

Article

Open Access

Onychostoma macrolepis, a cyprinid fish, displays a unique pattern of temperature-dependent sex determination

Tian Gao¹, Fei-Long Wang², Su-Ying Ma¹, Fan Bai¹, Lin-Yao Gan¹, Ming-Hui Li¹, Xing-Yong Liu¹, De-Shou Wang^{1,*}, Li-Na Sun^{1,*}

¹ Integrative Science Center of Germplasm Creation in Western China (CHONGQING) Science City, Key Laboratory of Freshwater Fish Reproduction and Development (Ministry of Education), Key Laboratory of Aquatic Science of Chongqing, Chongqing Technology Innovation Center of Breeding, School of Life Sciences, Southwest University, Chongqing 400715, China

² Anhui Province Key Laboratory of Pollution Damage and Biological Control for Huaihe River Basin, Fuyang Normal University, Fuyang, Anhui 236037, China

ABSTRACT

Current evidence supports high temperature-induced masculinization as the primary temperature-dependent sex determination (TSD) pattern in fish. To date, no study has reported bidirectional TSD in a fish species, in which low temperature induces masculinization while high temperature induces feminization. In this study, *Onychostoma macrolepis* exhibited a distinctive bidirectional TSD pattern, with a balanced 1:1 sex ratio at 24°C (moderate temperature), over 80% female development at 28°C (high temperature), and approximately 75% male development at 20°C (low temperature). Transcriptomic analyses revealed *kdm6bb* and calcium channel genes as key regulators of TSD among the differentially expressed genes between high and low temperature groups. Consistently, administration of calcium influx and *Kdm6bb* inhibitors effectively blocked high temperature-induced feminization and low temperature-induced masculinization, respectively. These findings indicate that temperature perception in *O. macrolepis* is mediated through calcium signaling, which regulates the expression of the epigenetic modifier *kdm6bb* and consequently modulates sex determination. Unexpectedly, canonical male pathway genes such as *dmrt1* and *gsdf* showed higher expression in the high temperature group than in the low temperature group at 50 days after fertilization (daf). Administration of aromatase inhibitors failed to induce sex reversal at either temperature, whereas extremely low concentrations of testosterone/methyltestosterone (T/MT) and 17β-estradiol (E2) successfully induced sex reversal under high and low

temperature conditions, respectively. These results suggest that downstream regulatory pathways controlling sexual differentiation in *O. macrolepis* are distinct from conventional TSD models. The discovery of this bidirectional mechanism establishes *O. macrolepis* as an exceptional model for elucidating the molecular and physiological bases of temperature-driven sex determination and underscores the ecological risks of global warming for aquatic vertebrates.

Keywords: *Onychostoma macrolepis*; TSD; Gonadal transcriptome; Inhibitor treatment; Hormone treatment

INTRODUCTION

Onychostoma macrolepis, a cyprinid fish endemic to China, is recognized for its high percentage of muscle proteins and unsaturated fatty acids (Chen et al., 2019), making it both nutritionally valuable and widely favored as a food fish. Despite its economic and ecological importance, *O. macrolepis* has experienced marked declines in wild populations in recent years, leading to its designation as a Grade II protected species in China in 2021. In response, large-scale artificial breeding initiatives have been implemented in Shaanxi, Sichuan, and other regions in China, with efforts focused on germplasm conservation through habitat protection and stock enhancement. However, marked sex ratio distortions have arisen in aquaculture populations of *O. macrolepis*, with female-to-male ratios as high as 5:1, suggesting the potential for environmental sex determination (ESD). Given that broodstock for artificial breeding were originally captured from wild populations, similar imbalances may occur in natural habitats, potentially disrupting reproductive dynamics and limiting population recovery.

Fish, as the most diverse group of vertebrates, display

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/4.0/>), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Copyright ©2025 Editorial Office of Zoological Research, Kunming Institute of Zoology, Chinese Academy of Sciences

Received: 21 July 2025; Accepted: 02 September 2025; Online: 03 September 2025

Foundation items: This work was supported by the National Key Research and Development Program of China (2022YFD1201600), National Natural Science Foundation of China (32473156, 32530106, 32373106), and Chongqing Fishery Technology Innovation Union (CQFTIU202501-7)

*Corresponding authors, wdeshou@swu.edu.cn; sunlina@swu.edu.cn

extraordinary plasticity in sex determination mechanisms, which can involve both genetic and environmental factors. Temperature-dependent sex determination (TSD) has been documented in at least 77 fish species, 44 of which lack identified sex chromosomes or defined sex-determining genes (Kitano et al., 2024). Three primary patterns of thermally influenced sex determination have been recognized: Type I, the most widespread, involves masculinization at high temperatures and/or feminization at low temperatures (72 species); Type II includes rare cases of feminization at high temperatures (two species) or masculinization at low temperatures (one species); Type III features masculinization at both thermal extremes (two species). To date, no fish species has been reported to exhibit a bidirectional TSD pattern in which low temperatures induce masculinization and high temperatures induce feminization.

In teleosts exhibiting TSD, temperature influences sexual fate through two principal mechanisms: stress hormone-mediated signaling or epigenetic modification. Cortisol, the primary glucocorticoid synthesized by the adrenal cortex/head kidney in vertebrates, rises sharply under thermal stress and is widely considered a central mediator of high temperature-induced masculinization. In pejerrey (*Odontesthes bonariensis*), elevated cortisol levels under high temperatures enhance production of the androgen 11-ketotestosterone (11-KT), a critical driver of masculinization (Hattori et al., 2009). In Japanese flounder (*Paralichthys olivaceus*), cortisol suppresses cAMP-dependent activation of *cyp19a1*, thereby inducing female-to-male sex reversal (Yamaguchi et al., 2010). Similarly, in medaka (*Oryzias latipes*), thermally elevated cortisol inhibits proliferation of female-type germ cells and triggers masculinization of XX individuals through the activation of *gsdf* and *amhr2* (Hara et al., 2021). Epigenetic modification constitutes an additional pathway through which temperature modulates sexual differentiation. In European sea bass (*Dicentrarchus labrax*), high temperature-driven masculinization is associated with hypermethylation of the *cyp19a1a* promoter and transcriptional repression of the gene (Navarro-Martin et al., 2011). In half-smooth tongue sole (*Cynoglossus semilaevis*), ZW females exhibit natural hypermethylation and low expression of *dmrt1*, a pattern that can be erased by high temperature incubation during sex determination, leading to masculinization of ZW females (Chen et al., 2014). More recently, studies on Nile tilapia (*Oreochromis niloticus*) have revealed that temperature modulates the alternative splicing of *kdm6bb*, a histone demethylase gene, thereby altering its regulatory influence on male pathway genes during sex differentiation (Yao et al., 2023).

The present study identified *O. macrolepis* as a cyprinid species exhibiting a unique bidirectional TSD pattern, characterized by high temperature-induced feminization and low temperature-induced masculinization. Comparative transcriptomic analysis between temperature groups revealed *kdm6bb* and calcium channel genes as pivotal regulators of thermal sex differentiation. Pharmacological inhibition of calcium influx and Kdm6bb activity effectively abolished high temperature-induced feminization and low temperature-induced masculinization, respectively. Gonadal transcriptomic profiling combined with sex hormone treatments further demonstrated that estrogen plays an important role in sex differentiation, although the downstream female and male signaling pathways differ markedly from those observed in

conventional TSD models. Collectively, these findings suggest that *O. macrolepis* mediates temperature perception through calcium signaling, thereby regulating the expression of *kdm6bb* and modulating its sex determination.

MATERIALS AND METHODS

Animals

Onychostoma macrolepis fries and adults aged 4 months, 1 year, 2 years, and 4 years were obtained from the Green Water Ecological Agriculture Company (China). Fries were reared in freshwater at 24°C under a natural photoperiod and fed three times a day with a commercial diet (Shengsu, China). All animal experiments were conducted in accordance with the regulations of the Guide for Care and Use of Laboratory Animals and were approved by the Committee of Laboratory Animal Experimentation at Southwest University (IACUC No.20181015-12).

Temperature treatment experiments

Under standard conditions, fries and juveniles were maintained at 24°C. Each experimental group consisted of 50 individuals per tank. Temperature treatment protocols were as follows:

Experiment 1: (1) 20°C-10d: Temperature was reduced to 20°C beginning at 10 days after fertilization (daf) and maintained until 120 daf. (2) 24°C: Temperature was maintained at 24°C throughout. (3) 28°C-10d: Temperature was increased to 28°C from 10 daf and maintained until 120 daf.

Experiment 2: (1) 20°C-10d: Temperature was reduced to 20°C from 10 daf and maintained until 60 daf. (2) 20°C-20d: Temperature was reduced to 20°C from 20 daf and maintained until 60 daf. (3) 20°C-30d: Temperature was reduced to 20°C from 30 daf and maintained until 60 daf. (4) 20°C-40d: Temperature was reduced to 20°C from 40 daf and maintained until 60 daf.

Following temperature treatment, all groups were gradually returned to 24°C.

Inhibitor and hormone treatment experiments

Fries and juveniles were typically maintained at 24°C under standard rearing conditions. For all experiments, each treatment group comprised 50 individuals housed in a single tank. Two experiments were designed to evaluate the effects of specific inhibitors and sex hormones under different temperature conditions:

Experiment 1: (1) 20°C-Control: Temperature was reduced to 20°C from 10 daf and maintained until 60 daf; fish were fed a standard commercial diet. (2) 20°C-GSK-J1: Temperature was reduced to 20°C from 10 daf and maintained until 60 daf; GSK-J1 (100 µg/g diet), an inhibitor of *Kdm6b* that blocks enzymatic activity by targeting 2-oxoglutarate and Fe²⁺ cofactors (Bao et al., 2017; Kruidenier et al., 2012), was incorporated into the feed. (3) 28°C-Control: Temperature was increased to 28°C from 10 daf and maintained until 60 daf; fish were fed a standard commercial diet. (4) 28°C-amlodipine: Temperature was increased to 28°C from 10 daf and maintained until 60 daf; amlodipine (100 µg/g diet), an L-type calcium channel blocker (Mullasser et al., 2024; Nanda et al., 2024; Þorsteinsson et al., 2025), was added to the feed. (5) 28°C-flufenamic acid: Temperature was increased to 28°C from 10 daf and maintained until 60 daf; flufenamic acid (100 µg/g diet), which affects multiple ion channels including

calcium channels (Guinamard et al., 2013; Oh et al., 2008), was included in the feed.

Experiment 2: (1) 20°C-Control: Temperature was reduced to 20°C from 10 daf and maintained until 60 daf; fish received a standard commercial diet. (2) 20°C-E2: Temperature was reduced to 20°C from 10 daf and maintained until 60 daf; 17 β -estradiol (E2, 20 μ g/g diet) was added to the feed. (3) 28°C-Control: Temperature was increased to 28°C from 10 daf and maintained until 60 daf; fish were fed a standard commercial diet. (4) 28°C-MT: Temperature was increased to 28°C from 10 daf and maintained until 60 daf; methyltestosterone (MT, 20 μ g/g diet) was added to the feed. (5) 28°C-T: Temperature was increased to 28°C from 10 daf and maintained until 60 daf; testosterone (T, 20 μ g/g diet) was added to the feed.

Following all treatments, water temperatures were gradually returned to 24°C. All groups were then maintained on a standard commercial diet until sampling. Hormones and inhibitors were dissolved in 95% ethanol, uniformly sprayed onto the feed, thoroughly mixed, air-dried, and administered to the experimental groups. Control groups were fed the same diet treated with 95% ethanol alone.

Gonadal histological examination

Gonads were dissected from randomly sampled fish at 120 daf. Samples were fixed in Bouin's solution for 24 h at room temperature, dehydrated, embedded in paraffin, and sectioned at a thickness of 5 μ m, followed by hematoxylin and eosin (H&E) staining for sex identification. Photographs were taken using an Olympus BX51 light microscope (Japan).

RNA preparation, library construction, sequencing, and assembly

For fish subjected to low (20°C) and high (28°C) temperature treatment, gonads were collected at 30 and 50 daf. Due to the extremely small size of the gonads at these stages, dissection included the peritoneal region. Gonads from 10 individuals were pooled per sample, with three biological replicates collected for each time point. Total RNA was extracted using TRIzol reagent, following the manufacturer's protocols. RNA quality was assessed using a NanoPhotometer® spectrophotometer. cDNA libraries were constructed using the NEBNext® Ultra™ RNA Library Construction Kit (NEB, USA). Poly(A) mRNA was enriched with oligo(dT) magnetic beads and fragmented using fragmentation buffer. Double-stranded cDNA was synthesized using the Invitrogen cDNA Synthesis Kit (Invitrogen, USA). After repair of the 3' ends, DNA was connected to the Illumina sequencing adapter. Library fragments were purified using the AMPure XP system (Beckman Coulter, USA) to select 370–420 bp cDNA fragments. Polymerase chain reaction (PCR) was then performed using Phusion High-Fidelity DNA Polymerase, universal PCR primers, and index (X) primers. Finally, the PCR products were purified using the AMPure XP system and library quality was assessed using an Agilent 2100 Bioanalyzer. Libraries meeting quality criteria were pooled based on effective concentration and desired sequencing depth and sequenced using the Illumina platform with 150 bp paired-end reads.

Low-quality reads were removed prior to downstream analysis. Clean reads were aligned to the *O. macrolepis* reference genome (GCF_012432095.1_ASM1243209v1) using HISAT2 with default parameters. Gene-level read counts were obtained using Feature Counts v.1.5.0-p3. Gene

expression levels were quantified as fragments per kilobase of transcript per million mapped reads (FPKM) based on gene length and mapped read counts (Trapnell et al., 2010). Differential expression analysis between ovaries and testes at each stage was performed using DESeq2 (Love et al., 2014), with $|\log_2(\text{fold change})| > 2$ and $P_{\text{adj}} < 0.05$ used to define significantly differentially expressed genes (DEGs). Volcano plots, Gene Ontology (GO) enrichment, and heatmaps were generated via the Bioinformatics online platform (<https://www.bioinformatics.com.cn>; accessed 10 December 2024).

For *O. macrolepis* at four additional developmental stages (4 months, 1 year, 2 years, and 4 years), gonads were dissected and divided into two portions. A small portion was fixed, embedded, sectioned, and stained with H&E for sex identification. The remaining tissue was flash-frozen in liquid nitrogen and stored at -80°C for RNA analysis. For each stage, ovaries and testes from three individuals were pooled as one sample, with three biological replicates per age group. RNA extraction, quality assessment, and sequencing procedures were performed as described above.

Quantitative real-time polymerase chain reaction (qPCR) at different gonadal development stages

Gonads were collected from fish exposed to low (20°C) and high (28°C) temperature treatments at two developmental stages (30 and 50 daf, $n=3$ individuals per group per stage) for qPCR analysis. Total RNA was isolated using RNAiso Plus (Takara, China) following the manufacturer's instructions and treated with DNase I (RNase free) (Invitrogen, USA). RNA concentrations were quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). First-strand cDNA was synthesized using the PrimeScript RT Master Mix Perfect Real Time Kit (Takara, China) in accordance with the manufacturer's instructions. qPCR was conducted on the ABI 7500 Real-Time PCR System (Applied Biosystems, Germany) using SYBR Premix Ex Taq™ II (Takara, China). Relative mRNA expression levels were evaluated using the $2^{-\Delta\Delta C_t}$ approach (Livak & Schmittgen, 2001). Primer sequences used for qPCR are listed in Supplementary Table S1.

Fluorescence *in situ* hybridization (FISH)

Gonads were dissected from fish at 30 and 50 daf, fixed overnight at 4°C in 4% paraformaldehyde (PFA) prepared in 0.1 mol/L phosphate buffer (pH 7.4), and embedded in paraffin. Tissue sections were cut at a thickness of 5 μ m. Gonadal sex was confirmed by H&E staining and microscopic examination. Sense and antisense digoxigenin-labeled RNA probes were transcribed *in vitro* from linearized pGEM-T Easy-cyp19a1a/kdm6bb cDNA using an RNA Labeling Kit (Roche, Germany). Rabbit polyclonal antibodies against Vasa (2 mg/mL, 1:1 000), previously generated and validated (Jiang et al., 2016), were used for germ cell identification. FISH was performed as described previously (Li et al., 2020).

Statistical analysis

Gene expression data are presented as mean $\pm$ standard deviation (SD). Two-tailed independent Student's *t*-test was used to determine significant differences between two groups. One-way analysis of variance (ANOVA), followed by Tukey multiple comparison, was chosen for tests involving more than two groups. Statistical analyses were performed using GraphPad Prism v.8.0.2 (GraphPad Software, USA). In all analyses, $P < 0.05$ was considered to be significantly different.

RESULTS

Identification of TSD and critical period for low temperature-induced masculinization in *O. macrolepis*

Gonadal development was assessed histologically from 15 to 60 daf (Supplementary Figure S1). No discernible morphological differences were observed between the sexes at 15 or 30 daf (Supplementary Figure S1A, B). By 45 daf, ovarian cavities became visible, followed by the onset of meiosis at 50 daf and the appearance of oocytes at 60 daf. In contrast, testes at the same stages had not yet initiated meiosis, although germ cell numbers increased progressively from 45 to 60 daf (Supplementary Figure S1C–E). To evaluate TSD, fries were reared at 24°C until 10 daf and then transferred to 20°C, 24°C, or 28°C (Figure 1A). Sex was determined at 120 daf based on gonadal histology. A balanced 1:1 sex ratio was observed at 24°C, whereas rearing at 20°C resulted in an exceptionally high proportion of males (>75%), and at 28°C resulted in an exceptionally high proportion of females (>80%) (Figure 1B). At 120 daf, male gonads contained abundant spermatogonia, while female gonads were predominantly composed of early-stage oocytes. Temperature treatments did not visibly alter gonadal morphology in either sex (Figure 1C).

To define the sensitive period for low temperature-induced masculinization, a series of 20°C exposure windows were applied from 10 to 60 daf (Figure 1D). Results showed that low-temperature treatment from 10 to 60 daf and from 20 to 60 daf consistently induced male-biased sex ratios exceeding 75%, with no obvious difference between the two groups. However, exposure from 30–60 daf yielded a reduced

proportion of males, and treatment from 40–60 daf resulted in an approximately equal sex ratio (Figure 1E). These results delineate a critical window for low temperature-induced masculinization between 20 and 40 daf in *O. macrolepis*.

Screening of key genes underlying TSD in *O. macrolepis*

Transcriptome profiling identified 606 and 1 244 DEGs in the gonads of fish reared at high (28°C) and low (20°C) temperatures at 30 and 50 daf, respectively. Among these, 337 and 704 genes up-regulated under high temperature, while 269 and 540 were up-regulated under low temperature at the respective time points (Figure 2A, B). Comparative analysis revealed 259 co-DEGs shared across both developmental stages (Figure 2C). GO enrichment analysis of these co-DEGs showed significant associations across biological processes (BP), molecular functions (MF), and cellular components (CC). Enriched BP terms included developmental processes, hormone response, reproductive regulation, molecular function modulation, connective tissue development, hormone level regulation, transmembrane transport, and temperature stimulus response. Enriched MF terms were primarily associated with extracellular space, extracellular regions, hemoglobin complexes, and extracellular matrix components. Enriched CC terms included transmembrane transporters, hormones, voltage-gated channels (especially calcium channels), molecular function suppression, protein binding, and receptor-ligand activity (Figure 2D). Notably, several functionally relevant genes, including female pathway genes (*foxl2a* and *foxl2b*), key steroidogenic enzyme genes (*cyp19a1a*, *hsd17b1*, and *cyp11c1*), epigenetic factor mediating TSD (*kdm6bb*), and

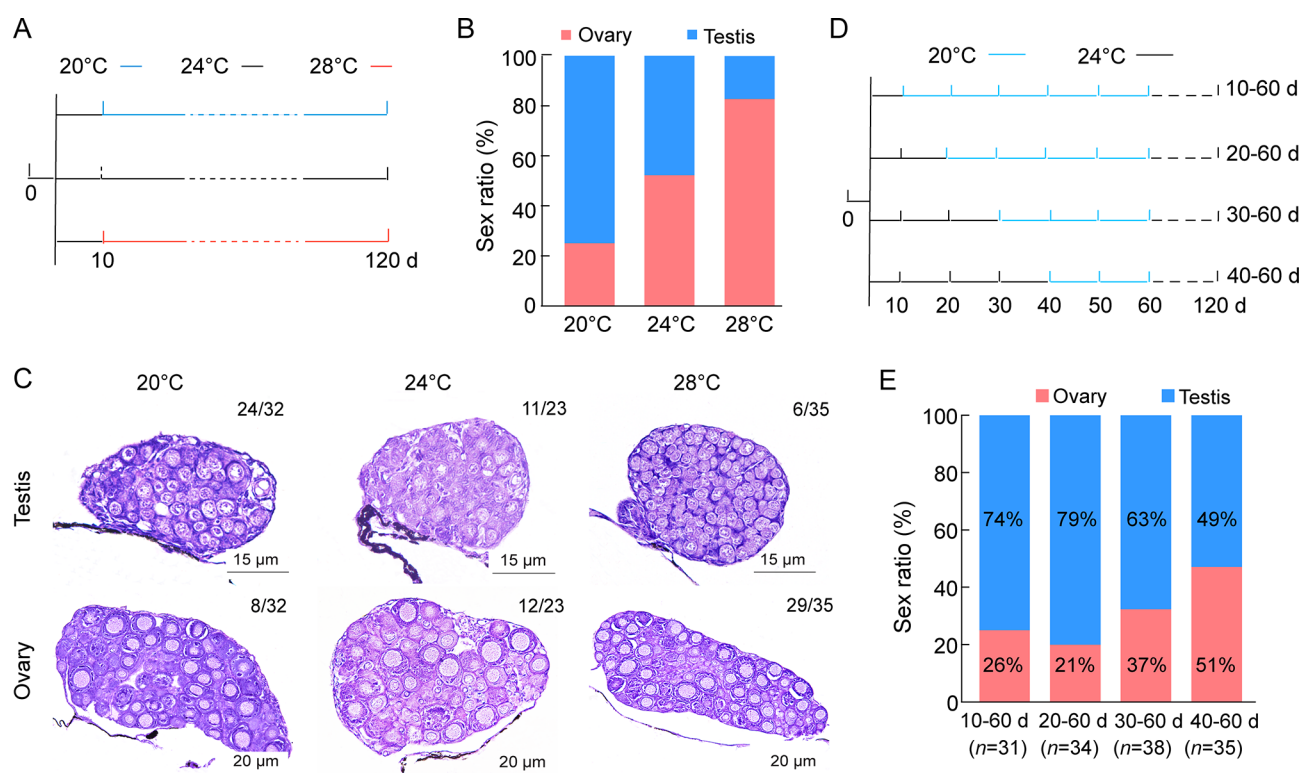


Figure 1 Identification of temperature-dependent sex determination (TSD) and the critical period for low temperature-induced masculinization in *Onychostoma macrolepis*

A, B: Temperature gradient experiments and resultant sex ratio at 120 daf showing that high temperature-induced feminization and low temperature-induced masculinization. C: Gonadal histology of fish (120 daf) from temperature gradient experiments. D, E: Low temperature treatments with different time windows and resultant sex ratio at 120 daf. daf: Days after fertilization.

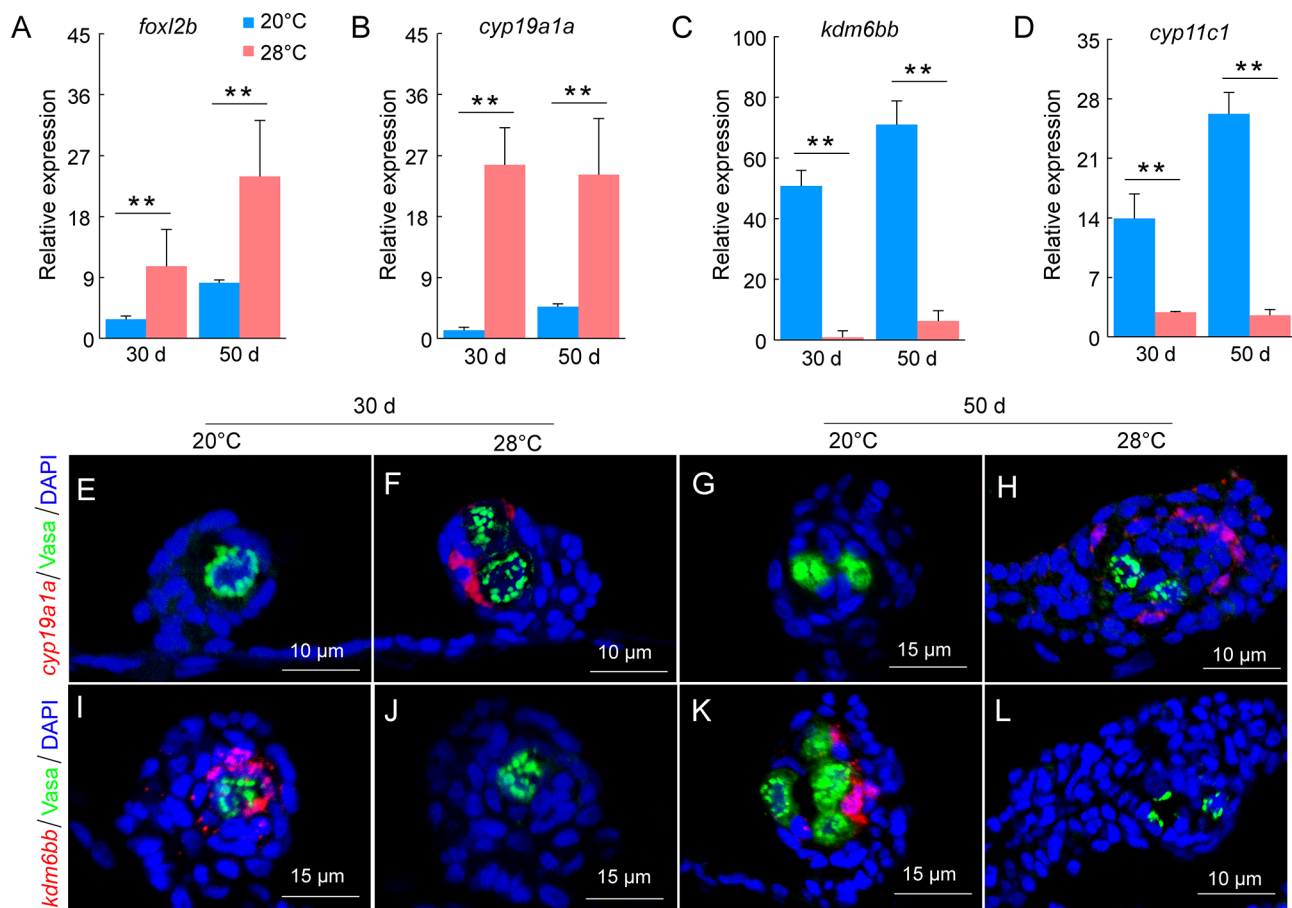


Figure 3 Validation of differentially expressed genes (DEGs) in the gonads of *Onychostoma macrolepis* based qPCR and FISH analyses

A–D: Expression levels of *foxl2b*, *cyp19a1a*, *kdm6bb*, and *cyp11c1* were verified by qPCR in the gonads of high- and low-temperature groups at 30 and 50 daf. Expression was normalized to β -actin. Values are presented as mean $\pm$ SD ($n=3$). **: $P<0.01$ by two-tailed independent Student's t -test. E–H: FISH analysis showing *cyp19a1a* mRNA expression in the gonads of the high-temperature group. Red fluorescence represents *cyp19a1a* signaling. I–L: FISH analysis showing *kdm6bb* mRNA expression in the gonads of the low-temperature group. Red fluorescence represents *kdm6bb* signaling. Green fluorescence represents Vasa signaling. Nuclei were counterstained with DAPI.

during the TSD-sensitive period. Notably, the canonical male pathway genes *dmrt1*, *amh*, and *gsdf* did not show elevated expression at low (male-inducing) temperature during the TSD-critical period but were up-regulated during later testicular differentiation and were paradoxically more highly expressed under high (female-inducing) temperature during the TSD window. The epigenetic regulator *kdm6bb* was consistently enriched at low temperature during early sex differentiation and maintained testis-biased expression through later development (Figure 4A). In contrast, its paralog *kdm6ba* exhibited low expression without significant temperature-dependent differences (Supplementary Figure S2). The chromatin-associated gene *hmgb3a*, involved in gametogenesis in mammals, was strongly and persistently up-regulated under low temperature in males. Other meiosis marker genes, including *dazl*, *ddx4*, and *sycp3*, initially displayed female-biased expression but shifted to testis-specific enrichment with spermatogenesis (Figure 4B). High temperature exposure induced robust expression of heat shock response genes *dnaja*, *hsp70*-like, and *hsp90aa1.2* during the TSD-critical period, while *dnaja* lost sexual dimorphism at later stages and *hsp90aa1.2* remained testis-biased (Figure 4C). Voltage-gated calcium channel genes *cacng3b*, *cacng5a*, and *cacng6b* were predominantly expressed in testicular tissue throughout all examined stages (Figure 4D). Estrogen synthesis enzymes *hsd17b1* and

cyp19a1a showed female-specific up-regulation under high temperature during the TSD critical period and persisted thereafter. Conversely, *cyp11c1*, a key androgen synthesis enzyme gene, was predominantly expressed in males. Although *ar*, *esr2a*, and *esr2b* were expressed in both thermal groups during early development, all three showed higher expression in the testes than in the ovaries at later stages (Figure 4E). In addition, *stat3* expression was elevated in high-temperature gonads at both 30 and 50 daf (Supplementary Figure S3), highlighting *stat3* as a thermosensitive gene during the TSD window.

Calcium signaling and *kdm6bb* mediate TSD in *O. macrolepis*

To functionally assess the roles of calcium signaling and *kdm6bb* in TSD, pharmacological inhibitor experiments were conducted (Figure 5A). Administration of the Kdm6bb inhibitor GSK-J1 at low temperature significantly shifted the sex ratio, increasing the proportion of females from 20% in the control group to 56% (Figure 5B). Conversely, inhibition of calcium influx at high temperature increased the proportion of males from 25% in the control group to 56% and 74% in the flufenamic acid and amlodipine treatment groups, respectively (Figure 5C). At 120 daf, testicular tissue from all inhibitor-treated males (GSK-J1, flufenamic acid, and amlodipine) appeared histologically indistinguishable from that of the

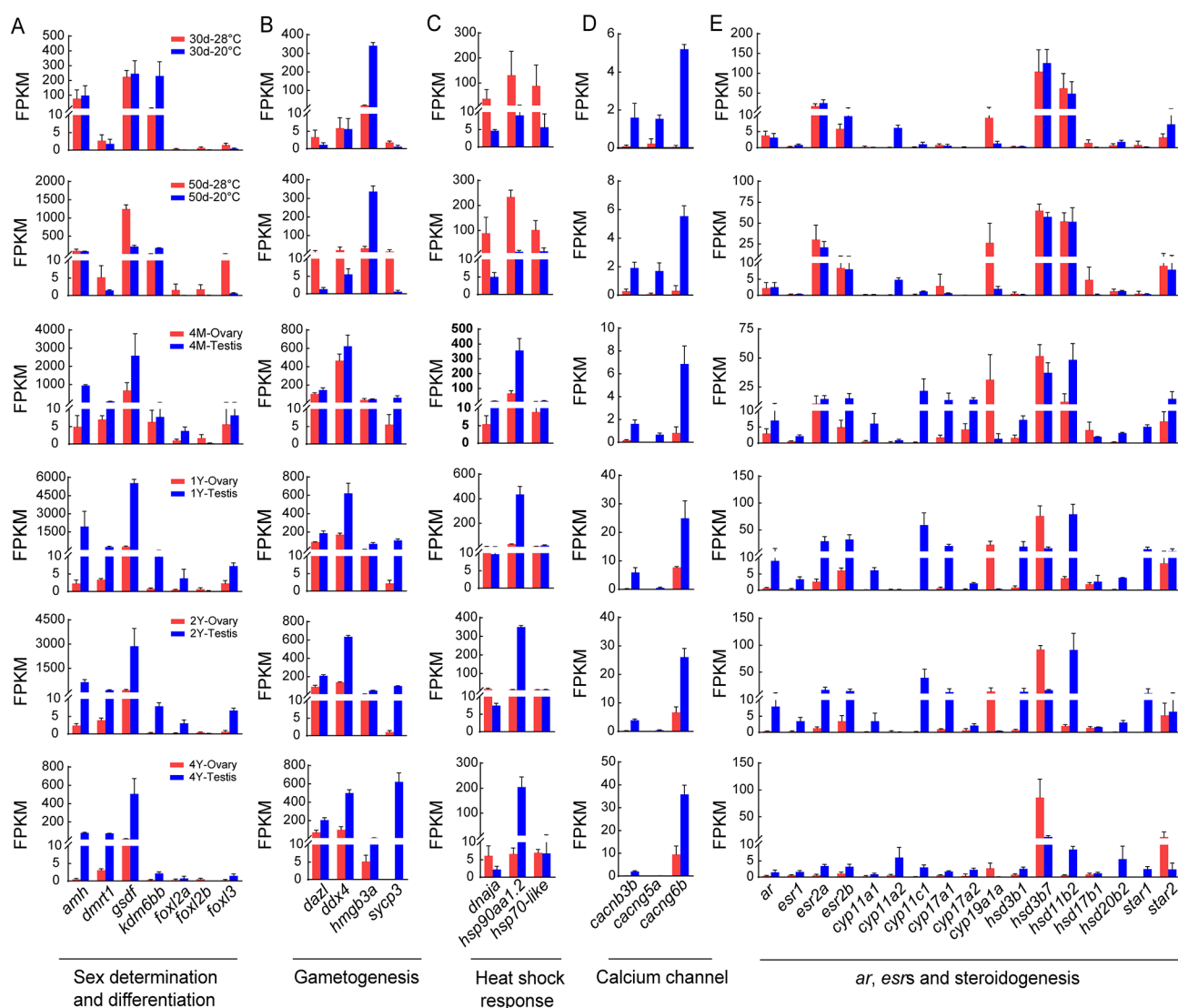


Figure 4 FPKM of genes involved in sex determination and differentiation, heat shock response, calcium channel, and steroidogenesis (including *esrs* and *ar*)

FPKM, fragments per kilobase of transcript per million mapped reads. Values are presented as mean $\pm$ SD ($n=3$). 4M: 4 months old; 1, 2, and 4Y: 1, 2, and 4 years old.

corresponding controls. However, a subset of ovaries from GSK-J1-treated fish exhibited atypical testis-like morphology, characterized by rounded profiles and the absence of ovarian cavities, yet still contained developing oocytes. Similar structural alterations were observed in ovaries from calcium influx inhibitor-treated fish (Figure 5D).

Estrogen modulates TSD in *O. macrolepis*

To further investigate the role of estrogen in gonadal fate under thermal influence, exogenous hormone treatments were administered (Figure 6A). At low temperature, E2 exposure successfully counteracted masculinization, increasing the proportion of females from 20% in controls to 50%; the remaining individuals exhibited undeveloped gonads (Figure 6B). Aromatase inhibitor (letrozole and exemestane) administration produced 77% and 24% males at low and high temperatures, respectively (Supplementary Table S2). Conversely, androgen administration at high temperature reversed feminization, producing over 75% males relative to 20% in untreated controls (Figure 6B). Gonadal histological examination at 120 daf revealed that androgen-administered fish exhibited testes with significantly increased

spermatogonia populations compared to the control, while estrogen-administered fish showed reduced ovarian size. In addition, fish treated with androgens showed testis-like ovarian morphology without ovarian cavities. Notably, hormone administration also resulted in undeveloped gonads characterized by complete depletion of germ cells (Figure 6C). qPCR analysis demonstrated marked down-regulation of steroidogenic pathway genes (*star1*, *cyp11a1*, *cyp11c1*, *hsd3b1*, *cyp17a1*, and *cyp19a1a*) in androgen-treated testes, with MT exhibiting stronger inhibitory effects than T. Notably, *dmrt1* was significantly up-regulated in the T-treated group but not in the MT-treated group, potentially reflecting a higher incidence of underdeveloped gonads in the latter. Expression of *kdm6bb* remained consistently elevated in all androgen-treated groups, comparable to levels in normal males (Figure 6D).

DISCUSSION

Unique bidirectional TSD pattern in *O. macrolepis*

TSD is typically categorized into three major patterns: Type I,

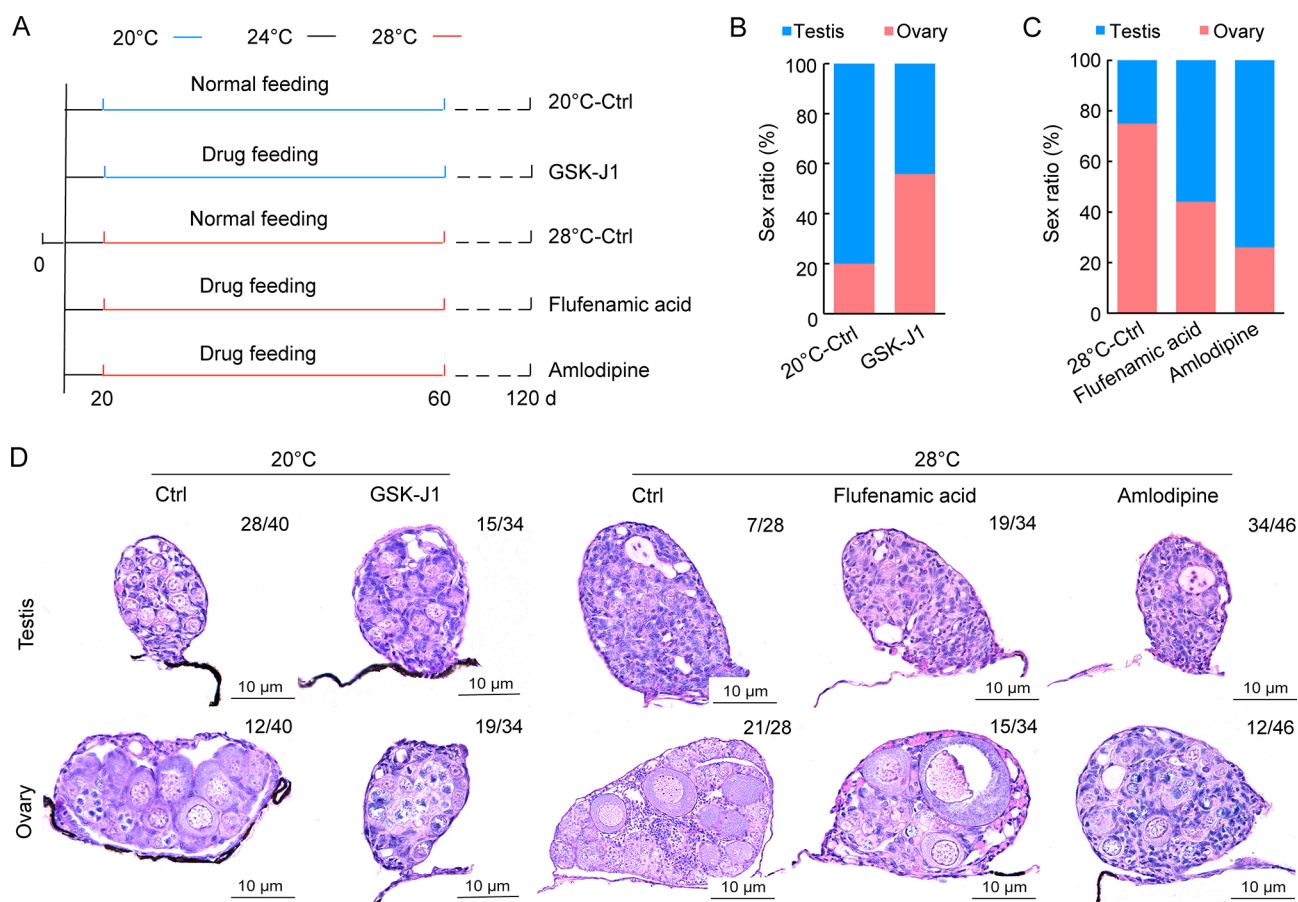


Figure 5 Inhibition of calcium influx and Kdm6bb blocked the feminization and masculinization of *Onychostoma macrolepis* induced by high and low temperature, respectively

A: Fish raised at 28°C were supplementally fed calcium influx inhibitors, flufenamic acid, or amlodipine, while fish raised at 20°C were supplementally fed a Kdm6bb inhibitor (GSK-J1). Both the 20°C and 28°C control groups were fed a normal diet. B, C: Sex ratio of each group at 120 daf. D: Gonadal histology and quantification of each group at 120 daf. Ctrl: Control.

characterized by masculinization at high temperature and/or feminization at low temperature; Type II, characterized by feminization at high temperature or masculinization at low temperature; and Type III, marked by masculinization at both thermal extremes. Among these, Type I is by far the most prevalent, accounting for approximately 90% of all reported TSD cases in fish (Kitano et al., 2024). In contrast, only a few species exhibit Type II or III responses, including channel catfish (*Ictalurus punctatus*) (Bao et al., 2019), fugu (*Takifugu rubripes*) (Zhou et al., 2019), and dwarf gourami (*Trichogaster lalius*) (Ramee et al., 2020), which exhibit high temperature-induced feminization or low temperature-induced masculinization (Type II), and *Onychostoma barbatulum* (Tseng et al., 2017), Japanese flounder (Yamamoto, 1999), and southern flounder (*Paralichthys lethostigma*) (Type III) (Montalvo et al., 2012). All known Type II examples involve unidirectional temperature effects on sex ratios. In contrast, *O. macrolepis* exhibited a previously unreported bidirectional TSD pattern characterized by low temperature-induced masculinization and high temperature-induced feminization, with approximately 75% males at 20°C, 50% males and 50% females at 24°C, and over 80% females at 28°C. While similar TSD patterns have been observed in reptiles, such as the red-eared slider (*Trachemys scripta*), which produces exclusively male or female offspring under extreme temperatures, the response in *O. macrolepis* is incomplete—male individuals still occurred at high female-producing temperatures and vice

versa. This incomplete bias suggests the potential existence of a cryptic genetic sex determination (GSD) system that maintains a baseline proportion of each sex regardless of environmental input. Alternatively, the temperatures applied in this study may have been insufficient to trigger complete sex reversal. Broader thermal gradients and genomic investigations are needed to determine whether *O. macrolepis* possesses a mixed GSD-TSD system or a highly plastic environmental mechanism. Moreover, the rarity of this bidirectional TSD pattern among teleosts raises important questions about its evolutionary origin and ecological significance.

Calcium signaling and Kdm6bb mediate TSD in *O. macrolepis*

Current studies suggest that TSD in fish is primarily mediated by either cortisol-mediated signaling or epigenetic modification. Under thermal stress, elevated cortisol levels typically drive male differentiation by activating genes that initiate testis development and/or suppress the expression of female pathway genes. In *O. macrolepis*, *cyp11c1* was markedly upregulated at low temperature, whereas *cyp21a*, a key gene in cortisol biosynthesis, was nearly undetectable at both 30 and 50 daf. This expression pattern indicates that androgen 11-KT, instead of cortisol pathway, might be involved in the low temperature-induced male sex determination. Epigenetic regulation, including DNA

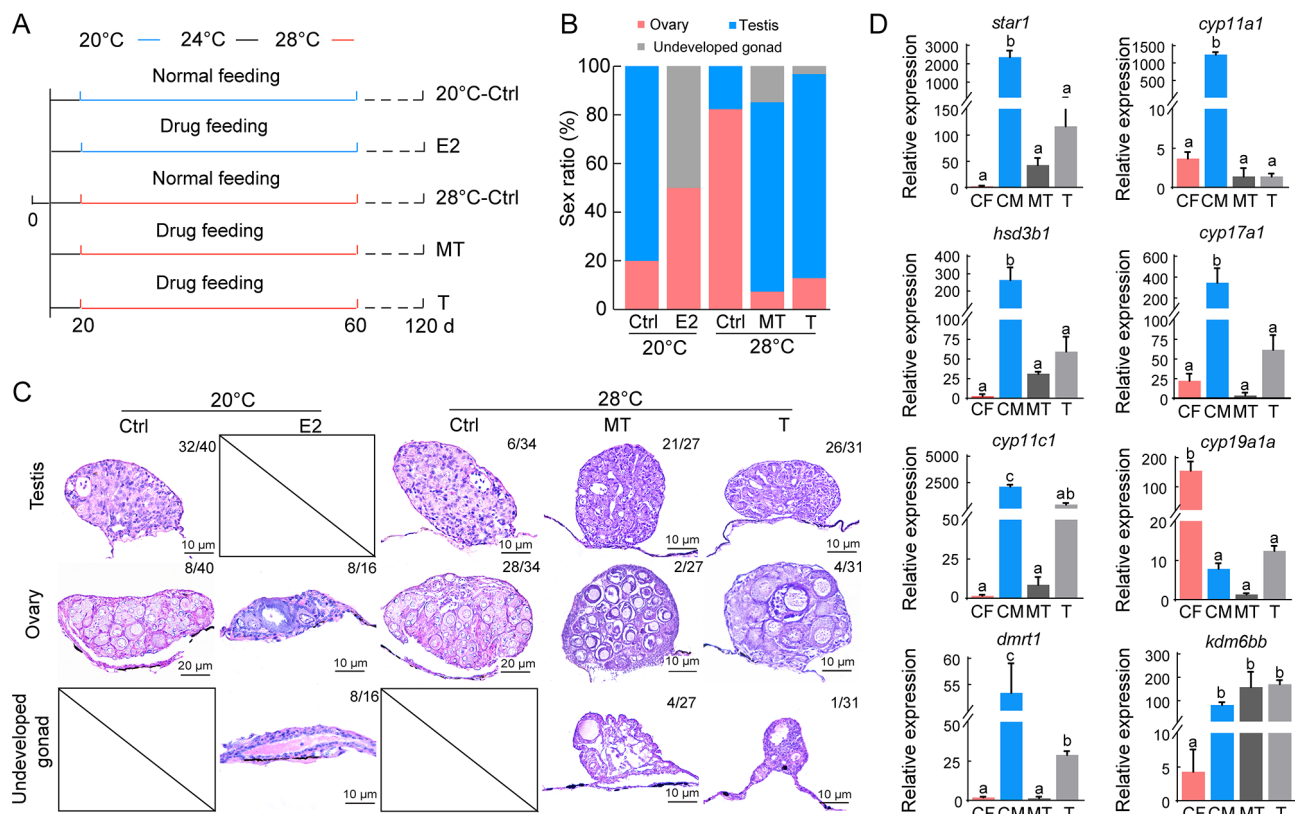


Figure 6 Androgen and estrogen administration rescued high- and low-temperature-induced feminization and masculinization in *Onychostoma macrolepis*, respectively

A: Fish raised at 28°C were supplementally fed androgen, MT, and T, while fish raised at 20°C were supplementally fed estrogen, E2. Both the 20°C and 28°C control groups were fed a normal diet. B: Sex ratio of each group at 120 daf. C: Gonadal histology and quantification of each group at 120 daf. D: qPCR analysis of steroid synthase, *dmrt1*, and *kdm6bb* in the gonads of control females, MT-treated fish, T-treated fish, and control males at 120 daf. Expression was normalized to β -actin. Data are expressed as mean $\pm$ SD. Different letters above error bar indicate statistical differences at $P < 0.05$ as determined by one-way ANOVA followed by Tukey test. E2: 17 β -estradiol; MT: Methyltestosterone; T: Testosterone; Ctrl: Control; CF: Control female; CM: Control male.

methylation and histone modification, has been implicated in TSD across multiple species (Chen et al., 2014; Navarro-Martín et al., 2011; Yao et al., 2023). Recently, alternative splicing of *kdm6bb* has been suggested to play a critical role in temperature-sensitive sex differentiation in Nile tilapia (Yao et al., 2023). Although such splicing variation was not detected in *O. macrolepis*, transcriptomic analysis identified *kdm6bb* as significantly up-regulated in the gonads of low temperature-induced males. Functional experiments further demonstrated that inhibition of calcium influx suppressed high temperature-induced feminization, while inhibition of Kdm6bb enzymatic activity blocked low temperature-induced masculinization. These results indicate that thermal cues in *O. macrolepis* are perceived via calcium signaling, which regulates the expression of the epigenetic modifier *kdm6bb*, thereby directing sex-specific developmental trajectories. This mechanistic framework mirrors that of the red-eared slider turtle, a model TSD species in which high temperatures trigger calcium-dependent phosphorylation of STAT3, leading to suppression of *Kdm6b* and downstream inhibition of the male pathway gene *Dmrt1* (Ge et al., 2018; Weber et al., 2020). Although differential phosphorylation of Stat3 could not be confirmed at the protein level in this study, transcriptomic data from *O. macrolepis* revealed significantly higher *stat3* expression in gonads under high temperature at both 30 and 50 daf, supporting a potential role for *stat3* in temperature-sensitive gonadal regulation. This constitutes the first reported

case in fish where the molecular framework of TSD aligns with that established in reptiles, notably the red-eared slider turtle. In that species, transient receptor potential (TRP) channels such as TRPV4 are up-regulated during the early thermosensitive window at feminizing temperatures (31°C) (Weber et al., 2020), contributing to calcium influx that modulates downstream signaling. In *O. macrolepis*, several calcium channel subunit genes (*cacng3b*, *cacng6b*, and *cacng5a*) were up-regulated under low temperature, male-producing conditions. However, the functional contribution of these specific subunits to calcium dynamics and their potential regulatory role in the thermal sensitivity of sex determination in *O. macrolepis* remains to be elucidated.

Distinct downstream pathways of female and male differentiation in the TSD of *O. macrolepis*

Estrogen is a pivotal regulator of sex determination and differentiation in fish, irrespective of whether the mechanism follows genetic or environmental control (Nagahama et al., 2021). Early transcriptomic analyses in tilapia revealed that *cyp19a1a*, which encodes aromatase—the key enzyme catalyzing estrogen biosynthesis—and *esrs* genes are expressed in XX gonads prior to sexual differentiation (Tao et al., 2013). In *O. macrolepis*, gonadal transcriptomic analyses showed that *cyp19a1a* and its upstream regulators *foxl2a* and *foxl2b* were strongly up-regulated under high temperature at 30 daf, coinciding with the critical window for

TSD. Estrogen receptor genes were expressed at comparable levels in both temperature groups at this stage, and exogenous estrogen exposure induced complete feminization even under masculinizing low temperature. Conversely, androgen treatment induced masculinization by broadly repressing steroidogenic gene expression, particularly *cyp19a1a*, consistent with mechanisms described in tilapia (Bhandari et al., 2006). In cultured HEK293 cells, MT has been shown to inhibit both basal and Sf-1-activated *cyp19a1a* promoter activity via ARa and ARb (Dai et al., 2024), whereas in Nile tilapia, Foxl2 enhances and Dmrt1 suppresses Sf-1-dependent activation of the same promoter (Wang et al., 2007, 2010). Similarly, in the red-eared slider turtle, Foxl2 exhibits temperature-dependent, female-specific expression prior to gonadal differentiation, with functional assays showing that Foxl2 overexpression under male-producing temperatures triggers male-to-female sex reversal, whereas Foxl2 knockout under female-producing temperatures leads to masculinization, accompanied by either strong activation or suppression of endogenous estrogen synthesis (Ma et al., 2022; Wu et al., 2024). Collectively, these findings indicate that the regulation of estrogen biosynthesis represents a conserved axis governing gonadal fate across both GSD and TSD systems.

However, two central issues remain to be clarified regarding TSD in *O. macrolepis*. First, the male-associated genes *dmrt1* and *gsdf* exhibited unexpectedly higher expression under high temperature at 50 daf. The biological significance in this atypical expression pattern is intriguing and critical. Whether these genes potentially involved in bipotential gonad development or playing other non-canonical roles at early stages of *O. macrolepis* are unknown. Moreover, inhibition of aromatase using letrozole and exemestane failed to induce female-to-male sex reversal at either temperature, while exogenous E2 treatment at low temperature successfully induced feminization. Whether estrogen plays a critical role in TSD of *O. macrolepis* needs further investigation. These findings suggest that the downstream female and male pathways might be unique for TSD in *O. macrolepis*. Further research on the signaling cascade connecting epigenetic modifiers and key gonadal differentiation factors are needed to reveal the molecular mechanism for such a unique TSD pattern.

Adaptive significance of TSD and implications for conservation of wild *O. macrolepis* population

The Charnov-Bull model provides the most widely accepted theoretical framework for understanding ESD, proposing that ESD is adaptive when fitness is influenced by an interaction between sex and environmental conditions (Charnov & Bull, 1977). In Atlantic silversides (*Menidia menidia*), for instance, offspring hatching early in the breeding season develop as females under low temperatures, whereas those hatching later in warmer waters differentiate as males. Because females attain larger body sizes and early-hatched individuals have longer growth periods, this thermal pattern enhances reproductive fitness by optimizing female growth and fecundity (Conover & Fleisher, 1986; Conover & Kynard, 1984). A similar principle applies to sea turtles, where TSD is thought to confer adaptive flexibility under climatic stress. High incubation temperatures elevate embryo and hatchling mortality in sea turtles, and TSD may compensate for this loss by elevating the proportion of female offspring, yet

simultaneously bias sex ratios toward females, buffering the impacts of adverse climatic conditions and enhancing future reproductive potential (Hays et al., 2014; Santidrián Tomillo et al., 2015; Santidrián Tomillo & Spotila, 2020; Valverde et al., 2010).

In *O. macrolepis*, females mature later (4–5 years) and attain larger body sizes than males, which typically mature within 2–3 years. Spawning occurs from April to July, when ambient water temperatures fluctuate between 17°C and 26°C (Qu et al., 2019), overlapping with peak seasonal warming during juvenile growth. The observed TSD pattern—favoring female differentiation at elevated temperatures—may thus serve an adaptive function analogous to that in sea turtles, increasing the proportion of females under thermally stressful conditions to secure future reproductive capacity. Nevertheless, temperature influences a wide range of developmental and physiological processes beyond gonadal differentiation, shaping traits that affect survival, metabolism, and life-history strategies across the lifespan (Noble et al., 2018). Consequently, elucidating the adaptive significance of TSD in *O. macrolepis* will require integrative studies encompassing not only reproductive output but also growth performance, thermal tolerance, and other temperature-sensitive fitness traits relevant to population persistence under ongoing climatic change. In this study, undeveloped gonads were observed in a subset of *O. macrolepis* individuals from all hormone-treated groups, most likely resulting from excessive steroid exposure. Similar pathological outcomes caused by high or prolonged MT exposure have been observed in many fish species (Abed Elmdoust et al., 2011; El-Greisy & El-Gamal, 2012; Fatima et al., 2016; Luckenbach & Fairgrieve, 2016; Mohammad Nejad & Mousavi Sabet, 2022; Pavlov et al., 2016; Turan et al., 2006). However, reports of comparable effects induced by excessive estrogen remain rare. The observed developmental arrest may reflect either disruption of endogenous endocrine signaling by exogenous steroids or direct cytotoxicity to gonadal tissue (Devlin & Nagahama, 2002). A clear depletion of germ cells was observed in the undeveloped gonads of *O. macrolepis* following E2 and MT treatment, which implying the toxic effects of high-dose steroids on germ cells. Such outcomes likely arise from interference with the hypothalamus-pituitary-gonad (HPG) axis, impairing gonadal differentiation and maturation (Liu et al., 2023). The molecular basis of this developmental inhibition under excessive or prolonged E2 or MT exposure remains to be elucidated.

In Nile tilapia, typical concentrations of MT and E2 used to induce sex reversal reach approximately 150 µg/g diet (Shi et al., 2017), whereas in other teleosts, hormone concentrations as low as 50 µg/g diet rarely cause gonadal regression (Liu et al., 2021; Nagy et al., 1981). In contrast, exposure of *O. macrolepis* to only 20 µg/g diet of T and MT still resulted in undeveloped gonads, indicating an exceptional sensitivity to hormonal perturbation. Severe sex ratio deviations have emerged during aquaculture practices, with female-to-male ratios reaching up to 5:1 in *O. macrolepis*. As broodstock for artificial breeding were captured from wild populations, which implies that wild populations may also suffer from sex ratio imbalance, potentially contributing to the reduction in the replenishment of resources for this species in its natural habitat. For the effective conservation of *O. macrolepis*, it is critical to integrate habitat temperature monitoring with comprehensive screening for environmental

endocrine disruptors in wild habitats. Collectively, these findings establish *O. macrolepis* as a powerful model for elucidating temperature-driven mechanisms of sex determination and underscore the broader ecological risks posed by climate warming and anthropogenic interference in aquatic ecosystems.

DATA AVAILABILITY

All raw sequencing data generated in this study have been submitted to the NCBI SRA database (BioProjectID PRJNA1225507), Genome Sequence Archive (GSA) of the China National Center for Bioinformation (CRA048404), and Science Data Bank (10.57760/sciencedb.j00139.00248).

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTION

D.S.W. and L.N.S. conceived and designed the experiments and edited the manuscript; T.G., F.L.W., and S.Y.M. performed the experiments; L.Y.G. and F.B. analyzed the data; M.H.L. and X.Y.L. contributed to animal experimental resources; T.G. drafted the manuscript. All authors read and approved the final version of the manuscript.

REFERENCES

Abed Elmdoust AR, Farahmand H, Rafiee G, et al. 2011. Masculinization of blue hap (*Sciaenochromis ahlil*) treated with 17 α -methyltestosterone. *Journal of Agricultural Science and Technology*, **13**: 173–180.

Bao BE, He YZ, Tang DM, et al. 2017. Inhibition of H3K27me3 histone demethylase activity prevents the proliferative regeneration of zebrafish lateral line neuromasts. *Frontiers in Molecular Neuroscience*, **10**: 51.

Bao LS, Tian CX, Liu SK, et al. 2019. The Y chromosome sequence of the channel catfish suggests novel sex determination mechanisms in teleost fish. *BMC Biology*, **17**(1): 6.

Bhandari RK, Nakamura M, Kobayashi T, et al. 2006. Suppression of steroidogenic enzyme expression during androgen-induced sex reversal in Nile tilapia (*Oreochromis niloticus*). *General and Comparative Endocrinology*, **145**(1): 20–24.

Charnov EL, Bull J. 1977. When is sex environmentally determined?. *Nature*, **266**(5605): 828–830.

Chen SL, Zhang GJ, Shao CW, et al. 2014. Whole-genome sequence of a flatfish provides insights into ZW sex chromosome evolution and adaptation to a benthic lifestyle. *Nature Genetics*, **46**(3): 253–260.

Chen SW, Chen YY, Qu GS. 2019. Analysis and evaluation of nutritional composition of *Onychostoma macrolepis* in Qinling-Bashan Mountain area. *Biotic Resources*, **41**(2): 112–118. (in Chinese)

Conover DO, Fleisher MH. 1986. Temperature-sensitive period of sex determination in the Atlantic silverside, *Menidia menidia*. *Canadian Journal of Fisheries and Aquatic Sciences*, **43**(3): 514–520.

Conover DO, Kynard BE. 1984. Field and laboratory observations of spawning periodicity and behavior of a northern population of the Atlantic silverside, *Menidia menidia* (Pisces: Atherinidae). *Environmental Biology of Fishes*, **11**(3): 161–171.

Dai SF, Li M, Yuan J, et al. 2024. *dmrt1* is responsible for androgen-induced masculinization in Nile tilapia. *Genes*, **15**(9): 1238.

Devlin RH, Nagahama Y. 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture*, **208**(3–4): 191–364.

El-Greisy ZA, El-Gamal AE. 2012. Monosex production of tilapia, *Oreochromis niloticus* using different doses of 17 α -methyltestosterone with

respect to the degree of sex stability after one year of treatment. *Egyptian Journal of Aquatic Research*, **38**(1): 59–66.

Fatima S, Adams M, Wilkinson R. 2016. Sex reversal of brook trout (*Salvelinus fontinalis*) by 17 α -methyltestosterone exposure: a serial experimental approach to determine optimal timing and delivery regimes. *Animal Reproduction Science*, **175**: 39–47.

Ge CT, Ye J, Weber C, et al. 2018. The histone demethylase KDM6B regulates temperature-dependent sex determination in a turtle species. *Science*, **360**(6389): 645–648.

Guinamard R, Simard C, Del Negro C. 2013. Flufenamic acid as an ion channel modulator. *Pharmacology & Therapeutics*, **138**(2): 272–284.

Hara S, Sawamura R, Kitano T. 2021. Cortisol induces masculinization of XX medaka through gonadal soma-derived growth factor (GSDF) and anti-Müllerian hormone receptor type 2 (AMHR2). *Fisheries Science*, **87**(1): 85–91.

Hattori RS, Fernandino JI, Kishii A, et al. 2009. Cortisol-induced masculinization: does thermal stress affect gonadal fate in pejerrey, a teleost fish with temperature-dependent sex determination?. *PLoS One*, **4**(8): e6548.

Hays GC, Mazaris AD, Schofield G. 2014. Different male vs. female breeding periodicity helps mitigate offspring sex ratio skews in sea turtles. *Frontiers in Marine Science*, **1**: 43.

Jiang DN, Yang HH, Li MH, et al. 2016. *gsdf* is a downstream gene of *dmrt1* that functions in the male sex determination pathway of the Nile tilapia. *Molecular Reproduction and Development*, **83**(6): 497–508.

Kitano J, Ansai S, Takehana Y, et al. 2024. Diversity and convergence of sex-determination mechanisms in teleost fish. *Annual Review of Animal Biosciences*, **12**(1): 233–259.

Kruidenier L, Chung CW, Cheng ZJ, et al. 2012. A selective jumonji H3K27 demethylase inhibitor modulates the proinflammatory macrophage response. *Nature*, **488**(7411): 404–408.

Li MH, Liu XY, Dai SF, et al. 2020. Regulation of spermatogenesis and reproductive capacity by Igf3 in tilapia. *Cellular and Molecular Life Sciences*, **77**(23): 4921–4938.

Liu S, Xu P, Liu XG, et al. 2021. Production of neo-male mandarin fish *Siniperca chuatsi* by masculinization with orally administered 17 α -methyltestosterone. *Aquaculture*, **530**: 735904.

Liu SZ, Chen Y, Li TY, et al. 2023. Effects of 17 α -Methyltestosterone on the transcriptome and sex hormones in the brain of *Gobiocypris rarus*. *International Journal of Molecular Sciences*, **24**(4): 3571.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta$ CT Method. *Methods*, **25**(4): 402–408.

Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, **15**(12): 550.

Luckenbach JA, Fairgrieve WT. 2016. Gonadal sex differentiation and effects of dietary methyltestosterone treatment in sablefish (*Anoplopoma fimbria*). *Fish Physiology and Biochemistry*, **42**(1): 233–248.

Ma XH, Liu F, Chen QR, et al. 2022. *Foxl2* is required for the initiation of the female pathway in a temperature-dependent sex determination system in *Trachemys scripta*. *Development*, **149**(13): dev200863.

Mohammad Nejad M, Mousavi Sabet SH. 2022. Comparative sterilizing between livebearer and oviparous fishes by oral treatment of 17 α -methyl testosterone; case study on guppy (*Poecilia reticulata*) and convict cichlid (*Cichlasoma nigrofasciatum*). *Journal of Animal Environment*, **14**(1): 205–208.

Montalvo AJ, Faulk CK, Holt GJ. 2012. Sex determination in southern flounder, *Paralichthys lethostigma*, from the Texas Gulf coast. *Journal of Experimental Marine Biology and Ecology*, **432–433**: 186–190.

Mullasser S, Jadav R, Hans A, et al. 2024. Amlodipine for glaucoma: a

zebrafish model. *Current Science*, **127**(1).

Nagahama Y, Chakraborty T, Paul-Prasanth B, et al. 2021. Sex determination, gonadal sex differentiation, and plasticity in vertebrate species. *Physiological Reviews*, **101**(3): 1237–1308.

Nagy A, Bercsényi M, Csányi V. 1981. Sex reversal in carp (*Cyprinus carpio*) by oral administration of methyltestosterone. *Canadian Journal of Fisheries and Aquatic Sciences*, **38**(6): 725–728.

Nanda A, Aparna S, Sahoo RN, et al. 2024. Amlodipine attenuates carrageenan-induced oxidative stress targeting transsynaptic neuronal damage by promoting survival of retinal ganglion cells in adult zebrafish (*Danio rerio*). *Annals of Neurosciences*, 09727531241246671.

Navarro-Martín L, Viñas J, Ribas L, et al. 2011. DNA methylation of the gonadal aromatase (*cyp19a*) promoter is involved in temperature-dependent sex ratio shifts in the european sea bass. *PLoS Genetics*, **7**(12): e1002447.

Noble DWA, Stenhouse V, Schwanz LE. 2018. Developmental temperatures and phenotypic plasticity in reptiles: a systematic review and meta-analysis. *Biological Reviews*, **93**(1): 72–97.

Oh SJ, Park JH, Han S, et al. 2008. Development of selective blockers for Ca²⁺-activated Cl⁻ channel using *Xenopus laevis* oocytes with an improved drug screening strategy. *Molecular Brain*, **1**(1): 14.

Pavlov ED, Ganzha EV, Ponomareva VY, et al. 2016. Effect of methyltestosterone on physiological state and rheoreaction of rainbow trout *Parasalmo mykiss* (= *Oncorhynchus mykiss*) under unfavorable keeping conditions. *Journal of Ichthyology*, **56**(6): 904–915.

Porsteinsson H, Baukman HA, Sveinsdóttir HS, et al. 2025. Validation of L-type calcium channel blocker amlodipine as a novel ADHD treatment through cross-species analysis, drug-target mendelian randomization, and clinical evidence from medical records. *Neuropsychopharmacology*, **50**(7): 1145–1155.

Qu GS, Cai ZW, Huang Y, et al. 2019. Investigation and protection of germplasm resources of *Varicorhinus macrolepis* Qinba mountain area. *Hubei Agricultural Science*, **58**(18): 93–97. (in Chinese)

Ramee SW, Lipscomb TN, DiMaggio MA. 2020. Evaluation of the effect of larval stocking density, salinity, and temperature on stress response and sex differentiation in the dwarf gourami and Rosy barb. *Aquaculture Reports*, **16**: 100287.

Santidrián Tomillo P, Genovart M, Paladino FV, et al. 2015. Climate change overruns resilience conferred by temperature-dependent sex determination in sea turtles and threatens their survival. *Global Change Biology*, **21**(8): 2980–2988.

Santidrián Tomillo P, Spotila JR. 2020. Temperature-dependent sex determination in sea turtles in the context of climate change: uncovering the adaptive significance. *BioEssays*, **42**(11): 2000146.

Shi HJ, Gao T, Liu ZL, et al. 2017. Blockage of androgen and administration

of estrogen induce transdifferentiation of testis into ovary. *Journal of Endocrinology*, **233**(1): 65–80.

Tao WJ, Yuan J, Zhou LY, et al. 2013. Characterization of gonadal transcriptomes from Nile tilapia (*Oreochromis niloticus*) Reveals Differentially Expressed Genes. *PLoS One*, **8**(5): e63604.

Trapnell C, Williams BA, Pertea G, et al. 2010. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nature Biotechnology*, **28**(5): 511–515.

Tseng MC, Yang DH, Yen TB. 2017. Comparative study on hatching rate, survival rate, and feminization of *Onychostoma barbatulum* (Pellegrin, 1908) at different temperatures and examining sex change by gonad and karyotype analyses. *Zoological Studies*, **56**: e16.

Turan F, Cek S, Atik E. 2006. Production of monosex male guppy, *Poecilia reticulata*, by 17 α -methyltestosterone. *Aquaculture Research*, **37**(2): 200–203.

Valverde RA, Wingard S, Gómez F, et al. 2010. Field lethal incubation temperature of olive ridley sea turtle *Lepidochelys olivacea* embryos at a mass nesting rookery. *Endangered Species Research*, **12**(1): 77–86.

Wang DS, Kobayashi T, Zhou LY, et al. 2007. Foxl2 up-regulates aromatase gene transcription in a female-specific manner by binding to the promoter as well as interacting with ad4 binding protein/steroidogenic factor 1. *Molecular Endocrinology*, **21**(3): 712–725.

Wang DS, Zhou LY, Kobayashi T, et al. 2010. Doublesex- and mab-3-related transcription factor-1 repression of aromatase transcription, a possible mechanism favoring the male pathway in tilapia. *Endocrinology*, **151**(3): 1331–1340.

Weber C, Zhou YJ, Lee JG, et al. 2020. Temperature-dependent sex determination is mediated by pSTAT3 repression of *Kdm6b*. *Science*, **368**(6488): 303–306.

Wu PF, Wang XF, Ge CT, et al. 2024. pSTAT3 activation of *Foxl2* initiates the female pathway underlying temperature-dependent sex determination. *Proceedings of the National Academy of Sciences of the United States of America*, **121**(37): e2401752121.

Yamaguchi T, Yoshinaga N, Yazawa T, et al. 2010. Cortisol is involved in temperature-dependent sex determination in the Japanese flounder. *Endocrinology*, **151**(8): 3900–3908.

Yamamoto E. 1999. Studies on sex-manipulation and production of cloned populations in hirame, *Paralichthys olivaceus* (Temminck et Schlegel). *Aquaculture*, **173**(1-4): 235–246.

Yao ZL, Fang QF, Li JY, et al. 2023. Alternative splicing of histone demethylase *Kdm6bb* mediates temperature-induced sex reversal in the Nile tilapia. *Current Biology*, **33**(23): 5057–5070. e5.

Zhou H, Zhuang ZX, Zhang R, et al. 2019. Temperature-control-induced masculinization in tiger puffer *Takifugu rubripes*. *Journal of Oceanology and Limnology*, **37**(3): 1125–1135.