CHAPTER IV

Validation of XX/XY Sex Determination and All-Female Production Via 17α-Methyltestosterone–Induced Neomales in Snakeskin Gourami (*Trichopodus Pectoralis*)

4.1 Abstract

The snakeskin gourami (*Trichopodus pectoralis*) exhibits pronounced female-biased sexual size dimorphism, making all-female production highly desirable for aquaculture. The use of neomale broodstock represents an indirect strategy for achieving monosex populations while avoiding hormone application during grow-out. This study aimed to evaluate the effectiveness of 17α-methyltestosterone (MT)-induced sex reversal for neomale production, genetic sex identification, and reproductive performance in snakeskin gourami. Sex reversal was induced by feeding fry with an MT-supplemented diet (MT-100) from 7 to 97 days post-hatching (dph), while control fish received a basal diet. Sex-specific SNP markers were applied to distinguish homozygous females (XX), heterozygous males (XY), MT-treated males (XY), and MT-derived neomales (XX). Gonadal development and reproductive performance were assessed using squash methods, histological analysis, progeny testing, and controlled breeding experiments. Although MT-treated male broodstock exhibited reduced reproductive performance compared with control males, they retained the ability to produce functional sperm and achieve successful fertilization. Crosses between control males or MT-treated males and wild-type females produced progeny with an approximately 1:1 sex ratio. In contrast, crosses between MT-derived neomales (XX) and females (XX) consistently produced 100% female offspring. Genotypic analysis of progeny using PACE SNP assays confirmed that neomales transmitted the XX genotype. Collectively, these findings demonstrate that MT-induced neomales function as phenotypic males and provide strong evidence that snakeskin gourami follows an XX/XY sex determination system. This study establishes an integrated framework combining hormonal sex reversal, molecular marker-assisted genotyping, and neomale-

based breeding, providing a practical and sustainable strategy for hormone-free all-female production in female-biased aquaculture species.

Keywords: snakeskin gourami, 17α-methyltestosterone, sex reversal, neomales, all-female production.

4.2 Introduction

Among vertebrates, sexual dimorphism is widely found in teleosts, which occasionally influences traits of commercial significance, such as growth rate, metabolic and physiological parameters, and external morphology (Devlin and Nagahama, 2002; Liu et al., 2024). For example, growth-related sexual dimorphism is observed in Nile tilapia (*Oreochromis niloticus*), zig-zag eel (*Mastacembelus armatus*), and red Tilapia (*Oreochromis spp.*), in which males grow faster than females (Asad et al., 2024; Lu et al., 2024; Yostawonkul et al., 2023). In contrast, mature females are larger than males in species such as the large yellow croaker (*Larimichthys crocea*), rainbow trout (*Oncorhynchus mykiss*), and snakeskin gourami (*Trichopodus pectoralis*) (Kabpha et al., 2023; Mu et al., 2024; Van Der Honing, 2001). In addition, plasma metabolites, hematology, and immune parameters revealed clear sex-specific differences in Nile tilapia, snakeskin gourami, yellowtail kingfish (*Seriola lalandi*), Brown trout (*Salmo trutta fario*), and giant kokopu (*Galaxias argenteus*), which showed distinct physiological profiles between males and females (Phumyu et al., 2012; Kabpha et al., 2023; Lulijwa et al., 2021; Marijani et al., 2017; Sheikh et al., 2022; Valero et al., 2024). Indeed, it was demonstrated that sex-related phenotypic differences in fish are obvious during adulthood, particularly in the maturation stage, suggesting that sex hormones would be mainly involved in sexual dimorphism (Kawajiri et al., 2015). Furthermore, sexually dimorphic gene expression in the gonads has been demonstrated in both juvenile and adult stages demonstrating that several genes are involved in sex determination and differentiation snakeskin gourami, Tongue Sole (*Cynoglossus semilaevis*), loach, half-smooth tongue sole, yellow catfish, and Nile tilapia (Boonanuntanasarn et al., 2020; Jantawongsri et al., 2025; Luo et al., 2022; Wang et al., 2016). Therefore, sexual dimorphic traits and their regulation in fish have been

intensively studied not only for improving aquaculture-related traits but also for advancing fundamental studies on genetic sex determination and physiological mechanisms of sex differentiation (Baroiller and D'Cotta, 2001; Piferrer et al., 1994).

Sex differentiation in fish is highly plastic, being influenced by genetic factors and environmental cues such as exogenous hormones and population density (Guiguen et al., 2010; Heule et al., 2014; Okutsu et al., 2015). Indeed, several oviparous fish offer sex reversal manipulation during the larval stage (at the labile period of gonad development). Particularly, exogenous sex hormone treatment influencing sex reversal can be manipulated during early gonadal development through the administration of exogenous androgens, and estrogen, such as 17α-methyltestosterone (MT), and estrogen, such as 17β-estradiol (E2), respectively, in fish (El-Greisy and El-Gamal, 2012; Liu et al., 2021, 2024; Mu et al., 2024; Teal et al., 2024; Universidade Federal de Santa Catarina et al., 2018). In addition, sex reversed fish exhibited physiological appearances similar to their naturally sexual traits. For instance, in Nile Tilapia, dietary MT treatment can produce a high proportion (~95%) of male populations, and the resulting sex-reversed males exhibit growth performance and physiological characteristics equivalent to those of naturally males (Asad et al., 2024). Similarly, E2 feminization in green sunfish (*Lepomis cyanellus*) and red shiner (*Cyprinella lutrensis*) produced fully functional phenotypic females, which showed similar survival rate, growth, and ovary development to those of control females (Teal et al., 2024, 2023). Moreover, the cryopreserved semen of MT sex-reversed males showed fertilization rates comparable to those of normal males, demonstrating that sex-reversed males in rainbow trout possess equivalent reproductive performance to genetic males (Ninhaus-Silveira et al., 2006). Therefore, sex reversal using exogenous sex hormone treatment has been applied in fish to gain sexual benefits for a monosex population in aquaculture-related fish.

The snakeskin gourami is a commercially important freshwater species native to South Asia. FAO statistics indicate that global production reached approximately 30,500 tonnes in 2021, with Thailand being a leading contributor (Sutthakiet et al., 2024). From an economic perspective, the female snakeskin gourami grows approximately 1.2–1.3 times faster than males under intensive culture (Kabpha et al., 2023; Panthum et al., 2021). This sexual dimorphism in growth highlights the advantage of producing all-female stocks for aquaculture productivity. It was demonstrated that dietary

supplementation of E2 feeding to snakeskin gourami fry could produce an all-female population, which proved highly responsive to hormonal manipulation, and the resulting sex-reversed females exhibit fast growth and physiological characteristics resembling those of control females. (Kabpha et al., 2023). A previous study in our laboratory aimed to evaluate the effects of MT-induced sex reversal on sex ratio, growth performance, several physiological parameters, including blood metabolites, hematological indices, and immune response. We demonstrated that MT-100 was optimum for the production of sex reversed male. Also, MT-induced males and control males exhibited similar gene expression profiles in the testes, whereas both groups showed differentially expressed genes compared with the female ovary. An indirect strategy for producing all-female populations in fish with an XX/XY sex determination system involves the use of neomale broodstock crossed with normal females (Figure 4.1A). This approach has attracted increasing interest as a potential alternative to direct hormonal treatments during grow-out stages. However, the practical application of this strategy requires reliable tools for the identification of genetic sex and the validation of sex-reversed individuals. In our previous study, sex-specific DNA markers were developed, which were able to identify the genotype of homozygous females and heterozygous males, and proposed its XX/XY sex determination system (Chapter III). Therefore, this study aimed to use sex-specific DNA markers for genetic sex identification in snakeskin gourami and to assess the potential of neomale broodstock in monosex breeding programs. By integrating molecular sex identification with controlled breeding strategies, this work seeks to provide a practical and sustainable approach for hormone-free all-female production in fish species with an XX/XY sex determination system.

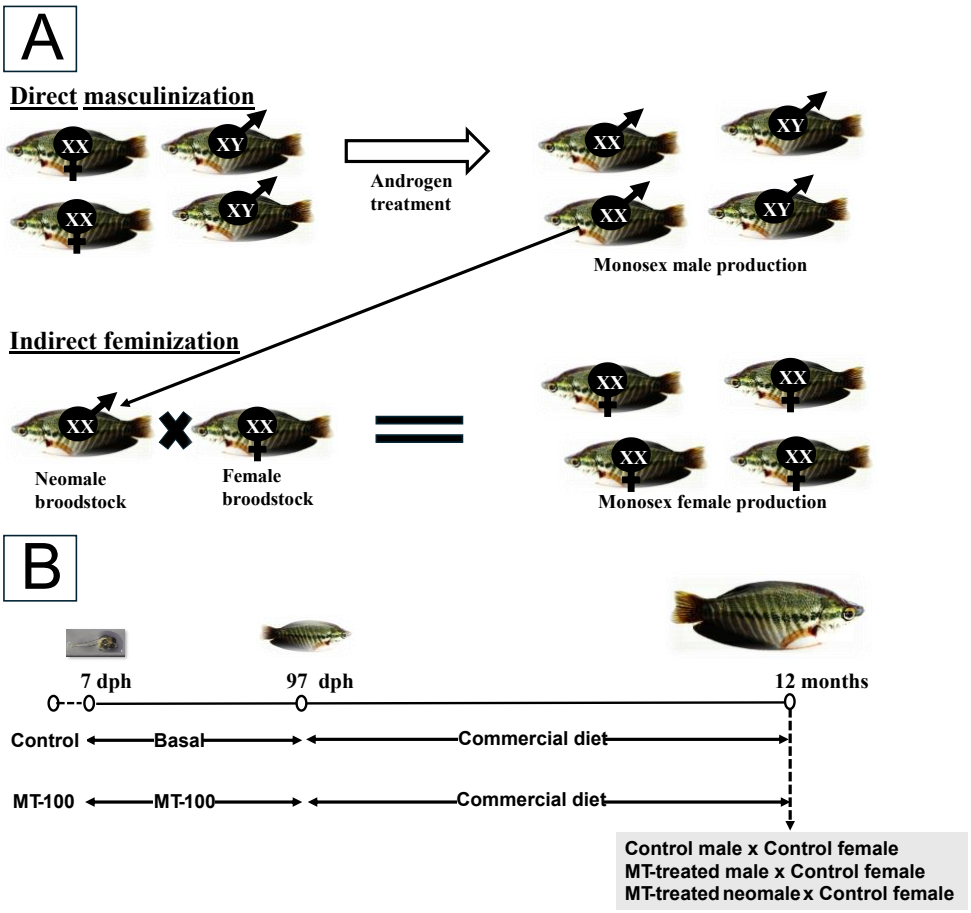

Figure 4.1 Experimental plan for MT-induced neomale production in snakeskin gourami. 7-dph fry were fed a basal diet (control) or MT-100 for 90 days. Subsequently, the fingerlings were cultured through mature stage to become bloodstock. Control male, MT-treated male and MT-treated neomale were mated with control female, and the progeny were cultured for 3 months for sex determination. Using SNP_19, SNP_20 and SNP_21, genotypes of control male, MT-treated male and MT-treated neomale were identified using Sanger sequencing and PACE SNP. Sex determination in progeny was performed using squash method and histological analysis. PACE SNP analysis of SNP_19, SNP_20 and SNP_21 was conducted to identify genotypes of progenies.

4.3 Materials and methods

4.3.1 Ethics statement

All protocols related to the experimental fish in this study were approved by the Ethics Committee of the Suranaree University of Technology Animal Care and Use Committee (approval no. 1731039).

4.3.2 Fish preparation

The snakeskin gourami was obtained from commercial farms in Samut Sakhon and subsequently transported to and maintained at the Suranaree University of Technology (SUT) farm in Nakhon Ratchasima, Thailand. Fish were acclimated before experimentation and reared in a 0.04-hectare earthen pond under ambient photoperiod conditions in freshwater. During the culture period, fish were fed to apparent satiation twice daily (10:00 and 16:00) with a commercial formulated diet (Aquafeed, Thai Union Feedmill, Samut Sakhon, Thailand) containing 40% crude protein and 6% lipid.

4.3.3 Neomale broodstock production

The experimental plan for neomale production in this study is shown in figure 4.1B. Fish breeding (1 male: 1 female) was conducted in an aerated concrete pond (2 m × 2 m × 1 m) containing floating aquatic vegetation to support bubble nest formation. Spawning was induced by hormonal injection. Female broodstock received two intramuscular injections: an initial priming dose of 10 μg kg^{-1} body weight of luteinizing hormone-releasing hormone analog (LHRHa; Suprefact, Hoechst, Germany) combined with 5 mg kg^{-1} domperidone (Motilium-M, OLIC, Bangkok, Thailand), followed 12 h later by a resolving dose of 10 μg kg^{-1} LHRHa and 5 mg kg^{-1} domperidone. Male broodstock received a single intramuscular injection of 10 μg kg^{-1} LHRHa combined with 5 mg kg^{-1} domperidone. Fertilized eggs were observed after 14 hours post-spawning, and the broodstock were subsequently removed. Larvae were reared in the same tanks until 6 dph. The offspring were divided into two ponds for control and MT-reversed fish. The control fry was fed with a free-MT diet while the MT-reversed fish were fed with the MT-100 diet until three months. Subsequently, 200 fish were transferred into hapas (2 × 2 × 2 m), which were set up in an earthen pond and fed with the commercial diet (32% CP and 4 % fat) through the mature stage (12 months).

4.3.4 Progeny testing

Progeny testing was conducted using three mating groups: (i) control males crossed with wild-type (WT) females, (ii) MT-treated males paired with WT females, and (iii) MT-derived neomales crossed with WT females. For the control group, a total of 36 male broodstock were selected for breeding with WT females, hormonal induction using LHRHa and domperidone, as previously described. Among these, 27 males successfully produced offspring. Fin clips were collected from six control males and six control female broodstock for genotyping, and their progeny were reared for subsequent sex determination.

In the MT-100 treatment group, 40 MT-treated males were selected for breeding with WT females using the same hormonal induction protocol. Of these, 13 males successfully produced offspring, and fin samples were collected from all reproductively active individuals for genotyping. A total of seven progeny populations were obtained from MT-treated males and reared for sex determination at three months post-hatching. In addition, six MT-treated males were identified as neomales, and their progeny were also cultured for sex determination.

Fish breeding was conducted in mating tanks (2 m × 2 m × 1 m) with gentle aeration. Fourteen hours after spawning, the broodstock were removed from the tanks to prevent egg and larval predation. Larvae were initially reared in the spawning tanks. Subsequently, larvae (7 dph) were fed a commercial diet containing 40% protein and 8% fat until 30 dph. Thereafter, fingerlings from all experimental groups were transferred to 2 × 2 × 2 m^3 hapas installed within a 0.08-ha earthen pond. Fish were hand-fed a commercial diet containing 35% protein and 4% lipid for a further three months. At 97 dph, individuals were randomly sampled for fin tissue collection for genotyping, and gonads were collected for sex determination using the squash method (Menu et al., 2005) and histological examination. Throughout the rearing period, ambient air temperatures ranged from 22.0–35.0°C, while water temperatures were maintained between 28.5–30.0°C.

4.3.5 Histological examination of the gonads

Sex determination was performed through histological examination of gonadal tissue. Samples were fixed in Bouin's solution at 4°C for 16 hours. Sex ratio was determined in fish at 3 months of age (n = 60 fish per replicate; six replications). Followed by dehydration through a graded ethanol series (70% to 100%). The tissues were then embedded in paraffin, sectioned at 5 µm, and mounted on glass slides. Sections were subsequently dewaxed, rehydrated through xylene and ethanol series, and stained with hematoxylin and eosin (H&E). Gonadal morphology was examined using a digital upright microscope (Olympus BX53, Tokyo, Japan).

4.3.6 Genotypic analysis using sex-specific SNP

Based on a previous study, sex-specific SNP markers (SNP_19, SNP_20, and SNP_21) were developed to distinguish heterozygous males and homozygous females in snakeskin gourami (Chapter III). In the present study, Sanger sequencing and PACE SNP assays were employed to determine the genotypic sex of control males, MT-treated males, MT-derived neomale broodstock, and their offspring. Genomic DNA (gDNA) was extracted from fin tissue (approximately 100 mg) using the standard phenol-chloroform method. Briefly, fin samples were minced and incubated in lysis buffer containing proteinase K at 55°C until complete tissue digestion was achieved. Following lysis, an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1, v/v/v) was added, and the mixture was gently vortexed and centrifuged to separate the aqueous and organic phases. The upper aqueous phase containing DNA was carefully transferred to a new tube and further extracted with chloroform to remove residual phenol. gDNA was subsequently precipitated by the addition of cold absolute ethanol in the presence of sodium acetate, followed by centrifugation. The resulting DNA pellet was washed with 70% ethanol, air-dried, and resuspended in TE buffer. DNA concentration and purity were assessed using a NanoDrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) before downstream applications. Primer sequences, annealing temperatures, and expected amplicon sizes are presented in (Table 4.1). PCR amplification was carried out using TaKaRa Ex Taq polymerase (TaKaRa Bio, Shiga, Japan) in a total reaction volume of 25 µL, consisting 2.5 µL 10 × Ex Taq Buffer, 2.0 µL dNTP Mixture (2.5 mM each), 0.125 µL Ex Taq polymerase (5 U μL^{-1}), 2.5 µL each of forward and reverse primers (2 µM), 2.5 µL gDNA template (50ng μL^{-1}), and 12.875 µL nuclease-

free water. PCR was performed under the following cycling conditions: initial denaturation at 95°C for 3 min; 35 cycles of 95°C for 30 s, annealing at the primer-specific optimal temperature (55–65°C) for 30 s, and extension at 72°C for 25 s; followed by a final extension at 72°C for 10 min. The target PCR products were excised from agarose gels and purified using the QIAEX® II Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Purified products were subjected to Sanger sequencing to identify nucleotide variation at the target SNP locus.

PACE SNP assays (PCR Allele Competitive Extension) were employed as a high-throughput genotyping approach, in which allele-specific fluorescent probes competitively extend during PCR, enabling rapid and accurate genotype discrimination based on fluorescence signal patterns. SNP genotyping was performed using the PACE® 2.0 Genotyping Master Mix (3CR Bioscience, Harlow, UK) according to the manufacturer's instructions. Each reaction in a total volume of 5 µL, containing 2.5 µL of 2× PACE 2.0 Genotyping Master Mix, 0.1 µL of custom-designed Assay Mix (comprising two allele-specific forward primers at 12 µM each and one common reverse primer at 30 µM), and 20 ng of gDNA template. PCR amplification was performed under the following cycling conditions: initial enzyme activation at 94°C for 15 min; followed by 10 cycles of 94°C for 20 s and 65–57°C for 60 s with a touchdown of 0.8°C per cycle; followed by 30 cycles of 94°C for 20s and 57°C for 60s. Following amplification, endpoint fluorescence signals of FAM and HEX were measured at below 40°C using a LightCycler® 480 real-time PCR platform (Roche, Germany). Genotypes were assigned based on relative FAM and HEX fluorescence intensities, and allelic discrimination cluster plots were generated for genotype calling. DNA-free reactions were included as negative controls, which exhibited no detectable fluorescence, confirming the absence of cross-contamination.

4.3.7 Data analysis

All statistical analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). Chi-square (χ^2) tests were used to assess deviations from the expected 1:1 sex ratio in MT-treated and control groups and to compare sex ratios among progeny derived from control males, MT-treated males, and MT-treated neomales. A P-value < 0.05 was considered statistically significant.

Table 4.1 Sequences of primers used for Sanger sequencing and PACE genotyping.

Name	Primers (5′-3′)	Tm (°C)	Products (bp)	Application
SNP_19, 20	F: AATACGACTCACTATAGGGCCAGGGCTGTAACTCTGGACT R: GCACCTTGATCAAAGCACTCTCCA	63	551	Sanger sequencing
SNP_21	F: GAGACAGCTCTAGGGGCTCT R: TGCTCCTGTTTGGCCAGAAG	60	518	Sanger sequencing
SNP_19, 20 (PACE)	CRP:CTACTAATGCATTAAATGTCCTAAGCTCCC ASP-GC:GAAGGTGACCAAGTTCATGCTAATATTGAGCATTTACAATACTGGACATGC ASP-TT:GAAGGTCGGAGTCAACGGATTAATATTGAGCATTTACAATACTGGACATTT	60	100	PACE genotyping
SNP_21 (PACE)	CRP:ATAGTGTGGAGCTGGCCACCATTAT ASP-G:GAAGGTGACCAAGTTCATGCTTATAATGACTCACCACTCCATGAAAC ASP-A:GAAGGTCGGAGTCAACGGATTTTATAATGACTCACCACTCCATGAAAT	60	195 (ASP-G) 196 (ASP-A)	PACE genotyping

Abbreviation: Tm (°C) =Annealing temperature (°C); bp= base pair; ASP=Allele-specific forward primers; CRP=Common reverse primer; PACE=PCR Allele Competitive Extension.

Note: The underlined sequence AATACGACTCACTATAGGG represents the T7 universal sequencing tag added to the 5′end of primers. The universal FAM (green) and HEX (orange) fluorescent tails were appended to the 5′ends of allele-specific primers for PACE genotyping.

4.4 Results

4.4.1 Genotypic identification of control males, MT-treated males, MT-derived neomales, and females broodstock by Sanger sequencing

Table 4.2 shows that among 36 selected mature control males, 27 individuals successfully produced offspring, corresponding to a mating success rate of 75.0%. In contrast, only 13 of 40 MT-treated males (32.5%) were able to produce offspring. For progeny testing, six offspring from each mating group, including control males, MT-treated males, and MT-treated neomales, were selected and reared for 90 days for subsequent sex ratio determination. The genotypes of all broodstock, including control males, MT-treated males, and MT-derived neomales and females, were determined using PCR amplification followed by Sanger sequencing. These results were consistent with previous findings indicating that snakeskin gourami possesses an XX/XY sex determination system (Chapter III). Genotyping results showed that control males and MT-treated males were heterozygous at the SNP_19 (G/T), SNP_20 (C/T), and SNP_21 (G/A; complementary T/C detected in antisense sequencing), whereas MT-derived neomales and females were homozygous at the SNP_19 (G/G), SNP_20 (C/C), and SNP_21 (G/G) loci (Figure 4.2, 4.3, and 4.4).

Table 4.2 Number of control male and MT-treated male broodstock that were used for progeny testing.

Parameters	Control group		MT-100 group	
	Males	females	males	Control females
No. of broodstock	36	36	40	40
No. of pairs with hatching	27	27	13	13
No. of pairs with no hatching	9	9	27	27
Mating success (%)	75.0	75.0	32.5	32.5

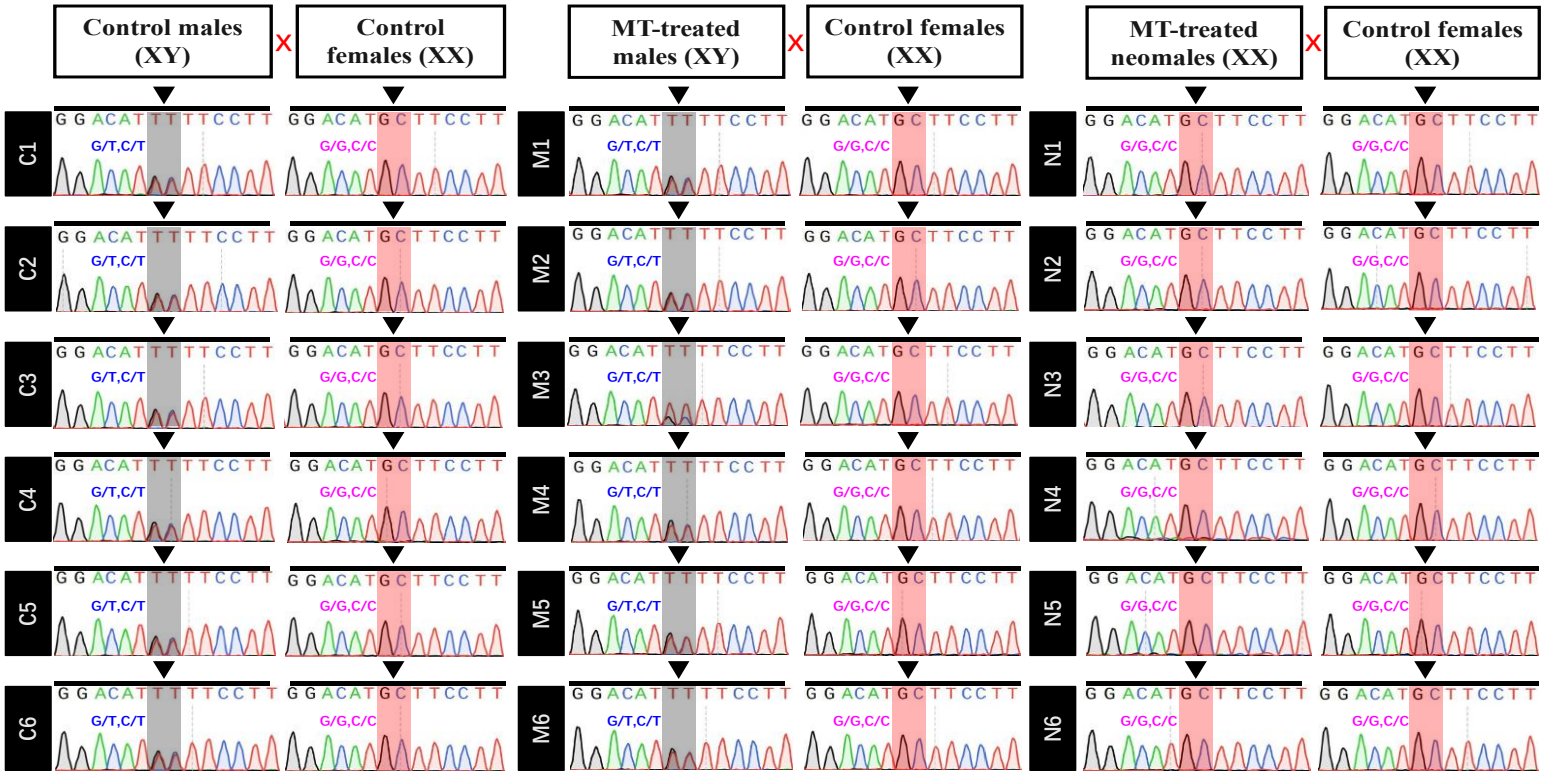

Figure 4.2 Identification of sexual genotypes at SNP_19 and SNP_20 in broodstock using Sanger sequencing. Representative chromatograms from control males × control females (C1–C6), MT-treated males × control females (M1–M6), and MT-treated neomales × control females (N1–N6) showed that control males and MT-treated males were consistently heterozygous at SNP_19 (G/T) and SNP_20 (C/T), whereas control females and MT-treated neomales were homozygous at both loci (G/G and C/C). Abbreviations: C = control group; M = MT-treated male; N = MT-treated neomale.

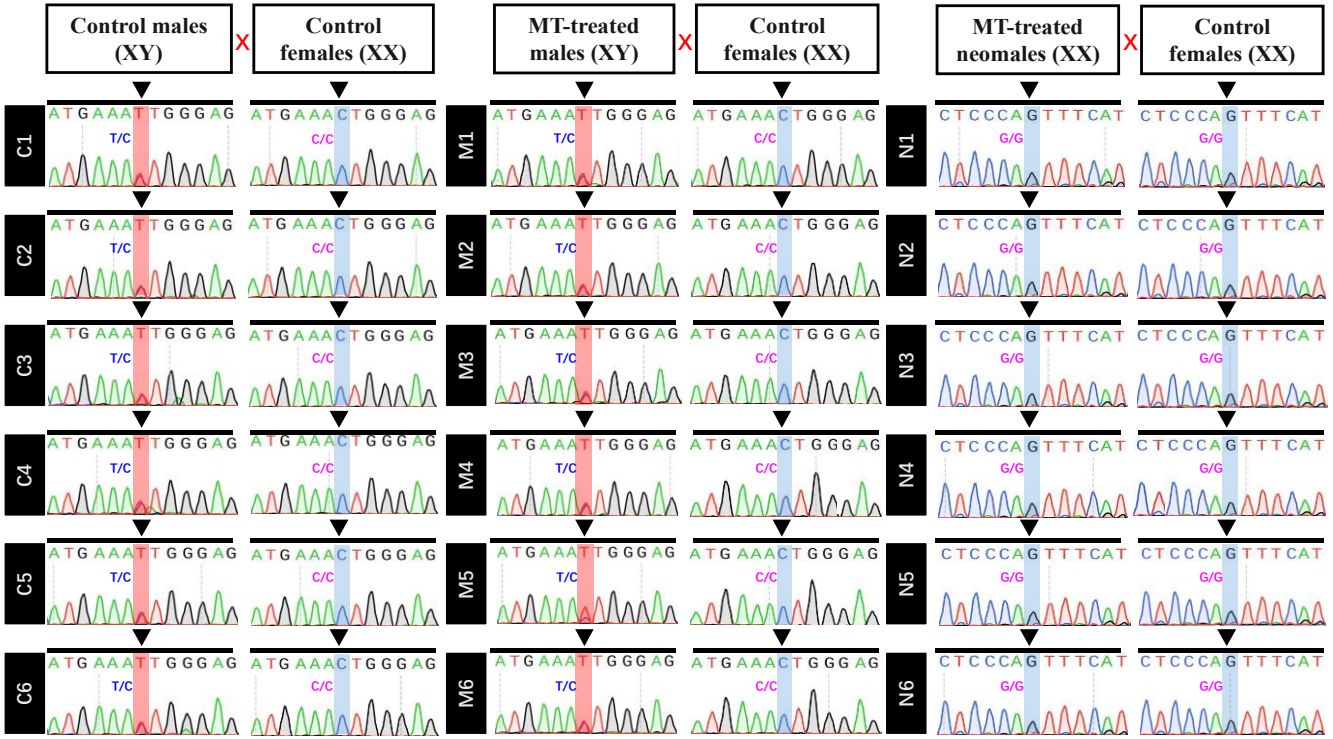

Figure 4.3 Identification of sexual genotypes at SNP_21 in broodstock using Sanger sequencing. Representative chromatograms from control males × control females (C1–C6), MT-treated males × control females (M1–M6), and MT-treated neomales × control females (N1–N6) showed that males were consistently heterozygous at SNP_21 (T/C, complementary to G/A), whereas females and neomales were homozygous (C/C, complementary to G/G). Abbreviations: C = control group; M = MT-treated male; N = MT-treated neomale.

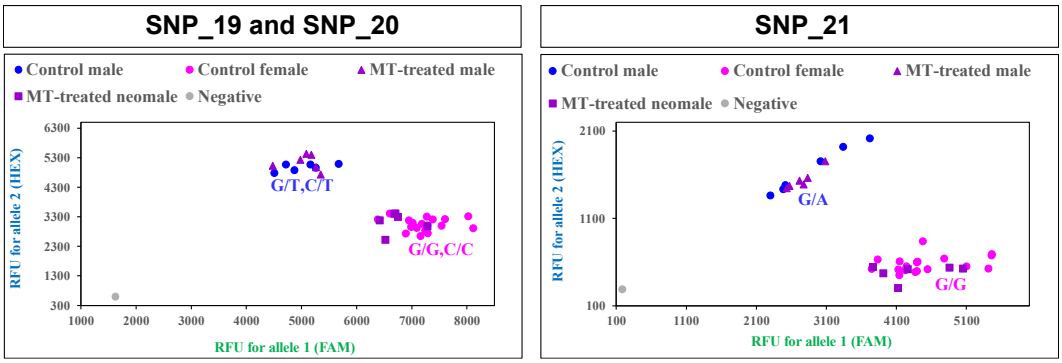

Figure 4.4 Genotypic validation of sex-specific SNPs (SNP_19, SNP_20, and SNP_21) in broodstock using PACE assays. Scatter plots illustrate relative fluorescence unit (RFU) signal intensities for allele 1 (FAM, x-axis) and allele 2 (HEX, y-axis). Each point represents an individual fish (pink = female; blue = male; purple = MT-treated male and MT-treated neomale; gray = negative control). SNP_19, SNP_20, and SNP_21 showed homozygous females and heterozygous males.

4.4.2 Genotypic identification of control males, MT-treated males, MT-treated neomales, and females using PACE genotyping

Consistent with the genotyping results obtained by Sanger sequencing, broodstock genotypes (control males, MT-males, MT-neomales, and females) were further validated using PACE SNP assays. The PACE genotyping results clearly discriminated genotypic classes among broodstock groups. Specifically, control males and MT-treated males consistently exhibited heterozygous genotypes. In contrast, MT-treated neomales and females displayed homozygous genotypes at the SNP_19 (G/G), SNP_20 (C/C), and SNP_21 (G/G) loci (Figure 4.4). No discordance was observed between genotypes determined by PACE SNP assays and those obtained by Sanger sequencing, confirming the robustness and reliability of the PACE-based genotyping approach for genetic sex identification in snakeskin gourami.

4.4.3 Progeny testing of control males, MT-treated males, and MT-derived neomales using the squash method and histological analysis

Using the squash method, the gonadal sex of progeny was preliminarily assessed based on cellular morphology. As shown in (Figure 4.5A and B), ovarian and

testicular tissues were clearly identified in female and male progeny, respectively. To validate the accuracy of the squash method, histological analysis was subsequently performed on corresponding samples, confirming ovarian structures in female progeny and testicular structures in male progeny. In female progeny, squash preparations revealed numerous rounded oocytes with distinct cellular boundaries (Figure 4.5A), which closely corresponded to the ovarian morphology observed in histological sections. Histological examination showed ovaries containing oogonia (OG) and previtellogenic oocytes (PVO), indicative of immature ovarian development (Figure 4.5 D, F, G, and H). In male progeny, the squash method displayed dense aggregates of spermatogenic cells, predominantly spermatocytes (Figure 4.5B). Consistently, histological sections revealed well-organized lobular testes containing spermatogonia (SG), primary spermatocytes (PSC), secondary spermatocytes (SSC), and a small proportion of spermatids (ST) distributed throughout the testicular tissue (Figure 4.5C and E). Similar gonadal characteristics were observed in progeny derived from control males, MT-treated males, and MT-derived neomales, all of which exhibited representative features of immature ovaries or testes at the juvenile stage (Figure 4.5C–H). The strong concordance between squash preparations and histological observations demonstrates that, by 3 months post-hatching, gonadal sex in snakeskin gourami progeny can be reliably distinguished based on distinct gonadal morphology. These results confirm the accuracy and practicality of the squash method as a rapid and efficient tool for early sex determination and large-scale progeny screening.

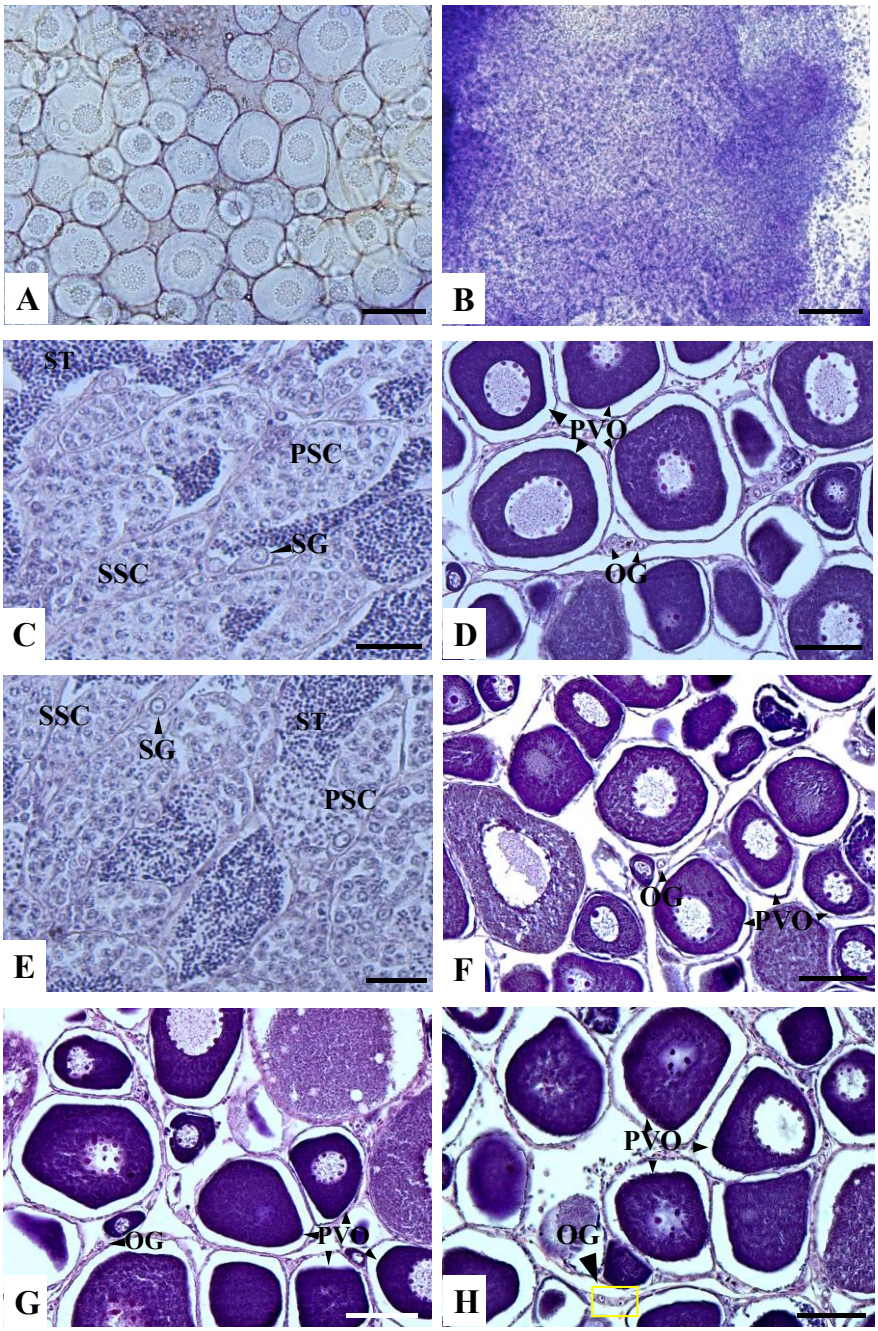

Figure 4.5 Histological and squash-based characterization of gonadal development in progeny derived from neomale experiments in snakeskin gourami. (A) ovary of fingerlings at three months determination using squash method. (B) testis of fingerlings at three months of determination using the squash method. (C) testis of fingerlings at three months of determination using histological analysis in the control group offspring. (D) ovary of fingerlings at three months using histological analysis in the control group offspring. (E) testis

of fingerlings at three months of determination using histological analysis in MT-treated male offspring. (F) ovary of fingerlings at three months of determination using histological analysis in MT-treated male offspring. (G) ovary of fingerlings at three months of determination using histological analysis in MT-treated neomale offspring. (H) ovary of fingerlings at three months of determination using histological analysis in MT-treated neomale offspring. The observed ovarian cell types included oogonia (OG) and previtellogenic oocytes (PVO). The observed testicular cell types include spermatogonia (SG), primary spermatocytes (PSC), secondary spermatocytes (SSC), and spermatids (ST). Scale bars represent 20 μm (C, E), 50 μm (A, D, F, G, H), and 100 μm (B).

4.4.4 Progeny testing of control males, MT-treated males, and MT-derived neomales using PACE genotyping

In addition, progeny was further subjected to PACE SNP assays to determine their genotypic sex. PACE genotyping results showed that progeny derived from control males and MT-treated males comprised both heterozygous male at the SNP_19 (G/T), SNP_20 (C/T), and SNP_21 (G/A) loci (Figure 4.6) and homozygous female at the SNP_19 (G/G), SNP_20 (C/C), and SNP_21(G/G) loci (Figure 4.6), whereas all progeny produced by MT-derived neomales exhibited a homozygous at the SNP_19 (G/G), SNP_20 (C/C), and SNP_21(G/G) loci (Figure 4.6). The genotypic results obtained from PACE assays were fully consistent with the phenotypic sex assignments determined by histological examination and squash method, and a summary of sex determination outcomes obtained using these methods is presented in (Table 4.3). In the control group, progeny derived from XY males crossed with XX females displayed a sex ratio close to 1:1, with female and male proportions of 50.83 ± 4.05% and 49.17 ± 4.05%, respectively. Statistical analysis revealed no significant deviation from the expected Mendelian ratio (χ^2 test, $P > 0.05$). Similarly, progeny from MT-treated XY males also exhibited an approximately equal sex ratio, with 49.45 ± 2.02% females and 50.56 ± 2.02% males, and no significant deviation from the 1:1 expectation was detected (χ^2 test, $P > 0.05$). In contrast, progeny produced by MT-derived neomales (XX genotype) crossed with XX females exhibited a strongly female-biased sex ratio,

with 100% female offspring observed. This sex ratio showed a highly significant deviation from the expected 1:1 ratio (χ^2 test, $P < 0.001$). Collectively, these results demonstrate that MT-derived neomales function as phenotypic males capable of successful fertilization, while transmitting a female (XX) genotype to all offspring, thereby enabling the production of an all-female progeny population.

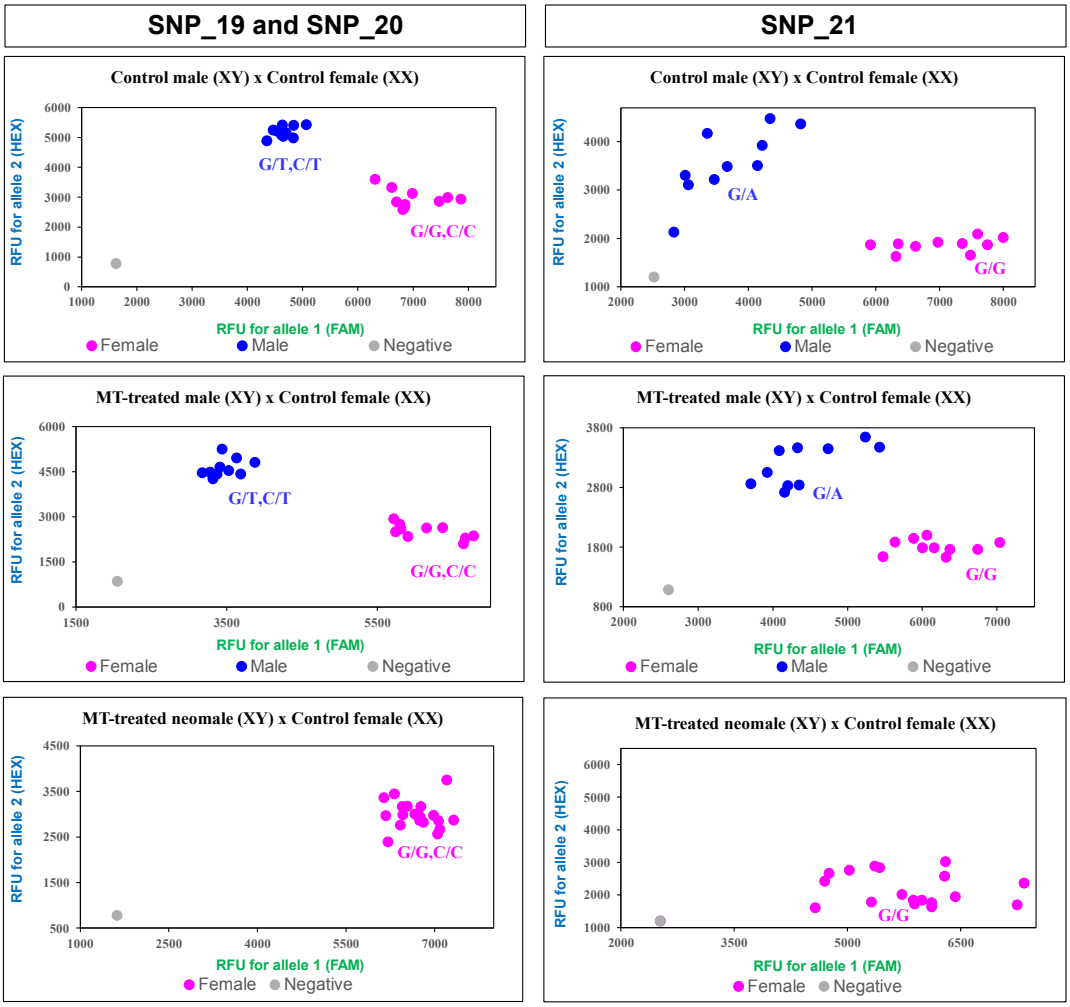

Figure 4.6 Segregation analysis of sex-specific SNPs (SNP_19, SNP_20, and SNP_21) in progeny using PACE assays. Scatter plots illustrate RFU signal intensities for allele 1 (FAM, x-axis) and allele 2 (HEX, y-axis). Each point represents an individual fish. Genotype distributions in progeny were consistent with the parental genotype patterns.

Table 4.3 Sex ratio and zootechnical characteristics of progeny produced from control females crossed with control males, MT-treated males, and MT-treated neomales.

	Body weight (g)	Total length (cm)	No. of males	No. of females	Male (%)	Female (%)	χ^2 *P*-value
Control male[1]							
C 1	7.13 ± 3.72	7.77 ± 1.24	29	31	48.33	51.67	0.7384
C 2	8.48 ± 5.12	8.15 ± 1.76	31	29	51.67	48.33	0.7384
C 3	10.53 ± 4.65	8.72 ± 1.08	33	27	55.00	45.00	0.3173
C 4	10.38 ± 5.30	9.03 ± 1.18	28	32	46.67	53.33	0.5054
C 5	10.28 ± 4.83	8.79 ± 1.47	26	34	43.33	56.67	0.1822
C 6	8.24 ± 9.87	7.58 ± 1.89	30	30	50.00	50.00	1.0000
Mean ± SD					*49.17 ± 4.05*	*50.83 ± 4.05*	
MT-treated male[1]							
M 1	4.74 ± 3.91	6.4 ± 1.62	32	28	53.33	46.67	0.5054
M 2	12.83 ± 5.32	9.34 ± 1.36	30	30	50.00	50.00	1.0000
M 3	7.75 ± 4.13	7.71 ± 1.25	31	29	51.67	48.33	0.7384
M 4	9.27 ± 4.34	8.08 ± 1.55	31	29	51.67	48.33	0.7384
M 5	10.22 ± 4.24	8.78 ± 1.12	29	31	48.33	51.67	0.7384
M 6	5.75 ± 3.15	7.10 ± 1.41	29	31	48.33	51.67	0.7384
Mean ± SD					*50.56 ± 2.02*	*49.45 ± 2.02*	
MT-treated neomale[1]							
N 1	11.00 ± 6.61	8.83 ± 1.54	0	60	0.00	100.00	<0.0000
N 2	13.27 ± 6.86	9.27 ± 1.72	0	60	0.00	100.00	<0.0000
N 3	13.18 ± 8.65	9.32 ± 1.76	0	60	0.00	100.00	<0.0000
N 4	10.94 ± 6.61	8.81 ± 1.54	0	60	0.00	100.00	<0.0000
N 5	12.97 ± 7.22	9.21 ± 1.80	0	60	0.00	100.00	<0.0000
N 6	13.17 ± 9.94	9.25 ± 1.97	0	60	0.00	100.00	<0.0000
Mean ± SD					*0.00 ± 0.00*	*100.00 ± 0.00*	

Abbreviations: C = control male; M = MT-treated male; N = MT-treated neomale. Genotypes of broodstock were determined using SNP_19, SNP_20, and SNP_21 (Figures 4.2–4.4). Gonadal sex of progeny was determined by squash method and histological analysis (Figure 4.5). Progeny genotypes were confirmed using PACE assays (Figure 4.6). Sex ratio deviations from the expected 1:1 ratio were analyzed using χ^2 tests. Values are presented as mean ± SD. All neomale-derived progeny showed significant deviation from 1:1 ($P < 0.001$).

4.5 Discussion

In the present study, sex-specific DNA markers enabled accurate identification of neomale genotypes, providing a reliable molecular tool for selective breeding. MT-derived neomales were successfully applied to generate an all-female progeny population in snakeskin gourami. Consistent with previous studies, indirect crosses between neomales and wild-type (WT) female broodstock have been widely used to produce all-female populations in teleost fishes, underscoring the effectiveness and broad applicability of neomale-based monosex breeding approaches (Luckenbach et al., 2017; Mu et al., 2024; Zhai et al., 2022). Notably, crosses involving MT-derived neomales consistently yielded exclusively female offspring, further validating the effectiveness of this neomale-based breeding strategy. These results provide direct evidence that MT-derived neomales function as phenotypic males, despite possessing a female (XX) genotype. Notably, MT-derived neomales were capable of normal spermatogenesis and successful fertilization, indicating that their reproductive performance is comparable to that of natural males. The production of exclusively female progeny further confirms the genetic female identity of neomales and demonstrates their effectiveness as functional broodstock for indirect, hormone-free monosex production.

4.5.1 MT-induced masculinization and molecular identification of neomales in snakeskin gourami

In teleost fishes, monosex populations have been widely produced through hormonal sex reversal, typically by administering sex hormones to sexually undifferentiated fry via the diet (Asad et al., 2024; El-Greisy and El-Gamal, 2012; Galbreath et al., 2003; Universidade Federal de Santa Catarina et al., 2018). Accordingly, the present study induced masculinization by feeding sexually undifferentiated fry a diet supplemented with the optimum dose of MT-100 diet. We produced MT-treated male fry and cultured them through the mature stage, providing MT-treated male broodstock. Note that control broodstock was also produced by feeding fry (7-97 dph) with a basal diet, which included both male and female broodstock. The resulting MT-treated fish exhibited male-like biological characteristics that persisted into adulthood, indicating effective and stable masculinization. Previous studies have reported that all-female production in snakeskin gourami can be achieved by hormonal treatment

during the early developmental period of 7–97 dph, and that estrogen-treated fish display growth performance comparable to genetic females, which show superior growth relative to males at adulthood (Kabpha et al., 2023). Building on these findings, the present study successfully produced a monosex male population using an optimized MT dosage, providing suitable broodstock for subsequent neomale-based breeding.

In our previous work, sex-specific SNP markers were developed to distinguish homozygous females from heterozygous males, with females exhibiting SNP_19 (G/G), SNP_20 (C/C), and SNP_21 (G/G) genotypes and males displaying SNP_19 (G/T), SNP_20 (C/T), and SNP_21 (G/A) genotypes (Chapter III). Using these markers in combination with Sanger sequencing and PACE SNP assays, the present study reliably identified the genotypic sex of broodstock, including females, control males, MT-treated males (XY), and MT-derived neomales (XX). Genotyping results demonstrated that MT-derived neomales shared identical genotypes with natural females, whereas both control males and MT-treated males exhibited heterozygous genotypes across all three loci. Consistent with reports in other fish species, such as rainbow trout and common carp (*Cyprinus carpio*), the application of molecular sex markers to identify neomales provides a powerful strategy for producing all-female populations through indirect breeding approaches (Colihueque et al., 2024; Xie et al., 2025).

4.5.2 Neomale-based all-female production and reproductive performance of MT-treated broodstock

Notably, MT-derived neomales were capable of generating exclusively female progeny, providing an effective indirect strategy for all-female production in snakeskin gourami. Accordingly, the present study employed a neomale-based breeding approach to achieve all-female progeny without the direct application of sex hormones during the grow-out phase. In fish species with an XX/XY sex determination system, hormonally induced neomale broodstock have been widely applied to generate all-female populations, thereby offering a practical means to eliminate the need for hormonal sex reversal in commercial production (Mu et al., 2024). The use of neomales as functional male broodstock for all-female production has also been successfully demonstrated in other species, including large yellow croaker and sablefish, highlighting the broader applicability of this strategy (Luckenbach et al., 2017;

Mu et al., 2024). Collectively, these findings establish a biotechnological framework for sustainable, hormone-free monosex aquaculture, with significant potential for future large-scale application.

According to previous reports, MT-treated broodstock in XX/XY sex determination systems may comprise both genetic males (XY) and sex-reversed neomales (Liu et al., 2021). In our study, progeny testing was performed to confirm male and neomale broodstock. Our results showed that 75% of the control males could produce offspring, while only 32.5% of the MT-treated males were able to produce offspring. These findings demonstrated that MT-treated males faced a reduction in reproductive performance. Therefore, further research and development are required to improve the reproductive performance of MT-treated male broodstock.

4.5.3 Progeny testing confirms the XX/XY sex determination system and the effectiveness of neomale-based all-female production

For progeny testing, histology of gonadal tissue and PACE SNP genotyping were applied to identify both phenotypic sex and genotypic sex, respectively. Our results showed that control male (XY × XX) mating and MT-treated male (XY × XX) mating produced the expected Mendelian 1:1 sex ratio, reaffirming the XX/XY sex determination system in snakeskin gourami. Expectedly, offspring derived from neomales (XX × XX) mating were uniformly female. The successful generation of all-female progeny through crosses between XX neomales and XX females confirms that MT-induced masculinization effectively produced functional male gametes, while maintaining a genetically female (XX) genotype in the offspring. These findings are consistent with previous reports in other teleost species, such as yellow croaker, common carp (Cyprinus carpio), and yellow drum (Nibea albiflora), in which neomale-based breeding strategies have also been successfully applied to generate all-female populations (Mu et al., 2024; Xie et al., Xu et al., 2018; 2026; Zhai et al., 2022).

Therefore, a combination of sex-specific DNA markers and MT-sex reversed male production could offer the effectiveness of neomale production technology in snakeskin gourami. This biotechnological approach provides a practical, hormone-free strategy for producing all-female populations. Future research is required to develop this technology at a farm scale for the commercialization of all-female snakeskin gourami farming.

4.6 Conclusion

In the present study, MT-treated male broodstock retained the ability to produce functional male gametes and successfully fertilize eggs, despite exhibiting reduced reproductive performance. Using sex-specific SNP markers, MT-derived neomales carrying a genetically female (XX) genotype were accurately identified and distinguished from genetic males. Subsequent crosses between XX neomales and XX females consistently produced all-female progeny, confirming both the functional male phenotype of neomales and the genetic basis of all-female inheritance. Compared with direct hormone application during early development, the neomale-based approach enables hormone-free production of all-female offspring, offering clear advantages in terms of food safety, regulatory acceptance, and sustainability. Overall, this study establishes an integrated biotechnological framework that combines hormonal sex reversal, molecular marker-assisted genotyping, and controlled breeding. Importantly, the consistent genotypic patterns and inheritance outcomes observed in progeny testing collectively provide strong evidence that snakeskin gourami possesses an XX/XY sex determination system, thereby supporting the practical application of neomale-based strategies for all-female production in this and other commercially important teleost species.

4.7 Acknowledgements

This work was supported by (i) Suranaree University of Technology (SUT), (ii) Thailand Science Research and Innovation (TSRI), and (iii) National Science, Research and Innovation Fund (NSRF) (FF3-303-68-12-11(F)). The authors also express their gratitude for the funding support from the Agricultural Research Development Agency (Public Organisation) (PRP6705031040).

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