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Examination of sex-related differences in intestinal and gonadal lipid metabolism in the sea cucumber *Apostichopus japonicus*

Shuangyan Zhang [a,b,c,d,e,f], Xiaoshang Ru [a,b,c,d,f], Libin Zhang [a,b,c,d,e,f,*], David Gonçalves [g], Hongsheng Yang [a,b,c,d,e,f,h]

[a] CAS Key Laboratory of Marine Ecology and Environmental Sciences, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China
[b] Laboratory for Marine Ecology and Environmental Science, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266237, China
[c] Center for Ocean Mega-Science, Chinese Academy of Sciences, Qingdao 266071, China
[d] CAS Engineering Laboratory for Marine Ranching, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China
[e] University of Chinese Academy of Sciences, Beijing 100049, China
[f] Shandong Province Key Laboratory of Experimental Marine Biology, Qingdao 266071, China
[g] Institute of Science and Environment, University of Saint Joseph, Macao SAR 999078, China
[h] The Innovation of Seed Design, Chinese Academy of Sciences, Wuhan 430000, China

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ABSTRACT

Reproduction of the sea cucumber *Apostichopus japonicus* is critical for aquaculture production. Gonadal development is the basis of reproduction, and lipids, which are among the main nutrients required for gonadal development, directly affect reproduction. We investigated whether gonadal and intestinal lipid metabolism differed between male and female *A. japonicus*. Transcriptome analysis of the intestines of sexually mature male and female wild-caught individuals revealed differences in gene expression, with 27 and 39 genes being up-regulated in females and males, respectively. In particular, the expression of the fatty acid synthase gene was higher in males than in females. Metabolome analysis of the gonads identified 141 metabolites that were up-regulated and 175 metabolites that were down-regulated in the testes compared with the ovaries in the positive/negative mode of an LC-MS/MS analysis. A variety of polyunsaturated fatty acids were found at higher concentrations in the testes than in the ovaries. 16 s rDNA sequencing analysis showed that the composition and structure of the intestinal microbiota were similar between males and females. These results suggest that sex differences in intestinal metabolism of *A. japonicus* are not due to differences in the microbiota, and we speculate that gonadal metabolism may be related to intestinal morphology. This information might be useful in improving the reproductive efficiency of sea cucumbers in captivity.

1. Introduction

The sea cucumber *Apostichopus japonicus* is an important aquaculture species in China. In recent years, the production area of sea cucumbers has been expanding, and their cultivation technology has been continuously improving(2021 China Fishery Statistical Yearbook, 2022). Aquatic animals, such as sea cucumbers, require many essential nutrients to maintain growth, reproductive, and other physiological functions, and providing them with a balanced feed is vital for the production of high-quality individuals (Prabu et al., 2017).

Reproduction is a key part of the overall production cycle of sea cucumbers in aquaculture. Proper metabolism and energy supply are important for the normal development of gametes, and for covering the significant physiological costs of reproduction (Fowler and Williams, 2017), with both nutrient acquisition and energy storage being important (Jonsson, 1997). However, males and females often differ in their gonadal investment, and female reproduction is usually energetically more demanding than male reproduction in sea cucumbers (Muthiga and Kawaka, 2009). This suggests that there may be differences in gonadal metabolic patterns between the sexes.

Gonadal development forms the basis of reproduction and is a prerequisite for obtaining high-quality gametes for aquaculture. As one of the main nutrients required for gonadal development, lipids directly affect reproduction. Lipids and their derivatives are used as nutrients by

* Corresponding author at: CAS Key Laboratory of Marine Ecology and Environmental Sciences, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China.
E-mail address: zhanglibin@qdio.ac.cn (L. Zhang).

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most organisms, and can serve as energy sources or be stored. They also play an important role in the structure of cell membranes. Among them, the decomposition of triglycerides produces glycerol and fatty acids, and the oxidation of fatty acids is a high-energy process. Lipids closely related to gonadal development include various fatty acids, such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), arachidonic acid (ARA), and sterols (Nhan et al., 2020; Soudant et al., 1996; Xu et al., 2017). High levels of EPA, DHA and ARA in feed can improve male reproductive performance and sperm quality (Chimsung, 2014).

Compared with terrestrial ecosystems, aquatic ecosystems are characterized by a relatively high content of long-chain n-3 polyunsaturated fatty acids (PUFAs), which mainly include EPA, DHA, and linolenic acid (Hixson et al., 2015). Bell and Tocher (2009) reported that invertebrates seemed to have the ability to synthesize PUFAs, and PUFAs in crabs are key fatty acids for lipid transport from the hepatopancreas to the gonadal gland during gonadal development (Ghazali et al., 2017; Li et al., 2011). The digestion and decomposition of lipids not only provides energy for gonadal development, but it also participates in the process of reproductive endocrine regulation (Arukwe, 2008; Jiang et al., 2009). Certain lipids, such as cholesterol, are hormone precursors, which are involved in sex hormone synthesis. Therefore, it is critical to understand the relationship between lipids and gonadal function to improve the breeding efficiency of aquaculture organisms.

In most mammals, the metabolic homeostasis system differs between the sexes (Mauvais-Jarvis et al., 2017). For example, Hudry et al. (2016) found sex differences in the expression of metabolic genes in animal intestines, which may be a significant factor leading to sex differences in other organs. The intestine is an important part of the digestive system, and may serve as a connection for communication with other organs in certain organisms. For example, Hudry et al. (2019) reported that intestinal citrate can be locally transferred from the R4 midgut region to the adjacent testis by Indy transporter to maintain male gamete maturation in fruit flies (*Drosophila*). This indicates that there may be a bidirectional communication pattern between the intestine and the gonads and that physiological functions in specific areas of the intestine may differ between the sexes to meet the reproductive needs of the organism. Among aquatic animals, crabs can transport lipids from the hepatopancreas to the gonads through transporters and transferases (Wen et al., 2001). This suggests that aquatic invertebrates may also have tissue communication capabilities similar to terrestrial animals to promote gonadal development.

The complex microbial community in the intestine has potential effects on host physiology (Huttenhower et al., 2012). Microorganisms are affected by the host and the environment. They are involved in the homeostasis of the intestine, such as immune regulation, metabolism, and in particular, lipid metabolism which has been the focus of research (Roeselers et al., 2011). The composition and structure of the intestinal microbial community varies among organisms due to environmental influences and diet, among other factors (Wong and Rawls, 2012). Compared with terrestrial animals, there are more Fusobacteria and Proteobacteria in the intestinal microbiomes of aquatic animals (Kostic et al., 2013). An experiment in which zebrafish (*Danio rerio*) were fed diets with different fat content, revealed that a high fat diet affected the intestinal microflora of males and females differently, suggesting that sex has an important influence on the composition of the intestinal microflora of this species (Navarro-Barrón et al., 2019). In addition to bidirectional regulation, intestinal microbes participate in physiological processes such as metabolism and immune regulation in the host (Flint et al., 2012). Bäckhed et al. (2007) found that intestinal microbes in mice were related to host obesity, indicating that microbes can affect the accumulation of fat in tissues, and Min et al. (2019) reported a sex-specific microbial community corresponding to fat distribution. Semova et al. (2012) found that intestinal microbes play an important role in lipid metabolism in zebrafish. However, how these microorganisms regulate lipid metabolism within the host remains unclear.

This study set out to investigate whether the lipid metabolic processes of *A. japonicus* differ between males and females during gonadal maturation. Transcriptome analysis of the intestines was conducted to search for sex-biased differentially expressed genes (DEGs) related to lipid metabolism, and liquid chromatography with tandem mass spectrometry (LC-MS/MS) analyses were performed to identify differential metabolites in ovaries and testes. The intestinal microbial communities of male and female *A. japonicus* using 16 s rDNA sequencing. Nutrition and feeding are the primary means of achieving sustainable aquaculture of sea cucumbers, and our results provide insights into the possible relationships between the expression of metabolic genes in the intestines and gonadal metabolites in *A. japonicus*. Ultimately, this work may contribute to improving the breeding efficiency of cultured sea cucumbers.

2. Methods

2.1. Sample collection

All of the sea cucumbers used in this experiment were derived from wild stocks. Male and female *A. japonicus* weighing 150–200 g, and approximately 3 years old, were collected from Laizhou Bay, Shangdong, China in May 2020. There were no significant differences in body weight of the selected sea cucumbers by sex. The sea cucumbers were acclimatized in a laboratory aquarium for 3 days. The aquarium was continuously ventilated, and the dissolved oxygen content maintained at 8.5 mg/L. The salinity and pH were kept at 30‰ and 8.0, respectively. A sediment mixture was fed three times a day. Sea cucumbers were immersed in ice-cold seawater for anaesthesia before being sacrificed and sampled. The biological characteristics of the gonads were observed using a light microscope, and the sex and developmental stage of the gonads of individuals were determined based on their anatomy. Individual sea cucumbers at gonadal maturity were finally selected for analysis. By weighing the intestines and gonads, it was found that female sea cucumbers had significantly heavier gonads than males. The intestines and gonads were removed from the specimens, quickly frozen in liquid nitrogen, and stored at −80 °C prior to analysis. Hereafter, female intestines are referred to as IFs and gonads as GFs, and those of males are referred to as IMs and GMs, respectively.

2.2. Transcriptome analysis of DEGs in sea cucumber intestines

2.2.1. RNA extraction and cDNA library construction

Nine female and nine male sea cucumbers were selected for analysis. Intestine samples for each sex were pooled into groups of three, resulting in three replicates for both males and females. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The integrity of the RNA was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and checked by agarose gel electrophoresis. After the total RNA was extracted, the mRNA was enriched using Oligo dT beads. The enriched mRNA was fragmented into short fragments using fragmentation buffer and then reverse transcribed into cDNA using random primers.

2.2.2. Transcriptome sequencing, quality control, and sequence alignment

The cDNA fragments were purified using a QiaQuick PCR Extraction Kit (Qiagen, Venlo, Netherlands) and sequenced using an Illumina HiSeq2500 instrument (San Diego, CA, USA). To obtain high-quality reads, fastp 0.18.0 was used to remove raw reads containing adapters or low-quality bases (Chen et al., 2018). For genome-guided assembly, we used the HISAT2.2.4 package (Kim et al., 2015) to map reads from RNA-seq samples against the *A. japonicus* reference genome. Mapped reads for each sample were assembled using StringTie v1.3.1 through a reference-based method (Pertea et al., 2016; Pertea et al., 2015).

2.2.3. Gene expression and pathway enrichment analysis

The transcriptome raw data has been uploaded to NCBI (BioProject: PRJNA768534). For each transcription region, the fragments per kilobase of transcript per million mapped reads value was calculated to quantify differences in expression levels between males and females using the StringTie software. RNA differential expression analysis was performed using DESeq2 to compare different groups (Love et al., 2014). Genes with a false discovery rate (FDR) < 0.05 and absolute fold change (log2fc) ≥ 2 were considered to be differentially expressed. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to identify the significantly enriched metabolic pathways and signal transduction pathways relative to the genome background.

2.2.4. Quantitative real-time PCR (qRT-PCR) analysis

Five genes (gene-BSL78_02090, gene-BSL78_04869, gene-BSL78_06830, gene-BSL78_15322, and gene-BSL78_28188) were selected based on their transcriptomic expression profile in the intestine for validation using qRT-PCR. RNA extracted from transcriptome sequencing was used in qRT-PCR. First-strand cDNA was synthesized according to the instructions of the TaKaRa PrimeScript RT kit with SYBR Green I Master Mix. All primers used for qRT-PCR were designed using the Sangon Biotech primer design tool (https://www.sangon.com/primerDesign). All qRT-PCR analyses were performed using a Takara PrimeScript RT kit with SYBR Green I Master Mix in 20 μL of reaction mixture containing 10 μL of TB Green, 1 μL of cDNA template, 0.5 μL of forward primer, 0.5 μL of reverse primer, and 8 μL of ddH2O. All reactions followed kit instructions. The qRT-PCR data were analyzed using the formula $R = 2^{-\Delta\Delta CT}$ to calculate relative gene expression levels ($\log_2$(female (F)/male (M))) in different tissues of *A. japonicus* of different sexes. The *Grb2* gene served as the reference. ANOVA was used for analysis of variance of the data, and a significance level of $p < 0.05$ was considered to be statistically significant.

2.3. Metabolomic analysis of differential metabolites in sea cucumber gonads

2.3.1. Metabolite extraction

For metabolite extraction, 1000 μL of extraction solvent containing the internal standard (acetonitrile: methanol: water, 2:2:1) were added to Eppendorf tubes containing 50 mg of sea cucumber gonads. The samples were swirled for 30 s, homogenized at 45 Hz for 4 min, and sonicated in an ice-water bath for 5 min; this homogenizing and sonicating process was repeated three times. Samples were then incubated at −20 °C for 1 h and centrifuged at 12,000 rpm at 4 °C for 15 min. The supernatant was transferred to an LC-MS bottle and stored at −80 °C for UHPLC-QE Orbitrap/MS analysis.

2.3.2. LC-MS/MS analysis

LC-MS/MS analyses was performed by Gene Denovo Biotechnology Co. (Guangzhou, China) using a UHPLC system (1290, Agilent Technologies), UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm), and Q Exactive Orbitrap MS (Thermo Fisher Scientific). In the mobile phase A, the positive was 0.1% formic acid aqueous solution and the negative was 5 mmol/L ammonium acetate aqueous solution, and the mobile phase B was acetonitrile. The flow rate and injection volume were 0.5 mL/min and 2 μL, respectively. The elution gradient was set to 1% B at 0 min, 1% B at 1 min, 99% B at 8 min, 99% B at 10 min, 1% B at 10.1 min, and 1% B at 12 min. The QE mass spectrometer registered MS/MS spectra on an information-dependent basis (IDA). Acquisition software (Xcalibur 4.0.27, Thermo) continuously evaluated full scan measures of the MS data as collected and recorded the acquisition of MS/MS spectra. In the ESI, capillary temperature was set at 320 °C; flow rates of sheath gas and auxiliary gas were 45 Arb and 15 Arb; full MS resolution and MS/MS resolution were 70,000 and 17,500, respectively; and the collision energy was 20/40/60 eV. In the model, the spray voltage was 3.8 kV (positive mode (POS)) or − 3.1 kV (negative mode (NEG)).

2.3.3. Data pre-processing

MS raw data were converted to mzML format using ProteoWizard, and then processed using the R package XCMS (version 3.2) for retention time alignment, peak detection, and peak matching. Data were filtered when the number of samples containing a metabolite was <50% of all samples in a group. Each sample was normalized to an internal standard. Then, missing values were replaced by half of the minimum value found in the dataset by default. Pre-processing results generated a data matrix, including retention time, mass-to-charge ratio values, and peak intensity. After data processing using the MS/MS database, peak annotation was performed using OSI-SMMS (version 1.0, Dalian Chem Data Solution Information Technology Co. Ltd.).

2.3.4. Differential metabolites and KEGG pathway analysis

Orthogonal partial least-squares discrimination analysis (OPLS-DA) was used for the multivariate statistical analysis, and relevant information was concentrated in the first predictive component. The differential metabolites were analyzed using the OPLS-DA results. A variable importance in projection (VIP) score of the OPLS model was applied to rank the metabolites that best distinguished between the two groups. The threshold of the VIP was set to 1. *t*-tests were used to screen for differential metabolites. Metabolites with a t-test p value <0.05 and VIP ≥ 1 were considered to be differential metabolites between the two groups. Metabolites were mapped to KEGG metabolic pathways for pathway enrichment analysis (Kanehisa et al., 2007), which identified metabolic pathways or signal transduction pathways that were significantly enriched in differential metabolites compared with the entire background.

2.4. 16s rDNA sequencing analysis of microbial diversity in sea cucumber intestines

2.4.1. DNA extraction, PCR amplification of 16S rRNA genes, and sequencing

16s rDNA sequencing analysis was conducted using the three male and three female intestinal replicates (each replicate was a mixture of material from three sea cucumbers). A QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) was used to extract DNA from the intestines following the manufacturer's instructions. DNA purity was evaluated on 1% agarose gels, and a NanoDrop 2100 device (Thermo Fisher Scientific) was used to measure the DNA concentration. Each sample was adjusted to 1 ng/μL with sterile water. PCR amplification of 16S rRNA genes and sequencing was carried out by Omicsmart (Guangdong, China). PCR amplification was performed before sequencing, and a barcoded sequencing approach was used to study the bacterial composition of each digestive tract sample. Briefly, the 515f/806r primer targeting the V4 region of the bacterial 16S rRNA gene was used for PCR amplification of the DNA samples. The PCR products were purified using a Gene JET Gel Extraction kit, and an Ion Plus Fragment Library Kit was used to construct the sequencing libraries according to the protocol in the handbook. The library quality was monitored using a Qubit 2.0 Fluorometer and a Bioanalyzer 2100 system, and the library was sequenced using the Ion S5TMXL platform.

2.4.2. Community composition, indicator taxa, alpha diversity analysis, and function prediction

The composition of the bacterial taxa found in the samples was analyzed using Uparse software to cluster the Effective Tags of all samples. The abundance statistics of each taxonomic group were visualized using Krona (version 2.6) (Ondov et al., 2011). The stacked bar plot of the community composition was generated using the R project ggplot2 package (version 2.2.1). Between-group Venn analysis was performed using the R project Venn Diagram package (version 1.6.16). The Shannon index and Simpson's evenness index were calculated in QIIME (version 1.9.1). The alpha index comparison between groups was conducted using Welch's *t*-test. The KEGG pathway analysis of the

operational taxonomic units (OTUs) was conducted using Tax4Fun (version 1.0) (Aßhauer et al., 2015). Analysis of function difference between groups was conducted using Welch's t-test, the Wilcoxon rank test, the Kruskal-Wallis H test, and Tukey's honest significant difference test in the R project Vegan package (version 2.5.3).

3. Results

3.1. Analysis of the intestinal transcriptome of female and male sea cucumber

3.1.1. DEG analysis

The transcriptomes of male and female intestine samples were analyzed for DEGs to identify enriched genes. Of the 24,865 genes expressed in intestines, 21,131 genes were commonly expressed in both males and females, 2468 were only expressed in females, and 1266 were only expressed in males (Fig. 1A). Additionally, 27 genes were up-regulated in females and 39 genes were up-regulated in males (Fig. 1B, FDR $\leq$ 0.05 and log2fc $>$ 1).

3.1.2. Pathway enrichment analysis

The KEGG enrichment analysis results showed that DEGs were mainly related to the endocrine and digestive systems (Fig. 2A). In terms of metabolism, the enriched pathways were mainly related to lipid metabolism, and genes were also enriched in signal transduction. The main gene that was significantly enriched in the pathway was for fatty acid synthase (*FASN*). The top 10 pathways identified by GO enrichment analysis were acyl-[acyl-carrier-protein] hydrolase activity, thiolester hydrolase activity, FASN activity, response to interleukin-4, 3-hydroxyacyl-[acyl-carrier-protein] dehydratase activity, negative regulation of the developmental process, regulation of isoprenoid metabolic process, acyl-CoA metabolic process, thioester metabolic process, and protein ADP-ribosylation (Fig. 2B). The molecular function classifications of these 10 pathways were related to fatty acid synthesis and metabolism, and this is mainly the result of enrichment of the *FASN* gene.

3.1.3. qRT-PCR validation

To validate the results of the transcriptomic analysis, five genes were selected for validation using qRT-PCR, with the *Grb2* gene serving as the reference. The expression patterns obtained by qRT-PCR were consistent with those obtained by transcriptome analysis (Table 1).

3.2. Analysis of metabolites in the gonads of male and female sea cucumbers

3.2.1. Multivariate statistical analysis and differential metabolite analysis

OPLS-DA showed that the metabolites in the gonads of male and female sea cucumbers differed significantly, as indicated by differences identified in the POS and NEG modes of the LC-MS/MS analysis (Fig. 3A). In the POS mode, 75 metabolites were up-regulated, and 44 metabolites were down-regulated in GM samples compared with GF samples. In the NEG mode, 66 metabolites were up-regulated, and 131 metabolites were down-regulated in GM samples compared with GF samples.

3.2.2. Enrichment analysis of differential metabolites

The KEGG analysis showed that the main metabolic pathways enriched with the different metabolites in the gonads of sea cucumbers were amino acid metabolism, lipid metabolism, and carbohydrate metabolism, and the system involved was the digestive system. Filtering the differential metabolites enriched in the lipid metabolism pathway (KEGG B class) revealed sex differences in choline and lysophosphatidylcholine (LysoPC 15:0) in the POS mode, while acetoacetic acid, glyceric acid, taurine, cis-9-palmitoleic acid, oleic acid, EPA, ARA, DHA, and LysoPC (14:0) in the NEG mode were all related to lipid metabolism. The heat map of these metabolites is shown in Fig. 3B.

3.3. Analysis of intestinal 16s rDNA sequencing of male and female sea cucumbers

3.3.1. Species composition and biomarker taxa analysis

To study the composition of the bacterial taxa of the samples, we used Uparse software to cluster the Effective Tags of all samples, in which the default settings provide 97% identity to cluster the sequences into OTUs, and calculated the absolute abundance and relative information of the tags for each OTU in each sample. To analyze the distribution of sea cucumber intestinal microbes, we selected the top 10 groups (phyla and genera) in terms of mean abundance in all samples (Fig. 4). The other known organisms were classified as Others, and unknown microbes were marked as Unclassified. The top 10 phyla were Proteobacteria, Bacteroidetes, Planctomycetes, Firmicutes, Verrucomicrobia, Epsilonbacteraeota, Actinobacteria, Cyanobacteria, Fusobacteria, and Patescibacteria (Fig. 4A), and the most abundant genera were *Brevibacillus*, *Halioglobus*, *Vibrio*, *Ilumatobacter*, *Actibacter*, *Bacillus*, *Sulfurospirillum*, *Rhodopirellula*, *Chryseobacterium*, and *Shimia* (Fig. 4B).

There were three unique phyla in IM samples (Spirochaetes, Nanoarchaeaeota, and Armatimonadetes) (Fig. 4C); 73 unique genera in IF samples (Fig. 4D); 90 unique genera in IM samples; and 252 genera

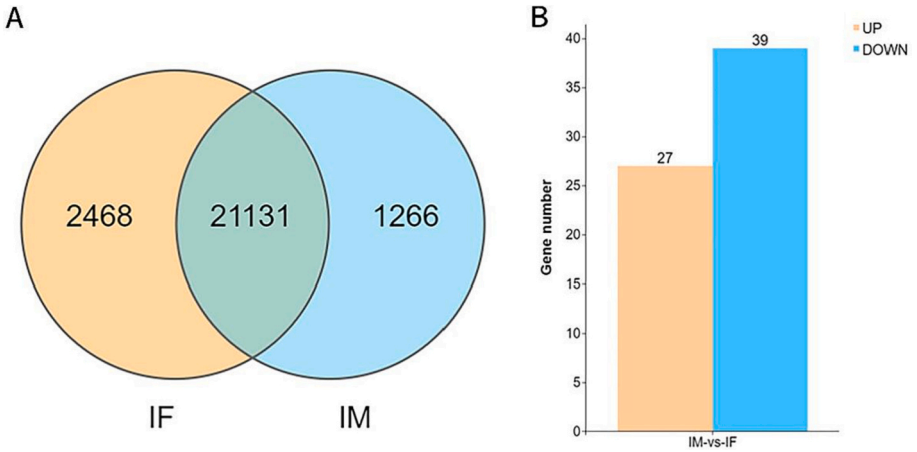
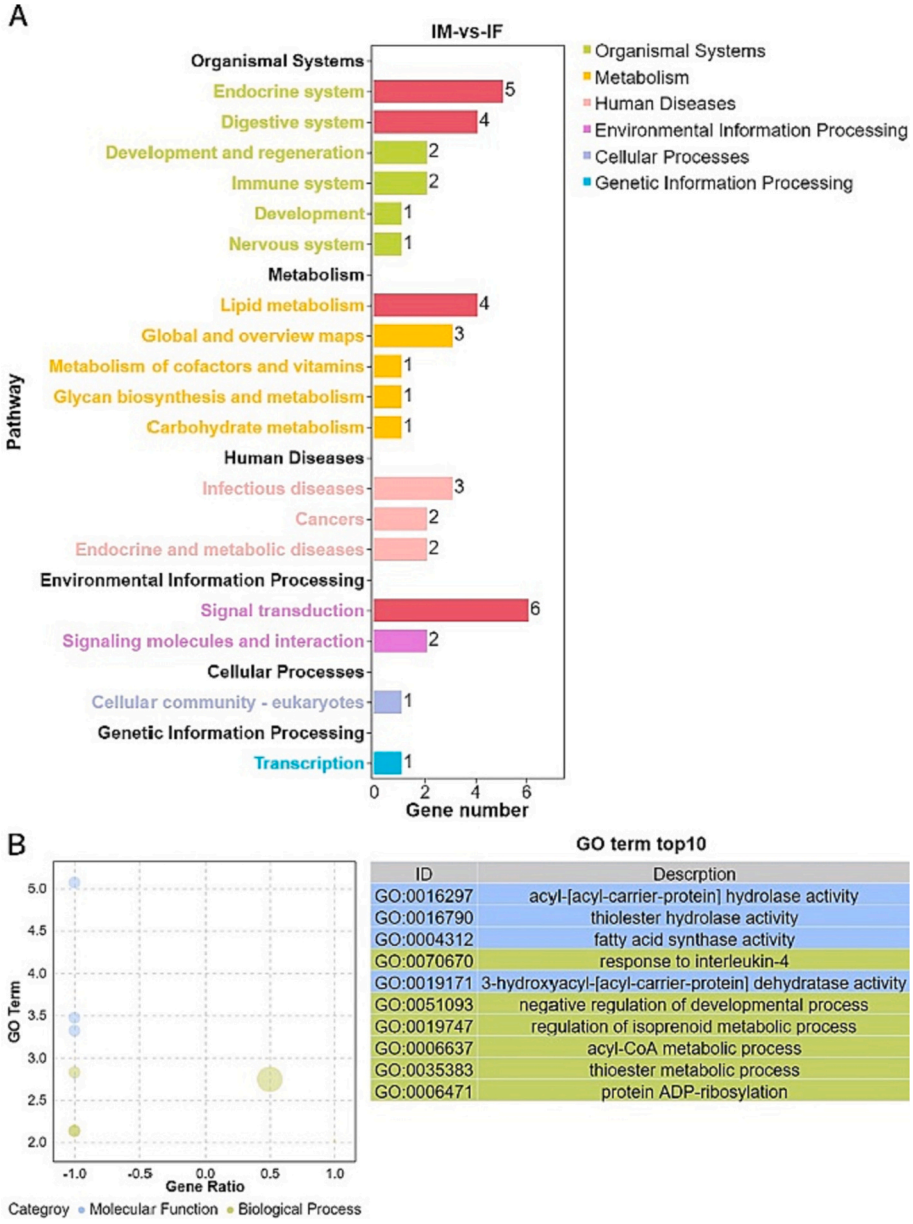


Fig. 1. Gene expression in the intestines of sea cucumbers. A) Venn diagram of gene expression in male (IM) and female (IF) intestines; B) histogram of DEGs in IM and IF; IM-vs-IF analysis revealed whether gene expression in IF was up-regulated or down-regulated relative to IM.



ID	Descrption
GO:0016297	acyl-[acyl-carrier-protein] hydrolase activity
GO:0016790	thiolester hydrolase activity
GO:0004312	fatty acid synthase activity
GO:0070670	response to interleukin-4
GO:0019171	3-hydroxyacyl-[acyl-carrier-protein] dehydratase activity
GO:0051093	negative regulation of developmental process
GO:0019747	regulation of isoprenoid metabolic process
GO:0006637	acyl-CoA metabolic process
GO:0035383	thioester metabolic process
GO:0006471	protein ADP-ribosylation

Fig. 2. Enrichment analysis of DEGs in male (IM) and female (IF) intestines. A) Chart showing the number of DEG enrichment pathways identified by KEGG analysis. The column marked in dark red is the classification column of the main enrichment pathway; B) the bubble chart showing the top 10 DEG-enriched terms identified by GO analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Validation of the RNA-seq data by qRT-PCR.

Gene id	Description	qRT-PCR (log_2(F/M))	RNA-seq (log_2(F/M)
gene-BSL78_02090	Lactase-phlorizin hydrolase [*A. japonicus*]	−2.357*	−1.201**
gene-BSL78_04869	Cytoplasmic protein NCK2 [*Astyanax mexicanus*]	−2.435*	−1.418**
gene-BSL78_06830	Alpha-mannosidase 2C1-like [*Acanthaster planci*]	−1.957*	−1.138*
gene-BSL78_15322	Fatty acid synthase [*Strongylocentrotus purpuratus*]	−1.343**	−1.406**
gene-BSL78_28188	Fibropellin-3 isoform X2 [*S. purpuratus*]	−3.164*	−2.049*

F/M: Whether the gene expression of female intestines (IFs) compared with male intestines (IMs) was up-regulated or down-regulated.
* $p < 0.05$.
** $p < 0.01$.

common to both males and females. The unique genera in IM samples included *Spirochaeta_2* in the unique phylum Spirochaetes.

3.3.2. Microbial alpha-diversity and function prediction analysis

Alpha diversity can indicate taxon abundance in the habitat, and it is usually calculated using species richness and species evenness (Fig. 5A, B). In IF samples, the Shannon and Simpson index values were 8.85289

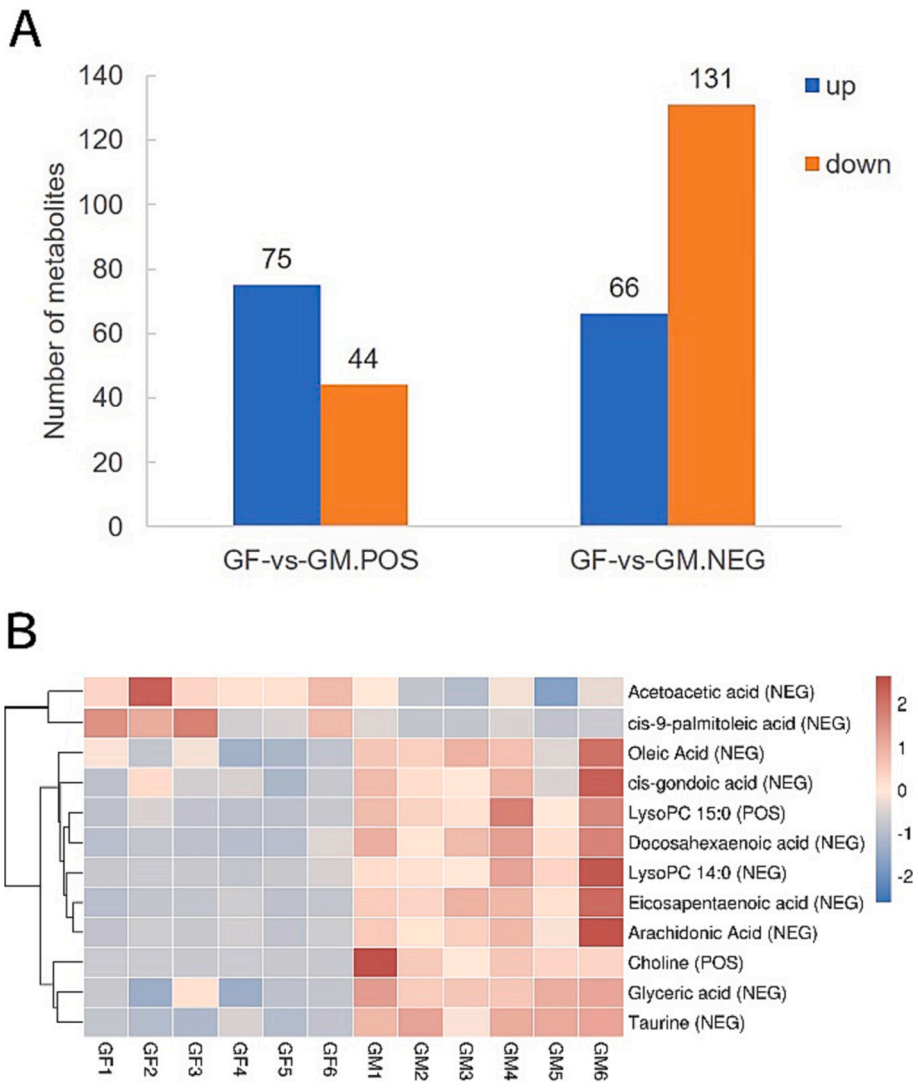


Fig. 3. Differential metabolites in female gonads (GFs) and male gonads (GMs). A) Differential metabolite statistics for POS and NEG modes; B) heatmap of metabolites related to lipid metabolism (KEGG B class).

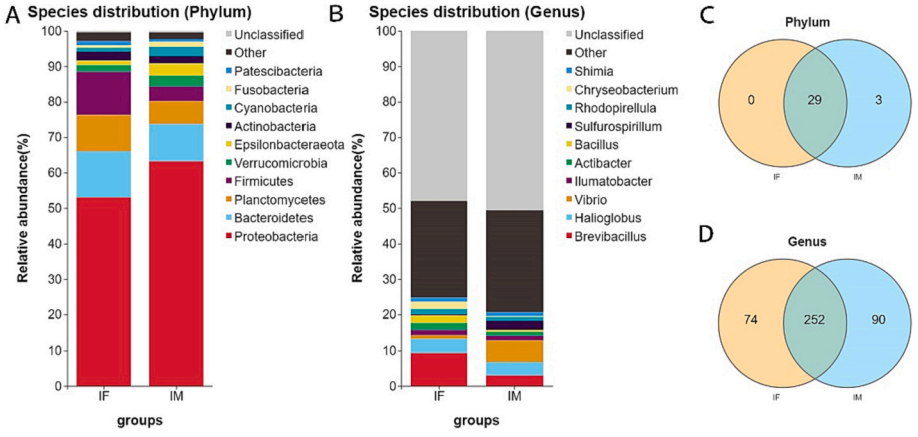


Fig. 4. Species composition and biomarker species of intestinal microbes in *A. japonicus*. A) Top 10 phyla of intestinal microbes; B) top 10 genera of intestinal microbes; C) the number of phyla of intestinal microbes in male intestines (IMs) and female intestines (IFs); D) the number of genera of intestinal microbes in IMs and IFs.

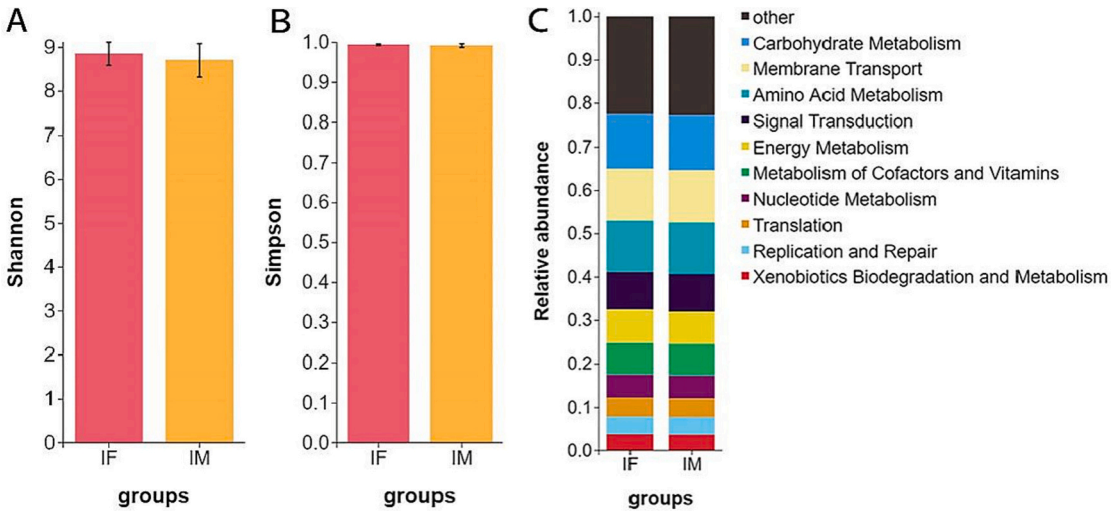


Fig. 5. The alpha diversity and function prediction determined by Tax4Fun of *A. japonica* intestinal microbes. A) Shannon index of alpha diversity; B) Simpson index of alpha diversity; C) top 10 function predictions by Tax4Fun in male intestines (IMs) and female intestines (IFs).

and 0.993724, respectively, while the values for IM samples were 8.705248 and 0.991648. Tax4Fun was used for function prediction, and the results showed 37 enrichment pathways. Fig. 5C shows the top 10 pathways enriched in the two groups, which included carbohydrate metabolism, membrane transport, amino acid metabolism, signal transduction, energy metabolism, metabolism of cofactors and vitamins, nucleotide metabolism, translation, replication and repair, and xenobiotics biodegradation and metabolism.

4. Discussion

This study shows differences in the metabolic profile of both intestines and gonads of *A. japonicus*. The results are discussed regarding the possible relationship between the intestine and gonadal metabolism in the sea cucumber. Analysis of DEGs in the intestines found that more genes were expressed in the intestines of female *A. japonicus*, but the number of up-regulated genes was larger in male intestines. A number of DEGs between the two groups were related to metabolism. Mauvais-Jarvis et al. (2017) reported that the metabolic homeostasis system in most mammals can be affected by sex, and Hudry et al. (2016) found sex differences in the expression of metabolic genes in the intestine, which may lead to other differences in the body. There is also a relationship between growth and metabolism. As far as lipids are concerned, moderately increasing dietary lipid levels can increase growth rate, but excessive lipid intake will impair growth performance (Chatzifotis et al., 2010; Kim and Lee, 2005). In order to better explain the sex differences in sea cucumber lipid metabolism, we selected and unified the body weight and gonadal development stage samples. In our study, the fatty acid-related gene *FASN*, which plays a critical role in lipid metabolism, was up-regulated in male intestines. Fatty acids are synthesized when an organism needs to obtain energy from the diet for storage. Recent studies of lipid metabolism in aquatic animals focusing on the effects of feed and environmental stress (Guan et al., 2016; Lee et al., 2018; Liu et al., 2018; Liu et al., 2016), reported that *FASN* was an important indicator of whether lipid metabolism was disturbed. Guan et al. (2018) studied the role of bisphenol A (BPA), an environmental estrogen pollutant, in lipid metabolism in the fish *Gobiocypris rarus* and explored its mechanism of action. They concluded that BPA affects lipid metabolism by inhibiting the β-oxidation of fatty acids, interfering that the expression of *FASN* changes the binding ability of sterol regulatory element binding protein 1. They also reported that when fat content was high and carbohydrate level was low, *FASN* expression was down-regulated. Yang et al. (2017) reported that adding vitamin A to the diet can increase the activity of lipid metabolism-related enzymes and the expression of *FASN*. Our finding of a male-bias in the expression of *FASN* in the intestines of *A. japonicus* suggests that there may be sex differences in intestinal lipid metabolism.

As expected, LC-MS/MS analysis of the gonads of *A. japonicus* revealed the presence of many different lipid metabolites. In fish, lipids are used not only for energy storage but also as an important structural part of mature gonads, and Henderson and Almatar (1989) reported that lipid content may be directly related to fertility. Choline is a water-soluble vitamin that is a bound constituent of cell membranes and the precursor of the neurotransmitter acetylcholine, which can effectively regulate fat metabolism. Taurine has been shown to have a reproductive function, regulating mating behaviour by regulating testosterone levels (Chacur et al., 2013; Yang et al., 2013). In aquatic animals, studies of taurine have mainly focussed on its effect on larval growth, and its reduction of fat deposition in fish (Guo et al., 2018; Wang et al., 2017). Oleic acid plays an important role in membrane structure and catabolism. In a study of walleyes, it was found that the level of oleic acid was significantly higher in males than in females, which was related to the influence of reproductive requirements on the biochemical composition of the two sexes(Johnston et al., 2020). Choline deficiency can result in impaired lipid metabolism and even cause growth retardation (Wilson and Poe, 1988). We found that choline content was higher in the testes than in the ovaries, and that the LysoPC concentration was about eight times higher in male gonads than in female gonads. LysoPC is an intermediate product of lecithin metabolism and can be further catalyzed into lysolecithin. In contrast to our results, Cubero-Leon et al. (2012) found that the LysoPC content of female mussels *Mytilus edulis* was higher than in males, possibly due to the activity of phospholipase A2 and the renewal rate of lecithin. Phospholipase A2 can catalyze DHA to generate free DHA, and some DHA can act as LysoPC in an organism (Kita et al., 2018). Essential fatty acids are important nutrients that affect parental reproductive performance and larval quality. Among them, PUFAs, such as DHA, EPA and ARA, are essential nutrients in nutrition (Fei et al., 2021). ARA is a precursor of prostaglandins, and plays a crucial role in regulating physiological processes, such as reproduction and immunity (Cha et al., 2006). Chimsung (2014) found that EPA, DHA and ARA content were related to male reproductive performance and sperm quality. High levels of DHA and EPA were found in fish testes, and were positively correlated with sperm density and sperm motility (Jeong et al., 2002). Wen et al. (2001) found that lipid transport from the hepatopancreas to the gonads was common in crabs through analysis of the changes in lipid composition and content in gonads and hepatopancreas during gonadal development. The study further found that a variety of proteins and enzymes in the

hepatopancreas were involved in lipid transport, such as fatty acid transporters, fatty acid binding proteins, and acyltransferases (Li et al., 2011). In addition, sex differences in strategies for DHA synthesis and delivery to specific tissues, such as the gonads, have been reported in birds (Surai et al., 1999). Martínez-Pita et al. (2012) found sex-related differences in PUFA levels in the clam *Donax trunculus*, with lower levels in females than in males. In this study, the levels of DHA, EPA, and ARA were significantly higher in the testes, possibly resulting in sex differences in lipid metabolism.

FASN can catalyze the synthesis of long-chain fatty acids from acetyl-CoA and malonyl-CoA. Then, PUFA, such as DHA, can be produced by the catalysis of fatty acid desaturase and elongase. In studies of zebrafish, Jiang et al. (2019) found that increased levels of PUFAs may be caused by increased fatty acid synthesis. Ji et al. (2006) reported that the fatty acid composition of the gonads was positively correlated with feeding. Therefore, metabolites in the gonads of *A. japonicus* may be affected by conditions in the intestines. Regarding the intestine, its metabolism was influenced by microbes. Many types of microorganisms inhabit the intestines of sea cucumbers. Compared with terrestrial animals, there are more Fusobacteria and Proteobacteria in the intestinal microbiome of aquatic animals. In studies of the intestinal microbes of *A. japonicus* at different growth stages, Zhang et al. (2013) found that Firmicutes, Proteobacteria, and Actinobacteria were abundant, which is consistent with the experimental results of our study. The microorganisms in the intestines of *A. japonicus* were not only abundant in number and variety, but also functioned to produce digestive enzymes. Tax4Fun function prediction results showed that these microorganisms had a critical influence on several important processes, such as the metabolism of carbohydrates, amino acids, and energy. We did not detect sex differences in the intestinal microflora structure of male and female sea cucumbers, likely due to the non-selective feeding of these organisms. Sea cucumbers are generalist feeders and as such the environment greatly affects the composition of the intestinal microbiome. Because male and female sea cucumbers in our study lived in the same environment, they showed no significant differences in their intestinal microbial community structure, suggesting that other differences between males and females, such as endocrine activity, did not affect the intestinal microbiome. Because we did not detect sex differences in the intestinal microbiome of *A. japonicus*, variation in DEGs between males and females, including *FASN*, were probably not caused by microbial action. Differences in digestion, absorption, and transformation of nutrients in the intestines may underlie the sex differences in intestinal metabolism. Since the fatty acid composition of the gonads was related to feeding, our study found that *FASN* was up-regulated in the intestines of male *A. japonicus*, while PUFAs were also found to be up-regulated in the gonads. This suggests that different metabolites in sea cucumber gonads may be related to intestinal metabolism, but the details of this relationship remain unknown and require further study.

5. Conclusions

In this study, we found that male and female *A. japonicus* have different lipid metabolic profiles in the gonads and in the intestines, which was probably related to differences in reproductive investment and physiology. The expression of *FASN* in the male intestine was up-regulated relative to that in females, and the content of PUFAs such as DHA, EPA, and ARA were higher in the testes than in the ovaries. Therefore, we speculate that sex differences in sea cucumber gonadal metabolites may be related to intestinal metabolism. The mechanisms of interaction between the intestines and gonads of sea cucumbers requires further exploration and this information may be relevant for improving the reproductive efficiency of sea cucumbers in aquacultural settings, principally by improving their diet and nutrition.

CRediT authorship contribution statement

Shuangyan Zhang: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Xiaoshang Ru:** Investigation, Methodology. **Libin Zhang:** Conceptualization, Funding acquisition, Writing – review & editing. **David Gonçalves:** Funding acquisition, Writing – review & editing. **Hongsheng Yang:** Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Data availability

I have shared the BioProject number (PRJNA768534) of the data to this article.

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Appendix A. Supplementary data

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