CHAPTER V

Evidence for an XX/XY Sex Determination System in Snakeskin Gourami (*Trichopodus Pectoralis*) Revealed by 17β-Estradiol-Induced Neofemale

5.1 Abstract

In snakeskin gourami (*Trichopodus pectoralis*), females exhibit superior growth performance compared with males, highlighting the economic potential of all-female stocks in aquaculture. However, despite previous indications, additional genetic and functional evidence is still required to conclusively define the underlying sex determination system in this species. This study aimed to functionally validate neofemale- and supermale-based breeding strategies in snakeskin gourami through integrated progeny testing and sex-specific DNA marker analysis. in snakeskin gourami through integrated progeny testing and sex-specific DNA marker analysis. Neofemales were produced by dietary administration of 17β-estradiol (E2), and their genetic sex was confirmed using sex-linked SNP markers (SNP_19, SNP_20, and SNP_21). Progeny derived from control females, E2-treated females, and E2-treated neofemales were evaluated using a combination of squash methods, histological examination, and sex ratio analysis. Control and E2-treated females produced offspring with balanced 1:1 sex ratios, whereas crosses involving neofemales (XY genotype) resulted in a male-biased progeny ratio consistent with Mendelian expectations under an XX/XY system, confirming that neofemales function as fertile phenotypic females while retaining a male genetic constitution. Offspring from neofemales were further reared to identify supermales, which were confirmed by SNP genotyping and progeny testing. Crosses between supermales and wild-type females produced exclusively male offspring, demonstrating that supermales are fully fertile and transmit Y-linked sex-determining factors to all progenies. Notably, gonadal sex identified by the squash method showed complete concordance with histological observations at three months post-hatching, indicating that this approach provides a rapid, low-cost, and reliable method for large-

scale progeny sex identification. Collectively, the integration of molecular markers, progeny testing, and efficient phenotypic screening offers a practical framework for controlled monosex production and provides robust functional evidence supporting an XX/XY sex determination system in snakeskin gourami.

Keywords: Snakeskin gourami (*Trichopodus pectoralis*); Sex determination; Neofemale and supermale; Sex-specific SNP markers; Monosex production.

5.2 Introduction

Sex determination mechanisms in teleosts are remarkably diverse, ranging from classical male-heterogametic (XY) and female-heterogametic (ZW) systems to more complex arrangements such as X_1X_2Y, ZW_1W_2, and X0/XX (Fang et al., 2025). The identification of these systems has relied on a variety of cytogenetic, molecular, or genomic approaches, including karyotyping, fluorescent in situ hybridization (FISH), microsatellite-based linkage mapping, high-density SNP genotyping, and high-throughput sequencing technologies (e.g., RAD-seq, GWAS, and whole-genome assembly) (Sember et al., 2021; Whyte et al., 2007; Woram et al., 2003; Yang et al., 2024). Sex determination and sex control strategies represent critical components in modern aquaculture, particularly for species exhibiting pronounced sexual dimorphism in growth performance, physiology, or economic value, for instance, snakeskin gourami, half-smooth tongue sole (*Cynoglossus semilaevis*), largemouth bass (*Micropterus salmoides*), mandarin fish (*Siniperca chuatsi*), and sablefish (*Anoplopoma fimbria*), females generally grow faster, making them the favored sex for commercial culture (Bi et al., 2020; Kabpha et al., 2023; Luckenbach et al., 2017; Zhou and Chen, 2016). In contrast, species such as Northern snakehead (*Channa argus*), zig-zag eel (*Mastacembelus armatus*), and Nile tilapia (*Oreochromis* spp.) exhibit male-biased growth, which renders males more desirable for aquaculture purposes (Asad et al., 2024; Lu et al., 2024; Wang et al., 2019). In teleost fish, genetic sex determination systems are highly diverse and frequently interact with environmental or hormone factors, resulting in remarkable plasticity during early gonadal development (Devlin and Nagahama, 2002; Baroiller and D'Cotta, 2001). This plasticity has enabled the widespread application of hormonal sex reversal

techniques to manipulate phenotypic sex for monosex production, thereby improving production efficiency and stock uniformity in aquaculture systems (Pandian and Sheela, 1995; Piferrer, 2001).

In species with an XX/XY sex determination system, Direct monosex production using hormones has been successfully applied in species such as silver barb (*Puntius gonionotus* Bleeker), Medaka (*Oryzias latipes*), and black rockfish (*Sebastes schlegelii*) (Gaxheri et al., 2025; Scholz et al., 2003; Pongthana et al., 1999;). In particular, indirect monosex production based on sex-reversed broodstock of neomales or neofemales-derived supermales allow monosex populations to be generated without direct hormone exposure during the grow-out phase (Figure 5.1 A), like large yellow croaker (*Larimichthys crocea*), Common Carp (*Cyprinus carpio* L.), and mandarin fish (*Siniperca chuatsi*) (Liu et al., 2024; Mu, et al., 2024; Zhai et al., 2022). This approach addresses both regulatory and consumer concerns regarding hormone use in aquaculture. regarding hormone use in aquaculture (Penman and McAndrew, 2000; Devlin and Nagahama, 2002). However, the successful implementation of such strategies critically depends on two prerequisites: (i) confirmation that sex-reversed individuals are reproductively functional, and (ii) reliable identification of their underlying genetic sex. [Grab your reader's attention with a great quote from the document or use this space to emphasize a key point. To place this text box anywhere on the page, just drag it.]

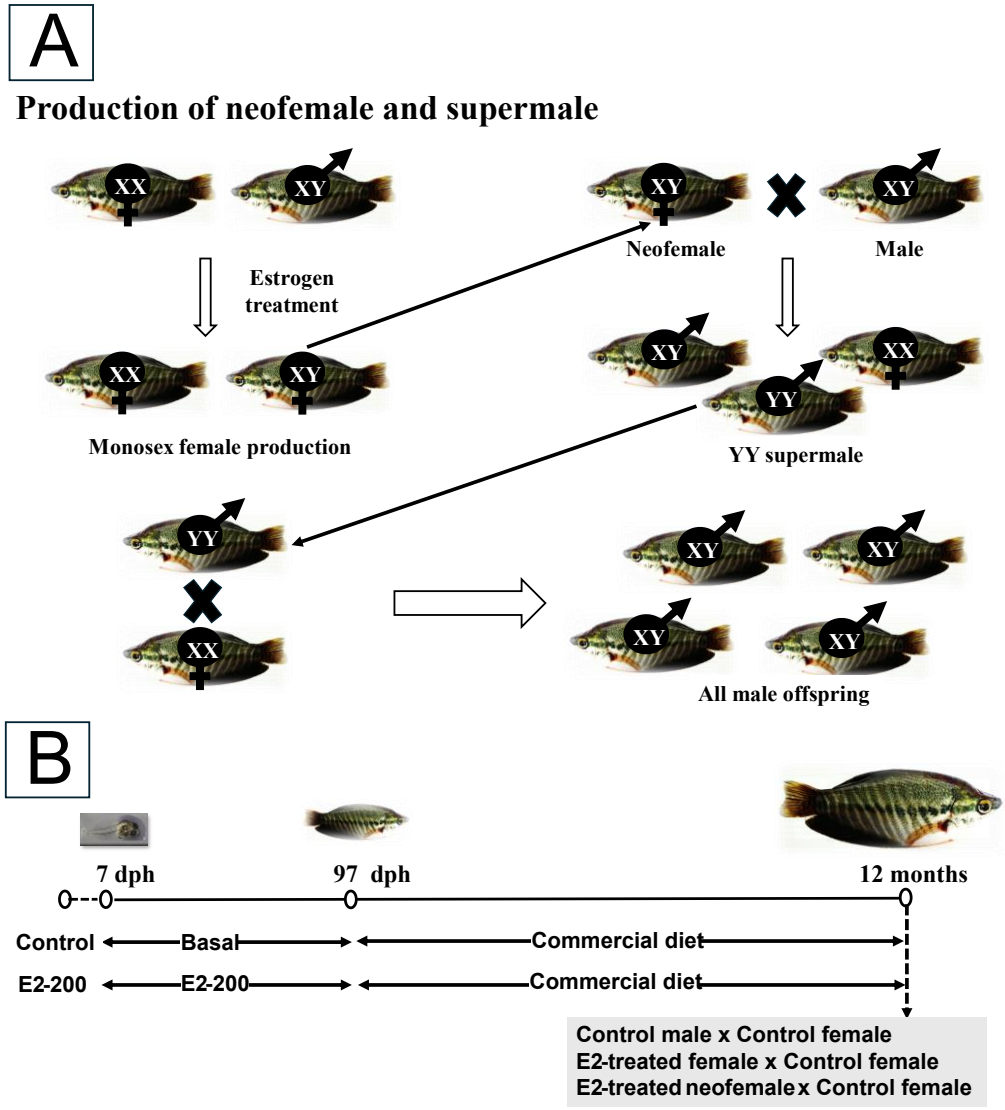

Figure 5.1 Experimental plan for E2-induced neofemale production in snakeskin gourami. **(A)** Schematic overview of sex reversal and breeding strategies under an XX/XY sex determination system, illustrating E2-induced neofemale production, supermale (YY) generation, and expected offspring sex outcomes. **(B)** Experimental workflow showing E2 treatment of 7-dph fry, broodstock development, controlled crosses, and progeny sex identification using sex-specific SNP markers (SNP_19, SNP_20, and SNP_21), squash method, and histological analysis.

Snakeskin gourami (*Trichopodus pectoralis*) is a commercially important freshwater species in Southeast Asia, where females exhibit superior growth performance compared with males (Panthum et al., 2021; Kabpha et al., 2023). Previous studies have demonstrated that dietary estrogen (E2) or androgen treatments can effectively induce sex reversal in this species, producing phenotypic females or males with growth and physiological characteristics comparable to those of natural counterparts (Kabpha et al., 2023). In a previous study, the physiological performance and gonadal development of hormonally sex-reversed broodstock were characterized, providing evidence that sex reversal does not impair reproductive potential (Chapter IV). Nevertheless, phenotypic observations alone are insufficient to distinguish true genetic females from sex-reversed individuals, particularly in broodstock intended for selective breeding.

Recent advances in molecular genetics have enabled the development of sex-specific DNA markers that allow unambiguous identification of genetic sex, independent of phenotypic appearance, such as Nile tilapia (*Oreochromis niloticus*), Spotted Knifejaw *(Oplegnathus punctatus), and* snakehead *(Channa maculata)* (Ma et al., 2022; Sun, ea al., 2014; Yang, et al., 2020) . In Chapter III, sex-linked SNP markers were developed for snakeskin gourami, enabling discrimination between homozygous females and heterozygous males and supporting an XX/XY sex determination system. While these markers provide a powerful tool for broodstock classification, their practical value must be validated through progeny testing to establish a direct link between parental genotype, reproductive performance, and offspring sex ratios.

Therefore, the primary objective of Chapter V was to integrate molecular sex identification with progeny testing to functionally validate neofemale and supermale broodstock in snakeskin gourami. Specifically, this chapter aims to (i) evaluate the reproductive performance of neofemales and supermales, (ii) assess gonadal development and sex differentiation in their offspring using squash method and histological approaches (Menu et al., 2005), and (iii) verify the consistency between offspring sex ratios and parental genotypes using sex-specific SNP markers. By combining phenotypic, histological, and molecular evidence, this chapter provides a comprehensive assessment of the feasibility of neofemale- and supermale-based

breeding strategies and delivers robust confirmation of an XX/XY sex determination system in snakeskin gourami.

5.3 Materials and methods

5.3.1 Ethics statement

All experimental procedures involving fish were reviewed and approved by the Animal Care and Use Committee of Suranaree University of Technology (approval no. 1731039).

5.3.2 Fish breeding and larval production

Sexually mature broodstock of snakeskin gourami (*Trichopodus pectoralis*) were obtained from commercial aquaculture farms located in Samut Sakhon Province, Thailand, and subsequently transported to the experimental facilities of the Suranaree University of Technology (SUT) farm in Nakhon Ratchasima, Thailand. Upon arrival, fish were acclimated to local rearing conditions prior to experimental use. Broodstock were maintained in a 0.04-ha earthen pond under ambient environmental conditions, including natural photoperiod and temperature fluctuations. Fish were fed a commercial formulated diet (Aquafeed, Thai Union Feedmill, Samut Sakhon, Thailand) containing 40% crude protein and 6% crude lipid. Feeding was conducted twice daily at 10:00 and 16:00 h to apparent satiation throughout the conditioning period. Artificial spawning was performed in five aerated concrete tanks (2 m × 2 m × 1 m), each filled with freshwater and equipped with floating aquatic vegetation to promote bubble nest formation, a characteristic reproductive behavior of this species. Each spawning tank was stocked with four males weighing 140–160 g and two females weighing 180–200 g, maintaining a male-to-female ratio of 2:1.

To induce ovulation, females were hormonally stimulated using a two-step intramuscular injection protocol. The initial injection consisted of 10 μg kg^{-1} body weight luteinizing hormone-releasing hormone analog (LHRHa; Suprefact, Hoechst, Germany) combined with 5 mg kg^{-1} domperidone (Motilium-M, OLIC, Bangkok, Thailand). A second injection containing 20 μg kg^{-1} LHRHa and 5 mg kg^{-1} domperidone was administered 12 h later. Males received a single intramuscular injection of LHRHa (10–20 μg kg^{-1} body weight) in combination with 5 mg kg^{-1} domperidone to

synchronize spermiation. Spawning activity was monitored regularly, and fertilized eggs were observed approximately 14 h after spawning. Following confirmation of fertilization, broodstock were promptly removed from the tanks to prevent egg predation. Fertilized eggs and newly hatched larvae were maintained in the same tanks under continuous aeration. Larvae were reared until 6 days post-hatching (dph), after which they were transferred into hapas for subsequent sex-reversal experiments.

5.3.3 Production of neofemales

Experimental diets were prepared by incorporating E2 (Sigma-Aldrich Chemical Co., St Louis, MO, USA) into a commercial diet (protein 50%, lipids 15%). The two treatment groups were as follows: basal diet (control), and 200 mg kg−1 (E-200) E2-supplemented diets. To prepare the hormonal treatment diets, E-200 was dissolved in 95% ethanol (240 mL) and sprayed over the commercial diet (1 kg). After evaporation of ethanol for 6 h in the dark at room temperature (26°C), the experimental diet was kept at 4°C until use.

Figure 5.1 B illustrates the experimental plan used in this study. For sex reversal experiment, the experimental units comprised 10 hapas (fine mesh aquatic dip net cages: 10 × 5 × 0.8 m) located in a cement pond (10 × 5 × 0.8 m3) with aeration and maintained at a 12:12 h light–dark cycle. Seven dph swim-up fry (n = 6 000) were transferred and randomly distributed to each hapa (600 fry/ hapa). The fish continued to grow in the same hapas and were fed basal diet and hormone-treated diets until 97 dph (Figure 5.1 B). The fish were fed four times daily (0900, 1130, 1400, and 1630) with experimental diets at 30% BW/day (month one), 20% BW/day (month two), and 15% BW/day (month three). A flow-through water-exchange system was used to replace one-third of the water weekly. Air and water temperatures were measured daily during the experimental period and were 23.0–33.0°C and 28.0–29.0°C, respectively. Throughout the sex reversal period, dissolved oxygen (DO) and pH were determined weekly using a DO and pH meter, respectively, and were within acceptable ranges of 4.50–6.83 mg L−1 and 7.75–8.10, respectively.

After three months of the sex reversal treatment (97 dph), the experimental fish were transferred to a cage for growth to become broodstock (Figure 5.1 B). Six cages; 4 × 8 × 1 m) were placed in two earthen ponds (0.08 ha) under a 12:12 h light–dark cycle. Each cage was released with 700 fish. Fish were hand-fed a

commercial diet (protein 28%, lipids 4%) twice daily ad libitum through the age of 12 months. Water was added to maintain a depth of 1 m. Air and water temperatures were measured daily during the experimental period and were 20–36°C and 28.3–29.9°C, respectively. DO and pH were determined weekly and were within acceptable ranges of 3.52–5.82 mg L^{-1} and 6.80–6.92, respectively.

5.3.4 Progeny testing of control group and E2-treated female group, and E2-treated neofemale group

Progeny testing was conducted in three mating groups including (i) control males crossed with wildtype (WT) females, (ii) E2-treated females paired with WT males, and (iii) E2-treated females paired with WT males. Control males (36 fish) were sampled for breeding with WT females using LHRHa and domperidone injection, and twenty-seven fish could produce offspring. Six male broodstock were sampled, and fin samples were collected for genotyping. Their offspring were cultured for sex determination. For E-200, fish (38 fish) were sampling for breeding with WT males using LHRHa and domperidone injection, and thirteen fish could produce offspring. The E2-treated fish which were able to produce offspring were sampling for fin collection for genotyping. 30 progeny populations were obtained from E2-treated females, and six progeny population were sampling for culture through 3 months for sex determination. The other six E2-treated females were neofemales, and their progeny were 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. Subsequently, larvae (7 dph) were fed a commercial diet (40% protein and 8% fat) until 30 dph. Thereafter, fingerlings from all 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 (35% protein and 4% lipid) through 3 months. At three months, individuals were randomly sampled for fin tissue collection, and gonads were collected for sex determination using squash method (Menu et al., 2005) and histological examination. Throughout the rearing period, air temperatures ranged from 22.0–35.0°C, while water temperatures were between 28.5–30.0°C.

5.3.5 Histological examination of the gonads

Sex determination was performed through histological analysis 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). Fixed tissues were subjected to ethanol dehydration (70% to 100%), embedded in paraffin, and sectioned at 5 μm. The sections were mounted on glass slides, dewaxed, rehydrated with ethanol and xylene, and stained with hematoxylin and eosin. Gonadal morphology was then examined using a digital upright microscope (Olympus BX53, Tokyo, Japan).

5.3.6 Genotypic analysis using sex-specific SNP

Based on a previous study, sex-specific SNP markers, including SNP_19, SNP_20, and SNP_21, were developed to identify heterozygous males and homozygous females in snakeskin gourami (Chapter III). In this study, we used Sanger sequencing to determine the genotypic sex of control males, E2-treated females, E2-treated neofemales 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 5.1. PCR amplification was performed using TaKaRa Ex Taq polymerase (TaKaRa Bio, Shiga, Japan) in a total reaction volume of 25 μL. Each reaction mixture contained 2.5 μL 10× Ex Taq Buffer, 2 μL dNTP Mixture (2.5 mM each), 0.125 μL TaKaRa Ex Taq polymerase (5 U/μL), 2.5 μL forward primer (2 μM), 2.5 μL reverse primer (2 μM), 2.5 μL DNA template (50ng μL-1), and 12.875 μL nuclease-free water. PCR was performed under the following conditions: 95°C for 3 min; 35 cycles of 95°C for 30 s, annealing at the optimal temperature for each primer pair (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 protocol for sequencing. Sanger sequencing was used to determine nucleotide variation at the target SNP locus.

5.3.7 Production of supermale

After progeny testing, the offspring from the neofemales were transferred to a new hapa for growth. 2 hapas (2 × 2 × 2 m^3) were placed in an earthen pond (0.08 ha) under a 12:12 hours light-dark cycle. Fish were hand -fed a commercial diet (protein 28%, lipids 4 %) twice per day ad libitum through the age of 12 months. Water was added to maintain a depth of 1 m. Air and water temperatures were measured daily during the experimental period and were 20 – 36°C and 28.3–29.9°C, respectively. DO, and pH were determined weekly and were within acceptable ranges of 3.52–5.82mgL-1 and 6.80–6.92, respectively.

5.3.8 Progeny testing of the control male group and supermale group

Progeny testing was performed using two mating groups: (i) control males crossed with wild-type (WT) females, and (ii) supermales crossed with WT females. In the control group, five breeding pairs were induced to spawn using LHRHa and domperidone injections. Fin clips were collected from all broodstock for genotyping before breeding, and the resulting offspring were reared until 3 months of age for sex determination. Similarly, five supermale broodstock were paired with WT females and induced to spawn using the same hormonal treatment. All five mating pairs successfully produced offspring. Fin samples were collected from the broodstock for genotyping, and five progeny populations were reared to 3 months post-hatching for subsequent 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. Subsequently, larvae (7 dph) were fed a commercial diet (40% protein and 8% fat) until 30 dph. Thereafter, fingerlings from all groups were transferred to 2 × 2 × 2 m^3 hapas installed within a 0.08-ha earthen pond. Fish were hand-fed commercial diet (35% protein and 4% lipid) for 3 months. At three months, individuals were randomly sampled for fin tissue collection, and gonads were collected for sex determination using the squash method (Menu et al., 2005) and histological

examination. Throughout the rearing period, air temperatures ranged from 22.0–35.0°C, while water temperatures were between 28.5–30.0°C.

5.3.9 Histological analysis of gonads

Sex identification was conducted by histological analysis of gonadal tissues. Samples were preserved in Bouin's fixative at 4°C for 16 h. The sex ratio was assessed in fish at three months of age (n = 60 individuals per replicate, with six replicates). After fixation, tissues were dehydrated using a graded ethanol series ranging from 70% to 100%, embedded in paraffin, and sectioned at a thickness of 5 µm. The sections were mounted on glass slides, followed by dewaxing and rehydration through xylene and ethanol gradients. Subsequently, the samples were stained with hematoxylin and eosin (H&E). Gonadal structures were observed under a digital upright microscope (Olympus BX53, Tokyo, Japan).

5.3.10 Genotypic analysis using sex-specific SNP

Similarly, the Sanger sequencing was used to determine the genotypic sex of control males, supermale broodstock, and their offspring (Chapter III). gDNA extraction was performed as described previously. Primer sequences, annealing temperatures, and expected amplicon sizes are presented in Table 5.1. PCR amplification and Sanger sequencing were performed as described previously.

Table 5.1 Sequences of primers used for Sanger sequencing.

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

Abbreviation: Tm (°C) = Annealing temperature (°C); bp= base pair.

Note: The underlined sequence AATACGACTCACTATAGGG represents the T7 universal sequencing tag added to the 5′end of primers.

5.3.11 Data analysis

All statistical analyses were performed using SPSS software version 22.0 (SPSS Inc., Chicago, IL, USA). Chi-square (χ^2) goodness-of-fit tests were applied to assess deviations from the expected 1:1 sex ratio in the E2-treated female, E2-treated neofemale, control, and supermale groups. In addition, chi-square tests were used to compare the sex ratios of progeny among the E2-treated female, E2-treated neofemale, control, and supermale groups. Differences were considered statistically significant at $P < 0.05$.

5.4 Results

5.4.1 Genotypic identification of control females, E2-treated females, E2-treated neofemales, and males broodstock by Sanger sequencing

Table 5.2 shows that, among the 36 selected mature control males, 27 individuals successfully produced offspring, corresponding to a mating success rate of 75.0%. Similarly, 30 out of 38 E2-treated individuals were fertile, yielding a mating success rate of 78.0%. For progeny testing, sixty offspring from each mating group—control males, E2-treated females, and E2-treated neofemales—were randomly selected and reared for 90 days, after which sex ratios were determined. The genotypes of all broodstock, including control females, E2-treated females, E2-treated neofemales, and males, were identified by PCR amplification followed by Sanger sequencing. Consistent with previous findings demonstrating an XX/XY sex determination system in snakeskin gourami (Chapter III), genotyping analysis revealed that control females and E2-treated females were homozygous at SNP_19 (G/G), SNP_20 (C/C), and SNP_21 (G/G; complementary C/C detected in antisense sequencing). In contrast, E2-treated neofemales and control males exhibited heterozygous genotypes at SNP_19 (G/T), SNP_20 (C/T), and SNP_21 (G/A; C/T detected in antisense sequencing) (Figure 5.2 and 5.3).

Table 5.2 Number of control male and E2-terated female broodstock that were used for progeny testing.

Parameters	Control group		E2-200 group	
	Males	females	females	Control males
No. of broodstock	36	36	38	38
No. of pairs with hatching	27	27	30	30
No. of pairs with no hatching	9	9	8	8
Mating success (%)	75.0	75.0	78.95	78.95

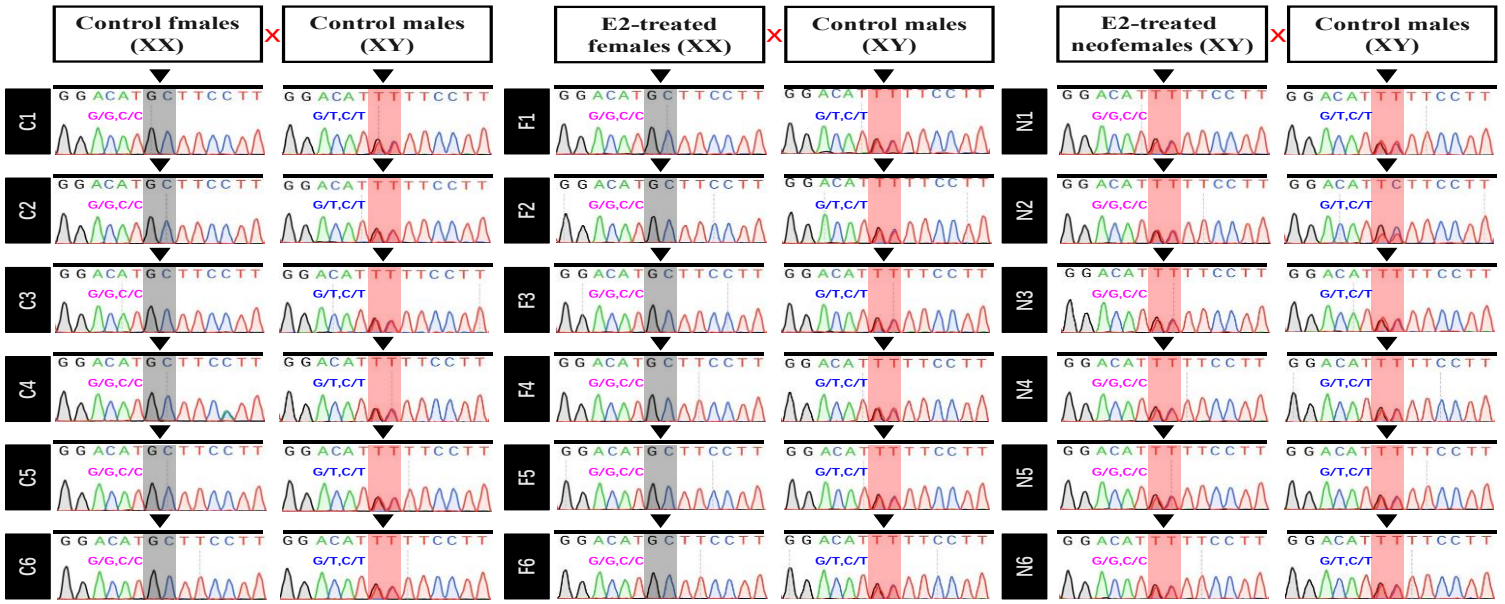

Figure 5.2 Identification of sexual genotypes at SNP_19 and SNP_20 in broodstock using Sanger sequencing. Representative chromatograms from control females (XX) × control males (XY) (C1–C6), E2-treated females (XX) × control males (XY) (F1–F6), and E2-treated neofemales (XY) × control males (XY) (N1–N6) showed that control females and E2-treated females were consistently homozygous at SNP_19 (G/G) and SNP_20 (C/C), whereas control males and E2-treated neofemales were heterozygous at both loci (SNP_19: G/T; SNP_20: C/T). These results confirm that E2-treated neofemales retained a male (XY) genetic constitution despite exhibiting a female phenotype. Abbreviations: C = control group; F = E2-treated female; N = E2-treated neofemale.

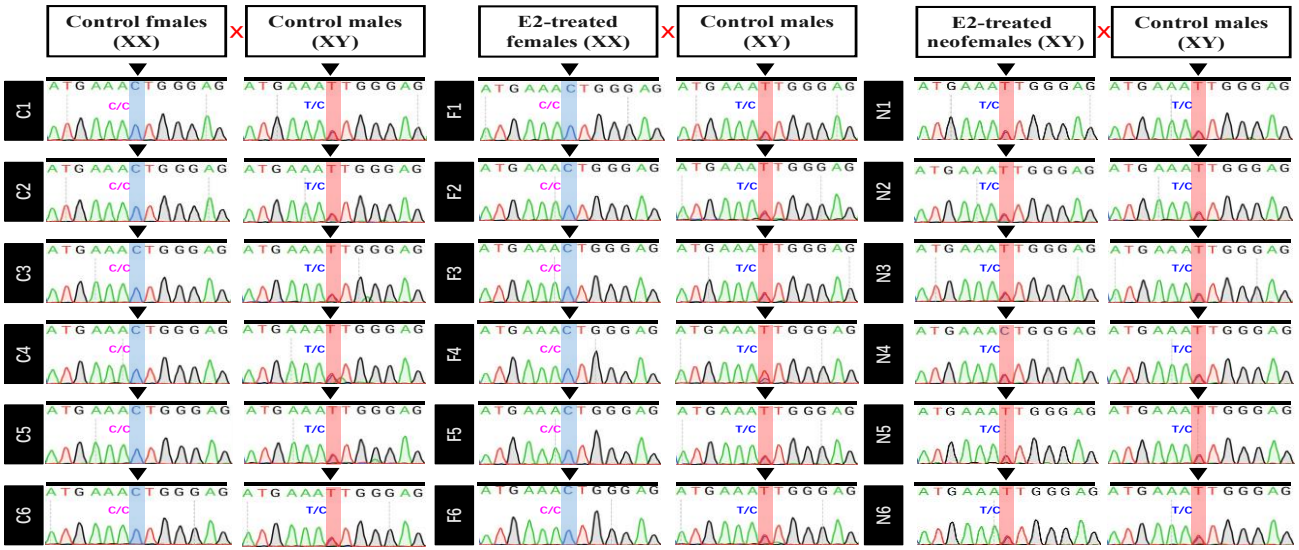

Figure 5.3 Identification of sexual genotypes at SNP_21 in broodstock using Sanger sequencing. Representative chromatograms from control females (XX) × control males (XY) (C1–C6), E2-treated females (XX) × control males (XY) (F1–F6), and E2-treated neofemales (XY) × control males (XY) (N1–N6) showed that control females and E2-treated females were consistently homozygous at SNP_21 (G/G; complementary C/C detected in antisense sequencing), whereas control males and E2-treated neofemales were heterozygous at SNP_21 (G/A; complementary C/T detected in antisense sequencing). These results confirm that E2-treated neofemales retained a male (XY) genetic constitution despite complete phenotypic feminization. Abbreviations: C = control group; F = E2-treated female; N = E2-treated neofemale.

5.4.2 Progeny testing of control females, E2-treated females, and E2-treated neofemales using the squash method and histological analysis

Gonadal sex of progeny was initially determined using the squash method based on cellular morphological characteristics. As illustrated in (Figure 5.4 A and B), ovarian and testicular tissues were readily distinguishable in female and male progeny, respectively. To verify the reliability of this approach, corresponding samples were further examined by histological analysis, which consistently confirmed ovarian structures in females and testicular structures in males. In female progeny, squash preparations revealed abundant round oocytes with clear cellular boundaries (Figure 5.4 A), closely matching the ovarian features observed in histological sections. Histological examination demonstrated ovaries composed primarily of oogonia (OG) and previtellogenic oocytes (PVO), indicative of an immature ovarian stage (Figure 5.4 C, E, and G). In contrast, squash preparations from male progeny showed compact clusters of spermatogenic cells, mainly spermatocytes (Figure 5.4 B). Consistently, histological sections revealed well-defined lobular testes containing spermatogonia (SG), primary spermatocytes (PSC), secondary spermatocytes (SSC), and a small number of spermatids (ST) distributed throughout the testicular tissue (Figure 5.4 D, F, and H). Comparable gonadal characteristics were observed among progeny derived from control males, E2-treated females, and E2-treated neofemales, all of which exhibited typical features of immature ovaries or testes at the juvenile stage (Figure 5.4 C–H). The high concordance between squash observations and histological findings indicates that gonadal sex in snakeskin gourami progeny can be reliably distinguished by distinct gonadal morphology at 3 months post-hatching. These results confirm that the squash method represents a rapid, accurate, and efficient approach for early sex identification and large-scale progeny screening.

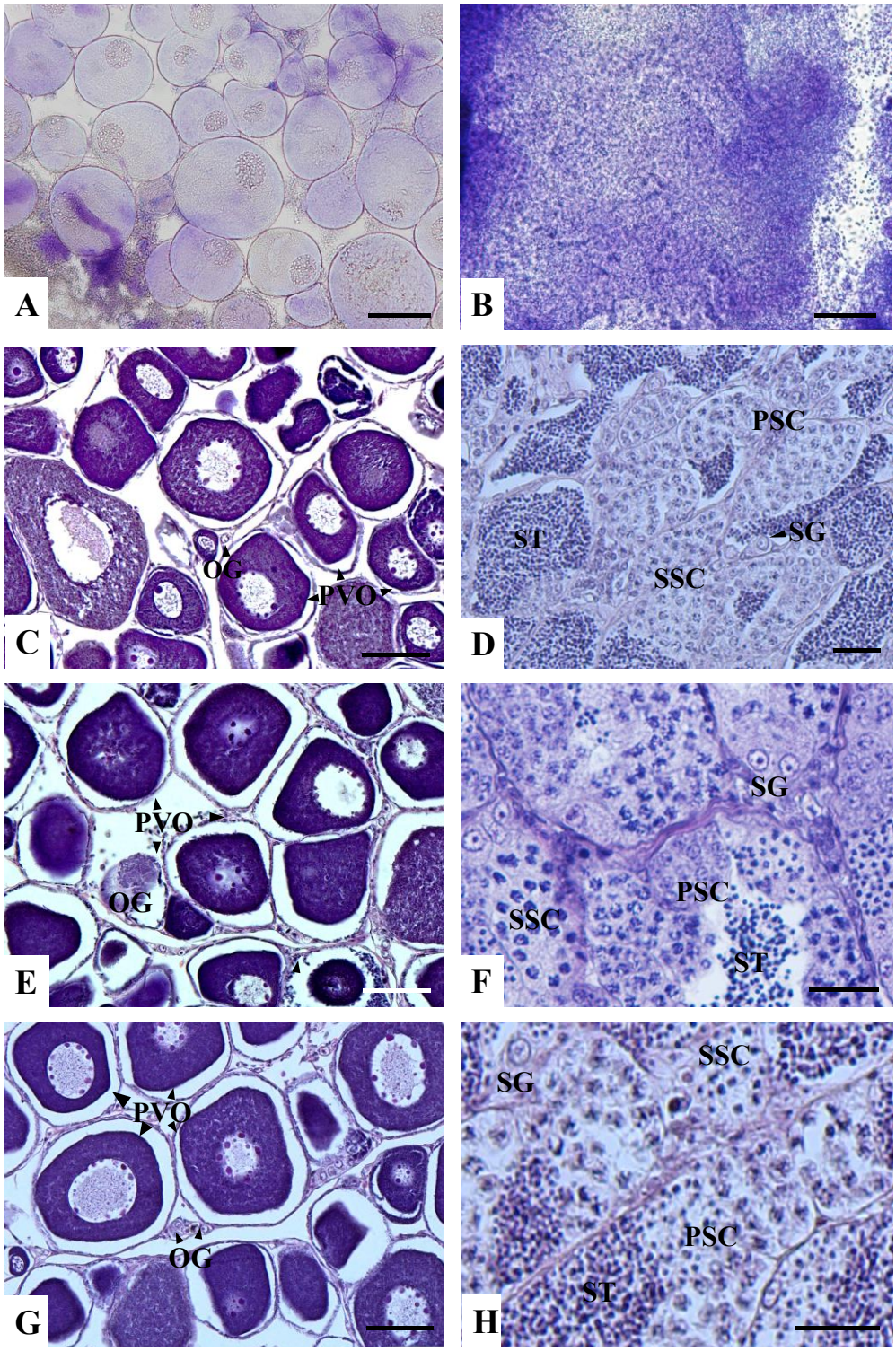

Figure 5.4 Histological and squash-based characterization of gonadal development in progeny derived from neofemale experiments in snakeskin gourami. (A) Ovarian tissue of progeny at three months was identified by the squash

method, showing numerous rounded oocytes. (B) Testicular tissue of progeny at three months was identified by the squash method, characterized by dense clusters of spermatogenic cells. (C) Histological section of the ovary from control group offspring at three months. (D) Histological section of testis from control group offspring at three months. (E) Histological section of the ovary from E2-treated female offspring at three months. (F) Histological section of testis from E2-treated female offspring at three months. (G) Histological section of the ovary from E2-treated neofemale offspring at three months. (H) Histological section of testis from E2-treated neofemale offspring at three months. 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 100 μm (A, B), 50 μm (C, E, G), and 20 μm (D, F, H).

A summary of sex determination results obtained using these methods is presented in (Table 5.3). In the control group, progeny produced by crosses between XY males and XX females displayed an approximately balanced sex ratio, with male and female proportions of 48.33 ± 3.94% and 51.67 ± 3.94%, respectively, showing no significant deviation from the expected Mendelian 1:1 ratio (χ^2 test, $P > 0.05$). Similarly, offspring derived from E2-treated XX females exhibited a comparable sex distribution, with 47.50 ± 5.03% males and 52.50 ± 5.03% females, and no significant departure from the expected ratio was detected (χ^2 test, $P > 0.05$). In contrast, progeny obtained from crosses between E2-treated neofemales (XY genotype) and XY males showed a pronounced male-biased sex ratio consistent with the expected 3:1 male-to-female ratio, with no significant deviation from this expectation (χ^2 test, $P > 0.05$). Taken together, these findings demonstrate that E2-treated neofemales function as fertile phenotypic females.

Table 5.3 Sex ratio and body measurements of progeny derived from control, E2-treated female, neofemale broodstock.

Group	progeny Body weight (g)	Total length (cm)	No. of males	No. of females	Male (%)	Female (%)	χ^2 *P*-value
Control female							
C 1	8.65 ± 4.99	8.24 ± 1.69	32	28	53.33	46.67	0.6056
C 2	6.86 ± 5.6	7.39 ± 1.93	28	32	46.67	53.33	0.6056
C 3	13.22 ± 5.23	9.47 ± 1.31	30	30	50.00	50.00	1.0000
C 4	5.45 ± 3.16	6.95 ± 1.43	27	33	45.00	55.00	0.4386
C 5	5.10 ± 2.58	7.06 ± 1.16	31	29	51.67	48.33	0.7963
C 6	15.82 ± 11.45	9.58 ± 2.17	26	34	43.33	56.67	0.3017
Mean ± SD					*48.33 ± 3.94*	*51.67 ± 3.94*	
ES-treated female							
F 1	8.95 ± 3.5	8.22 ± 0.89	24	36	40.00	60.00	0.1213
F 2	10.47 ± 5.95	8.64 ± 1.55	27	33	45.00	55.00	0.4386
F 3	19.47 ± 8.27	10.84 ± 1.61	30	30	50.00	50.00	1.0000
F 4	12.23 ± 8.39	8.69 ± 2.01	33	27	55.00	45.00	0.4386
F 5	10.8 ± 3.84	8.83 ± 1.06	28	32	46.67	53.33	0.6056
F 6	6.85 ± 2.81	7.7 ± 1.02	29	31	48.33	51.67	0.7963
Mean ± SD					*47.50 ± 5.03*	*52.50 ± 5.03*	
E2-treated neofemale							
N 1	13.02 ± 6.04	9.21 ± 1.48	46	14	76.67	23.33	0.7656
N 2	8.88 ± 3.8	8.49 ± 1.14	46	14	76.67	23.33	0.7656
N 3	11.62 ± 6.58	8.96 ± 1.7	45	15	75.00	25.00	1.0000
N 4	8.63 ± 3.8	8.29 ± 0.95	42	18	70.00	30.00	0.3711
N 5	8.12 ± 3.85	8.14 ± 1.4	42	18	70.00	30.00	0.3711
N 6	12.45 ± 7.32	9.01 ± 2.06	43	17	71.67	28.33	0.5510
Mean ± SD					*73.34 ± 3.16*	*26.67 ± 3.16*	

Abbreviations: C = Control group; F = ES-treated female; N = ES-treated neofemale

Note: Values are expressed as mean ± SD. The χ^2 test was used to assess deviations from the expected 1:1 sex ratio in the control group. Statistical significance was set at $p < 0.05$.

5.4.3 Reproductive performance and genotypic identification of control males and supermales

To evaluate reproductive capacity, control males and YY supermales were subjected to progeny testing. As summarized in Table 5.4, control males showed

a mating success rate of 75.0%, with 27 out of 36 pairs successfully producing hatched larvae. In contrast, all supermales successfully produced offspring, achieving a 100% mating success rate. These results indicate that YY supermales retained normal fertility. Genotypic identification of broodstock was subsequently performed by PCR amplification followed by Sanger sequencing. Consistent with the established XX/XY sex determination system in snakeskin gourami (Chapter III), control males exhibited heterozygous genotypes at SNP_19 (G/T), SNP_20 (C/T), and SNP_21 (G/A). In contrast, supermales were homozygous at these loci, displaying SNP_19 (T/T), SNP_20 (T/T), and SNP_21 (A/A). All control females were homozygous at SNP_19 (G/G), SNP_20 (C/C), and SNP_21 (G/G) (Figures 5.5 and 5.6). These results confirm the successful production of YY supermales and validate the reliability of the identified sex-specific SNP markers.

Table 5.4 Number of control male broodstock and neofemale-derived supermale broodstock used in progeny testing.

Parameters	Control group		Supermale group	
	Males	females	Supermale	Control females
No. of broodstock	36	36	5	5
No. of pairs with hatching	27	27	5	5
No. of pairs with no hatching	9	9	0	0
Mating success (%)	75.0	75.0	100	100

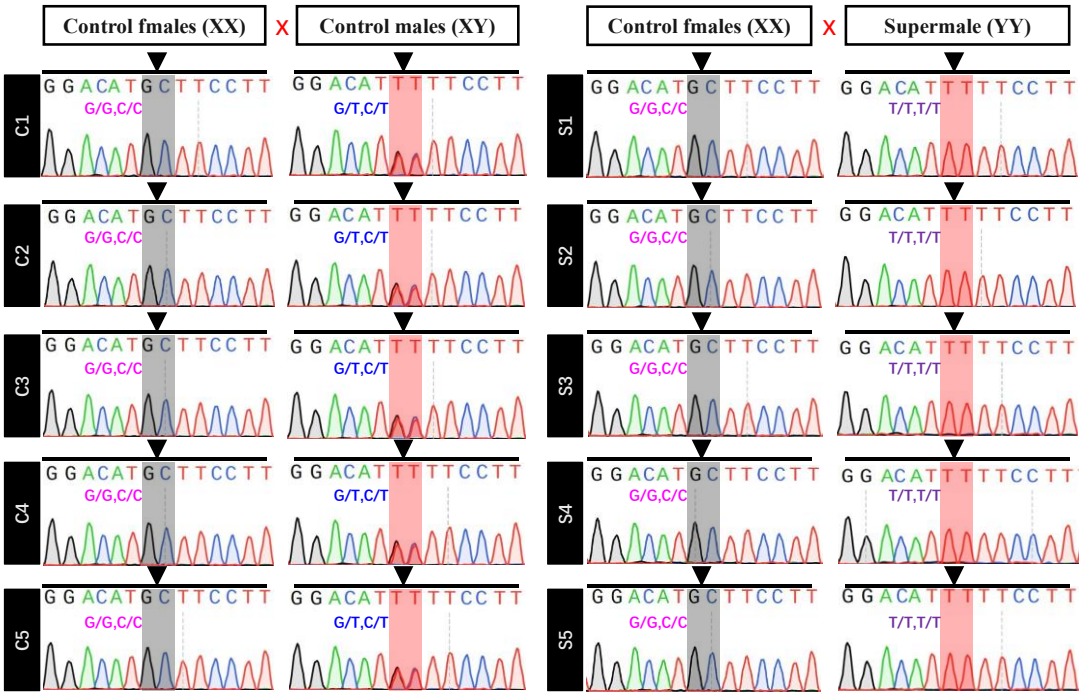

Figure 5.5 Identification of control males and supermales based on sex-specific SNP markers (SNP_19 and SNP_20) using Sanger sequencing Representative chromatograms from control females (XX) × control males (XY) (C1–C5) and control females (XX) × supermales (YY) (S1–S5) showed that control males were consistently heterozygous at SNP_19 (G/T) and SNP_20 (C/T), whereas supermales were homozygous at both loci (SNP_19: T/T; SNP_20: T/T). In contrast, control females were homozygous at SNP_19 (G/G) and SNP_20 (C/C). These results confirm that supermales retained a homozygous genotype pattern distinct from control males, validating the successful production of YY supermales.

Abbreviations: C = control group; S = supermale group.

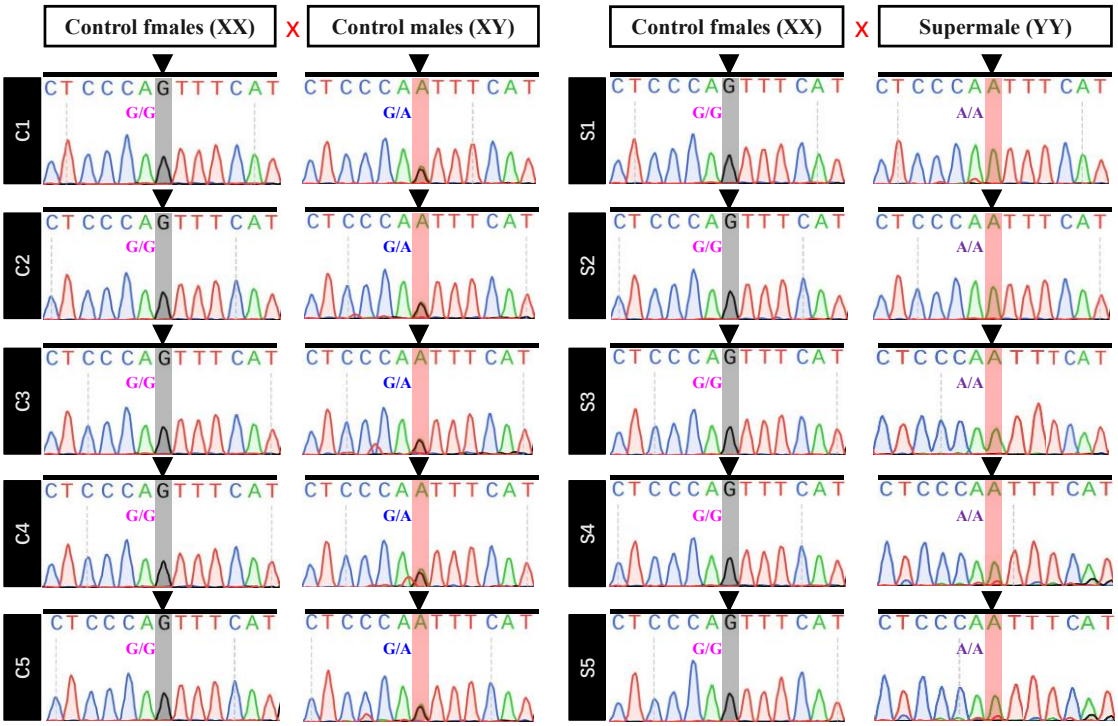

Figure 5.6 Identification of control male and supermale genotypes using the sex-specific SNP marker SNP_21 by Sanger sequencing. Representative chromatograms from control females (XX) × control males (XY) (C1–C5) and control females (XX) × supermales (YY) (S1–S5) showed that control males were consistently heterozygous at SNP_21 (G/A), whereas supermales were homozygous at SNP_21 (A/A). In contrast, control females were homozygous at SNP_21 (G/G). These results confirm that supermales retained a homozygous genotype pattern distinct from control males, providing further molecular validation of successful YY supermale production.

Abbreviations: C = control group; S = supermale group.

5.4.4 Progeny testing of control males and supermale using the squash method and histological analysis

Gonadal sex of progeny was initially assessed using the squash method based on cellular morphology. As shown in (Figure 5.7 A and B), ovarian and testicular tissues were clearly distinguishable in female and male progeny, respectively. To evaluate the reliability of this method, representative samples were further subjected

to histological examination, which consistently confirmed ovarian structures in females and testicular structures in males. In female progeny, squash preparations revealed numerous rounded oocytes with well-defined cellular boundaries (Figure 5.7 A), corresponding closely to ovarian morphology observed in histological sections. Histological analysis showed ovaries primarily composed of oogonia (OG) and previtellogenic oocytes (PVO), indicating an immature ovarian stage (Figure 5.7 C). In contrast, squash preparations from male progeny displayed dense clusters of spermatogenic cells, predominantly spermatocytes (Figure 5.7 B). Consistently, histological sections revealed well-organized lobular testes containing SG, PSC, SSC, and a small number of ST distributed throughout the testicular tissue (Figure 5.7 D-F). Similar gonadal features were observed in progeny derived from control males (Figure 5 .7 C and D), and supermales (Figure 5.7 E and F), all of which exhibited typical characteristics of immature testes at the juvenile stage. The strong agreement between squash observations and histological findings indicates that gonadal sex in snakeskin gourami progeny can be reliably identified based on gonadal morphology at 3 months post-hatching.

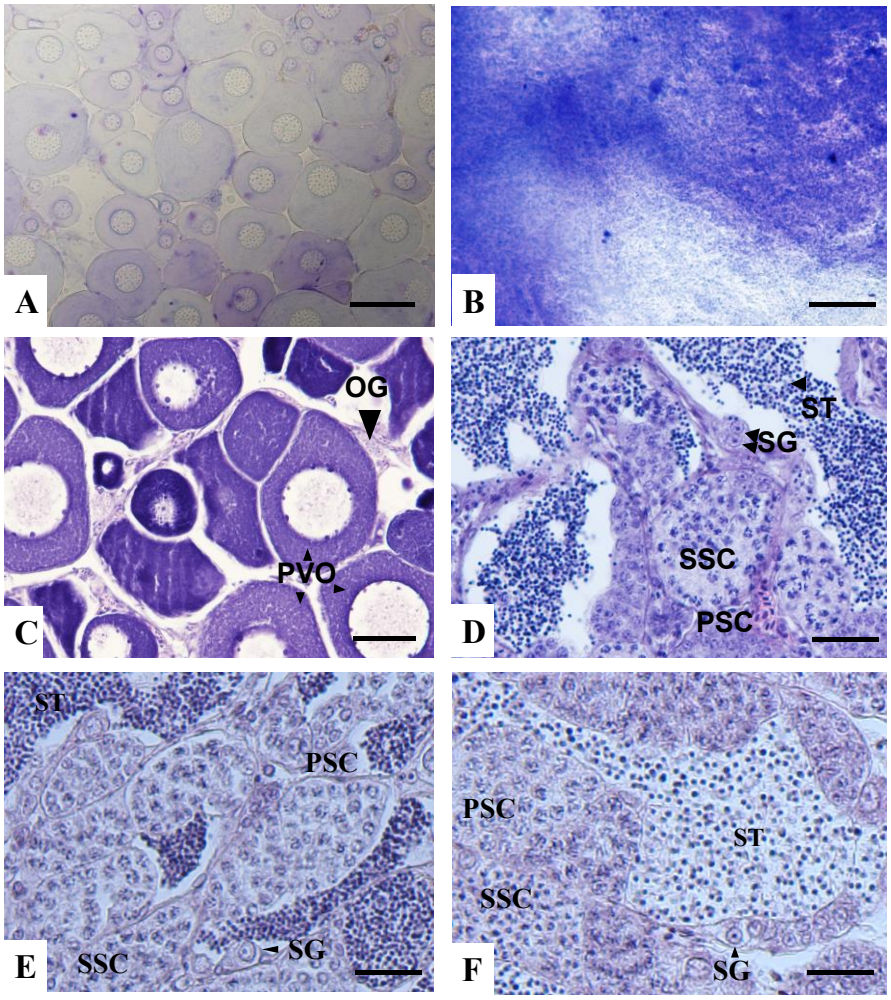

Figure 5.7 Histological and squash-based characterization of gonadal development in progeny derived from supermale experiments in snakeskin gourami. (A) Squash preparation of the ovary from progeny at three months, showing numerous rounded oocytes with clear cellular boundaries. (B) Squash preparation of testis from progeny at three months, characterized by dense clusters of spermatogenic cells. (C) Histological section of ovary from progeny derived from control males at three months. (D) Histological section of testis from progeny derived from control males at three months. (E and F) Histological section of testis from progeny derived from supermales at three months. The observed ovarian cell types included OG and PVO. The observed testicular cell types include SG, PSC, SSC, and ST. Scale bars represent 100 μm (A, B), 50 μm (C), and 20 μm (E, F).

A summary of sex determination results obtained using these approaches is presented in (Table 5.5). In the control group, progeny produced from crosses between XY males and XX females exhibited an approximately balanced sex ratio, with 51.33 ± 5.05% males and 48.67 ± 5.05% females, showing no significant deviation from the expected Mendelian 1:1 ratio (χ^2 test, $P > 0.05$). In contrast, offspring derived from supermale (YY) males displayed an exclusively male phenotype, with 100.00 ± 0.00% males and no females detected, representing a highly significant deviation from the 1:1 expectation (χ^2 test, $P < 0.001$). Taken together, these results demonstrate that supermales function as fertile phenotypic males and provide further confirmation that sex determination in snakeskin gourami follows an XX/XY genetic system.

Table 5.5 Sex ratio and body measurements of progeny derived from control, supermale broodstock**.**

Group	progeny Body weight (g)	Total length (cm)	No. of males	No. of females	Male (%)	Female (%)	χ^2 *P*-value
Control							
C 1	7.13±3.72	7.77±1.24	29	31	48.33	51.67	0.7963
C 2	9.4±3.22	8.62±1.12	35	25	58.33	41.67	0.1976
C 3	10.53±4.65	8.72±1.08	33	27	55.00	45.00	0.4386
C 4	10.38±5.3	9.03±1.18	28	32	46.67	53.33	0.6056
C 5	11.5±5.59	9.16±1.4	29	31	48.33	51.67	0.7963
					51.33 ± 5.05	*48.67 ± 5.05*	
Supermale							
S1	6.38 ± 4.06	7.26 ± 1.4	60	0	100.00	0.00	<0.0000
S2	12.2 ± 5.92	9.15 ± 1.57	60	0	100.00	0.00	<0.0000
S3	8.8 ± 4.3	8.44 ± 0.98	60	0	100.00	0.00	<0.0000
S4	8.38 ± 2.43	8.47 ± 0.75	60	0	100.00	0.00	<0.0000
S5	8.06 ± 3.76	8.13 ± 1.36	60	0	100.00	0.00	<0.0000
Mean ± SD					*100.00 ± 0.00*	*0.00 ± 0.00*	

Abbreviations: C = Control group; S = Supermale group.

Note: Values are expressed as mean ± SD. The χ^2 test was used to assess deviations from the expected 1:1 sex ratio in the control group and supermales.

Statistical significance was set at $p < 0.05$.

5.5 Discussion

Sex determination and control are key research areas in aquaculture biology, particularly for economically significant species that show sexual dimorphism in growth, survival, or market value. In snakeskin gourami (*Trichopodus pectoralis*), previous studies have suggested a genetic XX/XY sex determination system (Panthum et al., 2021); however, the lack of stable sex-specific markers and validated breeding strategies has limited the practical application of sex control in this species. The present study integrates endocrine manipulation, progeny testing, gonadal histology, and sex-specific SNP genotyping to establish a complete and verifiable pathway to produce functional neofemales and supermales. By combining phenotypic, histological, and molecular evidence, this work provides a closed-loop validation of genetic sex determination and demonstrates a feasible strategy for controlled monosex production in snakeskin gourami.

5.5.1 Molecular validation of estrogen-induced sex reversal using sex-specific SNP

A previous study reported that dietary administration of 200 mg kg^{-1} E2 successfully produced an all-female population of snakeskin gourami (Kabpha et al., 2023). In the present study, the same E2 dosage was adopted to induce feminization and generate an all-female population of this species. One of the primary objectives of this study was to evaluate whether dietary E2 administration could effectively induce sex reversal while maintaining normal gametogenesis and fertilization capacity in treated broodstock. The observed mating success rate of E2-treated broodstock (78.0%) was comparable to that of untreated control males (75.0%), indicating that estrogen exposure during early developmental stages did not adversely affect subsequent gonadal maturation or fertility. This finding is consistent with previous studies in teleost fish demonstrating that estrogen-induced sex reversal, when applied within a critical developmental window, can redirect gonadal differentiation while preserving reproductive function (Pandian and Sheela, 1995; Devlin and Nagahama, 2002; Piferrer, 2001).

In our previous study, 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). In this study, control females and E2-treated females consistently exhibited homozygosity at all three sex-specific loci (SNP_19, SNP_20, and SNP_21), whereas E2-treated neofemales and males displayed heterozygous genotypes at the same loci. This clear and reproducible genotype–phenotype correspondence strongly supports the presence of an XX/XY sex determination system in this species and demonstrates the reliability of these SNP markers for distinguishing genetic sex independent of phenotypic appearance. Importantly, the identification of heterozygous genotypes in E2-treated neofemales provides unambiguous molecular confirmation that these individuals are genetic males (XY) that have undergone complete phenotypic feminization. This distinction is particularly critical, as phenotypic observations alone cannot reliably differentiate between true genetic females and sex-reversed individuals. The application of sex-specific SNP markers, therefore, eliminates ambiguity in broodstock classification and ensures accurate interpretation of progeny testing results. Similar marker-assisted approaches have been widely adopted in other teleost species to validate sex reversal and to avoid misclassification caused by environmental or hormonal influences on gonadal development (Huang et al., 2024; Ou et al., 2025; Penman et al., 2008; Yang et al., 2024). The consistency of SNP genotypes across control males, E2-treated neofemales, and phenotypic females further indicates that estrogen treatment altered gonadal differentiation without modifying the underlying genetic sex. the SNP markers demonstrated clear diagnostic reliability and are compatible with routine PCR and Sanger sequencing workflows, making them practical tools for broodstock screening and genetic management in aquaculture operations.

5.5.2 Functional validation of E2-induced neofemales through phenotypic and progeny sex ratio analyses

The combined application of the squash method and histological analysis provided a robust framework for phenotypic sex identification in juvenile snakeskin gourami progeny. This approach has previously been applied successfully for sex identification in juvenile fishes, demonstrating its broad applicability across teleost species (Cungihan et al., 2024; Menu et al., 2005). The clear correspondence between cellular morphology observed in squash preparations and gonadal structures revealed

by histological sections indicates that gonadal differentiation at three months post-hatching is sufficiently advanced to allow reliable sex discrimination, consistent with typical patterns of juvenile gonadal development reported in teleost fishes. The strong concordance between these two independent methods confirms that the squash technique represents a rapid, accurate, and practical tool for early sex determination, particularly well-suited for large-scale progeny screening in breeding programs. Importantly, comparable gonadal morphologies were observed among progeny derived from control males, E2-treated females, and E2-treated neofemales, indicating that parental E2 treatment did not induce abnormal gonadal development in the offspring. This finding suggests that estrogen-induced sex reversal in broodstock does not exert transgenerational effects on gonadal differentiation, which is similar to the common snook (*Centropomus undecimalis*) (Contreras-García et al., 2023).

Progeny sex ratio analyses provided additional genetic insight into the functional role of E2-treated neofemales. As expected under an XX/XY sex determination system, crosses between control XY males and XX females yielded an approximately balanced 1:1 sex ratio, confirming normal Mendelian segregation. Similarly, progeny produced by E2-treated XX females also exhibited sex ratios indistinguishable from the control group, indicating that estrogen treatment did not alter the genetic sex composition of these females. In contrast, crosses involving E2-treated neofemales (XY genotype) and XY males resulted in a pronounced male-biased sex ratio consistent with the theoretical 3:1 male-to-female expectation. The absence of significant deviation from this ratio provides strong genetic evidence that neofemales produce functional gametes carrying either X or Y chromosomes.

Collectively, the concordant phenotypic and sex ratio data demonstrate that E2-treated neofemales function as fully fertile phenotypic females while retaining a male (XY) genetic constitution. This dual validation—phenotypic sex identification supported by progeny testing—provides a critical link between molecular genotyping and reproductive performance. The ability of neofemales to generate predictable sex ratios in their offspring establishes a solid foundation for subsequent supermale (YY) production and confirms the feasibility of applying controlled sex reversal strategies in snakeskin gourami aquaculture.

5.5.3 Validation of sex-specific SNP markers for genetic sex identification in broodstock

Accurate identification of genetic sex is a fundamental prerequisite for the establishment of monosex breeding programs and for elucidating the underlying mechanisms of sex determination in aquaculture species (Liu et al., 2024; Zhang et al., 2021). In the present study, Sanger sequencing was employed to validate the genotypes of control males, neomales, and female broodstock in snakeskin gourami, with particular emphasis on assessing the practical reliability of SNP_19, SNP_20, and SNP_21 as sex-linked molecular markers (Chapter III, VI). The clear and non-overlapping genotype patterns observed among the broodstock groups provide strong support for the applicability of these SNPs in broodstock management and selective breeding programs. Specifically, control males consistently exhibited heterozygosity at SNP_19 (G/T), SNP_20 (C/T), and SNP_21 (G/A), whereas females showed homozygosity at SNP_19 (G/G), SNP_20 (C/C), and SNP_21 (G/G). In contrast, supermales displayed homozygous genotypes at all three loci, namely SNP_19 (T/T), SNP_20 (T/T), and SNP_21 (A/A). This distinct genotype configuration conforms to expectations under an XX/XY sex determination system and complements the inheritance-based evidence presented in Chapters III and VI. Importantly, the present analysis focuses on marker validation at the broodstock level, thereby avoiding redundancy with earlier segregation analyses. Comparable patterns have been widely reported in teleost fish, in which hormonally sex-reversed individuals retain their original chromosomal sex and can be reliably distinguished using sex-linked molecular markers (Devlin & Nagahama, 2002; Piferrer, 2001). The consistency of genotypes observed in neomales across all examined SNP loci indicates that these markers are not affected by hormonal treatment, underscoring their robustness and long-term reliability for breeding applications. In conclusion, the present findings demonstrate that SNP_19, SNP_20, and SNP_21 constitute a reliable and application-ready marker set for genetic sex identification in snakeskin gourami. Moreover, these results provide additional molecular evidence supporting an XX/XY sex determination system in this species. The integration of these SNP markers into selective breeding strategies is expected to enhance the efficiency, predictability, and scalability of all-female population production in snakeskin gourami aquaculture.

5.5.4 Functional confirmation of the XX/XY sex determination system through progeny testing

Building upon the molecular evidence obtained from SNP-based genotyping of broodstock, progeny testing provides an essential phenotypic and functional validation of sex determination in snakeskin gourami. While sex-linked SNP markers reliably distinguish genetic sex at the broodstock level, the evaluation of gonadal development and sex ratios in offspring offers direct confirmation of how these genotypes are translated into reproductive outcomes. In the present study, gonadal sex of progeny derived from control males and supermales was successfully identified using the squash method, with histological analysis serving as a confirmatory approach. The high degree of consistency between squash preparations and histological sections demonstrates that gonadal differentiation in snakeskin gourami is sufficiently advanced at three months post-hatching to allow reliable morphological sex identification. Female progeny displayed characteristic ovarian features, including rounded oocytes in squash preparations and ovaries dominated by oogonia and previtellogenic oocytes in histological sections. Conversely, male progeny exhibited dense clusters of spermatogenic cells in squash preparations and well-organized lobular testes containing multiple stages of spermatogenesis. Similar agreement between squash and histological observations has been reported in other teleost species, supporting the utility of this combined approach for juvenile sex assessment (Guerrero and Shelton, 1974; Wang et al., 2008).

Importantly, progeny testing results further substantiated the genetic sex determination pattern inferred from SNP genotyping. Crosses between control XY males and XX females produced progeny with an approximately balanced sex ratio, consistent with Mendelian expectations under an XX/XY system. In contrast, progeny derived from supermales exhibited a uniform male phenotype, with no females detected. This extreme deviation from the expected 1:1 ratio provides compelling functional evidence that supermales act as fertile phenotypic males and transmit a Y-linked sex-determining factor to all offspring.

From an applied perspective, the successful use of the squash method combined with histological validation offers a practical and efficient strategy for large-scale sex identification in breeding programs. When integrated with the SNP-based

genotyping results presented earlier, these findings establish a clear and coherent link between genotype, phenotype, and reproductive outcome. Such concordance across molecular, histological, and progeny-testing approaches greatly strengthens confidence in the inferred XX/XY sex determination system in snakeskin gourami.

5.6 Conclusion

This study establishes a reliable framework for sex control in snakeskin gourami by integrating hormonal manipulation, molecular genotyping, gonadal histology, and progeny testing. Dietary E2 treatment effectively induced stable phenotypic sex reversal without compromising reproductive capacity. Both neofemales and supermales were fully fertile and produced viable offspring with predictable sex ratios, confirming that hormonal sex reversal alters gonadal phenotype while preserving genetic sex. Sex-specific SNP markers (SNP_19, SNP_20, and SNP_21) consistently distinguished genetic sex among control broodstock, neofemales, and supermales, regardless of phenotypic appearance, demonstrating their robustness for broodstock identification and breeding management. In addition, the combined use of the squash method and histological analysis enabled accurate sex identification in juvenile progeny at three months post-hatching. The strong concordance between these approaches indicates that the squash method is a rapid and practical tool suitable for large-scale progeny sex screening.

Collectively, these results provide compelling molecular, phenotypic, and reproductive evidence supporting an XX/XY sex determination system in snakeskin gourami.

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