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Genetic and metabolic insights into sexual dimorphism in the flexor carpi radialis of Asiatic toads (*Bufo gargarizans*) associated with amplexus behavior

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Abstract

Background Sexual dimorphism, a widespread phenomenon across the animal kingdom, encompasses differences between sexes in size, morphology, and physiological traits. In this study, we investigated sexual dimorphism in the flexor carpi radialis (FCR) muscle, which is critical for amplexus in Asiatic toads (*Bufo gargarizans*), using integrated transcriptomic and metabolomic approaches.

Results Male toads exhibited significantly larger FCR muscles, reflecting enhanced muscle function required for sustained amplexus. Transcriptomic analysis identified 818 differentially expressed genes (DEGs) between sexes, with 389 upregulated and 429 downregulated in males, predominantly associated with muscle contraction, sarcomere organization, and energy metabolism. Metabolomic profiling revealed 69 differentially expressed metabolites (DEMs), with male-biased enrichment in pathways involved in protein synthesis and degradation, energy metabolism, and material transport. Integrated analysis pinpointed key metabolic pathways—such as glycine, serine, and threonine metabolism; alanine, aspartate, and glutamate metabolism; fatty acid degradation; and the tricarboxylic acid (TCA) cycle—as central to the observed sexual dimorphism. Among these, the genes *AGXT*, *ACADL*, *ACAT1*, *MDH2*, and *SUCLG2* emerged as pivotal regulators.

Conclusions Collectively, these findings provide novel insights into the genetic and metabolic basis of sexual dimorphism in *B. gargarizans*, offering a deeper understanding of the evolutionary mechanisms driving sex-specific traits in vertebrates.

Keywords Sexual dimorphism, Transcriptomic, Metabolomic, Muscle, Amplexus

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Background

Sexual dimorphism refers to intraspecific variations in morphology, physiology, and behavior between males and females, and is widespread among both invertebrates and vertebrates [1, 2]. This phenomenon, driven by evolutionary forces such as sexual and natural selection, is pivotal in enhancing sex-specific fitness, particularly regarding reproductive success [3]. Given that males and females share a largely identical genome, with the exception of sex chromosome-specific genes, the need for understanding the molecular mechanisms underlying sexual dimorphism under various selective pressures has been extensively emphasized [4, 5]. Furthermore, investigating these mechanisms not only aids in elucidating the adaptive functions of dimorphic traits but also reveals how species develop specialized structures and behaviors to optimize reproductive roles.

Among vertebrates, amphibians are an ideal group to explore the evolution and functional significance of sexual dimorphism traits due to their pronounced sex-biased differences in size, coloration, and vocalizations [3, 6, 7]. A particularly illustrative example is the amplexus behavior observed in most anurans. During the breeding period, males tightly grasp females from the back using specialized forelimb muscles to ensure female fidelity and increase reproductive success [8, 9]. The flexor carpi radialis (FCR) muscle, one of the amplectant muscles, plays a crucial role in this clasping behavior. Evidence indicates that the FCR mass and the length of the attached forelimb bone in adult male Asiatic toads are significantly greater than in adult females [10, 11]. Similar findings have been observed in other anuran species [12–15]. Despite similar muscle fiber type compositions between sexes, the male FCR muscles have greater oxidative capacity compared to those of the female [14, 16]. Furthermore, under simulated natural contraction conditions, the male FCR muscles exhibit higher fatigue resistance and produce greater force compared to female FCR muscles [12, 17]. These morphological and physiological differences enable males to maintain amplexus for hours or even days, thereby increasing their changes of fertilizing eggs.

Sexual dimorphism in amphibians is often closely associated with traits influenced by sexual selection, such as musculature adapted for reproductive behaviors [10, 15]. Several studies have demonstrated the pivotal role of androgens, particularly testosterone, in shaping sexual dimorphism in muscle mass and contractile properties [18–21]. However, sexual dimorphism might also emerge from more complex mechanisms, such as developmental organizational effects and tissue-specific decoupling from hormonal regulation, which enable traits to evolve independently in response to distinct selective pressures [22, 23]. Although phenotypic and hormonal differences between sexes in amphibians are well documented, the molecular regulatory networks underpinning sex-specific gene expression and the metabolic adaptations driving these traits remain poorly understood. Recent advancements in molecular biology, particularly transcriptomics and metabolomics, offer powerful tools to investigate the genetic pathways and metabolic processes involved in sexual dimorphism [1, 24–27]. For instance, studies on *Pelophylax esculentus* have revealed sexually dimorphic gene expression patterns in various brain regions. Genes involved in the synthesis and function of sex hormones, such as *hsd17b1*, *cyp19a1*, and *esr1*, are expressed at higher levels in reproductive females compared to males, indicating a possible link between hormone regulation and sex-biased traits in amphibians [28]. Furthermore, in male *Leptobrachium leishanense*, the development of nuptial spines on the maxillary skin—a feature absent in females—appears to play a role in male combat, female attraction, or nest guarding during the breeding season [29, 30]. A previous study integrating genomic and transcriptomic analyses has identified a cluster of male-biased, duplicated hair keratin genes specifically expressed in the maxillary skin, potentially contributing to the development of these nuptial spines [31]. Advancements in omics and functional studies will likely offer deeper insights into the evolutionary mechanisms driving sexual dimorphism in amphibians.

The Asiatic toad (*Bufo gargarizans*) is widely distributed across East Asia, encompassing regions such as China, Russia, and the Korean Peninsula [32]. In Nanchong City, Sichuan, China, this species typically enters hibernation between November and December and emerges in mid-March of the following year [33]. Notably, *B. gargarizans* exhibits an explosive breeding strategy, with a reproductive period lasting approximately 25–30 days from December to February [34]. During this time, hibernation is temporarily interrupted to facilitate reproduction, after which dormancy resumes. Given the critical role of sexual dimorphism traits in the reproductive success of amphibians, especially in the context of amplexus behavior, the FCR muscle in *B. gargarizans* presents an ideal model for investigating the genetic and metabolic foundations of sexual dimorphism. By integrating transcriptomic and metabolomic approaches, this study aims to elucidate the molecular mechanisms that drive the pronounced differences in male and female FCR muscles. Our research will not only provide novel insights into the specific pathways regulating these traits but also contribute to a broader understanding of the evolutionary forces shaping sexual dimorphism across vertebrates.

Results

Sexual dimorphism in the flexor carpi radialis of *B. gargarizans*

Compared to females, male *B. gargarizans* exhibited significantly larger flexor carpi radialis (FCR) muscles, which are critical for amplexus during the breeding season (Fig. 1A and B). The mean wet and dry masses of the FCR muscle were substantially greater in males than in females. These differences were significant, independent of snout-vent length (SVL), as shown by an ANCOVA ($P < 0.001$) (Fig. 1C and D).

Identification of genes expressed differentially between male and female toads

A transcriptomic analysis was conducted to investigate differential gene expression patterns in the FCR muscles of male and female groups. The distribution of FCR samples was initially determined using a principal component analysis (PCA). The PCA results demonstrated that principal components 1 and 2 accounted for 32.32% and 9.05% of the variances, respectively, indicating substantial distinctions between the male and female groups (Fig. 2A). Employing a criterion of $|\log_2 FC| \geq 2$ and an adjusted q-value < 0.05, a total of 818 differentially expressed genes (DEGs) were identified in the male group compared to the female group. Of these DEGs, 389 were upregulated, while 429 were downregulated in the FCR muscles of males compared to those of females (Fig. 2B, Table S1).

GO and KEGG analysis of DEGs

GO term and KEGG pathway enrichment analysis were further performed to elucidate the biological functions of DEGs associated with sexual dimorphism in the FCR muscles of Asiatic toads.

A total of 2,246 GO terms were identified for the DEGs. The top 10 terms in the biological process, cellular component, and molecular function categories were selected for detailed analysis (Fig. 2C). In the biological process category, terms such as sarcomere organization, muscle contraction, microtubule-based movement, and skeletal muscle thin filament assembly were prominent,

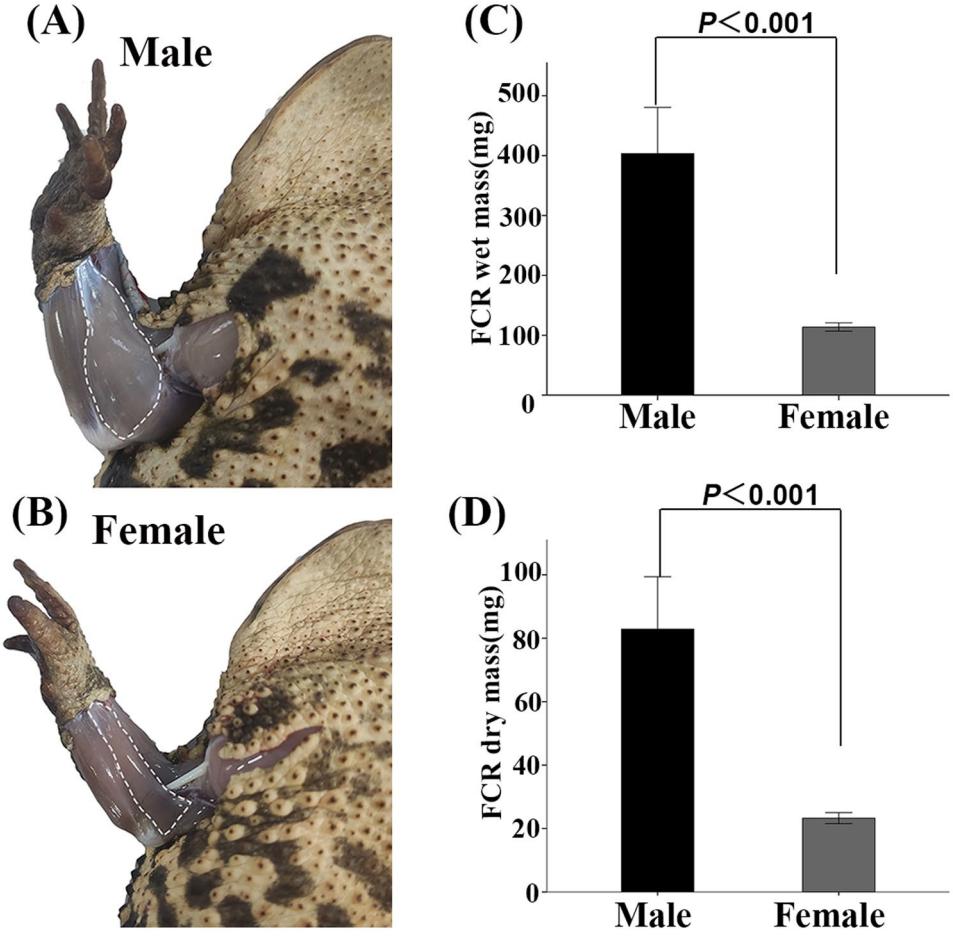

Fig. 1 (**A**) and (**B**) Illustration of the location and sexual dimorphism of the flexor carpi radialis (FCR) in *B. gargarizans* from a ventral view, with the FCR shown within the white dashed box. (**C**) and (**D**) Comparative analysis of FCR wet and dry mass between males and females, independent of SVL ($n = 9$). Significant differences are indicated by $P < 0.001$

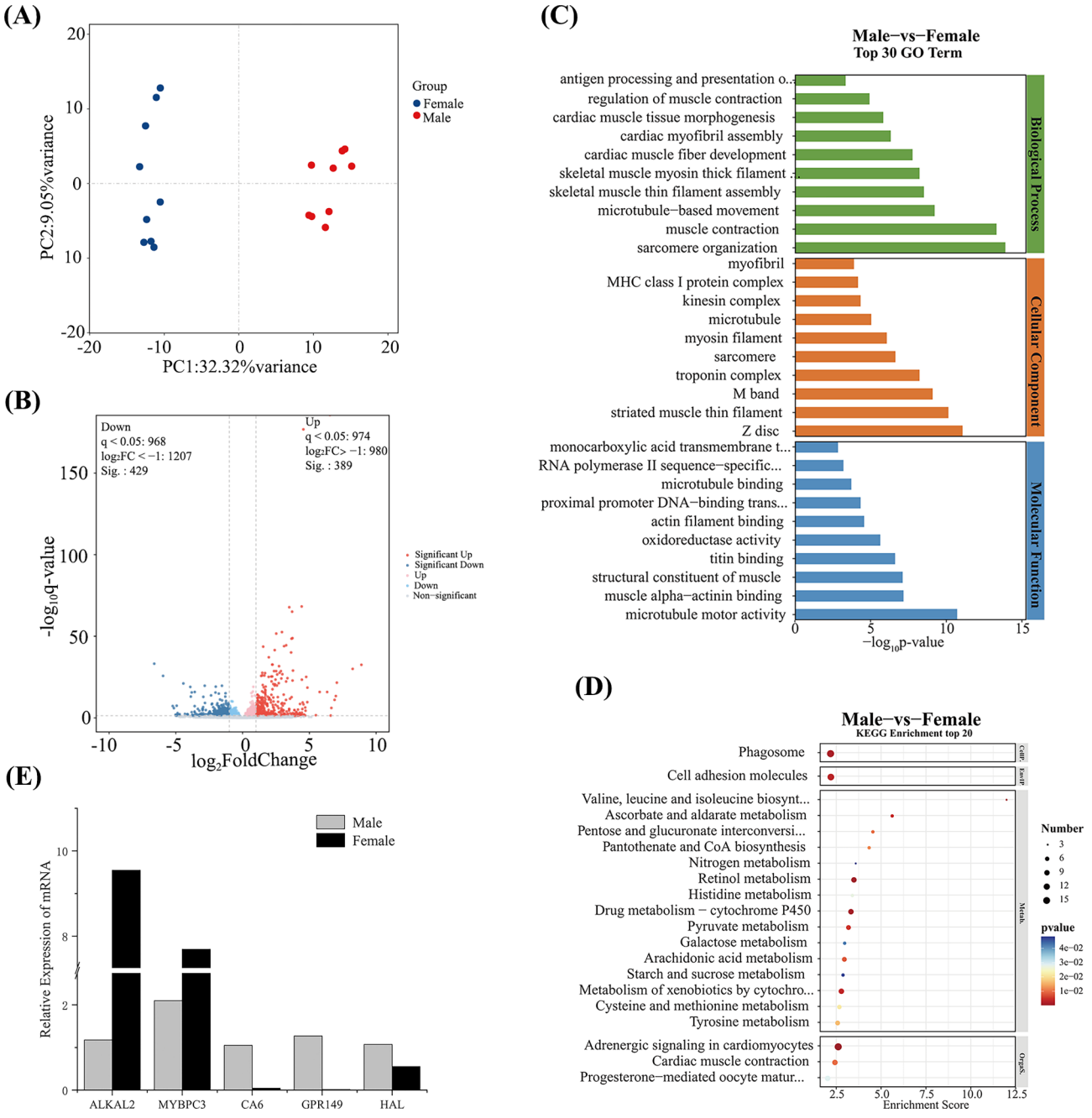

Fig. 2 (**A**) Principal component analysis (PCA) score plot distinguishing male and female *B. gargarizans*, showing clear clustering between the two sexes. (**B**) Volcano plot illustrating the genes that are expressed differentially ((DEGs)) in males relative to females. Red and blue dots represent significantly upregulated and downregulated genes, respectively. DEGs were defined by a threshold of$|\log_2 FC| \geq 2$ and an adjusted q-value < 0.05 ($n = 9$). (**C**) Gene Ontology (GO) enrichment analysis and (**D**) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the identified DEGs. (**E**) qRT-PCR validation of five DEGs (*ALKAL2*, *MYBPC3*, *CA6*, *GPR149*, *HAL*), consistent with RNA-seq results

indicating significant differences in the development and contraction processes of FCR between males and females. In the cellular component category, terms like sarcomere, troponin complex, M band, striated muscle thin filament, and Z disc were highlighted, underscoring variations in the structural elements of FCR between males and females. The molecular function category included terms such as microtubule motor activity, muscle alpha-actinin binding, structural constituent of muscle, titin binding, and oxidoreductase activity, suggesting notable differences in the molecular activities essential for FCR function and regulation between males and females.

The KEGG analysis revealed enrichment of DEGs in 128 pathways, with the top 20 pathways highlighted for

display (Fig. 2D). Notable changes were observed in the phagosome and cell adhesion molecule pathways. Additionally, 15 of the top 20 pathways were enriched in various metabolic processes, including valine, leucine, and isoleucine biosynthesis, retinol metabolism, and drug metabolism via cytochrome P450. These pathways may be involved in muscle regeneration, structural composition, and metabolism. Collectively, our findings highlight the essential roles of DEGs in biological processes and pathways that potentially influence sexual dimorphism in the FCR muscles of Asiatic toads.

Validation of RNA-seq data using qRT-PCR

To validate the transcriptomic analysis results, 5 genes (*ALKAL2*, *MYBPC3*, *CA6*, *GPR149*, and *HAL*) were randomly selected for quantitative real-time polymerase chain reaction (qRT-PCR). The expression profiles of these genes measured by qRT-PCR were in close agreement with the RNA-seq data (Fig. 2E, Table S1), further confirming the reliability of the RNA-seq results.

Metabolomics analysis of male and female toads

A metabolomic analysis was performed to discern between the metabolic profiles of the FCR muscles between of male and female toads. PCA and OPLS-DA analysis revealed a clear separation between the male and female groups (Fig. 3A and B), indicating distinct metabolic characteristics of FCR muscles in males compared to females. A total of 69 differential metabolites (DEMs) were identified between the groups based on VIP > 1 and

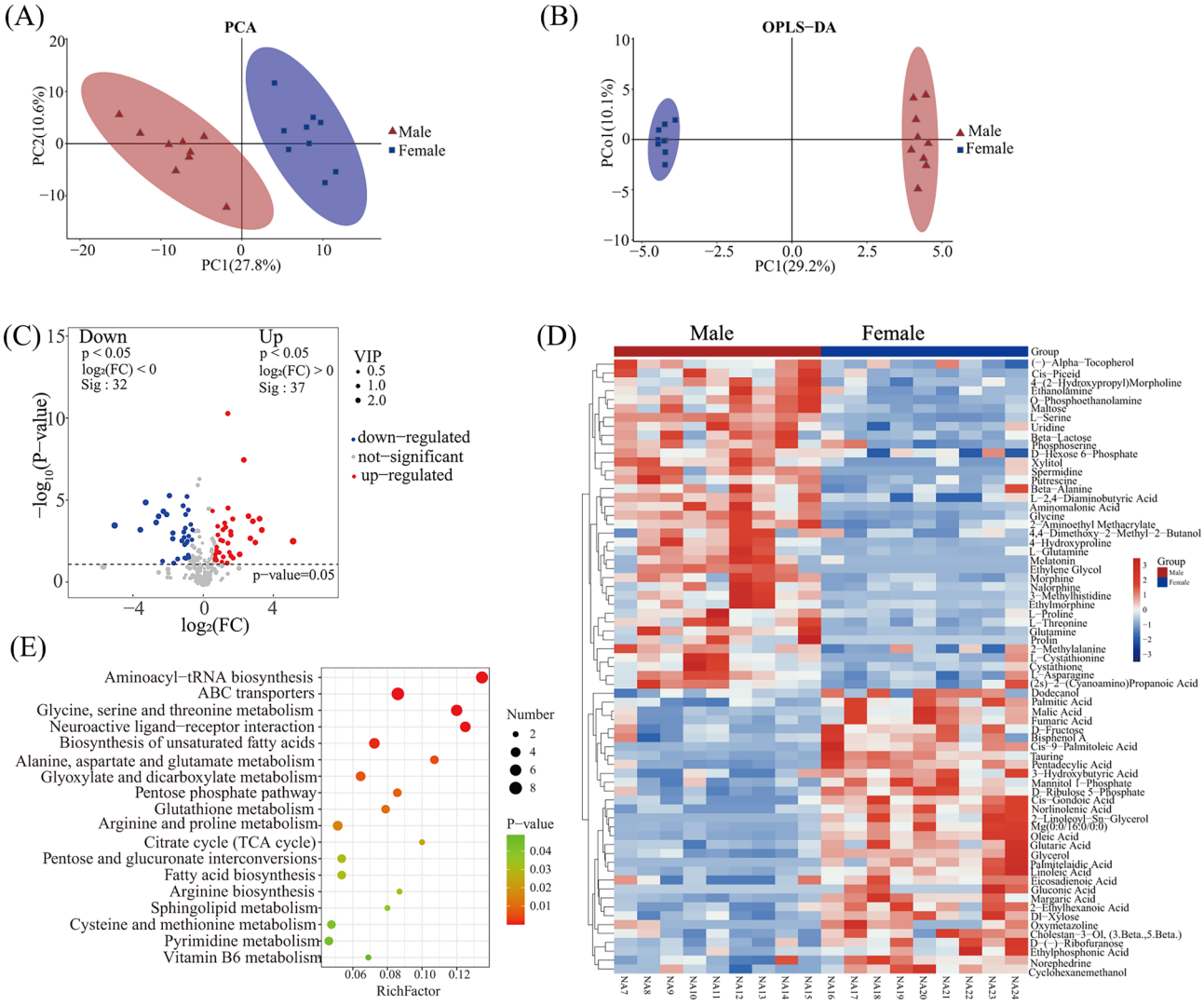

Fig. 3 (**A**) PCA and (**B**) OPLS-DA score plots differentiating male and female *B. gargarizans*, showing clear clustering between the two sexes. (**C**) Volcano plot illustrating the differentially expressed metabolites (DEMs) of males relative to females. Red and blue dots represent significantly upregulated and downregulated metabolites, respectively. (**D**) Heat map of DEMs in male and female *B. gargarizans*. Color intensity corresponds to metabolite concentration, with red indicating higher levels and blue indicating lower levels. DEMs were identified based on VIP > 1 and $P < 0.05$ ($n = 9$). (**E**) KEGG pathway enrichment analysis of the identified DEMs

$P < 0.05$, with 37 metabolites upregulated and 32 downregulated in males compared to females (Fig. 3C and D; Table S2). Among the 69 DEMs, 25 were organic acids and derivatives involved in the TCA cycle and amino acid metabolism, 12 were organic acids and derivatives involved in glycometabolism, 16 were lipids and lipid-like molecules involved in fatty acid metabolism and antioxidant protection, and 16 were other substances.

Subsequent KEGG pathway annotation of the DEMs identified 52 metabolic pathways, of which 18 exhibited significant differences ($P < 0.05$) between the male and female groups. As shown in Fig. 3E, the DEMs were primarily enriched in pathways associated with protein synthesis (aminoacyl-tRNA biosynthesis), amino acid metabolism (glycine, serine, and threonine metabolism; alanine, aspartate, and glutamate metabolism; arginine and proline metabolism; arginine biosynthesis), fatty acid biosynthesis and metabolism (biosynthesis of unsaturated fatty acids, fatty acid biosynthesis), and glutathione metabolism. Collectively, these differences in metabolic pathways related to protein synthesis and degradation, energy metabolism, and material transport suggest a metabolic basis for sexual dimorphism in the FCR muscles.

Integrative transcriptomic and metabolomic analysis

To investigate the network interactions between differentially expressed genes (DEGs) and differentially expressed metabolites (DEMs), we conducted a comprehensive association analysis of metabolomic and transcriptomic data. The correlation analysis of DEGs and DEMs identified L-serine, glycine, 4-hydroxyproline, oleic acid, morphine, and several other metabolites as key nodes within the correlation network (Fig. 4A). Subsequently, DEGs and DEMs between male and female groups were mapped to 42 common pathways using KEGG pathway analysis (Fig. 4B). Among these, 4 pathways exhibited particularly notable enrichment: glycine, serine, and threonine metabolism; alanine, aspartate, and glutamate metabolism; fatty acid degradation; and TCA cycle. Within the glycine, serine, and threonine metabolism pathway, *AGXT* expression was significantly higher in male FCR muscles, coinciding with elevated glycine and serine levels, suggestive of enhanced protein synthesis (Fig. 4C, Table S2). Additionally, *AGXT* was involved in the regulation of the alanine, aspartate, and glutamate metabolism pathway (Figure S1). In the fatty acid degradation pathway, the downregulation of two upstream metabolites, hexadecanoate and glutarate, was associated with the upregulation of *ACADL* and the downregulation of *ACAT1* in male FCR muscles (Figure S2). Within the TCA cycle pathway, two upstream DEGs, *MDH2* and *SUCLG2*, exhibited positive correlations with the downstream metabolites fumarate and (S)-malate, respectively, potentially contributing to sex-specific differences in FCR muscle energy supply (Figure S3). Taken together, the interactions between DEGs and DEMs within these common pathways appear to play a crucial role in the regulation of amino acid and fatty acid metabolism and the energy supply process, likely contributing to the observed sexual dimorphism in FCR muscles.

Discussion

Amplexus is a crucial reproductive behavior in amphibians, characterized by a male grasping and embracing a female around the waist or under the arms. The FCR muscle is central to amplexus, because it allows males to maintain a firm grip on females during this reproductive behavior. This muscle exhibits pronounced sexual dimorphism in both morphology and physiology, yet the molecular mechanisms underlying these differences remain largely uncharacterized. To our knowledge, this is the first study to integrate transcriptomic and metabolomic analyses to uncover key genes, metabolites, and associated pathways driving sexually dimorphic traits in the amplectant muscles of amphibians. We identified 818 DEGs and 69 DEMs, which were analyzed through a comprehensive association approach. This revealed several key pathways, such as glycine, serine, and threonine metabolism; alanine, aspartate, and glutamate metabolism; fatty acid degradation, and the TCA cycle. These pathways are pivotal in regulating the metabolic and functional differences observed between the sexes in the FCR muscles. Notably, five key DEGs -*AGXT*, *ACADL*, *ACAT1*, *MDH2*, and *SUCLG2*-emerged as central regulators within these pathways, highlighting their critical roles in driving the metabolic adaptations required for sexual dimorphism in *B. gargarizans*.

Sexual dimorphism in the FCR muscles of *B. gargarizans*

Sexual dimorphism in skeletal muscle structure and function is common across vertebrates, reflecting distinct selective pressures on males and females. Key traits such as muscle mass, myofiber composition, contractile strength, and resistance to fatigue have been extensively studied [35–39]. In mammals like gorillas (*Gorilla beringei*) and lions (*Panthera leo*), males exhibit significantly larger muscles than females, aiding in dominance during territorial disputes and mate competition [40–42]. Similarly, in many male anurans, the evolution of enlarged forelimb and hindlimb muscles is essential for clasping females during amplexus and fending off rivals, thus ensuring reproductive success [11, 43–46]. In line with these findings, our study revealed that male *B. gargarizans* exhibit greater FCR muscle mass compared to females, supporting the role of sexual selection in driving the evolution of muscle dimorphism in this species.

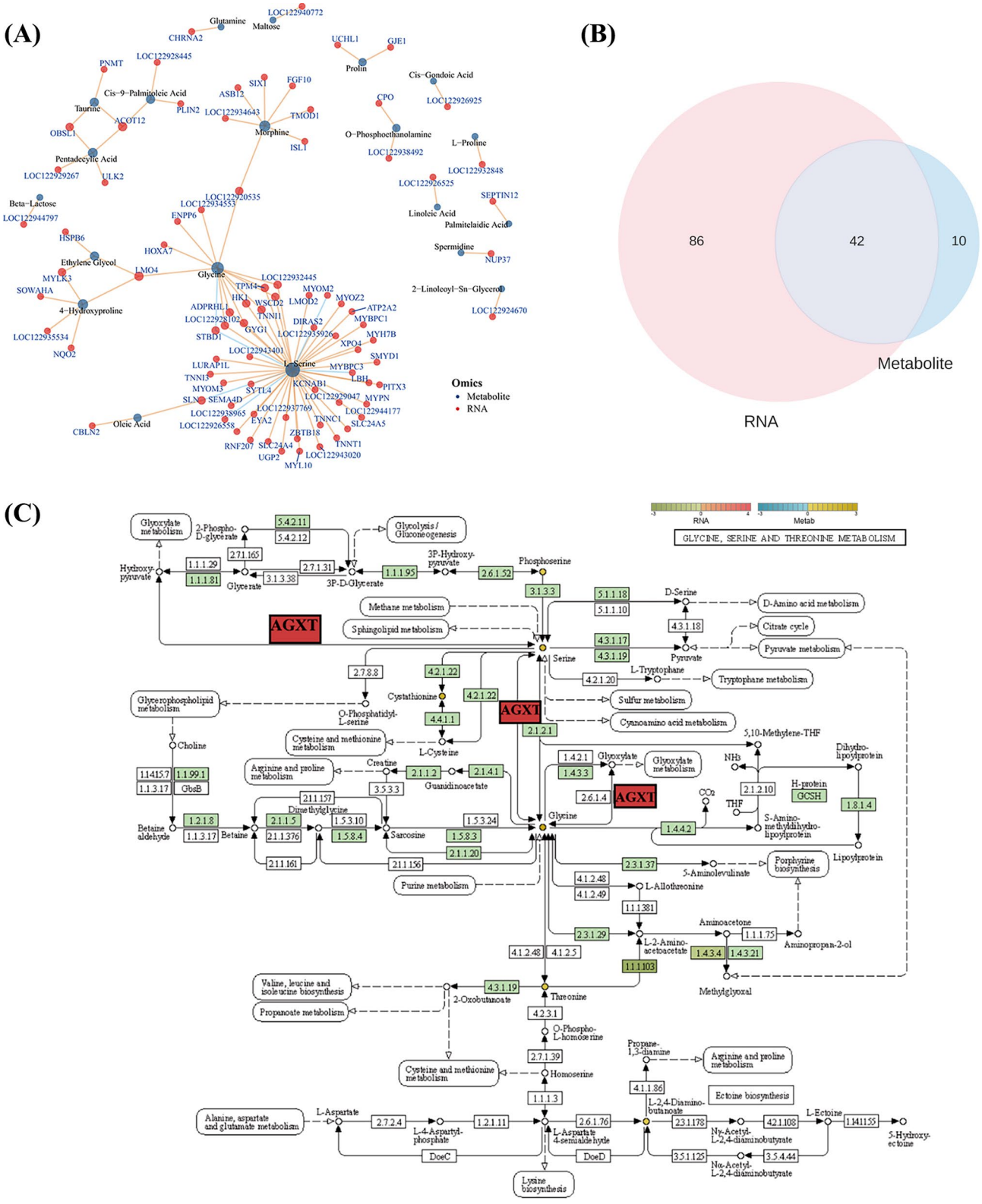

Fig. 4 (**A**) Correlation network analysis of DEGs and DEMs. Yellow lines represent positive correlations between DEGs and DEMs, while blue lines represent negative correlations. Blue labels indicate gene nodes, and black labels indicate metabolite nodes. (**B**) Venn diagram showing the overlap of pathways mapped to both DEGs and DEMs. (**C**) The glycine, serine, and threonine metabolism pathway, regulated by the DEG *AGXT*

Differential gene expression associated with sexual dimorphism in the FCR muscle

Our results from the GO and KEGG pathway analyses of DEGs revealed a strong association between DEGs and sexually dimorphic traits. Notably, DEGs were significantly enriched in GO terms related to muscle structure and function, such as sarcomere organization, muscle contraction, M band, Z disc, and oxidoreductase activity. The sarcomere, the basic contractile unit of skeletal muscle, is composed of interdigitating thick and thin filaments. Two transverse structures, the Z-disc and the M-band, anchor these filaments to the titin filament system [47–49]. Muscle contraction is driven by the sliding of these filaments past each other. Therefore, DEGs affecting the number, diameter, or structure of myofilaments could result in functional differences in sarcomere performance, consequently influencing muscle contractility. DEGs like *ACTN2*, *LMOD2*, *MYBPC1*, *MYOM1*, and *OBSL1* are linked to muscle atrophy and myasthenia, suggesting their potential role in modulating muscle integrity and function within the FCR muscle [50–53]. Furthermore, several DEGs, such as *NQO2*, *STEAP1*, and *KCNAB1*, were linked to oxidoreductase activity, which plays a key role in energy metabolism and antioxidant capacity. These genes may represent adaptations in males to meet the heightened muscular demands associated with prolonged amplexus [54–56]. The findings suggest that oxidative stress regulation and energy efficiency are critical factors in male muscle function under reproductive pressure. Our KEGG pathway analysis further underscored the role of DEGs in cellular processes such as phagocytosis, cell adhesion, and metabolic pathways. The phagosome pathway, for instance, is crucial for muscle regeneration, as it facilitates the removal of cellular debris and apoptotic cells, thereby promoting an environment conducive to tissue repair [57, 58]. Similarly, cell adhesion molecules (CAMs) contribute to satellite cell activation and promote muscle myogenesis and repair [59, 60]. Given that 15 of the top 20 pathways were related to metabolism, it is reasonable to speculate that gene expression differences drive metabolic variations between sexes, with males potentially requiring a more energy-efficient muscle function to sustain prolonged amplexus. Collectively, genes expressed differently between the sexes may underpin the observed sexual dimorphism in the amplectant muscles of *B. gargarizans*. This highlights the roles of muscle structure, energy metabolism, and regenerative processes in shaping these functional differences.

Metabolic differences between male and female *B. gargarizans*

Building on the transcriptomic insights, the metabolomic analysis further elucidates the molecular basis underlying sexual dimorphism in the FCR muscle of *B. gargarizans*. The majority of DEMs were associated with amino acid, carbohydrate, and fatty acid metabolic pathways, suggesting a coordinated metabolic adaptation in males to meet the energy demands of reproductive behavior. These results reveal conserved metabolic strategies observed across vertebrates, including humans and rodents, where sex-dependent adaptations optimize muscular activity for reproduction and survival [61–64]. A total of 25 DEMs were involved in amino acid metabolism, likely functioning as both an energy source through the TCA cycle and as substrates for protein synthesis and muscle repair critical for maintaining muscle integrity [65–67]. These findings align with studies in human skeletal muscle, where amino acid utilization is an important adaptive feature during prolonged or high-intensity activities. For instance, glycine and serine are known to play pivotal roles in oxidative metabolism and cellular repair [61, 62]. Furthermore, 12 DEMs related to glycometabolism emphasize the critical role of glucose in energy production. These metabolites not only provide immediate energy via glycolysis but also contribute to the synthesis of essential intermediates for various anabolic processes, indicating a possible adaptation in glucose utilization and storage mechanisms to meet the prolonged energetic demands of male reproductive behavior [68, 69]. 16 DEMs involved in fatty acid metabolism were identified, highlighting the role of lipids as an alternative energy resource. The upregulation of fatty acid metabolism in males suggests a shift towards lipid utilization during prolonged muscle activity, allowing for more efficient energy production over extended time frames. In addition, fatty acid metabolism likely contributes to the mitigation of oxidative stress, protecting the muscles from oxidative damage and maintaining muscle integrity during amplexus [70, 71]. The KEGG pathway enrichment analysis revealed that many of the identified DEMs are involved in protein synthesis and degradation, energy metabolism, and material transport. This indicates that male FCR muscles undergo higher rates of protein turnover, facilitating muscle repair and adaptation to the physical demands of prolonged mating behaviors. Overall, our metabolomic findings align with the physiological traits observed in males, suggesting that these metabolic changes are critical for sustaining the extended muscular activity required for reproductive success.

Integrative analysis of gene-metabolite networks in FCR sexual dimorphism

The integrated analysis of transcriptomic and metabolomic data further provide new explanations into the molecular networks driving sexual dimorphism in the FCR muscle of *B. gargarizans*. DEMs, including L-serine, glycine, and several other metabolites, emerged as key

nodes within the gene-to-metabolite network. Notably, L-serine and glycine are pivotal in intermediary metabolism, protein synthesis, and the neurotransmission process [72–74]. Additionally, four KEGG pathways (glycine, serine, and threonine metabolism; alanine, aspartate, and glutamate metabolism; fatty acid degradation; and the TCA cycle) were significantly enriched and they were all related to amino acid, fatty acid metabolism, and energy production. Five genes (*AGXT*, *ACADL*, *ACAT1*, *MDH2*, and *SUCLG2*) were implicated in the regulation of these pathways. *AGXT*, encoding alanine-glyoxylate aminotransferase, facilitates the transamination of alanine, glycine, and glyoxylate, producing glycine and pyruvate. Glycine plays a critical role in protein synthesis, while pyruvate serves as a key intermediate in gluconeogenesis, positioning *AGXT* as a central regulator of amino acid balance and protein synthesis [75–77]. *ACADL* encodes Long-chain specific acyl-CoA dehydrogenase, catalyzing the initial step of mitochondrial beta-oxidation of straight-chain fatty acid, an aerobic process critical for fatty acids into acetyl-CoA and supplying energy to skeletal muscles and the heart [78]. Mutations in *ACADL* are known to cause long-chain acyl-CoA dehydrogenase deficiency, resulting in nonketotic hypoglycemia [79]. Conversely, *ACAT1*, which encodes acetyl-CoA acetyltransferase, plays a major role in ketone body metabolism [80]. Evidence from skeletal muscle methylome-transcriptome integration studies in humans has demonstrated that sex differences in muscle are, in part, regulated by epigenetic mechanisms, which influence the expression of genes involved in substrate metabolism, such as fatty acid oxidation and glycolysis [61]. In our study, *ACADL* was upregulated, while *ACAT1* was downregulated in male FCR muscles, suggesting that males rely more on direct fatty acid oxidation than on ketogenesis for energy during amplexus. Furthermore, *MDH2* and *SUCLG2*, both involved in the TCA cycle, were positively correlated with downstream metabolites fumarate and (S)-malate, respectively. These genes encode enzymes essential for the TCA cycle, a central metabolic pathway responsible for ATP production through the oxidation of acetyl-CoA (Miller et al., 2011; Martinez-Reyes & Chandel, 2020). Our findings highlight the critical interactions between DEGs and DEMs, emphasizing their roles in amino acid and fatty acid metabolism, as well as energy supply mechanisms. These gene-to-metabolite networks likely underlie the molecular basis of sexual dimorphism observed in the FCR muscles of *B. gargarizans*.

Conclusions

Sexual differences in phenotypic and physiological traits can be attributed to variations in gene expression and metabolite profiles. This study uncovered a molecular network underlying sexual dimorphism in the FCR muscles of *B. gargarizans* through an integrated transcriptomic and metabolomic analysis. This analysis identified five key genes (*AGXT*, *ACADL*, *ACAT1*, *MDH2*, and *SUCLG2*) involved four significantly enriched pathways linked to amino acid metabolism, fatty acid metabolism, and energy production. These findings offer new insights into the molecular basis of sex-specific muscle phenotypes and energy demands in amphibians, enhancing our understanding of reproductive behavior and its physiological adaptations.

Methods

Sample Preparation and measurement

A total of 18 sexually mature Asiatic toads (*Bufo gargarizans*), comprising 9 males and 9 females, all confirmed to be 4 years of age, were collected on the evening of 12 October 2022 from agricultural fields in Nanchong City, China. The age of the toads was determined using the skeletochronological method as described in our previous study [32]. All individuals were euthanized via double pithing, following the Institutional Animal Care and Use Committee of China West Normal University (approval code: CWNU2021D006). The snout-vent length (SVL) of each toad was measured to the nearest 0.1 mm using precision calipers. For subsequent analyses, the flexor carpi radialis (FCR) muscle from the right forelimb was rapidly excised and snap-frozen in liquid nitrogen for both transcriptomic and metabolomic profiling. In contrast, the FCR from the left forelimb was reserved for weight determination as described in previous studies [15].

RNA sequencing and differentially expressed genes analysis

Total RNA was extracted from FCR muscles using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's guidelines. Libraries were constructed using the VAHTS Universal V6 RNA-seq Library Prep Kit according to standard protocols. Sequencing was performed on an Illumina NovaSeq 6000 platform, generating 150 bp paired-end reads. OE Biotech Co., Ltd. (Shanghai, China) conducted the transcriptome sequencing and subsequent analyses. Raw fastq reads were firstly processed using fastp to remove low-quality sequences. The resulting clean reads were mapped to the reference genome using HISAT2 [81]. Each gene's expression level was quantified in FPKM, and gene read counts were derived using HTSeq-count. Principal Component Analysis (PCA) was conducted using R software (v3.2.0) to assess the biological replicability of samples. Differential expression analysis was performed with DESeq2, applying a significance threshold of Q value < 0.05 and $|\log_2 FC| \geq 2$ for differentially expressed genes (DEGs). Hierarchical clustering of DEGs was executed with R (v3.2.0) to elucidate gene expression patterns across different groups

Table 1 List of primers used in this study

Genes	Forward primer sequence	Reverse primer sequence
GAPDH	CTAAGGCTGTAGGCAAAGTCATCCC	CCAAGCGAGCAGTCAGGTCAAC
ALKAL2	GACTGACTCCAATAACCTGACTGAC	TCAATGTCCTCCTTGTCCTCTGG
CA6	AGTCGCCTACTAAGTGCTCAAGAG	AGGATGACACCGCCAGATACAG
GPR149	TCGGATAACCAGGAACGGCTAC	AGCAGGATTATCAGGAGACAGAGAC
HAL	GCTTACTGGACCTGGATGACAAAC	ATCACATCGCCTTCCACAACAAC
MYBPC3	TCAGAGGTTGTGGTCGGAGATTC	TCACTAGCGGCGATGTCTACG

and samples. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of DEGs were also conducted using R (v3.2.0) to identify significantly enriched terms.

qRT-PCR validation of gene expression

Total RNA was extracted from the FCR muscles using the method described above. The extracted RNA was then reverse transcribed into first-strand complementary DNA (cDNA) using the PrimeScript RT reagent Kit (RR047A, TaKaRa, China). RT-qPCR was conducted using the TB Green Premix (RR820A, TaKaRa, China) on the QuantStudio TM3 system (ThermoFisher, CA, USA). All reactions were performed in triplicate to ensure reliability and reproducibility. RT-qPCR conditions were as follows: 95 °C for 230 s, followed by 45 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. To normalize the data, relative RNA expression levels were calibrated against the endogenous control GAPDH. The sequences of the primers used in this study are listed in Table 1.

Metabolomic analysis

Metabolites were extracted from the FCR samples following protocols outlined in previous studies [82], and analyzed using a gas chromatography system (Agilent 7890B) coupled with a mass spectrometry detector (5977 AMSD, Agilent Technologies, CA, USA). The raw GC/MS data were first converted to the.abf format for streamlined processing. Peak detection, identification, alignment, and normalization were subsequently performed using MS-DIAL, referencing the LUG database. Following normalization and peak merging, a data matrix comprising sample information, retention times, and signal intensities was generated. This matrix was imported into R for Principal Component Analysis (PCA) to evaluate sample distribution and analysis consistency. Orthogonal Partial Least-Squares-Discriminant Analysis (OPLS-DA) was then conducted to identify differentially expressed metabolites (DEMs), using 7-fold cross-validation and 200 response permutation tests to prevent overfitting. Variable Importance of Projection (VIP) scores were calculated to assess the contribution of each metabolite to group separation, with significance confirmed by a two-tailed Student's t-test. Metabolites with VIP > 1 and $P < 0.05$ were considered significant and mapped to relevant biological pathways using the KEGG Pathway Library.

Integrative transcriptome and metabolome analysis

To identify key candidate genes and pathways associated with sexual dimorphism in *B. gargarizans*, an integrative transcriptomic and metabolomic analysis was conducted. Pearson correlation coefficients (PCC) were calculated for DEGs and DEMs using the Hmisc package in R (version 4.4.0) to assess their correlations. The top 100 DEGs and DEMs with the highest PCC values were mapped to a correlation network, from which a correlation network diagram was generated to visualize the interactions. Furthermore, both DEGs and DEMs were mapped to the KEGG pathway database, identifying common pathways that revealed distinct features shared between the transcriptomic and metabolomic datasets.

Statistical analyses

Differences in muscle mass between the sexes were tested by the analysis of covariance (ANCOVA) with muscle mass as the dependent variable, sex as the independent variable, and SVL as a covariate. The significance level was set as 0.05, and data presented as mean ± SD. Statistical analysis was conducted using SPSS 23.0. The analysis of transcriptomic and metabolomic data between sexes was conducted as detailed in the preceding section.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11392-5.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Not applicable.

Author contributions

Conceptualization, C.Y. and Z.M.; Formal analysis, H.M., and L.J.; Project administration, H.M., L.J. and L.Z.; Funding acquisition, Z.M., H.M. and L.J.; Writing—original draft, C.Y. and Z.M. All authors have read and agreed to the published version of the manuscript.

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Data availability
The raw RNA-seq data are available at the Genome Sequence Archive (GSA) under the accession number CRA019873. The raw metabolic data have been deposited in Open Archive for Miscellaneous Data (OMIX), China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences, and are publicly accessible under the accession number OMIX007758 via the following link: https://ngdc.cncb.ac.cn/omix/release/OMIX007758.

Declarations

Ethics approval and consent to participate
All animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of China West Normal University (approval code: CWNU2021D006).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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