BMC Genomics

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Identification of sex-biased and gonadal developmental miRNAs during critical windows of early gonadal differentiation in the Chinese giant salamander (*Andrias davidianus*)

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Abstract

The Chinese giant salamander (CGS), *Andrias davidianus*, the largest living amphibian, is of significant conservation importance for its wild populations and serves as a valuable economic species in aquaculture. Given that male CGS are larger than female, understanding the mechanisms behind gonadal differentiation and development is critical for optimizing production in aquaculture. This study conducted small RNA-seq on male and female gonads during two key differentiation windows in CGS. PCA analysis revealed distinct clustering within groups and clear differentiation between groups. A total of 374 miRNAs were identified, including 162 known and 212 novel miRNAs. Differential expression and enrichment analysis across the two time points identified nine sex-biased miRNAs involved in CGS gonadal differentiation, including let-7b-3p, miR-3529-3p, miR-34c-5p, miR-10-5p, miR-7-5p, and four novel miRNAs, and network of these nine sex-biased miRNAs with their target genes were constructed. Furthermore, our findings suggest that male and female gonadal development processes are likely regulated by distinct miRNAs. This study provides a comprehensive analysis of sex-biased and gonadal development related miRNAs in CGS, offering a theoretical foundation for enhancing CGS aquaculture productivity through targeted sex ratio management.

Keywords *Andrias davidianus*, Small RNA-seq, Sex-biased, Gonadal development

Background

Aquatic animals, often referred to as "blue food," are playing an increasingly important role in global food security [1, 2]. Among fish and other aquatic species, sex is one of the most valuable traits, as there are distinct sexual dimorphisms in growth, size, and other characteristics [3]. Therefore, creating monosex populations (all-male or all-female) or producing populations with higher proportions of males or females can significantly enhance aquaculture efficiency [4]. Consequently, further exploration of the sex determination mechanisms and sex control methods in aquaculture species will continue to drive sustainable aquaculture practices and global food supply [5]. However, the concept of sex determination remains a subject of debate [6, 7]. A recent perspective challenges the "Top-level" sex determiner model, suggesting the idea of "Turtles all the way up!"—that no gene truly sits at the top of the regulatory hierarchy because its expression is controlled by another layer of upstream regulatory factors [7]. Increasing evidence highlights the critical role of non-coding RNAs in sex determination and gonadal

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differentiation, further supporting the notion of higher-level regulatory factors above genes. For example, the transposon *DRBX1* of the sex-determining gene *DMRT1* plays a key role in sex determination in *Betta splendens* [8], and Ca2 + plays a pivotal role in sex determination in both *Trachemys scripta* and *Pogona vitticeps* [9, 10].

MicroRNAs (miRNAs) are small, non-coding RNA molecules that play critical roles in the regulation of gene expression at posttranscription level [11]. These miRNAs can function as key regulators by modulating the expression of genes associated with sex differentiation, influencing pathways that drive the development of male or female characteristics. miRNA regulation of gene expression follows a complex network, where a single miRNA can control the expression of hundreds of target genes, while a single gene can be simultaneously regulated by multiple miRNAs. miRNAs play a crucial role in reproduction by regulating the synthesis and function of sex steroid hormones [12]. In recent years, an increasing body of evidence has highlighted the involvement of miRNAs in various biological processes, including sex determination and gonadal development [13]. Studies on mammals have established miRNAs as key regulators of biologically significant traits with economic relevance. Their development has advanced significantly in diagnostics and therapeutics [14]. However, despite the ecological and commercial significance of aquaculture, research on miRNAs in this field remains largely exploratory. Further investigation into the role of miRNAs in aquaculture reproduction could enhance breeding techniques, contributing to the holistic development of the aquaculture sector [15]. miRNAs have been shown to play a significant role in gonadal differentiation, exhibiting notable sex bias [16, 17]. However, the existence of a "sex-determining miRNA" still requires further experimental validation. Current studies on miRNAs in aquaculture species primarily focus on fish, crustaceans, and other species. For instance, significant differences in miRNA expression have been observed between males and pseudomales in *Cynoglossus semilaevis* [16]. Additionally, miRNAs also show notable differences between normal males and females in *Portunus trituberculatus* [17]. However, despite amphibians being an important group of aquaculture species [18, 19], reports on sex-biased miRNAs in amphibians remain limited.

The Chinese giant salamander (CGS), *Andrias davidianus*, is the largest and with long evolutionary history species in the world. Wild populations of CGS are critically important for conservation efforts [20], while captive populations are recognized for their significant value in high-quality aquaculture [18, 19]. Studying the sex of CGS can effectively enhance reproductive efficiency, supporting a sufficient population size for strategic field releases. Furthermore, given that male CGS are larger and exhibit faster development [21], maintaining a male-biased captive population can significantly enhance economic returns in aquaculture. Current research on sex-related mechanisms in the CGS is predominantly concentrated at mRNA level [22, 23]. Similar to zebrafish, the timing of gonadal development differs between the male and female in the CGS [24]. To fully understand these dynamics, it is crucial to explore the critical windows of gonadal differentiation prior to the maintenance of adult gonads, which is essential for advancing sex related studies. Our recent study, which integrates histological phenotypic analysis with molecular data, has identified distinct differences in the timing of gonadal differentiation between male and female CGS. Specifically, around 210 days post hatching (dph) marks the critical window for gonadal differentiation in females, while this key period occurs at approximately 270 dph in males [25]. Focusing on these two critical periods offers valuable insights into the mechanisms underlying sex determination and gonadal differentiation in CGS, and exploring miRNAs in CGS is expected to clarify their regulatory impact on mRNA. This also has important implications for artificial control of sex ratios in CGS populations. Increasing the production of larger male CGS can significantly enhance both the overall yield and the economic benefits of CGS cultivation. Previous studies have conducted small RNA sequencing exclusively on 3-year-old male CGS and 4-year-old male and female CGS [26, 27]. While these findings may provide insights into the miRNAs required for gonadal maintenance in CGS, they do not capture the miRNA expression patterns during the critical periods of gonadal differentiation and development. Research on miRNAs during the early gonadal differentiation window in CGS remains unexplored.

This study performed small RNA sequencing at two critical windows of CGS gonadal differentiation, identified in previous research, with the goal of exploring the differential expression patterns of miRNAs involved in male and female gonadal differentiation and identifying miRNAs associated with CGS gonadal development. This is the first to investigate male and female gonads of CGS across multiple time points from the miRNA perspective, allowing for a more precise exploration of miRNAs associated with sexual dimorphism and gonadal development in CGS. The findings aim to lay the groundwork for molecular regulation of CGS breeding in aquaculture and to enhance production efficiency.

Materials and methods

Sample collection

All CGS used in this study were captive-bred individuals sourced from the Yunxing Professional Breeding

Cooperative of Chinese Giant Salamander in Ya'an, Sichuan Province. These CGS were offspring of the same parental group. We anesthetized the CGS using MS-222, a commonly used aquaculture anesthetic, dissolved in water at a concentration of 1 mL per 20 L. Once the CGS were fully anesthetized, cervical vertebral dislocation was performed before proceeding with sampling. Samples were collected at 210 and 270 dph. F210 and M210 represent female and male gonads at 210 dph, respectively, while F270 and M270 represent female and male gonads at 270 dph, respectively. Tail tip tissue was first collected from each sample, and DNA was extracted using a Tiangen DNA extraction kit. Subsequently, PCR amplification was performed using the CGS female-specific primer *adf340* to determine the sex of each sample [27] (Table 1). Then, Gonadal tissues were then collected under a dissecting microscope. Due to the small size of CGS gonads, tissues from three individuals were pooled into a single sample. A total of six male and six female gonadal samples were collected at each stage, with three samples designated for RNA-seq and three for qPCR analysis. All samples were immediately frozen in liquid nitrogen for ten minutes and then stored at −80 °C until RNA isolation.

RNA Isolation, library construction and small RNA sequencing

Total RNA was extracted from gonadal tissues using the RNAiso™ Plus RNA Kit (TaKaRa) following the manufacturer's protocol, and RNA integrity was assessed using the Agilent 5400 Fragment Analyzer (Agilent, USA). Small RNA libraries were prepared from 2 μg of total RNA per sample using the NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, USA). PCR amplification was conducted with LongAmp Taq 2X Master Mix, SR Primer for Illumina, and Index Primer. PCR products were purified on an 8% polyacrylamide gel, and DNA fragments of 140–160 bp were recovered and dissolved in 8 μL elution buffer. Library quality was assessed using the Agilent Bioanalyzer 2100 system with DNA High Sensitivity Chips. Index-coded samples were clustered using the cBot Cluster Generation System with the TruSeg SR Cluster Kit v3-cBot-HS (Illumina) following the manufacturer's protocol. After clustering, libraries were sequenced on the NovaSeq 6000 platform, producing 50 bp single-end reads.

Table 1 Primer for sex identification

Gene name	Primer sequence (5′to 3′)
adf340s	TTAACGGCCCTAACACCAGG
adf340a	GGTTTAGGGCGGCTCTGATT

Quality control, miRNA Identification and target gene prediction

To obtain clean reads, raw reads in fastq format were processed using Perl and Python scripts. Reads containing poly-N, poly-A/T/G/C sequences, 5' adapter contamination, or missing 3' adapters or insert tags were removed. The 3' adapter sequences were trimmed, and Q20, Q30, GC content were calculated. Reads shorter than 18 bp or longer than 35 bp were discarded.

The processed small RNA reads were mapped to the CGS gonad transcriptome [13](SRA accession number: PRJNA1029821) using Bowtie (https://bowtie-bio.sourceforge.net/), allowing for up to one mismatch base. Known miRNAs were identified by aligning the mapped reads to the mature miRNAs of two amphibian species, *Xenopus laevis* and *Xenopus tropicalis*, as well as *Homo sapiens*, which has the highest number of known miRNAs among vertebrates, sourced from miRBase 22.0 (http://www.mirbase.org/). Prior to novel miRNA prediction, reads originating from protein-coding genes, repeat sequences, rRNA, tRNA, snRNA, and snoRNA were removed. Small RNAs were classified as ncRNAs using the Rfam database (http://rfam.xfam.org/), and repeat sequences were filtered out with RepeatMasker (https://www.repeatmasker.org/). Additionally, reads mapped to introns or exons, representing mRNA fragments, were excluded. Novel miRNAs were then predicted by integrating miREvo [28] and miRDeep2 [29].

The target gene prediction of miRNA was performed by miRanda (http://www.miranda.org/), Targetscan (http://www.targetscan.org/), and RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/). To investigate the biological functions and pathways associated with these candidate target genes, enrichment analyses were conducted using Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG). The mRNA–miRNA network relationships were visualized using Cytoscape software.

Identification of differentially expressed miRNAs (DEMs)

MiRNA expression levels were quantified using transcripts per million (TPM). Principal component analysis (PCA) was performed in R to evaluate sample relationships and clustering patterns based on gene expression profiles. Differential expression analysis was carried out in *R* using the DESeq2 package [30], with miRNAs exhibiting a $|\log2[\text{fold change}]| \geq 1$ and p-value < 0.05 between any two groups considered as differentially expressed miRNAs (DEMs). The Venn diagram was created using Evenn (http://www.ehbio.com/test/venn/#/).

Table 2 The primers used in RT-qPCR

Name	RTprimer(specific) Sequence (5'–>3')	reverse primer	forward primers
let-7b-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGGAAG	GTCGTATCCAGTGCAGGGT	ACCACCGCTATACAACCTACT
miR-10b-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCACAAA	GTCGTATCCAGTGCAGGGT	AACACGCTACCCTGTAGAACC
miR-34c-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCAATC	GTCGTATCCAGTGCAGGGT	AACCGGAGGCAGTGTAGTTAG
miR-3529-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGGAAG	GTCGTATCCAGTGCAGGGT	AAGCGCCTAACAACAAAATCACTA
miR-7-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACAAC	GTCGTATCCAGTGCAGGGT	AACCGGTGGAAGACTAGTGATT
novel_157	CAGTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTT	CAGTGCAGGGTCCGAGGT	AACAAGATCCCACCGCTGC
novel_261	CAGTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTT	CAGTGCAGGGTCCGAGGT	AACAGACACCTGGTGCTGTAGG
novel_28	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAAATC	GTCGTATCCAGTGCAGGGT	AACCGGAGGCAGTGTAGTTAG
novel_60	CAGTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTT	CAGTGCAGGGTCCGAGGT	AACACGCATCTCAGCAGTGC
U6	AACGCTTCACGAATTTGCGT	AACGCTTCACGAATTTGCGT	CTCGCTTCGGCAGCACA

Real-time quantitative PCR verification

RNA was reverse transcribed into cDNA using the PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Japan), following the manufacturer's protocol. The primers used are listed in Table 2, with U6 serving as the internal reference gene. All experiments were performed in triplicate, and relative mRNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method. Data visualization was done using GraphPad Prism 8.0.

Results

Characteristics of the small-RNA sequencing data

Small-RNA sequencing was performed on male and female gonadal samples of CGS at two critical developmental stages, 210 and 270 dph. A total of 12 libraries were generated, producing approximately 153.8 million raw reads. Following quality control, including the removal of low-quality reads and the retention of reads between 18–35 bp in length, approximately 149.8 million high-quality clean reads were retained. All samples exhibited a Q30 score above 96.55%, with GC content ranging from 50.26% to 54.95%. These metrics confirm that the sequencing data are of high quality and suitable for downstream analyses (Table 3).

In this study, a total of 374 miRNAs were identified, including 162 known miRNAs and 212 novel miRNAs. At both 210 dph and 270 dph, the number of miRNAs identified in the female gonads was higher than in the male gonads. In male gonads, more miRNAs were identified at 210 dph compared to 270 dph, whereas the number of miRNAs in female gonads showed no significant difference between the two time points (Table 4). Additional non-coding RNAs identified in CGS gonads, including rRNAs, tRNAs, snRNAs, and snoRNAs, are presented in Table S1.

Table 3 Summary statistics for the small RNA-seq data from each sample

Sample	Total Reads	Clean Reads	Q30	GC content
F270_1	11,427,796	11,198,870 (98.00%)	96.87%	54.24%
F270_2	14,863,311	14,649,398 (98.56%)	97.69%	54.95%
F270_3	11,404,355	11,085,164 (97.20%)	97.13%	51.70%
M270_1	15,114,719	14,909,915 (98.65%)	97.93%	51.30%
M270_2	15,316,915	14,891,029 (97.22%)	96.75%	51.51%
M270_3	11,737,293	11,317,147 (96.42%)	96.98%	51.59%
F210_1	12,412,807	12,088,601 (97.39%)	96.55%	52.69%
F210_2	11,890,043	11,362,999 (95.57%)	97.32%	52.37%
F210_3	12,380,885	11,727,540 (94.72%)	97.34%	53.39%
M210_1	8,349,200	8,081,113 (96.79%)	97.20%	52.12%
M210_2	13,712,928	13,517,419 (98.57%)	97.60%	50.26%
M210_3	15,212,466	14,989,363 (98.53%)	97.31%	53.48%

Table 4 Number of miRNAs identified in this study

Sample	miRNAs	Known_miRNA	Novel_miRNA
M210_1	265	116	149
M210_2	259	114	145
M210_3	265	122	143
M270_1	208	87	121
M270_2	185	86	99
M270_3	207	97	110
F210_1	271	121	150
F210_2	313	131	182
F210_3	218	96	122
F270_1	283	115	168
F270_2	245	107	138
F270_3	251	106	145
Total	374	162	212

Identification of DEMs between female and male at different stages

To assess the correlation of miRNA expression between samples, PCA analysis was conducted using the TPM values of 374 expressed miRNAs. The results indicated that the biological replicates clustered within distinct, independent ellipses, while different ellipses represented different groups, demonstrating a clear separation between groups (Fig. 1). This finding suggests that the sequencing results are suitable for subsequent analyses.

Pairwise comparisons were performed between male and female samples at 210 dph and 270 dph to identify DEMs between the two groups (Fig. 2A-B). Down-regulated DEMs are indicative of male-specific miRNAs, whereas up-regulated DEMs signify female-specific miRNAs. At 210 dph, a total of 14 male-specific miRNAs were identified (6 known miRNAs and 8 novel miRNAs), along with 12 female-specific miRNAs (7 known miRNAs and 5 novel miRNAs). At 270 dph, 19 male-specific miRNAs were identified (7 known miRNAs and 12 novel miRNAs), as well as 11 female-specific miRNAs (7 known miRNAs and 4 novel miRNAs) (Fig. 2C). Among all DEMs, 10 were found to exhibit differential expression at both 210 dph and 270 dph. Additionally, 16 DEMs showed differential expression exclusively at 210 dph, while 20 DEMs were uniquely differentially expressed at 270 dph (Fig. 2D).

Enrichment analysis of predicted target genes

To further explore the functions of the target genes of DEMs, target gene prediction was performed for the identified DEMs. We conducted enrichment analyses separately for the target genes corresponding to DEMs that were differentially expressed exclusively at 210 dph, exclusively at 270 dph, and at both 210 dph and 270 dph (Fig. 3A).

The red line marks miRNAs that were differentially expressed exclusively at 210 dph, with 16 miRNAs predicted to target 7,824 genes. GO enrichment analysis revealed that these target genes were primarily involved in lipid biosynthetic processes (GO:0008610), response to bacterium (GO:0009617), and defense response to bacterium (GO:0042742) under the Biological Process (BP) category. In the Cellular Component (CC) category, the genes were mainly associated with the photosystem (GO:0009521) and protein kinase complex

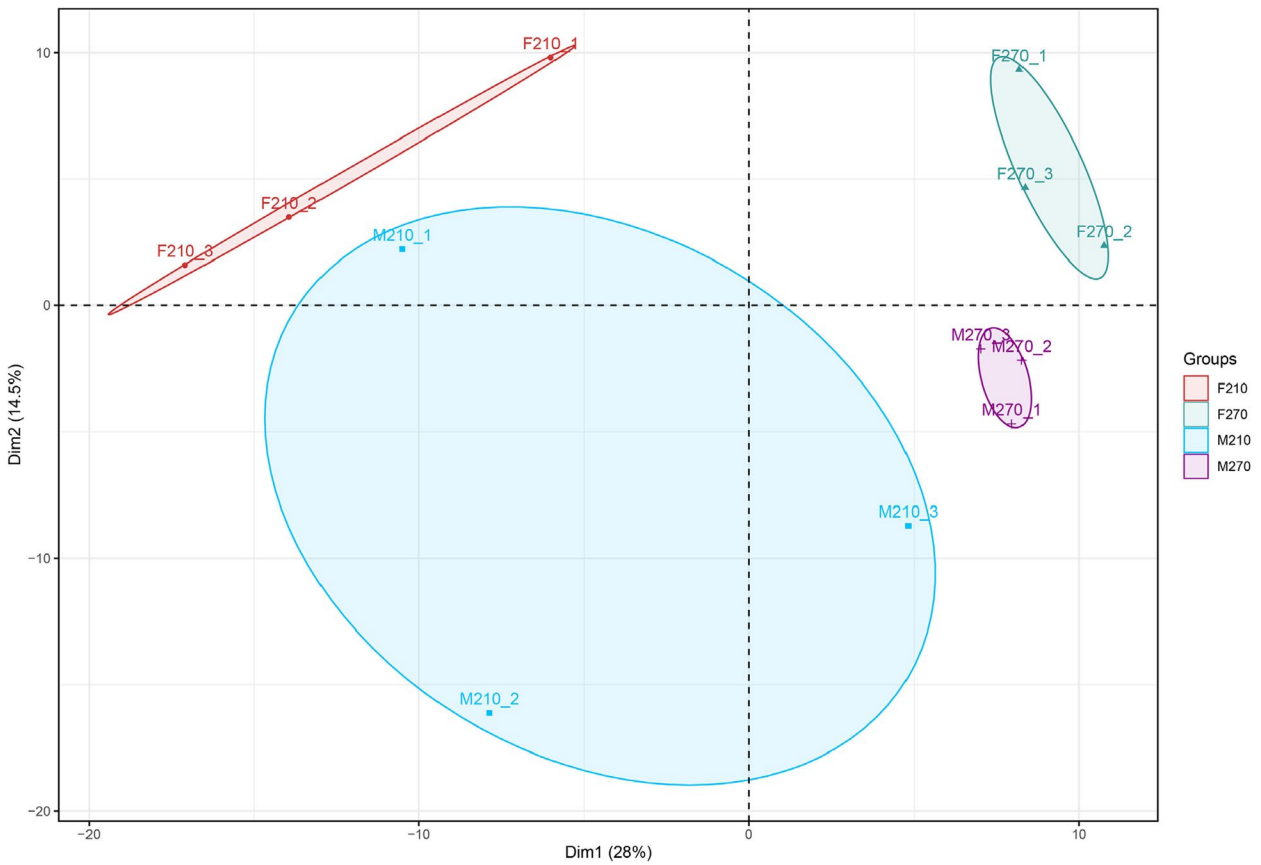

Fig. 1 Principal component analysis results of the twelve samples from the four groups in this study

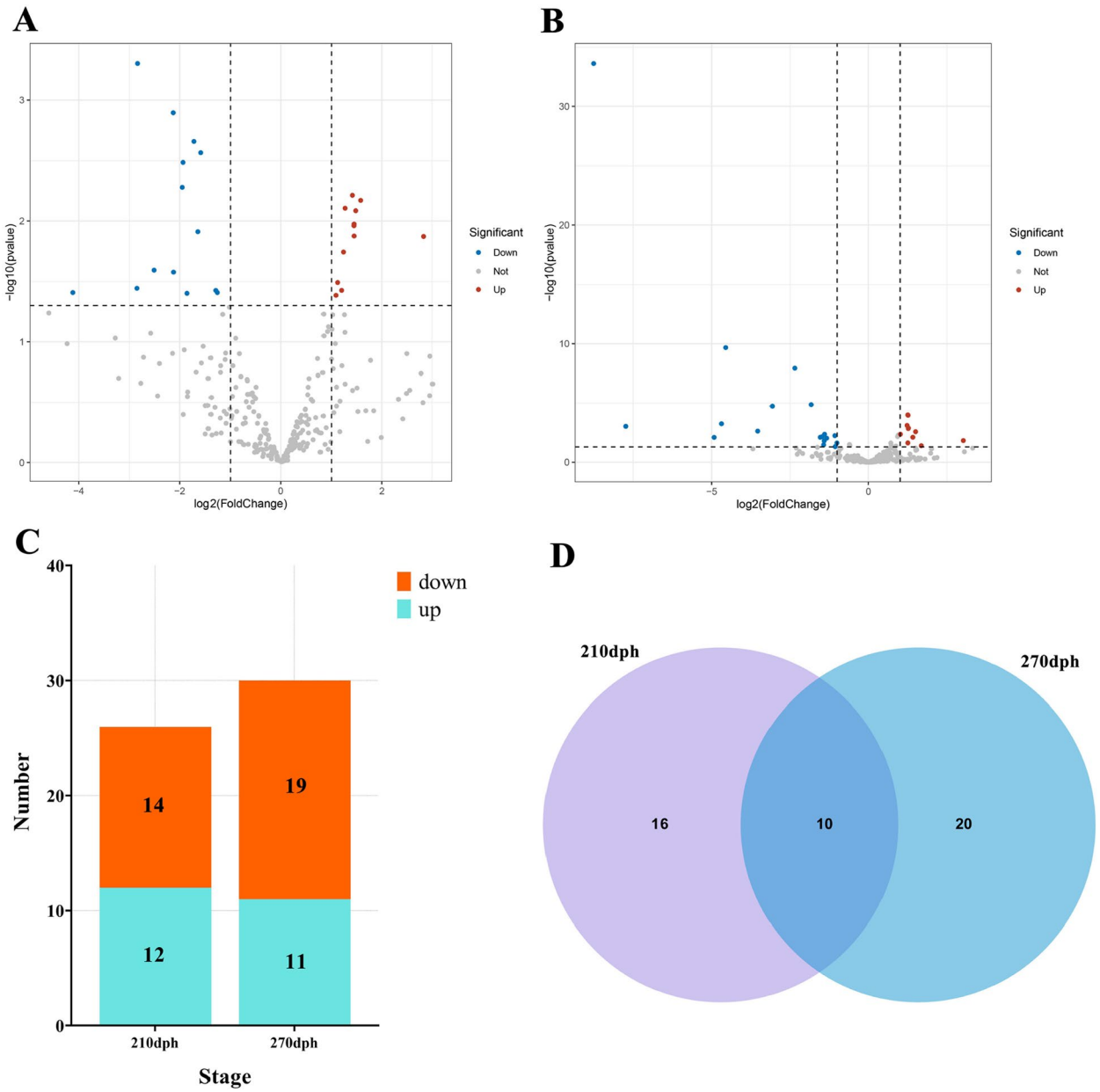

Fig. 2 Volcano of differentially expressed miRNAs (DEMs) between males and females at 210dph (**A**) and 270dph (**B**). Numbers of DEMs for pairwise comparisons between males and females at two stages (**C**). Venn diagram of DEMs in two stages (**D**). Down: down-regulated DEMs and representative male-specific miRNAs. Up: up-regulated DEMs and representative female-specific miRNAs

(GO:1,902,911). Under Molecular Function (MF), the targets were significantly enriched for hydrolase activity, specifically acting on glycosyl bonds (GO:0016798), and acyltransferase activity (GO:0016746) (Fig. 3B). KEGG pathway analysis demonstrated notable enrichment in the axon guidance pathway (ko04360) and the PI3K-Akt signaling pathway (ko04360) (Fig. S1A).

In contrast, the blue line highlights miRNAs differentially expressed only at 270 dph, with 20 miRNAs predicted to target 8,587 genes. GO enrichment analysis for these targets predominantly implicated chromatin organization (GO:0006325) and cellular developmental processes (GO:0048869) in the BP category. In the CC category, the target genes were mainly associated with the endoplasmic reticulum (GO:0005783) and endoplasmic reticulum membrane (GO:0005789). For MF, significant enrichment was observed in glycosyltransferase activity (GO:0016757) and metallopeptidase activity

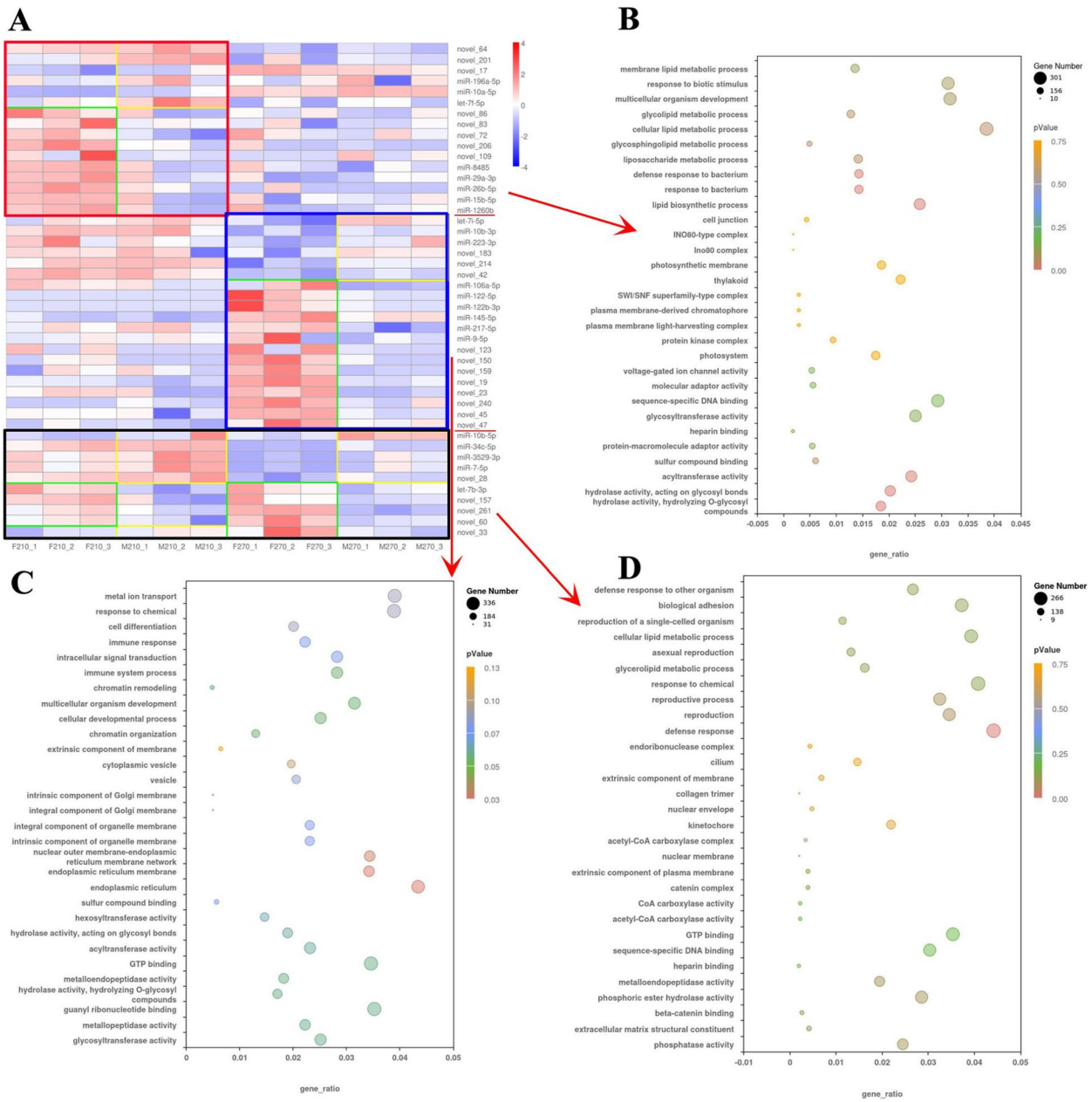

Fig. 3 Heatmap of all sex-biased DEMs (**A**). The red box indicates miRNAs differentially expressed only at 210 dph; the blue box indicates miRNAs differentially expressed only at 270 dph; the black box indicates miRNAs differentially expressed at both 210 dph and 270 dph; the green box indicates miRNAs highly expressed in male gonads; and the yellow box indicates miRNAs highly expressed in female gonads. **B**: GO enrichment results of target genes corresponding to miRNAs differentially expressed only at 210 dph; **C**: GO enrichment results of target genes corresponding to miRNAs differentially expressed only at 270 dph; **D**: GO enrichment results of target genes corresponding to miRNAs differentially expressed at both 210 dph and 270 dph

(GO:0008237) (Fig. 3C). KEGG pathway analysis showed enrichment in the PI3K-Akt signaling pathway (ko04151) and the Phospholipase D signaling pathway (ko04072) (Fig. S1B).

The black line indicates miRNAs that were differentially expressed at both 210 dph and 270 dph, with 10 miRNAs predicted to target 6,028 genes. GO enrichment analysis of these target genes highlighted significant involvement in reproduction (GO:0000003), reproductive process (GO:0022414), sexual reproduction (GO:0019953), and even asexual reproduction (GO:0019954) within the BP category. In the CC category, the target genes were

mainly linked to the catenin complex (GO:0016342) and the extrinsic component of the plasma membrane (GO:0019897). For MF, significant enrichment was observed in beta-catenin binding (GO:0008013) and phosphatase activity (GO:0016791) (Fig. 3D). KEGG pathway analysis revealed enrichment in the AMPK signaling pathway (ko04152) and the PI3K-Akt signaling pathway (ko04151) (Fig. S1C). Notably, the target genes of this miRNA group were significantly enriched in several well-defined sex-related terms. Among these, novel_33 showed a male-biased expression tendency at 210 dph, which shifted towards female-biased expression at 270 dph. Given this, we propose that the role of novel_33 in sex determination and gonadal differentiation is likely minimal, prompting us to direct more focus towards the other nine sex-biased miRNAs in this group in subsequent analyses.

Integrated analysis of the sex-biased miRNAs and corresponding target genes

Based on the functional enrichment results, we propose that the nine sex-biased miRNAs differentially expressed at both 210 and 270 dph are most closely linked to CGS sex determination and gonadal differentiation. The mature sequences of these nine sex-biased miRNAs are presented in Table S2. To further investigate, we plan to explore the co-expression networks of these sex-biased miRNAs and their corresponding target genes. In our previous study, we identified female-specific and male-specific genes associated with CGS (Tables S2 and S4 in reference 13), ultimately identifying 114 sex-related genes linked to CGS. By intersecting these genes with the predicted targets of the DEMs from the current study, we identified a set of CGS-related target genes involved in sex determination and gonadal differentiation. Given that miRNAs negatively regulate the expression of their target genes, most interacting miRNAs and mRNAs exhibited inverse expression patterns in the gonads.

Among the male-biased miRNAs, we identified one known miRNA and three novel miRNAs, collectively regulating 65 target genes (Fig. 4A and Table S3). Four target genes (*XIAP*, *NSD1*, *TBL1X* and *WNK2*) were associated with all four miRNAs. Additionally, novel_357 had the highest number of associated target genes, with a total of 39. The miRNA-mRNA co-expression correlation heatmap shows that let-7b-3p negatively regulates male-related mRNAs *BMP6*, *FHL2*, and *FGF23* at 210 and 270 dph. Novel_28 negatively regulates male-related mRNAs such as *TBX1*, *SOX9,* and *STAR*. Novel_60 negatively regulates *SOX9*, *FGF23*, and *FGF7,* while novel_261 negatively regulates *STAR*, *FGF23*, and *FGF7*. The downregulation of these miRNAs leads to an increase in the expression of their target mRNAs, thereby inducing the activation of male pathways (Fig. 5).

In the female-biased miRNAs, four known miRNAs and one novel miRNA were associated with 76 target genes (Fig. 4B and Table S4). Both miR-34c-5p and novel_28 were associated with the highest number of target genes, each regulating 47 genes. The *SOX9* gene was regulated by four miRNAs, excluding miR-10-5p. *TBX1* showed a strong association with miR-10-5p and miR-3529-3p. Additionally, the male sex-related gene *DMRT1* exhibited an inverse regulatory relationship with miR-7-5p. The miRNA-mRNA co-expression correlation heatmap shows that miR-10b-5p, miR-34c-5p, and novel_28 negatively regulate the female-related gene *RSPO1*, while miR-34c-5p and novel_28 exhibit a negative regulatory relationship with *ZP3* and *ZP4*. MiR-7-5p negatively regulates *GDF9*. Notably, the female sex-determining candidate gene *TRPM1*, which we identified in a previous study, is negatively regulated by miR-3529-3p. The downregulation of these miRNAs leads to an increase in the expression of their target mRNAs, thereby inducing the activation of female pathways.

Identification of DEMs in gonadal development

To investigate miRNAs associated with gonadal development during the critical windows of gonadal differentiation in the CGS, we analysed miRNAs related to male and female gonadal development by comparing 270 dph with 210 dph (Fig. 6A-B). In males, 74 DEMs were identified, including 30 upregulated and 44 downregulated DEMs. In females, a total of 68 DEMs were identified, comprising 20 upregulated and 48 downregulated DEMs (Fig. 6C). Among all gonadal development related DEMs, 29 were identified exclusively in males, while 23 were found only in females. Additionally, 45 miRNAs exhibited differential expression in both sexes (Fig. 6D).

To further explore the functions of the target genes of above DEMs, target gene prediction was performed for the identified DEMs. We conducted enrichment analyses separately for the target genes corresponding to DEMs that were differentially expressed exclusively in male, exclusively in female, and at both in male and female (Fig. 7A). GO enrichment analysis revealed that the target genes of DEMs identified exclusively in males were primarily enriched in processes such as cell differentiation (GO:0030154), cellular developmental processes (GO:0048869), cell development (GO:0048468), GTP binding (GO:0005525), guanyl ribonucleotide binding (GO:0032561) and guanyl nucleotide binding (GO:0019001) (Fig. 7B). In contrast, the target genes of DEMs identified exclusively in females were primarily enriched in pathways such as intracellular signal transduction (GO:0035556), cell wall organization or

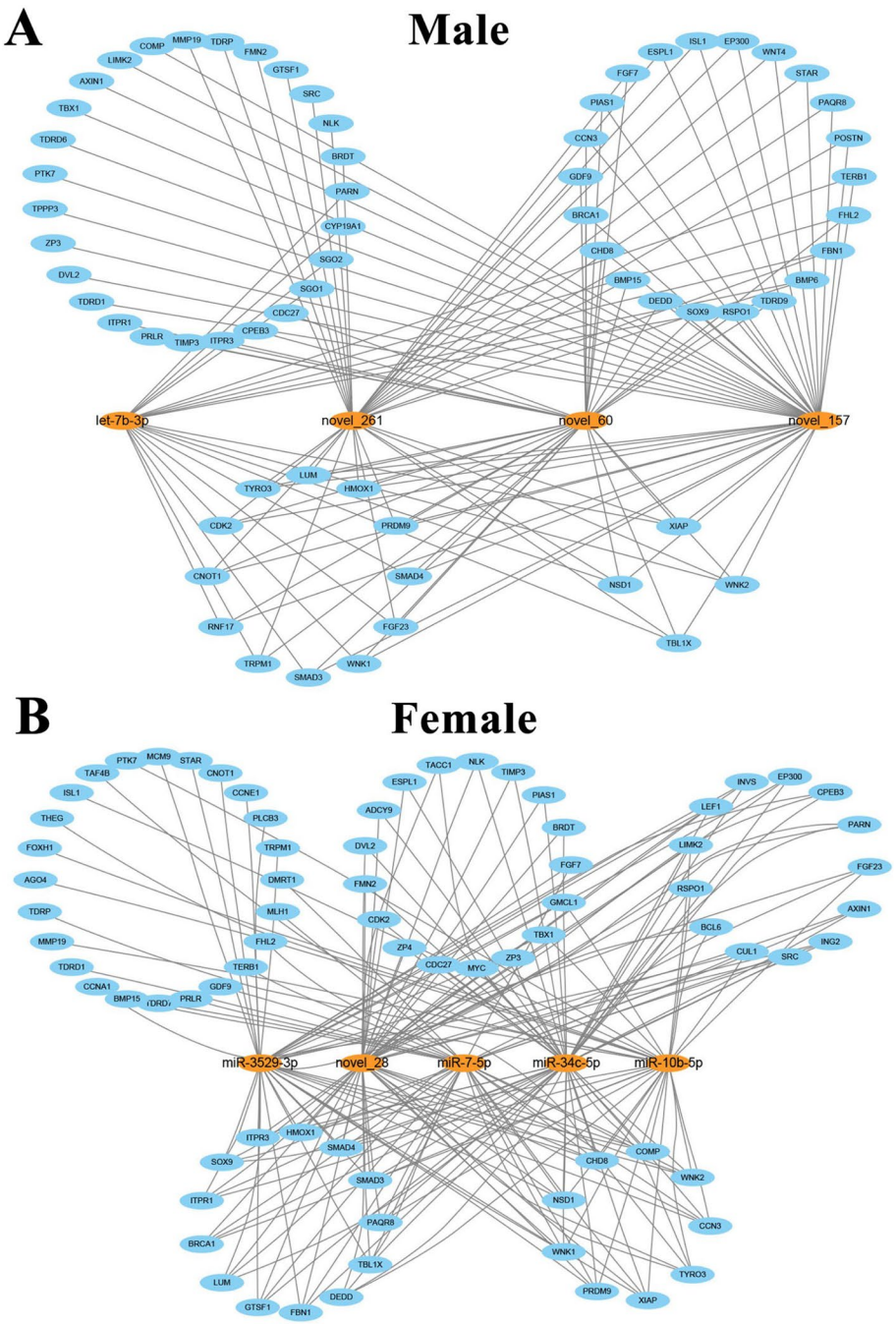

Fig. 4 The network of nine sex-biased miRNAs and their target genes. **A**: Network of four miRNAs with sixty-five target genes. **B**: Network of five miRNAs with seventy-six target genes. Orange represents miRNAs; blue represents mRNAs

biogenesis (GO:0071554), calcium-mediated signaling (GO:0019722), endosome formation (GO:0005768), and phosphatase activity (GO:0016791) (Fig. 7C). Additionally, the target genes of DEMs expressed in both males and females were primarily enriched in processes such as disaccharide metabolic processes (GO:0005984), transcription by RNA polymerase II (GO:0006366), plasma membrane protein complexes (GO:0098797), thylakoid (GO:0009579), metalloendopeptidase activity (GO:0004222), and hydrolase activity, hydrolyzing O-glycosyl compounds (GO:0004553) (Fig. 7D). KEGG enrichment analysis revealed that the target genes from

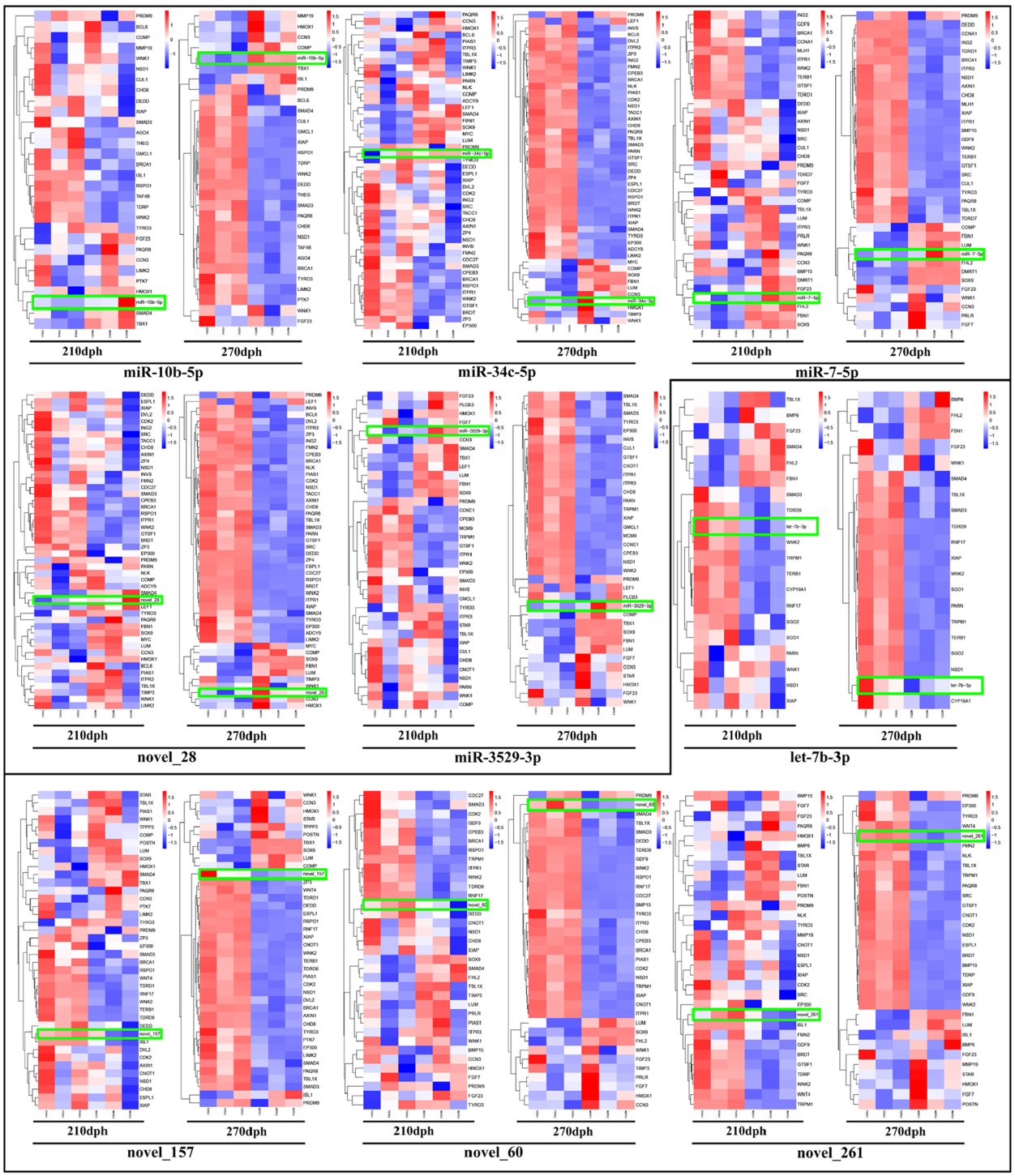

Fig. 5 Nine sex-biased miRNAs and their target genes expression correlation heatmap. The green boxes highlight the positions of the sex-biased miRNAs

all three groups were enriched in pathways involving GTP-binding proteins (ko04151) and the PI3K-Akt signaling pathway (ko04031) (Fig. S2).

Validation of sex-biased miRNAs

To validate the reliability of the small RNA-seq data, we selected above nine sex-biased miRNAs, including

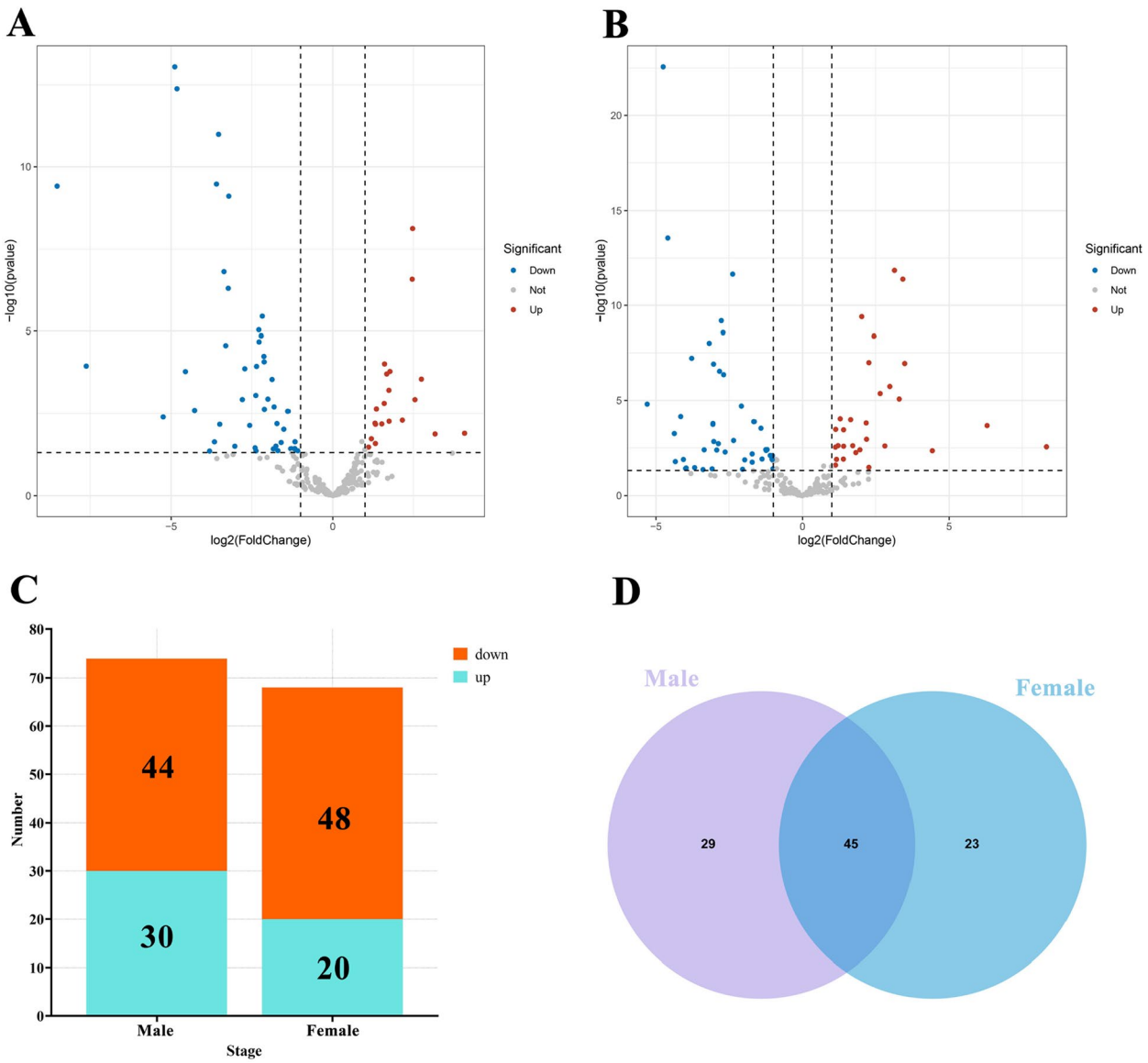

Fig. 6 Volcano of differentially expressed miRNAs (DEMs) between two stages at male (**A**) and female (**B**). Numbers of DEMs for pairwise comparisons between two stages at male and female (**C**). Venn diagram of DEMs in male and female (**D**)

five known miRNAs (let-7b-3p, miR-34c-5p, miR-7-5p, miR-3529-3p and miR-10b-5p) and four novel miRNAs (novel_261, novel_60, novel_157, novel_28), from two pivotal stages of early gonadal differentiation (210 dph and 270 dph) to perform RT-qPCR analysis. Consequently, it indicates that the RT-qPCR results are consistent with the small RNA-seq results, confirming the reliability and accuracy of the small RNA-seq data analysis (Fig. 8).

Discussion

The discovery of miRNAs has been a landmark achievement in molecular biology, significantly advancing our understanding of gene regulation, as evidenced by the awarding of the 2024 Nobel Prize in Physiology or Medicine to Victor Ambros and Gary Ruvkun, the pioneers of miRNA research (https://www.nobelprize.org/prizes/medicine/2024/press-release/). However, research on sex-related miRNAs in amphibians, particularly in

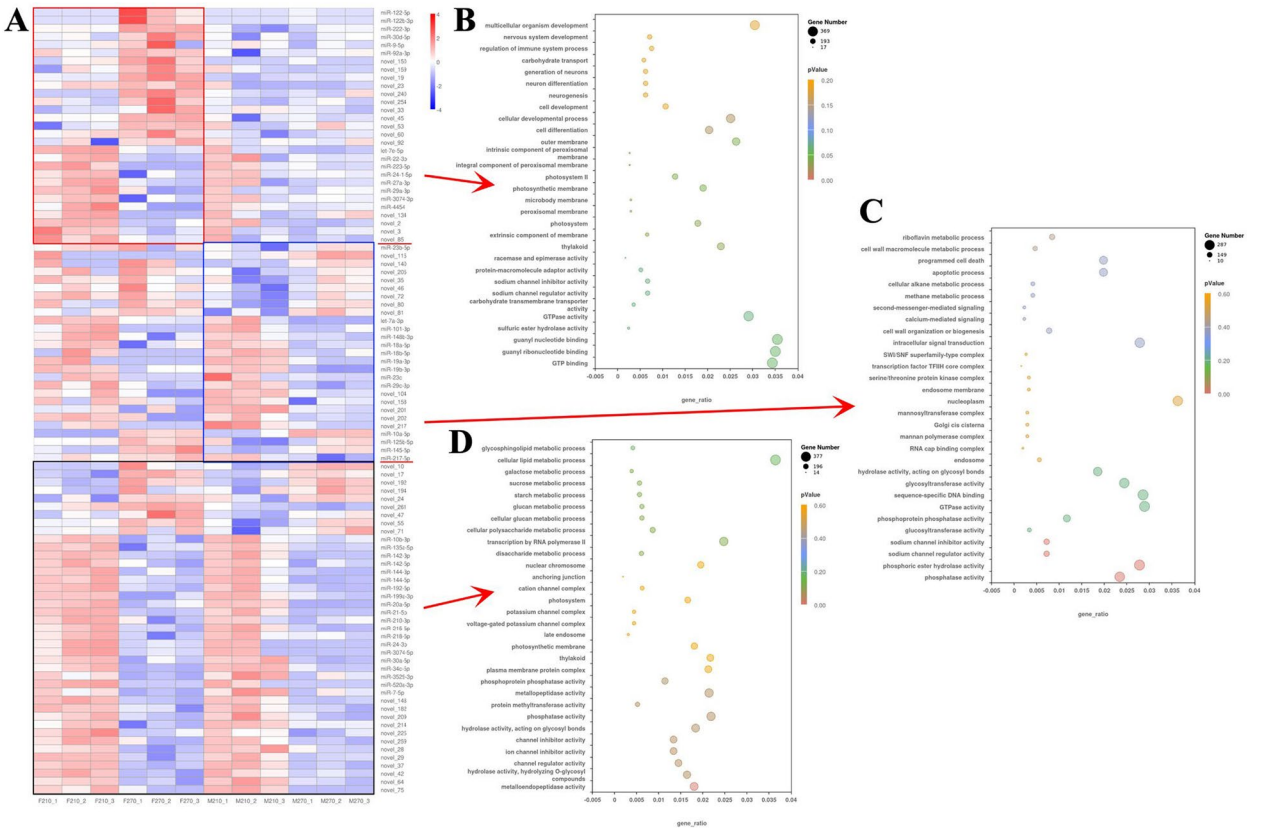

Fig. 7 Heatmap of all gonadal developmental DEMs (**A**). The red box indicates miRNAs differentially expressed only in male; the blue box indicates miRNAs differentially expressed only in female; the black box indicates miRNAs differentially expressed in both male and female. **B**: GO enrichment results of target genes corresponding to miRNAs differentially expressed only in male; **C**: GO enrichment results of target genes corresponding to miRNAs differentially expressed only in female; **D**: GO enrichment results of target genes corresponding to miRNAs differentially expressed in both male and female

Caudata species, remains extremely limited. Previous studies comparing miRNAs in the ovaries and testes of four-year-old CGS identified miR-451, miR-10c, miR-101, miR-202, miR-7a, and miR-499 as potential key regulators in sex differentiation, gametogenesis, and development in CGS [26]. However, recently studies using both histological and molecular data have shown that sex differentiation in CGS is completed by 270 dph [21, 25]. At four years of age, CGS have clearly surpassed this developmental stage, leading us to conclude that the relationship between the aforementioned miRNAs and gonadal differentiation as well as development is quite limited. Moreover, while Chen et al. used transcriptomic data from *Xenopus laevis* as their reference sequence, this study analyzes miRNAs using transcriptomic data from CGS of the same age and sex, resulting in more accurate and reliable findings. Previous analyses of miRNAs in the testes of CGS have revealed high expression levels of miR-99b-5p, miR-10c, and miR-36 [27]. However, those studies were conducted on three-year-old CGS, which also may not accurately reflect the true processes of testicular development and differentiation. Moreover, prior research has demonstrated that the relationship between differentially expressed genes exhibiting binary expression patterns at a single time point and gonadal differentiation is quite limited [31]. This limitation likely extends to miRNAs as well. Consequently, investigating dynamic changes in gonadal differentiation and development solely from a single time point offers a constrained perspective. In earlier studies, the limitations of age were largely due to the inability to determine the sex of CGS at early stages based on phenotype. However, the development of sex markers has significantly aided research on early gonadal differentiation in CGS [32], enabling more precise analysis during critical windows of gonadal differentiation. In our recently study, we identified two critical windows of early gonadal differentiation in CGS at 210 and 270 dph [25]. Building upon these pivotal time points, the present study aims to further explore the potential sex regulatory roles of miRNAs in CGS, thereby providing deeper insights into the molecular mechanisms underlying amphibian sex differentiation.

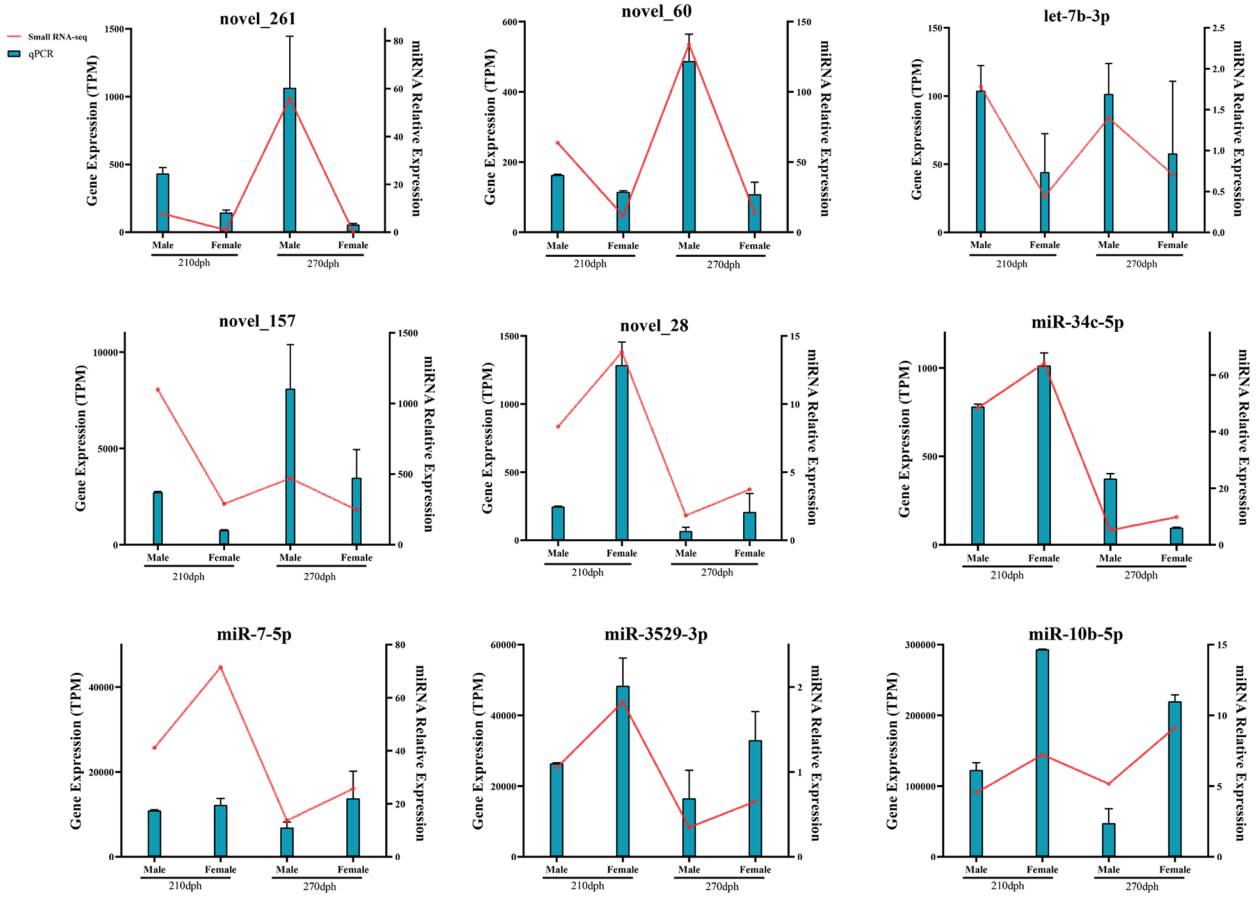

Fig. 8 Comparison of small RNA-seq and qRT-PCR results of nine sex-biased miRNAs in the 210dph and 270dph

In this study, a total of 162 known miRNAs and 212 novel miRNAs were identified from twelve samples. This may be attributed to the relatively small number of known miRNAs in amphibians; in miRBase 22.0 (http://www.mirbase.org/), the only two amphibian species listed, *Xenopus laevis* and *X. tropicalis*, have just 148 and 196 known miRNAs, respectively. A similar trend, where the number of known miRNAs is less than that of novel miRNAs, has also been observed in respiratory-related tissues of the CGS, including the gills, lungs, and skin [33]. Notably, the number of miRNAs identified in male individuals at 270 dph was significantly lower than that at 210 dph (Table 4). Given that miRNAs exert a negative regulatory effect on mRNA, our previous research indicated that 270 dph represents a critical window for male gonadal differentiation [25]. Consequently, the demand for mRNA production during gonadal differentiation at 270 dph necessitates a reduction in the number of miRNAs that suppress mRNA expression. In females, while previous studies identified 210 dph as the window for gonadal differentiation, no significant difference in the number of miRNAs was observed between 210 and 270 dph. We speculate that the window for female gonadal differentiation may occur even earlier than 210 dph. Additionally, the miRNA-mRNA co-expression correlation heatmap shows that at 270dph, the negative regulatory relationships between miRNAs and mRNAs are more clearly defined compared to 210dph (Fig. 5). This suggests that the period between 210 and 270dph may involve a significant recruitment of gonadal differentiation-related miRNAs in the gonads of CGS, which in turn participate in the regulation of gonadal differentiation through mRNA modulation.

Previous studies on *Pogona vitticeps* gonads found that differentially expressed genes identified at a single time point were predominantly enriched in muscle-related terms, leaving the authors questioning any direct association with sex determination [31]. Gonadal differentiation is recognized as a dynamic process [34], suggesting that identifying sex-specific genes at a single time point may have considerable limitations. In this study, we identified differentially expressed miRNAs (DEMs) in both male and female gonads at two critical time points, 210 dph and 270 dph. We found 16 miRNAs exhibiting

differential expression only at 210 dph, 20 miRNAs only at 270 dph, and 10 miRNAs at both time points. We then performed enrichment analysis on the target genes of these three groups of DEMs. The results revealed that miRNAs differentially expressed at both time points were significantly enriched in sex related terms, whereas those specific to a single time point showed no clear enrichment in sex related terms. This suggests that miRNAs with differential expression across multiple time points may be most closely associated with gonadal differentiation. Accordingly, in subsequent analyses, we focused specifically on this subset of ten sex-biased miRNAs.

Among these ten sex-biased miRNAs that exhibited differential expression at both time points, novel_33 displayed varying sex biases in each stage. However, as this is a novel miRNA, its specific function remains undetermined based on current knowledge. Therefore, novel_33 was not a focus in this study. Should future research yield new insights, we will provide a more detailed analysis and interpretation. In the male-biased miRNAs, only one known miRNA was identified: let-7b-3p. The let-7 family was first discovered as a crucial developmental regulator in nematodes and is one of the earliest known microRNAs. Let-7 has regulatory roles in both testes and ovaries, and it has been shown to influence spermatogenesis and oocyte maturation [35, 36]. Additionally, let-7 is involved in the initial regulation of sexual maturation [37]. High expression levels of let-7 have been observed in the ovaries and testes of *Portunus trituberculatus* [17]. Let-7 negatively regulates ovarian development in *Megalobrama amblycephala*, with let-7a/b/d being the most critical [38]. In *Litopenaeus vannamei* and *Oplegnathus punctatus*, let-7 shows higher relative expression in the ovaries [39, 40]. However, in *Pelteobagrus fulvidraco* and *Acanthopagrus latus*, let-7 exhibits a male-biased expression pattern [41, 42]. The expression level of let-7a ranks among the top 10 in the semen of *Cynoglossus semilaevis* [16]. Similar to the latter, is highly expressed in the male gonads of CGS. In combination with findings from a prior mRNA transcriptome study [25], the targeting relationships between the nine DEMs identified in this study and sex-related genes in CGS were investigated. let-7b-3p targets the female-specific differentiation marker gene *CYP19A1*, as well as the sex determination candidate gene *TRPM1* identified in our previous research. This suggests that let-7b-3p may negatively regulate female-related genes such as *CYP19A1* and *TRPM1* in CGS, facilitating differentiation along the male gonadal pathway. In contrast to our findings regarding the targeting of female genes, previous studies have shown that let-7b can negatively regulate male-related genes, such as *IAG* and *DSX*, in *Eriocheir sinensis* [43]. Similarly, in *Paralichthys olivaceus*, the let-7 family regulates *CBX2*, thereby influencing the expression of the *SF1* gene in testes [44]. This further highlights the varying regulatory roles of the let-7 family in gonadal differentiation across different species. In the female-biased miRNAs, four known miRNAs, namely miR-3529-3p, miR-34c-5p, miR-10-5p, and miR-7-5p, were identified. Notably, research on miR-3529 in relation to sex differentiation remains unexplored. In CGS, miR-3529-3p is predicted to target several genes linked to male gonadal differentiation, including *TBX1*, *SOX9*, *STAR* and *FGF7*. This suggests that miR-3529-3p may negatively regulate these male differentiation genes, thereby promoting female differentiation in CGS. However, its regulatory patterns and mechanisms require further investigation. In *Alligator sinensis*, miR-10a and miR-10b have been shown to target and regulate *ADCY4* and *FGFR2*, thereby influencing the process of mid-TSP stage [45]. In aquaculture, the expression of miR-10 is exclusively biased towards the testes in *A. latus*, and has been reported only in this species [41]. In contrast, miR-10 exhibits a female-biased expression in the CGS of this study. miR-34 has been shown to play an indirect role in sex differentiation and testicular development of *Scylla paramamosain* by regulating *DMRT1*, *iDMRT2*, *DSX*, and *DMRT-like* genes [46]. Additionally, miR-34 may indirectly modulate reproduction in *S. paramamosain* by binding to the 3′-UTRs of *MIH*, *CHH*, *ECR*, and *FAMET* genes and suppressing their expression [47]. Similar to let-7, miR-34 has been shown to downregulate the expression of *IAG* in *Eriocheir sinensis* [43]. In CGS, miR-34 similarly targets male-related genes, such as *SOX9*, aligning with previous findings. *DMRT1* is an important gene involved in male gonadal differentiation, and in this study, it is targeted and regulated by miR-7-5p. Additionally, miR-7-5p also targets other crucial genes related to male gonadal differentiation, such as *SOX9* and *FGFFGF7*. This highlights the significant role of miR-7 in the gonadal differentiation of CGS. Previous studies have similarly implicated miR-7 in the regulation of gonadal development [41, 48]. Several novel miRNAs identified in this study appear to play an important role in gonadal differentiation. This research also provides detailed sequence information for these miRNAs, though further studies are required to elucidate their specific functions and regulatory patterns on mRNA.

In our study on gonadal development, we identified 74 DEMs in males and 68 in females. These DEMs are associated with GTP-binding proteins and the PI3K-Akt signaling pathway. Of these, 45 DEMs were present in both sexes, with their target genes primarily enriched in terms related to metabolism, transcription, and enzyme activity. The 29 DEMs unique to males were mostly enriched in categories related to cellular differentiation and development, often involving guanosine binding processes.

Gonadal development is inherently a multicellular developmental process [34], and these male-specific categories suggest progression in gonadal development. In contrast, the 23 DEMs unique to females were linked to intracellular signal transduction and calcium signaling. Ca^{2+} plays a key role in gonadal development, sex differentiation, and even sex reversal [9, 10, 49]. Therefore, we propose that male and female gonadal development processes are regulated by distinct miRNAs rather than the same ones. Moreover, GTP-binding proteins and the PI3K-Akt signaling pathway likely play a significant role in the gonadal development of CGS.

Conclusions

In summary, this study represents the first investigation of miRNAs associated with gonadal differentiation in CGS at two critical windows, further confirming that 210 dph and 270 dph are key periods for CGS gonadal differentiation. We identified nine common DEMs between these periods as sex-biased miRNAs of CGS gonadal differentiation, including let-7b-3p, miR-3529-3p, miR-34c-5p, miR-10-5p, miR-7-5p, and four novel miRNAs. However, our analysis also indicates that different miRNAs, rather than the same ones, may regulate the development of male and female gonads. This study provides insights into sex-biased and gonadal development related miRNAs in CGS, offering valuable references for understanding CGS sex determination and gonadal development. Additionally, it establishes an important theoretical foundation for advancing CGS aquaculture and improving production efficiency.

Abbreviations

CGS	Chinese giant salamander
dph	Days post hatching
miRNA	MicroRNA
ncRNA	Non-coding RNA
DEMs	Differentially Expressed miRNAs
RT-qPCR	Real-Time quantitative PCR

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11419-x.

Supplementary Material 1.

Supplementary Material 2.

Acknowledgements

We are very thankful for Yunxing Professional Breeding Cooperative of Giant Salamander in Ya'an, Sichuan Province for their breeding work and are equally grateful to Dr. Martinson Ankrah Twumasi in Sichuan Agricultural University for his modification work in the language of this paper.

Authors' contributions

S.J.Y. and Q.W. conceived and designed the study. G.X.Z. and Q.W. supervised the work. X.L.W., H.Y., F.Y., L.S.X., Z.J.J., and S.J.Y. collected samples and conducted experiments. S.J.Y. analyzed the data and drafted the manuscript. S.J.Y. and Q.W. revised the manuscript. All authors have reviewed and approved the final version.

Funding

This study is supported to Wang Q by Applied Basic Project of Sichuan Science and Technology Department (2022NSFSC0122). Additional support is provided by the National Natural Science Foundation of China (NSFC32270477) to Zhu GX.

Data availability

The raw small RNA-seq reads are available in the NCBI SRA (accession number: PRJNA1182843; https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1182843).

Ethics approval and consent to participate

The sampling location is not privately owned or protected, and no specific permission is required for sample collection. Animal handling and euthanasia followed Institutional Animal Care and Use Committee protocols, approved by the Animal Care and Use Committee of Sichuan Agricultural University (license number SKY-2021116003). The methods applied in this study were carried out in strict accordance with the Laboratory Animal Management Principles of China.

Consent for publication

Not Applicable.

Competing interests

The authors declare no competing interests.

Received: 21 November 2024 Accepted: 28 February 2025
Published online: 12 March 2025

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