [15]—exhibit size dimorphism between the sexes, with males growing larger than females after reaching maturity. In this study, the GI of cultured PBTs during the reproductive season was significantly higher in females than in males, suggesting that females require more energy for gonadal maturation than males. These results indicate that in cultured PBTs that reach sexual maturity at 4 years of age, sexual dimorphism in growth performance may be due to differences in energy investment for gonadal maturation between the sexes: female PBTs allocate more energy for reproduction after sexual maturation, and are thus smaller in body size than males.

Sawada et al. [8] reported that there is no difference in body size of cultured PBT between sexes until 5 years of age. Although the sample number at each sampling point is unclear, and there are no data available regarding the sexual maturation status of sampled fish in the previous study; it is speculated that the difference in maturation characteristics between hatchery-produced and captive-reared PBTs is one possible cause of the difference in results between the study by Sawada et al. [8] and this study. Sawada et al. [8] examined cultured PBTs that were reared from fertilized eggs, while this study examined cultured PBTs originally from wild-caught seedlings. According to the study by Higuchi et al. [39], hatchery-produced PBTs seem to have different maturation characteristics from captive-reared PBTs. For instance, all males of hatchery-produced PBTs reach sexual maturation at 3 years of age [39], while the maturation rate of captive-reared male PBTs at 3-4 years of age are relatively low (20–75%) as reported in the study by Seoka et al. [41] and this study. Furthermore, the gonadosomatic indexes (GSI) of male hatchery-produced PBTs were comparable to or higher than those of females [39]. Sexual dimorphism of growth performance was observed at earlier life stages in this study than in previous studies that examined wild stocks, although the data on the body size of PBT at this age were



(a)



(b)

FIGURE 4: Plasma levels of 17β-estradiol (E2) (a) and 11-ketotestosterone (11-KT) (b) in each genotypic sex of the Pacific bluefin tuna in the aromatase inhibitor (AI)-treated and nontreated (control) groups. The genotypic sex of individuals was identified using male-specific DNA markers. Data are presented as the mean ± standard error of the mean ($n = 8$ fish). Different letters indicate significant differences between group ($P < 0.05$, two-way ANOVA followed by Tukey's multiple comparison test).

limited in previous studies [15, 16]. Furthermore, our results showed that there was no significant difference in BW including GW between the sexes at the reproductive season of 4 years old (August 2021), although it tended to be larger in males than in females, suggesting that the effect of the weight of removed organs, including the gill, gut, and gonad, in body size of cultured PBT for each sex at harvest needs to be considered for a deeper understanding of the sexual dimorphic growth of PBT in aquacultural settings. To address those issues and completely characterize the sexual dimorphic growth of cultured PBT, further data on the growth performance of aquaculture-produced PBTs, including both hatchery-produced and captive-reared PBTs, need to be collected for each sex in the future study.

Our results show that male cultured PBTs exhibit higher growth performance than females, suggesting that mono-sex male production may be a prominent tool for improving the productivity of closed-cycle PBT aquaculture by shortening production times and reducing production costs. Here, we show that the expression of *cyp19a1a* increases specifically in the gonads of genotypic females during sex differentiation in PBT. Our results suggest that aromatase plays an important role in ovarian differentiation in PBT, as reported in many other fish species [23, 24], and that the suppression of aromatase activity would be effective in inducing the sex reversal of genotypic females into phenotypic males. Based on this finding, we orally administered letrozole, a third-generation nonsteroidal AI commonly used in marine aquaculture-target species [32, 33], into sexually undifferentiated PBT to induce masculinization in genotypic females. Histological analysis revealed that the gonads of all genotypic females in the AI-treated group showed testicular characteristics. Furthermore, we detected the expression of *dmrt1* (a key determinant of testicular development) and *cyp11b* (encoding a key enzyme in androgen synthesis) in the sex-reversed gonads of genotypic females but not in the gonads of genotypic females in the control group. Moreover, serum levels of 11-KT among genotypic females in the AI-treated group were significantly higher than those among genotypic females in the control group. In contrast, the *cyp19a1a* expression was not detected in the sex-reversed gonads of genotypic females but was detected in the gonads of genotypic females in the control group. Furthermore, serum levels of E2 among genotypic females in the AI-treated group tended to be lower than those among genotypic females and even those among genotypic males in the control group. These results indicate that AI administration suppressed the aromatase-catalyzed synthesis of estrogen, resulting in the sex reversal of genotypic females into phenotypic males at the molecular level in PBT.

It is known that the activation of the endogenous sex steroid production pathway is important for maintaining sex-reversed status in fish [23]. In cases of masculinization, endogenous androgen production is critical for maintaining male sex status after a female-to-male sex change, even after treatment with exogenous sex steroids or AI is terminated [23]. In this study, the high expression levels of *cyp11b* and high serum levels of 11-KT among genotypic females in the AI-treated group were comparable with those of genotypic males in the control group. These results indicate that the endogenous androgen production pathway in genotypically female PBTs in the AI-treated group was activated to a level comparable to that in genotypic males. Oral administration of AI (letrozole) was reported to induce complete masculinization in rice field eel (*Monopterus albus*): the endogenous androgen production pathway of sexually reversed fish was significantly up-regulated compared to that of the nontreated group, the sex-reversed status of AI-treated fish was maintained after cessation of AI treatment, and sexually reversed fish produced functional sperm [43]. Sexual fate maintenance after the termination of AI treatment was also observed in other fish species such as Atlantic halibut (*Hippoglossus hippoglossus*) [44], Senegalese sole (*Solea senegalensis*) [45], blue drum, and yellow drum [33]. Similarly, the reversed sex of genotypically female PBTs may be maintained even when exogenous AI treatment is terminated.

Several methods have been developed for administering sex steroids and AI, including direct injection, direct immersion, and dietary supplementation [26]. However, direct injection is difficult in PBT because it is very difficult to handle this fish, which have extremely delicate skin that can be easily damaged [21]. Furthermore, PBT juveniles at the stage of gonadal sex differentiation (approximately

40–70 dph) [30] are generally reared in offshore sea net cages, which helps avoid death due to collision with the walls of the indoor tank in aquaculture operations [2]. As such, the administration of sex steroids and AI through direct immersion is not practical in PBT. For these reasons, it is desirable to use an oral administration method for the sex manipulation of PBT in practice. However, this approach is known to have certain limitations. The hormones may degrade during storage or as part of the normal digestive process of the fish [10]. Moreover, there may be variability in dosage among individuals due to a nonuniform concentration of the hormone in the feed [10]. Behavioral hierarchies among fish can also influence their feeding rates, leading to differences in the amount of chemicals ingested by the fish [10]. In this study, however, we achieved 100% masculinization of genotypic females via oral administration of AI in both trials. Furthermore, AI treatment did not negatively affect the survival or growth of the fish. Incidentally, other than sex steroid and AI administration, successful masculinization by, for example, temperature treatment during gonadal sex differentiation and cross-species hybridization have been reported [10, 46]. However, it is difficult to perform temperature treatment on PBT juveniles at the gonadal sex differentiation stage since they are generally reared in offshore sea net cages, as discussed above. Furthermore, it is difficult to perform artificial insemination in mature PBT bloodstocks for producing cross-species hybrids, owing to their large body size and very delicate skin, which can be easily damaged [21]. Hence, our results indicate that oral AI administration is the most effective and practical method for inducing masculinization in genotypically female PBT in an aquaculture.

In this study, we showed that oral AI administration is effective for inducing sex reversal of PBT from genotypic females to phenotypic males. However, the use of letrozole on aquaculture fish as food is legally restricted, due to potential health risks. Instead of letrozole, the use of a safer AI is required to establish mono-sex male PBT production systems. Phytochemicals are derived from plants and can potentially be used to inhibit the aromatase enzyme [47]. Previously, chrysin (5, 7-dihydroxyflavone), which is a polyphenolic flavonoid with a high concentration in honey and propolis, was reported to have high aromatase inhibitory potency [47, 48]. It has been shown that chrysin binds to the active site of aromatase as a competitive inhibitor regarding substrate [49], similar to other AIs including letrozole [31]. Because of the acceptable safety [50], it is expected that oral administration of chrysin would be a practical method to produce mono-sex male stocks in PBT, and this should be tested with priority in future research.

In this study, we showed that expression levels of *cyp19a1a* specifically increased in the gonads of the genotypic female during sex differentiation. We also demonstrated that suppression of aromatase activity by AI administration results in the sex reversal of genotypic females into phenotypic males. These results indicate that estrogen synthesis by aromatase is essential for ovarian differentiation in PBT. Furthermore, our results strongly suggest that suppression of estrogen synthesis is an essential prerequisite for testicular differentiation in this species. To date, studies have identified master sex-determining factors in a few fish species [51]. In particular, the role of a sex-determining factor in suppressing estrogen synthesis has been well-studied in rainbow trout (*Oncorhynchus mykiss*) [52, 53], which have a male heterogametic system (XX: female and XY: male) [54] as with PBT [34, 55, 56]. SdY, the sex-determining factor located on the Y chromosome in salmonid fishes, specifically interacts with Foxl2, which is a transcription factor controlling *cyp19a1a* expression [52]. This interaction of SdY with Foxl2 completely prevents estrogen synthesis by blocking a positive regulatory loop that controls *cyp19a1a* expression in differentiating male gonads. By blocking this positive regulatory loop very early in the sex differentiation process, the interaction of SdY with Foxl2 triggers testicular differentiation [52]. Similarly, sex-determining factors located on the Y chromosome may block estrogen synthesis and cause the development of testes in PBT. In fact, a recent study has identified *sult1st6y* (a homolog of the estrogen sulfotransferase gene) as a candidate sex determination gene in PBT [57]. It is generally known that endogenous estrogens are deactivated by sulfation reactions of sulfotransferases [58]. Nakamura et al. [57] suggested that *sult1st6y* may inhibit ovarian differentiation by depleting active estrogens in male PBT gonads at the initiation of sex development, resulting in testicular differentiation. Our results strongly support this promising hypothesis. Further studies to investigate sex determination and subsequent differentiation mechanisms of PBT, particularly the expression pattern and function of *sult1st6y*, are required.

Although the mechanisms underlying sex differentiation have been well studied in various aquaculture target fishes [23, 24], information on this phenomenon in tuna species is scarce, owing to the difficulty in obtaining artificial seedlings that can be used for experiments. To gain a deeper understanding of reproductive biology in tuna species, we have recently studied crucial reproductive aspects of PBT, as a model of tuna species, particularly during the early life stages, based on our closed-cycle aquaculture technique [30, 59, 60]. Among them, we succeeded in revealing the timing and morphological characteristics of gonadal sex differentiation in PBT [30]. The present study provides additional knowledge on sex differentiation mechanisms of PBT, including a female-specific increase of *cyp19a1a* expression, the essential role of aromatase in ovarian differentiation, and up-regulation of 11-KT production in males. The results obtained in PBT could provide valuable data toward understanding the reproductive biology, particularly sex differentiation mechanisms, in tuna species generally.

In conclusion, we observed sexual dimorphism in the body size of cultured PBT at harvest, that is, the FL and BW of males were larger than that of females during the reproductive season at 4 years of age. We also found that aromatase plays a critical role in ovarian differentiation in PBT. Moreover, we demonstrated that sex can be controlled in the PBT through the conventional masculinization method, oral AI administration, which resulted in 100% sex reversal of genotypic females into phenotypic males. This study provides a basis for

the large-scale production of mono-sex male PBT stocks, which would help improve the productivity of closed-cycle aquaculture of this economically important species by shortening production times and reducing production costs. In fact, according to their high market value based on the market value of aquaculture-produced PBT in Japan (approximately, 3,000 yen/kg BW) and the average BW of each sex obtained in this study, it is calculated that mono-sex male PBT production will result in significant increase in aquaculture profits compared with a conventional sex-mixed production system (approximately, 10% increase at the reproductive season of 4 years old). Moreover, the production of mono-sex male stocks may also be effective in avoiding the deterioration of flesh quality in aquaculture-produced PBT since we recently revealed that the reduction of muscle fat content associated with sexual maturation occurs more frequently in females than in males at maturity (Higuchi et al., unpublished data). The authors acknowledge that further studies on completely characterizing the sexually dimorphic growth of cultured PBT and demonstrating the gonadal development and growth performance of AI-treated PBTs until adulthood are needed. In addition, further examination to reveal the survival performance of AI-treated PBTs is important, since the survival rate of AI-treated groups tended to be higher than that of nontreated groups in both trials. If AI-treated PBTs show higher survival performance at early developmental stages, mono-sex male PBT aquaculture will also be meaningful in the context of efficient fingerling PBT production.

Our results can be further applied to the development of techniques to induce sex reversal of genotypic males to phenotypic females. An increase in female ratios in aquaculture populations via artificial sex manipulation also seems to be important in improving the productivity of closed-cycle PBT aquaculture. Namely, female-rich broodstock management allows for large amounts of high-quality fertilized eggs to be obtained. In addition, sex manipulation techniques have been widely used as powerful tools to elucidate sex determination and differentiation mechanisms in fish [23, 24]. Therefore, the masculinization technique established in this study will also be valuable for ongoing investigations focused on sex determination and differentiation in PBT.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical Approval

All experiments were performed in accordance with the Guidelines for the care and use of Live fish of the Fisheries Technology Institute, Japan Fisheries Research and Education Agency, and were approved by the Institutional Animal Care and Use Committee of Fisheries Technology Institute.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Takao Hayashida was involved in conceptualization and investigation and wrote the original draft. Kentaro Higuchi was involved in conceptualization and investigation and wrote, reviewed, and edited the manuscript. Kogen Okita was responsible for investigation. Yukinori Kazeto was involved in conceptualizationand wrote, reviewed, and edited the manuscript. Masaomi Hamasaki was involved in resources. Sota Yoshikawa was involved in resources. Toshinori Takashi was responsible for investigation, and Koichiro Gen was involved in supervision.

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